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Plasticity in Structural and Functional Interactions between the Phosphoprotein and Nucleoprotein of Measles Virus

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Background: Binding of the MeV C-terminal disordered domain of the nucleoprotein (NTAIL) to the X domain (XD) of the phosphoprotein mediates recruitment of the polymerase.

Results: NTAIL amino acid substitutions that reduce NTAIL-XD affinity and/or NTAIL α-helical folding do not affect polymerase rates but strongly affect infectivity.

Conclusion: MeV polymerase tolerates NTAIL amino acid substitutions.

Significance: NTAIL sequence plays a role in optimal infectivity.

The measles virus (MeV) phosphoprotein (P) tethers the polymerase to the nucleocapsid template for transcription and genome replication. Binding of P to nucleocapsid is mediated by the X domain of P (XD) and a conserved sequence (Box-2) within the C-terminal domain of the nucleoprotein (NTAIL). XD binding induces NTAIL α-helical folding, which in turn has been proposed to stabilize the polymerase-nucleocapsid complex, with cycles of binding and release required for transcription and genome replication. The current work directly assessed the relationships among XD-induced NTAIL folding, XD-NTAIL binding affinity, and polymerase activity. Amino acid substitutions that abolished XD-induced NTAIL α-helical folding were created within Box-2 of Edmonston MeV NTAIL. Polymerase activity in minireplicons was maintained despite a 35-fold decrease in XD-NTAIL binding affinity or reduction/loss of XD-induced NTAIL α-helical folding. Recombinant infectious virus was recovered for all mutants, and transcriptase elongation rates remained within a 1.7-fold range of parent virus. Box-2 mutations did however impose a significant cost to infectivity, reflected in an increase in the amount of input genome required to match the infectivity of parent virus. Diminished infectivity could not be attributed to changes in virion protein composition or production of defective interfering particles, where changes from parent virus were within a 3-fold range. The results indicated that MeV polymerase activity, but not infectivity, tolerates amino acid changes in the XD-binding region of the nucleoprotein. Selectional pressure for conservation of the Box-2 sequence may thus reflect a role in assuring the fidelity of polymerase functions or the assembly of viral particles required for optimal infectivity.

Measles virus (MeV) is a member of the Paramyxoviridae family within the Mononegavirales order. Mononegavirales are nonsegmented negative-stranded RNA viruses that share a complex and unique mode of transcription and replication (1, 2). Virions are enveloped, and the single strand of genomic RNA is encapsidated by the nucleoprotein (N). For MeV, the envelope contains hemagglutinin (H) and a fusion glycoprotein (F) that mediates tissue targeting and MeV entry (3–5). This process is coordinated by the matrix (M) protein, which also mediates packaging of nucleocapsid into virions by interacting with both glycoprotein tails and nucleoprotein (6). Nucleocapsids are filamentous, consisting of genomic RNA encapsidated into a regular helical array of N monomers. The structure of MeV N has been modeled (7) into the electron density of MeV nucleocapsids, using the crystal structure of the N protein from the respiratory syncytial virus as template (8). This ribonucleoprotein complex, rather than naked RNA, is the template for both transcription and replication. These latter activities are carried out by the RNA-dependent RNA polymerase that is composed of the large (L) protein and of the phosphoprotein (P). Once the viral ribonucleoprotein complexes are released into the cytoplasm of infected cells, the transcription of viral genes occurs using endogenous NTPs as substrate. Following primary transcription, the polymerase switches to a processive

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# This article contains supplemental Figs. S1 and S2 and Table S1.

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Amino Acid Substitutions within P-binding Region of MeV \( ^{\text{NTAIL}} \)

mode and ignores the gene junctions to synthesize a full, complementary strand of genome length. This positive-stranded RNA (antigenome) does not serve as a template for transcription; its unique role is to provide an intermediate in genome replication. The intracellular concentration of the \( \text{N} \) protein is the main element controlling the relative level of transcription versus replication (9). When \( \text{N} \) is limiting, the polymerase functions preferentially as a transcriptase, thus leading to an increase in the intracellular concentration of viral proteins including \( \text{N} \). When \( \text{N} \) levels are high enough to allow encapsidation of the nascent RNA chain, the polymerase functions preferentially as a replicase (see Refs. 10–12 for reviews on transcription and replication).

The \( \text{L} \) protein does not directly bind viral genomic RNA during transcription and genome replication but instead is tethered onto the viral ribonucleoprotein template via oligomers of \( \text{P} \). As such, the \( \text{P} \) protein is an essential polymerase co-factor. The exact oligomeric state of \( \text{MeV P} \) is not known. By analogy with the closely related Sendai virus (13, 14), it is thought to be tetrameric, contrary to the dimeric \( \text{P} \) proteins of rabies and vesicular stomatitis viruses (15, 16). The \( \text{P} \) oligomers simultaneously bind to \( \text{L} \) (via the \( \text{P} \) multimerization domain) and to the exposed C-terminal domain of \( \text{N} \) (\( \text{NTAIL} \); amino acids 459–502) via the C-terminal X domain of \( \text{P} \) (XD; amino acids 459–507) (17–26). Progressive movement of the polymerase along its template thus is thought to require cycles of XD-\( \text{NTAIL} \) binding and release (see Ref. 27 and references cited therein).

\( \text{NTAIL} \) is an intrinsically disordered domain (12, 18) that undergoes \( \alpha \)-helical folding upon binding to XD (17–26). Structure prediction and available experimental observations indicate that the disordered nature of the C-terminal region of \( \text{N} \) is a conserved feature within members of the Paramyxovirinae subfamily (29–32). Like all intrinsically disordered proteins (33–38), \( \text{NTAIL} \) exists as a dynamic ensemble of interconverting conformers under physiological conditions of pH and salinity and in the absence of XD (24–26). Despite the overall paucity of \( \text{NTAIL} \) in hydrophobic residues and the concomitant enrichment in polar and charged residues typical of intrinsically disordered proteins (39, 40), \( \text{NTAIL} \) possesses two conserved hydrophobic patches: Box-2 (amino acids 489–506) and Box-3 (amino acids 517–525) (Fig. 1A). Box-2 contains a predicted \( \alpha \)-helical folding upon binding to XD (17–26). The latter is characterized by an equilibrium dissociation constant \( (K_D) \) of \( \sim 100 \text{nM} \), although the relationship between induced folding and binding affinity between \( \text{NTAIL} \) and XD has not been directly tested.

A proposed link among induced folding, binding affinity, and polymerase function has been suggested by functional analysis of \( \text{NTAIL} \) variants in the context of the full-length \( \text{N} \) protein (47). Strikingly, amino acids located C-terminally to Box-2 were found to impose constraint on viral polymerase function, as judged from minireplicon studies using \( \text{N} \) constructs with C-terminal truncations (47). Progressively larger deletions up to amino acid 501 result in progressively larger increases in minireplicon reporter gene expression, after which there is a precipitous loss of polymerase activity. These findings, combined with the observed loss of conformational freedom of Box-3 upon binding to XD, support the view that XD-induced structural changes in \( \text{NTAIL} \) select for Box-3 conformers, stabilizing the XD-\( \text{NTAIL} \) complex. In turn, stability of the XD-\( \text{NTAIL} \)-XD complex (measured by the XD-\( \text{NTAIL} \)-binding affinity) would impose constraint on the cycles of binding and release that are required for the polymerase to move along the template. Understanding these structure-function relationships will be essential in defining the mechanistic basis by which host and viral factors may enhance polymerase function.

