Novel role of the central unstructured Paramyxovirus nucleopcapsid protein tail domain

Vidhi Deepak Thakkar

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The paramyxovirus replication machinery comprises the viral large (L) and phospho-(P) proteins in addition to the nucleocapsid (N) protein that encapsidates the single stranded RNA genome. Common to paramyxovirus N proteins is a C-terminal tail (Ntail), which contributes to docking of the polymerase complex to the genome through defined interaction domains. The central Ntail section is structurally disordered and thought to provide spatial flexibility required for productive interaction of the polymerase with the encapsidated viral genome, but its mechanistic role and relevance for successful virus replication is untested. Focusing initially on members of the morbillivirus genus, a series of Measles virus (MeV) and Canine distemper virus (CDV) N proteins were generated with internal deletions in the unstructured tail section. N proteins with
large tail truncations remained bioactive in mono- and polycistronic minireplicon assays and supported efficient replication of recombinant viruses. Bioactivity of Ntail mutants extended to N proteins derived from highly pathogenic Nipah virus (NiV), a member of the henipavirus genus. To probe an effect of Ntail truncations on viral pathogenesis, recombinant CDVs were analyzed in a lethal CDV/ferret model of morbillivirus disease. The recombinant viruses displayed different stages of attenuation ranging from ameliorated clinical symptoms to complete survival of infected animals, depending on the molecular nature of the Ntail truncation. Reinfection of surviving animals with pathogenic CDV revealed robust protection against a lethal challenge. The highly attenuated was genetically stable after extensive ex vivo passaging and recovery from infected animals. Mechanistically, gradual viral attenuation coincided with stepwise-altered viral transcriptase activity in infected cells. These results identify the central Ntail section as a determinant for viral pathogenesis and establish a novel platform to engineer gradual virus attenuation for next-generation paramyxovirus vaccine design.

INDEX WORDS: Paramyxoviruses, Nucleoprotein, Nucleocapsid, MeV, CDV
NOVEL ROLE OF THE CENTRAL UNSTRUCTURED PARAMYXOVIRUS NUCLEOCAPSID PROTEIN TAIL DOMAIN

by

VIDHI DEEPAK THAKKAR

A Dissertation Submitted in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy in the College of Arts and Sciences Georgia State University

2018
NOVEL ROLE OF THE CENTRAL UNSTRUCTURED PARAMYXOVIRUS NUCLEOCAPSID PROTEIN TAIL DOMAIN

by

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DEDICATION

This dissertation is entirely dedicated to my parents and my little brother as they have been the strongest pillars throughout my journey as a Ph.D student. They have motivated me, supported me and have made innumerable sacrifices to let me grab some of the best opportunities in this country. I will be eternally grateful to them for everything that I have in my life and shall always remain indebted to them. I would especially like to thank my dearest brother, Kirtan, for being my pillow, my punching bag and my go-to guy – I have always shared my anxieties and problems with him. He has been a great source of inspiration in my life and has made things seem easier when they were difficult. My brother keeps teaching me a lot of things and the most important thing that will stay with me forever is that “Success and Smiles go a long long way”. Although my family has been miles apart from me in India, I have always received their love, support and encouragement all the way.

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<th>Term</th>
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<tr>
<td>Acute disseminated encephalomyelitis</td>
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<td>Alveolar macrophages</td>
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<td>Bovine disease virus</td>
<td>BDV</td>
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<td>Canine distemper virus</td>
<td>CDV</td>
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<tr>
<td>Dendritic cells</td>
<td>DC</td>
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<td>Human respiratory syncytial virus</td>
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<td>Measles virus</td>
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<tr>
<td>Measles inclusion body encephalitis</td>
<td>MIBE</td>
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<tr>
<td>Molecular recognition element</td>
<td>MORE</td>
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<td>Mumps virus</td>
<td>MuV</td>
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<td>Nipah virus</td>
<td>NiV</td>
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<tr>
<td>Nucleoprotein</td>
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<td>Phosphoprotein</td>
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<tr>
<td>Polymerase</td>
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<tr>
<td>Ribonucleoprotein complex</td>
<td>RNP</td>
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<td>RNA-dependent-RNA polymerase</td>
<td>RdRp</td>
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<tr>
<td>RNA polymerase</td>
<td>RNAP</td>
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<tr>
<td>Signaling lymphocyte activation molecule</td>
<td>SLAM</td>
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<tr>
<td>Subacute sclerosing panencephalitis</td>
<td>SSPE</td>
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<td>Vesicular stomatitis virus</td>
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1 INTRODUCTION

1.1 Paramyxoviridae family

Mononegavirales (non-segmented, negative sense RNA viruses) comprises eight major families, which include human and animal pathogens: 1) Bornaviridae (for example- Borna disease virus (BDV)), 2) Filoviridae (for example-Ebola virus, Marburg virus), 3) Paramyxoviridae (for example- Measles virus (MeV), Canine distemper virus (CDV), Mumps virus (MuV), Henipaviruses (Hendra virus (HeV) and Nipah virus (NiV)), 4) Pneumoviridae (for example- human respiratory syncytial virus (RSV)), 5) Rhabdoviridae (for example- rabies virus, vesicular stomatitis virus (VSV)), 6) Mymonaviridae (Sclerotimonaviruses), 7) Nyamiviridae (for example- Nyavirus), 8) Sunviridae (Sunshine virus). Paramyxoviridae family is further divided into different genera including Rubulaviruses (for example- MuV), Respiroviruses, Henipaviruses (for example- NiV), Morbilliviruses (for example- MeV and CDV), and Avulaviruses, Aquaparamyxoviruses and Ferlaviruses (1)

Members of the Paramyxoviridae family are mostly pathogenic, contagious and infect a variety of hosts through the respiratory route. Paramyxoviridae virions are generally pleomorphic in shape and 150-350nm in diameter. Paramyxoviruses infect cells through fusion with the plasma membrane. They induce cell-cell fusion, thereby creating giant multinucleated cells (syncytia) (2)

Paramyxoviridae genomes are similar in gene order and intergenic junctions separate the genes. Some differences in these genomes are their different attachment proteins. Respiroviruses and Rubulaviruses have hemagglutinin-neuraminidase [HN] attachment protein, while
Morbilliviruses have hemagglutinin [H] and Henipaviruses have G as their attachment protein in different genera of Paramyxoviridae subfamily. The envelope of the MeV virion contains two glycoproteins: F or the fusion protein, which promotes fusion of the viral and host cell membranes, and H—which is the viral attachment protein. The matrix protein (M protein) forms an electron-dense layer underlying the viral bilayer. M regulates MeV RNA synthesis and particle assembly by interacting with the nucleoprotein [N] as well as the lumenal tails of H and F (2-4).

All members of paramyxoviridae share a common mechanism of RNA synthesis. Viral RNA synthesis occurs in the cytoplasm and involves the N, phosphoprotein (P) and large protein (L). The viral polymerase (RNA-dependent RNA-polymerase/RdRp) is a hetero-oligomer composed of the L and P proteins. L performs all of the enzymatic activities while P acts as a co-factor. P chaperones newly synthesized N protein (N0) to the RNA template during replication (5-7), binds L, and also interacts with the nucleocapsid/ribonucleoprotein (RNP) template to mediate transcription and replication, respectively (8).

Polymerization initiation can be performed in different ways for different single stranded RNA viruses. There are two kinds of different mechanisms: de novo and primer-dependent initiation. In addition to MeV, VSV and RSV demonstrates de novo synthesis. In case of VSV and RSV, the polymerase preloads with the first two and three nucleotides respectively (9, 10). Some positive sense RNA viruses use a primer-dependent initiation mechanism, while a cap snatching mechanism is used by Influenza. The endonuclease cleaves capped RNAs to generate the primers that initiate viral RNA transcription (11), but in case of replication it uses a de novo
mechanism (12). The switch mechanism between transcription and replication is not fully understood. For example, the RSV polymerase can initiate polymerization at either +1 or +3 position (13). It has also been proved that the regulation of transcription and replication also depends on host proteins as shown with the leader region of MeV polymerase (14).

MeV P encodes two additional nonstructural proteins- the C and V proteins. Alternative translation initiation or RNA editing, respectively, expresses these proteins. By inhibiting the interferon signaling, C and V interfere with the innate host immune response (3, 4). The C protein interferes with IFN induction via its regulatory role in viral RNA synthesis. Viral RNAs accumulate in cells infected with MeV-(ΔC) virus, possibly stimulating host innate immune responses (15, 16). V protein is formed by the insertion of a non-templated guanine nucleotide at a precise location, called an “editing site,” which generates an mRNA which differs from that of P with an altered ORF downstream of the editing site. Thus, due to this specific mechanism, the N-terminal domain of P and V are identical, whereas their C-terminal domains are unique (17). The V protein blocks the JAK–STAT signalling pathway by interacting with STAT1 and STAT2 (18).

Some members of the Paramyxoviridae family are responsible for pediatric morbidity and mortality. Vaccines have been successful in providing protection from some paramyxoviruses, such as MeV and MuV. MeV is one of the most readily transmitted communicable diseases infecting children. There is an effective Measles-Mumps-Rubella vaccine recommended by the World Health Organization (WHO). WHO recommends two doses of the MMR vaccine- the primary dose is administered to children within 9-15 months of age and the booster dose is given between 2-5 years of age. Exposure to either wild type or attenuated
MeV in form of the MMR vaccine delivers a lifetime protection mediated by antibodies delivered from memory B cells (19, 20). However, natural boosting is required to maintain protection since antibodies induced by the vaccine wane over time.

The highly efficacious MMR vaccine has currently failed to eliminate MeV worldwide. A herd immunity of 95% is required in order to prevent MeV endemic outbreaks (21) because MeV typically reemerges first when vaccination coverage in a population drops (22). On the other hand, a fraudulent article reports that the MMR vaccine is linked to the development of autism in 1998 and this has led to many anti-vaccination campaigns (23). Although the research has been proven flawed (24, 25), many parents are still scared to vaccinate their child.

Unfortunately, no vaccine exists for the newly emerging paramyxoviruses like NiV (26), despite major research efforts. The Hendra vaccine has been shown to be cross-protective against NiV infection in the non-human primate model (27). A NiV-specific monoclonal antibody has been shown to be protective in non-human primates (28). The efficacy of some vaccines (such as CDV vaccine) still remains questionable (29). Thus, there is an urgent need for the development of new and highly efficient strategies to prevent infections and provide potent therapeutic treatment to treat many outbreaks that still occur.

1.2 Morbillivirus genus

1.2.1 Measles virus (MeV)

For many Morbilliviruses, the tropism and tissue distribution is found to be similar. There are three main receptors used by MeV for attachment and cell entry. The first is CD46, a
ubiquitously expressed receptor on all nucleated cells. Its physiological function is to protect the cells from attack of invading pathogens by regulating complement activation and also regulates innate and acquired immune responses. The vaccine and laboratory-adapted MeV strains use CD46 for attachment and entry (30, 31). The second is the signaling lymphocyte activation molecule (SLAM/CD150), expressed on immune cells like the activated T and B cells, macrophages and mature dendritic cells (DC’s). SLAM functions by interacting with another SLAM molecule on the adjacent cell and undergoes its tyrosine phosphorylation to produce T-helper cytokines: interleukin (IL)-4 and IL-13 (32, 33). The functions and distribution of SLAM receptor proves the lymphotropic and immunosuppressive nature of MeV. The third receptor is Nectin-4/PVRL4, which is expressed on epithelial cells located on the basolateral side of the airway epithelium (34, 35). Other receptors that have been identified are DC-specific ICAM-3 grabbing non-integrin (DC-SIGN) and neuronal cell-specific Neurokinin-1 (NK-1) (36).

MeV is transmitted via the respiratory route and infects resident DC’s and alveolar macrophages (AM’s) in the lungs. Infected DCs and AM’s transport MeV to the draining lymph nodes where they infect the lymphocytes via the SLAM receptor. A transient fever at 3-6 days post MeV infection coincides with primary viremia. The virus is largely lymphotropic and spreads through lymphoid organs and tissues such as spleen or thymus as well as the liver, skin, tonsils and respiratory mucosal surfaces. This causes the secondary viremia (37). High fever coincides with viremia and other symptoms such as cough, coryza, Kopliks’s spots occur 10-12 days post infection. The trademark maculopapular rash occurs later at around 14-15 days post infection. This occurs because of the infiltration of cytokines into sites of MeV replication (38).
Nectin-4/PVRL4 receptor is involved in MeV spread through virus infected immune cells to the basolateral side of the airway epithelium. This finally results in virus shedding (39). A large number of infected cells present in the respiratory tract cause the symptoms such as coughing and sneezing and sometimes pneumonia. This allows the virus to undergo host-to-host transmission, which contributes to MeV outbreaks (40).

MeV causes suppression of the adaptive immune response. Some studies show decreased IL-12 and increased IL-4, IL-10 production that changes Th1 response to a prolonged Th2 response. The infection also causes T cell non-responsiveness due to virus-induced immaturity of infected DC’s (41-45). MeV infection also suppresses the proliferation of lymphocytes (46). This unresponsiveness can be caused by contact of lymphocytes with the viral glycoprotein on MeV virions or MeV-infected cells, independently of virus replication in lymphocytes. It may also be caused due to the H protein interaction with SLAM on lymphocytes (47). At the same time, MeV induces strong immune responses that confer lifelong immunity (48). This contradiction is known as the ‘measles paradox’. The lymphocyte numbers generally return to normal within a week after the disappearance of MeV clinical symptoms, while the immune suppression extends for several weeks to months. It is therefore believed that MeV immune suppression mainly results from depletion of immune cell subsets, but this is in turn masked by the rapid proliferation of MV-specific lymphocytes (49).

Additionally, MeV-induced autoimmune demyelination of neurons in the brain, known as acute disseminated encephalomyelitis (ADEM), can occur within 2 weeks after the onset of infection. Progressive measles inclusion body encephalitis (MIBE) can arise after the virus has
cleared from the host (50, 51). Another fatal disease of the central nervous system (CNS) is the subacute sclerosing panencephalitis (SSPE). SSPE can occur several years later after infection and is caused by demyelination and presence of MeV in neuronal cells of the brain (52). As of today, it is unclear how MeV infects neurons within the CNS (53) and circulating virus specific antibodies alone fail to clear the virus.

1.2.2 Canine distemper virus (CDV)

CDV, another member of Morbillivirus genus, is a common cause of morbidity and mortality in unvaccinated domestic or feral dogs. It also affects other carnivores such as cats, lions, tigers, bears and is also very common in some endangered species and other aquatic and terrestrial carnivores (54-60).

The use of SLAM receptor is common to the Morbillivirus genus. CDV uses dog SLAM as its principal receptor for viral entry. Like MeV, CDV also spreads by aerosol and causes primary and secondary viremia. CDV infection is also mainly lymphotropic. It causes clinical symptoms such as fever, rash, conjunctivitis, pneumonitis, and neurological diseases. In the case of CDV infection, acute encephalomyelitis is often observed. Also, symptoms like hyperceratosis of footpads and epithelium of the nasal tract are very often observed. CDV shows the same disease profile in ferrets as MeV causes in humans. Although, the live-attenuated CDV vaccines are fully apathogenic in dogs, they can cause severe clinical distemper in sensitive species such as black-footed ferrets or other carnivores (29).
Animal models:

CDV disease is very severe and leads to 100% mortality in ferrets after intranasal infection. Therefore, the lethal CDV-ferret animal model (29) is the most commonly used small-animal model system for the members of the Morbillivirus genus. It is used as the surrogate model as there is no other suitable animal model for MeV except the macaques, which are inaccessible, expensive and do not show all the classic symptoms except mild rash and transient lymphopenia (61, 62). Rodent models like mice expressing transgenic CD46 (63, 64) or cotton rats (65) do not replicate the exact MeV pathogenesis and virulence.

1.3 Henipavirus genus

1.3.1 Nipah virus (NiV)

Henipaviruses have a broad species tropism including flying fox species, fruit bat species which are the reservoirs for the viruses in this genus (66). NiV is a highly lethal zoonotic paramyxovirus. The natural infection occurs in pigs and is amplified in them. The first outbreak occurred in peninsular Malaysia in 1998. NiV first infected pig farmers working in contact with pigs and this was the primary cause of the outbreak. Numerous cases of encephalitis among pig farmers were reported. The pigs had been infected through consumption of contaminated fruit from infected fruit bats (*Pteropus spp.*). Pig-human transmission occurred accidentally. Infection in humans is found to be severe (67-71) Subsequent deadlier outbreaks occurred in Bangladesh and India. Human-to-human transmission in home or hospital settings has also been documented (72, 73) High case fatality rate by NiV infection emphasizes the urgent need for prophylactic or therapeutic medical treatments.
Ephrin-B2 or B3 is known to be the functional receptor for Henipavirus genus and is found on arteries, arterioles and capillaries in various organs. The viruses attach to the receptor and infect host cells using the attachment G and the fusion F proteins (74, 75). The common symptoms include fever, headache, drowsiness, hypertension, and encephalitis. Atypical pneumonia and acute severe respiratory syndrome is also common (17). No vaccines are available to treat NiV infection and therefore, it is very important to develop a safe and efficacious vaccine (76).

1.4 Replication machinery of Paramyxoviridae members

Negative-sense viruses (NSV) contain RNP complexes, which are formed when the genomic as well as the antigenomic RNA are encapsidated by the viral N protein. Paramyxoviruses forms a characteristic herringbone structure in EM images. RNP’s act as the template for both transcription and replication. In addition, P and L proteins are also involved in both transcription and replication, but the two processes are different (77).

![Figure 1: Schematic representation of MeV phosphoprotein (not drawn to scale)](image)

having a N-terminal (PNT) and a C-terminal (PCT) including a tetramerization domain (PMD) and a C-terminal RNP binding domain (P-XD).

MeV P protein consists of 400-600 amino acids and its physiological oligomer is the tetramer. It is very highly phosphorylated protein. P is the polymerase cofactor and links the L polymerase to the RNP template. It comprises of L (polymerase) binding site and N binding site.
The MeV P protein is comprised of an N-terminal (PNT) domain (1-230aa) and a C-terminal (PCT) domain (231-507aa) as shown in figure 1. PNT chaperones newly synthesized N protein (N⁰) to the RNA template during replication. PNT also holds N monomers in an open conformation and prevents premature oligomerization (7). PCT includes a tetramerization domain (PMD) and a C-terminal RNP binding domain (P-XD) that mediates high-affinity interactions with the N protein (78-80).

MeV L protein is a 2200 AA (220 to 250 kDa) protein and its structural organization and location of individual enzymatic activities are very well understood. The L protein contains all enzymatic (capping, methylation, adenylation, RNA binding) activities associated with mRNA synthesis and genome replication. The L protein is comprised of six domains- domain II considered to be associated with RNA binding, domain III harbors the predicted catalytic center for phosphodiester bond formation and domains V and VI postulated to mediate capping functions (81-83).

Viral replication of Paramyxoviridae takes place in the host cell cytoplasm after viral entry and uncoating of the genome into the cell. For both transcription and replication, the viral polymerase complex initiates polymerization at the 3’ end of the RNP (84). N wraps around 6 nucleotides of RNA and follows the rule of six. These genomes are of negative polarity and thus the viral RdRp transcribes into mRNA first, which gets capped and polyadenylated by the L protein. 3’ and a 5’ noncoding leader and trailer regions, respectively, flank the coding regions of the non-segmented genome. The viral polymerase promoter is present in the noncoding regions and gene start/stop signals are in the intergenic junctions that separate each of the individual
genes. For both transcription and replication, the viral polymerase complex exclusively initiates polymerization at the 3’ end of the N encapsidated RNA (84). In the case of transcription, the MeV RdRp complex scans and polymerizes along the template until it encounters the polyadenylation signal to produce monocistronic N mRNA at position 55. The complex then re-initiates to transcribe the next gene. Some premature falling of the polymerase complex with increase in template length occurs and creates a gradient of gene expression in which the genes that are closer to the promoter are more abundant. The likelihood of premature detachment increases with template length, resulting in lower mRNA levels of downstream genes, which creates the transcription gradient. When enough N protein is produced to encapsidate the nascent RNA, the RdRp complex switches from transcription to replication. In replication mode, the MeV RdRp complex ignores the intergenic junctions in between genes. It produces a complete plus polarity copy of the genome (antigenome), which is concurrently encapsidated by N (8). The polymerase initiates antigenome production from the leader region. The RdRp complex does not recognize any non-encapsidated RNA (85).

1.5 Nucleoprotein

N proteins from Paramyxoviridae members are approximately 500 or more amino acids in length. Their function is to encapsidate the viral genomic RNA to form RNP. N does not bind to any cellular RNA or viral mRNAs. Unassembled N interacts with P called the N⁰-P complex that involves the interaction between Ncore and PNT. This complex helps in guiding N to newly synthesized genomic RNA prevents N from nonspecifically binding cellular RNA. However, when used in heterologous systems, and in the absence of other viral proteins, N will bind cellular RNAs and form structures that are similar to authentic viral RNP (86, 87).
Figure 2: Cryo-EM structure of the MeV N\textsubscript{core} RNA nucleocapsid at near-atomic resolution (From: Irina Gutsche et al. Science 2015;348:704-707)

(A) Schematic of MeV N (navy blue, NTD arm; blue, NTD; salmon, CTD; yellow, CTD arm). The same color code (with RNA in green) is used for the rest of the figure. (B and C) Isosurface representation of the cryo-EM 3D reconstruction of the helical nucleocapsid: (B) front view, (C) cutaway view.

Recently, a 4.3Å resolution structure of the MeV helical nucleocapsid was solved using cryo-electron microscopy. This structure clearly defined the interactions between MeV N and the encapsidated RNA. The RNA thread winds around the MeV nucleocapsid: inside the cavity between the N-terminal domain (NTD) of the N-core and the C-terminal domain (CTD) of the N-core as shown in figure 2. A hinge region between NTD and CTD may allow for an open-closed transition during RNP formation (88).
Figure 3: Schematic representation of Paramyxoviridae nucleoproteins (not drawn to scale) having a conserved N-core and a flexible N-tail.

All paramyxoviridae N proteins have a conserved amino terminal domain, known as N-core, and a flexible and poorly conserved, intrinsically disordered and is highly flexible C-terminal domain, known as N-tail (as shown in figure 3). MeV-N features a 400-residue amino-terminal RNA-binding core (N-core) and a 125-residue carboxy-terminal tail domain (N-tail). The presence or absence of the N-tail affects the overall spatial organization of the RNP. It has been demonstrated that complete removal of the N-tail by trypsin digestion leads to complete loss of RdRp bioactivity. This is due to the decrease in the MeV N:RNA diameter and pitch that causes extreme rigidity (89). Decrease in flexibility due to the decrease in the diameter may be affecting the RdRp processivity and this may lead to the loss of RdRp bioactivity.

Some studies show that the N-tail is essential for RdRp-mediated transcription and replication (90-93). There are conserved microdomains in N-tail (box 1-3) that are important for MeV replication. Box 1 (401-420a.a) binds to an uncharacterized nucleoprotein receptor (NR)
Box 2 (488-499a.a), also known as the molecular recognition element i.e. MoRE interacts with the X-domain (XD) in the C-terminal of the P protein (91). This interaction is thought to stabilize the RNP template which is very important for allowing the polymerase complex to progress along the template for transcription and replication of the virus (80). It is a reversible phenomenon since the interaction has to be made and broken to allow the polymerase to progress during transcription and replication unless P remains static and L moves (95). Box 3 (517-525a.a) stabilizes the MoRE-XD interaction (80, 96, 97). Furthermore, box 3 is thought to interact with the M protein (3) and with the heat shock proteins to facilitate efficient genome replication and incorporation of the genome into nascent particles (3, 98, 99).

In a recent study, it was demonstrated that the MeV nucleocapsid protein tail domain is dispensable for polymerase activity. The truncated N-tail mutant (N-Δ86) restored the polymerase complex activity in a minigenome system. In order to test the bioactivity of the central section of the N-tail region, a plasmid mutant was designed and the conserved N-tail boxes were kept intact. This MeV N-tail mutant showed higher polymerase activity as compared to its standard but the recombinant mutant virus was not replication competent. By qPCR analysis, it was also shown that the presence of fully N-tail truncated mutant affected the stability of RdRp binding to RNP’s and that the likelihood of abortive separation of RdRp from the RNP template was increased in the absence of the N-tail MoRE domain. Thus, it was concluded that the MoRE:P-XD interaction is required to prevent catastrophic premature polymerase termination (80).

In another study, the placement of the MoRE near the MeV N-tail terminus was shown
not to be important for virus replication and that MORE could be placed in the MeV conserved N-core. It was also confirmed that MORE is not important for loading of RdRp onto and/or RdRp progression along the RNP template (100). A regulatory role of the central section seems to exist and to define that was the main objective of the following study.

![Figure 4: Schematic representation of MORE-XD interaction in the presence of Morbilliviral N-standard and a central section truncated mutant.](image)

The main hypothesis of my Ph.D work was that the central section of the morbilliviral N-tail is not important for virus replication and would be responsible for a specific polymerase regulatory role. This is shown in the figure 4, which shows that by truncating the central section of the N-tail, the morbilliviral replication would not be affected.

In the subsequent chapters, a detailed overview of a minireplicon/ bioluminescent reporter assay systems is given as they are very commonly used to study gene expression as well as other cellular components and events that are involved in gene regulation.

**Advantages:** Such virus-free reporter assay systems have also been widely used for screening antiviral drugs. Many compounds have been tested for potential antiviral activity using reporter
assay systems (89). Various luciferase reporters are used for a high-throughput screening (HTS) protocol design that can identify paramyxovirus-specific, orthomyxovirus-specific, or broadly specific compounds in a single assay system (101). Furthermore, prior to rescuing a recombinant virus from its full-length cDNA, a minireplicon reporter system is used to verify the validity of the rescue protocol experimental process (102).

1.6 Monocistronic Minireplicon / Bioluminescent assays

Minireplicon/Bioluminescent assays depend on enzymes called luciferases. Luciferases or luciferase enzymes are classified as oxidative enzymes found in several species that enable the organisms that express them to eradicate bioluminescence. These enzymes emit a photon and in turn allow the oxidation of luciferins to form oxyluciferin. There are many structurally diverse luciferin substrates. Many bioluminescence-producing organisms are isolated in order to utilize them for this assay, including luciferases from fireflies and various marine organisms such as the sea pansy that belong to the families Lampyridae and Renillidae respectively (103, 104).

![Figure 5: Schematic representation of a bioluminescent assay system driven by T7 promoter and T7 polymerase](image-url)
1.6.1 Minigenome construct

Minigenomes are smaller in length and easier to manipulate. They usually only carry a single reporter gene open reading frame flanked by the leader and trailer as shown in figure 5 (105). Thus, the minigenome system is constructed to mimic viral gene expression in the same way.

Promoters are found upstream of target genes for specific polymerases. The sequence of the promoter controls the binding of the RNA polymerase and transcription factors; therefore these regions determine where and when the gene of interest will be expressed (106). The leader and the trailer contain the gene start and the gene stop signals. After transfection of the plasmid DNA into recipient cells, the plasmid DNAs are transcribed by the corresponding RNA polymerase to generate a negative-sense minigenome RNA, which is then encapsidated in the presence of the N. Next, with the help of P and L proteins, an encapsidated sense anti-minigenome RNA is produced using the negative-sense encapsidated minigenomic RNA as a template. The positive strand is in turn used as template to generate the negative strand as a key step in viral replication. The encapsidated negative RNA strand is transcribed into messenger RNA (mRNA) lacking the complete 5- and 3- untranslated regions. Finally, the transcribed mRNA is translated by host-cell machinery to produce the reporter proteins (84).

In a bioluminescent assay, cells are transfected with plasmids encoding for helper plasmids and the respective luciferase replicon reporter. Luciferase activities are determined 40 hours post-transfection using a luciferase substrate. Substrates are directly added to the cells and relative activities are quantified. The reporter protein's activity or fluorescence within a
transfected cell population is proportional to the steady-state mRNA level and thus gene expression.

1.6.2 Transcription in the cytoplasm: T7 systems

T7 polymerase has been used for recovery of negative-sense RNA viruses. T7-system undergoes gene expression and requires entry in cytoplasm. There have been many advantages of T7-based system: various cell lines (such as Human embryonic kidney (HEK)-293T, Baby Hamster Kidney (BHK)-T7) can be used that provide exogenous T7 (37). Such permissive cell lines can be also used for virus recovery but the choice of cell lines in important based on viral cell tropism. Earlier, cytoplasmic T7 was provided by using recombinant vaccinia virus (78) but many of its cytopathic effects were found to hamper the T7-dependent system (79). Furthermore, transiently transfected (89) or stable transfection systems (103) have also been used to prevent such cytopathic effects.

1.6.3 Transcription in the nucleus: pol I and pol II systems

Three RNA polymerases are present in eukaryotes: 1) RNA polymerase (pol) I synthesizes the rRNA, without generating 5’caps and 3’tails. 2) pol II synthesizes mRNA and 3) pol III synthesizes tRNA. Eukaryotic RNA polymerases- pol I and pol III assists cellular transcription that accounts to 80% of total RNA synthesis. Transcription by pol I to synthesize rRNA is localized to discrete sites called nucleoli (107).
Transcription of mRNA in mammalian cells via CMV promoter as a viral promoter or other eukaryotic promoters is dependent on cellular RNA polymerase II. Thus, expression of foreign genes by such vectors requires the entry of vectors into the nucleus (108).

The pol I-based system has gained attraction over the T7- based system in the development of reporter assays for segmented RNA viruses like influenza. It was first developed as a solution to generate this complex recombinant virus as RNA pol-I assists nuclear transcription (109, 110). Pol-I system has many advantages such as: 1) does not require exogenous T7 polymerase 2) does not need ribozyme cleavage (106).

