REPORT

The control of paramyxovirus genome hexamer length and mRNA editing

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ABSTRACT

The unusual ability of a human parainfluenza virus type 2 (hPIV2) nucleoprotein point mutation (NP^{Q202A}) to strongly enhance minigenome replication was found to depend on the absence of a functional, internal element of the bipartite replication promoter (CRII). This point mutation allows relatively robust CRII-minus minigenome replication in a CRII-independent manner, under conditions in which NP^{wt} is essentially inactive. The nature of the amino acid at position 202 apparently controls whether viral RNA-dependent RNA polymerase (vRdRp) can, or cannot, initiate RNA synthesis in a CRII-independent manner. By repressing genome synthesis when vRdRp cannot correctly interact with CRII, gln²⁰² of N, the only residue of the RNA-binding groove that contacts a nucleotide base in the N-RNA, acts as a gatekeeper for wild-type (CRII-dependent) RNA synthesis. This ensures that only hexamer-length genomes are replicated, and that the critical hexamer phase of the *cis*-acting mRNA editing sequence is maintained.

Keywords: paramyxoviruses; human parainfluenza virus type 2; nucleoprotein; bipartite replication promoters

INTRODUCTION

Each nucleoprotein (N or NP) protomer of paramyxovirus nucleocapsids (N/NP-RNAs) is associated with precisely 6 nucleotides (nt), and overall genome nucleotide length must be precisely a multiple of 6 (Egelman et al. 1989; Calain and Roux 1993; Kolakofsky et al. 1998, 2005). Paramyxovirus genome replication is governed by this “rule of six,” which is thought to act during promoter recognition and the initiation of RNA synthesis from the genome 3′ end. Because hexamer length genomes have a strong replicative advantage, this rule imposes a hexamer phase on the entire RNA genome. The structural basis for this rule, deduced from the cryo-EM reconstruction of the measles virus (MeV) N-RNA (Gutsche et al. 2015), is that, of the 6 nucleotides associated with each N protomer, three contiguous bases are stacked and pointed toward the solvent (or “out”; i.e., hexamer positions 5 and 6, and position 1 of the next N protomer) (Fig. 1). The other three bases are stacked and point toward the protein core (hexamer positions 2, 3, and 4; or “in”) (Fig. 1). This alternating arrangement of three-base stacks (positions 5,6,1^{out} 2,3,4ⁱⁿ 5,6,1^{out} 2,3,4ⁱⁿ) is imposed on the bound RNA by N protein structure, and is found throughout the N-RNAs, except presumably at their very ends (Gutsche et al. 2015).

Because paramyxovirus genomes are precisely of hexamer length, each nucleotide within *cis*-acting sequences also has

a precise hexamer position within each N protomer; and these positions are conserved within each genera. This is particularly important for 3′ end promoters that are bipartite in nature, being composed (at a minimum) of the first 12 nt at very 3′ genome end (critical region I or CRI). The second component (CRII) is found in the 5′ UTR of the first (N) gene and the 3′ UTR of the last (L) gene, and is simply cytosine at hexamer position 1 (hex¹) of hexamers 14, 15, and 16 (from the 3′ end) for Sendai virus (SeV), human parainfluenza virus type 3 (hPIV3) (respiroviruses), and MeV (a morbillivirus) (Pelet et al. 1996; Tapparel et al. 1998; Walpita 2004). Rubulavirus PIV5 CRII is also very simple, GC at hex⁵ and hex⁶ of hexamers 13, 14, and 15 (Fig. 1; Murphy et al. 1998; Murphy and Parks 1999). CRI and CRII are thus found on the same vertical face of the N-RNA helix, such that viral RNA-dependent RNA polymerase (vRdRp) presumably can simultaneously contact both elements of the promoter. Tellingly, CRI/CRII nt spacing must be exact, as the displacement of CRII tripartite repeat by even 1 nt position strongly reduces genome replication (Pelet et al. 1996; Murphy et al. 1998).