The objective of the present study was to establish directly the relationships among XD-induced \( \text{NTAIL} \) folding, complex stability, and polymerase activity. The hypothesis tested was that XD-induced \( \text{NTAIL} \) folding maintains XD-\( \text{NTAIL} \) binding affinity, which in turn regulates polymerase activity. Our approach was to create amino acid substitutions in the Box-2 helix-forming region that would reduce XD binding affinity and/or XD-induced conformational changes. Taking into account the fact that the \( \text{NTAIL} \)-XD interface is dominated by hydrophobic contacts, we introduced charged (i.e. acidic) amino acids within Box-2 without however replacing large hydrophobic residues that might be essential for the XD-\( \text{NTAIL} \) interaction. The side chains of the introduced residues were of a size comparable to that of the native side chain. Two \( \text{NTAIL} \) variants were created in which the acidic group was oriented slightly toward the interface between XD and the \( \alpha \)-helix encompassing residues 486–502 (Q499E and A502D), and one was created in which the acidic side group faced away from the interface (A500D) (Fig. 1, B and C). Two of these substitutions also lie within the region of Box-2 that is essential for polymerase function in minireplicons (i.e. they are located upstream amino acid 501), and one lies C-terminally to this region, targeting amino acids that dampen viral polymerase activity. The binding affinity of the \( \text{NTAIL} \) variants for XD was measured using isothermal titration calorimetry (ITC) to achieve reliable data for potentially low affinity interactions. In addition, the impact of these mutations on the biophysical properties of \( \text{NTAIL} \) was established, including the propensity for \( \alpha \)-helix for-
mation. The ability of the mutated N proteins to support viral polymerase function was tested in MeV minireplicons. The recombinant infectious Edmonston-based strain of MeV (Ed) was used to characterize the effect of the mutations on the level and kinetics of viral transcript and genome production.

**EXPERIMENTAL PROCEDURES**

**Generation of NTAIL Constructs and Purification of NTAIL Proteins**—Coding regions for all NTAIL constructs were obtained by PCR using as template the plasmid pDEST14/NTAIL-HNP, which encodes residues 401–525 of the Ed MeV N protein with an N-terminal hexahistidine tag (19). Each mutant construct was obtained using Turbo-Pfu polymerase (Stratagene) and a pair of complementary mutagenic primers (Invitrogen). After digestion of the PCR mixture with DpnI to remove the methylated DNA template, *Escherichia coli* was transformed with the amplified PCR product. The sequence of the primers used to generate the NTAIL Q499E, NTAIL A500D, NTAIL A502D, and NTAIL G503D coding constructs is provided in supplemental Table S1. *E. coli* strain DH5α (Stratagene) was used for selection and amplification of DNA constructs. Bidirectional sequence analysis of the coding region of all expression plasmids was performed (MilleGen).

The *E. coli* strain Rosetta (DE3)pLysS (Novagen) was used for expression of recombinant proteins. Because the MeV N gene contains several rare codons that are used with a very low frequency in proteins was performed (MilleGen).

The ability of the mutated N proteins to support viral replication was disrupted by sonication in 5 volumes (v/w) of buffer A (50 mM sodium phosphate, pH 7.0, 300 mM NaCl, 10 mM imidazole, and 1 mM phenylmethylsulfonyl fluoride (PMSF)) supplemented with lysozyme (0.1 mg/ml), DNase I (10 μg/ml), 20 mM MgSO4, and protease inhibitor mixture (Sigma; 50 μl/g cells). The lysate was clarified by centrifugation at 30,000 × g for 30 min. The clarified supernatant, as obtained from a 1-liter culture, was incubated for 1 h with 4 ml of chelating Sepharose Fast Flow resin preloaded with Ni2+ ions (GE Healthcare) equilibrated previously in buffer A. The resin was washed with buffer A containing 20 mM imidazole, and the NTAIL proteins were eluted in buffer A containing 250 mM imidazole. Eluates were analyzed by SDS-PAGE. Fractions containing the recombinant product were concentrated using centrifugal filtration (Centrificon Plus-20, 5000-Da molecular cutoff, Millipore). The proteins were then loaded onto a S200 HR 10/30 column (GE Healthcare) and eluted in 10 mM sodium phosphate, pH 8.

The apparent molecular mass values of proteins were based on elution profiles from gel filtration columns using low molecular weight and high molecular weight calibration kits (LMW/ HMW, GE Healthcare). The theoretical Stokes radii (R$_{s}$) of a native (R$_{s}$N) and fully unfolded (R$_{s}$U) protein were calculated according to log(R$_{s}$N) = 0.369log(MM) − 0.254 and log(R$_{s}$U) = 0.533log(MM) − 0.682, with MM being the molecular mass (in Daltons) and R$_{s}$ being expressed in Å.

Purification of histidine-tagged XD has been described previously (19). Protein concentrations were calculated by using either the theoretical absorption coefficients ε(μm$^{-1}$.cm$^{-1}$) at 280 nm as obtained using the program ProtParam at the Bio-Rad protein assay reagent (Bio-Rad).

**Dynamic Light Scattering**—Dynamic light scattering experiments were performed with a Zetasizer Nano-S (Malvern) at 20 °C. Protein samples were diluted in 10 mM sodium phosphate buffer, pH 7, to a final concentration of 170 μM (NTAIL A502D) or 2.4 mM (XD). The samples were filtered prior to taking the measurements (Mille syringe filters, 0.22 mm, Millipore). Diffusion coefficients were inferred from the analysis of the decay of the scattered intensity autocorrelation function. All calculations were performed using the software provided by the manufacturer.

**Isothermal Titration Calorimetry**—ITC experiments were carried out either on a VP-ITC or on an ITC 200 isothermal titration calorimeter (Microcal, Northampton, MA) at 20 °C. Protein pairs used in the binding analyses were dialyzed against 10 mM sodium phosphate, pH 7.0, 1 mM PMSF, and 0.02% sodium azide to minimize undesirable buffer-related effects. The dialysis buffer was used in all preliminary equilibration and washing steps. The same XD sample was used in all binding reactions. When the VP-ITC device was used, the concentration of NTAIL proteins was initially adjusted to 9–12 μM in the microcalorimeter cell (1.41 ml), and a 10-μl volume of XD (stock solution at 150 mM) was added from a computer-controlled 300-μl microsyringe at 5-min intervals. In the case of binding reactions to parent NTAIL and NTAIL Q499E, NTAIL was also used as the injectant (from a stock at 145–155 μM), added progressively to XD (at 12–14 μM).

For experiments carried out on the ITC 200 device, the concentration of parent NTAIL and A500D NTAIL proteins was initially adjusted to 20 μM in the microcalorimeter cell (0.2 ml), and XD (stock solution at 200 mM) was added from a computer-controlled 40-μl microsyringe via a total of 19 injections of 2 μl each at intervals of 180 s. For the NTAIL A502D-XD couple, numerous experiments were carried out in which XD at various concentrations (from 200 μM up to 2.4 mM) was added progressively in the microcalorimeter cell in which the concentration of the NTAIL variant ranged from 20 to 200 μM. Heat dilution of the ligand was taken into account from peaks measured after full saturation of the protein sample contained in the microcalorimeter cell by the ligand.

A theoretical titration curve was fitted to the experimental data using the Origin software (Microcal). This software uses
the relationship between the heat generated by each injection and \( \Delta H' \) (enthalpy change in cal mol\(^{-1}\)), \( K_a \) (association binding constant in M\(^{-1}\)), \( n \) (number of binding sites per monomer), total protein concentration, and free and total ligand concentrations. The variation in the entropy \( (S/H) \) was used to estimate the content of the \( \alpha \)-helical and disordered structures using as reference the protein set 7.

