JOURNAL OF VIROLOGY, Mar. 2010, p. 2629–2634 Vol. 84, No. 5
0022-538X/10/$12.00 doi:10.1128/JVI.02049-09

Role of Untranslated Regions in Regulation of Gene Expression, Replication, and Pathogenicity of Newcastle Disease Virus Expressing Green Fluorescent Protein[▽]

Shin-Hee Kim and Siba K. Samal*

Virginia-Maryland Regional College of Veterinary Medicine, University of Maryland, 8075 Greenmead Drive, College Park, Maryland 20742

Received 29 September 2009/Accepted 4 December 2009

To gain insight into the role of untranslated regions (UTRs) in regulation of foreign gene expression, replication, and pathogenicity of Newcastle disease virus (NDV), a green fluorescent protein (GFP) gene flanked by 5′ and 3′ UTRs of each NDV gene was individually expressed by recombinant NDVs. UTRs of each gene modulated GFP expression positively or negatively. In particular, UTRs of the M and F genes enhanced levels of GFP expression at the junction of the P and M genes without altering replication of NDV, suggesting that UTRs could be used for enhanced expression of a foreign gene by NDV.

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 transcriptional units (3′-NP-P-M-F-HN-L-5′) (9). Each transcriptional unit contains a major open reading frame flanked by short 5′ and 3′ untranslated regions (UTRs), which are followed by conserved transcriptional initiation and termination control sequences, known as gene start (GS) and gene end (GE), respectively. The UTRs of NDV vary in length and sequence. Transcription begins at a single promoter located at the 3′ leader end, and the genes are copied in a sequential and polar manner into individual mRNAs by a start-stop mechanism guided by GS and GE signals (9). By using a reverse genetics system, the NDV genome has been shown to accommodate insertion of an additional transcriptional unit expressing a foreign antigen, which has allowed NDV to be used as a vector for vaccines against human and animal pathogens (2, 7, 11, 18).

UTRs in viruses have been shown to play a role in the regulation of viral transcription and translation. In influenza A virus, UTRs contain the signals responsible for RNA replication, transcription, polyadenylation, and packaging of the RNA segments (19). In measles virus, the long 3′ UTR of M mRNA and 5′ UTR of F mRNA play important roles in replication and pathogenicity of the virus (14). In NDV, deletion of UTRs of the HN gene was shown to affect the HN mRNA transcription, translation, and pathogenicity (17). However, the role of other UTRs in NDV gene expression is unknown. We hypothesized that different NDV UTRs would lead to differential expression of a foreign gene. By using a reverse genetics system, we have evaluated the individual role of UTRs of each of the six NDV genes in expression of a foreign gene. We generated a series of recombinant NDVs expressing green fluorescent protein (GFP) gene flanked by 5′ and 3′ UTRs of each NDV gene (Fig. 1A). Each transcriptional unit was inserted between the P and M genes in a full-length antigenomic cDNA of NDV strain Beaudette C (BC), because this position is known to support stable expression of foreign genes without affecting virus replication (3, 11, 18). Recombinant BC viruses (rBCs) were recovered by using our standard protocol (8), and their GFP expression was monitored in virus-infected DF-1 cells using fluorescent microscopy. Among the recovered viruses, only rBC/GFP-HN UTRs and rBC/GFP-L UTRs expressed little or no GFP (data not shown), indicating that the HN and L UTRs might have an inhibitory effect on GFP expression at this insertion position. To investigate the contribution of 3′ UTR to viral gene expression, we replaced the 3′ UTR of the L gene with that of the NP gene in the GFP transcriptional unit (Fig. 1A). In order to determine whether the UTRs of downstream genes had an inhibitory effect on GFP expression at an upstream position, each GFP gene flanked by the HN or L UTRs was inserted between the HN and L genes in the NDV genome (Fig. 1B). The growth characteristics of the rBCs were examined by multicycle growth curve in DF-1 cells infected with a multiplicity of infection (MOI) of 0.01 PFU/cell (6). Our results showed similar kinetics of growth between the parental virus, rBC, and all rBCs containing the GFP gene at the junction of the P and M genes (Fig. 1A), suggesting that addition of different UTRs in the GFP transcriptional unit did not affect viral replication. In contrast, insertion of GFP gene flanked by the HN or L UTRs between the HN and L genes resulted in slow growth of the viruses up to 24 h postinfection, but their titers were similar to that of rBC thereafter (Fig. 1B).