The RNA in resting nonsegmented negative-strand RNA virus (nsNSV) N-RNAs is tightly bound to the N protomer chain predominantly via its ribose-PO₄ backbone, in a deep,

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FIGURE 1. Paramyxovirus bipartite genome promoters. Schematic diagram of NP-RNA 3' end and bipartite promoters of SeV (as a representative Respirivirus) and hPIV2 (as a representative Rubulavirus). The NP subunits (and the hexamer positions within each subunit) are numbered from the genome 3' end. Critical regions CRI and CR II indicate the conserved elements of the bipartite promoters. The interactions of the various RNA-binding residues of hPIV2/5 NP with the ribonucleotides at each hexamer position, numbered in their pentagonal ribose rings, are shown below. The amino acids found in each of the two RNA-binding pockets are indicated.

positively charged groove between the N-ter and C-ter domains of the nucleoprotein (Ruigrok et al. 2011). The X-ray crystal structure of PIV5 NP-RNA rings shows the same alternate orientation of these three-base stacks, and the RNA is bound to the PIV5 nucleoprotein in a similar fashion as the other nsNSV N-RNAs (Albertini et al. 2006; Green et al. 2006; Luo et al. 2007; Tawar et al. 2009; Alayyoubi et al. 2015). Ten residues of PIV5 NP make contact with the bound RNA; nine with the ribose-PO₄ backbone, and one, Q202, with the base at hex¹ (Alayyoubi et al. 2015). hPIV2 and PIV5 are closely related rubulaviruses, and their NP protein sequences, including these 10 residues, are highly conserved (Matsumoto et al. 2017). In studying the effect of individually mutating each of these residues to alanine on minigenome reporter gene expression, an interesting pattern emerged (Matsumoto et al. 2017). Mutation of those amino acids that contacted the ribose-PO₄ backbone of nucleotides whose bases pointed “in” were mostly neutral. Mutation of those residues that contacted the RNA backbone of nucleotides whose bases pointed out clearly inhibited reporter gene expression. However, mutation of Q202, in contrast, unexpectedly led to strongly enhanced reporter gene expression (>30-fold relative to NP^{wt}). This report examines the nature of the NP^{Q202A} mutation, and provides evidence that the nature of the amino

acid at this position controls whether vRdRp can, or cannot, initiate RNA synthesis in a CR II-independent manner. These experiments shed light on the mechanism by which CR II controls vRdRp initiation of RNA synthesis, and why the requirement for genome hexamer length, bipartite replication promoters and P gene mRNA editing, which are unique to paramyxoviruses among nsNSV, are linked.

RESULTS

Enhanced minigenome replication in the presence of NP^{Q202A} is due to the absence of a CR II element

NP participates in RNA synthesis in several ways. NP promoters form a chain that encapsidates the entire genome RNA, 6 nt at a time, forming helical NP-RNA nucleocapsids. Individual groups of promoters also need to be transiently displaced from the template RNA during RNA synthesis, so the template can be transcribed within the RdRp synthesis chamber. Moreover, for genome and antigenome (G/AG) synthesis, vRdRp's processivity on the template is likely coupled to the concurrent assembly of the nascent replicate RNA with NP, as nascent nucleocapsids are fully assembled (Gubbay et al. 2001).

