Sequence of events in measles virus replication: role of phosphoprotein-nucleocapsid interactions

Joanna Brunel¹, Damien Chopy¹, Marion Dosnon², Louis-Marie Bloyet¹, Patricia Devaux³, Erica Urzua¹, Roberto Cattaneo³, Sonia Longhi² and Denis Gerlier¹

¹Centre International de Recherche en Infectiologie, INSERM, U1111, CNRS, UMR5308, Université Lyon 1, ENS Lyon, CERVI, 21 Avenue Tony Garnier 69007 Lyon, France.
²Aix-Marseille Université, Architecture et Fonction des Macromolécules Biologiques (AFMB) UMR 7257, 13288, Marseille, France
³Department of Molecular Medicine, Mayo Clinic, Rochester MN 55905, USA

e.mails : Joanna Brunel - joanna.brunel@inserm.fr ; Damien Chopy - damienchopy@hotmail.fr ; Marion Dosnon - marion.dosnon@afmb.univ-mrs.fr ; Louis-Marie Bloyet - louis-marie.bloyet@inserm.fr ; Patricia Devaux - Devaux.Patricia@mayo.edu ; Erica Urzua - urzua.ERICA@gmail.com ; Roberto Cattaneo - cattaneo.roberto@mayo.edu ; Sonia Longhi - Sonia.Longhi@afmb.univ-mrs.fr ; and Denis Gerlier – denis.gerlier@inserm.fr.

Corresponding author: DG, ¹CIRI, INSERM U1111, CNRS, ENS Lyon, Université Lyon 1, CERVI, 21 Avenue Tony Garnier 69007 Lyon, France tel : +33(0)4 37 28 23 90, e.mail: denis.gerlier@inserm.fr

Short title: P to N binding mode for transcription.

Word count for Abstract: 215
Word count for Importance: 134
Word count for text : 6599

Footnotes :
Author contributions: DG, SL, and RC designed research. JB, DC, LMB, EU, and PD designed and/or contributed new reagents. JB, DC, EU, LMB and MD performed research. JB, DC, EU, LMB and DG analysed data. Every author drafted or revised the manuscript and they all approved the final version.

The authors declare no conflict of interest.

Keywords: RNA dependent RNA polymerase/ phosphoprotein/ nucleoprotein/ protein complementation/ measles virus

Abstract:

The genome of non-segmented negative strand RNA viruses is tightly embedded within a nucleocapsid made of a nucleoprotein (N) homopolymer. To ensure processive RNA synthesis, the viral polymerase L in complex with its co-factor phosphoprotein (P) binds the nucleocapsid that constitutes the functional template. Measles virus P and N interact through two binding sites. While binding of the P amino-terminus with the core of N (N\textsubscript{CORE}) prevents illegitimate encapsidation of cellular RNA, the interaction between their C-terminal domains, P\textsubscript{XD} and N\textsubscript{TAIL} is required for viral RNA synthesis. To investigate the binding dynamics between the two latter domains, the P\textsubscript{XD} F497 residue that makes multiple hydrophobic intramolecular interactions was mutated. Using a quantitative mammalian protein complementation assay, and recombinant viruses, we found that an increase in P\textsubscript{XD} to N\textsubscript{TAIL} binding strength is associated with a slower transcript accumulation rate and that abolishing the interaction renders the polymerase non-functional. The use of a newly developed system allowing conditional expression of wild type or mutated P genes, revealed that the loss of the P\textsubscript{XD}-N\textsubscript{TAIL} interaction results in reduced transcription by preformed transcriptases suggesting reduced engagement on the genomic template. These intracellular data indicate that the viral polymerase entry into and progression along its genomic template relies on a protein-protein interaction that serves as a tightly controlled dynamic anchor.

Importance:

Mononegavirales have a unique machinery to replicate RNA. Processivity of their polymerase is only achieved when the genome template is entirely embedded into a helical homopolymer of...
nucleoproteins that constitutes the nucleocapsid. The polymerase binds to the nucleocapsid template through the phosphoprotein. How the polymerase complex enters and travels along the nucleocapsid template to ensure uninterrupted synthesis of up to ~6700 nucleotide long messenger RNAs from 6 to 10 consecutive genes is unknown. Using a quantitative protein complementation assay and a biGene-biSilencing system allowing conditional expression of two P genes copies, the role of the P to N interaction in polymerase function was further characterized. We report here a dynamic protein anchoring mechanism that differs from all other known polymerases that rely only onto a sustained and direct binding to their nucleic acid template.

**Introduction**

Non-segmented negative strand RNA viruses (or *Mononegavirales*) share a unique transcription and replication machinery. When using naked genomic RNA as a template, the viral polymerase (L protein) displays poor processivity, with neosynthesized RNAs not exceeding few tens of nucleotides in length even in the presence of the polymerase cofactor, the phosphoprotein P (1). The functional template is the nucleocapsid made of the RNA genome tightly covered by a continuous helical homo-polymer of nucleoprotein (N), the structure of which is well conserved within the *Mononegavirales* order (2-5). Upon binding of the P-L complex to the nucleocapsid template, transcription initiates at the 3' genomic end, where the polymerase is switched on by recognizing the transcription promoter localized in the *leader* region. Transcription of the 6 to 10 genes occurs sequentially thanks to the intergenic regions containing stop and start signals (6). Upon their delivery into the cytoplasm, genomic nucleocapsids are immediately transcribed by ready to start transcriptases residing in the incoming virus particles. Linear transcript accumulation during this primary transcription (5-6 hours) is followed by exponential transcript accumulation consecutive to the recruitment of neosynthesized transcriptases on the same number of genomic nucleocapsid templates for the next 5-8 hours. When enough encapsidation substrate, called N⁰P (a soluble complex made of newly synthesized N protein and P), becomes available at ~12-14 hpi, replication (and secondary transcription, i.e. transcription from neosynthesized genomic nucleocapsid) starts (7). The nascent RNA copy is concomitantly encapsidated, a process possibly responsible for the uninterrupted RNA synthesis at the intergenic regions. To ensure an RNA synthesis processive enough to produce mRNAs of up to ~6.7 kb in length and of the ~16 kb genome,
measles virus polymerase has to be anchored in a sustained manner onto the nucleocapsid template. This anchoring is thought to rely on the interaction between the C-terminal domains of P (PXD) and N (NTAIL). This process should be dynamic so as to permit the progression along the nucleocapsid, where sequential and transient opening of every N subunit would allow the polymerase to access to nucleotides (6, 8).