1.7 Bicistronic and Tricistronic Reporter Assays

Multicistronic reporter assays can test the negotiation of intergenic junctions by the RdRp complex in negative sense non-segmented viral genomes. By using such multicistronic constructs for reporter based assays systems, we could determine if the transcription gradient is compromised in the subsequent genes. As monocistronic assay system does not consider the RdRP travel through the intergenic junctions, we were able to identify a system that could be used for testing the polymerase activity and the successful negotiation of intergenic junctions by the RdRp complex in negative sense non-segmented viral genomes.
Figure 6: Schematics of the bi- and tricistronic minigenome plasmids generated (firefly luciferase; nanoLuc, nanoluciferase; IGS- intergenic segment; Le- leader sequence; Tr, -trailer sequence; P_stopstop, MeV P protein–encoding ORF harboring a tandem stop codon after the 21st triplet) (100)

Firstly, the MeV bicistronic replicon was first cloned using monocistronic MeV replicon (111) as the template. Recombineering PCR technique was performed to generate a bicistronic construct cassette that consists of one intergenic junction between two reporters. Secondly, the bicstronic construct was used as a backbone to generate a tricistronic construct. This construct had two intergenic junctions placed in between three ORF’s as shown in figure 6.

**Limitations:** Despite the wide use of the reporter system, there are limitations to this approach. The viral infectious cycle environment is very different from the intracellular environment for the plasmid-based expression of helper plasmids. Many types of plasmids need to be transfected that can limit the efficiency of transfection and target gene expression and the ratio of plasmids need to be adjusted. Furthermore, a viral minigenome is shorter than its full-length genome. These limitations should be considered for designing a reporter assay system.
2 MATERIALS AND METHODS


2.1 Cell Culture

Human embryonic kidney (HEK293, CRL-3216; ATCC), Baby hamster kidney cells (C-13; ATCC) stably expressing T7 polymerase (BSR-T7/5, (112)) and African green monkey kidney epithelial cells (CCK-81; ATCC) stably expressing human or canine signaling lymphocytic activation molecule (Vero-hSLAM and Vero-cSLAM, respectively (113)) were maintained at 37°C and 5% CO2 in Dulbecco’s modified Eagle’s medium supplemented with 7.5% fetal bovine serum. All stable cell lines were incubated in the presence of G-418 (100 µg/ml) at every fifth passage. GeneJuice (Novagen) reagent was used for all transient transfections of cells.

2.2 Molecular biology

Plasmids encoding expression constructs of MeV strain Edmonston N, P, and L (103), MeV strain IC-B N, P, and L (62, 80), CDV strain Onderstepoort N, P, and L (29), and NiV N, P, and L (114) were previously described. Likewise, plasmids harboring full length cDNA copies of the MeV strain IC-B genome (62), CDV strain 5804PeH genome (115), the different MeV minireplicons (100), and shuttle vectors harboring MeV strain IC-B and CDV strain 5804PeH derived N ORFs were previously reported (80). A cloning strategy developed in our earlier work (80) was applied to generate all MeV, CDV, or NiV-encoding N genes with internal Ntail
truncations. Briefly, sets of PCR primers were engineered that flanked the specific nucleotides targeted for deletion and contained terminal Afe-I restriction sites in frame with the N ORF. Religation of the Afe-I digested PCR products reconstituted the expression plasmid, now replacing the targeted Ntail section with Ser-Ala residues encoded by the Afe-I site. All Ntail modifications were confirmed by DNA sequencing. In addition, all full-length genome plasmids were sequence-confirmed prior to recovery transfection of recombinant virions. To generate a NiV nano luciferase minireplicon reporter construct, the Nano luciferase gene was amplified using appropriate PCR primers and the resulting product cloned into an existing NiV replicon backbone (114) that was likewise PCR amplified using appropriate primers. The nano-luciferase amplicon was ligated to the replicon vector backbone using the NeBuilder kit in accordance with the manufacturer’s protocols (New England Biolabs), and the resulting plasmid sequence verified.

2.3 Immunoblotting

BSR-T7/5 cells transfected in a 12-well plate format (4x10^5 per well) with 2 µg of MeV or CDV N-encoding expression plasmid DNA were washed once 40 hours after transfection with phosphate buffered saline (PBS) and lysed in RIPA buffer (1% sodium deoxycholate, 1% NP-40, 150 mM NaCl, 50 mM Tris-Cl, pH 7.2, 10 mM EDTA, 50 mM NaF, 0.05% SDS, protease inhibitors [Roche]. Cleared lysates (20,000xg, 10 min, 4°C) were mixed with 5 x urea buffer (200 mM Tris, pH 6.8; 8 M urea; 5% sodium dodecyl sulfate (SDS); 0.1 mM EDTA; 0.03% bromphenol blue; 1.5% dithiothreitol). Samples were denatured for 30 min at 50°C, fractionated on 10 % SDS-PAGE gels, blotted on polyvinylidene difluoride (PVDF) membranes and subjected to enhanced chemiluminescence detection using specific antibodies directed against
MeV-N (MAB8905, Millipore), CDV (DV2-12, Bio-Rad), CDV-N-core (clone 1214) (116) and GAPDH (6C5, Ambion) as specified. Immunoblots were developed using a ChemiDoc digital imaging system (Bio-Rad), and the Image Lab software package (Bio-Rad) for image visualization. When applied, densitometry was carried out on non-saturated images with global background correction.

2.4 Minireplicon luciferase reporter assay

BSR-T7/5 cells (5,000 in a 96-well plate format) were transfected with plasmids encoding for IC-B-L (0.02 µg), IC-B-P (0.02 µg), IC-B-N (0.016 µg) and the respective MeV luciferase replicon reporter (0.044 µg). CDV minireplicon assay were performed accordingly using CDV helper plasmids. For NiV minireplicon experiments, cells were transfected with NiV-L (0.005 µg), NiV-P (0.005 µg), NiV-N (0.01 µg), and NiV nanoluciferase replicon reporter (0.06 µg) encoding plasmid DNA. Firefly or nano luciferase activities were determined 40 hours post-transfection in a Synergy H1 microplate reader (BioTek), using Bright-Glo or Nano-Glo luciferase substrate (Promega), respectively. Substrates were directly added to the cells and bioluminescence quantified after a 3-minute incubation for signal stabilization. Relative RdRp activities (relA) were determined on the basis of the formula % relA = (experimental - signal_{min})/(signal_{max} - signal_{min}) × 100, with signal_{max} corresponding to cells transfected with plasmids encoding the standard NiV proteins and signal_{min} representing cells that received equal amounts of empty vector (pUC-19) in place of the N-encoding plasmid. All experiments were performed in at least 3 independent replicates, each measured in nine dependent repeats.
2.5 Virus recovery

Recombinant MeV or CDV were recovered in BSR-T7/5 cells by transfecting 1.25 µg of the cDNA copy of the modified genome and IC-B-N (0.42 µg), IC-B-P (0.54 µg) and IC-B-L (0.55 µg). All recombinant CDV genomes harbored an additional transcription unit encoding the mKate fluorescent protein in pre-L ORF position, which does not affect viral pathogenicity (115). Transfected cells were overlaid 48 hours after transfection onto Vero-hSLAM or Vero-cSLAM cells and emerging infectious particles were passaged in Vero-hSLAM or Vero-cSLAM cells, respectively. Integrity of newly rescued virus strains was confirmed by extracting total RNA from infected cells (RNeasy mini kit, Quiagen) and generating cDNA copies using random hexamer primers and Superscript III reverse transcriptase (Invitrogen). Modified genome regions were amplified using appropriate primers and subjected to Sanger sequencing.

2.6 Preparation of virus stocks

MeV and CDV virus stocks were prepared by infecting Vero-hSLAM or Vero-cSLAM cells at a multiplicity of infection (MOI) of 0.01 50% tissue culture infectious dose (TCID\_50) units per cell, followed by incubation at 37°C. When microscopically observed virus-induced cytopathicity reached approximately 90%, cell-associated progeny particles were released through freeze/thaw cycles and titers determined by TCID\_50 titration on Vero-hSLAM or Vero-cSLAM cells as described (117).

2.7 Multi step virus growth curves

Prior to infection for multi-step growth curves, viral stocks were diluted to approximately $1 \times 10^4$ TCID\_50 units/ml and exact titers determined in a separate aliquot by TCID\_50 titration.
Vero-hSLAM or Vero-cSLAM cells (1 × 10⁵ per well in a 12 well format) were infected with the different MeV or CDV strains at a MOI of 0.01 TCID₅₀ units per cell for 1 hour and the inoculum replaced with DMEM growth medium. Individual wells were harvested in 12-hour intervals and cell-associated progeny virus titers determined by TCID₅₀ titration. At least three independent growth curves were generated for each virus strain examined. Virus-induced cytopathicity in infected cells was documented using an inverted fluorescence microscope (Nikon) equipped with digital imaging package.

3 RESULTS

3.1 RSV minireplicon under pol1 control


Various luciferase reporters are used for a high-throughput screening (HTS) protocol design that can identify paramyxovirus-specific, orthomyxovirus-specific, or broadly specific compounds in a single assay system (101). The goal was to develop pol-I reporter system for use in RSV nucleoside analog testing.

As discussed earlier, pol-I system has many advantages such as: 1) the pol I enzyme is expressed in the nucleolus of all eukaryotic cells and therefore does not need to be provided in trans. 2) transcripts generated by the pol I constructs have precise viral ends, i.e., they lack the
5′ cap structure and the 3′ poly A tail (119, 120). 3) the pol I system does not have the potential inherent disadvantage of vaccinia virus cytopathocity (119, 120).

**Experimental Results:** Firstly, TOPO-cloning was performed using the RSV primers to generate RSV leader-Firefly-trailor casette.

RSV forward 5′-CGTCTCCTATTACGAGAAAAAAAGTGTCAAAAAC-3′

RSV reverse 5′-CGTCTCGGGGGACGGGAAAAATGCGTACAAC-3′

The resulting product was cloned into an existing replicon by using BsmB-I restriction site. Based on a described pT7-RSV-luciferase minigenome reporter (121), this RSV minigenome construct (pHH-RSV-repl-firefly) was generated under the control of the constitutive RNA pol I promoter (as shown in the figure 7). This construct was then tested using varied amounts (0.8-2.5ug) for reporter activity in order to test the amount of reporter to be used subsequently. To determine the relative luciferase reporter activities, Human embryonic kidney (HEK293/293T) cells were used at 28 and 50h post-transfection because it has high pol-1 activity inherently.
Figure 7: Monocistronic pol-1 based RSV minigenome

A. Schematics of the Pol-I minigenome RSV plasmids generated (firefly- firefly luciferase; Le, leader sequence; Tr, trailer sequence.

B. Graphic map of the pol-I minigenome RSV plasmid generated

RNA pol-I terminator:

```
AAAAAAGAGGTCCAGAGTGCCCAGTTCCGCGC
CGGGGGGGGGGGGGGGGGGGGACACTTTTCGGACATCTGGTCGACCTCCAGCATCGG
GGG
```
3’ non-coding- Leader:
agtagaaacaggGTAGATAATCACTCACTGAGTGACATC

Firefly Luciferase- ORF:

```
atggaagacgcaccaaaaaacataaagagaagggcccggcggccattctatctctctagatgtaggatggaacaggaacagtgcata
aggctatgaagacgccaaaaacataaagggcccgttacatgtgagaagctgaacgtggtatcttcatcctcaatctcatttctgt
ctatgagctgacaaaaagcgctgacatgtgagaagctgaacgtggtatcttcatcctcaatctcatttctgt
tgatcgtgacaaaaagcgctgacatgtgagaagctgaacgtggtatcttcatcctcaatctcatttctgt
tgatcgtgacaaaaagcgctgacatgtgagaagctgaacgtggtatcttcatcctcaatctcatttctgt
tgatcgtgacaaaaagcgctgacatgtgagaagctgaacgtggtatcttcatcctcaatctcatttctgt
tgatcgtgacaaaaagcgctgacatgtgagaagctgaacgtggtatcttcatcctcaatctcatttctgt
tgatcgtgacaaaaagcgctgacatgtgagaagctgaacgtggtatcttcatcctcaatctcatttctgt
tgatcgtgacaaaaagcgctgacatgtgagaagctgaacgtggtatcttcatcctcaatctcatttctgt
tgatcgtgacaaaaagcgctgacatgtgagaagctgaacgtggtatcttcatcctcaatctcatttctgt
tgatcgtgacaaaaagcgctgacatgtgagaagctgaacgtggtatcttcatcctcaatctcatttctgt
tgatcgtgacaaaaagcgctgacatgtgagaagctgaacgtggtatcttcatcctcaatctcatttctgt
tgatcgtgacaaaaagcgctgacatgtgagaagctgaacgtggtatcttcatcctcaatctcatttctgt
tgatcgtgacaaaaagcgctgacatgtgagaagctgaacgtggtatcttcatcctcaatctcatttctgt
tgatcgtgacaaaaagcgctgacatgtgagaagctgaacgtggtatcttcatcctcaatctcatttctgt
tgatcgtgacaaaaagcgctgacatgtgagaagctgaacgtggtatcttcatcctcaatctcatttctgt
tgatcgtgacaaaaagcgctgacatgtgagaagctgaacgtggtatcttcatcctcaatctcatttctgt
tgatcgtgacaaaaagcgctgacatgtgagaagctgaacgtggtatcttcatcctcaatctcatttctgt
tgatcgtgacaaaaagcgctgacatgtgagaagctgaacgtggtatcttcatcctcaatctcatttctgt
tgatcgtgacaaaaagcgctgacatgtgagaagctgaacgtggtatcttcatcctcaatctcatttctgt
tgatcgtgacaaaaagcgctgacatgtgagaagctgaacgtggtatcttcatcctcaatctcatttctgt
tgatcgtgacaaaaagcgctgacatgtgagaagctgaacgtggtatcttcatcctcaatctcatttctgt
tgatcgtgacaaaaagcgctgacatgtgagaagctgaacgtggtatcttcatcctcaatctcatttctgt
tgatcgtgacaaaaagcgctgacatgtgagaagctgaacgtggtatcttcatcctcaatctcatttctgt
tgatcgtgacaaaaagcgctgacatgtgagaagctgaacgtggtatcttcatcctcaatctcatttctgt
tgatcgtgacaaaaagcgctgacatgtgagaagctgaacgtggtatcttcatcctcaatctcatttctgt
tgatcgtgacaaaaagcgctgacatgtgagaagctgaacgtggtatcttcatcctcaatctcatttctgt
tgatcgtgacaaaaagcgctgacatgtgagaagctgaacgtggtatcttcatcctcaatctcatttctgt
tgatcgtgacaaaaagcgctgacatgtgagaagctgaacgtggtatcttcatcctcaatctcatttctgt
```
5’ non-coding- Trailer:
AGAAAAATACccttgcttctact

RNA pol-I promoter:
aataACCCGGCGGCCAAA

HEK293/293T cells were cotransfected with 0.8ug, 1.5ug, 2ug or 2.5ug pol-I reporter with optimized ratios of RSV helper plasmids (N, P, M2 (0.4ug/well each) and L (0.2ug/well)) under CMV promoter control (122). Luciferase reporter activities were determined at 28 and 50 h after transfection activity in order to test the amount of reporter to be used subsequently.

Figure 8: Minireplicon analysis of a firefly luciferase-RSV reporter in 293T cell line at 28 and 50h post-transfection.

*Activity represents relative luciferase reporter activities. Values represent averages of two dependent experiment, determined as ± SD.*
The pol-I reporter was reported to be active with 2ug at 50 hours post transfection as shown in the figure 8. This experiment showed technical errors as the reporter showed increasing activity with increasing amounts of reporter for 28 hours post transfection. This was not observed for 50 hours post infection activity profile. But this reporter was active and was used further in RSV reporter assays in nucleoside analog testing.

3.2 Polycistronic minireplicons


The natural paramyxovirus transcription gradient of mRNA synthesis has been shown to affect by the structurally disordered central Ntail section through two synergistic effects- 1) promoting the initiation of the transcriptase complex and 2) reducing the success of the transcriptase to negotiate the entire genome (123). To address whether N-tail mutants allow RdRp to efficiently negotiate the intergenic junctions in the viral genome, a process that involves the nontemplated polyadenylation of the newly synthesized mRNA, migration of the RdRp complex to the next downstream transcription start sequence, and reinitiation of RNA synthesis, we generated a novel firefly luciferase and nanoluciferase bi- and tricistronic minigenome reporter plasmids.

Pseudotemplated addition of nucleotides or RNA editing, as described earlier, occurs in P. The P reading frame was chosen because of its RNA editing site, which makes the RdRp stutter
and may enhance the likelihood for premature termination of replication in order to evaluate the successful negotiation of intergenic junctions and to determine the polymerase activity of different MeV N-tail mutants in Morbilliviruses with the help of multicistronic minireplicons since the monocistronic minireplicons cannot address this question.

The bicistronic reporter harbors the N/P open reading frame (ORF) intergenic junction of the MeV genome between the luciferase reporter genes, whereas the tricistronic plasmids contains the entire MeV P ORF including the N/P and P/M intergenic junctions. Firstly, the MeV bicistronic replicon was cloned using monocistronic MeV replicon (111) as the template. Recombineering PCR technique was used with the help of Aat II and Avr II sites to create a firefly luciferase–(P/M-IGS)–gaussi cassette. But, this bicistronic construct did not show any appreciable activity for the Gaussi luciferase reporter and the construct was then exchanged for the nanoluciferase reporter construct. By cloning with the help of Pac I and Avr II sites, this bicistronic cassette was introduced it into an existing monocistronic MeV minigenome (111).

The bicistronic construct served as the backbone for the tricistronic variant. Again by recombineering PCR, a second intergenic junction cassette was cloned into the former with the help of Pac I and Aat II sites. An eGFP protein (with the same intergenic junctions as the others N-P and P-M), Pstopstop or P_ORF without the editing site-(1200bp shorter) P-(ΔCV) was placed as the 2nd ORF in between two intergenic junctions. In the case of Pstopstop, a tandem stop codon was introduced into the ORF to avoid influencing minireplicon activity through additional P protein originating from the tricistronic minigenome, The stop codon was inserted 21 triplets downstream of the start codon.
Figure 9: Bi- and Tricistronic minigenome assays -quantitating negotiation of intergenic junctions in the presence of transiently expressed MeV N standard and mutant plasmid MeV NΔ439-482.

(contributed by K.Wabbel and R.K.P- from Institute for Biomedical Sciences, Georgia State University)

A) Minireplicon analysis of the MeV N mutant using a Bicistronic minigenome reporter. Activity represents normalized (to the mutant) ratio – second ORF (nano luciferase) versus first ORF (firefly luciferase) reporter activities of each biological replicate. Values represent averages of at least three independent experiments, determined in nonuplets each ± SEM. Shown is a comparison of two out of three data sets generated. Experimental variation was assessed through one-way analysis of variance (ANOVA) combined with Tukey’s multiple comparison post test (*P < 0.05; **P < 0.01; ***P < 0.001; NS, not significant). B) Schematic of the tricistronic minigenome plasmids generated (firefly, firefly luciferase; nanoLuc, nanoluciferase; IGS, intergenic segment; Le, leader sequence; Tr, trailer sequence; eGFP, P_stopstop and P-ORF – without the non-structural proteins C and V – P-(ΔCV) – 2nd ORF. C) Minireplicon analysis of the MeV N mutant using a Tri-cistronic minigenome reporter. Activity represents normalized (to
Bi- and Tricistronic minigenome reporter assay measures the negotiation of intergenic junctions in the presence of the MeV-N mutant was first tested as above (figure 9). In this protocol, BHK-T7 cells were cotransfected with either reporter and helper plasmids under T7 promoter control. For analysis of the minigenome data, data sets obtained for each reporter were normalized in the presence of the mutant N. Later, the ratios of normalized nanoluciferase versus firefly luciferase signals for standard N was calculated. In the presence of the different constructs, this kind of approach enables us to appreciate the relative efficiency with which the RdRp complex accesses the downstream relative to the upstream reporter.

In case of bicistronic construct, the relative reporter expression levels of MeV N-(Δ439-482) were higher than standard N. In the case of tricistronic P-stopstop construct, the relative reporter expression levels did not differ significantly. For viral foreign proteins like eGFP and a highly truncated P that lacks the reading frames for C and V – (P-(ΔCV), significantly lower transcription efficiency in the presence of the N mutant was observed. Whether this finding is due to the shortened reading frames (both tricistronic constructs are shorter than the original P ORF) or due to RdRp processivity is further needed to be determined. It was demonstrated that through minireplicon assays that the mutant N did not affect RNA editing in the P open reading
frame. These results show that the non-structural proteins C and V do not influence the multicistronic RdRp activity and also shows successful negotiation of intergenic junctions by the RdRp complex in the absence of the unstructured morbilliviral Ntail section.

3.3 Paramyxovirus Nucleocapsid Protein Tail Domain Modulation

The functional importance of the structurally disordered central Ntail sections for paramyxovirus polymerase activity is currently mechanistically poorly understood, although it was thought that it provides structural flexibility to MoRE for recruitment of the polymerase complex (124, 125).

![Schematic of the morbillivirus Ntail organization](image)

**Figure 10:** Schematic of the morbillivirus Ntail organization

*(model by R. M Cox - from Institute for Biomedical Sciences, Georgia State University)*

A) Model of the full-length measles N protein. The Ntail domain (134 amino acids) missing from the cryoEM reconstruction of Ncore (blue-grey; PDB 4UFT) was added using Coot for
relative length illustration only. Conserved box regions are highlighted in yellow, orange, and green according to their position in the linear sequence. Heat maps (red) represented the predicted degree of structural disorder. Cartoon representations of the assembled MeV nucleocapsid assume a perpendicular orientation of Ntail to the axis of the helical RNP assembly (right). Ntail originates at the inner surface of the RNP and box1 residues are predicted to be buried between the rungs of the helix. B, C) Models of Ntail mutants after partial (B) or nearly complete (C) removal of the disordered central Ntail section. The truncations posit box2 and 3 regions in close proximity to the trunk of the RNP assembly. D) Sequence alignment of the MeV and CDV Ntail domains. Box1-3 domains are color-coded as described in (A). Truncation donor (red) and acceptor (green) residues explored in this study are highlighted.Alignments were generated using T-Coffee [69] and structural models of MeV Ntail variants created in Pymol [70].

To test whether the morbillivirus unstructured central Ntail section is required for virus replication, we first designed a series of progressively larger internal tail deletions in MeV N, commencing with the removal of residues between positions 439 and 482, located just upstream of the conserved MoRE and box3 and extending to elimination of most of the structurally disordered residues between box1 and MoRE (figures 10A-C). Figure 10D provides an overview of all morbillivirus (MeV and CDV) Ntail truncations targeted. The largest deletion in MeV N eliminated all of Ntail between residues 399 and 482, which also includes the conserved box1 region at the N-terminal origin of the tail. Naturally, gradual removal of the central Ntail residues will position the C-terminal end of Ntail harboring MoRE and box3 in immediate proximity of the trunk of the helical RNP assembly, ultimately predicting the placement of MoRE...
immediately proximal to the interface between consecutive turns of the RNP helix.

**Figure 11: Bioactivity of MeV and CDV N protein mutants with different length deletions in the disordered central tail section.**

A, C) Steady state levels of MeV (A) and CDV N (C) protein mutants in cells BHK-T7 cells transfected with N protein-encoding plasmid DNA. Immunoblots were decorated with specific antibodies directed against MeV and CDV N protein, respectively, or cellular GAPDH for
sample processing control. Numbers represent means of densitometry quantitations of three biological repeats ± SEM; all uncropped blots are shown in supplementary figures 5 and 6. B, D) Monocistronic minigenome assays testing bioactivity of the N protein mutants specified in (A) and (C). Symbols represent relative luciferase units of each biological replicate, determined each in nine technical replicates and normalized for values measured for standard N protein. Columns show sample means ± SEM; P values are based on one-way ANOVA with Tukey’s multiple comparison post hoc test, F: 18.1 (B) and 37 (D). E, F) Tricistronic minigenome assays -quantitating negotiation of intergenic junctions in the presence of the N protein mutants (contributed by K.Wabbel, J. Sourimant and R.K.P- from Institute for Biomedical Sciences, Georgia State University). Symbols represent third ORF (nano luciferase) versus first ORF (firefly luciferase) reporter activities of each biological replicate, each measured in nine technical replicates. Columns show samples means ± SEM; P values are based on one-way ANOVA with Tukey’s multiple comparison post hoc test, F: 30.6 (E) and 4.8 (F); NS: not significant).

Western blot analyses of cell lysates after transient transfection with plasmids encoding these mutants confirmed stable expression of all modified N proteins and revealed a gradual increase in electrophoretic mobility with expanding truncation size as anticipated (figure 11A). The two constructs harboring partial or complete deletions of box1 (NΔ399-482 and NΔ409-482), resulted in substantially reduced or no appreciable minireplicon activity, respectively. All other mutants supported efficient RdRp activity in a monocistronic minireplicon reporter assay (figure 11B). While average relative RdRp activities varied slightly compared to those observed in the presence of standard MeV N, none of these changes was statistically significant.
Encouraged by these results, we generated a comparable series of CDV N deletion mutants informed by the Ntail linear sequence alignments as outlined (figure 10D). Resembling the corresponding MeV N mutants, expression levels and bioactivity of CDV NΔ441-479 and NΔ425-479 was indistinguishable from that of standard CDV N in Western blotting and minireplicon assays (figures 11C and D). However, larger truncations (CDV NΔ423-479 and NΔ421-479, respectively) resulted in significantly enhanced minireplicon activities. As noted for MeV, truncation encroaching into the box1 section abolished CDV N bioactivity.

Since monocistronic minireplicons cannot address whether the viral transcriptase retains the ability to successfully negotiate intergenic junctions, we employed a tricistronic minireplicon construct that contains two intergenic junctions and distinct firefly and nano luciferase reporter genes in the first and third reading frame position, respectively. In this assay, the relative ratio of downstream versus upstream reporter activity serves as an indicator for the efficiency with which the RdRp complex advances through intergenic junctions and reinitiates mRNA synthesis. When tested in the presence of a subset of the MeV (figure 11E) and CDV (figure 11F) Ntail mutants, only the MeV NΔ420-482 construct returned a slight (approximately 25%) but statistically significant relative reduction of third ORF expression. In the presence of all other constructs, relative reporter expression levels did not differ significantly from those observed with standard N, suggesting successful negotiation of intergenic junctions by the RdRp complex in the absence of the unstructured Ntail section.
**Figure 12: Bioactivity of corresponding Henipavirus N protein mutants**

(Performed in assistance with R. M Cox, Institute for Biomedical Sciences, Georgia State University)

A) Schematic of the NiV Ntail organization and disorder prediction, color-coded as in supplementary figure 1A. Ntail truncations eliminate the predicted helical box4 near the center of Ntail. B) Sequence alignment of the MeV and NiV Ntail domains, color-coded as in (A). The individual truncation donor (red) and acceptor (green) sites are highlighted. Alignments were generated using T-Coffee. C) Minigenome activity analysis of the NiV N protein mutants. Symbols represent individual biological replicates, each determined in nine technical replicates. Columns show sample means ± SEM; P values are based on one-way ANOVA with Tukey’s multiple comparison post hoc test, F: 19.1; df1: 2, df2: 6.

To evaluate whether continued RdRp activity in the absence of the central Ntail section extends to paramyxoviruses outside the morbillivirus genus, we applied an equivalent truncation strategy to the Ntail of highly pathogenic NiV, a member of the recently established henipavirus genus (figures 12A and B). Although the organization of the NiV tail is predicted to be more complex
than that of the morbilliviruses, featuring an additional box4 in the central Ntail section (126), NiV RdRp also readily accepted template RNA encapsidated by tail-truncated N (figure 12C). Whereas a NiV NΔ424-471 mutant protein with large truncation showed standard NiV N-like activity in minireplicon assays, bioactivity of the NiV N construct harboring a shorter NΔ443-471 truncation was also significantly increased.

![Image](image.png)

**Figure 13:** Recovery of recombinant MeV and CDV expressing N protein mutants.
A, B) Electrophoretic mobility profiles of N proteins expressed by the different recMeV (A) and recCDV (B) strains. Whole cell lysates of infected Vero-hSLAM and Vero-cSLAM cells, respectively, were subjected to immunoblotting and detection with specific antibodies directed against the MeV or CDV N protein. C, F) Multi-step growth curves of the different recMeV (C) and recCDV (F) strains recovered. Vero-hSLAM and Vero-cSLAM cells, respectively, were infected at an MOI of 0.01 TCID$_{50}$ units/cell, followed by sampling and titration of cell-associated progeny virus at the indicated time points. Values represent means of three independent experiments ± SEM. D, G) Regression modeling of growth profiles shown in (C) and (F). *(contributed by R.K.P- from Institute for Biomedical Sciences, Georgia State University). Bindslev’s population growth four-parameter variable slope model was applied (PDT$_{\text{max}}$, maximal population doubling time; Titer$_{\text{max}}$, titer corresponding to the top plateau of the regression models; values in parentheses specify 95% confidence intervals; * denotes non-overlapping confidence intervals relative to standard recMeV; NS, overlapping confidence intervals). E, H) Cytopathic effect associated with the different recMeV and recCDV strains *(contributed by R.M Cox- from Institute for Biomedical Sciences, Georgia State University). Microphotographs of infected cells were taken at the specified times post-infection at a magnification of 200×.

RdRp activity in minireplicon assays is a necessary albeit not sufficient function to support a full viral replication cycle. We also substituted the N protein encoding ORFs in cDNA genome copies of MeV and CDV strains that are based on pathogenic viral isolates, MeV-IC-B (62) and CDV-5804PeH (115), with two different Ntail truncations each, MeV NΔ439-482 and NΔ420-482, and CDV NΔ441-479 and NΔ425-479, respectively. These specific truncations were
selected on the basis that in each case they represent the shortest and largest internal tail truncation that did not significantly alter N bioactivity in the original monocistronic minireplicon assay. The corresponding recombinant viruses were recovered readily after rescue transfection and overlay onto receptor-positive Vero-humanSLAM (Vero-hSLAM) and Vero-canineSLAM (Vero-cSLAM) cells, respectively. DNA sequencing after RT-PCR amplification of recovered virus genomes and Western blot analyses of infected cell lysates (figures 13A and B) confirmed the presence of the respective Ntail truncations in the recombinant viruses.