We previously reported that mutation of NP^{Q202} to ala (NP^{Q202A}) led to strongly enhanced minigenome replication and reporter gene expression in a cell culture system (Matsumoto et al. 2017). The minigenome used in this study, however, was subsequently found to be missing the region containing the CRII element of the antigenome promoter (AG/CRII^{minus}). When minigenomes containing both wt promoters (AG^{wt}) were reexamined, NP^{wt} again very poorly promoted Rluc expression from Rluc-AG/CRII^{minus}, whereas NP^{Q202A} showed robust activity (black bars, Fig. 2). However, when the minigenomes contained both wt replication promoters (Rluc-AG^{wt}), NP^{Q202A} had no obvious phenotype; NP^{wt} performed as well as NP^{Q202A} in Rluc expression (Fig. 2). Tellingly, in both cases, Rluc expression levels were almost twice that of the AG/CRII^{minus}/NP^{Q202A} combination. The unusual phenotype of NP^{Q202A} is thus “synthetic,” as it depends on the absence of a CRII element. The more important finding was that NP^{Q202A}, in contrast to NP^{wt}, promotes Rluc expression independent of AG/CRII. The most straightforward interpretation of these results is that whereas NP^{wt} supported minigenome replication requires both elements of the bipartite promoter, NP^{Q202A} has acquired the ability to support minigenome replication in the absence of AG/CRII.

This result is reminiscent of the analogous phenotype of NP^{Q202A} when the CRII element of the genomic promoter is inactivated (G/CRII^{minus}) in the background of AG/

CRII^{minus}, i.e., when both CRII elements are absent. G/CRII^{minus} was inactivated not by its deletion (like that of AG/CRII^{minus}), but by displacing its tripartite repeats from their precise locations, either via successive 6 nt deletions, or 1 to 5 nt deletions, both within the leader region (Matsumoto et al. 2017). As found previously, these AG/CRII^{minus}/G/CRII^{minus} minigenomes displayed clear Rluc activity only when driven by NP^{Q202A} (up to 60% of that of AG/CRII^{minus} that lacks only one CRII element). Thus, NP^{Q202A} similarly allows relatively robust RNA synthesis from the “mutant” G/CRII^{minus} promoter under conditions where NP^{wt} is again essentially inactive. NP^{Q202A} has thus similarly acquired the ability to support minigenome replication independent of CRII of the genomic promoter. This property of NP^{Q202A}, of supporting RNA synthesis from CRII^{minus} promoters under conditions where NP^{wt} is inactive, can more clearly be seen with minigenomes that contain the same AG/CRII^{minus} (trailer) promoter on both genomes and antigenomes (Fig. 3). As the Rluc genes of these copyback-like minigenomes lack a gene-start signal, they are essentially templates only for genome replication.

NP^{Q202} and the initiation of genome replication

Minigenomes that lack a CRII element are essentially inactive when NP^{wt} supports their replication (Pelet et al. 1996).

FIGURE 2. Luciferase expressions during NP^{wt} or NP^{Q202A} supported replication of hPIV2 minigenomes with or without the internal element of the antigenomic promoter (AG^{CRII}). hPIV2 minigenomes with or without CRII were subjected to a minireplicon assay supported by NP^{wt} or NP^{Q202A}. The Rluc expressions from minigenomes were normalized to an internal control Rluc expression, and relative values are shown (NP^{wt} with CRII^{minus} = 1). Data represent means and standard deviations from three separate experiments.

FIGURE 3. Replication of NP^{wt} or NP^{Q202A}-supported minigenomes lacking both CRII elements. Antigenome replication levels of trailer-trailer copyback-like minigenome (A), and a minigenome whose gene start signal is deleted (B) are shown. NP-encapsidated NP-RNAs were isolated by immunoprecipitation after replication. The relative amounts of antigenome were measured by qRT-PCR and shown as relative values (NP wt = 1). Data represent means and standard deviations from three separate experiments.

Mutation of NP^{Q202} to ala, however, allows relatively robust RLuc expression from CRII^{minus} minigenomes. Thus, unlike NP^{wt}-supported minigenome replication where CRII is important for vRdRp to initiate at CRI, vRdRp in NP^{Q202A}-supported minigenome replication can apparently initiate at CRI in a CRII-independent fashion. As gln/ala²⁰² may be interacting with every base at hex¹ of their respective NP-RNAs, these different interactions might well affect NP-RNA structure. The CRI *cis*-acting sequences of resting NP^{Q202A}-RNAs may then be more accessible to vRdRp than those in NP^{wt}-RNAs, and vRdRp may no longer be as dependent on CRII interaction to facilitate the initiation of RNA synthesis.