PXD folds into a three antiparallel \( \alpha \)-helical bundle delineating a hydrophobic groove into which a Molecular Recognition Element of helical nature (\( \alpha \)-MoRE, aa 486-504) located within NTAIL dynamically binds (9-11). In the free state, the side chain of PXD residue F497 is involved in a network of intramolecular hydrophobic interactions, with an additional intramolecular contact being established when PXD is bound to the \( \alpha \)-MoRE of NTAIL. As such, this residue provides a good target for modulating PXD binding to NTAIL. It was therefore chosen for substitution in view of binding and functional studies.

To perform these studies, we implemented a quantitative protein complementation assay working in mammalian cells (12, 13), and designed an assay allowing the analysis of the function of P variants in the context of viral infection. This latter approach is based on the demonstrated ability of a constitutively expressed siRNA to repress expression of a viral gene (14, 15). We combined this method with duplication of the viral gene of interest and conditional selective silencing of one of the two gene copies, and named it biGene-biSilencing. Thanks to the exclusive expression of the wild type (wt) copy of the P gene in cells expressing a siRNA targeting the mutated P gene copy, bi-P viruses with wt phenotype were successfully rescued. Upon infection of another cell line that specifically prevents the expression of the wt P gene copy, the function of the mutated P gene was explored.

Through the combination of these methods, we show that the viral mRNA accumulation rate and the kinetics of virus production depend on optimal binding strength between PXD and NTAIL. Furthermore, a PXD point mutant unable to bind NTAIL not only fails to support viral transcription but blocks primary transcription by incoming transcriptases. These findings thus highlight how a protein-protein interaction serves as a tightly controlled dynamic anchor for the viral polymerase entry and/or progression along its genomic template.

Methods
Plasmid construction. The pSP-161 lentiviral vectors coding the puromycin resistance gene under the control of the SV40 promoter and the small hairpin RNA (shRNA) targeting either the P mRNA sequence GGACACCTCTCAAGCATCAT or the GFP mRNA sequence GAACGGCATCAAGGTGAA (14) under the control of the pol III H1 promoter were built by sub-cloning synthetic oligonucleotides using the Gateway™ technology (16).

The plasmid p(+) MVNSe (17) with two unique cloning sites (BsiWI, AatII) added into the 5'UTR of the P gene was used as the MeV genome backbone. This plasmid encodes a laboratory measles virus strain, derived from the Edmonston strain that uses both CD150 and CD46 as a receptor, and hence can be grown in either Vero or VeroSLAM cells leading to syncytia formation in both cell lines. This strain exhibits a reduced growth rate possibly because of a mutation in the V protein, and it proved to be very useful since, in preliminary experiments, it was found to be much more sensitive to the silencing effect against P mRNA than the vaccine Moraten strain. MeV genomic plasmids were built either by a two-step subcloning via an intermediate vector as described previously (18) or by direct recombination of one or two PCR fragments using the vaccinia virus-derived recombinase according to the InFusion™ user manual (Clontech). To build biG-biS recombinant viruses, the P gene was duplicated in P1 and P2 in gene position 2 and 3, respectively. P1 was rendered resistant to P RNAi by introducing silent mutations (lower-case letters, GGcCACCTtagctcaATCAT and tagged with an N-terminal Flag peptide and three copies of the GFP RNAi target sequence in the 3' UTR of its mRNA. P2 was tagged with an N-terminal HA peptide and three copies of the P RNAi target sequence in the 3'UTR. Mutations into the X domain of P (D497 and A497) were introduced by subcloning PCR amplified fragments from a prokaryotic P_{XO} vector mutagenized with QuikChange® Site-Directed Mutagenesis Kit from Stratagene. The previously described pCG-P eukaryotic expression vector (18) was also used as a backbone for the transient expression of P protein and mutants by transfection.

To avoid the inconvenience of unwanted amino acid intrinsically added by the Gateway™ cloning system, the two original expression vectors used for Gaussia luciferase based protein complementation assay (12) were modified into pCI-glu1 and pCI-glu2 to allow subcloning into the unique NotI site: the Gateway insert was eliminated without changing the flanking vector sequence in order to keep unchanged the linker bridging glu domains and inserts. P^*, P-D497 and N_{401-525} and P_{376-507} fragments were subcloned downstream Gaussia glu1 and/or glu2 domains by InFusion™.
recombination of PCR-amplified fragments. All plasmids and viruses (N, P₁, P₂, M and L gene) were verified by sequencing the subcloned PCR fragments or cDNA obtained by RT-PCR performed on virus stocks. All plasmids have been deposited in the Addgene plasmid repository service.

**Cell lines and viruses.** Cells were cultured in Dulbecco's Medium Eagle's Modified (DMEM) (Life Technologies) supplemented with 10% of heat inactivated (30 min at 56°C) fetal bovine serum, 1% L-Glutamine and 10 µg/ml gentamycin at 37°C and 5% CO₂. Medium of 293-3-46 helper cells (17) was supplemented with 1.2 mg of G418/ml.

Vero (si2) and Vero-SLAM (19) (si1) cells stably expressing shRNA targeting the P and GFP mRNAs, respectively were derived by transduction using lentivectors. Infectious non-replicative retroviral particles were produced in 293T cells (20). Briefly, 4.10⁶ cells were co-transfected by 8 µg of pSP-161 coding for shRNA anti-P or anti-GFP mRNA, 8 µg pCMVΔR8.91 and 4 µg pMD2.VSVG, the two later coding for retroviral packaging and vesicular stomatitis virus envelope G protein, respectively, using ProFection Mammalian Transfection System (PROMEGA). Two days later, 3-fold dilutions of the supernatant were used to infect 293T, Vero and Vero-SLAM cells. Transduced cells were selected by adding puromycin (2.5 µg/ml for 293T and 10 µg/ml for Vero cells) the day after the infection. After cloning by limiting dilutions, one 293T-derived si2, one 293T-derived si1, one Vero-derived si2 and one VeroSLAM-derived si1 clone able to efficiently silence the transient expression of a GFP-P (18) hybrid construct were selected. Attempts to get si2 and si1 on an identical Vero cell background failed since Vero cells were poorly susceptible to transduction by lentivectors. Fortunately, the growth of a recombinant MeV with an RNAi resistant P gene in si2 and si1 cell lines was comparable.

To rescue recombinant viruses, the helper cell line 293-3-46 stably expressing T7 polymerase, MeV N and P was transfected using the ProFection kit (Promega) with two plasmids coding for the MeV genome and MeV-L protein (pEMCLA) (17). Three days after transfection, cells were overlaid on either Vero (single P gene virus) or Vero-si2 cells (bi-P virus). Upon appearance, isolated syncytia were picked and individually propagated on relevant Vero (single P virus) or Vero-si2 (bi-P virus) cells.