To gain insight into the effect of the NDV UTRs on regulation of GFP gene expression, we determined its transcription and translation levels in virus-infected DF-1 cells. Transcription of GFP gene was measured by quantitative reverse transcriptase PCR (RT-PCR). The results were normalized to β-actin and expressed as fold difference relative to the mRNA level

* Corresponding author. Mailing address: Virginia-Maryland Regional College of Veterinary Medicine, University of Maryland, 8075 Greenmead Drive, College Park, MD 20742. Phone: (301) 314-6813. Fax: (301) 314-6855. E-mail: ssamal@umd.edu.

▽ Published ahead of print on 16 December 2009.

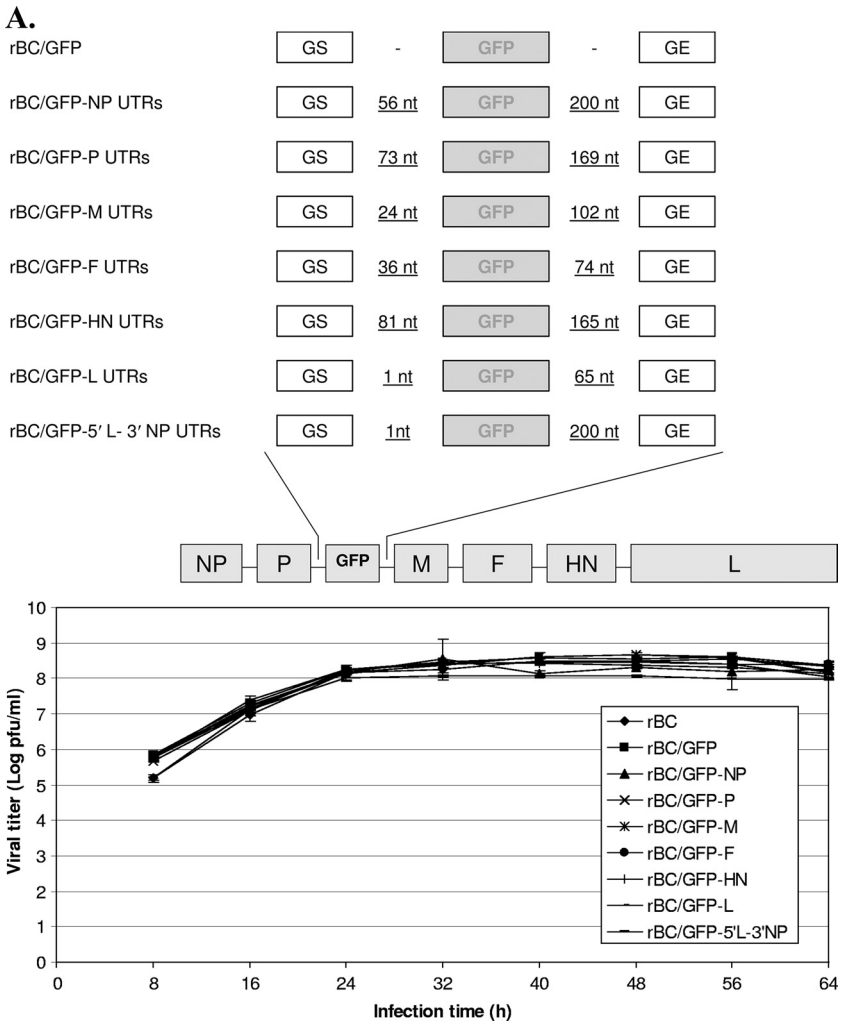

FIG. 1. Generation and *in vitro* replication of rBCs containing GFP genes flanked by UTRs of the six NDV genes. GFP gene was flanked by a conserved gene start (GS) and 5′ UTR of individual NDV gene upstream and 3′ UTR of corresponding NDV gene and a conserved gene end (GE) downstream. Each transcriptional unit was inserted between the P and M genes (A) or between the HN and L genes (B) in the NDV genome. *In vitro* replication of the recovered viruses was determined in virus-infected DF-1 cells at an MOI of 0.01. The viral titers were determined by plaque assay.