Multiple step growth curves revealed a parent virus-like replication profile for recMeV-IC-B NΔ439-482 (figure 13C), whereas recMeV-IC-B NΔ420-482 with the larger Ntail truncation showed an initial 12-hour growth delay. Modeling of growth profiles confirmed that maximal population doubling times of recMeV-IC-B NΔ420-482 were significantly longer than those of standard recMeV-IC-B, but differences between recMeV-IC-B NΔ439-482 and the parent strain remained not significant (figure 13D). Microphotographs of the infected cell populations at different times after infection demonstrated that cytopathic effects corroborated the virus titer-based growth profiles (figure 13E). Interestingly, the growth pattern of the MeV mutants was inversed in the case of the recombinant CDV virus strains (figure 13F). recCDV-5804PeH NΔ425-479 carrying the larger truncation showed significantly shorter maximal doubling times than recCDV-5804PeH NΔ441-479 (figure 13G). However, maximal growth rates of both mutant strains lagged behind that of the parental recCDV-5804PeH, although growth profile modeling did not reveal significant differences in peak progeny titers reached. Visual examination of viral cytopathicity again showed only small differences between the two mutant strains and the parental recCDV (figure 13H).
These data demonstrate that the unstructured central Ntail section is not associated with an essential RdRp function required for virus replication, establish distinct effects of different length truncations on viral fitness in cell culture, and indicate that the actual impact of internal Ntail truncations on virus growth is not necessarily directly proportional to the length of the deletion but must be individually determined.

Figure 14: Pathogenesis of the recCDV mutants in the ferret model
(contributed by B. Sawatsky, and V. Messling- from Veterinary Medicine Division, Paul-Ehrlich-Institut, Federal Institute for Vaccines and Biomedicines, Langen, Germany and R. Budaszewski-
A) Cell-associated viremia titers after intranasal infection of animals with the different recCDV strains. Symbols represent TCID50 units in 106 isolated PBMCs for each biological replicate; lines connect sample means (recCDV NΔ425-479-infected animals n = 4; recCDV NΔ441-479-infected animals n = 7; recCDV-5804PeH-infected animals n = 4); P values are based on two-way ANOVA with Tukey’s multiple comparison post hoc test; F: 2.9; df1: 2, df2: 10. B) Survival curves. Statistical significance of differences between the curves was assessed through Mantel-Cox test, predicted median survival is shown; df: 2. C) Virulence index for standard and mutant recCDV-5804PeH strains. Each box represents one animal, clinical score index ranges from high (black), to intermediate (grey) and absent (white). D) Body weight changes in the infected animals. Weight is expressed as the percentage of the initial weight at the day of infection. Symbols represent individual biological replicates; lines connect sample means; P values are based on two-way ANOVA with Sidak’s multiple comparison post hoc test; F: 7.5; df1: 1, df2: 9. E) Fever curves of infected animals. Symbols represent body temperature for each biological replicate; lines connect sample means; P values are based on two-way ANOVA with Sidak’s multiple comparison post hoc test; F: 38; df1: 1, df2: 189. F) Lymphopenia assessment. Symbols represent mean leukocyte counts per cubic millimeter of blood for each biological replicate; lines connect sample means; P values are based on two-way ANOVA with Tukey’s multiple comparison post hoc test; F: 0.91; df1: 2, df2: 10. G) Nonspecific PBMC proliferation capacity. Symbols represent mean rations of 5-bromo-2’ deoxyuridine (BrdU) incorporation relative to nonstimulated PBMCs for each biological replicate; lines connect sample means; P values are based on two-way ANOVA with Tukey’s multiple comparison post hoc test; F: 0.42; df1: 2, df2: 11.
To evaluate the importance of Ntail for viral pathogenesis, we capitalized on the lethal CDV/ferret model that recapitulates key features of human morbillivirus disease such as host invasion strategy, tissue tropism, and replication profile (127). Ferrets were infected intranasally with \(2 \times 10^5\) TCID\(_{50}\) units of standard recCDV-5804PeH, recCDV-5804PeH N\(\Delta\)441-479, or recCDV-5804PeH N\(\Delta\)425-479, and clinical signs, PBMC-associated viremia titers, white blood cell counts, and lymphocyte proliferation response were monitored in regular intervals. Consistent with our previous experiences with the model (128), peak viremia titers were reached seven days post-infection, followed by a rapid decline in viral load in animals infected with either of the N mutant viruses (figure 14A). All animals infected with standard recCDV-5804PeH succumbed to the disease by day 14. By contrast, the group that had received recCDV-5804PeH N\(\Delta\)441-479 and 75\% of animals in the recCDV-5804PeH N\(\Delta\)425-479 group survived the infection (figure 14B). In recovering animals, viremia fully subsided 21 (recCDV-5804PeH N\(\Delta\)441-479) and 35 (recCDV-5804PeH N\(\Delta\)425-479) days after infection, respectively.

Animals infected with the parental virus experienced severe disease with extensive rash, substantial weight loss, and high fever (figures 14C-E). By comparison, disease progression was less aggressive in animals infected with the recCDV-5804PeH N\(\Delta\)425-479 mutant virus and mild in animals of the recCDV-5804PeH N\(\Delta\)441-479 group. Specifically, recCDV-5804PeH N\(\Delta\)441-479-infected ferrets showed a benign, more localized rash and only transiently lost a moderate (<10\%) amount of body weight before making a full recovery by conclusion of the study (figure 11D). Fever peaked in these animals two to three days earlier and at a lower level than in recCDV-5804PeH and recCDV-5804PeH N\(\Delta\)425-479-infected animals, and resolved within the second week after infection (figure 14E). In contrast, recCDV-5804PeH N\(\Delta\)425-479 infected
ferrets presented with a more prolonged weight loss and fever fully resolved only in the third week after infection.

Acute lymphopenia and temporary lack of lymphocyte responsiveness to stimulation constitute a hallmark complication associated with morbillivirus infections (129). When assessing immune competence of animals infected with the different viruses, we noted significantly milder lymphopenia early after infection in the recCDV-5804PeH NΔ441-479 group compared to animals that had received standard recCDV-5804PeH or recCDV-5804PeH NΔ425-479 (figure 14F). However, lymphocytes derived from animals of all groups showed a similar decline in proliferation responsiveness during the first two weeks after infection (figure 14G). Proliferation response improved in all surviving animals only at 35 days post-infection, although we noted a temporary rebound in recCDV-5804PeH NΔ441-479-infected ferrets at the 21-day time point.
Figure 15: Immune responses to recombinant Ntail deletion mutant viruses

(Contributed by B. Sawatsky, and V. von Messling- from Veterinary Medicine Division, Paul-Ehrlich-Institut, Federal Institute for Vaccines and Biomedicines, Langen, Germany and R. Budaszewski- from Paul-Ehrlich-Institut and Federal University of Rio Grande do Sul, Porto Alegre, Brazil)
A) Relative levels of type I IFN and Mx-1 message present in PBMCs isolated at days three and seven after infection of animals with the different CDV recombinants. Symbols represent the relative fold-change in mRNA level normalized to uninfected naive controls (day 0) for each biological replicate. Horizontal lines and error bars represent mean values ± SEM; t-tests were used for pairwise comparisons; one-way ANOVA with Sidak’s multiple comparison post hoc test was applied to Mx-1 day 7 fold-change analysis; F: 1.96; df1: 8, df2: 27. B) Neutralizing antibody responses in plasma samples. Antibody titers are shown as the reciprocal of the highest dilution in which CPE was observed. Symbols represent individual biological replicates, lines connect sample means. P values are based on two-way ANOVA with Sidak’s multiple comparison post hoc test; F: 4.1; df1: 1, df2: 6. C) Intranasal challenge of surviving animals from figure 4 with 2 x 10^5 TCID50 units of standard recCDV-5804PeH. Survival curves of animals after rechallenge at day 49 post-infection. For comparison, CDV-naïve ferrets were challenged in parallel (n = 3: recCDV NΔ425-479; n = 4: recCDV NΔ441-479; n = 4: recCDV-5804PeH). D) Cell-associated viremia titers in rechallenged animals. Symbols represent TCID50 units in 106 isolated PBMCs for each biological replicate, lines connect sample means; P values are based on two-way ANOVA with Tukey’s multiple comparison post hoc test; F: 1448; df1: 2, df2: 8. E) Lymphopenia assessment in rechallenged animals. Symbols represent leukocyte counts per cubic millimeter of blood for each biological replicate, lines connect sample means; P values are based on two-way ANOVA with Tukey’s multiple comparison post hoc test; F: 60.4; df1: 2, df2: 8.
Animals infected with either mutant virus mounted comparable type I interferon responses, reaching approximately 10-fold induction levels in IFN-α, β and Mx-1 message, the latter representing one of the major interferon-stimulated genes in response to CDV infection (figure 15A).

In the same manner, anti-CDV -5804PeH antibody responses were robust in animals of either group, although interestingly neutralizing antibody titers induced by the more attenuated recCDV-5804PeH NΔ441-479 peaked slightly higher than those found in animals of the recCDV-5804PeH NΔ425-479 group (figure 15B). To determine whether antibody titers mounted by the surviving animals infected with the Ntail modified recombinants were protective, we re-challenged with a lethal dose of standard recCDV-5804PeH at 49 days after the original infection. All challenged animals survived (figure 15C) and none developed appreciable viremia (figure 15D), showed clinical signs, or experienced severe lymphopenia (figure 15E).

These results highlight a role of the unstructured Ntail section in paramyxovirus pathogenesis. Gradual shortening of the tail induces different degrees of viral attenuation, although not with direct proportionality. Importantly, all surviving animals in the CDV/ferret model were completely protected against a lethal challenge with standard CDV, underscoring efficient immunization by the Ntail-modified recombinant strains.
Table 1: Sequence analysis of recCDV strains after passage in cell culture and through ferrets

(contributed by N. Makhsous, A. Greninger- from Virology Division, Department of Laboratory Medicine, University of Washington, Seattle, WA and with M. Russ and J. Sourimant- from Institute for Biomedical Sciences, Georgia State University)

To assess the genetic stability of the recCDV with Ntail truncations, we subjected viral RNA preparations to deep sequencing before and after 10-11 passages in cell culture, and determined N ORF consensus sequences in viral RNA extracted from PBMCs harvested from ferrets seven days after infection through Sanger sequencing (table 1). Neither standard recCDV-5804PeH nor
recCDV-5804PeH NΔ441-479 showed any changes in the N nor P ORF compared to the genome cDNA plasmids that were used for virus recovery. In contrast, recCDV-5804PeH NΔ425-479 carried a glutamate to glutamine substitution at N residue 156 that was dominant in the viral population after four passages in cell culture, and acquired an alanine to aspartate exchange at N residue 410 that became increasingly fixed during cell culture passaging. All recCDV-5804PeH NΔ425-479 recovered from infected ferrets at the peak of viremia likewise contained both N substitutions.

Comparison with N sequences representing a variety of different circulating CDV strains and isolates revealed that the N ORF is fully sequence conserved at position 156 and shows only more conservative changes than the aspartate substitution at residue 410 (figure 16).
Figure 16: Alignment of a diverse pool of CDV N sequences representing clinical viral isolates and laboratory-adapted strains. (analyses by R.K.P, Institute for Biomedical Sciences, Georgia State University)

Shown are strain identifiers, database access codes, and the areas surrounding the two residues in the N protein (156 and 410, respectively) featured in table 1. Alignments were created using T-Coffee and outputs rendered with ESPript 3.0.

None of the mutants or standard recCDV harbored any coding mutations in the P and L ORFs, with the exception of a single recCDV-5804PeH NΔ441-479 passaging line that carried a leucine to proline substitution at L residue 2175 with approximately 50% allele frequency after 15 passages in cell culture (table 1). Since none of the other recCDV-5804PeH NΔ441-479 lines analyzed in parallel showed allele variation at this position, this mutation most likely represents a stochastic event that became partially fixed in the genome.
Figure 17: Analysis of RNA populations present in cells infected with Ntail mutant viruses in comparison with the parental strain.

(Contributed by R. M. Cox, Institute for Biomedical Sciences, Georgia State University)

A, B) MiSeq analysis of viral mRNA editing in the P ORF after infection of cells with recCDV NΔ441-479 (A), recMeV NΔ439-482 (B), or the corresponding parent virus strains. Values represent a minimum of 91,741 reads each and are expressed as mean percentage of the
differentially edited mRNAs relative to the total transcripts from the P ORF ± SEM. C) qRT-PCR quantitation of relative CDV genome copy numbers in cells infected with the recCDV Ntail mutants compared to standard recCDV. First strand synthesis with specific primers directed at the viral genome UTR, followed by amplification of a section of the N ORF. D, E) qRT-PCR quantitation of relative CDV N mRNA (D) and L mRNA (E) copy numbers present in RNA preparations as in (C), compared preparations from cells infected with standard recCDV. First stand synthesis with oligo(dT) primers, followed by amplification of sections of the N and L ORF. F to H) qRT-PCR quantitations of RNA preparations as in (C) of the relative ratios of L versus N encoding mRNAs (F), and of intergenic sequence (IGS) N/P (G) and mKate/L (H) encoding polycistronic mRNAs versus mRNAs encoding the IGS-preceding ORF. First stand synthesis with oligo(dT) primers, followed by amplification of sections of the N, mKate, and L ORF, respectively, or across the specified IGS sections. In (C) to (H), symbols represent individual values of three biological repeats analyzed in two technical repeats each. Columns show mean values ± SEM. P values are based on one-way ANOVAs with Tukey’s multiple comparison post hoc test; F ratios and df values are shown for each graph.

To further elucidate the mechanistic basis for the altered CDV pathogenesis profiles, we analyzed viral RNA populations synthesized in cells infected with the different recombinant virus strains. Co-transcriptional paramyxovirus mRNA editing results in the expression of two additional proteins, V and W, from the viral P ORF through the insertion of non-templated G residues at an editing site (130-133). Of these P ORF products, the CDV V protein serves as the major suppressor of the host-cell innate antiviral response. RNA editing is thought to result from backsliding of the RdRp complex on the RNP template (130, 134, 135), which requires structural
flexibility that could be mediated by the flexible central Ntail section. Since impaired V protein expression causes viral attenuation (136), we employed a MiSeq assay to quantify the relative ratios of P, V and W-encoding mRNAs in infected cells. recCDV-5804PeH NΔ441-479 and the corresponding recMeV-IC-B-NΔ439-482 were selected for this analysis based on the superior level of attenuation of this shorter CDV truncation mutant in the ferret model. Relative mRNA distributions were comparable between MeV and CDV, but we noted only minor changes when mutant and the corresponding parent viruses were compared (figures 17A and B).

Our recent characterization of an MeV recombinant that expressed a mutant N with MoRE relocated into Ncore and partially truncated tail revealed a steepened viral mRNA transcription gradient in infected cells (100). However, this MeV recombinant was temperature-sensitive and unable to replicate under physiological conditions. Based on this finding, we hypothesized that attenuation of the recCDV Ntail mutants in the ferret model may alternatively result from deregulated viral transcriptase activity, although more subtle than that experienced with the MoRE-relocated MeV recombinant. Using an RT-qPCR-based approach, we quantified viral genome copies in infected cells, determined relative N-encoding and L-encoding mRNA levels in cells infected with the mutant versus parental virus strains, examined relative ratios of L protein to N protein-encoding mRNAs produced by each virus strain, and calculated the relative frequencies with which polycistronic viral mRNAs are synthesized by each recombinant during replication.

Viral genome copy numbers of both mutant recCDV strains were reduced by approximately 19 to 29% at the end of the replication cycle compared to standard recCDV (figure 17C). By
contrast, N protein-encoding mRNA levels of either mutant strain were increased approximately 1.8-fold relative to standard recCDV (figure 17D). Interestingly, when we examined relative mRNA levels of the downstream-most positioned L protein ORF, we noted that this 1.8-fold increase was maintained in recCDV-5804PeH NΔ425-479 infected cells, but boosted to an approximately 3-fold relative excess in cells harboring recCDV-5804PeH NΔ441-479 (figure 17E). Analyzing ratios of L protein-encoding versus N protein-encoding mRNAs synthesized by each virus strain revealed a significant increase in relative amounts of L protein-encoding mRNAs only in cells infected with recCDV-5804PeH NΔ441-479, while essentially identical ratios were obtained for standard recCDV and the less attenuated recCDV-5804peH NΔ425-479 (figure 17F).

The higher relative L mRNA level produced by recCDV-5804PeH NΔ441-479 could reflect a relative increase in bona fide L message due to a lowered premature termination ratio of the advancing polymerase complex or due to a higher proportion of non-productive polycistronic mRNAs. To differentiate between these alternatives, we evaluated the relative content of polycistronic message generated at the first (figure 17G) and last (figure 17H) intergenic sequence (IGS) present in the recCDV genomes. Depending on the individual IGS examined, replication of the standard recCDV-5804PeH and recCDV-5804P NΔ425-479 strains resulted in the synthesis of 2-10% polycistronic message relative to the total message synthesized for the preceding ORF. At either IGS, however, we noted a significantly higher ratio of polycistronic message present in cells infected with the recCDV-5804PeH NΔ441-479 mutant strain. Sequence analysis of the polycistronic message after RT-PCR amplification of an N/P message fragment harboring the IGS revealed straight read-throughs of the transcriptase complex, lacking
any non-templated residues at the poly-adenylation site. These results implicate the structurally disordered central Ntail section in affecting paramyxovirus transcriptase function on two different levels. Both Ntail mutant strains show heightened transcriptase activity relative to the parental recCDV strain. In addition, the more severely attenuated recCDV-5804peH NΔ441-479 further disturbs the relative ratio of viral message in infected cells by generating a higher proportion of non-productive polycistronic mRNAs.

**Figure 18:** Predicted location of candidate compensatory mutation to the ND425-479 truncation in a model of the CDV RNP assembly.

*(model by R. M Cox, Institute for Biomedical Sciences, Georgia State University)*

A) The E156Q mutation (red spheres) in Ncore is located near the position at which Ntails (yellow circles) are postulated to protrude from the RNP assembly. The homology model of the CDV nucleocapsid was created using the SWISS-Model homology-modeling server based on the structure of the MeV Ncore assembly (PDB ID: 4UFT). B) Side-view of consecutive rungs of the helical CDV RNP assembly. No structural information is available for the position of Ntail, but box1 residues (yellow squares) are predicted to locate close to the outer surface of the RNP or
between consecutive turns of the RNP helix (shown in the model). The position of the A410D substitution near the center of box1 is highlighted (red square).

The observations demonstrate that the NΔ441-479 truncation is genetically stable over a number of generations in cell culture and after \textit{in vivo} passage of the recombinant strain. Efficient growth of recCDV-5804PeH NΔ425-479 appears to be linked to the presence of two compensatory mutations, one located in Ncore (residue 156) and the second in box1 of Ntail (residue 410). Localization of CDV N residue 156 in a structural model of the morbillivirus RNP assembly posits this substitution at the C-terminal end of a flexible loop in Ncore (100), orientated towards the interface between consecutive turns of the RNP helix (figure 18) and in proximity to the site where Ntail is thought to emerge from the RNP core (88).

\textbf{Supplemental Results}

We wanted to determine the virus recovery of N-(Δ439-482) mutant virus in the MeV-Edmonston (Edm) vaccine strain. MeV-Edm vaccine strain has been extensively used in our laboratory. The truncations have been transferred to the MeV clinical isolate IC-B strain as shown in the \textit{Appendix 1 – Thakkar et al (2018)}. The hypothesis was that the kinetics of Edm mutant viruses would be different from the clinical isolate IC-B mutant viruses with the same truncation.
Figure 19: Recovery and expression analysis of recombinant Edmonston-MeV expressing N mutant.

A-D) Multi-step growth curves of recMeV N(Δ439-482) cell-associated (A) or supernatant (B) virus recovered at 37°C respectively, and recMeV N(Δ439-482) cell-associated (C) or supernatant (D) virus recovered at 32°C respectively, as compared to the standard. Vero-hSLAM cells were infected at an MOI of 0.01 TCID$_{50}$ units/cell, followed by sampling and titration of cell-associated/supernatent progeny virus at the indicated time points. Values represent means of at least three independent experiments ± SEM. E, F) Expression levels of growth-curve cell lysates of cell-associated virus strains recovered at 37°C (E) and at 32°C (F). Whole cell lysates
of infected Vero-hSLAM were subjected to immunoblotting and detection with specific antibodies directed against the MeV N protein.

To determine the importance of the unstructured Ntail section in virus replication in the Edmonston strain, we substituted the N protein encoding ORFs in cDNA genome copies of MeV-Edmonston vaccine strain with MeV NΔ439-482. As discussed earlier, RdRp activity in minireplicon assays is not a necessary function to support viral replication cycle of that specific mutant. Surprisingly, the corresponding recombinant mutant virus was recovered readily after rescue transfection and overlay onto receptor-positive Vero-humanSLAM (Vero-hSLAM). DNA sequencing after RT-PCR amplification of recovered virus genomes confirmed these viruses. Multiple step growth curves were performed at 37°C and at 32°C (figure 19) with non-GFP containing viruses.

These results demonstrate that the growth-kinetics at 37°C revealed an initial 12-hour growth delay replication profile for recMeV-Edm- NΔ439-482 for the cell-associated and supernatant recombinant viruses as compared with its standard. On the other hand, the peak titers for recMeV-Edm- NΔ439-482 showed a 2-day delay profile at 32°C with respect to the cell-associated and supernatant viruses. This was replicated in the whole cell lysates of these viruses.
Figure 20: Recovery of recombinant Edmonston-MeV (GFP) expressing N mutants.

A-C) Multi-step growth curves of recMeV-GFP N(Δ427-482) and recMeV-GFP N(Δ420-482) cell-associated viruses recovered at 37°C (A), at 32°C (B) or at 39.5°C (C). Vero-hSLAM were infected at an MOI of 0.01 TCID₅₀ units/cell, followed by sampling and titration of cell-associated progeny virus at the indicated time points. Values represent means of at least three independent experiments ± SEM.

Secondly, we substituted the N protein encoding ORFs in c-DNA genome copies of MeV-Edm strain with MeV-GFP NΔ427-482 and with MeV-GFP NΔ420-482 respectively. MV recombinants expressing the green fluorescent protein (GFP) have been used to monitor the kinetics of the virus in the cell. We have therefore placed the GFP-ORF in the first ORF before the N-protein ORF in order to observe them over a period of time. The corresponding recombinant mutant viruses were recovered readily after rescue transfection and overlay onto receptor-positive Vero-humanSLAM (Vero-hSLAM). DNA sequencing after RT-PCR amplification of recovered virus genomes. Multiple step growth curves were performed at 37°C, 32°C and at 39.5°C (figure 20) with GFP containing viruses.
These results demonstrate that the peak titers at 37° showed a 2 and 1.5-days delay profile respectively, with respect to the standard C for recMeV-Edm-GFP NΔ427-482 and recMeV-Edm-GFP NΔ420-482 respectively. The growth-kinetics of recMeV-Edm-GFP NΔ427-482 and recMeV-Edm-GFP NΔ420-482 at 32°C showed a day-delay profile with half a log less titer for the former than the latter. Additionally, at 39.5°C, recMeV-Edm-GFP NΔ427-482 mutant virus showed temperature-sensitivity, while recMeV-Edm-GFP NΔ420-482 mutant virus peaked its titers a day later as compared to its corresponding standard.

This proves that the MeV replication is tolerant towards changes in both central Ntail sequence and its length but there is more pronounced effect in the Edmonston strain than in the clinical isolate IC-B strain. This also may be due to varying degrees of susceptibility of infection, relative innate immune responses or also as discussed earlier difference in usage of receptors in both the strains (the IC-B strain uses SLAM as a receptor, whereas the Edmonston strain can use both SLAM and CD46 as receptors). Although, there is no correlation between the intrinsically disordered central N-tail truncation and its specific viral fitness; further experiments are needed to test these mutants in vivo, specifically in rhesus or cynomolgus macaques.

4 CONTINUED STUDIES

In order to probe the functional and mechanistic role of the central section of CDV-Ntail, we have many questions that have still remain unanswered such as: 1) can we truncate further down in the central CDV-N-tail section? 2) Can we truncate the CDV-N-tail section without an artificial Afe-I site? 3) Is there a motif in the central section of the CDV-N-tail? If yes, is the motif really important for viral replication? 4) Are the serine residues in the central section of
CDV-N-tail phosphorylated? And are these residues important for virus rescue? Therefore, in order to evaluate the functional role of the central section of the CDV N-tail further we continued additional studies on the central section of the CDV N-tail.

Because of the minigenome data of CDV NΔ423 and CDV NΔ421 in the previous study, we substituted the N protein encoding ORFs in cDNA genome copies of CDV strain CDV-5804PeH. We also removed the artificial Afe-1 site and substituted the CDV NΔ425-479 plasmid mutant with alanine and aspartic acid residues to create: CDVNΔ425-SERS, CDVNΔ425-SERA, CDVNΔ425-AERS, CDVNΔ425-AERA, CDVNΔ425-SERD, CDVNΔ425-DERS, CDVNΔ425-DERD, respectively. The corresponding recombinant viruses were recovered readily after rescue transfection and overlay onto receptor-positive Vero-canineSLAM (Vero-cSLAM) cells. DNA sequencing after RT-PCR amplification of recovered virus genomes confirmed CDV NΔ425-SERA and CDV NΔ425-DERD mutants but showed a mutation in box 1 of CDV NΔ425-SERS mutant recombinant virus (as shown in Table 2). This led us to believe that the Afe-1 restriction site (SA) replaces the hypothesized natural phosphorylation site in the central section of CDV-N-tail.
<table>
<thead>
<tr>
<th>CDV truncations</th>
<th>422-425&lt;sup&gt;th&lt;/sup&gt; amino acids</th>
<th>Afe site (SA)</th>
<th>Titer (pfu/ml)</th>
<th>RTPCR confirmation</th>
</tr>
</thead>
<tbody>
<tr>
<td>CDV NΔ 425afe</td>
<td>SERS</td>
<td>✓</td>
<td>1.5 × 10^6</td>
<td>✓</td>
</tr>
<tr>
<td>CDV NΔ 425+SERS-noafeP3</td>
<td>SERS</td>
<td>✓</td>
<td>2.0 × 10^5</td>
<td>✓ (mut. in box 1 S→P)</td>
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<tr>
<td>CDV NΔ 425-SERA-noafe</td>
<td>SERA</td>
<td>×</td>
<td>8.76 × 10^5</td>
<td>✓</td>
</tr>
<tr>
<td>CDV NΔ 425-DERD-noafe</td>
<td>DERD</td>
<td>×</td>
<td>1.1 × 10^6</td>
<td>✓</td>
</tr>
<tr>
<td>CDV NΔ 423afe</td>
<td>SE(SA)</td>
<td>✓</td>
<td>3.54 × 10^4</td>
<td>✓</td>
</tr>
<tr>
<td>CDV NΔ 421afe</td>
<td>(SA)</td>
<td>✓</td>
<td>6.57 × 10^6</td>
<td>✓</td>
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</tbody>
</table>

**Table 2: Summary of recCDV N mutant viruses**

When we substituted N protein encoding ORF in cDNA genome copies of CDV strain CDV-5804PeH (wild type (wt)), with the CDV N_AERA (CDV5804-N422-425Ala) and CDV NΔ425-AERA, we could recover the former recombinant virus readily, but not the latter, after rescue transfection and overlay onto receptor-positive Vero-canineSLAM (Vero-cSLAM) cells. Finally, Vero-cSLAM cells were infected with CDV-5804PeH and CDV N_AERA at an MOI of 0.01 TCID<sub>50</sub> units/cell, followed by sampling and titration of cell-associated progeny virus at the indicated time points. The growth profiles overlapped as expected.

In the case of CDV N_AERA, we believe that the downstream serine residues would be functional to regulate the phosphorylation activity we hypothesize in the central section of the CDV N-tail. And in the case of CDV NΔ425-AERA, the hypothesized phosphorylation site is important for CDV replication.
5 DISCUSSION

Bioluminescent assays are very commonly used to study gene expression as well as other cellular components and events that are involved in gene regulation. These assays are very sensitive—allowing quantification of even small changes in transcription— and the availability of results within minutes makes it appealing. They are extremely simple to use and relatively inexpensive.

In bacteria, there is only one enzyme, however, eukaryotes have many polymerases that are each responsible for a specific subset of RNAs. Various promoters are selected depending upon whether viral transcription occurs in the nucleus or the cytoplasm during replication of the virus. Although the majority of the RNA viruses replicate in the cytoplasm, the plasmids of this protocol undergo nuclear transcription and the transcripts then undergo the downstream processes in the cytoplasm. When human cells were cotransfected with different amounts of pol-I reporter plasmid and with optimized ratios of RSV helper plasmids (N, P, M2 – (0.4ug/well each) and L (0.2ug/well). This was done in order to test the amount of reporter to be used subsequently. The reporter was found to be active but the amount of reporter activity was not directly proportional to the amount of reporter plasmid used at 50 hours post transfection as seen in 28 hours post transfection. Although, the pol-I reporter assay showed some technical errors and as the data was based on dependent replicates, the reporter was subsequently used in RSV nucleoside analog testing successfully.

Since monocistronic minireplicons cannot address whether the viral transcriptase retains the ability to successfully negotiate intergenic junctions, we employed a multicistronic reporter assay
We first generated a novel firefly luciferase and gaussia luciferase containing bicistronic minigenome constructs plasmids to address whether N-tail mutants allow RdRp to efficiently negotiate the intergenic junctions in the viral genome. This kind of minigenome construct did not show any appreciable signal and therefore we then generated firefly and nanoluciferase containing bi- and tricistronic minigenome reporter.