Virus stock was produced after a second passage at MOI=0.03 in the relevant cell line. This stock was checked for lack of mycoplasma contamination, has its N, P₁, P₂, M, and L genes sequenced and was titrated on the relevant host cell before use. In some experiments, the previously described MeV-GFP with GFP expressed from an additional transcription unit in first position (21) was also used.
Analysis of virus protein expression and replication. Parental Vero, si1 and si2 cells were infected at indicated MOI with recombinant viruses with or without addition of 10 μg/ml of fusion inhibitor peptide z-IFG to prevent syncytia formation. Viral protein expression was determined by flow cytometry analysis of cells labelled with the Y503 anti-F monoclonal antibody and/or GFP detection, detection of the expression of viral N (cl25 antibody), P (49.21 antibody), HA-P2 (anti-HA antibody, Sigma), Flag-P1 (anti-Flag antibody, Sigma) and cellular GAPDH (MAB 374 antibody, Chemicon) protein by western blot revealed by chemoluminescence as detailed previously (22, 23). Unexpectedly, when the P protein was tagged with the HA peptide, its recognition by 49.21 mAb anti-P was reduced. Efficient recognition was restored using a cell lysis buffer supplemented with 6 M urea. While P denatured after boiling in the Laemmli sample buffer migrates in SDS-PAGE as a doublet, upon denaturation in the presence of urea, P migrates as a single band. This explains the differential P migration behaviour according to the absence or presence of urea in the lysis buffer. Protein contents of virus particles were also quantified using a dot-blot assay revealed by monoclonal antibodies specific for N, P, M proteins, Flag, and HA peptides as previously described (24, 25). In some experiments, the luminescent signal was quantified from imaging using Quantity-One software (Biorad) and viral protein level were given in % of the signal observed for the studied protein expressed from the control bi-Pwt virus infecting the same host cell (Vero, si1 or si2 cells). Virus production was measured after freeze-thaw cycles of infected cells using the TCID50 titration assay. Pwt, P-D497 and P-A497 variants were also transiently expressed after transfection of 6.10⁵ cells with corresponding pCG vectors and their expression was determined by western blot. Contamination of virus stock with internal deletion and copyback defective interfering (DI) mini genomes were assessed according to (26). Quantification of MeV genome and mRNA contents of infected cells were performed by RT-qPCR essentially as described in (27) with the following minor modification. Negative strand genome was reverse transcribed using sense 5’ tagged-N primer 5’-gcagggcaatctcaatatcaggGTGATCAAAGTGAAGACTGAGCT-3’ and the cDNA was PCR quantified using sense tag primer 5’-gcagggcaatctcaatatcagg-3’ and antisense N primer GCTGACCTCGACTGTCTCCT to overcome the non-optimal efficacy of the L-Tr and L primers used previously (ref?). For genome, results were expressed as copy number/μg RNA and for transcripts either as polymerized nt number/genome copy, or viral transcript/μg RNA after normalization for the
genome copy contents of each sample. For trans-complementation studies, si2 (293T background) cells were infected at MOI=1 during 1 hour with addition of 10 µg/ml of fusion inhibitor peptide, then transfected using jetPRIME (Polyplus). Two days later, the percentage of infected cells was determined by flow cytometry analysis of virus encoded GFP expression or viral F expression labelled with anti-F antibody.

**Protein complementation Assay.** Gaussia luciferase-based complementation assay and data analysis (normalized luminescent ratio, NLR) were performed according to (12). Original pSPICA-N1-GW and pSPICA-N2-GW plasmids, kindly provided by Y. Jacob, were modified by replacing the Gateway™ recombination sequences by a unique NotI site in which the constructs were subcloned using the Infusion™ recombination technology (Clontech). The linker sequence downstream the glu1 and glu2 domains were unchanged. NLR was calculated by dividing the luciferase value of the two chimeric partners by the sum of the luciferase value of every chimeric partner mixed with the other "empty" glu domain.

**Results**

**Design of P\(_{\text{XD}}\) substitutions in view of functional studies.** In the crystal structure of P\(_{\text{XD}}\), the aromatic side chain of F497 is part of an intramolecular hydrophobic network involving residues I464, I468, L481, L484, L485, I488 and L501 from the three \(\alpha\)-helical segments (Fig. 1, left panels) (9). In the bound form, an additional interaction involving the side chain of L494 occurs (9, 10). We thus reasoned that targeting F497 for substitution with either an aspartic or an alanine residue would perturb the N\(_{\text{TAIL}}\)-P\(_{\text{XD}}\) interaction, thus offering the opportunity to assess the impact of an altered N\(_{\text{TAIL}}\)-P\(_{\text{XD}}\) interaction strength on polymerase function. Indeed molecular modelling of P\(_{\text{XD}}\) variants bearing the F497D and F497A substitutions predicted complete or partial loss of these hydrophobic interactions, respectively (Fig. 1, middle and right panels).

The D497 and A497 P\(_{\text{XD}}\) variants were found to be not properly expressed in *E. coli*, thus precluding direct determination of their binding strength towards N\(_{\text{TAIL}}\) using purified protein domains. In parallel experiments, full-length P proteins harbouring these substitutions were readily expressed in mammalian cells, alone or in combination with the N protein (Fig. 2). This led us to switch to another
expression system to evaluate the strength of the P\textsubscript{XD}-N\textsubscript{TAIL} interaction. We selected an improved version of the *Gaussia* luciferase protein complementation assay (PCA) (12) because of its broad signal range and of the possibility to measure protein-protein interactions at steady state (13).

In a first step, we addressed the question as to whether this assay intracellularly reproduces known binding properties of P and truncated P proteins towards the N protein. P and N bind to each other via both their N and C termini, hence, N+P exhibited the highest normalized luminescence ratio (NLR) that was taken as the reference (100%) (Fig. 3A). When only the N-terminal disordered region of P (PNT-sp-glu1) was used, the NLR signal was reduced to 46%, while the use of monomeric P C-terminal region (i.e. linker-XD) led to a much lower binding ability (reduced by 92.2%). The substitution of the spacer by VCT with the glu domain grafted at its N-terminus (glu1-PNT-VCT construct, i.e. V protein) further decreased PNT binding to N by ~3 times. Reduced binding is due to the presence of the glu domain at the N-terminus since multiple combinations of glu1 or glu2 grafted at the N terminus of PNT invariably reduced by ~3 times the luciferase signal upon interaction with N-glu1/2 constructs. This suggests that although the P protein can tolerate extension at its N terminus with the ~30 kDa GFP protein (18), such an N-terminal graft could significantly modulate its ability to bind to N.

When the construct encompassing XD was tetramerized by the addition of the P multimerization domain (PMD) (PMD-XD construct) (28, 29), the NLR signal was increased 3.8 times (as expected because of enhanced avidity), while the PMD domain on its own exhibited only a background signal.