of rBC using the comparative threshold cycle ($\Delta\Delta C_T$) method (10). A comparison of GFP transcription level between the GFP virus without UTRs (rBC/GFP) and GFP viruses containing UTRs showed that, between the P and M genes, the M and F UTRs increased GFP transcription by 36% and 19%, respectively, whereas the HN and L UTRs reduced its transcription by 71% and 79%, respectively ($P < 0.05$) (Fig. 2A). The reduced transcription level of the GFP gene inserted between the HN and L genes (rBC/GFP-AgeI) was probably due to the polar effect of transcription (2, 5). However, addition of the HN or L UTRs into this transcriptional unit enhanced GFP transcription. Furthermore, our results showed enhanced GFP transcription by replacement of 3′ UTR of the L gene with that of the NP gene in GFP transcriptional unit between the P and M genes, suggesting that the 3′ UTR probably plays a distinct role in viral transcription. UTRs of viral genes contain binding sites required for viral polymerase complex or host factors (4). For instances, 3′ UTRs of viral genes often contain regulatory elements (i.e., activator/repressor binding sites) that negatively or positively modulate gene expression. Myb-like transcription regulators are also found in 5′ UTRs of viral genes. Therefore, both 5′ and 3′ UTRs of each NDV gene could modulate a foreign gene transcription differently, depending upon their interaction with RNA-binding proteins and cellular proteins.

The 5′ UTRs of viral mRNAs can regulate translation efficiency by forming secondary structures and interacting with internal ribosome entry sites (15). The 3′ UTRs of viral mRNAs can also regulate translation efficiency by affecting mRNA stability (12). We first determined GFP production in virus-infected DF-1 cells by Western blot analysis. We were able to clearly detect GFP production only by six rBCs containing GFP transcriptional units between the P and M genes. Among them, rBC/GFP-M, rBC/GFP-F, and rBC/GFP-5′L-3′NP produced high levels of GFP. To further determine the effect of UTRs on GFP translation, we quantitated the level of