In case of the bi and varied tricistronic constructs, the relative reporter expression levels of MeV N-(Δ439-482) did not differ significantly as compared to the standard. In fact, the bicistronic assay showed higher RdRp activity with the mutant as compared to the standard. In the case of tri-P-stopstop construct, the relative reporter expression levels did not differ significantly but was shown to be lower in case of mutant with respect to tri-eGFP and tri-(P-(ΔCV)). Since both- tri-eGFP and tri-(P-(ΔCV)) are shorter than tri-P-stopstop, it seems to be possible that the RdRp processivity depended on the extra residues missing. These results also led us to believe that the non-structural proteins C and V do not influence the multicistronic RdRp activity and show successful negotiation of intergenic junctions by the RdRp complex in the absence of the unstructured Ntail section. Additionally, we have also employed such a multicistronic (both bi- and tricistronic) reporter based system to determine RdRp activity in different morbilliviral N-tail truncated constructs with increasingly large internal truncations of central residues.

We have also generated a series of MeV, CDV, and NiV mutant N proteins with increasingly large internal truncations of central Ntail residues, each ending immediately upstream of MoRE near the Ntail C-terminus. Characterization of these constructs revealed the
role of Ntail in RdRp bioactivity in biochemical assays and morbilliviral fitness and growth kinetics in cell culture including CDV viral pathogenesis analysis. To probe an effect of Ntail truncations on viral pathogenesis, recombinant CDVs were analyzed in a lethal CDV/ferret model of morbillivirus disease. The recombinant viruses displayed different stages of attenuation ranging from ameliorated clinical symptoms to complete survival of infected animals, depending on the molecular nature of the Ntail truncation. Reinfection of surviving animals with pathogenic CDV revealed robust protection against a lethal challenge. The highly attenuated virus was genetically stable after \textit{ex vivo} passaging and recovery from infected animals. Mechanistically, gradual viral attenuation coincided with stepwise-altered viral transcriptase activity in infected cells. These results identify the central Ntail section as a determinant for viral pathogenesis and establish a novel platform to engineer gradual virus attenuation for next-generation paramyxovirus vaccine design.

We can summarize our study using the following model:

\begin{figure}
\centering
\includegraphics[width=\textwidth]{model}
\caption{Models of the mechanistic basis for the impact of Ntail on RdRp transcriptase activity.}
\end{figure}
Upon initialization at the terminal promoter, the viral P-L polymerase complex first synthesizes a short Leader RNA (as shown in the figure 21: L is depicted in grey, P tetramers are shown in blue, orange and yellow circles at the end of Ntails represent MoRE and box3, respectively). Encapsidation of this Leader by N proteins switches the polymerase into replicase mode in which it ignores intergenic junctions and generates a full-length encapsidated copy of the viral genome. If not encapsidated, Leader is released by the polymerase complexes, which then travels in transcriptase mode towards the first gene start site and proceeds generating viral mRNAs. In either case, advancing of the RdRp along the genome involves the release of existing N-MoRE/P-L interactions, Ntail reordering in front of the polymerase complex, and local de-encapsidation of the viral RNA. A) In the first model, removal of the central Ntail section may reduce the encapsidation efficiency of the nascent Leader RNA, causing a transcriptase bias of the polymerase complex (thick horizontal arrow). B) Alternatively, elimination of the central Ntail section may reduce the need for local Ntail ordering ahead of the advancing polymerase complex, narrowing the time window available for Leader encapsidation by accelerating Leader transcription (staggered horizontal arrows) promoting polymerase switch to transcriptase mode.

The significance of this study can be summarized as follows:

**New Vaccination Strategy:** The first attenuated live vaccine for MeV was developed by adaptation of Edm strain to chick embryo fibroblasts but this vaccine was further passaged to develop a more attenuated and non-reactogenic vaccine (137). Limitations to MeV vaccines do exist including the potential for interference from maternal antibodies in young children.
preventing a strong protective response (138, 139). This has led to the re-emergence of MeV infection in highly vaccinated populations (140).

Different individual point mutations have also been tested to engineer attenuation to develop new vaccine strategies. But, this kind of strategy always has the risk of spontaneous reversion to its pathogenic form. To overcome this, DNA vaccines have been proposed as an alternative vaccination strategy for infants and in vivo studies recently. These have demonstrated their potential utility (141, 142) as they elicit strong protective humoral and cell-mediated immune response (143, 144). Although such vaccines are effective and relatively inexpensive to produce, problems associated with vaccination regime and delivery routes need to be resolved.

With the help of an attenuation strategy, we can engineer the unstructured Ntail section in order to modulate the varying degrees of attenuation to develop the next-generation vaccines. This attenuation strategy by Ntail truncation may also be applicable to various other paramyxovirus candidates as we have shown proof-of-concept in minireplicon systems for highly pathogenic NiV. The main advantage of using an attenuation strategy for the development of vaccines for closely related Paramyxoviruses is that it will be highly unlikely for the virus to revert back and turn pathogenic as it cannot de novo synthesize the deleted residues of the nucleoprotein. This kind of tunable attenuation can be very helpful for the development of an efficient vaccines against highly pathogenic members of this family that do not have effective prophylactic measures to date.
Revising the current model of polymerase recruitment and progress along the template and protein interactions: Our current understanding of Paramyxoviridae replication and how N, P and L proteins interact is incomplete. It is also critically important to understand and revise the current model of the proposed interactions between the proteins, in order to aid in the development of effective antiviral treatments. In our study, N proteins with large tail truncations remained bioactive in mono- and polycistronic minireplicon assays and supported efficient replication of recombinant viruses. Bioactivity of Ntail mutants extended to N proteins derived from highly pathogenic Nipah virus. We have hypothesized the predicted location of candidate compensatory mutation to the NΔ425-479 truncation, thereby suggesting the cross-talk of N-core and N-tail, in a model of the CDV RNP assembly. As shown in the figure 21, the truncation in the central Ntail section may allow acceleration of the advancing polymerase complex. This may reduce the time window available for encapsidation and may accelerate transcription.

Mechanistic role of the central section of the N-tail: Knowledge of the replication machinery and the protein-protein interactions involved is very critical for the study and development of effective prophylactic/therapeutic strategies. This study helped us to gain deeper insights into their role in RdRp-mediated transcription and replication. Although the MeV N-tail is considered dispensable for polymerase activity, it is considered to be important for stabilization of the P-L complex onto RNP template (80) By making truncations in the central section of the N-tail, we altered the molecular nature of the interface between N-terminal Ntail residues and the RNP rungs. We noted two different degrees of viral attenuation, depending on the extent of the central CDV-Ntail truncation. By analyzing the quantitations of viral RNA populations present in these virus-infected cells, we observed a single versus double hit effect of the individual modifications
in the N-tail on polymerase function. The first hit is termed by showing a partial shift of polymerase activity from replicase to transcriptase mode compared to the parental recCDV strain. This change was seen in cells infected with either CDV mutant virus. In addition to the first hit, the recCDV NΔ441-479 recombinant also showed a second hit. The second hit shows a relative higher proportion of polycistronic viral message, which directly affects the gradient of functional viral mRNAs available in infected cells.

Currently, we cannot conclude whether these intrinsically disordered N-tails have developed recently or whether they were present in the N proteins of an ancestral mononegavirales. As there is an experimental confirmation that N-tail MORE can be readily moved into paramyxovirus Ncore, we assume that ancestral nucleoproteins were tailless and then gradually the N-tail developed in the modern viruses. As the N-tails developed, the nucleoproteins gained novel N functions such as interactions with host cell factors, to fine-tune viral polymerase activities, to regulate the natural paramyxovirus transcription gradient of mRNA synthesis. These functions seem to explain the evolution of the unstructured Ntail region.

To summarize, we have confirmed that the truncations in the CDV central N-tail section are possible. The additional CDV central N-tail truncated NΔ425 substitutions do not affect RdRp activity. But, the central N-tail truncated mutant viruses closer to the RNP leads to accumulation of some compensatory mutations. We are further investigating the role of a hypothesized motif present in the central section of the CDV-N-tail that may regulate the viral activity. This will give us a better understanding of the repeated serine residues (possibly a motif) present in the central section of the N-tail and help us to definitely revise the current model of RdRp
recruitment and progress along the template. The double-substituted aspartic-acid (CDV-NΔ425_DERD) does not hamper virus rescue but CDV-NΔ425_SERS only grows further with compensatory mutation in box 1 (so far seen). The negative charges/S421 phosphorylation at the region near RNP in the N-tail seems to be important for viral replication. I hypothesize that the 421S residue is phosphorylated and this phosphorylated site between the box 1 and 2 and the presence of all the conserved boxes are main factors important for efficient CDV replication.

The modeling of the cross-talk between morbilliviral N-tail residues and the N-core loop region seems to be very interesting. Further experiments are needed to characterize the role of compensatory mutations in central unstructured N-tail mutants closer to RNP in the future. The effect of compensatory mutations in terms of minireplicon activity, viral fitness, viral pathogenesis and transcription/replication balance needs to be further determined. In the future, the influence of charges and/or S421 phosphorylation on the RNP surface needs to be identified. It may be possible that this is applicable only to the interferon-negative Vero-dog SLAM cells and that the actual hosts (carnivores) show different results. Further in-vivo experiments are needed to prove this.
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APPENDIX

The Unstructured Paramyxovirus Nucleocapsid Protein Tail Domain Modulates Viral Pathogenesis through Regulation of Transcriptase Activity

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ABSTRACT The paramyxovirus replication machinery comprises the viral large (L) protein and phosphoprotein (P)-protein (L-P) in addition to the nucleocapsid (N) protein, which encapsidates the single-stranded RNA genome. Common to paramyxovirus N proteins is a C-terminal tail (Ntail). The mechanistic role and relevance for virus replication of the structurally disordered central Ntail section are unknown. Focusing initially on members of the Morbillivirus genus, a series of measles virus (MeV) and canine distemper virus (CDV) N proteins were generated with internal deletions in the unstructured tail section. N proteins with large tail truncations remained bioactive in mono- and polycistronic minireplicon assays and supported efficient replication of recombinant viruses. Bioactivity of Ntail mutants extended to N proteins derived from highly pathogenic Nipah virus. To probe an effect of Ntail truncations on viral pathogenesis, recombinant CDVs were analyzed in a lethal CDV/ferret model of morbillivirus disease. The recombinant viruses displayed different stages of attenuation ranging from ameliorated clinical symptoms to complete survival of infected animals, depending on the molecular nature of the Ntail truncation. Reinfection of surviving animals with pathogenic CDV revealed robust protection against a lethal challenge. The highly attenuated virus was genetically stable after ex vivo passaging and recovery from infected animals. Mechanistically, gradual viral attenuation coincided with stepwise altered viral transcriptase activity in infected cells. These results identify the central Ntail section as a determinant for viral pathogenesis and establish a novel platform to engineer gradual virus attenuation for next-generation paramyxovirus vaccine design.

IMPORTANCE Investigating the role of the paramyxovirus N protein tail domain (Ntail) in virus replication, we demonstrated in this study that the structurally disordered central Ntail region is a determinant for viral pathogenesis. We show that internal deletions in this Ntail region of up to 55 amino acids in length are compatible with efficient replication of recombinant viruses in cell culture but result in gradual viral attenuation in a lethal canine distemper virus (CDV)/ferret model. Mechanistically, we demonstrate a role of the intact Ntail region in the regulation of viral transcriptase activity. Recombinant viruses with Ntail truncations induce protective immunity against lethal challenge of ferrets with pathogenic CDV. This identification of the unstructured central Ntail domain as a nonessential paramyxovirus pathogenesis factor establishes a foundation for harnessing Ntail truncations for vaccine engineering against emerging and reemerging members of the paramyxovirus family.

KEYWORDS RdRp complex, measles virus, nucleocapsid protein, paramyxovirus, viral pathogenesis, virus replication

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The paramyxovirus family comprises a multitude of major human and animal respiratory pathogens, such as measles virus (MeV), canine distemper virus (CDV), the parainfluenzaviruses, mumps virus (MuV), and the recently emerged highly pathogenic Hendra and Nipah (NiV) viruses (1). Together with the rhadbo-, borna-, filo-, and pneumo-viruses, the paramyxoviruses belong to the order Mononegavirales, which is characterized by nonsegmented negative-polarity RNA genomes that are encapsidated by viral nucleocapsid (N) proteins into helical ribonucleoprotein (RNP) complexes (2–5). Only encapsidated viral RNA serves as a template for the viral RNA-dependent RNA polymerase (RdRp) complex that functions as both the replicase and transcriptase of the viral genome.

Virus-encoded components forming the RdRp are the large (L) protein and its obligatory cofactor, the tetrameric phosphoprotein (P protein). Of these, the L protein harbors all catalytic centers required for RNA polymerization, mRNA capping and methylation, and miRNA polyadenylation, while the P protein binds to both the L and N proteins. This interaction is thought to tether the P-L polymerase to the RNP and trigger transient local release of the encapsidated RNA that gives L protein access to the template (6–13). In addition, the P protein prevents nonproductive aggregation of newly synthesized N proteins through the formation of cytosolic N-P complexes and chaperoning of N protein to nascent genomic and antigenic RNA emerging from the polymerase complex for cotranscriptional encapsidation (14–17).

Paramyxovirus N proteins feature an N-terminal core domain of approximately 400 residues that is responsible for RNA encapsidation and a structurally largely disordered C-terminal tail of approximately 120 to 150 amino acids. Functionally analogous Ncore domains are present in all Mononegavirales. However, only members of the bornavirus, filovirus, and paramyxovirus families contain unstructured Ntail extensions, whereas N proteins of the closely related rhabdo- and pneumoviruses are composed of Ncore only (18). Crystal structure data of N-P complexes of MeV and NiV have revealed that the protein–protein interface consists of a short stretch of residues positioned at the N terminus of the P protein, the P peptide, binding to Ncore (15, 17). However, the initial recruitment of paramyxovirus P-L polymerase to the RNP genome was thought to require an interaction between a C-terminal binding domain in the P protein, designated P-XD, and Ntail (5, 19, 20).

In the case of MeV and CDV, two well-characterized paramyxoviruses of the *Morbillivirus* genus, Ntails harbor three short conserved microdomains, designated box 1 to box 3, that are positioned at its N-terminal (box 1) and C-terminal (box 2 and box 3) ends, respectively (Fig. 1A). Of these domains, box 2 contains a molecular recognition element (MoRE) that mediates interaction with the P-XD through an induced-fit protein–protein interaction (6, 8, 9, 21, 22). The adjacent box 3 of Ntail binds to the viral matrix (M) protein, aiding proper particle assembly (23, 24). In addition, Ntail is reportedly recognized by host cell interferon regulator factor 3 (IRF-3) (25) and box 2 and box 3 residues can interact with the major inducible heat shock protein HSP72 (26).

In contrast, the molecular role of the N-terminal box 1 is less defined (27, 28). Cryo-electron microscopy-based reconstructions of the MeV RNP assembly positioned the Ntail origin at the internal surface of the RNP (29), thus requiring tail residues to protrude toward the outer surface between the rungs of the RNP helix. In fact, not only box 1 residues but also the first approximately 50 amino acids of Ntail were proposed to be buried based on nuclear magnetic resonance (NMR) predictions, leaving only MeV N protein residues 450 to 525 to extend away from the RNP core structure (29, 30). Consistent with this model, proteolytic removal of all of Ntail triggered a major conformational change in the RNP assembly, resulting in a rigidified helix with decreased pitch and diameter compared to those of the native complex. This rigidified helix is considered to be bioactive for replication (31).

The three conserved box domains are separated by the highly variable central Ntail section of approximately 60 amino acids located in MeV from positions 420 to 480, which is considered to be structurally disordered (8, 20, 29, 30, 32). Since none of the
**FIG 1** MeV and CDV N protein mutants with different length deletions in the disordered central tail section. (A) Morbillivirus N protein organization. The Ntail domain missing from the cryo-electron microscopic reconstruction of Ncore (blue-gray; PDB code 4UFT) was added using (Continued on next page)
other paramyxovirus proteins are known to harbor domains of comparable size that are dispensable for virus viability, it was assumed that the central Ntail section also serves a critical role in viral polymerase function. Representing a long-standing view, for instance, a fly-casting model entailed that the unstructured section extends spatial flexibility to MoRE, which was suggested to be required for interaction with P-L polymerase and efficient recruitment of the polymerase complex to the RNP template (33, 34).

When testing this model experimentally, however, we recently demonstrated that MoRE can be physically relocated into Ncore in a recombinant MeV (18), revealing efficient virus replication in the absence of Ntail-mediated spatial flexibility of MoRE. In contrast, combining MoRE relocation with a partial deletion of the central Ntail section resulted in a severely temperature-sensitive virus that was replication defective under physiological growth conditions. Cells infected with this recombinant contained a significantly larger amount of nonproductive polycistronic viral miRNAs. These results may highlight a previously unappreciated role of the unstructured Ntail section in proper mRNA polyadenylation and release, or they could simply be due to unrelated but synergistic adverse effects of MoRE relocation combined with partial central Ntail truncation.

To better define the role of the unstructured Ntail section in virus replication, we explored in this study the effect of internal Ntail truncations on RdRp activity in minireplicon assays and recombinant virus replication, and we employed the lethal CDV/ferret model of morbillivirus disease to query a relation between Ntail-controlled viral polymerase activity and pathogenesis. Our experiments identified the unstructured Ntail section as a nonessential paramyxovirus pathogenesis factor that contributes to viral fitness through ensuring properly balanced viral transcriptase activity. We provide proof of concept that Ntail modifications can be harnessed to engineer genetically stable attenuated paramyxovirus recombinants for next-generation vaccine design.

RESULTS

The functional importance of the structurally disordered central Ntail regions for paramyxovirus polymerase activity is currently mechanistically poorly understood (33, 34). To test whether the morbillivirus unstructured central Ntail section is required for virus replication, we designed a series of progressively larger internal tail deletions in MeV N protein, ultimately extending to eliminating most of the structurally disordered residues between box 1 and MoRE (Fig. 1A). Figure 1B provides an overview of the morbillivirus (MeV and CDV) Ntail truncations targeted. The largest deletion in MeV N protein eliminated all of Ntail from residues 399 to 482, which also includes the conserved box 1 region at the N-terminal origin of the tail. Naturally, gradual removal of the central Ntail residues positions the C-terminal end of Ntail harboring MoRE and box 3 in immediate proximity of the trunk of the helical RNP assembly.

Bioactivity of transiently expressed MeV and CDV Ntail mutant proteins.

Western blot analyses of cell lysates after transient expression of the different Ntail

FIG 1 Legend (Continued)

Cost for length illustration, assuming a perpendicular orientation of Ntail to the axis of the helical RNP assembly (right). Conserved box regions are highlighted in yellow, orange, and green. Heat maps (red) represented the predicted degree of structural disorder. The lower portion shows a model of an Ntail mutant after removal of the disordered central Ntail section. The truncation points box 1 and 3 regions in close proximity to the trunk of the RNP assembly. (B) Sequence alignment of the MeV and CDV Ntail domains. Box 1 to 3 domains are color-coded as described for panel A. Truncation donor (red) and acceptor (green) residues explored in this study are highlighted; residues in the structurally disordered region are underlined. Alignments were generated using T-Coffee (69). (C and E) Stability levels of MeV (C) and CDV N (E) protein mutants in transiently transfected BSR T7/5's cells. Immunoblots were developed with specific antibodies directed against MeV and CDV N protein, respectively, or cellular GAPDH. Numbers represent means of densitometry quantities of three biological repeats ± SEM, MW, molecular weight, in thousands. (D and F) Polycistronic minigenome assays with N protein mutants specified in panels C and E. Symbols represent relative luciferase units of each biological replicate and normalized for standard N protein. Columns show sample means ± SEM; one-way ANOVA with Tukey's post hoc test was used. (G and H) Tricistronic minigenome assays. Symbols represent mean relative reporter activity ratios of each biological replicate, with error bars showing SEM; one-way ANOVA with Tukey's post hoc test was used. NS, not significant.
mutants confirmed stable expression of all modified N proteins and revealed the anticipated gradual increase in electrophoretic mobility with expanding truncation size (Fig. 1C). The two constructs harboring partial or complete deletions of box 1 (NΔ399–482 and NΔ409–482) showed substantially reduced and abrogated bioactivity, respectively, in minireplicon reporter assays. All other mutants supported efficient RdRp activity, and none of the small variations from activity observed in the presence of standard N protein was statistically significant.

Encouraged by these results, we generated a comparable series of CDV N deletion mutants informed by the Ntail linear sequence alignments (Fig. 1B). Resembling the corresponding MeV N protein mutant, expression levels and bioactivity of CDV NΔ441–479 and NΔ425–479 were indistinguishable from those of standard CDV N protein in Western blotting and minireplicon assays (Fig. 1E and F). However, larger truncations (CDV NΔ423–479 and NΔ421–479, respectively) resulted in significantly enhanced (approximately 2-fold) minireplicon activities. As noted for MeV, truncation encroaching into the box 1 section abolished CDV N protein bioactivity.

To address whether the viral RdRp successfully negotiates intergenic junctions in the presence of the mutant N proteins, we employed a recently described tricistronic minireplicon construct (Fig. 3D in reference 1B) shows a schematic of the construct) that contains two intergenic junctions and distinct firefly luciferase and nanoluciferase reporter genes in the first and third reading frame positions, respectively. In this assay, the relative ratio of downstream versus upstream reporter activity represents the relative efficiency with which the RdRp complex advances through intergenic junctions and reinitiates mRNA synthesis. When tested against a subset of the MeV (Fig. 1G) and CDV (Fig. 1H) Ntail mutants, only the MeV NΔ420–482 construct returned a slight (approximately 25%), but statistically significant, relative reduction of third open reading frame (ORF) expression compared to the relative reporter ratios observed with standard N protein. These results suggest successful negotiation of intergenic junctions by the RdRp complex in the absence of the unstructured Ntail section.

**Effect of Ntail truncations on NIV bioactivity.** To evaluate whether continued RdRp activity in the absence of the central Ntail section extends to paramyxoviruses outside the morbillivirus genus, we applied an equivalent truncation strategy to the Ntail of highly pathogenic NIV, a member of the henipavirus genus (Fig. 2A and B).
Although the organization of the NIV tail is more complex than that of the morbilli-viruses, featuring an additional box4 in the central Ntail section (11), NIV RdRp readily accepted template RNA encapsidated by tail-truncated N proteins (Fig. 2C). The NIV Ntail modifications did not substantially affect N protein steady-state levels in immunoblots (Fig. 2D). Whereas the NIV N423–471 mutant protein with the larger truncation showed standard NIV N-like activity in minireplicon assays, bioactivity of the NIV N construct harboring a shorter (N442–471) truncation was significantly increased.

**Recovery of recombinant MeV and CDV encoding tail-truncated N proteins.** RdRp activity in minireplicon assays is necessary but not sufficient for support of a full viral replication cycle. To test a role of the unstructured Ntail section in virus replication, we exchanged the N protein-encoding ORFs in cDNA copies of the genomes of two pathogenic viral isolates, MeV-IC-B (35) and CDV-5804PeH (36). Two different Ntail truncations each were inserted, MeV N439–482 and N420–482, and CDV N441–479 and N425–479, respectively, which in each case represent the shortest and largest internal tail truncation that did not significantly alter N protein bioactivity in the monocistronic minireplicon assays. The corresponding recombinant viruses were recovered readily on receptor-positive Vero-human SLAM (Vero-hSLAM) and Vero-canine SLAM (Vero-cSLAM) cells, respectively. DNA sequencing after reverse transcription-PCR (RT-PCR) amplification of recovered virus genomes and Western blot analyses of infected cell lysates (Fig. 3A and B) confirmed the presence of the respective Ntail truncations in the recombinant viruses.

Multiple-step growth curves revealed parent virus-like replication of recMeV-IC-B N439–482 (Fig. 3C), whereas recMeV-IC-B N420–482 with the larger Ntail truncation showed an initial 12-h growth delay. Modeling of growth profiles confirmed that the maximal population doubling times of recMeV-IC-B N420–482 were significantly longer than those of standard recMeV-IC-B, but differences between recMeV-IC-B N439–482 and the parent strain remained not significant (Fig. 3D).

This growth pattern of the MeV mutants was inversely in the recombinant CDV strains (Fig. 3E), recCDV N425–479 carrying the larger Ntail truncation showed significantly shorter maximal doubling times than recCDV N441–479 (Fig. 3F). While peak growth rates of both mutant strains lagged behind that of the parental recCDV-5804PeH, growth profile modeling did not reveal significant differences in final progeny titers reached. These data demonstrate that the unstructured central Ntail section is not associated with an essential RdRp function required for virus replication, establish distinct effects of different length truncations on viral fitness in cell culture, and indicate that the actual impact of internal Ntail truncations on virus growth is not proportional to the length of the deletion but must be individually determined.

In preparation of in vivo pathogenesis testing in the lethal CDV/ferret model, which recapitulates key features of human measles (37), we determined stimulation of the type I interferon response by the different CDV mutants in cultured cells. While recCDV N425–479 did not significantly change levels of induction of alpha interferon (IFN-α), IFN-β, or a subset of selected interferon-stimulated genes (ISGs) compared to those in cells infected with standard recCDV-5804PeH, significantly higher levels of induction of IFN-α, IFN-β, and ISGs were found after infection with recCDV N441–479 (Fig. 3G). These data suggest stronger stimulation or impaired downregulation of the host innate antiviral response by recCDV N441–479.

**Pathogenesis of Ntail-mutated CDV in ferrets.** Ferrets were infected intranasally with 2 × 10^5.50 tissue culture infectious dose (TCID₅₀) units of standard recCDV-5804PeH or the two recCDV Ntail mutants, and clinical signs, peripheral blood mononuclear cell (PBMC)-associated viremia titers, white blood cell counts, and lymphocyte proliferation response were monitored in regular intervals. Consistent with our previous experiences with the model (33), peak viremia titers were reached 7 days postinfection, followed by a rapid decline in viral load in animals infected with either of the N mutant viruses (Fig. 4A). All animals infected with standard recCDV-5804PeH succumbed to the disease by day 14. In contrast, the group that had received recCDV N441–479 and 75% of animals in the recCDV N425–479 group survived the infection (Fig. 4B). In
recovering animals, viremia fully subsided 21 (recCDV N441–479) and 35 (recCDV N425–479) days after infection.

Animals infected with the parental virus experienced severe disease with extensive rash, substantial weight loss, and high fever (Fig. 4C to E). In comparison, disease progression was less aggressive in animals infected with the recCDV N425–479
mutant virus and mild in animals of the recCDV N441-479 group. Specifically, recCDV N441-479-infected ferrets showed a benign, localized rash and only transiently lost a moderate (~10%) amount of body weight before making a full recovery (Fig. 4D). Fever peaked in these animals 2 to 3 days earlier and at a lower level than in recCDV-5804PeH
and recCDV ND425–479-infected animals and resolved within the second week after infection (Fig. 4E). In contrast, recCDV ND425–479-infected ferrets presented with a longer weight loss period and fever fully resolved only in the third week after infection.

Acute lymphopenia and temporary lack of lymphocyte responsiveness to stimulation are hallmarks of morbillivirus infections (39). When assessing immune competence of animals in the different groups, we noted significantly milder lymphopenia early after infection in the recCDV ND441–479 group than in ferrets that had received standard recCDV-S804PeH or recCDV ND425–479 (Fig. 4F). However, lymphocytes derived from animals of all groups showed similar declines in proliferation responsiveness during the first 2 weeks after infection (Fig. 4G). Proliferation response improved in all surviving animals only at 35 days postinfection, although we noted a temporary rebound in cells extracted from recCDV ND441–479-infected ferrets at the 21-day time point.

**Ferret immune response to Ntail-truncated CDVs.** Ferrets infected with the two mutant viruses mounted overall comparable type I interferon responses, reaching approximately 10-fold induction levels in IFN-β and MX-1 message, the latter representing one of the major ISGs in the ferret response to CDV infection (Fig. 5A). Reversing our results obtained in cultured cells, however, PBMcs derived from recCDV ND441–479-infected animals showed significantly higher induction levels in IFN-β and ISG message at day 7 postinoculation than at day 3.

Anti-CDV antibody responses were robust in animals of either group, although neutralizing antibody titers induced by the more attenuated recCDV ND441–479 peaked slightly higher than those found in animals of the recCDV ND425–479 group (Fig. 5B). Consistent with our past experience (38), low-level neutralizing antibodies were detectable in ferrets inoculated with standard recCDV-S804PeH at day 7, but titers did not reach robust levels by the time animals succumbed to the infection. To determine whether immune responses mounted by the surviving animals infected with the Ntail mutant viruses were protective, we challenged them with a lethal dose of standard recCDV-S804PeH at 49 days after the original infection. All challenged animals survived (Fig. 5C), and none developed appreciable viremia (Fig. 5D), showed clinical signs, or experienced severe lymphopenia (Fig. 5E).

These results identify the unstructured Ntail section as a determinant of paramyxovirus pathogenesis. Gradual shortening of the tail induces different degrees of viral attenuation, although not with direct proportionality. Importantly, all surviving animals in the CDV-ferret model were completely protected against a lethal challenge with standard CDV, underscoring efficient induction of functional immune responses by the Ntail-modified recombinants.

**Genetic stability of Ntail truncation in cell culture and in vivo.** To assess the genetic stability of the Ntail-truncated CDV, we subjected viral RNA preparations to deep sequencing before and after 10 or 11 passages in cell culture and determined N ORF consensus sequences in viral RNA extracted from PBMcs harvested from ferrets 7 days after infection through Sanger sequencing (Table 1). Neither standard recCDV-S804PeH nor recCDV ND441–479 showed any consensus changes in the N or P protein ORF compared to the corresponding genomic cDNA plasmids. However, recCDV ND425–479 carried a glutamate-to-glutamine substitution at N residue 156 that was dominant in the viral population after four cell culture passages and acquired an alanine-to-aspartate exchange at N residue 410 that became increasingly fixed during passaging. All recCDV ND425–479 recovered from infected ferrets at the peak of viremia contained both substitutions. Comparison with N protein sequences revealing a variety of different circulating CDV strains and isolates revealed that the N protein ORF is sequence conserved at position 156 and shows only conservative variations from the aspartate substitution at residue 410 (i.e., threonine or alanine residues in circulating strains).

