Parainfluenza virus chimeric mini-replicons indicate a novel regulatory element in the leader promoter

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Gene expression of paramyxoviruses is regulated by genome-encoded cis-acting elements; however, whether all the required elements for viral growth have been identified is not clear. Using a mini-replicon system, it has been shown that human parainfluenza virus type 2 (hPIV2) polymerase can recognize the promoter elements of parainfluenza virus type 5 (PIV5), but reporter activity is lower in this case. We constructed a series of luciferase-encoding chimeric PIV2/5 mini-genomes that are basically hPIV2, but whose leader (le), mRNA start signal and trailer sequence are partially replaced with those of PIV5. Studies of the chimeric PIV2/5 mini-replicons demonstrated that replacement of hPIV2 le with PIV5 le results in remarkably weak luciferase expression. Further mutagenesis identified the responsible region as positions 25–30 of the PIV5 le. Using recombinant hPIV2, the impact of this region on viral life cycles was assessed. Insertion of the mutation at this region facilitated viral growth, genomic replication and mRNA transcription at the early stage of infection, which elicited severe cell damage. In contrast, at the late infection stage it caused a reduction in viral transcription. Here, we identify a novel cis-acting element in the internal region of an le sequence that is involved in the regulation of polymerase, and which contributes to maintaining a balance between viral growth and cytotoxicity.

INTRODUCTION

The Paramyxoviridae family, a major group of non-segmented, negative-strand RNA viruses (nsNSV), exhibits a common feature of mRNA transcription and genome replication (Lamb & Parks, 2013). The genome contains six to ten tandemly linked genes, which are separated by junctional sequences composed of gene start (GS) and gene end (GE) signals that regulate mRNA transcription and 3’ poly (A) addition, respectively. Both ends of the genome contain additional nucleotides (nt), such as a 3’ leader sequence (le) and a 5’ trailer sequence (tr). The genome is encapsidated by nucleoprotein (NP) and acts as a template for RNA-dependent RNA polymerase (RdRp) composed of large protein (L) and phosphoprotein (P). The RdRp binds to the extreme 3’ end of the genome and firstly produces a short uncapped le RNA. Subsequently, capped and polyadenylated mRNAs encoding each viral protein are generated. After translation of these mRNAs, genome replication begins. During replication, RdRp initiates at the extreme 3’ end of the genome, ignores all junctional sequences and synthesizes a full-length complementary antigenome. The antigenome is entirely encapsidated by NP, and functions as a template for the synthesis of progeny genomes. These genomes can be used for the synthesis of further viral mRNA, and can act as a template for further replication, or can be assembled into infectious particles. All RNA synthetic events during these processes are regulated by cis-acting elements encoded in the genome and antigenome.

Human parainfluenza virus type 2 (hPIV2) is a major respiratory pathogen and a member of the Rubulavirus genus of the family Paramyxoviridae, which includes simian virus 41 (SV41), hPIV4, mumps virus (MuV) and PIV5. The gene order of hPIV2 is 3’ > NP > P/V > M > F > HN > L > 5’. Two critical cis-acting elements have been identified in the replication promoter of hPIV2 and PIV5 (Murphy et al., 1998; Murphy & Parks, 1999; Keller & Parks, 2003), and also confirmed in other members of the Paramyxovirinae (Tapparel et al., 1998; Hoffman & Banerjee, 2000; Walpita, 2004; Gander et al., 2011): promoter element I (PrE I) and promoter element II (PrE II). PrE I is present at the 3’ end of the genome and antigenome comprising the terminal 19 nt, and PrE II is present 73–90 nt from the 3’ end. It consists of repeated 3’-NNNGAC-5’ motifs, in hPIV2 and PIV5 (Murphy et al., 1998; Murphy & Parks, 1999). The Importance of the spacing and primary sequence between PrE I and PrE II for the replication promoter has also been

Two supplementary tables are available with the online Supplementary Material.
confirmed (Keller et al., 2001; Keller & Parks, 2003). Conserved sequences at the beginning and end of each gene modulate the activity of RdRp during transcription (Rassa et al., 2000; Whelan et al., 2004), whereas these signals are ignored during replication. These distinct elements for replication and transcription coordinate viral RNA synthetic events to achieve efficient growth. However, whether all the regulatory elements required have been clarified has not yet been confirmed. Identification of additional regulatory elements will lead to a detailed understanding of viral gene expression mechanisms.