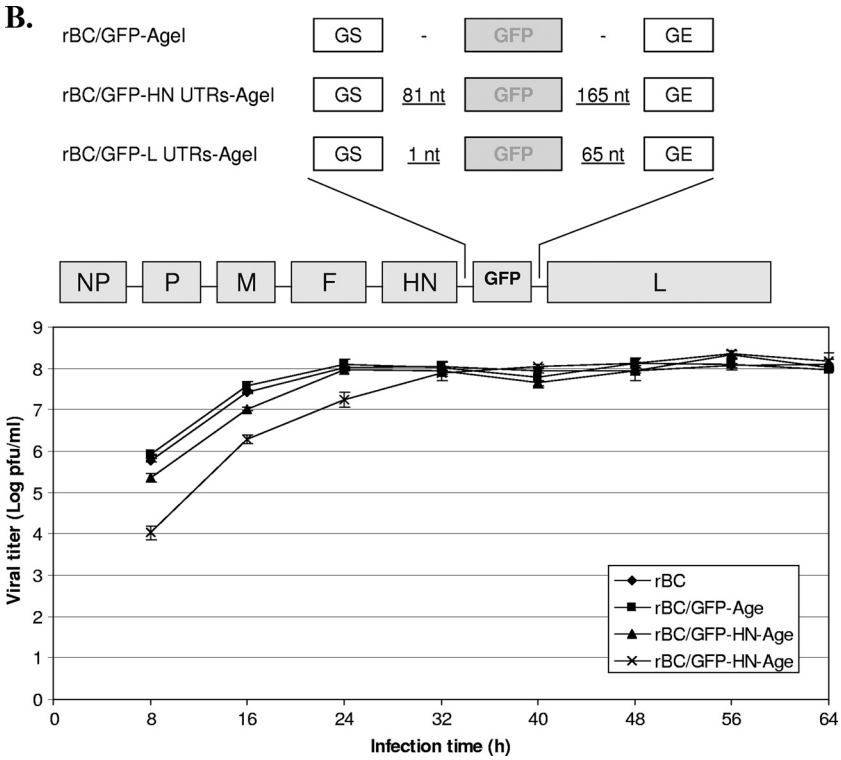

FIG. 1—*Continued.*

GFP production in the virus-infected cells by flow cytometry (FACS Aria II; BD Bioscience). In general, levels of GFP transcription and translation in each GFP transcriptional unit were correlated. Consistently, most rBCs containing the GFP gene between the P and M genes expressed high levels of GFP in virus-infected cells (98 to 99% positive) (Fig. 2B and C). In contrast, the percentage of cells expressing GFP was low in infected cells with GFP viruses containing HN or L UTRs. We also observed similar patterns of GFP expression by these viruses using fluorescent microscopy (data not shown). Mean fluorescence intensity analysis indicated that UTRs of the P, M, and F genes enhanced GFP production in virus-infected DF-1 cells. Interestingly, relocation of each transcriptional unit containing UTRs of the HN gene or L gene from upstream to downstream position resulted in an increase in level of GFP expression despite the relatively low level of GFP gene transcription shown in Fig. 2A. Our results showed that UTRs of the HN and L genes negatively modulated GFP expression (mean fluorescence intensities, 149 and 45, respectively) between the P and M genes but positively modulated GFP expression (304 and 1,072, respectively) between the HN and L genes. This result suggests that UTRs of the HN and L genes would contribute to enhanced translation efficiency at a downstream position. Overall, UTRs of the M and L genes produced the highest level of GFP at upstream positions of the M and L genes, respectively, suggesting that these UTRs at their original positions would be optimal for regulation of gene expression. It is possible that NDV has evolved to have unique UTRs for each gene, which are most effective in regulating gene expression only at that position.

Insertion of an additional transcriptional unit into the NDV genome might affect efficient incorporation of structural proteins into virions (16). Therefore, sucrose gradient-purified rBCs were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis (17) (Fig. 3A). Our results showed that insertion of the GFP transcriptional units between the P and M genes did not affect incorporation of viral protein into the virions by measuring the ratios of the HN and L proteins to the M protein (Fig. 3B). However, we found significant reduction of the HN and L proteins incorporated into the virions of rBC/GFP HN UTRs-AgeI and rBC/GFP L UTRs-AgeI, respectively, compared to those of rBC ($P < 0.05$).

We further determined the effect of UTRs on the pathogenicity of rBCs expressing GFP in embryonated chicken eggs and 1-day-old chicks (Table 1). Mean death time (MDT) was determined as the mean time (h) for the minimum lethal dose of virus to kill all the embryos when inoculated into 9-day-old specific pathogen free (SPF) embryonated chicken eggs (1). The effect of the HN and L UTRs on the pathogenicity of NDV in 1-day-old SPF chicks was also determined by intracerebral pathogenicity index (ICPI) test (1). The scale of the ICPI value in evaluating the virulence of NDV strains is from 0.00 (avirulent strains) to 2.00 (highly virulent NDV strains). Both MDT and ICPI values indicated that insertion of different GFP transcriptional units between the P and M genes did not affect pathogenicity of NDV. However, insertion of the GFP gene with HN and L UTRs (rBC/GFP HN UTRs-AgeI and rBC/GFP L UTRs-AgeI) between the HN and L genes decreased the pathogenicity of the viruses (ICPI, 1.07 and 0.96, respectively), probably due to reduced synthesis and/or incorporation of the HN and L proteins into virions (13, 17). Interest-