None of the CDV recombinants harbored coding mutations in the P and L protein ORFs, with the exception of a single recCDV ND441–479 passaging line that carried a
leucine-to-proline substitution at L protein residue 2175 with approximately 50% allele frequency after 15 passages in cell culture (Table 1). Since all other recCDV ND441–479 lines analyzed in parallel lacked any allele variation at this position, this mutation most likely represents a stochastic event that became partially fixed in the genome.

These observations demonstrate that the ND441–479 truncation is genetically stable over a number of generations in cell culture and after in vivo passage of the recombinant strain. Efficient growth of recCDV ND425–479 appears to be linked to the presence of two compensatory mutations, one located in Ncrr (residue 156) and the second in box 1 of Nnrr (residue 410). Localization of CDV N residue 156 in a structural model of the morbillivirus RNP assembly posits this substitution at the
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*aa: amino acid; NA: not applicable; TC: tissue culture; NC: no change from previously published sequence (5a); ND: not determined based on few allele variations after tissue culture passages (exceptions are noted). Boldface indicates deviations from the input consensus sequence.

**The input cDNA and ferret DNA sequences were determined through Sanger sequencing. “TC” indicates that the virus was passaged in tissue culture and that its sequence was determined through deep sequencing. P_{n,m}, the number (n) of consecutive passages (P) carried out in a independent parallel assessments.

*Not determined because the sequence was not subject to in vitro amplification or PCR-based modification because it was the last sequence verified.

**Virus was extracted after in vivo passage from hamster PMCs. The input viruses for animal studies were TC-Pr (recCDV NA441–479) and TC-Pr (recCDV NA475–479).

*Determined for each animal (n = 4) after Sanger sequencing of virus extracted from PMCs.

C-terminal end of a flexible loop in Ncore (18), orientated toward the interface between consecutive turns of the RNP helix (Fig. 6) where the N tails are thought to emerge from the RNP core (40). This substitution may thus highlight direct cross talk between Ntail residues located immediately downstream of position 425 and in the Ncore loop region harboring residue 156.

Quantitation of viral RNA populations in cells infected with Ntail mutant MeV or CDV. To further elucidate the mechanistic basis for the altered CDV pathogenesis profiles, we analyzed viral RNA populations synthesized in cells infected with the different recombinant virus strains. Cotranscriptional paramyxovirus mRNA editing results in the expression of two additional proteins, V and W proteins, from the viral P protein ORF through the deletion of nontemplated G residues at an editing site (41–44). RNA editing is thought to result from backsliding of the RdRP complex on the RNP template (41, 45, 46), which requires structural flexibility that could be mediated by the flexible central Ntail section. Since impaired V protein expression causes viral attenuation (47), we employed a MiSeq assay to quantify the relative ratios of P, V, and W protein-encoding RNAs in infected cells. Only recCDV NA441–479 and the corresponding recMeV-IC8-Na439–482 were analyzed, based on higher attenuation of this shorter CDV truncation mutant in the ferret model. Relative mRNA distributions were comparable between MeV and CDV, but we noted only minor changes when mutant and the corresponding parent viruses were compared (Fig. 7A and B).

We therefore examined whether attenuation of the recCDV Ntail mutants in ferrets may alternatively result from deregulated viral RdRP activity. Using an RT-quantitative PCR (qPCR)–based approach, we quantified viral genome copies in infected cells, determined relative N and L protein-encoding mRNA levels, examined relative ratios of

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L to N protein-encoding mRNAs, and calculated the relative frequencies with which polycistronic viral mRNAs are synthesized by the different recombinants.

Viral genome copy numbers of both mutant recCDV strains were reduced by approximately 19 to 29% at the end of the replication cycle compared to standard virus (Fig. 7C). In contrast, relative N protein-encoding mRNA levels of either mutant strain were increased approximately 1.8-fold (Fig. 7D). When we examined mRNA levels of the downstream-most positioned L protein ORF, we noted that this 1.8-fold relative increase was maintained in recCDV NΔ425–479-infected cells but boosted to an approximately 3-fold relative excess in the case of recCDV NΔ441–479 (Fig. 7E). Replication of recCDV NΔ441–479 furthermore results in a significant increase in the amounts of L protein-encoding message relative to N protein mRNAs, while essentially identical ratios of L to N protein message were obtained for standard recCDV and the less attenuated recCDV NΔ425–479 (Fig. 7F).

This higher relative L protein mRNA level produced by recCDV NΔ441–479 may reflect an increase in bona fide L protein message due to a lowered premature termination rate of RdRp or a higher proportion of nonproductive polycistronic mRNAs. We therefore quantified the relative content of polycistronic message generated at the first (Fig. 7G) and last (Fig. 7H) intergenic sequences (IGSs) in the recCDV genomes. Depending on the IGS examined, replication of standard recCDV-S804Peh and recCDV NΔ425–479 produced 2 to 10% polycistronic message relative to total message synthesized for the preceding ORF. At each IGS examined, however, we noted a significantly higher ratio of polycistronic message present in cells infected with the recCDV NΔ441–479 mutant strain.

These results implicate the structurally disordered central Ntail section in affecting paramyxovirus transcriptase function on two levels. Both Ntail mutant strains show heightened transcriptase activity relative to that of the parental recCDV strain. In addition, the more severely attenuated recCDV NΔ441–479 further disturbs the relative ratio of viral message in infected cells by generating a higher proportion of nonproductive polycistronic mRNAs.
FIG 7 | RNA populations present in cells infected with Ntai1 mutant viruses. (A and B) MiSeq analysis of viral P mRNA editing by recCDV N5441-479 (A) and recMeV N5439-482 (B), or the corresponding parent virus. Values represent a mean percentage of 93,741 reads each and are expressed as mean percentage of the differentially edited mRNA relative to total P ORF transcripts = SEM. (C) qRT-PCR quantitation of relative CDV genome copy numbers in cells infected with the recCDV Ntai1 mutants. First-strand synthesis was done with specific primers binding to the viral genome untranslated region (UTR). (D and E) qRT-PCR quantitation of relative CDV N mRNA (D) and L mRNA (E) copy numbers present in RNA preparations as in panel C. First-strand synthesis was done with oligo(dT) primers. (F to H) qRT-PCR quantitations of viral N preemptions as in panel C of the relative ratios of L and N protein-encoding mRNAs (F) and of polycistronic mRNA covering the N/P (G) and mKarsL (H) intergenic sequence (IGS). First-strand synthesis was done with oligo(dT) primers. In panels C to H, symbols represent individual values of three biological repeats analyzed in two technical replicates each. Columns show means = SEM; one-way ANOVA with Tukey’s post hoc test was performed.

DISCUSSION

Our characterization of Ntai1 truncation mutants revealed three major effects of the unstructured Ntai1 region on RdRp activity, viral fitness in cell culture, and viral pathogenesis.

First, all Ntai1 mutants with truncations in the disordered central tail section remained bioactive in monocistronic and, tested for MeV and CDV constructs only, tricistronic minireplicon assays. Only MeV-derived Ntai1 mutants showed a slightly impaired ability to transcribe the downstream reporter ORF in the tricistronic assay.
demonstrating that the central Ntail domain is not essential for negotiation of the IGS sections. In contrast, truncations eliminating the conserved box1 caused a decline in RdRp activity, which is most likely due to an impaired protein-protein interface between box1 and Ncore. The compensatory mutation found at Ncore position 156 in recCDV-NΔ425−479 reinforces this hypothesis of a direct cross talk between Ncore and tail residues. Altering the molecular nature of the interface between N-terminal Ntail residues and the RNP rungs could possibly affect local structural rearrangements that are required to transiently provide polymerase access to the genomic RNA.

Second, recombinant MeV and CDV replicate productively in the absence of the central Ntail section in cell culture. This surprising finding could not be extrapolated from the minireplicon data, since in our experience sustained RdRp activity in minireplicon assays is necessary but not sufficient for completion of a viral replication cycle (48). Intriguingly, the extent of the internal deletion affected viral growth kinetics, but no direct proportional correlation exists between the number of residues removed and fitness penalty. Our study demonstrates, however, that morbillivirus replication is tolerant toward changes in both central Ntail sequence and length, which is consistent with the notion that N proteins of ancestral Alphacoronaviruses may have lacked an unstructured tail domain (18).

Third, consistent with this proposed role of the central Ntail section as a regulatory element for optimal viral polymerase activity, we found that Ntail is a determinant for paramyxovirus pathogenesis in vivo. Depending on the extent of the central Ntail truncation, we found different degrees of viral attenuation. Our quantitations of viral RNA populations present in infected cells linked this differential loss in pathogenesis to a single versus double hit on polymerase function. The first hit is experienced by both CDV mutants and entails a partial shift of polymerase activity from transcriptase to transcriptase mode, evidenced by a higher relative proportion of first ORF-encoded viral message and lower relative genome copy numbers in cells infected with either mutant compared to standard recCDV-5804PeH. The decision of whether the paramyxovirus RdRp complex proceeds as transcriptase or replicase after initialization of de novo RNA synthesis depends on the concurrent encapsidation of a short leader RNA strand with N protein (49). Ntail may directly participate in the structural reorganization required for transfer of N proteins from N−P complexes to the nascent RNA for encapsidation (45). Tail shortening may affect the efficiency of this reaction, driving the RdRp complexes into transcriptase mode (Fig. 8A). Alternatively, Ntail could modulate the time window available for leader RNA encapsidation by slowing the progress of the advancing polymerase complex. Cryo-electron microscopy (12) and NMR spectroscopy studies (29) suggest that binding of the RdRp complex to the viral RNP involves local Ntail ordering. RNPs with shortened Ntails could mimic a permanently ordered state, potentially accelerating advance of the RdRp complex along the RNP and thus narrowing the window of opportunity for efficient leader RNA encapsidation (Fig. 8B). At later stages of the replication cycle, the ensuing relative excess of viral message may alleviate transcriptase bias of the polymerase complex to some degree, restoring sufficient replicase function in the presence of Ntail truncations for successful genome replication. It is noteworthy that the Ntail deletions did not simply reduce all RdRp activity. Our data thus show that the disordered central Ntail section is not required for initial polymerase loading onto the RNP template, eliminating a central prediction of the original model of Ntail attraction of P−L polymerase to the RNP (33, 34).

As a second hit experienced only by recCDV NΔ441−479, we noted a larger amount of polycistronic viral message. Since only the first ORF of a polycistronic paramyxovirus mRNA can be translated (49), this hit directly affects the viral protein transcription gradient established in a recCDV NΔ441−479-infected cell. This finding implicates the unstructured central Ntail section in the proper recognition of viral gene end sequences located near the beginning of each intergenic junction. Paramyxovirus mRNA polyadenylation is not templated but is thought to be achieved through backsliding of the RdRp complex on the RNP template, which also entails slippage of the nascent mRNA relative to the template strand (45). Possibly, Ntail is involved in providing additional
structural flexibility to optimize backsliding efficiency. However, the recCDV N0425–479 strain, with the larger truncation, was not subject to the second hit on transcriptase function. While providing a mechanistic explanation for the higher relative fitness of this mutant strain both in cell culture and in vivo compared to recCDV N0441–479, this finding suggests that the actual Ntai amino acid sequence rather than tail length determines how efficiently gene end signals are recognized by the transcriptase complex. The shorter N0425–479 tail may provide higher structural flexibility than the N0441–479 mutant.

Although rational viral attenuation for vaccine design has gained considerable traction in recent years (50, 51), we propose that three features in particular recommend the central paramyxovirus Ntai section as an attractive target for the engineering of next-generation recombinant vaccine strains: the promise of genetic stability, adjustability of attenuation, and application to related or emerging members of the family.

Engineering attenuation through individual point mutations or a small panel of mutations is straightforward but at risk of spontaneous reversion to the pathogenic form. A recent approach to mitigate the problem introduces a large number of changes, i.e., through codon deoptimization strategies (50), which generate sufficient redundancies to ensure that attenuation is maintained faithfully. However, calibrating the balance between residual pathogenicity and immunogenicity remains a challenge. Our identification of a role of the unstructured paramyxovirus Ntai section in modulating RdRps activities provides an opportunity to dial attenuation toward the desired balance. The CDV/ferret data provide proof of concept for the validity of the approach.

Large internal deletions in coding sequences are furthermore at very low risk of spontaneous reversion, since the high error rates of RdRps result from base mismatches rather than de novo creation of code (52) and homologous recombination events are rare in negative-sense RNA viruses with encapsidated genomes (53). In addition, sequence analysis of recCDV N0441–479 mutants after virus passage did not reveal any additional mutation or larger genome rearrangements in the N protein or its binding partner, the P protein. Efficient growth of the recCDV N0425–479 recombinant harboring the larger Ntai truncation appeared to require, however, the presence of two
candidate compensatory mutations. Future work will probe functional and mechanistic links between these substitutions and the Na\textsubscript{425→479} truncation, but the available data suggest that placing engineered N\textsubscript{t}ail truncation junctions too close to the RNP core calls for additional mutations and should therefore be avoided in the interest of genetic stability of the recombinant virus.

We furthermore expect that attenuation by N\textsubscript{tail} truncation will be applicable to different paramyxovirus targets. A safe and effective anti-M\textsubscript{e}V prophylaxis based on the live attenuated Me\textsubscript{e}V Edmonton strain exists, but CDV vaccine safety and efficacy remain problematic in species with unknown or high sensitivity, making the development of a next-generation vaccine with a known attenuation mechanism desirable (54, 55). Importantly, the NIH minireplicon data imply that the approach may be extendable to the highly pathogenic hemipneumoviruses, which are listed by the World Health Organization among the top emerging diseases likely to cause major epidemics (56, 57).

Provided that acceptable safety and immunogenicity profiles can be established and protection demonstrated in surrogate models (58), a broadly applicable, genetically stable, and tunable attenuation strategy could shorten vaccine development response times to clinically significant emerging or reemerging paramyxoviruses.

**MATERIALS AND METHODS**

**Cell culture.** Baby hamster kidney cells (BHK-21, ATCC) stably expressing 17 polymerase (BSR-T7/5 [79]), human coronavirus 229E N-protein (N371 [15]), and human coronavirus OC43 N-protein (OC43-ATCC) stably expressing human or canine lymphocytic activation molecule (Vero/vN5A or Vero-SLAM, respectively [62]) were maintained at 32°C and 5% CO\textsubscript{2} in Dulbecco’s modified Eagle’s medium (DME) supplemented with 7.5% fetal bovine serum. All stable cell lines were incubated in the presence of G-418 (100 \( \mu \)g/ml) at every fifth passage. Geneticin (Novagen) reagent was used for all transient transfections of cells.

**Molecular biology.** Plasmids encoding expression constructs of Me\textsubscript{e}V strain Edmonton N, P, and L proteins (83), Me\textsubscript{e}V strain W/971 P and L proteins (55, 48), CDV strain Underoot N, P, and L proteins (62), and NIV N, P, and L proteins (63) were previously described. Likewise, plasmids harboring full-length cDNA copies of the Me\textsubscript{e}V strain ICN L-protein (56), CDV strain 58048P2F3L-protein (56), and the different Me\textsubscript{e}V minireplicons (58) and shuttle vectors harboring Me\textsubscript{e}V strain ICN and CDV strain 58048P2F3L-derived N-protein ORFs were previously reported (48). A cloning strategy developed in our earlier work (48) was applied to generate all Me\textsubscript{e}V, CDV, and NIV N genes with internal N\textsubscript{tail} truncations. Briefly, sets of PCR primers were engineered that flanked the specific nucleotides targeted for deletion and contained terminal MfeI restriction sites in frame with the N-protein ORF. Religation of the MfeI-digested PCR products reconstituted the expression plasmid, now replacing the targeted N\textsubscript{tail} section with Ser/Ala residues encoded by the MfeI site. All N\textsubscript{tail} modifications were confirmed by DNA sequencing. In addition, all full-length genome plasmids were sequence confirmed prior to recovery transfection of recombinant virions. To generate an NIH nanoluciferase minireplicon reporter construct, the nanoluciferase gene was amplified using appropriate PCR primers and the resulting product cloned into an existing NIH replication backbone (49) that was likewise PCR amplified using appropriate primers. The nanoluciferase amplicon was ligated to the replicon vector backbone using the NotI/NotI pair in accordance with the manufacturer's protocols (New England Biolabs), and the resulting plasmid sequence was verified.

**Immunoblotting, SDS-PAGE, and antibodies.** BSR-T7/5 cells transfected in a 12-well plate format (4 × 10\textsuperscript{5} per well) with 2 \( \mu \)g of Me\textsubscript{e}V or CDV N-protein-encoding plasmid DNA were washed once 4 h after transfection with phosphate-buffered saline (PBS) and fixed in radiommunoprecipitation assay (RIPA) buffer (1% sodium deoxycholate, 1% NP-40, 150 mM Na\textsubscript{2}O, 50 mM Tris-C1 pH 7.2, 1 mM EDTA, 50 mM NaF, 0.02% sodium dodecyl sulfate (SDS), protease inhibitors [Roche]). Glutaraldehyde (concentration at 0.004% g and 4°C for 10 min) were mixed with 5× sample buffer (200 mM Tris-C1 pH 6.8, 8% urea, 5% SDS, 0.1% bromphenol blue, 1.5% 2-mercaptoethanol). Samples were denatured for 30 min at 50°C, fractionated on 10% SDS-PAGE gels, blotted on polyvinylidene difluoride (PVDF) membranes, and subjected to enhanced chemiluminescence detection using specific antibodies directed against Me\textsubscript{e}V N protein (AB20899; Millipore), CDV (D12-12; Bio-Rad), CDV None (clone 1210; 64), polyclonal whole NIH immune serum, or glyceraldehyde-3-phosphate dehydrogenase (GAPDH; IC; Ambion). Immunoblots were developed using a ChemiDoc digital imaging system (Bio-Rad) and the Image Lab software package (Bio-Rad) for image visualization. Densitometry was carried out on non-saturated images with global background correction. Positive and negative controls were included on each individual gel, and no normalization across different blots was carried out.

**Minireplicon luciferase reporter assay.** BSR-T7/5 cells (5,000 in a 96-well plate format) were transfected with plasmids encoding IC-N5 (0.02 \( \mu \)g), IC-P (0.02 \( \mu \)g), and IC-N5 (0.016 \( \mu \)g) and the respective Me\textsubscript{e}V luciferase reporter plasmid (0.004 \( \mu \)g). CDV minireplicon assays were performed accordingly with the helper plasmids. For NIH minireplicon experiments, cells were transfected with plasmid DNA encoding NIH N-protein (0.0005 \( \mu \)g), NIH P protein (0.005 \( \mu \)g), NIH N-protein (0.01 \( \mu \)g), and NIH nanoluciferase replicon reporter (0.006 \( \mu \)g). Firefly luciferase or nanoluciferase activities were determined 40 h posttransfection in a Synergy H1 microplate reader (Bio-Tek), using Bright-Glo or Nano-Glo luciferase substrate (Promega), respectively. Substrates were directly added to the cells; luciferase measurements were quenched after a 5-min incubation for...
signal stabilization. Relative RIIp activities (48), expressed as percentages, were determined on the basis of the formula \( \frac{\text{signal}_{\text{RIIp}}}{\text{signal}_{\text{RIIp}} + \text{signal}_{\text{wildtype}}} \times 100\% \). All experiments were performed in at least 5 independent replicates, each measured in nine independent repeats.

Virus recovery. Recombinant AV 1 or CDV was recovered in E50 E25 cells by transfecting 0.25 μg of the cDNA copy of the modified genome and I-Cre-N (0.42 μg), I-Cre-P (0.54 μg) and I-Cre-I (0.55 μg). All recombinant CDV genomes harbored an additional transcription unit encoding the firefly luciferase protein in pro–protease OVP position, which does not affect viral pathogenicity (49). Transfected cells were overlaid 48 hr after transfection onto Vero-BLA cells or Vero-E1 cells, and emerging infectious particles were passaged in Vero-BLA cells or Vero-E1 cells, respectively. All recombinant virus strains used in this study were recovered in the Plemper lab. The integrity of newly recovered virus strains was confirmed by extracting total RNA from infected cells (Blenny minilite, Qiagen) and generating cDNA copies using random hexamer primers and Superscript III reverse transcriptase (Invitrogen). Modified genome regions were amplified using appropriate primers and subjected to Sanger sequencing.

Preparation of virus stocks. AV 2 or CDV virus stocks were prepared by infecting Vero-BLA or Vero-CMV-3 cells at a multiplicity of infection (MOI) of 0.01 100% tissue culture infectious dose (TCID50) unit per cell, followed by incubation at 37°C. When microscopically observed virus-induced cytopathicity reached approximately 90%, cell-associated progeny particles were released through freeze-thaw cycles and titrated determined by TCID50 titration on Vero-BLA or Vero-CMV-3 cells as described previously (60).

Multistep Virus growth curves. Prior to infection for multistep growth curves, viral stocks were diluted to approximately 1 × 10^6 TCID50 unit/ml and exact titers determined in a separate aliquot by TCID50 titration. Vero-BLA cells (1 × 10^5) in a 12-well format were infected with the different MOI or CDV strains at an MOI of 0.01 TCID50 unit per cell for 1 hr and the inoculum was replaced with MEM. Individual wells were harvested at 12-h intervals, and cell-associated progeny virus titre determined by TCID50 titration. At least three independent growth curves were generated for each virus strain examined. Virus-induced cytopathicity in infected cells was documented using an inverted fluorescent microscope (Nikon) equipped with a digital imaging package.

CDV ferret studies. Male and female adult European ferrets (Mustela putorius foina) without immunity against CDV were used in this study. Animals were infected intranasally with 2 × 10^5 TCID50 units of recCDV-SH04/PBH per animal, and blood samples were collected from the jugular vein at the listed time points. Three parameters of viremia—peak, body temperature, and weight loss—were measured and graded based on a scale of 0 to 3. Scoring for peak viremia was as follows: 0, no rise; 1, localized rash; 2, generalised rash. Scoring for body temperature was as follows: 0, no fever; 1, temperature rise of 39.2°C and 2, temperature rise of 40°C. Scoring for weight loss compared to weight on day 0 was as follows: 0, no change; 1, 5% to 10%; 2, 10% to 15%; and 3, >15%. For white blood cell counts, 10^9/l of heparinized blood was diluted in 500 μl of 3% acetic acid and white blood cells were counted. Cell-associated viremia was quantified by first isolating total white blood cells, followed by addition to Vero-BLA cells in 10-fold dilution steps for TCID50 titration. Assays for proliferation activity of Ficoll-purified (GE Healthcare) PBMCs cells were stimulated with 0.2 μg of phytohemagglutinin (PHA, Sigma) for 24 hr, followed by incubation of 10 μl 5-bromo-2'-deoxyuridine (BrdU, Roche). After another 24 hr incubation period, cells were fixed and BrdU incorporation was quantified using a peroxidase-linked anti-BrdU antibody in a chemiluminescence assay (Roche). Signals were detected in a microplate luminometer (Polarstar, Sheriff and the extent of proliferation was expressed as the ratio of normalized to unstimulated cells.

Quantification of neutralizing antibodies. Neutralizing antibody titers were determined by mixing serial dilutions of ferret plasma collected at different time points with 10^6 TCID50 units of recCDV-SH04/PBH. Virus and plasma were incubated at 37°C for 20 min, followed by addition of Vero-BLA cells. Neutralizing titers are expressed as the reciprocal of the highest dilution at which no cytopathic effect (CPE) was observed after 4 days.

Cytokine mRNA induction analysis. Relative mRNA levels were determined in infected HeLa cells or ferret PBMCs, respectively, by semiquantitative real-time PCR analysis as described previously (62). Briefly, HeLa cells were spin inoculated (2000 rpm for 30 min) with an MOI of 1 and total RNA was isolated at 24 hr postinfection. For PBMCs, RNA was isolated from cells collected on days 3 and 7 after infection, or from CDV-naive ferrets representing day 0. In all cases, RNA was reverse-transcribed using the SuperScript III reverse transcriptase kit (Invitrogen), and 10 μl of the resulting cDNAs was subjected to real-time PCR using the Fast SYBR green master mix (Applied Biosystems) or Quantitative SYBR green PCR kit (Qiagen). GAPDH mRNA served as an internal control, and PBMC induction levels were normalized to the average of mock-infected cells (HeLa or average of the day 0 values (GAPDH)). The relative changes in transcription levels were calculated according to fold change in threshold cycle (2^-ΔΔCT) (63).

Deep sequencing of viral genomes. RNA was extracted from infected cells using the Zif viral RNA kit (Qiagen), according to the manufacturer’s instructions. Metagenomic next-generation sequencing libraries were constructed as described previously (67). Briefly, 20 μl of extracted RNA was reverse transcribed using SuperScript III reverse transcriptase (Thermo) and second-strand synthesis was performed using Sequenase v.2.0 (United). cDNA was purified using DNA Clean and Concentrate 5 (Zymo) and subjected to Nextera XT fragmentation (Illumina) followed by 10 cycles of PCR amplification and a 8.8× Ampure XP cleanup (Beckman Coulter). Libraries were sequenced on a 2 × 300 bp run on an Illumina MiSeq. Sequencing reads were adapter and quality trimmed (25/2) using cutadapt (68). Majority consensus genomes were called via mapping trimmed reads to the canine distemper virus...
reference genome (NC_001973) in Geneious v9.1, and allele frequencies were called via remapping trimmed reads to the majority consensus genome.

**MiSeq-based quantitation of relative mRNA editing efficiency.** Next-generation sequencing using MiSeq (Illumina) was performed to determine the ratio of P7/P8 transcripts in recΔ5V constructs. When CFE reached approximately 75 to 90%, total RNA was isolated from infected cells using the RNasey kit (Qiagen) and cDNAs of mRNA transcripts were synthesized using oligo(dT) primers and SuperScript III reverse transcriptase (Invitrogen). MiSeq primers with Illumina overhangs targeting a region of ~500 nucleotides surrounding the P gene editing site were used to PCR amplify cDNAs representing all P, V, and W protein transcripts. Sequences were queried for the relative ratios of P, V, and W protein transcripts for each sample.

**Profiling of viral RNA populations in infected cells.** To monitor relative quantities of viral genomic and mRNA populations during virus replication, Vero-CSLA1 cells were infected with the recΔ5V strains at an MOI of 0.1 TCID50 unit per cell and total RNA was isolated 24 h postinfection using the RNeasy kit (Qiagen). To quantify relative genome copy numbers, a specific primer located in the viral trailer sequence was used for first-strand cDNA synthesis with SuperScript III reverse transcriptase, while oligo(dT) primers were applied to determine relative amounts of message. Relative RNA ratios were determined in an Applied Biosystems 7500 real-time PCR system using Fast SYBR green master mix (Thermo Fisher Scientific) and appropriate primer pairs annealing in the L, N, and M1 protein and host GAPDH ORFs, respectively, or flanking the NPF or M2 Open reading frames as specified in the figure legends. To calculate relative difference in viral RNA populations or relative RNA present in standard recΔ5V-infected cells, threshold cycle values obtained for each sample were standardized to expression levels of cellular GAPDH as a reference, and then ΔΔCt values were determined by normalization of the standardized values to the individual reference RNA population as specified in the figure legends.

**Statistical analysis.** To assess experimental variation and the statistical significance of differences between sample means, one-way or two-way analysis of variance (ANOVA) was carried out in combination with Tukey’s or Sidak’s post hoc tests as specified in the figure legends, using the Prism (GraphPad) software package. For virus growth profiling, regression models were built using Bindy’s population growth four-parameter variable slope model. Results for individual biological replicates are shown whenever possible. Where appropriate, experimental uncertainties are identified by error bars, representing standard deviations (SD) or standard errors of the means (SEM) as specified in the figure legends.

**Ethics statement.** All animal experiments were approved by the responsible state authority, the Regierungspräsidium Darmstadt (approved no. F70/10/12), and carried out in compliance with the regulations of German animal protection law.

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Appendix II: The structurally disordered paramyxovirus nucleocapsid protein tail domain is a regulator of the mRNA transcription gradient. Science Advances (2017)
The structurally disordered paramyxovirus nucleocapsid protein tail domain is a regulator of the mRNA transcription gradient

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The paramyxovirus RNA-dependent RNA-polymerase (RdRp) complex loads onto the nucleocapsid protein (N)-encapsidated viral RNA genome for mRNA synthesis. Binding of the RdRp of measles virus (MeV), a paramyxovirus archetype, is mediated through interaction with a molecular recognition element (MRE) located near the end of the carboxy-terminal Ntaii domain. The structurally disordered central Ntaii section is thought to add positional flexibility to MoRe, but the functional importance of this Ntaii region for RNA polymerization is unclear. To address this question, we dissected functional elements of Ntaii by relocating MoRe into the Ntaii-encapsidating Ncere domain. Linker-scanning mutagenesis identified a microdomain in Ncere that tolerates insertions, MoRe relocated to Ncere supported efficient interaction with N, MoRe-deficient Ntaii had a dominant-negative effect on bioactivity that was alleviated by insertion of MoRe into Ncere, and recombinant MeV encoding N with relocated MoRe grew efficiently and remained capable of mRNA editing. MoRe in Ncere also restored viability of a recombinant lacking the disordered central Ntaii section, but this recombinant was temperature-sensitive, with reduced RdRp loading efficiency and a flattened transcription gradient. These results demonstrate that virus replication requires high-affinity RdRp binding sites in NtRNA, but productive RdRp binding is independent of positional flexibility of MoRe and cis-acting elements in Ntaii. Rather, the disordered central Ntaii section independent of the presence of MoRe in Ntaii steepens the paramyxovirus transcription gradient by promoting RdRp loading and preventing the formation of nonproductive polycistronic viral miRNAs. Disordered Ntaii may have evolved as a regulatory element to adjust paramyxovirus gene expression.