We used mini-replicons to study hPIV2 gene expression. The mini-genome used in this system consists of a reporter gene embedded by hPIV2 le, GS, GE and tr. We applied the system for confirming an inhibitory effect of hPIV2 V, an accessory protein, for RdRp activity and for identifying critical amino acids in hPIV2 L catalysis (Nishio et al., 2008, 2011; Matsumoto et al., 2015). In these processes, we reported that the RdRp complex of PIV5 can use the mini-genome of hPIV2, whereas the reporter expression is only 20% that of hPIV2 RdRp (Matsumoto et al., 2015). This suggests that a species of nsNSV can recognize regulatory sequences in a mini-genome from distinct, but closely related, viruses.

In the present study, we determined that hPIV2 RdRp can use the PIV5 mini-genome, and the reporter expression in this case is approximately 20% of that with the hPIV2 mini-genome, indicating that the RNA sequence in the PIV5 mini-genome determines the efficiency of hPIV2 RdRp. Identification of the signal responsible for the lower reporter expression in the PIV5 sequence will ultimately demonstrate a novel regulatory cis-acting element in the hPIV2 genome. We prepared a series of hPIV2 and PIV5 (PIV2/5) ‘chimeric’ mini-replicons, and measured reporter expression for hPIV2 RdRp. These experiments demonstrated that the leader sequence of PIV5 is sufficient to explain the lower reporter expression in the chimeric mini-replicon. Various mutagenesis experiments have shown that the regulatory signal is encoded in the internal region of the leader sequence.

RESULTS

Replacement of the leader sequence of the hPIV2 mini-genome with that of PIV5 results in weak reporter expression by hPIV2 RdRp

hPIV2 polymerase complex, NP, P and L, can utilize the PIV5 mini-genome encoding renilla luciferase (Rluc) as a template. The expression of Rluc from the PIV5 mini-genome was approximately 20% that from the hPIV2 mini-genome. This finding indicates that the RNA sequences of PIV5, such as le, GS and tr, cause weak Rluc expression by hPIV2 RdRp. To identify the sequences responsible, we generated three PIV2/5 chimeric mini-genomes that are based on the hPIV2 mini-genome, with le, GS and tr replaced with those of PIV5: PIV5/5 le, PIV5/5 GS and PIV5/5 tr, respectively (Fig. 1a). Their Rluc expression was examined using hPIV2 NP, P and L (Fig. 1b). The relative Rluc levels in the hPIV2 mini-genome were 200-fold higher than background (L-). The Rluc level in the PIV2/5 le was approximately 16% of that in the hPIV2, which is comparable to that of PIV5. The Rluc levels from both, PIV2/5 GS and PIV2/5 tr were approximately 134% and 59% that from hPIV2, respectively. These findings indicate that the sequence in PIV5 le is involved in the remarkably weak Rluc expression by hPIV2 RdRp.

Positions 25–30 nt of the leader sequence are responsible for the weak Rluc expression by hPIV2 RdRp

We focused next on PIV5 le, and looked for a region responsible for the weak Rluc expression by hPIV2 RdRp.

![Fig. 1. Luciferase expression by hPIV2 RdRp in the parainfluenza virus chimeric mini-replicon system. (a) Diagrams show PIV2/5 chimeric mini-genomes expressed by T7 polymerase as negative-sense RNA; the open boxes represent hPIV2 and the grey boxes represent PIV5 sequences. (b) Relative Rluc activity in the mini-replicon system by hPIV2 RdRp using chimeric hPIV2 mini-genomes with a PIV5 leader (PIV2/5 le), a gene start signal (PIV2/5 GS) and a trailer (PIV2/5 tr). Rluc expression from mini-genomes is normalized by an internal control of Fluc expression, and relative values are shown (hPIV2=1). L- indicates the result from hPIV2 mini-genomes without the hPIV2 L plasmid. Data represent means and standard deviations of experiments performed in triplicate.](http://jgv.microbiologyresearch.org)
All members of the *Paramyxodirinae* have a genome length that is a number divisible by six, and each six nucleotides are encapsidated by a single NP that makes the ‘rule of six’ (Kolakofsky et al., 1998). We generated a series of PIV2/5 chimeric mini-genomes with an le that was partially replaced with that of PIV5, with each six-bases: #1–#8, and examined them for Rluc expression by hPIV2 RdRp (Fig. 2a). Relative Rluc levels from #1, #2, #3, #5, #7 and #8 did not significantly differ from that of the parental hPIV2, whereas those from #4 and #6 were lower, by approximately 16% and 44% of the hPIV2, respectively. We generated a further mutant mini-genome with an le based on PIV5 and other regions from hPIV2. The partial region of PIV5 le with six bases each was replaced with that of hPIV2.