**Circular Dichroism (CD)**-Structural variations of \( N_{TAIL} \) proteins were measured as a function of changes in the initial far-UV CD spectrum following the addition of either increasing concentrations of 2,2,2-trifluoroethanol (TFE) (Sigma-Aldrich) or a 2-fold molar excess of XD or lysozyme (Sigma-Aldrich), where this latter served as a negative control. The CD spectra were recorded on a Jasco 810 dichrograph using 1-mm-thick quartz cells in 10 mM sodium phosphate, pH 7, at 20 °C. CD spectra, measured between 185 and 260 nm at 0.2 nm/min, were averaged from three independent acquisitions. Mean ellipticity values per residue (\( [\Theta] \)) were calculated as \( [\Theta] = 3300 m\Delta A/(lcn) \), where \( l \) (path length) = 0.1 cm, \( n \) = number of residues, \( m \) = molecular mass in daltons, and \( c = \) protein concentration expressed in mg/ml. Number of residues \( (n) \) is 132 for \( N_{TAIL} \) variants, 56 for XD, and 129 for lysozyme, and \( m \) values are 14,676 Da for \( N_{TAIL} \) variants, 6,690 Da for XD, and 14,300 Da for lysozyme. Protein concentrations of 0.1 mg/ml were used when recording the spectra of both individual and protein mixtures. In the case of protein mixtures, mean ellipticity values per residue (\( [\Theta] \)) were calculated as \( [\Theta] = 3300 m\Delta A/[(C_{n1}/m1) + (C_{n2}/m2)]l \), where \( l \) (path length) = 0.1 cm, \( n_1 \) or \( n_2 \) = number of residues, \( m_1 \) or \( m_2 \) = molecular mass in daltons, and \( c_1 \) or \( c_2 \) = protein concentration expressed in mg/ml for each of the two proteins in the mixture. The theoretical average ellipticity values per residue (\( [\Theta]_{avg} \)), assuming that neither disorder-to-order transitions nor secondary structure rearrangements occur, were calculated as follows: \( [\Theta]_{avg} = ([\Theta]_{n1} + ([\Theta]_{n2}))/n_1 + n_2 \), where \( [\Theta]_1 \) and \( [\Theta]_2 \) correspond to the measured mean ellipticity values per residue, \( n_1 \) and \( n_2 \) to the number of residues for each of the two proteins, and \( R \) to the excess molar ratio of protein 2.

The simulated CD spectrum of a mixture containing a 2-fold molar excess of XD and in which only 15% of \( N_{TAIL} \) binds to XD was generated. First, the CD spectrum of “folded” \( N_{TAIL} \) was obtained from the CD spectrum (in millidegrees) of a mixture containing native \( N_{TAIL} \) and XD in a 1:2 molar ratio upon subtraction of the XD contribution. Then, the CD spectrum of a mixture containing 15% folded \( N_{TAIL} \) and 85% unfolded (i.e. unbound) \( N_{TAIL} \) was obtained by summing up the spectrum of folded \( N_{TAIL} \) multiplied by 0.15 and the spectrum of unfolded (i.e. free) \( N_{TAIL} \) multiplied by 0.85. Finally, this latter spectrum was summed up to the spectrum of XD, and the mean ellipticity values per residue were calculated as described above.

The experimental data in the 185–260-nm range were analyzed using the DICHROWEB website, supported by grants to the Biotechnology and Biological Sciences Research Council Centre (BBSRC) for Protein and Membrane Structure and Dynamics (48, 49). The CDSSTR deconvolution method was used to estimate the content of the \( \alpha \)-helical and disordered structures using as reference the protein set 7.

**N Protein Mutagenesis and Minireplicon Reporter Gene Expression—Oligonucleotide-mediated site-directed mutagenesis of a MeV N gene cDNA (pT7MV-N expression plasmid) was performed as described previously (47). In brief, dut ung mutant E. coli RZ1032 was used to generate single-stranded pT7MV-N, which contains uracil instead of thymidine. Mutagenic oligonucleotide primers were then annealed to the single-stranded pT7MV-N, extended, and ligated in vitro, and the double-stranded plasmid with desired Box-2 mutations was used to transform E. coli DH5α. To facilitate the identification of the desired Box-2 mutations, mutagenic primers were designed to also incorporate nucleotide substitutions that did not affect coding but added or eliminated a unique restriction endonuclease site. The sequence of the primers used to generate the mutated N constructs is provided in supplemental Table S1. After the plasmids were screened by restriction endonuclease digestion, the inserts were sequenced bidirectionally to confirm the presence of desired Box-2 mutations and to rule out the presence of second site mutations. Resultant pT7-MV-N plasmids carrying the Box-2 mutations were tested for their ability to support MeV minireplicon reporter gene expression.

Minireplicon experiments were performed as described previously (47). Briefly, HEp-2 cells were cultured in 6-well plates at 37 °C, 5% CO\(_2\), in minimum essential medium containing Earle’s salts (1× MEM) and 10% fetal bovine serum. Calcium phosphate transfection of subconfluent monolayers was used to simultaneously introduce three plasmids that encode MeV N (0.8 μg of pT7MV-N, pT7MV-N Q499E, pT7MV-N A500D, pT7MV-N A502D, P (0.6 μg of pT7MV-P), and L (0.2 μg of pT7MV-L) proteins and one plasmid expressing MeV minigenomic RNA (0.2 μg of pMV107-CAT). The minigenome contained MeV genomic termini flanking the chloramphenicol acetyltransferase (CAT) coding sequence. Expression was driven by T7 polymerase encoded by a replication-deficient recombinant vaccinia virus MVAT7, introduced at a multiplicity of infection (m.o.i.) of 2. In addition, a T7-mediated luciferase expression vector (0.2 μg of pT7-GL3-Luc) was included as a control for transfection efficiency.

Cells were harvested at 30 h post-transfection into 250 μl of reporter lysis buffer. Twenty μl of cell lysate was added to a luciferase assay system (Promega) and fluorescence recorded on a PerkinElmer LS-5B luminescence spectrometer. Fifty μl of cell lysate was analyzed for CAT activity using a FAST CAT yellow (deoxy)chloramphenicol acetyltransferase assay kit (Molecular Probes Inc., Eugene, OR). CAT reaction products were resolved using silica gel thin layer chromatography, and the UV fluorescence intensity was quantified using an AlphaImager 2000 documentation and analysis system (AlphaEase software). Luciferase activity was used to normalize the CAT data.

**Generation and Characterization of Recombinant Infectious Ed MeV Incorporating Box-2 Mutations—Recombinant MeV encoding N protein Box-2 mutations were generated and rescued using previously described methods (51). Oligonucleotide site-directed mutagenesis (QuikChange II XL site-directed mutagenesis kit, catalog No. 200523) was performed on a 2310-bp subclone of the full-length MeV genomic cDNA, with
this latter contained in plasmid p+MV (52). The subclone included the complete N gene and 239 bp of the 5’ P gene coding region. The sequence of the primers used to generate the mutated MeV N subclones is provided in supplemental Table S1. To generate a full-length genomic cDNA containing the Box-2 mutations, p+MV and the genome cDNA subclones were digested with SfiI and SacII. The purified 2310-bp subclone was sequenced to rule out the presence of spurious second site mutations and then ligated into the 16,631-bp parent genomic cDNA. Resultant full-length MeV genome plasmids with Box-2 mutations (p+MV-NQ499E, p+MV-N A500D, or p+MV-N A502D) were amplified in XL10-Gold ultracompetent cells (Stratagene). Purified plasmids containing the desired mutations were identified by their unique restriction enzyme digestion patterns as described above for N. Infectious virus was rescued from Hep-2 cells as described previously (51). Cells were transfected with plasmids expressing plus sense MeV genomic RNA, and Ed N, P, and L messenger RNAs. T7 was provided by MVAT7. Viruses containing the Box-2 mutations were rescued on Vero cells in parallel with the parental virus (Ed N, derived from p+MV), plaque-purified, and used to generate low passage pools. Sequence analysis of genomic RNA from these viral pools confirmed the presence of the desired C-terminal N gene mutation.