RNA synthesis initiates at the 3' terminal uridine that is unique not only because of its free 3' OH group; it is presumably also the only base at hex¹ that is not part of a three-base stack. For RNA synthesis to initiate here, two or three 3' terminal NP protomers need to be separated from the template RNA, exposing 12–18 nt of naked RNA whose 3' end needs to end up at the active site within vRdRp. In NP^{wt}-RNAs, the presence of gln²⁰² somehow restricts initiation at CRI when vRdRp cannot also correctly interact with CRII. It is the apparent absence of gln²⁰² that relieves this restriction in CRII^{minus} minigenomes, as the replacement of gln²⁰² with several amino acids other than ala recapitulates the CRII-independent phenotype (Matsumoto et al. 2017). In this view, gln²⁰² acts negatively on initiation, and this inhibition is then neutralized by vRdRp/CRII interaction under wild-type conditions.

Carrying out CRII^{minus} minigenome replication when both NP^{wt} and NP^{Q202A} are present may be informative in examining this hypothesis. As NP^{wt}- and NP^{Q202A}-supported replication levels of wt minigenomes are very similar (open bars, Fig. 2), there is apparently little preference for either NP occupying each hexamer during NP-RNA assembly. The probability of each NP binding each hexamer will then simply depend on their relative concentrations. In this mixed NP system, we expect that the 3' terminal uridine of the NP-RNA must not be interacting with gln²⁰², as this interaction prevents the initiation of RNA synthesis in a CRII-independent manner. If this interaction, and/or similar interactions, determines whether vRdRp will initiate at CRI, then relative replication levels will vary relative to their NP concentrations in a manner that reflects this. For example, if this interaction with the 3' terminal uridine alone predominantly determines activity, when NP^{Q202A} is 1/4 of the total, replication levels will simply be 25% of that when only NP^{Q202A} is present. If two bases at hex¹ of two hexamers must be excluded from interaction with gln²⁰², relative replication levels will be (1/4)² or 6% of that when only NP^{Q202A} is present. Similarly, if three bases at hex¹ of three hexamers must be excluded, relative replication levels will be (1/4)³ (or 1.5%) of that when only NP^{Q202A} is present. The correspondence between the results of Figure 4 and these theoretical probabilities suggest that, on average, two bases at hex¹ of two hexamers must be excluded from interacting with gln²⁰² for vRdRp to initiate, according to this view. We appreciate that there are other possible interpretations of these results. Nevertheless, the finding that CRII^{minus} minigenome replication is hypersensitive to the presence of NP^{wt}/gln²⁰² is consistent with this residue acting in a complex negative manner. The ease with which the two 3' terminal NP protomers can be separated from the genome RNA during the initiation of RNA synthesis could then determine whether vRdRp can initiate in a CRII-independent manner.

DISCUSSION

NSV RdRps have a conserved architecture, with their synthesis chambers buried in the protein core that communicates

FIGURE 4. CRII^{minus} minigenome replication with mixtures of NP^{wt} and NP^{Q202A}. Various mixtures of NP^{Q202A} and NP^{wt} expressing plasmids, as indicated on the left, were used to support AG/CRII^{minus} minigenome replication, and the various Rluc expression levels were determined. The black bars show the % Rluc expression at each NP^{Q202A} vs NP^{wt} mixture relative to that of 100% Q202A. Data represent means and standard deviations from three separate experiments. The tricolored bar above each all-black data bar shows the expected relative Rluc values if one, two, or three gln202/hex1 interactions need to be excluded to permit RNA synthesis (see text).