![Image](https://example.com/image.png)

**Fig. 2.** Identification of the regulatory signal in the internal region of hPIV2 leader sequence. (a) Relative Rluc activity in the mini-replicon system using chimeric mini-genomes, whose hPIV2 leader sequence is partially replaced by that of PIV5 with each six-bases. The Rluc expressions from mini-genomes are normalized by an internal control Fluc expression, and relative values are shown (hPIV2=1). Data represent means and standard deviations of triplicate experiments. Accession numbers are: hPIV2 (ABI176531) and PIV5 (JQ743318). (b) Relative Rluc activity in the mini-replicon system using chimeric PIV2/5 le mini-genome, whose PIV5 leader sequence is partially restored to that of hPIV2 with each six-bases. The Rluc expressions from mini-genomes are normalized by an internal control Fluc expression, and relative values are shown (hPIV2=1, but it this omitted from this figure). Data represent means and standard deviations of triplicate experiments. (c) Relative Rluc activity in the mini-replicon system by hPIV2 RdRp using hPIV2 mini-genomes, whose le is deleted with each six-bases. The Rluc expressions from mini-genomes are normalized by an internal control Fluc expression, and relative values are shown (hPIV2=1). Data represent means and standard deviations of triplicate experiments.
(Fig. 2b). Although most mutants showed similar Rluc levels to PIV2/5 le, higher Rluc expressions were observed in #12 and #14, both mutated regions corresponded to #4 and #6 respectively, and this was more marked in #12. These findings demonstrate that the region responsible for weak Rluc expression by hPIV2 RdRp is the internal region, with the position at 25–30 nt (#4 the most essential).

To obtain more detailed information, we examined the deletion effect of le for Rluc expression by hPIV2 RdRp in the mini-replicon system. By generating deletion mutants with each six-bases (Fig. 2c, d#1–d#8), whose deleted regions are analogous to those of #1–#8 mutants, we examined Rluc expression by hPIV2 RdRp. The Rluc levels from all deletion mutants (d#1–d#8) were similar, with L− negative control (Fig. 2c). This is consistent with a previous study showing that the proper spacing between PrE I and PrE II is important (Murphy et al., 1998; Murphy & Parks, 1999). It is suggested that the primary sequences in the le region are determinants for the efficiency of hPIV2 RdRp activity.

To define the minimum nucleotides responsible for weak Rluc expression by hPIV2 RdRp in the #4 mutant, all candidate mini-genomes based on hPIV2 sequence, whose 25–30 nt region is individually replaced with those of PIV5 at single, double and triple nt level as indicated in Fig. 3, were generated and examined for Rluc expression by hPIV2 RdRp. Rluc expression from these mutants was lower than that from hPIV2 without #19. Reductions of Rluc levels were mild in the single nt mutants (#17, #18 and #20). Dramatic reductions were observed in the double and triple nt mutants, especially in #21 (approximately 17 % of hPIV2), #27 (19 %) and #29 (21 %). The Rluc levels from these mutants were comparable to that from

![Fig. 3. Identification of the minimum nucleotides responsible for the weak Rluc expression by hPIV2 RdRp in the #4 mutation. Relative Rluc activity in the mini-replicon system by hPIV2 RdRp using chimeric hPIV2 mini-genomes based on hPIV2 sequence, whose 25–30 nt region is partially replaced with those of PIV5 at single, double and triple nt level. The Rluc expressions from mini-genomes are normalized by an internal control Fluc expression, and relative values are shown (hPIV2=1). Data represent means and standard deviations of triplicate experiments.](http://jgv.microbiologyresearch.org)
the #4 mutant. The common mutation of #21, #27 and #29 is C26A27 → A26G27 substitution.