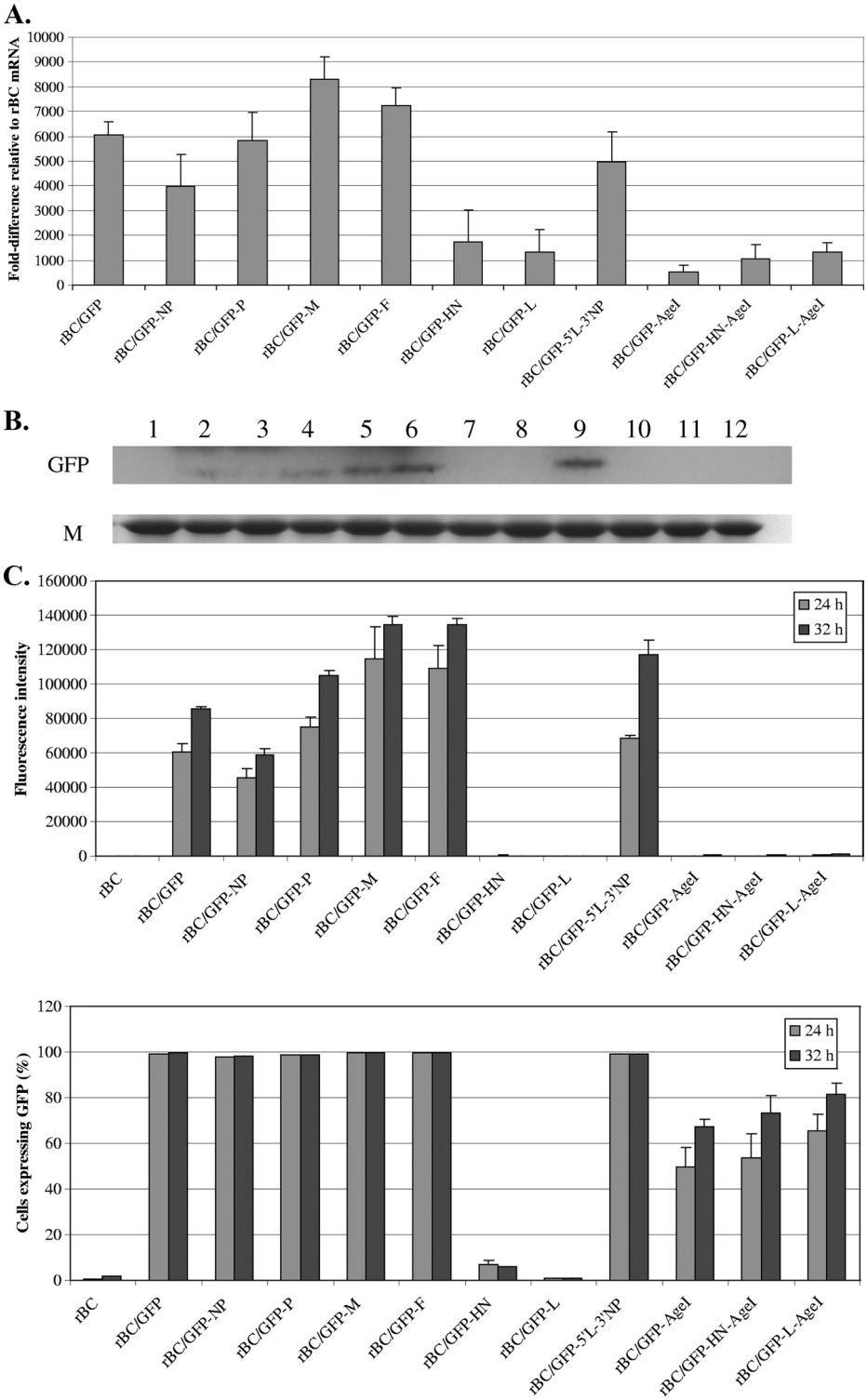

FIG. 2. Effect of the NDV UTRs on GFP transcription and translation in DF-1 cells infected with rBCs expressing GFP. (A) The levels of GFP mRNA transcription were determined by quantitative RT-PCR (qRT-PCR). DF-1 cells in 6-well plates were infected with rBCs at an MOI of 0.1. At 20 h postinfection, total RNAs were isolated using Trizol, treated with DNase to remove residual genomic DNA. The RNA (1.5 μg each) was reverse transcribed using SuperScript II. The prepared cDNA was subjected to qRT-PCR. Primers used for the GFP gene were the forward primer 5′-CGA CGG CAA CTA CAA GAC-3′ and the reverse primer 5′-TAG TTG TAC TCC AGC TTG TGC-3′. For internal normalization, the β-actin gene was amplified using the forward primer 5′-GAG AAA TTG TGC GTG ACA TCA-3′ and the reverse primer 5′-CCT GAA CCT CTC ATT GCC A-3′. (B) For Western blot analysis of GFP production, total proteins were collected from virus-infected DF-1 cells at 24 h postinfection, electrophoresed, transferred to a nitrocellulose membrane, and immunostained using a monoclonal antibody against GFP protein or the NDV M protein as a loading control. (C) GFP expression in virus-infected DF-1 cells at an MOI of 0.1 was quantitated by fluorescence-activated cell sorting analysis. At 24 h and 32 h postinfection, the cells were harvested, fixed with 4% paraformaldehyde, and analyzed by flow cytometry. Mean fluorescence intensity and percent GFP-positive cells were analyzed using Flowjo software. The analyses were independently performed three times.