Multistep growth curves for the recombinant viruses were established on subconfluent Vero cell monolayers in 6-well plates (m.o.i. = 0.01). Infection with each virus was performed in triplicate, and 1 ml of culture supernatant was collected at 12-h intervals between 24 and 72 h post-infection (h.p.i.). Progeny were titrated on Vero cells, reported as the 50% tissue culture infective dose (TCID50)/ml. Cell-free virus for Ed N, P and L viruses for Ed N and quantified by ImageQuant (Bio-Rad). The signal from the Box-2 mutations, p+MV and each of the three Box-2 mutants was purified from the supernatant of virus-infected Vero cells at 48 h.p.i. by sedimentation on a discontinuous sucrose gradient. Sucrose gradient-purified viruses were characterized by SYBR Green real time RT-PCR analysis of the L-trailer/F genome ratio as a measure of defective interfering particle content (9, 53).

Virion N, P, M, and F protein composition was also quantified by dot blot analysis according to a previously described procedure (54). Briefly, serial 2-fold dilutions of purified virus in 200 µl of phosphate-buffered saline were adsorbed onto a nitrocellulose sheet using a 96-well manifold and vacuum aspiration. After drying, the membrane was incubated in 50 mM Tris-HCl buffer, pH 7.5, and 150 mM NaCl containing 5% fat-free milk powder and 2% Tween 20 to permeabilize the virions. Then, the membrane was probed sequentially with mouse monoclonal antibodies against the viral proteins followed by incubation with a peroxidase-conjugated anti-lg antibody. The signal was captured using luminescence imaging of each spot and quantified by ImageQuant (Bio-Rad). The signal from serial dilutions of virus was used to calculate the relative viral protein content from the linear part of the titration curve, expressed as log(1/volume) as a function of log(luminescence) in arbitrary units. Results were expressed as the percentage of parental Ed virus titer values.

Viral mRNA and genome production was defined by Northern blot analysis of total cell RNA to assess the integrity of viral transcript and genome production and by SYBR Green real-time RT-PCR analysis to assess the kinetics of viral RNA production (9, 53). Vero cells were infected at an m.o.i. of 1.0, and total RNA was isolated at 0, 2, 4, 6, 8, 12, 18, and 24 h.p.i. (RNasy, Qiagen) followed by DNase I treatment (TURBO DNA-freeTM, Ambion). For RT-PCR analysis, one µg of total RNA was denatured at 70 °C for 5 min and reverse-transcribed using 0.4 µg of oligo(dT) primers (for mRNA RT-PCR reactions), random hexamer primers (for subsequent amplification of 18S RNA and the L gene/trailer region of the viral genome/antigenome), or negative strand-specific primers recognizing the F gene (for viral minus strand genome levels). These RT reactions included 25 nmol of dNTP and 1 µl of reverse transcriptase (RT from Stratagene) in a final volume of 20 µl. Reactions were incubated for 90 min at 48 °C. SYBR Green quantitative PCR was performed in a Roche 480 LightCycler using previously reported N, L-trailer, and negative strand F genespecific primers and PCR cycle conditions (9, 53). Serial dilutions of p+MV were used as the RT-PCR standard for genome levels, and in vitro transcribed N mRNA was the standard for transcript analysis. The calculated values for the MeV transcript and genome were adjusted for variation in 18S RNA levels. The viral polymerase elongation rate was defined for primary transcription as follows: [slope of linear increase in N transcript accumulation × nucleotides in N gene transcripts]/genome level/time period in which transcript levels were monitored.

RESULTS

Box-2 Mutations affect NTAIL Hydrodynamic Properties to Various Extents—We initially attempted four N amino acid substitutions, namely Gln-499, Ala-500, Ala-502, and Gly-503. Recombinant NTAILQ499E, A500D, and A502D proteins were readily expressed in E. coli, and their solubility was high, thus allowing recovery from the soluble fraction of the bacterial lysate. Conversely, expression and solubility of NTAILG503D was very poor. Attempts to increase both expression and solubility by modifying the temperature of induction and isopropyl β-D-thiogalactopyranoside concentration were unsuccessful and led to very low purification yields not compatible with the amounts required for the present study. Hence, production and characterization of the NTAIL G503D variant was not pursued. The NTAILQ499E, A500D, and A502D individual variants were purified to homogeneity (>95%) by immobilized metal affinity chromatography followed by gel filtration (Fig. 1D). The three NTAIL mutated proteins migrated in SDS-PAGE with an apparent molecular mass of 20 kDa (expected molecular mass is ~14.6 kDa). This abnormal migratory behavior is well documented for parental Ed MeV NTAIL (12, 18), having also been reported for all NTAIL variants described thus far (21) and being due to a rather high content of acidic residues, as frequently observed in intrinsically disordered proteins (55, 56).

The Q499E and A500D NTAIL variants were eluted from the gel filtration S200 column with a profile quite similar to that observed for the parental protein (see supplemental Fig. S1). Their inferred Stokes radii (~29 Å) are not consistent with the theoretical value (19 Å) expected for a globular conformation. Rather, the observed values are consistent with the theoretical value (28 Å) expected for a premolten globule state, as already
Amino Acid Substitutions within P-binding Region of MeV N_tail

FIGURE 1. Sequence and structural organization of the N protein C-terminal region that interacts with the P protein XD, indicating amino acid substitutions tested in the analysis ofXD-N_tail structural and functional interactions. A, top, structural organization of the N protein showing that it consists of an ordered N-terminal N_Core domain and an intrinsically disordered C-terminal N_Tail domain. The three N_tail regions (referred to as Box-1, -2, and -3) conserved among Morbillivirus members (50) are shown, where XD binds Box-2. Bottom, the Box-2 sequence is shown along with the amino acid substitutions generated in this study. The predicted α-MoRE and the XD-interacting sequence are shown. The region adopting an α-helical conformation in the N_tail-XD complex spans amino acids 486–502. B and C, lateral and end-on views, respectively, of the structure of XD in complex with residues 486–504 of N_tail. The side-chains of the substituted amino acids (displayed as sticks) are shown to illustrate their relative orientation with respect to XD. Substituted amino acid side groups face away from the groove created by the three β-helices of XD for A500D (Asp-500) and G503D (Asp-503) and toward the groove for Q499E (Gln-499) and A502D (Asp-502). The structural model was obtained by replacing the side chain of the native residue in the XD/Box-2 chimera (Protein Data Bank code 1T6O) by the side chain (most frequent conformer) of the corresponding introduced amino acid. The model was then energy-minimized to avoid steric clashes by using the GROMOS96 implementation of the Swiss-PdbViewer with default parameters. D, Coomassie Blue staining of an 18% SDS-PAGE loaded with NTAIL proteins purified from the soluble fraction of E. coli. M, molecular mass markers.

observed in the case of the parent Ed MeV N_tail. Thus, these mutated proteins share similar hydrodynamic properties with parent N_tail, being non-globular although possessing a certain residual compactness typical of the premolten globule state (39, 57–60).

Strikingly, the A502D variant displays a quite different behavior, eluting into two peaks at significantly higher elution volumes (see supplemental Fig. S1). SDS-PAGE and mass spectrometry analyses confirmed the presence of the N_tail protein in both peaks and ruled out possible degradation (data not shown). Therefore, the observed elution profile suggests that the N_tail A502D protein samples two major populations, both characterized by a much higher compactness with respect to the other N_tail proteins including the parental one. These two populations likely correspond to conformers adopting different collapsed states, with peak 2 containing the more collapsed species. Notably, the inferred Stokes radius for this latter peak is even smaller (~16 Å) than that expected for a fully folded form, implying a densely packed conformational state.

**Box-2 Mutations Reduce XD-N_tail Binding Affinity Only for the A502D Variant**—To estimate precisely the equilibrium dissociation constants and to ascertain possible differences in affinity for XD among the N_tail variant proteins, the N_tail-XD binding reaction was investigated by ITC, an approach that gives access to the stoichiometry, the equilibrium association constant, and the variation in enthalpy and entropy (61).