It is interesting that the hPIV2 mini-genome, whose le is entirely replaced with that of other species, is available for hPIV2 RdRp. We extended this for other members of the rubulaviruses: SV41, hPIV4 and MuV. Chimeric mini-genomes whose le is replaced with that of SV41, hPIV4 and MuV were generated and examined for Rluc expression by hPIV2 RdRp (Fig. 4). The Rluc levels from mini-genome having PIV5 and SV41 le were approximately 20%, whereas those from hPIV2 having hPIV4 le and MuV le were approximately 50% of hPIV2. The nucleotide C26A27 is not completely conserved in PIV5 and SV41, but partially and fully conserved in hPIV4 and MuV, respectively. This also supports the review that the di-nucleotide conservation at C26A27 of hPIV2 le is an important determinant for the efficiency of hPIV2 RdRp activity.

**Contribution of the internal leader signal to viral growth and gene expression**

To clarify the contribution of the internal leader region to intrinsic viral infection, recombinant hPIV2 (rPIV2) having mutations at le position 25–30 nt (#4 rPIV2), as well as wild-type (wt) rPIV2, were generated. Vero cells are highly susceptible to hPIV2 infection, which enables measurement of the maximum potential of rPIV2 growth efficiency. Vero cells were infected with these viruses at a multiplicity of infection (MOI) of 0.01. The supernatants were collected at 0, 24, 48 and 72 h post-infection, and titres were measured by a plaque assay (Fig. 5a). In contrast to the result from the mini-replicon system that the #4 mutation contributes to reduced luciferase expression, the titres of #4 rPIV2 were slightly higher than that of wt rPIV2. We also observed the cell fusions characteristic of hPIV2 infections, both in wt and #4 rPIV2-infected cells under light microscopy. Interestingly, this occurred more rapidly in #4 rPIV2-infected cells than that in wt rPIV2 (Fig. 5b). The infected Vero cells were collected for detection of hPIV2 proteins and β-actin by Western blotting (Fig. 5c). Expressions of NP, P and V proteins from #4 rPIV2 were slightly higher than those from wt rPIV2 at 24 and 48 h post-infection. Expressions of viral proteins, as well as β-actin, at 72 h in #4 rPIV2 were lower than those in wt rPIV2. Cell fusion may have altered the production and/or stability of total proteins. We next measured the copy numbers of viral RNAs, such as genome and anti-genome RNA, and all mRNAs by quantitative real-time PCR (qRT-PCR) (Fig. 5d and Table S1, available in the online Supplementary Material). At 24 and 48 h post-infection, the copy number of genome RNA was approximately five-fold higher than that of anti-genome RNA in both wt and #4 rPIV2. Genome and anti-genome RNA from #4 rPIV2 were higher than those from wt rPIV2. Copy numbers of mRNA were observed as a gradient in the following order: NP > P/V > M > F > HN > L. The copy numbers of each mRNA from #4 rPIV2 were higher than those from wt rPIV2 at 24 and

![Fig. 4. Effects of le exchange with other rubulaviruses on mini-replicon activity. Relative Rluc activity in the mini-replicon system by hPIV2 RdRp using hPIV2 mini-genomes, whose le is exchanged with those of PIV5, SV41, hPIV4 and MuV. The Rluc expressions from mini-genomes are normalized by an internal control Fluc expression, and relative values are shown (hPIV2=1). Data represent means and standard deviations of triplicate experiments. Accession numbers are: SV41 (NC_006428), hPIV4 (JQ241176) and MuV (AB744049). The conserved nt among all viruses are shown in bold. The positions at 26 and 27 nt are shown in the shaded box.](https://www.microbiologyresearch.org/article/1524)
48 h post-infection. In contrast, RNA expression patterns at 72 h were unique. Copy numbers of NP, P/V, M, F and HN mRNA from #4 rPIV2 were significantly lower than those from wt rPIV2, whereas L mRNA was not different between wt and #4 rPIV2-infected cells.