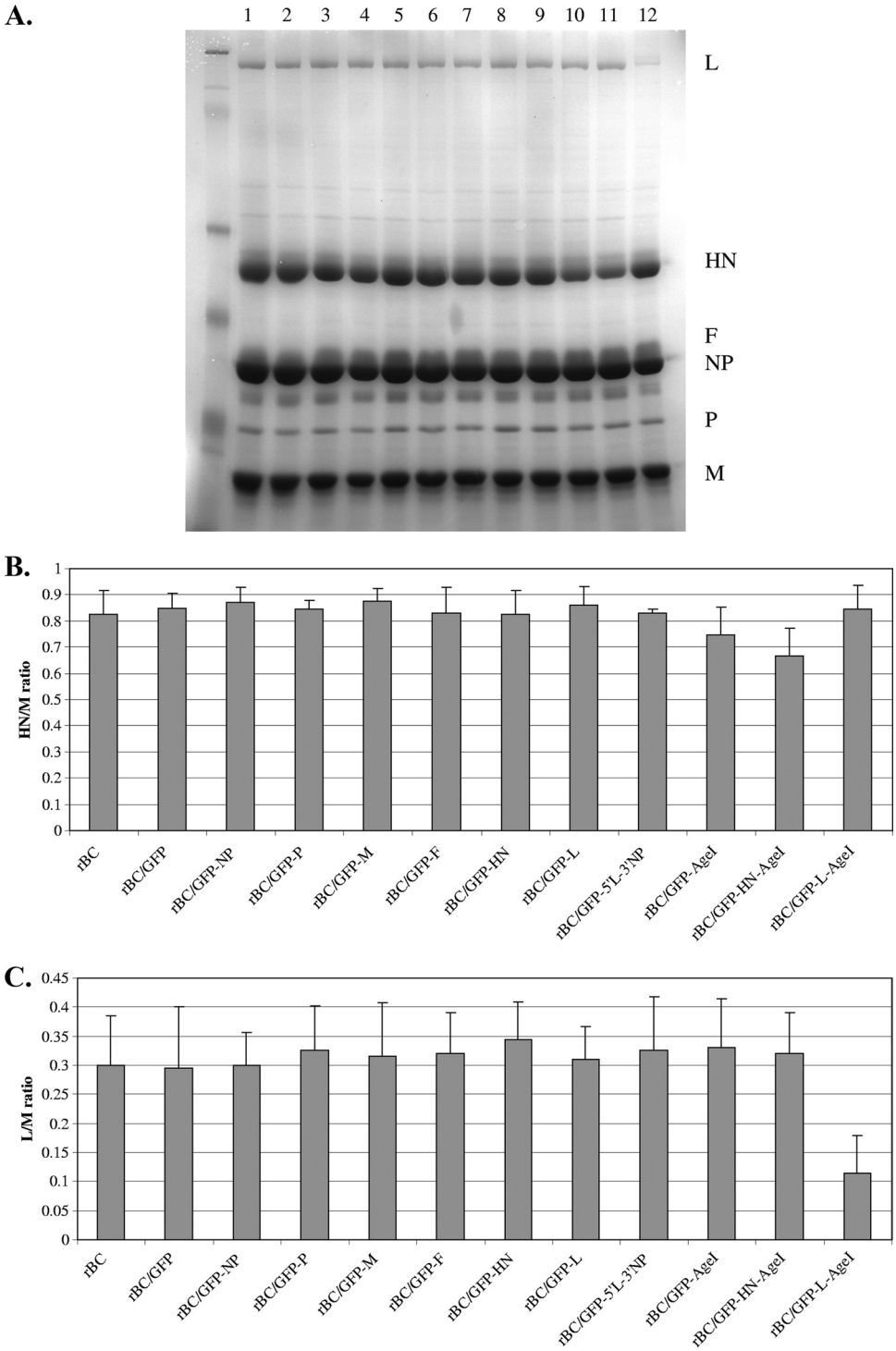

FIG. 3. Incorporation of structural proteins of rBCs expressing GFP into virions. (A) Viruses were harvested from allantoic fluids and purified through a 30% sucrose cushion. The viral proteins were separated on an SDS-PAGE gel (8%) and stained with Coomassie brilliant blue. (B and C) Ratios of the HN protein (B) or the L protein (C) to the M protein levels from rBC and rBCs expressing GFP were quantified. This assay was performed by determining the ratios of the P protein to M protein. Since similar levels of the P and M proteins were present among the different viruses, the levels of HN protein or L protein incorporation into the virus particles was measured by determining their ratios to the M protein. This analysis was independently performed three times.

ingly, insertion of the GFP gene without UTRs (rBC/GFP-AgeI) slightly increased the pathogenicity of the virus (ICPI, 1.70). It is not known how the insertion of GFP gene at this site increased the pathogenicity of the virus. However, it is possible that the presence of GFP gene sequences without UTRs might have enhanced the transcription of upstream or downstream gene.

In summary, we have evaluated the role of each NDV UTR in expression of a foreign gene by NDV. We showed that different

TABLE 1. Pathogenicity of the rBCs containing the GFP gene flanked by different UTRs in embryonated eggs and chicks