Then, the gene fragments encoding N[aa401-525] (N\textsubscript{TAIL}) and P[aa376-507] (thereafter called XD for simplicity but corresponding to the C-terminal fragment downstream PMD) were subcloned downstream the glu1 and glu2 halves of the luciferase. Co-expression of glu1-N\textsubscript{TAIL} and glu2-MeV-XD\textsuperscript{wt} constructs gave a small but reproducible PCA signal, while the glu2-MeV-XD-D497 and glu2-MeV-XD-A497 constructs elicited a very weak and an enhanced signal, respectively (Fig. 3B). However, both glu2-MeV-XD mutants were found to be poorly expressed (Fig. 3B, inset).

Oligomerization signals were added to both P\textsubscript{XD} and N\textsubscript{TAIL} domains both to improve P\textsubscript{XD} expression and to mimic the natural P oligomer to N oligomer interaction. P\textsubscript{XD} was tetramerized by generating a construct encompassing the natural P multimerization domain (P[aa301-507]) (29) yielding a construct referred to as MeV-PMD-MeV-XD, while N\textsubscript{TAIL} was either dimerized, or tetramerized by grafting a GCN4 peptide (30) or the PMD of the Sendai virus (SeV) P protein (31).
glu2-MeV-PMD-MeV-XD constructs were similarly expressed (Fig. 3C, inset). Together with the comparable expression of wt, A497 and D497 substituted P proteins (Fig. 2), this indicates that both substitutions at 497 position do not significantly interfere with proper folding and stability of the mutants proteins in these cells. The interaction levels of glu2-MeV-PMD-MeV-XD<sup>wt</sup>, glu2-MeV-PMD-MeV-XD-D497 and glu2-MeV-PMD-MeV-XD-A497 with dimeric (glu1-GCN4-MeV-N<sub>TAIL</sub>) (Fig. 3C, red bars) or tetrameric N<sub>TAIL</sub> (glu1-SeV-PMD-MeV-N<sub>TAIL</sub>) (Fig. 3D, E, red bars) were higher as compared to experiments with monomeric interacting pairs (Fig. 3B). Notably, the use of oligomeric partners led to the same profile in that the three P<sub>XD</sub> forms (i.e. wt, D497 and A497) led to medium, low/undetectable and high signals, respectively (Fig. 3C, D, E, red bars). The use of the entire N protein as binding partner resulted also in a similar profile (Fig. 3F).

There was a remarkable similarity in the NLR fold increase of the glu2 construct encoding the P<sub>XD</sub>-A497 variant over that encoding P<sub>XD</sub><sup>wt</sup> (average fold increase 1.5, p<0.005, Table 1). As controls, all three glu2-MeV-PMD-MeV-XD proteins displayed a low but equivalent background level of luciferase signal when co-expressed with a tetrameric glu1-SeV-PMD-SeV-N<sub>TAIL</sub> construct (Fig. 3G, red bars) while interaction of the latter with the homologous glu2-GCN4-SeV-XD construct gave a strong signal (Fig 3G, white bars). Likewise, the heterologous co-expression of glu2-PMD-SeV-XD with oligomeric glu1-GCN4-MeV-N<sub>TAIL</sub> proteins and that of glu2-GCN4-SeV-XD with oligomeric glu1-SeV-PMD-MeV-N<sub>TAIL</sub> proteins resulted in background signals (Fig. 3C and 3D respectively, white bars). Mixing two partners fused each to a GCN4 dimerization domain or to homologous PMD tetramerization domains resulted in very strong luciferase signals irrespective of whether the N<sub>TAIL</sub> and P<sub>XD</sub> partners were homologous or heterologous, indicating that luciferase reassembly was driven (and dominated) by high affinity coiled-coil interactions (Fig. 3C, D, E, and G, black bars).

**P-A497 MeV grows slowly, while rescue of P-D497 MeV fails even by trans-complementation.**

Recombinant genomes coding for viruses expressing P-D497 or P-A497 were built and transfected into rescue cells, where standard N, P and L proteins are provided in trans (17). Only the P-A497 virus was rescued, but it grew slowly (Fig. 4A). Lower viral protein expression was documented at 24 hpi (Fig. 4B). Virus production was delayed by one day, with A497 virus titer at 3 d.p.i. being similar to that of the wt virus at 2 d.p.i. (Fig. 4C, D; Table 1 for statistical analysis). Cells infected with either virus displayed equivalent amounts of genome copy/mg of total RNA that remained unchanged during the
first 8 hpi (Fig. 4E). Since the infection was done using identical MOI, this indicates that the two
viruses had equivalent contents of genomic RNA per infectious unit (p>0.35) (Table 1). The lower
replication kinetics of P-A497 MeV correlated with a ~1.6 fold reduction in the transcript accumulation
rate of the polymerase (p<0.0025) as measured during the linear accumulation of N, P, M and F
transcripts at early times post infection from this constant number of genome templates according to
(7) (Fig. 4F and Table 1). This growth phenotype could be attributed to the P-A497 substitution and not
to the acquisition of compensatory mutations as shown by the sequence identity of N, P, M and L
genes from our viral stock with those from the genomic plasmids used to rescue the viruses.

To analyse the P-D497 variant in a viral context, we sought to develop a system based on the
trans-complementation of a virus having its P gene expression repressed by RNAi. From an algorithm
predictor (from eurofins-mwg-operon) and screening, a single P mRNA sequence (+ strand) found to
be particularly suitable for RNAi targeting was stably expressed as a shRNA in a 293T cell clone (si2
cells). Unlike the parental 293T cell line, si2 cells resisted infection by a recombinant virus coding for
the green fluorescent protein (GFP) as shown by the very small percentage of cells expressing the cell
surface F or the GFP proteins (Fig. 5A) and by the undetectable levels of N and P proteins within cells
(Fig. 5A, inset) even at 96 hpi. As a control, the virus grew in si1 cells that constitutively express a
GFP-specific shRNA targeting the (+) strand (m)RNA, almost as well as in the parental cells (14, 32).
However, the onset of GFP expression was delayed reflecting the silencing effect on the viral GFP
mRNA (Fig. 5A). Virus production in si2 cells was strongly affected (Fig. 5B). The late virus production
in si2 cells at 72 hpi reflected incomplete repression of viral replication through RNA silencing since
the viral genome did not exhibit escape mutations within the si2 target region after sequencing the viral
genome that was identical to that of the parental virus (Genbank accession number KM054581).
Neither MeV N protein expression in cells undergoing active silencing of a cellular protein nor a
concurrent irrelevant silencing during MeV infection was found to be modified (Fig. 5C). This suggests
that there is little interference between MeV replication and silencing machinery.