We next examined rPIV2 growth in HeLa cells by infection at an MOI of 0.1. The supernatants collected at 0, 24, 48 and 72 h post-infection were subjected to the plaque assay. The titres of #4 rPIV2 were significantly higher than those of wt rPIV2 (Fig. 6a). Because the growth efficiency of...
hPIV2 in HeLa cells is weaker than that in Vero cells (Nishio et al., 2005), the difference of growth titres in HeLa cells between wt and #4 rPIV2 became clear. Apparent rounding cells, probably damaged cells, were observed in #4 rPIV2, but not obvious in wt rPIV2 at 48 and 72 h post-infection under light microscopy (Fig. 6b). The infected HeLa cells
were collected and subjected to Western blotting. Expressions of NP, P and V protein as well as β-actin were detected at 24, 48 and 72 h post-infection, and those of wt and #4 rPIV2 were at similar levels (Fig. 6c). Copy numbers of viral RNAs were quantified by qRT-PCR (Fig. 6d and Table S2). Although genome (and anti-genome) RNA of #4 rPIV2 were significantly higher than those of wt rPIV2 throughout the infection course, the dynamics of mRNA were different between wt and #4 rPIV2. At 24 and 48 h post-infection, copy numbers of most mRNAs from #4 rPIV2 were significantly higher than those from wt rPIV2. At 72 h post-infection, copy numbers of NP mRNA from #4 rPIV2 were lower than those from wt rPIV2. Other mRNAs from #4 rPIV2 were comparable to or slightly higher than those from wt rPIV2.

Although the differences in protein expression between wt and #4 rPIV2 were mild in both in Vero and HeLa cells, the high sensitivity of qRT-PCR to RNA calculation revealed the clear differences of the copy numbers of viral RNA products. The diminished pattern of mRNAs from #4 rPIV2 infection was observed for NP, P/V, M, F and HN in Vero cells and for NP in HeLa cells (Figs 5d and 6d). This cell type-specific difference is probably due to the growth phenotype of hPIV2 in both cell lines. The growth efficiency of hPIV2 in Vero cells was higher than that in HeLa cells (Figs 5a and 6a), indicating that the altered pattern of mRNA abundance was observed earlier in Vero cells. The reduction of transcripts from the gene proximal to the 3′ end of the genome observed in infected Vero cells at 72 h is thought to occur late in HeLa cells, whereas it could not be detected due to the technical difficulty caused by the strong cytopathic effects.

DISCUSSION

The purpose of this work was to identify novel regulatory elements in the hPIV2 genome by using a chimeric mini-replicon system. Based on the fact that hPIV2 RdRp can express luciferase in the mini-genome of PIV5 at lower levels, we have demonstrated that partial RNA sequences, such as 25–30nt (#4) and 37–42 nt (#6), of PIV5 are determinants of lower reporter activity. In particular, the #4 mutation resulted in a marked effect on mini-replicon activity. Functional cis-acting elements in the hPIV2 genome and antigenome were mapped due to their similarity to PIV5.

Additional mutagenesis established that the repeated 3′-NNNNNGC-5′ sequence motif within the PrE II is essential for PIV5 replication (Murphy & Parks, 1999), and this is also conserved in hPIV2. The structure of the PIV5 nucleocapsid showed that PrE I and PrE II are aligned on the same face of the helical nucleocapsid, leading to a proposal that both promoters function to create a binding site for RdRp (Murphy et al., 1998; Alayyoubi et al., 2015). The #4 and #6 regions are located in opposite positions to both promoters on the helical nucleocapsid, indicating that they are not directly involved in the RdRp binding site. Using a mini-genome system, a third promoter element in PIV5 was identified that is 51–66 bases from the 3′ terminus of the antigenome (Keller et al., 2001). Moreover, it has been shown that 20–50 nt of the PIV5 genome act to regulate replication negatively (Keller & Parks, 2003). Related elements have been studied in other paramyxoviruses, and their roles have been examined in detail for hPIV3 by using mini-replicon systems. The importance of PrE I (1–12 nt) and PrE II (79–96 nt) in the hPIV3 genome and antigenome for replication has been confirmed (Hoffman & Banerjee, 2000; Gander et al., 2011). The involvement of the internal space between PrE I and PrE II in replication has also been examined. The 13–39 nt of the hPIV3 antigenome contains complicated control signatures with both stimulatory and repressive elements, specifically the 21–28 nt act to decrease replication (Gander et al., 2011) while the 13–28 nt of the hPIV3 genome act to increase replication (Hoffman et al., 2006). Because many aspects of these promoters have been investigated using a copy-back DI genome and mini-genomes, which do not faithfully attribute a viral life cycle with a full-length genome, we examined the authentic functioning of the cis-acting element by generating recombinant viruses.