Virus	MDT (h)[a]	ICPI[b]
rBC	58	1.51
rBC/GFP	57	1.43
rBC/GFP-NP UTRs	58	
rBC/GFP-P UTRs	57	
rBC/GFP-M UTRs	56	
rBC/GFP-F UTRs	57	
rBC/GFP-HN UTRs	58	1.46
rBC/GFP-L UTRs	57	1.45
rBC/GFP-5′ L-3′ NP UTRs	56	
rBC/GFP-AgeI[c]	47	1.70
rBC/GFP-HN UTRs-AgeI	56	1.07
rBC/GFP-L UTRs-AgeI	66	0.96

[a] Mean time for the minimum lethal dose of virus to kill all the inoculated embryos. NDV strains were classified by the following criteria: virulent strains, taking <60 h to kill embryos; intermediate virulent strains, 60 to 90 h; and avirulent strains, >90 h.

[b] Pathogenicity of NDV in 1-day-old SPF chicks was evaluated by the ICPI value: virulent strains, 1.5 to 2.0; intermediate virulent strains, 1.0 to 1.5; and avirulent strains, 0.0 to 0.5. Seven selected viruses were tested for ICPI.

[c] Both MDT and ICPI were independently determined twice.

NDV UTRs affected the expression of the foreign gene differently. Furthermore, our results indicated that the NDV UTRs are not only gene specific but also position specific. For maximum expression of a foreign gene, it may be advantageous to have the open reading frame of the foreign gene flanked by the UTRs of the immediate downstream gene at the insertion site. These results may have implications in designing effective NDV vaccine vectors.

We thank Daniel Rockemann, Flavia Dias, Yunsheng Wang, and our laboratory members for excellent technical assistance.