with the outside via four channels (Reguera et al. 2016). That of vesicular stomatitis virus (VSV), the only nsNSV RdRp for which a high resolution structure is available (Liang et al. 2015), is similar and serves as a stand-in for paramyxovirus RdRp. Initiation of genome RNA synthesis requires the displacement of two or three 3' terminal Ns of the N-RNA, exposing 12–18 nt of the genome RNA. The 3' end of this genome RNA must then find its way to the active site within vRdRp; the entire pathway of the template through VSV RdRp would accommodate roughly 25 nt (Liang et al. 2015). The manner in which vRdRp carries out this complex task is unknown. We do not know whether RNA sequence affects how N is transiently separated from the template RNA during the initiation of RNA synthesis. However, a fusion construct of MeV P and N proteins can act to assemble nucleocapsid-like particles in vitro, and this assembly was strongly sequence-dependent (Milles et al. 2016). The displacement of the 3' proximal N protomers from the template RNA during the initiation of RNA synthesis might similarly be sequence-sensitive, and the hPIV2 polypyrimidine run from 3' U₄ to U₁₃ (Fig. 1) might play a role in this process.

The nucleotide bases of MeV and PIV5 N/NP-RNA structures are simply modeled as uracil, so the precise manner in which N can discriminate the various nucleotide bases is unclear. Nevertheless, there are significant interactions between nucleotide base moieties and the N/NP protein surface in both the MeV and PIV5 structures (Alayyoubi et al. 2015; Milles et al. 2016), including base-specific hydrogen bonding between Q202 and the uracil modeled at hex¹. Gln²⁰² could inhibit the initiation of RNA synthesis by strongly interacting with the *cis*-acting sequences of CRI, including the 3' uridine at hex¹, making the transient displacement of NP from the 3' terminal hexanucleotides more difficult. This inhibition would presumably be relieved by vRdRp interacting with

CRII, which could, for example, stabilize its interaction with the promoter and give vRdRp more time to get the job done. Conformational changes in vRdRp due to this interaction may also play a role (see below).

We have known for some time that vRdRp does not initiate RNA synthesis at the genome 3' end when the hexamer phase of CRII is displaced from its conserved position, but not how this selective initiation operates. These studies with NP^{Q202A} provide a clue. The requirement for genome hexamer length, bipartite replication promoters, and P gene mRNA editing are unique to paramyxoviruses. These three properties are presumably linked because the hexamer phase of the *cis*-acting mRNA editing sequence, as well as the sequence itself, controls mRNA editing and needs to be conserved (Iseni et al. 2002; Kolakofsky 2016). Hexamer phasing of *cis*-acting sequences, of course, requires hexamer length genomes, and this apparently requires a bipartite promoter. These bipartite promoters ensure that genomes with nonhexamer length insertions or deletions are poorly replicated, because such changes in genome length automatically alter the hexamer phase of the CRII tripartite repeat. Hexamer genome length and bipartite promoters are thus essential to maintain genome sequence phasing. Paramyxovirus bipartite promoters would operate by having a negative element acting at CRI (gln²⁰²) that needs to be neutralized by a positive element acting at CRII (vRdRp/tripartite repeat interaction), and this positive element is known to be sensitive to hexamer phase. The critical hexamer phase of the mRNA editing sequence, 2486 nt removed from the genome 3' end, is thus conserved by that of the tripartite repeat at CRII.

The need to conserve the hexamer phase of the P gene mRNA editing sequence apparently requires bipartite replication promoters and hexamer length genomes. However, none of the above explains why the NP^{Q202A} mutation is apparently so lethal that viruses harboring it have not been recovered from cDNA. It is hard to imagine how this lethality would result simply from allowing initiation of RNA synthesis in a CRII-independent manner, given how vigorously NP^{Q202A} drives minigenome replication. In our present understanding of paramyxovirus RNA synthesis, all synthesis begins at the genome 3' end, and each RdRp is then committed to the mutually exclusive tasks of mRNA synthesis or genome replication. This choice is thought to depend on whether leader RNA synthesis terminates and a capped mRNA is initiated at the first gene-start site, or whether leader RNA begins being assembled with NP before it terminates, and this RdRp is then locked into genome replication. Given these two very different forms of viral RNA synthesis, which presumably involve alternate RdRp conformations, each RdRp must remain committed to each process until that process has been completed. It is tempting to speculate that CRII plays a role in addition to that of a simple promoter element. In the absence of RdRp/CRII interaction during the initiation of 3' end RNA synthesis, the ability of RdRp to subsequently adopt the conformations of committed transcriptases and

replicases may be compromised. The Q202A mutation may thus cause the polymerase to be so error prone that infectious virus cannot be recovered.