We have demonstrated, using mini-replicon systems, that insertion of a #4 mutation in the leader promoter results in diminished luciferase expression by hPIV2 RdRp, which is consistent with the lower copy number of mRNA from #4 rPIV2 than wt rPIV2 observed 72 h post-infection. At the early infection stage (24 and 48 h), copy numbers of all viral RNAs from #4 rPIV2 were higher than those from wt rPIV2. At the late infection stage (72 h), although copy numbers of genomic and antigenomic RNA from #4 rPIV2 were higher than those from wt rPIV2, those of mRNAs proximal to the 3′ end of the genome were lower. This mRNA reduction might have been considered to be due to enhanced RNA degradation due to marked cell damage, but this suggestion is negated by the following observations: (i) the degree of mRNA reduction is highest in the genes proximal to the 3′ end of the genome; (ii) L mRNA, which has the lowest copy number and consists of the longest nucleotides among all the transcripts, is suggested to be the most sensitive to degradation, but was not diminished. These facts indicate a possibility that the lower copy number of mRNAs from #4 rPIV2 at the late infection stage may be due to marked cell damage; instead it can be explained by the attenuation of transcription, as shown by the result of mini-replicon assay. It is reasonable to consider that the #4 mutation hastens viral transcription in the early infection stage but attenuates it in the late infection stage. We examined whether adjustment of the level and ratio of protein-expressing plasmids in the mini-replicon system could specifically reconstitute the early events of viral infection. However, all attempts at modifying the amounts of NP, P and L plasmids resulted in lower luciferase expression in #4.
mutants (data not shown). Additionally, in the case of hPIV2 infection in the mini-genome expressing cells, proteins derived from infectious viruses recognize the mini-genome as a template that can properly reconstitute the natural condition of viral protein expression patterns. Although we attempted to obtain the reporter expression in the hPIV2 infected and mini-genome transfected cells, we could not confirm sufficient luciferase expression in all trials (data not shown). As the mini-genome is a short genomic mimic (1158 nt), the results may express specifically a point in time in the latter period of infection using a full-length genome (15654 nt). Alternatively, one difference between these two systems is that in viral infection, encapsidation of the nascent chain by NP is always coupled to L-directed RNA replication. In the mini-replicon system, the initial encapsidation of the plasmid-derived mini-genome takes place independently of L activity. This may obscure the true features of viral gene expression caused by #4 mutation: early up-regulation and late down-regulation of viral transcription. To completely reflect the initial events of infection by mini-replicon systems, more complicated conditions are required.

A probable reason for the observation that the degree of viral transcription changes according to the infection stage may be an alteration in the transcription–replication balance correlated with ongoing protein production. Genomic replication of nsNSV requires de novo NP production and simultaneous encapsidation of nascent RNA, whereas this is not the case for mRNA transcription (Vidal & Kolakofsky, 1989; Gubbay et al., 2001; Chen et al., 2015). The intracellular concentration of unassembled NP can determine the efficiency of transcription, synthesis of naked RNA and replication: synthesis of encapsidated RNA. At the early infection stage, newly synthesized NP is not abundant in infected cells, which results in both limited replication and preferential transcription. At the late infection stage, increased NP due to the accumulated transcripts enhances replication and, coincidently, reduces transcription. The #4 mutation may be marked affected by transcription repression induced by accumulated NP. If so, the process of plasmid-driven NP production in the mini-replicon system emphasizes the sensitivity of transcription attenuation caused by NP for #4 mutants. Such protein synthetic dynamics connected with viral growth would be required for the appearance of the unique phenotype of viral transcription and propagation observed in this study.