REFERENCES

1. **Alexander, D. J.** 1989. Newcastle disease, p. 114–120. *In* H. G. Purchase, L. H. Arp, C. H. Domermuth, and J. E. Pearson (ed.), A laboratory manual for the isolation and identification of avian pathogens, 3rd ed. The American Association of Avian Pathologists, Kendall/Hunt Publishing Company, Dubuque, IA.
2. **Bukreyev, A., M. H. Skiadopoulos, B. R. Murphy, and P. L. Collins.** 2006. Nonsegmented negative-strand viruses as vaccine vectors. J. Virol. **80:**10293–10306.
3. **Carnero, E., W. Li, A. V. Borderia, B. Moltedo, T. Moran, and A. García-Sastre.** 2009. Optimization of human immunodeficiency virus Gag expression by Newcastle disease virus vectors for the induction of potent immune responses. J. Virol. **83:**584–597.
4. **Edgil, Dianna, and E. Harris.** 2006. End-to-end communication in the modulation of translation by mammalian RNA viruses. Virus Res. **119:**43–51.
5. **He, B., R. G. Paterson, C. D. Ward, and R. A. Lamb.** 1997. Recovery of infectious SV5 from cloned DNA and expression of a foreign gene. Virology **237:**249–260.
6. **Huang, Z., A. Panda, S. Elankumaran, D. Govindarajan, D. Rockemann, and S. K. Samal.** 2004. The hemagglutinin neuraminidase protein of Newcastle disease virus determines tropism and virulence. J. Virol. **78:**4176–4184.
7. **Huang, Z., S. Elankumaran, A. S. Yunus, and S. K. Samal.** 2004. A recombinant Newcastle disease virus expressing VP2 protein of infectious Bursal disease virus protects against NDV and IBDV. J. Virol. **78:**10054–10063.
8. **Krishnamurthy, S., Z. Huang, and S. K. Samal.** 2000. Recovery of a virulent strain of Newcastle disease virus from cloned cDNA: expression of a foreign gene results in growth retardation and attenuation. Virology **278:**168–182.
9. **Lamb, R. A., and G. D. Parks.** 2007. Paramyxoviridae: the viruses and their replication, p. 1449–1496. *In* D. M. Knipe and P. M. Howley (ed.), Fields virology, 5th ed. Wolters Kluwer-Lippincott Williams & Wilkins, Philadelphia, PA.
10. **Le Nouλn, C., S. Munir, S. Losq, C. C. Winter, T. McCarty, D. A. Stephany, K. L. Holmes, A. Bukreyev, R. L. Rabin, P. L. Collins, and U. J. Buchholz.** 2009. Infection and maturation of monocyte-derived human dendritic cells by human respiratory syncytial virus, human metapneumovirus, and human parainfluenza virus type 3. Virology **385:**169–182.
11. **Nakaya, T., J. Cros, M. S. Park, Y. Nakaya, H. Zheng, A. Sagrera, E. Villar, A. García-Sastre, and P. Palese.** 2001. Recombinant Newcastle disease virus as a vaccine vector. J. Virol. **75:**11868–11873.
12. **Parks, C. L., R. A. Lerch, P. Walpita, H. P. Wang, M. S. Sidhu, and S. A. Udem.** 2001. Analysis of the noncoding regions of Measles virus strains in the Edmonston vaccine lineage. J. Virol. **75:**921–933.
13. **Rout, S. N., and S. K. Samal.** 2008. The large polymerase protein is associated with the virulence of Newcastle disease virus. J. Virol. **82:**7828–7836.
14. **Takeda, M., S. Ohno, F. Seki, Y. Nakatsu, M. Tahara, and Y. Yanagi.** 2005. Long untranslated regions of the measles virus M and F genes control virus replication and cytopathogenicity. J. Virol. **79:**14346–14354.
15. **Wang, C., P. Sarnow, and A. Siddiqui.** 1993. Translation of human hepatitis C virus RNA in cultured cells is mediated by an internal ribosome-binding mechanism. J. Virol. **67:**3338–3344.
16. **Wertz, G. W., R. Moudy, and L. A. Ball.** 2002. Adding genes to the RNA genome of vesicular stomatitis virus: positional effects on stability of expression J. Virol. **76:**7642–7650.
17. **Yan, Y., S. N. Rout, S. H. Kim, and S. K. Samal.** 2009. Role of untranslated regions of hemagglutinin-neuraminidase gene in replication and pathogenicity of Newcastle disease virus. J. Virol. **83:**5943–5946.
18. **Zhao, H. and B. P. H. Peeters.** 2003. Recombinant Newcastle disease virus as a viral vector: effect of genomic location of foreign gene on gene expression and virus replication. J. Gen. Virol. **84:**781–788.
19. **Zheng, H., P. Palese, and A. Garcia-Sastre.** 1996. Nonconserved nucleotides at the 3′ and 5′ ends of an influenza A virus RNA play an important role in viral RNA replication. Virology **217:**242–251.