We then attempted to rescue MeV growth in si2 cells by expressing in trans a P protein
harbouring silent mutations to resist silencing by si2 cells. This was technically challenging since
transfection reduced MeV replication efficiency (Fig. 5D): only 60% of cells transfected with empty
vector expressed the F protein at 2 d.p.i. after infection with MOI 4. Although well expressed in si2
cells (Fig. 5D, inset), the P construct did not improve virus growth in si2 (Fig. 5D). Similar results were
obtained after infection at MOI 1 except that the percentage of F expressing cells in the control was only 45%. Incidentally, this also indicates that the MeV P protein is not naturally endowed with inhibitory properties against the cellular silencing machinery.

Design of a biGene-biSilencing (biG-biS) cis-complementation system. We then sought to develop a cis-complementation assay based on a recombinant virus with a duplicated gene of interest. In this system each mRNA (+ strand) copy is artificially made sensitive to unrelated shRNAs (Fig. 6A). We know that silencing of P mRNA tagged in the 3'UTR with one copy of the si1 target is leaky in the context of a recombinant MeV because of overwhelming self-amplifying viral transcription (7). This is illustrated by the modest reduction in GFP expression from MeV-GFP grown into si1 (targeting GFP mRNA) cells (Fig. 5A). Thus, to ensure higher silencing levels, three copies of si1 (Fig. 6A, si1) and si2 (Fig. 6A, si2) target sequences were introduced into the 3'UTR of P1 and P2 copies of the P gene of a recombinant bi-P MeV, respectively. The P1 and P2 proteins were labelled by grafting an N-terminal Flag- and HA-peptide tag, respectively. Indeed, viable MeV tolerates having its P fused downstream to a polypeptide as long as GFP (18) and viable viruses with single P gene tagged with either N-terminal Flag or HA were also easily rescued (data not shown).

A virus with two copies (Flag-P1 & HA-P2) of wild-type P gene (bi-Pwt virus) was successfully rescued and amplified in Vero cells, which are very good producers of MeV and unable to produce type I interferon. This virus was then used to infect at a multiplicity of infection (MOI) of 1 three Vero cell lines: parental (Fig. 6B, Ø), si1 (Fig. 6B, si1) and si2 cells (Fig. 6B, si2). The three cell lines were equally infected as shown by a roughly similar expression level of intracellular N protein (Fig. 6B). While both Flag-P1 and HA-P2 proteins were expressed in parental cells, only HA-P2 and Flag-P1 were detected in si1 (si1, Fig. 6B) and si2 (si2, Fig. 6B), respectively. This illustrates the power of the double silencing system in controlling the selective expression of only one copy of the P gene. When P expression was determined using the 49.21 anti-P antibody that recognizes an epitope located upstream of P XD (33), both Flag-P1 and HA-P2 were detected. In addition, the virus production in si1 and si2 cells reached levels similar to those observed in the parental cells (data not shown).

Functional impact of the A497 and D497 substitutions in P XD. Both [Flag/P1wt + HA/P2-A497] and [Flag/P1wt + HA/P2-D497] bi-P viruses were successfully rescued in si2 cells allowing selective
expression of the P1 wt protein. The sequencing of N, P1, P2, M and L genes from all viral stocks did not reveal any nucleotide substitution when compared to the antigenomic plasmid backbone used for the rescue of each virus, thus excluding the selection of compensatory mutations. Reinfection of si2 cells with these bi-P viruses resulted in normal or slightly increased N expression, reduced expression of P (Fig. 7A, middle panel), slight reduction in the percentage of infected (F expressing) cells (Fig. 7B, white bars), and virus production levels similar to those observed with bi-P wt virus (Fig. 7C, white bars). Virions from both recombinant constructs incorporated similar levels of N and P proteins, mostly Flag/P1 wt with little HA/P2 proteins as observed for bi-P wt virions (Fig. 7D).

When grown in si1 cells, i.e. in conditions of selective expression of the mutated P2, [Flag/P1 wt + HA/P2-A497] bi-P MeV grew like the single P-A497 virus: with reduced expression of N and P (Fig. 7A right panel), reduced percentage of F expressing cells (Fig. 7B, black bar) and ~one log reduction in virus production (Fig. 7C (p1) black bar). Because of possible impact of P wt remnants brought by the incoming virus, a second passage of this virus into si1 cells was performed and resulted in a further ~1 log decrease in virus production at 2 d.p.i. (Fig. 7C (p2) black bar) that more closely mimicked the phenotype observed with the uni-P-A497 virus. Upon infection of parental Vero (Ø) cells that allowed the expression of both P1 wt and P2-A497 proteins to a similar extent (Fig. 7E), viral protein expression and virus production was comparable to those observed with the bi-P wt virus (Fig. 7A, left panel, and B & C, shaded bars). The wt-like and P-A497-like phenotype of the [Flag/P1 wt + HA/P2-A497] bi-P MeV when grown into si2 and si1 cells, respectively underlined the potential usefulness of the biG-biS system.

In agreement with the unsuccessful attempt to rescue the single P-D497 virus, when P2-D497 was selectively expressed from [Flag/P1 wt + HA/P2-D497] bi-P virus grown in si1 cells, viral protein expression and virus production were barely detectable: only trace amounts of viral proteins were detected (Fig. 7A, right panel, and B & C, black bars). Notably, the small amount of P1 wt that could be produced due to the small leakage of the silencing was not able to overcome this replication block at later times post-infection. Surprisingly, when both P1 wt and P2-D497 proteins were co-expressed by infecting parental Vero (Ø) cells, significant amount of N and P protein were detected (Fig. 7A, left panel) with almost every cell expressing the F protein (Fig. 7B, shaded bar). Furthermore, only ~1.5 log lower virus was produced (Fig. 7C, shaded bar) when compared to si2 cells allowing restricted expression of P1 wt protein or si1 cells infected with bi-P wt virus.
This intermediate phenotype of the [Flag/P1wt + HA/P2-D497] bi-P virus raised questions about the ability of Pwt and P-D497 to make heterotetramers that could display binding activity towards NTAIL. When assessed through PCA, the two P proteins were found to be able to form hetero-oligomers as well as homo-oligomers (Fig. 8B, inset). When co-expressed as hetero-oligomers via co-transfection of the two plasmids in a 1:1 ratio, PMD-XDwt/PMD-XD-D497 exhibited a binding ability to oligomeric NTAIL or N proteins similar to that of the homotetrameric PMD-XDwt (Fig. 1E, F). We conclude that the P-D497 variant has a too low affinity for NTAIL to sustain virus expression on its own, but apparently does not exert a dominant negative effect on Pwt.