INTRODUCTION
Paramyxoviruses are enveloped RNA viruses that are collectively responsible for major human and animal morbidity and mortality worldwide (1). In addition to measles virus (MeV), an archetype member of the family and member of the morbillivirus genus, the paramyxoviruses comprise major respiratory pathogens, such as mumps virus (MuV), the parainfluenzaviruses (PIV), Nipah (NiV) and Hendra (HeV) viruses, and canine distemper virus (CDV). Being part of the order mononegavirales, paramyxoviruses feature nonsegmented negative-stranded genomes that are encapsidated by the viral N protein, resulting in the formation of helical NtRNA ribonucleoprotein assemblies (2–5). Only the encapsidated RNA is recognized as a template by the viral RNA-dependent RNA-polymerase (RdRp) complex that is responsible for both transcription and replication of all viral RNA (6–8). Genome and antigenic encapsidation occurs concomitant to RNA synthesis, and RdRp switches into replicate mode only after a sufficiently large pool of N protein has been generated in infected cells (1, 5, 9).

Key components of the RdRp complex are the viral polymerase (L) protein and its essential cofactor, the phosphoprotein (P). The L protein mediates all enzymatic activities required for mRNA synthesis and genome production, such as nucleotide polymerization, mRNA capping, polyadenylation, and maturation, but does not interact directly with NtRNA. Rather, the P protein interacts with N and is thought to stabilize attachment of the advancing polymerase to the NtRNA (10–16) and also to deliver free N proteins to the replicase complex for encapsidation of the nascent strand (17–20). The MeV P protein is composed of an N-terminal (PNT) domain (amino acids 1 to 230) and a C-terminal (PCT) domain (amino acids 231 to 507). PNT is responsible for this chaperoning of newly synthesized N proteins to the replicase, holding N monomers in an open conformation and preventing premature oligomerization (19, 20). Major structural domains in PCT include a tetramericization domain mediating homooligomerization and a C-terminal NtRNA binding domain (P-XD) (10, 11, 21–25) that mediates high-affinity interactions with the N protein.

MeV N itself consists of a conserved core domain (Ncere) spanning residues 1 to 490 that mediates homo-oligomerization and RNA binding, and a 120–amino acid tail domain (Ntaii) that protrudes from the surface of the assembled viral NtRNA (5, 24). Ncere determines the spatial organization of the helical NtRNA complex, as evidenced by the atomic structures of the NiV, PIV5, and MeV Ncere that were recently solved (19, 20, 25, 26). In all cases, these structures revealed a separation of Ncere into an N-terminal and a C-terminal section that are separated by a groove accommodating the encapsidated RNA. Although the MeV Ntaii has not returned defined electron density in the NtRNA reconstruction and is considered to be largely structurally disordered (12, 27–30), the presence of the tail affects the overall spatial organization of the NtRNA. Complete removal of Ntaii by trypsin digestion decreases MeV NtRNA diameter and pitch, leading to increased rigidity (31) and the elimination of bioactivity. Multiple studies have suggested that the Ntaii protrudes through the interstitial space between successive turns of the NtRNA helix, leaving approximately 50 amino acids buried inside the helix and only the terminal residues 450 to 525 exposed on the NtRNA surface (10, 28, 30). Although most of the central region of the morbillivirus Ntaii is predicted to be disordered, three conserved microdomains or boxes have been identified (32). These comprise the N-terminal box 1 (residues 400 to 420) and the C-terminal box 2 (residues 589 to 506) and
box 3 (residues 517 to 525). Located within box 2 is an α-helical molecular recognition element (MoRE; residues 488 to 499).

Box 1 has been hypothesized to bind a cellular receptor (33, 34), but the nature of this receptor has remained obscure and the predicted position of box 1 between the rungs of the helical NRNA makes its availability for protein–protein interactions doubtful. Functional roles have been defined for both boxes 2 and 3. The former is thought to mediate high-affinity interactions with RdRps by engaging P-XD (10, 12, 13, 22), while the latter contributes to genome incorporation into nascent virions through direct interaction with the viral matrix (M) protein (35, 36). In addition, box 3 is thought to interact with cellular factors such as hsp70, which has been postulated to facilitate separation of MoRE and P-XD through cis-acting modulatory effects, enabling the RdRp complex to proceed along the genome (37, 38).

Two major roles in polymerase activity were originally attributed to this MoRE-P-XD interaction: initial loading of the RdRp complex onto the genome and prevention of premature termination of polymerization by tethering the advancing polymerase complex to the template (5, 29, 39). However, a recent study showed that deletion of the 86 C-terminal amino acids, including boxes 2 and 3 and all surface-exposed residues of the unstructured central Ntai, section, retained approximately 60% of standard MeV N bioactivity in minigenome assays, ruling out the possibility that MoRE is fundamentally required for RdRp loading onto the NRNA template (23). Despite the considerable bioactivity in minigenome assays, recombinant MoEN encoding this truncated N protein in place of standard N could not be recovered in this study. These data suggested that the MoRE-P-XD interaction is required to successfully master the template length of the viral genome compared to a minigenome reporter, preventing catastrophic premature polymerase termination (23).

Although these emerging data have begun to elucidate the contribution of the MoRE-P-XD interaction to polymerase activity, the functional role of the unstructured central Ntai section in paramyxovirus RNA replication is not understood. It was suggested that this section may provide positional flexibility of MoRE relative to the trunk of the NRNA helix, which is thought to facilitate the critical random encounter between MoRE and P-XD through a fly-casting mechanism (40) that allows the formation of high-affinity complexes. However, previous studies of recombintant virions to test these models were limited to the characterization of individual mutations inserted into the box 2 and/or 3 domains (36, 37, 39, 41), which imposes shortcomings. These studies are, by design, unable to explore the mechanistic importance of the disordered Ntai sections for polymerization, and the interpretation of results is compromised by the possibility that uncharacterized cis-acting elements in Ntai may enhance the effect of individual mutations. Thus, the multidomain architecture of the Ntai and the unstructured nature of the central Ntai section itself have prevented its functional characterization because it has been impossible to separate the contribution of the central section to polymerization from that of the MoRE-P-XD interaction.

Overcoming this obstacle was therefore the first aim of the present study. We developed a strategy to dissect Ntai into distinct functional elements while preserving bioactivity in both minigenome assays and the content of recombinant virus replication as the premise for the subsequent mechanistic characterization of the role of the individual Ntai elements in paramyxovirus replication. Relocating MoRE into Ncere may have allowed us to revert MeV to use an ancient mode of NRNA interaction (if the docking of P to a binding site in Ncere indeed represents the evolutionary older form of interaction).

RESULTS
Biobinformatics analyses have predicted several structured and disordered regions in the morbillivirus N protein (42, 43). In search of candidate Ncere sites that tolerate domain insertions, we enhanced the resolution of these previous domain predictions by subjecting the MeV N protein to a comprehensive meta-analysis of structurally disordered linker regions using a panel of distinct structure prediction algorithms. The individual in silico predictions were cross-referenced quantitatively, and the results were graphically plotted as a function of MeV-Edna N (Fig. 1A). In addition to the N terminus and the complete C-terminal Ntai, this analysis highlighted a microdomain in the N-terminal half of Ncere (residues 119 to 146) as a candidate interdomain region. A structural disorder analysis of N proteins derived from representatives of all morbilliviruses revealed that this pattern was mirrored by several families within the order, with the exception of the rhinoviruses and pseudorabies, which both completely lack a disordered Ntai domain (Fig. 1B).

Insertion scanning analysis of the nucleoprotein
To experimentally test the validity of the N protein domain architecture predictions, we launched a comprehensive linker insertion analysis, adding four amino acid peptide linkers (sequence GDAS) throughout the N protein in approximately 10- to 20-amino acid intervals, guided where possible by the local in silico predictions. Steady-state levels of all resulting N mutants were evaluated through immunoblotting after transient expression. Linker insertions were best tolerated throughout Ntai and, to some extent, in the N-terminal half of Ncere, whereas most mutants with an insertion in the C-terminal half of Ncere were barely detectable, suggesting severe misfolding followed by degradation (Fig. 1C). To assess the impact of linker insertions on N bioactivity, the mutants were tested in parallel in a monocytotropic MeV minigenome assay (44) using a firefly luciferase reporter to monitor RdRp transcription function (Fig. 1D). All N constructs harboring linker insertions in the Ntai maintained bioactivities from 70 to 130% of that of standard N, underscoring the unstructured nature of the Ntai (38, 42, 45, 46). In contrast, all insertions in Ncere abolished bioactivity, with the striking exception of an Ncere microdomain spanning residues 131 to 138 (Fig. 1D). Follow-up mapping of this putative interdomain region revealed that insertions at residues 131 and 133 were best tolerated, with no adverse effect on N bioactivity. Overlay of the activity data with the average prediction scores of the in silico analysis revealed a slightly elevated disorder propensity for N residues 115 to 140, but it is noteworthy that the overall correlation between the in silico predictions and experimental data was poor for the Ncere domain (Fig. 1D).

To explore the packaging capacity of the interdomain region of N residues 131 to 138, we inserted a larger 17-amino acid peptide encoding a HA epitope tag flanked by glycine linkers (sequence SGGGPPDYVPGPPGGS) at N positions 131 and 138, respectively. For reference, we also added this tag near the center of the flexible Ntai, at positions 436 and 469, respectively. Although both insertions in the tail were tolerated with reductions in bioactivity of less than 30% compared to standard N, introducing the tag at Ncere position 131 reduced bioactivity by approximately 90% and by 60% when placed at position 138 (Fig. 1E). These differences in bioactivity were not due to changes in protein expression rate and stability because all constructs reached comparable steady-state levels (Fig. 1F).

Relocation of MoRE into the MeV N core
On the basis of these results, we considered Ncere position 138 as the target for relocation of a 22-amino acid fragment (QDFQKSSQRSADALLRLQAMG)
including MoRE from Nt11. Applying in silico modeling based on the available reconstructions of standard MV Ncore and N-RNA assem-
brs (25), we simulated the structural consequences of this relocation in the context of an individual N monomer (Fig. 2, A and B), an isolated 12-membered N ring (Fig. 2, C and D), and the helical MV N-RNA assembly (Fig. 2, E and F). The SWISS-MODEL homology modeling server (47) was used to model disordered regions spanning N core residues 117 to 124 and 134 to 142. These simulations posed MoRE approximately 75 Å closer to the N-RNA helix trunk from its natural location near the end of the unstructured Nt11 (measurement based on radial extension of Nt11 from the N-RNA helix trunk) and predicted that lateral flexibility of the element is markedly restricted compared to standard N.

To explore MoRE relocation in cells, we generated five different N mutants in addition to the previously described N-Δnt11 construct that lacks the 86 C-terminal residues of N (Fig. 3A). First, we added
a second MoRE domain at Ncore position 138 to address whether MoRE in Ncore may have a dominant-negative effect on RdRp activity (N-2xMoRE). Second, we relocated MoRE from the tail to this Ncore position but left the Ntail architecture otherwise unchanged (Ncore-MoRE). Third, as a control, we deleted MoRE from the Ntail (N-noMoRE), resulting in an Ntail structure identical to that of Ncore-MoRE. Fourth, we added MoRE to Ncore position 138 in the context of the tail-truncated N-Δ68 variant (Ncore-MoRE-Δ68). Last, we reattached the terminal box 3 domain to the C terminus of this Ncore-MoRE-Δ68 mutant (Ncore-MoRE-Δ68-B3).

Immunoblotting of transiently expressed N demonstrated that all N mutants were expressed efficiently, reaching steady-state levels comparable to unmodified standard MoN (Fig. 3B). As before, minireplicon assays using the conventional monocistronic minigenome revealed that Ncore-MoRE retained bioactivity, supporting approximately 75% luciferase reporter activity compared to standard N. This activity level was virtually identical to that reached by N-2xMoRE, indicating that doubling the number of MoREs in N RNA does not inhibit polymerase processivity. In contrast, eliminating MoRE from the otherwise unchanged N protein (N-noMoRE) almost entirely abrogated RdRp activity (Fig. 3C). This result was consistent with the outcome of previous studies in which Ntail was truncated immediately upstream of MoRE (23, 30). As we have shown before, further Ntail shortening through truncation at position 440 (generating N-Δ68) partially restored bioactivity to approximately 65% that of standard N (23). The addition of MoRE to the core of N-Δ68 (Ncore-MoRE-Δ68) significantly boosted RdRp activity to levels indistinguishable to those achieved in the presence of standard N. However, addition of the conserved 8-amino acid box 3 region to the C terminus of Ncore-MoRE-Δ68 had a somewhat detrimental effect, reducing bioactivity back to the levels obtained with Ncore-MoRE and N-Δ68, respectively (Fig. 3C).

To address whether N RNAs with relocated MoRE allow RdRp to efficiently negotiate the intergenic junctions in the viral genome, a process that involves the nontemplated polyadenylation of the newly synthesized mRNA, migration of the RdRp complex to the next downstream transcription start sequence, and reinitiation of RNA synthesis, we generated novel firefly luciferase and nanoluciferase bi- and tricistronic minigenome reporter plasmids (Fig. 3D). The bicistronic reporter harbors the N/P open reading frame (ORF) intergenic junction of the MoV

Fig. 2. Structural models of standard MoV N and N featuring MoRE relocated into Ncore. MoRE is shown as a red cylinder or ribbon, respectively. (A and B) Side views of monomers of MoV N (A) (Protein Data Bank [PDB] 2-DN8) and MoV Ncore-MoRE (B) showing the predicted positions of MoRE in red. Residues shown in orange are predicted to be disordered in native MoV N. Compared to its position in a fully extended Ntail, relocation shifts MoRE approximately 75 Å toward Ncore. (C and D) Top views of a single ring of the helical MoV RNA assemblies featuring standard N (C) and Ncore-MoRE (D). Nproteins are colored, alternating in green and blue. RNA is shown in yellow. Derived trees represent fully extended Ntails, shown by vertical extension from the helix. (E and F) Side views of internal sections of assembled N RNA helices featuring standard N and Ncore-MoRE, as shown in (C) and (D), respectively. All images were rendered with MacPyMOL.
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Fig. 3. Activity characterization of transiently expressed MeV N mutants. (A) Schematic of the MeV N protein domain organization. Conserved boxes 1 to 3 in Nts1 [31 to 33] are highlighted, and the position of MoRE inserted into Nore (red box) is indicated. Numbers represent amino acid positions. (C) Steady-state levels of transiently expressed MeV N mutants schematically shown in (A). (B) Schematics of the bi- and tricistronic minigenome plasmids generated (firefly, firefly luciferase; nanoluc, nanoluciferase; G5, G5 oligonucleotide; IGS, intron segment; Le, leader sequence; P, stop codon; MeV, MeV protein-encoding ORF; P, P protein-encoding ORF harboring a tandem stop codon after the 21st triplet). (D) qRT-PCR-based comparison of transcription gradients experienced by polyomaviruses versus virus infection. qRT-PCR analysis of the RNA panel shown in (C) to determine the ratios of N- and L-encoding mRNAs. Values represent the percentage of downstream ORF mRNA relative to upstream ORF mRNA in each sample (MeV/MeV infection upstream ORF, N mRNA; downstream ORF, P mRNA; MeV minigenome upstream ORF; firefly luciferase mRNA; downstream ORF, nanoluciferase mRNA). Averages ± SEM of three independent experiments are shown, analyzed in duplicate each. Experimental variations were assessed through one-way ANOVA combined with Sidak’s multiple comparison post tests. (E) and (G) Assessment of the relative efficiency with which the downstream reporter ORF was transcribed in the presence of the different N mutants using the bicistronic (F) and tricistronic (G) minigenomes shown in (C). Relative reporter expression ratios represent averages of at least five independent experiments, determined in triplicate each ± SEM. Experimental variation was assessed through one-way ANOVA combined with Sidak’s multiple comparison post test.
cells (Fig. 3E), indicating that a transcription gradient resembling that of viral protein expression in infected cells is replicated by the polyctronic minireplicons.

For analysis of the polyctronic minigenome data, we first separately normalized the data sets obtained for each reporter for the signals obtained in the presence of standard N and then individually calculated the ratios of normalized nanoluciferase versus firefly luciferase signals for each N mutant. This approach enables us to appreciate the relative efficiency with which the RdRP complex accesses the downstream relative to the upstream reporter in the presence of the different N constructs, independent of the absolute differences in RdRP activity experienced by the different N mutants that are documented in Fig. 3C. When applied to the panel of Ncore mutants, we noted in all cases essentially unaltered firefly luciferase and nanoluciferase activity ratios compared to those observed with standard N (Fig. 3, F and G). This finding extended equally to the previously generated bioactive N-Δ86 mutant lacking any MoRE domain. Together, these results demonstrate that MoRE relocated into Ncore is functionally recognized by the RdRP complex and sufficient to support all activities required for the MoV RdRP transcriptase. Standard N-like bioactivity of Ncore-MoRE-Δ86 reveals that the physical separation of MoRE from its native environment, and hence from any hypothetical short-range regulatory activity that was speculatively attributed to box 3, does not impair N support of transcriptase activity.

Physical interaction of N MoRE mutants with P protein domains

Because the previously described N-Δ86 mutant lacking MoRE was bioactive in minireplicon assays but impaired in forming high-affinity interactions with the C-terminal PCT fragment of the MoV P protein harboring the MoRE-binding P-XD domain in coimmunoprecipitation experiments (23), we asked whether the relocation of MoRE into Ncore restores efficient interaction of N with PCT. All N mutants were subjected to a coimmunoprecipitation study using full-length P, the PCT, and the N-terminal PNT domain as targets. Ncore-MoRE coprecipitated P-Edm with an efficiency of approximately 75% that of standard N, whereas the corresponding Ncore-Δ86MoRE failed to appreciably precipitate P (Fig. 4A). As we previously reported, tail truncation partially restored the ability of N to interact with P in the absence of MoRE.

Coprecipitation of the PCT fragment with the Ncore-MoRE mutants essentially mirrored the results obtained for full-length P (Fig. 4B), thus demonstrating efficient interaction of the P-XD with the relocated MoRE. As observed previously, N-Δ86 without a MoRE added to Ncure did not interact appreciably with PCT, indicating that the MoRE-independent docking of P to tail-truncated N is mediated by residues in the N-terminal P fragment. Accordingly, only the two N mutants featuring the substantially truncated Δ86-tail architecture efficiently coprecipitated PNT (Fig. 4C). As expected, the N interaction with PNT, which lacks the XD domain, was not substantially affected by the presence or absence of MoRE in the different N mutants because all other N mutants did not coprecipitate PNT.

These results confirm that the relocated MoRE is recognized by and capable of engaging in high-affinity interaction with P-XD. Unstructured full-length Ntails lacking MoRE interfere with Ncure-to-P binding because both standard P and truncated PNT failed to interact with Ncure-MoRE (which contains the unstructured Ntail but not MoRE), but efficiently coprecipitated N-Δ86 lacking both MoRE and unstructured Ntail residues. However, only standard P, but not PNT, efficiently interacted with standard N, which naturally contains both MoRE and the unstructured Ntail. Because only the P-XD motif located in the P protein PCT domain complexes with MoRE, these data demonstrate by exclusion that the PNT region in standard P is capable of interacting with Ncure and that it is the presence of the unstructured Ntail residues that blocks this interaction in the absence of MoRE. Finally, the length of the unstructured Ntail region appears to modulate the strength of this negative effect on interaction because PNT coprecipitation efficiency was higher with tail-truncated N-Δ86 than with the longer N-Δ86-83.

Recovery of recombinant harboring Ncure-MoRE

Minireplicon assays provide basic insight into the functionality of RdRP and mRNA complexes, but are insufficient to determine whether all distinct bioactivity requirements for productive virus replication are met. For instance, N-Δ86 supports substantial minireplicon activity, but we were previously unable to recover MoV recombinants containing this mutant in place of standard N. To probe the full bioactivity spectrum of the Ncure-MoRE constructs, we transferred the five mutants into a complementary DNA (cDNA) copy of the MoEV-Edm genome. To facilitate the detection of emerging infectious centers and potentially poorly replicating recombinants, we used a genome version that also contains an enhanced green fluorescent protein (eGFP) ORF inserted in pre-N position (46).

Of the five different N mutants (N-2×MoRE, Ncure-MoRE, N-Δ86MoRE, Ncure-MoRE-Δ86, and Ncure-MoRE-Δ86-83), recombinants N-Δ86MoRE virions lacking MoRE could not be recovered, which is consistent with the lack of appreciable bioactivity of the N-Δ86MoRE mutant in the minireplicon assay (Fig. 5A). Strikingly, however, the corresponding recombinant Ncure-MoRE recombinant featuring the relocated MoRE domain was viable and could be readily amplified, similar to standard recombinant and the recombinant N-2×MoRE mutant with intact Ntail domain that were included as controls. In contrast, recombinant Ncure-MoRE-Δ86 could be recovered in principle, but the recombinant appeared to be severely growth-impaired, preventing the generation of a virus stock (Fig. 5A). However, the addition of the eight-residue box 3 to the truncated Ntail was sufficient to partially restore virus growth because we successfully recovered the corresponding recombinant Ncure-MoRE-Δ86-33 mutant strain. Despite initial rescue at 37°C, this mutant could only be efficiently amplified at 33°C, suggesting a temperature-sensitive phenotype (Fig. 5A).

The presence of the different modifications introduced into the N ORF in recovered recombinants was confirmed in all cases of successful virus amplification through RT-PCR and DNA sequencing. In addition, the electrophoretic mobility of the different N protein variants was assessed through immunoblotting of infected cell lysates (Fig. 5B). DNA sequencing did not reveal any additional point mutations that could have spontaneously occurred during virus rescue, and N mobility profiles were also consistent with that of our initial immuno-precipitation of cells transiently transfected with expression plasmids of the different N mutants.

To assess growth kinetics of the three recombinant-Ncure-MoRE mutants that could be amplified, we generated multistep growth curves at both 37°C and 32°C (Fig. 5, C and D). Maximal growth rate and peak titer of recombinant N-2×MoRE were virtually identical to those of standard recombinant at either temperature. The recombinant-Ncure-MoRE recombinant likewise showed robust replication under both conditions, but regression modeling revealed that maximal growth rates and peak titers remained behind those of standard recombinant (Fig. 5, C and D).
contrast, we could efficiently amplify the recMeV-Ncore-MoRE-506-B3 strain only at reduced temperature (32°C), confirming temperature sensitivity of this mutant (Fig. 3D). At the permissive temperature, however, growth rates and peak titers were essentially identical to those of recMeV-Ncore-MoRE, lagging slightly behind standard recMeV.

These results indicate that RdRP must be able to engage in high-affinity interactions with NRNA for virus stability. However, the precise physical environment of MoRE and the placement of native MoRE near the terminus of the unstructured Ntail region are not essential for fundamental activities required for successful virus replication, including loading of RdRP onto and/or RdRP progression along the NRNA genome.

**Contribution of the central, unstructured Ntail section to transcriptase processivity**

Notably, the closely related paramyxovirus and pneumovirus families, only the pneumoviridae have elongated Ntail domains (Fig. 1B). In addition, only the paramyxoviruses cotranslationally edit P ORF transcripts to express immunomodulatory nonstructural proteins (49–52), whereas the pneumoviridae encode the equivalent proteins in separate transcription units. mRNA editing involves pausing and backsliding of the polymerase complex, which is thought to require looping out of the nascent strand before polymerization continues (49, 53, 54). The unstructured central Ntail section could provide the flexibility required for looping out of the newly synthesized strand, whereas MoRE at the end of the unstructured tail could

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**Fig. 4. Interaction of transiently expressed N protein mutants with the MeV P protein.** (A to C) Coimmunoprecipitation analysis of standard MeV P protein (A) or C-terminal (B) and N-terminal (C) fragments of the MeV P protein only (PCT and PNT, respectively) with the different N constructs. Western blot (WB) of WCL and immunoprecipitation (IP) results are shown. Cellular GAPDH was detected as internal standard. Graphs depict densitometric quantitation of the relative coimmunoprecipitation efficiencies. Values represent averages of three independent experiments ± SD. Experimental variation was assessed through one-way ANOVA combined with Sidak’s multiple comparison post test (*P < 0.05; **P < 0.01; ***P < 0.001; NS, not significant; ND, not determined).
simultaneously tether the paused RdRp complex to the genome, preventing catastrophic premature termination.

Consequently, we asked whether the presence of a partially unstructured Ntail followed by the MoRE interaction site is causally linked to the ability to edit mRNA. Because the growth patterns of recMeV-Ncore-MoRE and recMeV-Ncore-MoRE-Δ86-B3 at permissive temperature closely resembled each other, only the former strain was included in this experiment. Total RNA was extracted from infected cells, and cDNA reverse transcriptions were subjected to next-generation sequencing to determine the relative ratio of mRNAs encoding

![Diagram](image)

Fig. 5. Growth profiles of recombinant MeV harboring N mutants with relocated MoRE. (A) Recovery of recMeV strains with modified N proteins in exchange of standard N and expressing eGFP from an additional transcription unit. Fluorescence microphotographs show cells transfected with virus recovery plasmids (Original recovery), after the first passage of infectious centers (Passage 1 infection), and after infection with cell-associated virus stocks (3rd infection). Images of fluorescent synchro show representative fields of view. All viruses were grown at physiological temperature, with the exception of recMeV-Ncore-MoRE (Δ86-B3), which was recovered and amplified at 32°C. Cells infected with this recombinant and incubated at 37°C are shown for comparison in the bottom-most panel. (B) Immunoblot analysis of WCL of cells infected with the specified recMeV strains. Cells infected with recMeV-Ncore-MoRE-Δ86-B3 were incubated at 32°C. C) and D) Growth curves of the viable recMeV mutant strains in comparison with standard recMeV. Cells were infected at a multiplicity of infection (MOI) of 0.03, followed by incubation at 37°C (C) or 32°C (D); respectively. Values show titers of cell-associated progeny virus; particles and represent averages of three independent repeats ± SD. For regression modeling, a linear relationship between parameters was applied (P0 every maximal population doubling time = Tdmax, y = Tdmax, titer corresponding to the top plateau of the regression model). Values in parentheses specify 95% confidence Intervals; * denotes non-overlapping 95% confidence intervals relative to standard recMeV N0, overlapping 95% confidence intervals, ND, not determined).
MeV P, V, or W, respectively. For each virus strain examined, a minimum of 27,469 distinct reads representing five independent infection experiments were analysed. Standard recMeV showed relative PV/NW mRNA ratios of 60%/32%/3% (Fig. 6A), which is consistent with previous estimates of the morbillivirus mRNA editing frequency (53). Both mutant viruses retained the ability to efficiently mRNA edit (recMeV-N26MeV, 69%/31%/3%; recMeV-NcoRE-MoRE, 69%/32%/3%), indicating that positional flexibility of MoRE relative to the NRNA binding is not required for the assembly of a functional mRNA editing machinery.

Therefore, we probed whether MoRE relocation and/or the presence of the unstructured central Ntail section alternatively affect the productivity of the RdRp complex moving along the NRNA template during virus replication. The paramyxovirus replication cycle comprises primary transcription of viral mRNAs from the incoming genomes early during infection, followed by synthesis of antigens and progeny genomes once a sufficient level of N protein has become available for encapsidation (53). Whereas the viral mRNA pool expands exponentially in later replication stages, primary transcription is characterized by linear growth. To examine transcription rates, we spin-inoculated cells with recMeV-NcoRE-MoRE or standard recMeV for synchronized infection and used TaqMan-based quantitation of viral N mRNA in a 30-hour window after warming of cells to 37°C (Fig. 6B). During the first 9 hours after infection, the N mRNA pools in cells infected with standard recMeV and recMeV-NcoRE-MoRE increased with similar rates. Subsequently, however, standard recMeV entered the exponential mRNA expansion phase approximately 10 hours earlier than recMeV-NcoRE-MoRE, resembling the virus growth profiles shown in Fig. 6C. When incubated at physiological temperature, no appreciable N mRNA pool expansion was detectable in cells infected with recMeV-NcoRE-MoRE-A86-B3. To appreciate viral RNA steady-state levels late in infection, we examined antigenic and N mRNA copies present in cells infected with recMeV, recMeV-NcoRE-MoRE, or recMeV-NcoRE-MoRE-A86-B3 and incubated for 72 hours at either 37°C or 32°C (recMeV and recMeV-NcoRE-MoRE-A86-B3 only). At this stage of infection, we detected statistically significant differences in viral RNA copy numbers only in cells infected with recMeV-NcoRE-MoRE-A86-B3 versus standard recMeV after incubation at nonpermissive temperature (Fig. 6C). Having thus verified the presence of both antigenic and mRNA pools in this sample set, we queried the RNA pool for the viral transcription gradient by determining the relative quantities of the MeV N and L protein-encoding mRNAs, respectively, through qRT-PCR (Fig. 6D). The relative quantities of L to N mRNAs in recMeV-NcoRE-MoRE-infected cells were indistinguishable from those obtained for standard recMeV. However, recMeV-NcoRE-MoRE-A86-B3-infected cells contained significantly higher relative levels of L mRNAs compared to standard recMeV-infected cells. This relative up-regulation of L mRNA copies was independent of incubation at permissive or restrictive temperature, indicating a flattened transcription gradient experienced only by the mutant that lacks the disordered central Ntail section.

To investigate the effect of a single intergenic sequence on the transcription gradient of this mutant strain, we compared P protein versus N protein mRNA levels of the recMeV-NcoRE-MoRE-A86-B3 mutant and standard recMeV strains (Fig. 6E). This experiment showed virtually identical P and N protein mRNA levels in recMeV-NcoRE-MoRE-A86-B3-infected cells, but relative P mRNA levels that were approximately 15% lower than N mRNA levels in recMeV-infected cells. However, the phenotype was naturally less pronounced over a single intergenic junction than over the length of the viral genome and was therefore statistically not robust.

At each intergenic junction, paramyxovirus polymerases fail to recognize gene-end signals with low frequency and generate polycistronic mRNAs, of which the downstream ORFs cannot be accessed by the host cell ribosome and are therefore nonproductive. To test whether the flattened transcription gradient of the recMeV-NcoRE-MoRE-A86-B3 strain reflects a higher relative amount of polycistronic mRNAs generated in cells infected by this mutant, we compared the amounts of mRNAs harboring N/P and H/L intergenic sequences relative to N protein and P protein mRNAs, respectively, in cells infected with recMeV-NcoRE-MoRE-A86-B3 or standard recMeV (Fig. 6E). In both cases, we found a significantly higher relative amount of polycistronic mRNAs in cells infected with the mutant strain than unmodified MeV.