There is a possible explanation to why the regulatory effect of the leader sequence in natural hPIV2 was attenuated at the early infection stage when compared to the #4 mutant. We observed that the #4 rPIV2-infected cells displayed marked cell fusion and rounding in Vero and HeLa cells, respectively. Excessive viral products, such as viral protein(s), RNA and infectious particles from #4 rPIV2, are plausibly the sources for this marked cytopathic effect. This implies that the significance of the internal leader signal is to keep the balance between virus amplification and cell damage by modulating RNA synthesis. This is supported by a report describing how substitution in the PrE I region of PIV5 led to elevated RNA synthesis, resulting in a more marked cytopathic effect (Manuse & Parks, 2009). There is a further possibility that the #4 region could be directly involved in the induction of cell damage by association with host factors. The trailer transcript of Sendai virus was shown to interact with a host protein, TIAR, which is involved in the induction of apoptosis and sequesters the protein for prevention of excessive apoptosis (Iseni et al., 2002). A sequence in the trailer region of the respiratory syncytial virus has an ability to inhibit the formation of stress granules (Hanley et al., 2010). These findings imply that non-coding promoter regions of nsNSV have the potential to regulate not only viral RNA synthesis, but also host responses, to ensure successful viral growth.

The #4 position is included in the regulatory element of the genome, as well as in the leader RNA transcript. The contribution of leader RNA transcript to viral RNA synthesis is worthy of consideration. Interactions of leader RNA transcript with host proteins such as La protein, glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and heterogeneous nuclear ribonucleoprotein were reported for vesicular stomatitis virus, rabies virus and hPIV3 (Kurilla et al., 1983, 1984; De et al., 1996; Gupta et al., 1998), and an involvement of GAPDH in hPIV3 transcription was suggested (Choudhary et al., 2000). We hypothesized that the intrinsic properties of leader RNA transcript from chimeric mini-genomes (and viruses) may affect viral gene expression. As RNA is a critical determinant for protein–RNA interaction, we have predicted the structures of hPIV2

![Image](http://rtools.cbrc.jp/centroidfold/). #1 to #8 are as in Fig. 2a.

**Fig. 7.** Prediction of the RNA secondary structure of leader RNAs. Prediction of RNA secondary structures is performed using the Centroidfold program (http://rtools.cbrc.jp/centroidfold/). #1 to #8 are as in Fig. 2a.
leader RNA (Fig. 7). Leader RNAs from wt hPIV2, PIV5 and most mutants, including #6, are predicted to possess a stem loop or pseudo-knot. However, the #4 mutant is predicted to show no RNA secondary structure. We propose that an unknown host factor may interact with hPIV2 leader RNA to modulate RdRp activity. Loss of RNA secondary structure in the #4 mutant leader transcript may alter protein–RNA affinity and result in a unique virus phenotype. Attempts to identify such a host factor are ongoing.

In this study, using the mini-replicon system, our data infer that a mutation in the internal leader region having the potential to control #4 mutation. These findings emphasize the importance of uncovering an unexpectedly complex obscure feature of the hPIV2 leader region; RNA affinity and result in a unique virus phenotype. Attempts to identify such a host factor are ongoing.

The hPIV2 Rluc mini-replicon assay was performed in BSR T7/5 cells cultured in 12-well plates. One-hundred thousand BSR T7/5 cells were seeded in 12-well plates. Plasmids hPIV2-Rluc (0.5 µg), pTM1-NP (0.375 µg), -P (0.2 µg) and -L (0.375 µg) or empty vector and Fluc (0.1 µg) were transfected using XtremeGENE HP (Roche). At 24 h post-transfection, Rluc and Fluc activities were measured using a Dual luciferase assay kit (Promega) according to the manufacturer’s instructions. All results obtained from Rluc were normalized according to the expression levels of Fluc.

### METHODS

#### Cells and antibodies.

BSR T7/5 cells (Buchholz et al., 1999) and HeLa cells were cultured in Dulbecco’s modified Eagle’s medium (Nissui) supplemented with 10% (v/v) fetal calf serum (FCS). Vero cells were cultured in Eagle’s minimal essential medium (MEM) (Nissui) supplemented with 10% (v/v) FCS. Monoclonal antibodies (mAbs) against hPIV2 NP (159–1) and P/V (315–1) were described previously (Nishio et al., 1997, 1999). Anti-β-actin antibody (2F3) was purchased from Wako (Wako).