To assess how transcript accumulation rate is influenced by the D497 mutation, the biP viruses were amplified in Vero cells. Virus production peaked at 1.22 x 10^7 (wt/wt) and 1.02 x 10^7 (wt/D497) TCID50/ml, with a similar kinetics. HeLa cells were then infected at MOI 1, and mRNA accumulation was measured 2, 4, 6 and 8 hours after infection. The wt/wt and wt/D497 biP viruses exhibited a remarkably similar genomic content (83 ± 41 and 62 ± 18 copies/μg of total RNA, respectively, p>0.15). This prompted us to analyse the viral protein contents of virions. Consistent with the similar genomic content, we did not detect any significant difference in the content of N, P, Flag-P1, HA-P2 and M proteins between the two biP viruses (data not shown). Comparison with wt uniP virus revealed a similar protein composition with a slight but not significant increase in P content for biP viruses. This similarity, associated to genome content per infectious unit of similar range, indicates that both biP and wt uniP viruses share a similar efficiency in assembling infectious virus. Moreover, both biP viruses were hardly contaminated with defective interfering (DI) nucleocapsids with only a possible slight contamination of the wt/wt virus with a single internal deletion DI (Fig. 8A). However, the transcript accumulation rate of wt/D497 biP virus measured within the windows of the first transcription step (i.e. 2-8 hpi) was reduced down to ~17% of that of the wt/wt biP virus (Fig. 8B). Accordingly, the replication start of wt/D497 biP virus was delayed by ~6 hours as revealed by the kinetics of genome accumulation, which then proceeds with the same rate as that of the wt/wt virus (Fig. 8C).

Negative effect of neo-synthesized D497 transcriptases on preformed Pwt transcriptases. Upon MeV entry, transcriptases located in the incoming particles transcribe the RNP ((7) and references herein). Thus, we analyzed primary transcription upon infection of si1 cells with wt/D497 biP virus produced in si2 cells. In those conditions, both wt/wt and wt/D497 biP virions are loaded with
polymerases that are exclusively and mostly made of P$^{wt}$, respectively (Fig. 7D). Indeed, over the first 12 hours post infection, the accumulation rate of N mRNA were similar for both biP viruses grown in si1 cells in the absence of viral (and cellular) protein synthesis (Fig. 9, empty symbols and dotted lines, compare A and B, note the similar slope in the equations). Over this time, the levels of genomic RNA remained unchanged (Fig. 9C,D). When the synthesis of D497 P protein was exclusively allowed from wt/D497 biP virus, the N mRNA transcript accumulation rate was reduced by ~2 fold as shown by the ratio of the slopes (Fig. 9B, full symbols and lines). In contrast, after the initial linear accumulation rate, N transcript accumulation from wt/wt virus rose exponentially (Fig. 9A, full symbols and lines). This latter phase is due to the recruitment of increasing amounts of neo-transcriptases by the constant amount of genome templates at least over the first ~12 hours (Fig. 9 C, full symbol and line), i.e. before the onset of wt/wt virus replication observed only at 18.5 hpi. Notably, at this late time point for the three other experimental settings, both N mRNA and cell associated genome levels dropped to low values illustrating the interruption of the virus replication cycle (Fig. 9 C, empty symbols & D, empty and full symbols that fully overlapped).

Discussion

The newly developed methodology to study phosphoprotein function herein described, together with the study of MeV P497 mutants yielded insights on how the interaction between P and N could govern MeV transcription. Previously, four different approaches have been used to study the polymerase activity of non-segmented negative strand RNA viruses (8, 34). The most popular relies on artificial minireplicons or minigenomes that encode a reporter gene. Spontaneous reconstitution of the first nucleocapsid relies on the complete coverage of a RNA minigenome by a continuous array of N subunits. This event is inefficient and many sub-genomic nucleocapsids are also made, severely limiting mechanistic studies (35, 36). The second assay, which relies on in vitro RNA synthesis with purified viral polymerase components and purified nucleocapsids made in cells as a template, is easily applied only to vesiculoviruses (1, 8, 37-39). The third strategy, consisting in substituting the wild type (wt) gene with a gene coding for a protein harbouring the substitution(s) of interest and in rescuing a mutant virus (see (24) for example), is limited to those modified proteins that are able to sustain a complete virus replication (see (18) for example). The fourth assay consists in building and rescuing a recombinant virus lacking expression of the P gene in a cell host that provides the lacking viral
polymerase component in trans (40, 41). However, trans-complementation results in inefficient viral amplification (40, 41). It remains unclear why trans-complementing polymerase components of paramyxoviruses work inefficiently, and further work will be needed to understand why exogenous viral component cannot be functionally integrated into the putative viral factory (42, 43). We developed here an alternative system to perform structure-function studies of a viral polymerase component, which we name biG-biS. This assay proved to be essential in revealing the inhibitory properties of the non-functional D497 P mutant on incoming preloaded transcriptases. Its power has also been documented in a previous study aimed at deciphering the mechanisms of a dominant negative mutant of the Sendai virus fusion glycoprotein (44).

The F497A substitution enhances the interaction strength with NTAIL by ~1.5 times, and reduces by 1.6-fold the transcript accumulation rate. This suggests a correlation between the PXD-NTAIL interaction strength and the displacement of the polymerase in the transcription mode along the nucleocapsid template, thereby explaining the slower growth of the A497 virus. We are confident that the interaction strength scale measured by PCA reflects the affinity between these two partners since, in another study, we found a correlation between PCA NLR values and affinity as determined using ITC and purified NTAIL variants and XD partners (Gerlier and Longhi, unpublished data). We previously documented that the NTAIL A502D substitution within the $\alpha$-MoRE, which leads to a 33-fold reduction in the binding affinity towards PXD, causes only a 1.7-fold reduction in the transcript accumulation rate (24). We concluded that there is a poor relationship between the interaction strength of the PXD-NTAIL pair and transcript accumulation rate (24). While those previous studies suggested lack of relationship between the interaction strength of the PXD-NTAIL pair and transcript accumulation rate, we propose here that tolerance of the polymerase to NTAIL substitutions applies only to a certain range of affinities: in spite of a pronounced drop in the affinity towards PXD brought by the A502D substitution, a PXD-NTAIL interaction with a $K_D$ in the $\mu$M range remains strong enough to ensure RNA synthesis by the polymerase. Conversely, an enhanced interaction, such as the one resulting from the PXD F497A substitution, might hinder the polymerase progression along the nucleocapsid template.

Furthermore, according to available structural data and our modelling, the side chain of PXD residue 497 does not contact NTAIL, and is rather only involved in intramolecular interactions that likely contribute to stabilize the triple $\alpha$-helical scaffold of PXD. Thus the stability of the PXD tertiary structure controls the ability of PXD to accommodate the $\alpha$-MoRE of NTAIL, which ultimately would result in
different levels of anchoring of the polymerase complex onto its template. That substitutions outside
the binding interface may affect the binding affinities of protein-ligand interactions through changes in
conformational entropy (i.e. fast internal dynamics) is well established (see (45) and references therein
cited). Further work is needed to understand how the dynamic interaction between NTAIL and PXD can
reflect their different tolerance to amino acid substitution.