Purified native N_tail at 10 μM was loaded into the sample cell of a VP-ITC microcalorimeter and titrated with XD (from a stock solution at 150 μM), achieving a XD-N_tail molar ratio of 3 at the end of the titration (Fig. 2). The data, following integration and correction for the heats of dilution, were fitted with a standard model allowing for a set of independent and equivalent binding sites. The estimates for the model parameters (Table 1) confirmed a 1:1 stoichiometry, in agreement with previous studies (12, 17–19, 22, 24, 45), and yielded a K_D of 124 ± 6 nm, in accord with our previously published data (19). In support of the reliability of these data, similar results were obtained when N_tail was the injectant (see supplemental Fig. S2). For all of the N_tail-XD pairs studied, the binding reaction was found to be enthalpy-driven (Table 1).

In the case of the binding reaction between XD and N_tail Q499E, a K_D value (159 ± 4 nm) approximating that observed with parent N_tail was obtained following injection of increasing amounts of N_tail (Fig. 2 and Table 1), with the K_D being slightly higher (230 ± 5 nm), but within the error range observed above, when XD was used as the injectant (see supplemental Fig. S2). The N_tail A500D variant yielded a similar affinity for XD, with a K_D of 177 ± 9 nm. In all cases, the stoichiometry was close to 1 and ΔH was approximately ~11 kcal/mol. In contrast, the N_tail A502D variant displayed a quite different behavior. Upon injection of XD into a 20 μM N_tail solution, saturation was not achieved even with a XD-N_tail molar ratio as high as 6, and a much lower ΔH value was obtained. In addition, the signal/noise ratio was very poor (data not shown). Subsequent experiments with N_tail A502D were therefore carried out using the ITC 200 device, which is better suited for studying binding reactions characterized by K_D val-
ues in the μM range, being less demanding in terms of protein amounts. To confirm that the inferred values could be compared with those determined using the VP-ITC device, we repeated the experiments with parent NTAIL and did indeed obtain similar binding parameters (data not shown). We then carried out binding studies in which the concentrations of both XD (used as injectant) and NTAIL A502D were tuned. The following combinations of initial concentrations were assayed: 60 μM NTAIL-600 μM XD, 80 μM NTAIL-500 μM XD, 100 μM NTAIL-500 μM XD, 100 μM NTAIL-1000 μM XD, 200 μM NTAIL-500 μM XD, and 200 μM NTAIL-2.4 mM XD (Fig. 2 and data not shown). In all of these experiments, a stoichiometry well below

FIGURE 2. ITC studies of binding reactions between NTAIL proteins and XD. Data are representative of at least two independent experiments. Data shown were obtained using the following initial concentrations: 12 μM NTAIL and 145 μM XD for the parent NTAIL-XD pair (NTAIL wt); 14 μM XD and 155 μM NTAIL for the NTAIL Q499E-XD pair; 10 μM NTAIL and 145 μM XD for the NTAIL A500D-XD pair; and 80 μM NTAIL and 500 μM XD for the NTAIL A502D-XD pair. Graphs shown in the bottom half of each panel correspond to integrated and corrected ITC data that were fitted to a single set of site models (i.e. all sites identical and equivalent). In each panel, the filled squares represent the experimental data, and the solid line corresponds to the model.
Amino Acid Substitutions within P-binding Region of MeV N₅TAIL

TABLE 1
Equilibrium dissociation constants and binding parameters for complex formation between XD and N₅TAIL variants as derived from ITC studies.

| Interacting pairs   | Stoichiometry | $K_D$ (nM) | $\Delta H$ (kcal mol⁻¹) | $\Delta S$ (kcal mol⁻¹ deg⁻¹) |
|---------------------|---------------|------------|-------------------------|-------------------------------|
| N₅TAIL wt-XD       | 1.06 ± 0.002  | 124 ± 6   | -11,610 ± 43            | -8.01                        |
| N₅TAIL Q499E-XD    | 1.08 ± 0.001  | 159 ± 4   | -11,400 ± 23            | -7.79                        |
| N₅TAIL A500D-XD    | 1.02 ± 0.003  | 177 ± 9   | -11,940 ± 80            | -9.83                        |
| N₅TAIL A502D-XD    | 0.26 ± 0.007  | 4.13 ± 0.52 | -8,472 ± 304           | -4.26                        |

FIGURE 3. Far-UV CD spectra and analysis of the $\alpha$-helical propensities of N₅TAIL proteins. Shown are far-UV CD spectra of parent N₅TAIL (A), N₅TAIL Q499E (B), N₅TAIL A500D (C), and N₅TAIL A502D (D) at 0.1 mg/ml in the presence of increasing concentrations of TFE (0, 10, 20, and 30%) recorded at 20°C. Each spectrum is the mean of three independent acquisitions. The insets show the $\alpha$-helical and unordered content at the various TFE concentrations estimated by CDSSTR (see “Experimental Procedures”). The error bar (10% of the value) in each panel corresponds to the experimentally determined standard deviation from three independent experiments.

1 (and typically close to 0.25) was obtained consistently, with a $K_D$ in the $\mu$m range (Fig. 2 and data not shown). Although the resultant binding curves typically lacked a plateau at the beginning of the titration (Fig. 2), these experiments clearly showed that the N₅TAIL A502D variant has a much lower affinity toward XD ($K_D$ of 4.13 ± 0.52 $\mu$m) relative to the other N₅TAIL variants.

Box-2 Mutations Impact XD-induced Folding of N₅TAIL to Varying Degrees—Far-UV CD was used to determine how the introduced acidic substitutions affect the overall secondary structure content of the N₅TAIL variants. Secondary structure predictions provided by PSI-PRED (62, 63) showed no differences among the N₅TAIL variants, with an $\alpha$-helix spanning residues 488–502 being predicted as the sole secondary structure element in all cases (data not shown). To directly assess the possible impact of the acidic substitutions on the N₅TAIL structure, the far-UV CD spectra of N₅TAIL variants were recorded at neutral pH. The CD spectra of mutated N₅TAIL proteins are very similar to that of parent N₅TAIL and all are typical of predominantly disordered proteins devoid of highly populated regular secondary structure, as seen by their large negative ellipticity at 200 nm and moderate ellipticity at 185 nm (Fig. 3). However, ellipticity values at 200 and 222 nm indicate that both mutated and parent N₅TAIL possess some residual regular secondary structure typical of the premolten globule state. The $\alpha$-helical
and unordered content of the NTAIL proteins, as derived by deconvolution of the spectra, showed that the NTAIL variants have an $\alpha$-helical content similar to parent NTAIL.

To further investigate the structural impact brought about by the acidic substitutions, the structural propensities of the NTAIL variants in the presence of TFE were analyzed. The solvent TFE mimics the hydrophobic environment experienced by proteins in protein-protein interactions and is therefore widely used as a probe to identify disordered regions that have a propensity to undergo induced folding (64–66). Previous studies have shown that the addition of increasing amounts of TFE to NTAIL triggers a gain of $\alpha$-helicity, with Box-2 playing a major role in this $\alpha$-helical transition (12, 18, 19). In the present work, CD spectra of mutated NTAIL proteins were recorded in the presence of increasing concentrations of TFE (Fig. 3), and the NTAIL $\alpha$-helical content was calculated. All proteins showed a gain of $\alpha$-helicity upon addition of TFE, as indicated by the characteristic maximum at 190 nm and minima at 208 and 222 nm, thus indicating that the acidic substitutions do not impair the ability of NTAIL to undergo $\alpha$-helical folding. Furthermore, the mutated NTAIL proteins have $\alpha$-helical propensities similar to the parent NTAIL protein based upon their $\alpha$-helical content at different TFE concentrations. Collectively, these experiments indicate that the acidic substitutions induce little, if any, structural perturbations within NTAIL.