#### Plasmid construction.

The renilla luciferase-expressing mini-genome plasmid of hPIV2 (hPIV2-Rluc) was constructed by modifying previously used hPIV2 mini-genomes (Nishio et al., 2008, 2011; Matsumoto et al., 2015). hPIV2-Rluc contained hPIV2 leader, 5' end of vRNA, 3' end of L mRNA, trailer and Rluc gene under the control of a T7 RNA polymerase promoter, whose transcript was expressed as the negative-sense RNA. hPIV2 NP, P and L genes cloned into a pTM1 vector, which contains a T7 promoter and an encephalomyocarditis virus internal ribosome entry site, were as described (Nishio et al., 2008). pTM1 firefly luciferase (Fluc) was also constructed. Chimeric mini-genomes were constructed by a standard PCR mutagenesis method, based on the hPIV2-Rluc whose leader (1–35 nt from 3' end of vRNA), 5' UTR of NP mRNA, 3' UTR of L mRNA, trailer and Fluc gene under the control of a T7 RNA polymerase promoter, whose transcript was expressed as the negative-sense RNA. hPIV2 NP, P and L genes cloned into a pTM1 vector, which contains a T7 promoter and an encephalomyocarditis virus internal ribosome entry site, were as described (Nishio et al., 2008). pTM1 firefly luciferase (Fluc) was also constructed. Chimeric mini-genomes were constructed by a standard PCR mutagenesis method, based on the hPIV2-Rluc whose leader (1–35 nt from 3' end of vRNA), 5' UTR of NP mRNA, 3' UTR of L mRNA, trailer and Fluc gene under the control of a T7 RNA polymerase promoter, whose transcript was expressed as the negative-sense RNA. hPIV2 NP, P and L genes cloned into a pTM1 vector, which contains a T7 promoter and an encephalomyocarditis virus internal ribosome entry site, were as described (Nishio et al., 2008).

#### Generation of recombinant hPIV2.

Two-hundred thousand BSR T7/5 cells were seeded in 6-well plates. Cells were transfected with wt or #4 pPIV2-GFP (2.5 µg) together with the following expression plasmids using XtremeGENE HP (Roche): pTM1-NP at 0.5 µg, pTM1-P at 0.25 µg and pTM1-L at 0.5 µg. After two or three days, viruses produced in the supernatant of transfected cells were further amplified in Vero cells, and virus recovery was confirmed from the expression of GFP in infected cells. Virus stock was prepared in Vero cells. Total RNA from infected cells was extracted using Isogene (Nippon Gene) for confirming the introduction of mutants by RT-PCR, followed by DNA sequencing analysis.

#### Virus growth kinetics.

Five-hundred thousand Vero or 170,000 HeLa cells cultured in 12-well plates were infected with wt or #4 pPIV2 at an MOI of 0.01 (for Vero) and 0.1 (for HeLa), and incubated at 37 °C in MEM without FCS. Supernatants were harvested at 0, 24, 48 and 72 h post-infection, and virus titres were determined by a plaque assay on Vero cells as described previously (Nishio et al., 2005, 2007).

#### Quantitative real-time RT-PCR.

Total RNAs were isolated from virus-infected Vero and HeLa cells using Isogene (Nippon Gene). cDNA synthesis was carried out using a PrimeScript RT reagent kit (Takara) with oligo-dT12-18 for mRNA or specific primers for both anti-genomic RNA (5'-ACCAAGGGGAAAAATCATATGT-3') and genomic RNA (5'-ACCAAGGGGAAAAATGATATGT-3'). qRT-PCR was performed using SsoAdvanced Universal SYBR Green Supermix (Bio-Rad). The primers for each gene are described in Table 1. A standard curve was generated from dilutions whose copy numbers were known, and the RNA per well of samples was quantified based on this standard curve.

#### Prediction of RNA secondary structures.

Secondary structures of the chimeric leader RNA were estimated by computational analysis using CentroidFold software (http://www.ncrna.org/centroidfold).

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