When the D497 P protein is produced exclusively or at least in large excess, it interferes with
the transcription activity by the pre-existing transcriptases interrupting the viral replication cycle. P-
D497 cannot bind to N via the PXD-NTAIL interaction but can still bind with N via the PNT-N\textsubscript{CORE}
interaction that appears much stronger than the former according to PCA measurements. The current
model of Mononegavirales transcription postulates an obligate entry for the P+L polymerase complex
at the genomic 3' end in order to be switched on for RNA synthesis. This is supported by functional
evidences (see (6) for review) and crystal structures of nucleocapsids from rabies (2), vesicular
stomatitis (3) and respiratory syncytial viruses (4), electron microscopy reconstructions of MeV
nucleocapsids (46, 47) and docking studies (48). PNT is the best candidate in allowing P+L to be
recruited at the 3' end of the nucleocapsid as inferred from the crystal structures of PNT peptides in
complex with the N protein with displacement of the RNA (49) and from functional studies with a P
protein having a large deletion within PCT (50). We have previously proposed that the early linear
accumulation phase of transcription relies on the very efficient re-initiation of long-living transcriptases
over several cycles of transcription along the entire genome (7). That P497D produced in a large
excess (i.e. almost exclusively) reduces the primary transcription by pre-formed P\textsubscript{wt} based
polymerases is indicative of a competition between P-D497 and P\textsubscript{wt} for binding to the 3' end of the
genomic nucleocapsid and/or to an out titration of the L component of resident P+L polymerase
complexes. It should be stressed here that the transcription was evaluated by measuring N mRNA
accumulation and not by directly quantifying the amounts of neosynthesized transcripts. This
explained why, during the first 6-8 hours of the transcription phase, we could only observe a linear
accumulation despite the likely recruitment of few neosynthesized P+L, the activity of which remains
negligible over that of the resident transcriptases. However, the restricted synthesis of newly
transcriptases that are made in small amounts from the inactive P-D497 seem to outcompete the
resident molecules, as suggested by the negative effect on transcript accumulation rate. Alternatively,
reassortment of P<sup>wt</sup>/P<sup>wt</sup> and D497/D497 homotetramers into inactive P<sup>wt</sup>/D497 heterotetramers could occur. However, this is unlikely owing to the very high cohesion of the PMD domain (28, 29).

Interestingly, the F497D substitution, which by itself no longer supports any transcription and consequently replication to allow virus growth, does not act as a dominant-negative over wt P when co-expressed at roughly similar levels. The reduced level of transcript accumulation rate of wt/D497 virus down to only ~17% that of wt/wt virus is higher than that expected from the 6.25% predicted wt/wt tetramers (random combination probability 1:16), but lower than that expected from the sum of wt/wt tetramers (6.25%) and 3<sup>wt</sup>:1<sup>D497</sup> heterotetramers (25%). This suggests that most if not all wt/D497 heterotetramers are inactive, and that the ratio of wt/wt P homotetramer (6.25%) may be underestimated. This would be the case if there is a higher propensity to homotetramerisation of successive nascent P subunits from the same P transcript (i.e. cis-homotetramerisation would be favoured over trans-tetramerization). Alternatively, we cannot exclude that the transcript accumulation rate is reduced by competition at the 3' genomic entry site by non-functional D497 containing tetramers. Furthermore, the replication of wt/D497 virus proceeds with kinetics similar to that of the wt/wt virus except for a ~6 hour delay as expected from lower kinetics of production of functional polymerases.

In conclusion, these results are fully consistent with a model where the RNA-dependent RNA polymerase of non-segmented negative strand RNA viruses attaches to the 3' end of genomic nucleocapsid via PNT interaction with N<sub>CORE</sub> and progresses along its genomic template through a tightly controlled dynamic anchor mediated by the P<sub>AD</sub>-N<sub>TAIL</sub> interaction. This is in contrast to all other known polymerases that rely only onto a sustained direct binding to their nucleic acid template.

**Acknowledgement:** We thank P. Pothier, R. Iggo, L. Roux, and Y. Jacob for providing us with useful reagents, S. Plumet for pioneering the MeV-P silencing work, C. Lazert and L. Drevet for technical help and J. Louber for helpful discussions. We are also indebted to the flow cytometry (T. Andrieu, S. Dussurgey) and qPCR (B. Blanquier) facilities of the SFR Biosciences Gerland-Lyon Sud (UMS344/US8).

**References**


Table 1. Transcript accumulation rates, genome contents and virus production of wt and mutant A497 virus

<table>
<thead>
<tr>
<th></th>
<th>Pwt</th>
<th></th>
<th>P-A497</th>
<th></th>
<th>Student t test</th>
</tr>
</thead>
<tbody>
<tr>
<td>NTAIL (PXD, % of wt, by PCA)</td>
<td>1 ± 0.22</td>
<td>18</td>
<td>1.5 ± 0.8</td>
<td>11</td>
<td>p&lt;0.005</td>
</tr>
<tr>
<td>Genome contents (copies/μg of RNA)</td>
<td>262 ± 46</td>
<td>5</td>
<td>272 ± 34</td>
<td>5</td>
<td>p&gt;0.35</td>
</tr>
<tr>
<td>Transcript accumulation rate (normalized slope)*</td>
<td>1.29 ± 0.15</td>
<td>4</td>
<td>0.79 ± 0.088</td>
<td>4</td>
<td>p&lt;0.0025</td>
</tr>
<tr>
<td>Virus titer 2 d.p.i. (log2)</td>
<td>20.6 ± 1.5</td>
<td>6</td>
<td>10.5 ± 1.8</td>
<td>6</td>
<td>p&lt;0.0025</td>
</tr>
<tr>
<td>Virus titer 2 (wt) &amp; 3 (A497) d.p.i. (log2)</td>
<td>19.8 ± 0.7</td>
<td>3</td>
<td>17.5 ± 0.3</td>
<td>3</td>
<td>p&gt;0.15</td>
</tr>
</tbody>
</table>

* values from N, P, M and F genes. Slopes were normalized on the N gene value from the corresponding virus to take into account the transcription gradient. Corresponding calculated wt/A497 slope mean ratio ± 1.7 ± 0.4.

n, number of independent trials.