Subsequent experiments determined whether the mutated NTAIL proteins retained the ability to undergo induced folding in the presence of XD. Far-UV CD spectra were recorded in the presence of a 2-fold molar excess of XD (filled circles). Under these conditions, the concentration of NTAIL proteins and of XD is 3.5 and 7 $\mu$M, respectively. The CD spectra of NTAIL proteins alone (black line) or XD alone (gray line), as well as the theoretical average curves (crosses) calculated by assuming that no structural variations occur are also shown. The dashed-line spectrum in A corresponds to the simulated spectrum of a mixture containing a 2-fold molar excess of XD in which only 15% of NTAIL binds to XD (see “Experimental Procedures”). Data are representative of three experimental trials.

FIGURE 4. Induced folding of NTAIL proteins in the presence of XD. Shown are far-UV CD spectra of parent NTAIL (A), NTAIL Q499E (B), NTAIL A500D (C), and NTAIL A502D (D) in the presence of a 2-fold molar excess of XD (filled circles). Under these conditions, the concentration of NTAIL proteins and of XD is 3.5 and 7 $\mu$M, respectively. The CD spectra of NTAIL proteins alone (black line) or XD alone (gray line), as well as the theoretical average curves (crosses) calculated by assuming that no structural variations occur are also shown. The dashed-line spectrum in A corresponds to the simulated spectrum of a mixture containing a 2-fold molar excess of XD in which only 15% of NTAIL binds to XD (see “Experimental Procedures”). Data are representative of three experimental trials.
Amino Acid Substitutions within P-binding Region of MeV N\textsubscript{TAIL}

corresponds to the spectrum that would be expected if no structural variations occurred, deviations from this curve indicate structural transitions. The observed deviations are consistent with an XD-induced \( \alpha \)-helical transition of parent N\textsubscript{TAIL} as judged by the much more pronounced minima at 208 and 222 nm and by the higher ellipticity at 190 nm of the experimentally observed spectrum compared with the corresponding theoretical average curve.

In the presence of a 2-fold molar excess of XD, the Q499E substitution dramatically decreases the ability of the N\textsubscript{TAIL} protein to undergo \( \alpha \)-helical folding upon binding to XD, based on the very low deviation of the experimental spectrum from the theoretical average curve (Fig. 4B). Under identical conditions, the A500D and the A502D substitutions fully abrogated XD-induced folding of N\textsubscript{TAIL} as judged by the superimposition of the experimental and average spectra (Fig. 4, C and D). It is noteworthy that we can rule out the possibility that \( \alpha \)-helical folding of N\textsubscript{TAIL} escaped detection because of insufficient complex formation. Indeed, under the experimental conditions we used, the XD concentration is \( \sim 7 \mu M \), a concentration well above the \( K_d \) displayed by parent, Q499E, and A500D N\textsubscript{TAIL} proteins. In the case of the A502D variant, the amount of N\textsubscript{TAIL} variant bound to XD would be estimated at 60% based on the total N\textsubscript{TAIL} and XD concentrations used in these studies (3.6 and 7 \( \mu M \), respectively) and on the \( K_d \) (4.13 \( \mu M \)). However, given the observed stoichiometry of 0.25 for the XD-N\textsubscript{TAIL} A502D variant, the total expected amount of complex formed under these conditions is actually 15% (i.e. one-fourth of 60%). Lack of XD-induced folding of the A502D variant does not arise from our inability to detect folding in the subset of N\textsubscript{TAIL} molecules that bind to XD. Instead, the results support a true lack of folding. This conclusion was based upon a simulated CD spectrum corresponding to a mixture containing a 2-fold molar excess of XD, where only 15% of N\textsubscript{TAIL} binds (and folds) to XD. As shown in Fig. 4A, this simulated spectrum still deviates from the spectrum that would be obtained in the event that no structural transitions were to take place.

Box-2 N\textsubscript{TAIL} Mutations Support Minireplicon Reporter Gene Expression—The impact of N\textsubscript{TAIL} mutations on viral polymerase function was tested using MeV minireplicons. Subconfluent monolayers of HEp-2 cells were transfected with plasmids supporting T7 RNA polymerase-mediated expression of N, P, and L proteins and a MeV minigenome. The N plasmid directed the expression of either the parent protein or a Box-2 mutant. The minigenomic RNA contained a CAT reporter gene, with CAT activity levels being corrected for variation in transfection efficiency and represented as a mean \( \pm \) S.D. relative to the activity supported by the Ed N protein, the latter being defined as 1.0. The expression levels supported by N A500D were elevated significantly relative to Ed N (\( p < 0.05 \), analysis of variance). Protein levels were comparable within cells based upon Western blot analysis of total cell protein. Representative results are illustrated, with GAPDH levels used as a loading control.

![Graph](image.png)

**FIGURE 5.** MeV minireplicon reporter gene expression (CAT) in HEp2 cells, where the N protein template function was provided by either parent N or one of the N proteins bearing Box-2 substitutions (i.e. Q499E, A500D, and A502D). Level of reporter gene expression is an average of 3–7 experimental trials, each trial representing transfections performed in triplicate. CAT activity levels were corrected for variation in transfection efficiency and expressed as a mean \( \pm \) S.D. relative to the activity supported by the Ed N protein, the latter being defined as 1.0. The expression levels supported by N A502D were elevated significantly relative to Ed N (\( p < 0.05 \), analysis of variance). Protein levels were comparable within cells based upon Western blot analysis of total cell protein. Representative results are illustrated, with GAPDH levels used as a loading control.

Both Mutant and Parent N\textsubscript{TAIL} Constructs Support Comparable Polymerase Elongation Rates, although There Is Diminished Infectivity of the Mutant Viruses—Minireplicon reporter gene expression does not discriminate between transcriptase and replicase functions of the viral polymerase. We are particularly interested in the relationship between XD-N\textsubscript{TAIL} interaction and transcription, given our ultimate goal of defining the mechanistic basis by which host factors enhance viral gene expression. Accordingly, recombinant infectious MeV was rescued in which the N\textsubscript{TAIL} variants were encoded by the viral genome. Parent N\textsubscript{TAIL} MeV was rescued in parallel. For the rescue, HEp-2 cells were transfected to express Ed N, P, and L proteins and the plus strand genomic RNA containing either the parent N gene or one of the mutated N genes encoding N (N\textsubscript{TAIL}) variants.

Vero cells were infected at an m.o.i. of 0.01, and cell-free infectious viral progeny release was monitored to define multiplex growth curves for each viral variant (Fig. 6). Peak progeny release occurred at 48 h.p.i. for parent N\textsubscript{TAIL} MeV, MeV N Q499E, and A502D exhibited similar kinetics for the elaboration of infectious viral progeny as Ed N, although there was a significant reduction in the magnitude of total and peak production, with the latter being reduced \( \sim 10 \)-fold. In contrast, peak progeny release was comparable for parent N\textsubscript{TAIL} and A500D virus, although that peak occurred at 60 h.p.i. for the mutant.
virus. Maximal cytopathic effects (i.e. 100% syncytial coverage) correlated to the time of peak infectious progeny release.

Virus released into the supernatant of virus-infected Vero cells at 48 h.p.i. was purified by sedimentation on a discontinuous sucrose gradient, and its composition (i.e. genome, antigenome, defective interfering particle, viral proteins) was analyzed. The total amount of genome per unit volume was increased by 3.3-, 3.5-, and 1.6-fold for Q499E, A500D, and A502D mutants, respectively, relative to parent NTAIL virus. When adjusted for infectivity titer, the results indicate reduced infectivity per unit genome by a factor of 25, 7, and 73 for Q499E, A500D, and A502D mutants, respectively. Contamination by internal deletion or copyback defective interfering particles, as determined by the ratio of genomic F segment to L-trailer segment, was only slightly increased for all mutants, with -fold changes for mutants relative to parent MeV being 1.2 for Q499E, 1.8 for A500D, and 2.3 for A502D.