These findings reveal that positional flexibility of native MoRE, mediated through the unstructured central Ntail section, has not evolved to enable mRNA editing. Independent of the relative position of MoRE, the central Ntail section instead critically affects RdRp transcriptase productivity twofold. Removal of the disordered residues reduces the initial accumulation of viral RNA and improves the likelihood that the transcriptase complex, once engaged, reaches downstream ORFs. The latter appears to achieve, at least in part, by an increased frequency of ignoring gene-end signals and generating polycistronic mRNAs in the absence of the disordered tail residues.

DISCUSSION

Recently solved NRNA and RdRp structures and substructures have advanced the spatial understanding of the monomeric measles polymerase machinery (10, 16, 20, 22, 25, 28, 36, 37). However, mechanistic insight, especially into the interplay between the RdRp complex and the encapsidating genomes, remains limited. This interaction is highly dynamic because the template must be locally de-encapsidated to expose the RNA to the advancing polymerase complex, followed by re-encapsulation as the polymerase progresses. N proteins of the paramyxoviruses contain, in addition to the RNA-encapsidating Ncore, a structurally mostly disordered Ntail domain proposed to have major functional importance for interaction with and advancing of RdRp (15, 30, 62, 66). However, related mononegavirales families such as the pneumoviruses and rhadoviruses lack equivalent unstructured tails, underscoring the fact that disordered Ntails are not a priori required to enable a monomeric measles RdRp to negotiate the NRNA template. Understanding the functional role of the disordered Ntail regions for paramyxovirus RNA synthesis and appreciating drivers of Ntail evolution was therefore the overarching goal of this study.

Previous approaches toward this goal were hampered by the challenge that disintegration of the Ntail into discrete functional domains disrupts viral viability, preventing their functional characterization in the physiologically relevant context of virus infection (23, 39). Although some recombiant viruses with individual point mutations in the conserved Ntail boxes were recovered (39), the insight gained from these mutants is inherently limited, and the interpretation of viral phenotypes is further complicated by the unknown contribution of cis-acting elements that are suspected to be present in Ntail (38, 39). The driving force for the development of the disordered Ntail region, which comprises approximately 50% of the overall length of the tail, and its function in virus replication remained therefore unexplored.

Because we had demonstrated in previous work that the presence of the RdRp-binding MoRE is required for virus viability, albeit not for
Fig. 6. Functional characterization of RdRp activity of the recMeV mutants with modified N proteins. (A) Next-generation sequencing analysis of P transcripts of the recMeV mutants and standard recMeV. All strains showed identical ratios of P, V, and W-encoding mRNAs, indicating unchanged mRNA editing activity. Values represent a minimum of 27,469 reads each and are expressed as percentage of the differentially edited mRNAs relative to the total transcripts from the P ORF. (B) TaqMan-based quantification of the N mRNA transcription rates in cells infected with standard recMeV or the specified mutant strains and incubated at 37°C. RNA extracts were normalized for 18S ribosomal RNA (rRNA), and N mRNA copy numbers are expressed relative to those present 1 hour after infection. The inset magnifies fold changes experienced in the initial 12-hour window after spin inoculation of cells. Values represent averages ± SEM of three independent experiments, analyzed in duplicate each. Shaded areas flanking the curves show 95% confidence intervals of the regression models. (C) TaqMan quantitation of MeV antigenome and N mRNA copy numbers in cells infected with standard recMeV or the specified mutant strains at 72 hours after infection. recMeV/Ncone-MoRE-S86-B3 was analyzed after incubation at both permissive and restrictive temperature. (D) and (E) qRT-PCR analyses of the RNA panel shown in (C) after first-strand synthesis using oligo(dT) primers to determine ratios of N- and L-encoding mRNAs (D), N- and P-encoding mRNAs (E), and the relative amount of intragenic sequence (ICS) containing polyhistidine mRNAs relative to the SGS-preceding ORF (F). Values represent the percentage of L mRNA relative to N mRNA in each sample (D) or the percentage of the specified target mRNA relative to the specified reference mRNA (E). For (C) to (E), averages ± SEM of three independent experiments are shown, analyzed in duplicate each. Experimental variations were assessed through one-way ANOVAs combined with Sidák’s multiple comparison post tests (∗P < 0.05; ∗∗∗P < 0.001; NS, not significant).
RdRp activity in minireplisome assays (23), we hypothesized that MoRE cannot be entirely removed from a viable virus, but it should be possible to physically separate the disordered Ntnl region and MoRE. We further hypothesized that a high-affinity RdRp binding site may have been present in Nore of an ancestral nonenveloped virus, providing the basis for our overarching assumption that it should be feasible to relocate MoRE into the Nore of modern MeV.

A linker-scanning mutagenesis revealed only a single candidate target site for insertions in Nore. However, suitability of this site for MoRE relocation was supported by a recent cryo-electron microscopy (cryo-EM)-based reconstruction of the MeV NRNA in near-atomic resolution (25). Here, low electron density was obtained for a segment spanning residues 133 to 142, suggesting a role as interdomain linker. On the basis of the comprehensive nature of our scan and the electron density maps of assembled MeV NRNA nucleocapsids, it is highly likely that this microdomain represents the only viable entry point for peptide insertions in Nore without losing N bioactivity. Structure simulations of MoRE in Nore predicted that positional flexibility of relocated MoRE would be largely eliminated and placed the element up to 7.5 Å closer toward the center of the NRNA helix. Fully consistent with these predictions, Nore-MoRE with relocated MoRE was as bioactive in minireplisome assays as the N-25MoRE reference construct harboring two MoRE domains and supported efficient replication of recombinant virus.

Four major mechanistic conclusions can be drawn from the characterization of the N proteins with relocated MoRE in functional assays and the context of recombinant viruses. First, high-affinity attachment points for RdRp on the NRNA helix are essential for virus replication, albeit not for RdRp polymerase function per se, because both Nore-MoRE and N-386 were equally bioactive in minireplisome assays but only the former supported recovery and growth of recombinant virions. This finding confirms that the MoRE binding sites are not fundamentally required for the original loading of the RdRp complex onto the NRNA template but are necessary to prevent premature termination of the advancing RdRp complex when the template length reaches full-genome size, as we have previously shown for the N-386 construct (23). The presence or absence of MoRE does not specifically affect the ability of the RdRp transcriptase to negotiate intergenic junctions and reinitiate mRNA synthesis, as was sometimes proposed (30, 42, 59), because the relative transcriptase efficiencies of the downstream reporter of both our newly generated bi- and tricistronic minigenome constructs in the presence of all bioactive N mutants including N-386 were identical to those obtained with standard N.

Second, however, the position of the high-affinity contact points near the end of the flexible Ntnl and downstream of the structurally disordered Ntnl section is optimal, rather than mechanistically required, as originally proposed (58–61). Successful recovery and amplification of both the recMeV-Nore-MoRE and recMeV-Nore-MoRE-386-B3 recombinants demonstrate that positional flexibility of MoRE and proposed cis-acting regulatory effects (58–60) are not essential for any of the diverse activities of RdRp in virus replication. Consistent with previous studies by our group and others of short Ntnl truncations eliminating native MoRE (23), the selective removal of only the MoRE from Ntnl (as in the N-nomore mutant) nearly completely abolishes N bioactivity. Before our analysis of N-386 (23), this observation formed the basis for the original model that places MoRE at the center of any productive interaction of MeV RdRp with the NRNA nucleocapsid (13, 38, 62, 63). In contrast to this MoRE-centric view, our data now demonstrate that it is not the absence of MoRE that prevents N-nomore bioactivity in minireplisome assays but rather the structurally disordered central Ntnl section that has a dominant negative effect on RdRp activity. Consequently, eliminating this disordered section through truncation restored minireplisome activity (23), demonstrating that morbillivirus P-E can productively interact with Nore in the absence of MoRE. Our coimmunoprecipitation studies revealing partially restored interaction of the N-terminal PNT fragment with N-386 and Nore-MoRE-386 confirm this conclusion biochemically. In the presence of the disordered Ntnl section, however, MoRE is required to override this dominant-negative effect, which can be achieved equally efficiently by MoRE located in Nore as in Ntnl.

Third, the presence of the conserved box 3 is mandatory for efficient virus replication, but box 3 does not directly contribute to polymerase activity: Nore-MoRE-386 was as active as standard N-EFn in minigenome assays, but only the corresponding recMeV-Nore-MoRE-386-B3 recombinant could be recovered and amplified, albeit at reduced temperature. MeV box 3 was demonstrated to interact with the viral M protein and thus be required for the interaction between viral genomes and envelope (35). The poor viability of recMeV-Nore-MoRE-386, which could be recovered initially but not expanded, could reflect impaired particle assembly, although recMeV lacking the M protein remain viable, albeit with a major growth phenotype (64). Notably, a possible contribution of Ntnl to correctly position box 3 for M binding and particle assembly could also be reflected by our observation that peak progeny titers of recMeV-Nore-MoRE-386-B3 at 37°C were approximately three to four orders of magnitude lower than those of standard recMeV, whereas antigenic RNA and N mRNA levels were reduced by only 1.5 orders of magnitude under these temperature conditions. In addition to vision assembly, box 3 has further been implicated in interacting with Hep!2 (37), and it could also be the disruption of this interaction or impaired contact with other host cell cofactors that prevents amplification of the recMeV-Nore-MoRE-386 recombinant.

Lastly, the structurally disordered central Ntnl section affects the natural paramyxovirus transcription gradient of mRNA synthesis (65) through two synergistic effects, promoting the initiation of the transcriptase complex and reducing the success of the transcriptase to negotiate the entire genome. However, the lower tendency of premature polymerase termination in the absence of the disordered tail resides coincides with an increased frequency of generating nonproductive polyalystronic mRNAs, suggesting that the central Ntnl section supports the transcriptase complex in correctly processing gene-end signals, possibly by providing necessary spatial flexibility to enable the nontemplated polyadenylation of newly synthesized mRNAs. The former conclusion, promotion of the initiation of the transcriptase complex, is based on our qRT-PCR assessment of the kinetic of mRNA expression and antigenic synthesis in virus-infected cells, whereas the latter, reduction of the ability of the transcriptase to negotiate entire genomes, is substantiated by the relative quantifications of N- and L-encoding mRNA steady-state levels and mRNAs containing intergenic junctions in cells infected with recMeV-Nore-MoRE (contains the disordered tail section) and recMeV-Nore-MoRE-386-B3 (lacks the disordered tail section), respectively. Therefore, we postulate that the structurally disordered Ntnl section adds a regulatory mechanism to ensure proper paramyxovirus protein expression. This modulation of the MeV transcription gradient is independent of the physical proximity of MoRE to the disordered residues but was significant only in the context of virus replication and not in our bi- and tricistronic minigenome assays. This dependence of the phenotype on virus replication could reflect the absence of the viral M protein from the minireplisome assays, given that MeV M not only may function as a particle
assembly organizer but, as recently suggested, also may be able to form tubular structures surrounding the NRNA nucleocapsid (65). Although the physiological significance of these structures is unknown, they could impair the accessibility of the NRNA genome through RdRp. The disordered section of the Ntail may compound the interaction of M with box 3 located at the distal end of the tail, thereby down-regulating the frequency with which these tubular M structures form, which could prevent a premature shutdown of genome access by RdRp.

At present, it cannot be definitively concluded whether the disordered Ntails represent a more recent development of the paramyoviruses or, instead, an ancestral feature of mononegaviruses N proteins. Of the modern mononegaviruses, viruses of three families feature disordered domains at the N protein C terminus, whereas two families lack an Ntail domain. On the basis of our experience that the high-affinity binding site for the RdRp complex can be readily moved into paramyxovirus Ncore, we favor the view that a tailless N may represent the developmental starting point. An RdRp binding site located near the C terminus of Ncore, as seen in modern rhabdoviruses, could have enabled tail formation through the acquisition of additional residues upstream of this contact site. The emerging tails may then have provided a platform to gain novel N functionality such as a broadened repertoire of interactions with host cell factors or to expand and optimize existing functionality without compromising the essential bioactivity of the highly structured Ncore. Providing an additional regulatory mechanism for balanced viral protein expression may have been a driving force for the evolution of the unstructured Ntail region. Selective pressure on the structurally disordered central MeV Ntail section has thus likely concentrated predominantly on maintaining the length of this region rather than the actual amino acid sequence. The readiness of MeV Ntail to tolerate random short transposon insertions without loss of virus viability corroborates this view (66, 67).

The presence or absence of MoRE from Ntail has no effect on the frequency of transcriptional paramyxovirus mRNA editing. We originally considered a direct link because the closely related pneumoviruses lack Ntail domains and express immunomodulatory nonstructural proteins from separate transcription units rather than through mRNA editing. Although viral countermeasures to the innate immune response are thus not directly impaired by the tail modifications, manipulating the mononegaviruses transcription gradient reportedly drives virus attenuation in vivo (60–71). We therefore expect that shortening the length of the disordered Ntail section may likewise modulate viral pathogenesis. The reduced fitness of the mutant MeV recombinants analyzed in this study and, in particular, the temperature-sensitive growth phenotype of recMeV-Ncore-MoRE-A66-B3 support this hypothesis, which is currently tested experimentally. If our predictions are met, varying the Ntail length may constitute a novel and universal approach toward engineering next-generation attenuated recombinant vaccine strains against existing and newly emerging pathogens of the paramyxovirus family.

MATERIALS AND METHODS

African green monkey kidney epithelial [CCK-81; American Type Culture Collection (ATCC)] cells stably expressing human signaling lymphocytic activation molecule (SLAM) (Vero/hSLAM) and baby hamster kidney (C13; ATCC) cells stably expressing T cell polyfunctional [B3R-T75] (72) were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum at 37°C and 5% CO2. Every fifth passage, both cell lines were incubated in the presence of G-418 (100 µg/ml). Here, recMeV (73) and expression plasmids based on the MeV-Edm or MeV-ICB strains were used. For virus stock preparation, vero-SLAM cells were infected at an MOI of 0.01 and incubated at 37°C, and cell-associated virus was released through two consecutive freeze-thaw cycles. Viral titers were determined by 50% tissue culture infectious dose (TCID50) (74). Cells were transfected using Lipofectamine 2000 (Invitrogen) or GeneJuice (Millipore) reagents according to the manufacturer's instructions. GeneJuice was used for all virus recovery transfections.

In silico analysis of MeV N domain organization

MeDor (75) was used to predict disordered domains in MeV-Edm N using the IUPred (76), GlobPlot2 (77), DisEMBL (78), FoldIndex (79), SPRITZ (80), and RONN (81) algorithms. Furthermore, MeV-Edm N was submitted to PONDR-FTI (82) and Disopred (83) for disorder predictions. To quantitatively assess the consensus of all algorithms, average values of GlobPlot2, FoldIndex, and DomCut (84) were transformed to positive integers, and positive output scores of all algorithms were normalized for identical hit cutoff values. All average scores were then transformed to a scale of 0 to 1 and plotted as a function of MeV-Edm N residues. MeV-Edm N secondary structure prediction was based on the StrBiB library of the Predgy program (85), embedded in the MeDor package (75). For the identification of disordered regions in MeV-Edm N based on DomCut (86), paramyxovirus N protein sequences were aligned using the ClustalW2 (86) and MUSCLE (87) algorithm. Results of three different settings were compared, and relative DomCut propensity scores were then averaged separately on the basis of the different sequence alignments: (i) different MeV genotypes [MeV-Edm (genotype A) (AF262901), MeV-Gambia (genotype B) (EU352949.1), MeV-Toulon (genotype C) (HM628964.1), MeV-Amsterdam (genotype G2) (EU098818.1), MeV-Illinois (genotype D) (EU150098.1), MeV-Alaska (genotype H2) (AY037043.1)], (ii) different morbilliviruses [MeV-Edm, MeV Gambia B2, MeV-Toulon, MeV-Alaska, MeV-Amsterdam, RPV-Kabete (X98291.3), CDV Onderstepoort (AF378705.1), CDV 5804 (AY360134.1), peste des petits ruminants virus Turkey 2000 (NC_005852), dolphin morbillivirus (NC_002263.1)], and (iii) members of all paramyxovirus genera [MeV-Edm, CDV 5804, NIV (NC_002281), HPIV type 1 C5 (NC_003461.1), HPIV type 3 L22 (NC_001764.2), HPIV type 2 (NC_003443.1), HPIV type 4 (NC_021928.1), NDV-B9201 (GenBank JF45007), Tupula paramyxovirus (NC_001951.1), RSV A2 (NC_001803.1), human metapneumovirus Sabina (NC_004142.2)].

Disorder and secondary structure prediction was performed using the MeDor package. The IUPred and FoldIndex algorithms were used for disorder prediction. Sequences used were as follows: RSV (accession: P54181.1), HMPV (accession: AAS26474.1), RAVV (accession: ABR11331.1), MeV ICB strain (accession: NP_696848.1), MeV Edmonston strain (accession: BAA69564.1), CDV 5804 strain (accession: AAQ06303.1), Nipah virus (accession: AE021372.1), Hendra virus (accession: NP_041061.1), Sendai virus (accession: Q07607.1), Mumps strain 88-9861 (accession: AAL76821.1), VSV Indiana strain (accession: ACK77580.1), Marburg virus (MARV) (accession: TP_00053153.1), RRV (accession: 15933_X), Ebola virus (EBOV) (accession: A01174.1), and Newcastle disease virus (NDV) (accession: ALR96387.1). Multiple sequence alignments and phylogenetic analysis were conducted using the Clustal Omega and ClustalW2, respectively, using
the neighbor-joining method and unweighted pair group method with arithmetic mean (UPGMA).

Computational modeling of MeV N:RNA with relocated MoREs

Homology models for MeV N:RNA with MoRE relocated to NcoI were obtained using the SWISS-MODEL homology modeling server (14) using the previously published MeV N:RNA structure (PDB ID: 1UFT) as a template. Images were manipulated and created using PyMOL (3M).

Molecular biology

For N domain screening, 4-amino acid (GDAS) linker insertion constructs were cloned with appropriate primers introducing a silent NsiI restriction site according to QuickChange protocol (Stratagene). Constructs were sequence-confirmed, and the presence of the NsiI restriction site was confirmed. For detection purposes, those constructs were tagged with a HA tag (SOGGYPDYVPDYA) at the C-terminus of N through PCR amplification using appropriate primer and religation using a silent NdeI restriction site in the tag sequence. The same strategy was applied to introduce the HA tag flanked by a short linker sequence (SOGGYPDYVPDYAGGGS) at various positions in the N protein. The MoRE domain with altered codon usage to native MoRE was inserted at residue 138 of the MeV-Edm N ORF. PCR was performed with primers containing overhanging sequences encoding for MoRE and terminal NsiI sites for religation. PCR metagenesis was then performed to remove the NsiI site. All newly generated constructs were sequence-confirmed. The MeV bicistronic replicon was first cloned into a pCR2.1-TOPO vector with the help of Aat II and Avr II sites by recombineering PCR, creating a firefly luciferase-(PM-KGS)-nanoluciferase cassette. This construct was then cloned into an existing monocistronic MeV minigenome (44) with the help of Pac I and Avr II sites. The resulting bicistronic minigenome thus features the PM-KGS between the firefly luciferase and nanoluciferase ORFs. This minigenome further served as a backbone for the bicistronic variant. A second intergenic junction cassette featuring the Ntp-KGS and P ORF was generated using recombineering PCR and cloned into the bicistronic backbone with the help of Pac I and Avr II sites. To avoid the expression of functional P protein from the central ORF, a tandem stop codon was inserted into the P reading frame 11 triplets downstream of the start codon. Thus, the final construct featured a bicistronic minigenome consisting of firefly luciferase-(PM-KGS)-P_stopstop-(PM-KGS)-nanoluciferase.

Minireplicon luciferase reporter assay

BSR-T7/5 cells (4 × 10⁵ per well) in a 12-well plate format were transfected with plasmid DNA encoding the MeV N construct and either MoV PfE or PSTAG with a C-terminal FLAG tag, or MoV PSTAG with an N-terminal FLAG tag. After 36 hours, cells were harvested and subjected to coimmunoprecipitation, as previously described (6). Following immunoprecipitation using an n-antibody, samples were fractionated on 10% SDS-PAGE gels, followed by immunoblotting and chemiluminescence detection, as described.

Virus recovery

Recombinant MeV was recovered in BSR-T7/5 cells by transfecting 5 μg of the cDNA copy of the modified genome and ICN-B (0.8 μg), ICN-P (0.6 μg), and ICN-L (0.5 μg) using Genejuice transfection reagent. Cells were overlaid 48 hours after transfection onto Vero/ HSAM cells, and emerging infectious particles were passaged twice in Vero/HSAM cells. Integrity of newly rescued virus was confirmed by extracting total RNA from virus-infected cells (RNasey Mini kit, Qiagen), and cDNA was created using random hexamer primers and SuperScript III reverse transcriptase (Invitrogen). Modified genome regions were amplified using appropriate primers and sequenced.

Multistep growth curves

Before infection for the multistep growth curve, viral stocks were diluted to approximately 1 × 10⁵ TCID₅₀/ml, and titers were recorvered by TCID₅₀ titration. Vero/HSAM cells (1 × 10⁵ per well in a 12-well format) were infected with the different MeV variants at an MOI of 0.01 TCID₅₀ per well for 1 hour, and the inoculum was replaced with growth medium. Every 12 hours, samples were harvested, cell-associated virions were released through two consecutive freeze-thaw cycles, and progeny virus titers were determined through TCID₅₀ titration.
Next-generation sequencing

Next-generation sequencing using MiSeq (Illumina) was performed to determine the ratio of 

PyN7V transcripts in recMeV constructs. When cDNA was isolated from infected cells using the RNeasy kit (Qiagen), and cDNAs of mRNA transcripts were synthesized using oligo(dT) primers and SuperScript III reverse transcriptase (Invitrogen). MiSeq primers with Illumina overhangs targeting a region of ~500 nucleotides surrounding the P gene-starting site were used to PCR amplify cDNAs representing all P, V, and W transcripts. Next-generation sequencing was performed by the Emory Integrated Genomics Core. Sequences were queried for the relative ratios of P, V, and W transcripts for each sample.

Determination of N-terminus transcription gradient

To determine mRNA ratios of cell-associated viruses, Vero/SLAM cells were infected with an MOI of 0.05 for all recombinant viruses. When cDNA was isolated as described previously, cDNAs and cDNAs were synthesized using oligo(dT) primers and SuperScript RT III reverse transcriptase. The mRNA ratios were determined using appropriate primer pairs annealing to the N, L, and GAPDH ORFs and PowerUp SYBR Green Master Mix (Thermo Fisher Scientific) using an Applied Biosystems 7500 Real-Time PCR system.

Quantitation of mRNA and antigenome copy numbers

To monitor mRNA and genome amplification in a one-step replication curve, Vero/SLAM cells were infected with an MOI of 0.2 and spin-inoculated (200g, 30 min, 4°C). For mRNA and genome amplification, total RNA was harvested hourly. To quantify mRNA copy numbers, oligo(dT)-primed cDNAs were synthesized, whereas for antigenome copy numbers, a specific primer located in the viral transcript sequence was used. qPCR was performed in the Applied Biosystems 7500 Real-Time PCR System using StepOnePlus Real-Time PCR System, TaqMan Fast Virus 1-Step Master Mix (Thermo Fisher Scientific), and specific primer pair 3 (99). To calculate mRNA copy numbers, a standard curve was created using a 1:4 dilution of 1×10^11 genomic DNA from a cell line. Counting was normalized on the basis of 18S RNA using a Euukaryotic 18S RNA Endogenous Control kit (Thermo Fisher Scientific).

Statistical analysis

To assess the statistical significance of differences between sample means, one-way ANOVA with Sidak’s multiple comparison post tests was applied using the Prism 7 software package (GraphPad). Student’s t-test for two independent samples was applied to determine the significance of the data. The results are presented as mean ± standard error of the mean (SEM) and were considered statistically significant at p < 0.05.

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Appendix III: Cross-resistance mechanism of respiratory syncytial virus against structurally diverse entry inhibitors. PNAS (2014)
Cross-resistance mechanism of respiratory syncytial virus against structurally diverse entry inhibitors


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Respiratory syncytial virus (RSV) is a leading pediatric pathogen that is responsible for a majority of infant hospitalizations due to viral disease. Despite its clinical importance, no vaccine prophylaxis against RSV disease or effective antiviral therapeutic is available. In this study, we established a robust high-throughput drug screening protocol by using a recombinant RSV reporter virus to expand the pool of RSV inhibitor candidates. Mechanistic characterization revealed that a potent newly identified inhibitor class blocks viral entry through specific targeting of the RSV fusion (F) protein. Resistance against this class was induced and revealed overlapping hotspots with diverse, previously identified RSV entry blockers at different stages of preclinical and clinical development. A structural and biochemical assessment of the mechanism of unique, broad RSV cross-resistance against structurally distinct entry inhibitors demonstrated that individual escape hotspots are located in immediate physical proximity in the metastable conformation of RSV F and that the resistance mutations lower the barrier for prefusion F triggering, resulting in an accelerated RSV entry kinetics. One resistant RSV recombinant remained fully pathogenic in a mouse model of RSV infection. By identifying molecular determinants governing the RSV entry machinery, this study spotlights a molecular mechanism of broad RSV resistance against entry inhibitors that may affect the impact of diverse viral entry inhibitors presently considered for clinical use and outlines a proactive design for future RSV drug discovery campaigns.

Significance

Respiratory syncytial virus (RSV) causes major disease in pediatric and elderly patients, urging the development of efficacious therapeutics. This study establishes a recombinant RSV reporter strain for drug discovery and identifies an entry inhibitor class targeting the viral fusion (F) protein. Biodimensional, structural, and functional characterization of the inhibitor spotlights two microdomains governing the conformational stability of prefusion F. Mutations in these domains cause broad cross-resistance against the compound and RSV entry inhibitors in preclinical and clinical development, without mandatory loss of in vivo pathogenicity, challenging the possible clinical benefit of current RSV entry inhibitor classes. Anti-RSV campaigns should better target postentry steps or proactively discern resistance to entry inhibitors. The resistant RSV reporter strain developed here establishes a strategy toward this goal.

Author contributions: M.L.M. and R.K.P. designed research; S.Y. and S.L. performed research; V.D.T. and M.L.M. contributed new reagents/analytic tools; D.V., S.L., M.L.M., and R.K.P. analyzed data; and R.K.P. wrote the paper.

Conflict of interest statement: M.L.M. and R.K.P. see inhibitors on a disclosure filing describing the use of recombinant A/V/AH.033 (H1N1) virus (H1N1) virus for drug discovery.

Distribution of these RSV strains and the pHR-NSV-NSV-NSV reporter plasmid is regulated by a material transfer agreement from Georgia State University. M.L.M. and Emory University are entitled to licensing fees derived from various agreements. Emory University has entered into related to products used in the research described in this paper. This study could affect his personal financial status. The terms of this agreement have been reviewed and approved by Emory University in accordance with its conflict of interest policies.

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peptide HIV entry inhibitor Fuzeon, interference with viral entry represents a clinically viable antiviral strategy (25).

High-resolution structures of RSV F trimers were solved in both the metastable prefusion (26) and final postfusion (27, 28) conformations, and show the hallmarks of class I viral fusion proteins. Each F monomer features two helical heptad repeat (HR) domains, one (HR-A) located adjacent to a membrane attack domain, the fusion peptide, and the other (HR-B) flanking the transmembrane domain. Although the HR-A is broken up into multiple distinct segments in the prefusion structure, triggering of the entry machinery leads to the assembly of an elongated HR-A triple-helix, insertion of the fusion peptide into the target membrane, and folding of the HR-B helices into the grooves of the HR-A coiled coil. In the resulting thermodynamically highly stable six-helix bundle (6H6B) structure, the fusion peptide and transmembrane domains align, and thus, the target and donor membranes, are brought into close proximity (29). Based on the appearance of resistance mutations in F protein HR domains and biochemical evidence, some of the most advanced RSV entry inhibitors were suggested to prevent the creation of fusion pores through competitive interference with 6H6B closure (21, 30).

Here, we describe a previously unidentified structural class of small-molecule RSV entry inhibitors through the development and implementation of an automated drug-screening assay. Resistance mutations located to two hotspots in the RSV F protein, setting the stage for a structural and mechanistic characterization of viral escape through molecular modeling, biochemical assays, and the generation of genetically controlled resistant RSV recombinants. Our results highlight molecular determinants that control triggering of the RSV membrane fusion machinery and outline a cross-resistance mechanism for broad RSV escape from structurally diverse entry inhibitors. We assessed the effect of resistance on viral pathogenesis in a small animal model of RSV infection to determine whether this escape mechanism may jeopardize the therapeutic potential of RSV drug candidates considered for clinical use (31).