MATERIALS AND METHODS

Cells

BSR T7/5 cells that constitutively expressed T7 RNA polymerases (Buchholz et al. 1999) were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum.

Plasmid construction

Rluc expressing hPIV2 minigenome (hPIV2-Rluc) composed of leader, 5'UTR of NP mRNA, 3'UTR of L mRNA, trailer and Rluc gene, which has been used as "normal" hPIV2 minigenome (Matsumoto et al. 2016, 2017), was represented as AG/CRII^{minus} in this study (hPIV2-Rluc AG/CRII^{minus}), because AG^{CRII} that is intrinsically included in the coding region of L had been deleted. The hPIV2-Rluc with AG^{CRII} (hPIV2 Rluc AG^{wt}) expressing plasmid was constructed by adding CRII to hPIV2-Rluc AG/CRII^{minus} by using a standard PCR mutagenesis method. Plasmids expressing trailer-trailer copyback-like minigenome and gene start signal deleted minigenome were constructed by modifying hPIV2-Rluc AG/CRII^{minus} by using a standard PCR mutagenesis method. All minigenomes were expressed as the negative sense RNA under the control of a T7 RNA polymerase promoter. hPIV2 NP^{wt} and NP^{Q202A}, P, L and firefly luciferase (Fluc) genes cloned into pTM1 vector, which contains a T7 promoter and an encephalomyocarditis virus internal ribosome entry site, were as described previously (Matsumoto et al. 2017).

hPIV2 minireplicon assay

The hPIV2 Rluc minireplicon 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 plate. Plasmids; hPIV2-Rluc (0.5 µg), pTM1-L (0.375 µg), -P (0.2 µg), and -NP (0.375 µg) or empty vector, and Fluc (0.1 µg) were transfected by using XtremeGENE HP (Roche). At 24 h post-transfection, the Rluc and Fluc activities were measured by using the Dual Luciferase Assay Kit (Promega) according to the manufacturer's instruction. All obtained results of Rluc were normalized by the expression levels of Fluc.

Analysis of antigenome replication during hPIV2 Rluc minireplicon assay

Quantification of antigenome was performed by quantitative real-time RT-PCR (qRT-PCR) using RNA sample immunoprecipitated by anti-NP mAb. At 48 h post-transfection, cells were lysed in lysis buffer (20 mM Tris-Cl [pH 8.0], 150 mM NaCl, 10% Glycerol and 1% Triton X-100) containing cOmplete Protease Inhibitor (Roche). The supernatants obtained by centrifugation were incubated with a mAb against hPIV2 NP (159-1) (Nishio et al. 1999) and protein A-Sepharose. The immunoprecipitated sample was subjected to RNA extraction using Isogen (Nippon Gene). To increase the efficiency of RNA extraction, Dr. GenTLE Precipitation Carrier (Takara) was used

according to the manufacturer's instruction. The cDNA synthesis was carried out by the PrimeScript RT Reagent Kit (Takara) with specific primer for antigenome RNA (5'-ACCAAGGGGAAATC AATATGTT-3'). For trailer-trailer copyback-like antigenome, an alternative primer (5'-CATTGTTGTTATTGTTTCATTTT-3') was used. qRT-PCR was performed by using SsoAdvanced Universal SYBR Green Supermix (Bio-Rad) using primers for Rluc gene (F: ATAAGTGGTCCGAGTGGTG, R: TAAGAAGAG GCCGCGTTACC).

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