Viral protein content per unit volume was greater for NTAIL mutants, with a 1.6–7.8-fold increase in N and P and a 5.1–25-fold increase in M relative to parent virus. The changes followed the order A500D < A502D ≈ Q499E. Although the P/N ratios were comparable between all viruses within the range of 0.53 to 0.75, the M/N ratios in mutants were increased by a factor of 1.5–3.3 relative to the parent virus, and M/P ratios were increased by 2.3–6.2-fold (Fig. 7B). Notably, this magnitude of increase in viral proteins was in general agreement with the increase in genome/antigenome RNA observed in the NTAIL mutants relative to parent NTAIL. Consequently, as with the genome analysis, the infectivity per unit of N protein was reduced by a factor of 94 and 99 for Q499E and A502D mutants, respectively, relative to the parent NTAIL virus, whereas that of the A500D mutant was comparable.

Viral production of transcript and genomic RNA was analyzed by Northern blot analysis after completion of a single virus replication cycle (24 h.p.i.) in Vero cells infected at an m.o.i. of 1.0. Increased cellular levels of both the promoter proximal N and promoter distal H transcripts and increased levels of full-length viral genomic RNA were observed for NTAIL mutants relative to parent NTAIL virus (Fig. 8). The differences were 1.5–1.9-fold for transcripts and 10–17-fold for genomic RNA. Kinetic analysis of viral RNA accumulation at 2, 4, 6, 8, 12, 18, and 24 h.p.i. measured by real time RT-PCR revealed that the much larger genome increases observed in Northern blots for NTAIL mutants reflected in part a larger input of genomic RNA. As expected from the known delay of virus replication (9), genome levels were constant during the first ~12 h.p.i. for all viruses, after which point linear increases occurred (data not shown). The average levels of this constant input genome between 2 and 8 h.p.i. show that for a single infectious unit, genome amounts for the NTAIL mutants exceed that of parent NTAIL virus by factors of 26, 7, and 57 for Q499E, A500D, and A502D, respectively (Fig. 9A). The results of quantification using L-trailer primers or primers specific to the minus strand F genome paralleled one another for all times points examined, thus excluding a major contamination of the virus stocks by subgenomic defective interfering particles and a significant excess of defective interfering particles production during virus infection (not shown). The 57-fold difference between A502D and parent NTAIL virus was statistically significant (p < 0.05, analysis of variance). This higher level of input genome for
mutant viruses is consistent with their reduced infectivity relative to the parent virus, given that the m.o.i. was constant. Transcript analysis focused on the N gene. Increases in transcript levels were linear between 0 and 6 h.p.i., with $R^2$ values of 0.98–0.99 (Fig. 9B). This defines the period of primary transcription (i.e. transcription of incoming genomic templates by preformed transcriptases) in which an apparent polymerase elongation rate can be calculated. During this phase, slopes describing the linear increase in transcript levels are used as a measure of polymerase (transcriptase) activity, corrected for variation in genome levels (9, 53). The calculation assumes that every incoming genome is transcriptionally active and that the number of active transcriptases is similar for every mutant. The calculated rate of parent NTAIL virus polymerase was 2.3 nucleotides/s, which closely approximates the 3.0 nucleotides/s reported for this virus and Hallé MeV strain on HeLa and 293T cells (9). Rates for the Box-2 mutant viruses were within a 1.7-fold range of that measured for parent NTAIL virus (Fig. 9C).

DISCUSSION

In this study, we created amino acid substitutions at three positions within the Box-2 region of NTAIL (e.g. the primary XD-binding site) with the specific purpose of assessing the possible relationships among NTAIL-XD binding affinity, XD-induced $\alpha$-helical folding of NTAIL, and polymerase activity. Contrary to our hypothesis, XD-induced folding was not required to maintain a high NTAIL binding affinity, and loss of XD-induced NTAIL-$\alpha$-helical folding or binding affinity had no significant effect on polymerase activity. Rather unexpectedly, however, these substitutions were found to affect virus infectivity strongly, thus arguing for a possible role for Box-2 in assuring the fidelity of polymerase activity or particle assembly.

Indeed, according to our results, MeV polymerase activity is maintained within a relatively narrow range when XD-induced $\alpha$-helical folding of NTAIL is reduced or eliminated, even when XD-NTAIL affinity is reduced by over 30-fold. The ITC experiments confirmed the 1:1 stoichiometry of the NTAIL-XD binding reaction and yielded a $K_d$ of 124 ± 6 nM, in accord with our previously published data (12, 17–19, 22, 24, 45). A recent study by Kingston and colleague (67) characterized the binding reactions between XD and synthetic peptides corresponding to amino acids 477–505 or 477–525 of N, deriving a $K_d$ that was either 7.4 or 15 μM, respectively. The discrepancy between our data and those of Kingston (67) likely reflects the use of full-length NTAIL versus NTAIL peptides, respectively. A $K_d$ for XD/full-length NTAIL binding reactions in the 100 nM range was obtained using surface plasmon resonance analysis, fluorescence spectroscopy, and ITC, supporting the view that it is the protein reactants, and not the analytical approach, that are
the basis for the discrepancy. \(N_{\text{Tail}}\) Q499E and A500D exhibited a binding affinity for XD that was comparable to parent \(N_{\text{Tail}}\), whereas the XD affinity of \(N_{\text{Tail}}\) A502D was significantly reduced. The latter may reflect conformational differences between A502D and the other constructs, with these differences being supported by size exclusion chromatography studies that point out a unique conformational behavior for \(N_{\text{Tail}}\) A502D. Indeed, this latter variant was found to populate two distinct conformational states, both characterized by a high degree of compactness. The higher extent of compactness of \(N_{\text{Tail}}\) A502D likely arises from the establishment of tertiary contacts (whether short or long range) and does not reflect an increased content in regular secondary structure, in accord with our recent results indicating that the content in regular secondary structure is not a major determinant of protein compaction (68). In agreement with this hypothesis, although both mutated and parent \(N_{\text{Tail}}\) possess some residual regular secondary structure typical of the premolten globule state, CD studies did show that \(N_{\text{Tail}}\) A502D possesses an \(\alpha\)-helical content that is even slightly lower than that of the other \(N_{\text{Tail}}\) proteins. This raises the possibility of a less stably configured prerecognition state as a possible basis for the reduced affinity of XD for \(N_{\text{Tail}}\) A502D. On the other hand, it is also conceivable that the more collapsed state adopted by \(N_{\text{Tail}}\) A502D could lead to the sequestering of the XD-binding site, thereby rendering this variant less competent to binding. That MoREs can escape binding to partners through functional misfolding of the intrinsically disordered protein has already been reported (69).

The stoichiometry below 1 that was observed for the \(N_{\text{Tail}}\) A502D-XD binding reaction likely reflects the reduced capacity of this \(N_{\text{Tail}}\) variant to bind to XD. A stoichiometry below 1 can reflect errors in the estimates of protein concentrations or the inability of a significant proportion of titrant to interact with the titrant. Because the same XD sample was used in all ITC studies, possible errors in the estimates of XD concentration that might have specifically confounded the analyses of \(N_{\text{Tail}}\) A502D could be ruled out. Likewise, errors in the estimate of the \(N_{\text{Tail}}\) A502D concentration could be excluded as judged by SDS-PAGE of \(N_{\text{Tail}}\) A502D could lead to the sequestering of the XD-binding site, thereby rendering this variant less competent to binding. That MoREs can escape binding to partners through functional misfolding of the intrinsically disordered protein has already been reported (69).

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Although CD studies under conditions mimicking the hydrophobic environment of protein-protein interactions (\textit{i.e.} in the presence of TFE) failed to unveil significant differences in the folding abilities of \(N_{\text{Tail}}\) proteins, studies carried out in the presence of the binding partner revealed that the acidic substitutions did impact the ability of \(N_{\text{Tail}}\) to undergo XD-induced
Amino Acid Substitutions within P-binding Region of MeV N\textsubscript{TAIL}

folding. The Q499E substitution significantly reduced the ability of N\textsubscript{TAIL} to undergo \(\alpha\)-helical folding in the presence of XD, and the A500D and A502D substitutions fully abrogated it. A plausible explanation for the observed discrepancy between studies making use of TFE \textit{versus} XD may reside in the global nature of the structural effects triggered by TFE (where TFE may promote folding of N\textsubscript{TAIL} sites other than Box-2, such as Box-1 (20)) and in the fact that TFE is a much less sensitive probe of local structural transitions than the true partner. This latter point is well illustrated by previous EPR studies showing that XD triggers a much more pronounced reduction in the mobility of spin labels grafted within Box-2 as compared with TFE (21).