**Results**

To identify novel anti-RSV drug candidates, we screened a 10,000-entry small-molecule library against a 10,928-entry compound library. Data for each compound were normalized, scaled, and are plotted by screening score (z score). The dashed line marks the hit selection cutoff (10 assay – 50% of hits maintained; >90% RSV inhibition at 0.5 μM concentration, respectively). Structures of compounds are shown in A. To identify compounds with higher efficacy against RSV, we used a high-throughput screening assay (10 nM, 0.5 μM, 2.5 μM, 5.0 μM, 10 μM). Values are means of three experiments ± SD. Where applicable, CHO cells were determined through four-parameter variable slope regression modeling. Values in parenthesis denote 95% confidence intervals. CHO concentrations are based on human metabolic activity after 24-hour exposure, highest concentration assessed 25 μM, n.d., not determined; SI, CHO/SI

**Fig. 1.** Discovery of a previously unidentified small-molecule class of RSV inhibitors. (A) Results of a high-throughput screen of RSV A241R-18a-4 cells against a 10,928-entry compound library. Data for each compound were normalized, scaled, and are plotted by screening score (z score). The dashed line marks the hit selection cutoff (10 assay – 50% of hits maintained; >90% RSV inhibition at 0.5 μM concentration, respectively). (B) Structures of compounds are shown in A. (C) Dose-response curves for infected compound GAP-3710 by compound GAP-3710 concentrations and p-test scores. In addition to the compound GAP-3710, we also screened for p-test scores. In addition to the compound GAP-3710, we also screened for p-test scores. In addition to the compound GAP-3710, we also screened for p-test scores. In addition to the compound GAP-3710, we also screened for p-test scores. In addition to the compound GAP-3710, we also screened for p-test scores. In addition to the compound GAP-3710, we also screened for p-test scores. In addition to the compound GAP-3710, we also screened for p-test scores. In addition to the compound GAP-3710, we also screened for p-test scores. In addition to the compound GAP-3710, we also screened for p-test scores. In addition to the compound GAP-3710, we also screened for p-test scores. In addition to the compound GAP-3710, we also screened for p-test scores. In addition to the compound GAP-3710, we also screened for p-test scores. In addition to the compound GAP-3710, we also screened for p-test scores.
JMN5-033 (34, remained potently inhibitory even when added 8 h after infection. This time-of-addition profile points toward inhibition of virus attachment or cell entry by the compound. For cross-

examination, we tested GPAR-3710 in two plasmid-based reporter assays that specifically measure biocytin staining of the viral entry (35) and polymerase (36) machinery, respectively. RSV F protein-mediated membrane fusion activity was specifically and potently inhibited by the compound in these assays (Fig. 2F), whereas activity in the viral RNA-dependent RNA polymerase (RdRp) complex remained unaffected (Fig. 2C). The perceived higher potency of the compound against live virus than transiently expressed F protein (95% inhibition in Fig. 1C vs. 80% inhibition in Fig. 2B) most likely originates from the potentiating effect of repeat opportunities for compound interference presented during multiple-step virus replication.

To directly monitor the effect of the inhibitor on the rate of viral entry, we established a quantitative RSV entry assay that measures virus-to-cell fusion in near real time (Fig. 2D). RSV particles were spin-precipitated on a monolayer of cells expressing either the amino- or carboxy-terminal halves of an EGFP-venililuciferase chimeric protein (37). Simultaneous fusion of the incoming viral particles with two target cell targets results in cell-cell mixing, restoring EGFP fluorescence, and venililuciferase activity. When executed in the presence of increasing GPAR-3710 concentrations, this assay revealed a significant, dose-dependent reduction of the RSV entry rate by the compound (Fig. 2D). Taken together, these observations characterize the GPAR-3710 scaffold as a previously unidentified class of small-molecule RSV entry inhibitors.

Escape Mutations Locate to the RSV F Protein. As a hallmark for pathogen-directed antiviral compounds, the experimental induction of viral escape from inhibition is typically straightforward. For RSV, resistance mutations generally locate to the viral protease and are naturally targeted by the compound. We predicted RSV escape from GPAR-3710 inhibition through gradual adaptation to growth in the presence of increasing compound concentrations. Robust resistance—defined by viral growth in the presence of 30 μM (≈120× EC50 concentration) of the compound—reliably appeared within a 3-4-d adaptation period. We concentrated on the viral entry machinery in search for the molecular basis for escape and determined the F protein sequences of six independently adopted RSV strains. Candidate mutations were built in an expression plasmid encoding the RSV line 19 (L19) F protein (38) through directed mutagenesis, followed by first-pass resistance testing in transient cell-to-cell fusion assays carried out in the presence of the compound (Fig. 3A).

In each of the six strains, we identified a single point mutation in the F protein that contributed to the phenotype (Fig. 3A). The mutations clustered in two linear microdomains (400 and 485) of RSV F, spanning residues 400–401 and 486–489, respectively. Mutations D401E and D489E were selected for transient cell-to-cell fusion assays in the presence and absence of the compound. In addition, we generated and analyzed an F401D/E198D double-mutant construct. All mutant F were hyperproductive compared with the standard L19-F protein (Fig. 3B). Particularly robust resistance was observed when the D489E mutation was present.

To verify the role of escape in the context of viral infection, we transferred the mutant F constructs into the genetically tractable cDNA background of a recombinant RSV A2, harboring the L19-F protein (32). In addition, we generated a cDNA construct containing the F401D/E198D double-mutant in the place of the parental L19-F. All three mutant RSVs were recovered successfully and showed resistance to GPAR-3710, based on efficient spread through cell monolayers in the presence of the compound (Fig. 3C). They also showed accelerated growth rates compared with standard recombinant RSV A2-L19F (Fig. 3D).

Fig. 3. Resistance profile of GPAR-3710. (A) Resistance mutants identified in the RSV F protein through viral adaptation. (B) Resistance quantification, using transiently expressed RSV L19F mutants, the DSP-based quantitative cell-to-cell fusion assay, and Venus luciferase substrate. Values represent mean ± standard error of the mean (SEM). Each measurement was performed in triplicate. (C) Drug response of RSV A2-L19F and RSV A2-L19F (D401E/E198D) incubated with viral inhibitors. (D) Growth curves of the recovered RSV recombinants at 37 °C. Cell-associated viral titers were determined through TOX assay by using DAPI-stained-derived fluorescence as readout. Values are means of three experiments ± SD.

Structural Basis for Cross-Resistance Against Diverse RSV F Inhibitors. Previous RSV drug discovery campaigns have yielded several structurally distinct, highly potent small-molecule entry inhibitor classes that reportedly likewise induced escape mutations in the F 392–401 and/or 486–489 microdomains (Table S1). Lead analogs of some of these inhibitor classes are at different stages of preclinical and clinical development. To quantify viral resistance, we generated dose–response curves for GPAR-3710 and, for comparison, a developmentally advanced RSV entry inhibitor, BMS-433771, against the three RSV recombinants (31). Mutations in either microdomain resulted in a more than 100-fold increased EC50 for either compound (Fig. 4A), confirming robust resistance.

Based on a biochemical target analysis, it was proposed that BMS-433771 populates a hydrophobic pocket in the HR-A triple helix that contains residue 489, preventing assembly of the 6H fusion core during F refolding into its postfusion conformation (30). Surprisingly, however, both resistance domains map to opposing ends of the rod-like postfusion F structure, separated by >100 Å from each other (Fig. 4B). Recently, the structure of RSV F was solved also in the in the metastable pseudovirus state (30). When we localized the hotspots in this structure, we found residues 401 and 489 to be positioned in close proximity of each other (<10 Å) at the base of the head domain in pseudovirus F (Fig. 4C). The close proximity of individual resistance hotspots in pseudovirus F was not limited to escape mutants specifically identified through viral adaptation to GPAR-3710, because both the F 392–394 and 389–401 microdomains are likewise located in immediate proximity to residues 486–489 in pseudovirus F (Fig. 4D).
Resistance Mutations Alter the Rate of F-Mediated Membrane Fusion.

Prompted by proximity of both resistance hotspots in prefusion F and the accelerated growth rate of the RSV recombinants, we asked whether mutations in either microdomain affect prefusion F refolding rates. Using the kinetic cell-to-cell fusion assay, we assessed the rates of fusion pore formation mediated by the different F mutants. At physiological temperature, maximal fusion rates of all three F mutants were increased compared with that of standard F (Fig. 5 A and B). To fully appreciate the altered refolding kinetics of the mutant F variants, we determined fusion rates under reduced energy conditions (32 °C incubation temperature). Then, none of the individual mutations showed a statistically significant accelerating effect on fusion kinetics. In contrast, the F_{PDAO1/PO0958} double mutant significantly boosted the fusion rate at 32 °C, indicating a temperature-sensitive phenotype (Fig. 5 B and C).

A densitometric analysis of whole-cell lysates and cell surface-expressed F material and immunoblotting demonstrated enhanced cell surface steady-state levels of the F_{PO0958} mutant compared with standard RSV L19-F (Fig. 5D). However, levels of the F_{PDAO1/PO0958} double mutant were slightly lower than those of F_{PO0958}, and intracellular transport rates of the double mutant and standard F remained essentially identical when cells were incubated at 32 °C (Fig. 5E).

These results suggest that higher bioactivity of the double mutant does not result from increased surface expression but indicate a synergistic effect of changes in each resistance hotspot on F bioactivity. To assess the broader applicability of this resistance model, we inactivated the inhibitor/escape mutant combination by generating F-K304R and F-EM60Y substitutions (reportedly mediating resistance to BMS-433771; Table S1) and testing resistance of these F constructs to inhibition by GPAR-3710. Each of these mutations caused a hyperthermophilic phenotype and mediated cross-resistance against GPAR-3710 (Fig. 5A–D). Remarkably, also an F-F341I substitution increased F cell-to-cell fusion activity and resulted in resistance to GPAR-3710 (Fig. 5A). This mutation is located in the F fusion peptide region and was previously reported only in conjunction with resistance to BMS-433771. Our results suggest that these fusion peptide mutations phenocopy the effect of substitutions in the F 392–401 and 486–489 microdomains, resulting in cross-resistance to diverse entry inhibitors.

Effect of Resistance Mutations on Viral Pathogenicity.

To test whether a reduced structural stability of the mutated prefusion F constitutes the underlying mechanism for resistance, we adapted to RSV F a recently established class I viral envelope protein fusion-core assay that biochemically monitors the formation of the thermodynamically stable 6H fusion core (35), which is indicative of fusion protein refolding into the prefusion conformation. Intact F trimers
were natively extracted from gradient-purified viral particles, followed by gel fractionation under mildly denaturing, nonreducing conditions. Presence of the stable GHH core in postfusion F complexes should be reflected by predominant migration of the extracted material as homotrimers, whereas metastable prefusion F trimers have a propensity to dissociate. Thermal refolding of standard prefusion F into the postfusion conformation through brief heat shock of particles at increasing temperatures before extraction resulted in a gradual rise in trimeric material (Fig. S5).

When we examined standard F and the three drug-resistant mutants in this assay, the mutant trimers predominantly migrated as stable trimers, whereas standard F was mostly monomeric (Fig. 6A). These findings spotlight that the resistance mutations reduce the structural stability of prefusion F complexes. To test whether this phenotype is mirrored by increased sensitivity of the reconstituted virions to thermal inactivation, we subjected viral preparations to a 24-h incubation step at different temperatures in the absence of target cells. The individual mutant strains showed an intermediate but significant reduction in titers compared with standard recRSV after incubation at 32–39 °C (recRSV A2-L19FΔp141Δp493) or 39 °C (recRSV A2-L19FΔp141Δp493), respectively (Fig. 6B). Moreover, temperature sensitivity was most pronounced in the case of the recRSV A2-L19FΔp141Δp493ΔΔp458 double mutant, because titers of this strain were significantly
lower over the whole temperature range assessed. Recombinant RSV harboring the resistance mutations specifically reported for BMS-433771 (recRSV A2-L159FΔp2Δ18 and recRSV A2-L19FΔp2Δ18) showed analogous phenotypes when subjected to these assays (Fig. S6, A and B), underscoring the broader applicability of escape mechanism from entry inhibitors.

Heightened thermosensitivity may coincide with lowered viral fitness in vivo, which could render drug-resistant variants clinically insignificant. We used an established mouse model for RSV infection (40) to assess pathogenicity of the mutant viruses. Only the two recombinants expressing single-mutant F variants that had emerged spontaneously during adaptation were subjected to this study. Lungs titers of BALB/c mice infected with recRSV A2-L159FΔp2Δ18 were slightly reduced compared with animals exposed to standard recRSV (Fig. 6C). However, viral loads of animals infected with recRSV A2-L19FΔp2Δ18 remained unchanged.

The induction of extensive mucus production is one of the key factors associated with RSV pathogenesis (41) and serves as an indicator for the severity of RSV disease in the mouse model (38, 42). When we infected animals with the two mutant recombinants and standard recRSV, the recRSV A2-L159FΔp2Δ18 mutant was only slightly mucogenic compared with mock-infected mice (Fig. 6D). In contrast, the recRSV A2-L19FΔp2Δ18 recombinant showed strong mucus induction at a level at least equivalent to that seen in lungs of infected animals infected with parental recRSV A2-L19F. Taken together, these results demonstrate that the individual F mutations, which mediate robust resistance of recombinant RSV to diverse entry inhibitors, are not necessarily associated with reduced viral pathogenicity in vivo.

**Discussion**

As a leading cause for infant hospitalization from viral respiratory disease, RSV has emerged in the past decade as a major target for the development of novel vaccines and therapeutics. Although formalin-inactivated RSV vaccines and subunit vaccines based on the G and F viral glycoproteins were associated in several past trials with disease enhancement (43–46), live attenuated vaccine candidates tested to date were—although safe in infants—poorly immunogenic, reflected by insufficient neutralizing antibody titers (47). Antiviral therapies may ameliorate disease in high-risk pediatric patients and possibly the elderly, especially because viral load in the early phase of clinical disease was found predictive for the severity of disease progression and the risk of life-threatening complications (16, 17).

Large-scale screening campaigns to identify novel therapeutic candidates against RSV were compromised thus far by the lack of appropriate reporter strains that were developed for robust automated drug discovery assays. Our study demonstrates the value of the recombinant RSV strain expressing renilla luciferase that we recently generated (32) for this task. Major advantages over conventional RSV-based assays explored for high-throughput campaigns are the broad dynamic range of the luciferase reporter, the availability of a full set of sub-infections for MOA characterization that are genetically matched to the screening strain; the option to readily assess resistance in genetically controlled viral recombinants by using an efficient reverse genetics system; and the high pathogenicity of the reporter strain in the mouse model compared with standard laboratory RSV strains (40), opening a straightforward path toward small-animal efficacy testing of lead candidates.

By design, the high-throughput assay developed for our screen has a higher propensity to identify early and intermediate stage inhibitors of the viral life cycle (i.e., inhibitors of viral attachment, fusion, and viral polymerase activity) than blockers of viral assembly and egress, because the latter would act downstream of luciferase reporter expression. Consistent with this assumption, the protocol yielded in the most prominent hit candidate a previously unidentified RSV-specific virus entry inhibitor class when tested in a 10,000-compound proof-of-concept campaign. Characterization of the hit compound in infection and sub-infection reporter assays and tracing of resistance to point mutations in the viral F protein confirmed interference with an F-mediated membrane merger as the underlying mechanism of anti-RSV activity. The RSV entry machinery emerges as highly susceptible to inhibition by small-molecule inhibitors, because structurally distinct lead compounds identified in several independent drug discovery campaigns all block membrane fusion (21–24). Possible reasons for this prevalence may include that entry inhibition poses lower demands on the compound than other inhibition strategies, because it does not mandate plasma membrane permeability of the inhibitor. In addition, paramyxovirus F proteins are comparably large in size and undergo—like other class I viral fusion proteins (29)—complex conformational rearrangements to mediate membrane merger. Combined, the result is the presentation of multiple druggable targets for small-molecule interference. In contrast to the F proteins of most members of the paramyxoviruses family (48), RSV F is capable of mediating not only fusion but also viral attachment (19, 20), which may further broaden the spectrum of possible drug targets.

Five of the previously identified RSV entry inhibitors were synthetically developed to therapeutic candidate level and are at different stages of preclinical and clinical assessment (21–34, 31). Photoaffinity labeling of antibodies implied physical binding of compounds representing two different inhibitor classes to F residue Y198 (21, 30), which is located in the HR-A domain and was proposed to reside in the immediate vicinity of HR-B residue D189 in a hydrophobic pocket in the viral G cleavage core (illustrated in Fig. S7). In addition to these two classes, resistance hotspots were determined also for the remaining three scaffolds and, in analogy to our characterization of GPPA 3710, in all cases included F residues in the 400 and/or 489 microdomains (22, 24). Although recognized as surprising that distinct chemical inhibitor classes supposedly target the same F microdomain with high affinity (30), previous studies concluded that all of these diverse compounds prevent RSV entry through docking into the same hydrophobic cavity around residue Y198 in the central HR-A triple helix (21, 30), which emerges transiently during assembly of the G/HN structure. Currently, none of the resistance mutations reported for any of these compounds maps to HR-A residues that surround Y198 and are implicated in forming the proposed target cavity, or any other position in the HR-A domain. However, this hypothesis was developed before the pre-fusion RSV F structure was solved, and previous work did not consider possible effects of resistance mutations on the conformational stability of pre-fusion F or the kinetics of viral entry.

Although biochemical data reveal direct binding of two inhibitor classes to F residue Y198 (21, 30), we propose based on three major lines of evidence—structural insight, biochemical characterization, and functional data—that unprecedented broad cross-resistance of RSV against multiple structurally diverse entry inhibitors is based on indirect escape. First, compound docking into postfusion RSV F structures failed to provide a mechanistic explanation for the hotspot around F residues 392–401 in resistance (30). We demonstrate that the 400 and 489 microdomains are located at opposing ends of the postfusion F structure, but are poised in close proximity to each other at the intersection of stalk and head domain in pre-fusion F. Interestingly, several studies investigating related paramyxovirus F proteins have identified this network of noncovalent interactions between pre-fusion F stalk and head as a major determinant for controlling the conformational stability of the trimmer (49, 50). We have furthermore demonstrated that point mutations in this region confer resistance against a small-molecule entry inhibitor of MoV that we have developed (51). Second, point mutations in either of the two resistance hotspots reduced the structural stability of the pre-fusion RSV F trimmer in biochemical assays and resulted in enhanced
spontaneous viral inactivation rates in the absence of target cells. Third, membrane fusion rates of resistant F proteins were enhanced compared with those of the parent trimer, indicating accelerated refolding of the complex from the prefusion to the postfusion confirmation.

Taken together, these observations support an effective mechanism of secondary RSV resistance, in which escape mutations accumulate in F microdomains that govern the structural stability of the envelope glycoprotein, leading to complex unfolding rates of the conformationally destabilized mutant F trimers are enhanced, resulting in a hyperfusogenic phenotype, and, possibly, a narrowed window of opportunity for small-molecule docking and interference with F trimers rearrangement leading to fusion pore formation. Different escape pathways were also identified for HIV resistance to the pertussis toxin inhibitor Fuzone (52, 53). However, Fuzone escape did not coincide with resistance to other entry inhibitors (54).

Acidic conformational change of RSV F and may amount to a substantial obstacle in the clinic.

Despite the reduction in prefusion F conformational stability, fitness of one of the resistant recRSVs was unchanged compared with the standard recRSV in a mouse pathogenesis model, whereas the other was appreciably attenuated. Recently established, this mouse model using the recRSV A2-L19F strain exhibits higher lung viral loads, more airway mucus, and more severe respiratory distress than the parental A2 and live 19 strains (35, 42), recapitulating key clinical parameters of infant RSV bronchiolitis (38, 40, 55). Although it is not determined yet whether pathogenicity of a resistant RSV-F mutants would be equal or in human host, efficient replication in particular of this recombinant in the mouse model raises concern that resistance mutations in the homologous protein of RSV may fail to become prevalent in circulating RSV strains should any of the entry inhibitors appear unique to RSV F and may amount to a substantial obstacle in the clinic.

Our data indicate that RSV entry inhibitors currently considered for clinical use are at risks to lose therapeutic benefit in the clinic because of rapidly emerging viral resistance. We propose that future RSV drug discovery campaigns be directed at inhibiting postentry steps of viral replication or be proactively designed to conceptually circumvent broad cross-resistance between entry inhibitors. For instance, using the resistant recRSV A2-L19F, viral escape was described in this study as the screening agent should have a high propensity to yield high candidates that either act postentry or, if mechanistically possible, block viral entry without being compromised by cross-resistance.

Materials and Methods

Cell Culture, Transfection, and Virus Stocks. All cell lines were maintained in DMEM supplemented with 7.5% (vol/vol) FBS at 37 °C and 5% CO2. Baby hamster kidney (BHK-21) cells stably expressing the E7 polyomavirus (EV 12/176aise, EV 12/176a) were incubated at every third passage in the presence of 500 μg/mL G418 (Genetex). Cell transfections were carried out by using Lipofectamine 2000 (Invitrogen) or GeneJuice reagent (EMD Millipore). Standard RSV virus stocks were prepared by infecting HEp-2 cells (ATCC HB-8050) at a multiplicity of infection (MOI) of 0.1 pfu per cell at 37 °C, followed by incubation at 32 °C for 7.5 h, and cell-associated progeny virus was released through one freeze-thaw cycle, and titers were determined by TCID50 titration or immunoprecipitation assay as described (26). RecRSV strain stocks were purified through ultracentrifugation through a 10%–60% (w/v) sucrose cushion at 35,000 rpm for 15 h at 4 °C. The virus-containing fraction was diluted in 1 mM Tris at pH 7.2, 100 mM NaCl, and 10 mM EDTA (TE buffer), passed at 60,000 × g for 30 min at 4 °C, and resuspended in the buffer.

Generation and Recovery of Recombinant RSV. Parent mutations were introduced through directed mutagenesis into a shuttle vector containing the RSV L19F ORF, followed by transfer of the modified shuttle plasmid 19F fragment into pBMY1RSV A2-L19F-rec (pBMY1RSV A2-L19F-rec) and pBMY1RSV A2-L19F-mKate2 (pBMY1RSV A2-L19F-mKate2). Recombinants were recovered as described (32) and subjected to RT-PCR and cDNA sequencing for confirmation of specific point mutations.

Compounds. All compounds were dissolved in dimethylsulfoxide (DMSO) and stored at −80 °C. Source compounds were obtained from Ambit (previously described p-nitrophenylphosphatase inhibitor O9517, ref. 57) and Vitas-M Laboratory or NCI (GPR-3710 stocks). The screening library was obtained from ChemDiv. For screening, 2 × 10^4 (96-well plate format) or 6 × 10^5 (384-well plate format) Hep-2 cells per well were seeded into solid-well microtiter plates. Test stocks dissolved in DMSO were added at 3 μM final concentration (final DMSO content was below 0.1% v/vol). As internal reference, four wells on each plate were treated with the pan-protein kinase inhibitor JNJ-237 (final concentration 1 μM) or vehicle (DMSO) only. Cells were infected with recRSV A2-L19F-rec (MOI = 0.2 pfu per cell) and screened for luciferase activities were quantified in a Synergy M1 (BioTek) multimode microplate reader at 44 to 48 h incubation.

HTS Data Analysis. Raw data sets were automatically reformatted and imported into the cellHTS application package (28, 59). Data were analyzed according to the protocol methods. Each value was normalized to the median for all compound wells of the plate, and normalized values were scaled to the median absolute deviation of the plate. The Seifdeer database package (American Chemical Society) was used to query chemical databases with hit candidate structures to evaluate known bioactivities of analogs, commercial availability, and free intellectual property space.

Dose-Response Curves, Efficacy, and Cytotoxicity. Cells infected (MOI = 0.05 pfu per well) with recRSV A2-L19F-rec, recRSV A2-L19F-add, and recRSV A2-L19F-mKate2, or GPR-3710-resistant variants thereof were incubated in the presence of serial dilutions of compound for 44 h, followed by titration of cell-associated progeny particles or quenched fluorescence as described. If possible, 50 or 50 percent effective concentrations (EC50 or EC90, respectively) were calculated based on four-parameter variable slope nonlinear regression modeling of mean values of at least three experiments. To quantify the effect of compound on cell metabolic activity, cells were incubated in a mixture of serial compound dilutions (μM, highest) for 44 h, then subjected to a nonradioactive cytoxality assay (CellToX; Promega) according to the manufacturer’s instructions. Assay values were normalized to vehicle (DMSO) controls according to the formula: 100 × (test value − control value) / (vehicle − control value).

Time-of-Addition Assays. Hep-2 cells were spin-inoculated (1000 × g, 30 min; 4 °C) and treated with 10 μM recRSV A2-L19F-rec. Compound was added at the specified times preinfection or postinfection, and luciferase activities determined 24 h after infection. "Representative samples received volume equivalents of vehicle (DMSO)."

Multiplex Reporter Assay. Based on a described p17-RSV-luciferase minigenome reporter (30), an RSV minigenome construct was generated under the control of the constitutive RNA pol I promoter (p17-RSV-rip). Mutant-7 cells were cotransfected with this plasmid and plasmids pRHE1, pRHE-M2, pRHE-M1, pRHE-M5, and pRHE-3C, and luciferase activities determined 24 h after transfection. "Representative samples received volume equivalents of vehicle (DMSO)."

Knockdown Cells. Cells were transfected with the plasmids encoding the shF3, shF9, or shF10 (37), respectively, mixed at equal ratios, and transfected with multiple cell culture substrate as described (38), and spin-inoculated with recRSV A2-L19F-rec (200 × g, 30 min; 4 °C). MOI = 6 pfu per cell in the presence of GPR-3710 or DMSO. Activity of reconstituted luciferase was quantified at the specified time points.

Knockdown Cells. Cells were transfected with the plasmids encoding the shF3, shF9, or shF10 (37), respectively, mixed at equal ratios, and injected into the mammary gland of mice. Animals were sacrificed at the specified time points.

Knockdown Cells. The shF3, shF9, or shF10 expression plasmids were transfected into 293T cells, and transfected cells were detached and reseeded in an equal ratio. Cells were then transfected with standard or mutant RSV L19F-expressing plasmid, loaded with Envluciferase substrate...
strats as above, and incubated at 32 °C or 37 °C. Lucifere activity was recorded at the specified time points.

**Microscopy.** Fluorescence micrographs were taken on a Zeiss Axio Observer.D1 inverted microscope at a magnification of 200x. For phase-contrast micrographs, a Nikon Diaphot 200 inverted microscope was used at a magnification of 20x.

**Virus Adaptation.** HEP-2 cells were infected with mockRSV A2-L198F-mKate2 at an MOI of 0.1 per cell. Infected cells were cultured in the presence of 0.1 µM GPMK at 37°C. An extensive red fluorescence emerged, and a cell monolayer was reinfected with 10-fold diluted cell-associated viruses in the presence of increasing concentrations of GPMK. RSV A2-L198F-mKate2 was extracted (ribonuclease and glycogen) from individually adhered cultures when GPMK 370 lutcented cultures were generated from 30 µM were tolerated, and DNA was generated by using random hexamer primers. The resulting PCR products were amplified and subjected to DNA sequencing. Candidate mutations were reinserted in RSV A2-L198F expression plasmids and subjected to cell-to-cell fusion assays in the presence of compound. Selected clones were reinserted in the pX floxedRSV A2-L198F-mKate2 plasmid background and the corresponding recombinants were recovered.

**Surface Biostatistics, SDSPAGE, and Immunoblotting.** Protein surface expression was determined as described (13) with the following modifications. 293T cells (8 x 10⁶ per well in a 12-well plate format) were transfected with 2 µg of plasmid DNA encoding the specified RSV V construct. Washed cells were lysed in 2% SDS, 50 mM Tris-HCl (pH 7.2), 1% sodium deoxycholate, 1% NP-40, 50 mM EDTA, 50 mM sodium fluoride, and protease inhibitors. Washed pellets were fractionated by SDS-PAGE. Blotted onto PVDF membranes (GE Healthcare), and p-protein material was immunostained by using the monoclonal antibody to RSV. Immunoblots were developed by using a ChemiDoc digital imaging system (Bio-Rad) and subjected to densitometry quantification by using the Image Lab software package (Bio-Rad).

**Fusion Core Activity.** Standard and mutant mockRSV A2-L198F were grown at 32 °C. Cell-free viral particles were harvested, pelleted, resuspended, and purified by ultracentrifugation through a 25% sucrose (w/v) stepwise sucrose gradient, and subjected to cold extraction of native plasma membrane proteins by using Native PAGE.より(101 mM Tris-Cl at pH 19.5, 0.1% SDS, 4% glycerol, 0.02% bromophenol blue, 0.1% digitonin, 25 mM sodium selenite, and deionized water). Extracts were mixed with Laemmli sample buffer with 0.05% SDS and were fractionated on a 10% (w/v) Tris-Acetate gradient gel (Life Technologies). Following immersion, as described above.

**Temperature Sensitivity Assay.** Standard and resistant mockRSV A2-L198F strains as specified were divided into equal aliquots, which either were frozen at -80 °C or incubated at the indicated temperature for 24 h followed by freezing, and remaining virus titers were determined by TCID₅₀ titration.

**In Vivo Infection.** BALB/c mice (Jackson Laboratories) were anesthetized by intraperitoneal injection of a ketamine/xylazine solution and injected intranasally with 1 x 10⁷ pfu of wildtype RSV A2-L198F, redRSV A2-L198F, or redRSV A2-L199F. All animal procedures were performed according to the guidelines of the Kunming University of Science and Technology Committee.

**Lung Tissues.** Mice were euthanized 4 days after infection (p.i.), and the left lung lobe was extracted, weighed, and homogenized by using a Dounce homogenizer (Bio-Rad). The homogenates were serially diluted, transferred to HEP-2 cells, and cells were cultured in 1 ml with minimum essential medium (MEM) containing 1% (v/v) fetal calf serum in a 37 °C incubator with 10% O₂ and 5% CO₂ solution. The supernatants were removed, and the cell lysates were visualized by immunodetection as described (40, 43).

**Mucin Expression.** Mice were euthanized with pentobarbital (100 mg/kg), and the heart-lung unit was harvested and fixed in 10% formalin. Lung tissues sections embedded in paraffin blocks were stained with periodic acid-Schiff (PAS) stain to visual mucin expression. EAC stained slides were digitally scanned by using a Zeiss Microscopy microscope (Carl Zeiss Microscopy).

**Statistical Analysis.** To determine the active concentrations of dose-response curves, four parameter variable slope regression modeling was performed by using the Prism (GraphPad) software package. Results are expressed as the percentage of infected macrophages with 95% asymmetrical confidence intervals. Statistical significance of differences between sample groups was assessed by oneway or two-way analysis of variance (ANOVA) in combination with Bonferroni’s multiple comparison posttests as described in the figure legends. Experimental uncertainties are identified by error bars, representing SD or SEM as specified.

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