It is noteworthy that the failure of XD to induce significant N\textsubscript{TAIL} A502D folding does not likely reflect the elevated \(K_D\) (relative to parent Ed N\textsubscript{TAIL}) given the molar excess of XD that was used in these experiments and the fact that a simulated CD demonstrated the ability to detect induced folding when only a subset of N\textsubscript{TAIL} binds XD. Likewise, the possibility that XD-induced \(\alpha\)-helical folding of N\textsubscript{TAIL} occurs but escapes detection due to possible differences between the time scale with which structural transitions take place and the time window of detection can be ruled out, given the low (below femtoseconds) time scale of CD and the typical nanosecond to microsecond time scale of structural transitions. As a consequence of this latter property, any CD spectrum can be resolved into the component spectra of the main conformer types (\(\alpha\)-helix, \(\beta\)-sheet, turns, and random coils).

Importantly, our results showed that XD-induced \(\alpha\)-helical folding of N\textsubscript{TAIL} is not a basis for the relatively high affinity of the XD-N\textsubscript{TAIL} complex (i.e. induced \(\alpha\)-helical folding of N\textsubscript{TAIL} is not strictly required to stabilize the complex). XD-induced \(\alpha\)-helical folding was significantly reduced for N\textsubscript{TAIL} Q499E and completely abrogated for A500D based upon the CD analyses, despite the fact that XD-N\textsubscript{TAIL} binding affinity was maintained at a level comparable to that of parent N\textsubscript{TAIL}. The finding that \(\alpha\)-helical folding is not strictly required for the formation of a stable complex implies that XD would be able to bind to N\textsubscript{TAIL} conformers that lack a transiently populated \(\alpha\)-helix, supporting previous spectroscopic data arguing for a mixed mechanism of N\textsubscript{TAIL}-XD complex formation relying on both conformational selection and folding after binding (24).

Minireplicon reporter gene expression and estimates of viral transcript elongation rates for A502D, where XD-N\textsubscript{TAIL} binding affinity was reduced by more than 30-fold relative to parent virus, also argues against the idea that the relatively high XD-N\textsubscript{TAIL} binding affinity imposes constraint on (i.e. slows down) viral transcriptase function. Minireplicon reporter gene expression was not significantly increased for A502D relative to the other viruses. In addition, the calculated transcriptase elongation rate was actually slightly reduced (i.e. the rate was reduced by 43% relative to parent virus), although the latter finding may in part reflect the fact that not all cytoplasmic nucleocapsids contribute equally to transcription. That P/N\textsubscript{TAIL} binding affinity is not the primary determinant of the relatively slow polymerase elongation rate of MeV is supported by observations in a related paramyxovirus: the polymerase elongation rate of Sendai virus is 1.7 nt/s, whereas the P/N complex has a \(K_D\) of 60 \(\mu\)M (70).

Despite the diminished capacity of isolated N\textsubscript{TAIL} A502D to bind XD, there was no apparent change in the P to N ratio in the composition of cell-free virions. Here, the oligomeric state of P interacting with the 2649 N monomers of an intact nucleocapsid may combine to assure that there are ample N\textsubscript{TAIL} conformers capable of engaging polymerase complexes to levels observed in parent virus. Likewise, globally the comparison of polymerase functions as transcriptase and replicase determined both in a minigenome assay and in the context of viable recombinant viruses indicates a lack of major impact of either less pronounced induced folding of N\textsubscript{TAIL} or a >30-fold reduction in N\textsubscript{TAIL} affinity to XD.

In contrast, the induced folding of N\textsubscript{TAIL} and maintenance of a relatively high N\textsubscript{TAIL}-XD binding affinity appears to be essential for maintaining optimal infectivity of MeV. This observation is consistent with the high degree to which amino acids targeted in the current work are conserved among measles isolates. Indeed, based on the analysis of more than 4000 MeV N protein sequences available in the databases, Box-2 is very poorly variable, and the lack of natural variants exhibiting the substitutions we have introduced argues for the amino acid sequence of Box-2 as being under a highly selective pressure. Consequently, one could predict that upon repeated passages \textit{in vitro} and/or \textit{in vivo} of the N mutants we have designed, a reversion to the parental sequence should be observed.

The drop in infectivity of N\textsubscript{TAIL} mutants could not be attributed to significant changes in RNA synthesis and N-encapsulation of genome, to increased defective interfering particle production, or to overt changes in the protein composition of the virus particles. Likewise, because dot blot analysis of the viral protein composition was performed by diluting intact virus particles (and not solubilized viruses), we can also exclude a major change in the size and polyplody (71, 72) of the virions based upon the relatively unchanged genome/N protein ratio.

The basis for reduced infectivity of the N\textsubscript{TAIL} mutants is currently unknown. Sequences C-terminal to N\textsubscript{TAIL} Box-2 may interact with other viral and/or cellular factors required to assure the efficient use of every nucleocapsid template and the fidelity of transcription and/or genome replication, where optimal interaction depends upon specific and highly conserved N\textsubscript{TAIL} conformers. Measles virus matrix protein interacts with the nucleocapsid (73–75) and promotes virus assembly and particle release (76). In addition, M negatively regulates virus transcription by binding to the C terminus N\textsubscript{TAIL} dileucine 523–524 motif (77, 78). Moreover, in virus particles and infected cells, many nucleocapsids are partially covered by the M protein (79–81), where discontinuous helices of M mediate nucleocapsid folding (72). Thus, for nucleocapsid delivered into the cytoplasm after virus entry, the incoming transcriptase will have to displace M polymers as it progresses downstream along the encapsidated genome. Likewise, later in the virus infection cycle, as M gene expression increases, the replicase will have to compete with the ongoing M polymerization. Because of the apparent heterogeneity of nucleocapsid coverage by the M protein (72), we can speculate that alteration of Box2-XD interactions can influence M and P competition for N\textsubscript{TAIL} binding.
Enhanced M binding, supported by the 1.5–3.3-fold increase in M/N ratios in virions, could increase the chance of producing nucleocapsid unsuitable for supporting an entire virus replication cycle. Further work is needed to support this hypothesis. As for the fidelity of polymerase function, it is possible that the error rate of the polymerase is increased, leading to subtle changes in the genome sequence that disrupt infectivity. This possibility will require full genome sequencing to illuminate.

Collectively, this work provides a new perspective for the study of viral polymerase activity and its modulation by viral and host factors. The currently accepted model whereby the N\textsubscript{TAIL}–XD interaction has to be relatively weak to allow the polymerase to cartwheel on the nucleocapsid template needs to be revisited in light of the present results. The N\textsubscript{TAIL} region C-terminal to Box-2 comprises a regulatory domain that can impose constraint on polymerase activity (47). Our results suggest that this regulatory domain acts not by directly influencing cycles of binding and release by the polymerase complex but rather by mediating the interaction with other factors that may positively or negatively influence polymerase function. Further development of our understanding of the relationship between XD-induced N\textsubscript{TAIL} folding and the fidelity and level of viral polymerase activity will require knowledge of the composition of the viral RNA-dependent RNA polymerase in transcriptase versus replicase mode. Indeed, the replicate and transcriptase of vesicular stomatitis virus, another Mononegavirales, have been shown to differ in both viral and cellular protein components (28).

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Amino Acid Substitutions within P-binding Region of MeV N\textsubscript{TAIL}

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