Note: μg of RNA stands for μg of total cellular + viral RNA from infected cells.
Figure Legends

Figure 1. (A) PXD residues in contact with residue 497 within the P XD structure (pdb 1OKS). Left: F497 makes hydrophobic contacts with PXD residues I464, I468, L481, L484, L485, I488 & L501. Middle and right panels: structural models of the D497 (middle) and A497 (right) PXD variants showing that while residue D497 does not establish any contacts, residue A497 makes a hydrophobic contact with PXD residue I488. (B) Residues contacting PXD residue 497 within the chimeric PXD/NTAIL α-MoRE structure (pdb 1T60, PXD in blue, NTAIL in red). Left: F497 makes an additional hydrophobic contact with PXD residue L494 in addition to those found in the unbound PXD structure (pdb 1OKS). Middle and right panels: structural models of the D497 (middle) and A497 (right) PXD variants in complex with the α-MoRE showing that while PXD residue D497 is not involved in any interaction (middle), residue A497 makes an additional hydrophobic contact with PXD L494 residue in addition to that involving PXD residue I488 occurring in the unbound form. The structural models were obtained by replacing the side chain of the native F497 residue either in the structure of PXD (pdb 1OKS) or in that of the the PXD/α-MoRE complex (pdb 1T60) by the side chain (most frequent conformer) of either Asp or Ala. The models were then energy minimized so as to avoid steric clashes by using the GROMOS96 implementation of the Swiss-PDB Viewer with default parameters.

Figure 2. Similar expression levels of P-D497 and P-A497 alone (Ø) or together with N protein one day after transfection of 293T cells. Western blot revealed by anti-P and anti-N monoclonal antibodies. GAPDH is a loading control.

Figure 3. Assessment of protein-protein interaction strength using the Gaussia luciferase-based protein complementation assay (PCA) in human 293T cells. (A) Interaction strength of full-length and truncated MeV P and V proteins (schemed on left) with the MeV N protein. PNT: P N-terminus; sp, spacer, PMD, P multimerization domain; XD, X domain; linker, linker region between PMD and XD; VCT, V C-terminus. The V protein is made by RNA editing at aa 231, hence it has a different C-terminal domain (VCT). Shown are the means of two independent experiments done in triplicates. Similar rankings were obtained in a third experiment. (B-G) Binding properties of PXD wt, PXD-D497 and PXD-A497 to NTAIL. (B) Interaction between monomeric glu1-MeV-NTAIL (aa401-525) with monomeric glu2-MeV-PXD (aa376-507) hybrid proteins with expression level of glu2-MeV-XD determined by
western blot (B, inset). (C) Interaction of glu1-GCN4-MeV-N\textsubscript{TAIL} with glu2-GCN4-MeV-XD (black bar) or with glu2-PMD-XD from MeV (MeV-P aa301-507, red bars) or SeV (P aa316-568, white bar) hybrid proteins with expression level of glu2-MeV-PMD-MeV-XD determined by western blot (C, inset). (D, E) Interaction of glu1-SeV-PMD-MeV-N\textsubscript{TAIL} with glu2-GCN4-XD from MeV (P aa376-507, red bar) or SeV-P (P aa 445-568, white bars) or tetrameric glu2-PMD-XD hybrid proteins from MeV (P aa 301-507, red bars) or SeV (P aa316-568, black bars) or a 1:1 mixture of glu2-MeV-PMD-MeV-XD\textsuperscript{wt} and glu2-MeV-PMD-MeV-XD-D497 (red bar “D497/wt”). (E) Repeat of D with additional testing of the 1:1 mixture of glu2-MeV-PMD-MeV-XD\textsuperscript{wt} (red bar “D497/wt”). (F) Same as E but using glu1-MeV-N as sparring partner instead of glu1-SeV-PMD-MeV-N\textsubscript{TAIL}. (G) Interaction of glu1-SeV-PMD-SeV-N\textsubscript{TAIL} with glu2-GCN4-XD from MeV (P aa376-507, red bar) or SeV-P (P aa 445-568, white bar) or glu2-PMD-XD hybrid proteins from MeV (P aa301-507, red bars) or SeV (P aa316-568, black bar). NLR values in panels B-G are the mean of three independent experimental replicates.

Figure 4. Growth characteristics of wt P and P-A497 virus. Percentage (%) of infected cells (A), expression of N and P (with expression of N and P expressed as % of wt N and P as estimated by western blot using an anti-N or an anti-P monoclonal antibody, respectively) at 24 hpi with MOI 1 (B), and virus production at 2 d.p.i. (C) or 2-4 d.p.i. (D). For cytometry analysis of F expression, cell-cell fusion was prevented by adding fusion inhibitor peptide z-FG after MeV infection. (E,F) Kinetics of RNA accumulation of viral genome (E) and of N mRNA (F) after infection with single P viruses. Error bars are standard deviations based on three experimental replicates. Genomic RNAs in (E) correspond to the virus inputs that remain constant before the replication start (see (7) for details). Slopes in (F) correspond to the transcript accumulation rate (according to (7)). See Table 1 for genomic inputs of both virus preparations and slope comparisons.

Figure 5. Inhibition of MeV infection by a shRNA targeting the P mRNA (si2) is not alleviated by the expression of a shRNA-resistant P protein \textit{in trans}. (A) Schematic representation of the MeV genome expressing GFP upstream the N gene (top scheme). Inhibition of the expression of F (filled symbols, continuous line), gfp (open symbols, dotted line) after infection (MOI 1) of parental 293T (Ø, diamonds) or 293T cells constitutively expressing a shRNA against P (si2, triangles) or GFP (si1, squares) mRNA. The inset shows the expression of N and P proteins. Protein expression was determined by
flow cytometry (curves) and western blot at 96 hpi (inset). For cytometry analysis of F expression, cell-cell fusion was prevented by adding fusion inhibitor peptide z-IFG after MeV infection. (B) Kinetics of virus production after infection (MOI 1) of parental (diamonds), si1 (squares) and si2 (triangles) cells and protein contents of purified virions collected at 96 hpi as determined by western blot (inset). (C) Neither an ongoing RNA silencing affects infection by MeV or MeV expressing luciferase as reporter gene, nor MeV infection inhibits an ongoing RNA silencing. Cells constitutively expressing a miRNA-based shRNA targeting the luciferase gene or endogenous TNPO3 gene were infected with MeV or MeV-luc virus (MOI 1) for one day. TNOP3 and virus N protein expression were determined by western blot. (D) Inability of P protein transiently expressed from a shRNA resistant transcript (rP) to restore MeV growth in si2 cells (MOI 4). Inset: protein expression, as determined by western blotting, showing resistance and sensitivity to si2 shRNA of the rP and P constructs, respectively. In the absence of urea, P migrates as a doublet due to its phosphorylation heterogeneity.

**Figure 6.** biG-biS assay: principle and proof of concept. (A) Antigenome (+ strand, 5’-3’) organization of recombinant “bi-P” MeV expressing two copies of the P gene: (wt) P1 with mRNA and protein tagged with 3 copies of the 21 nt target of shRNA #1 (si1) and Flag peptide, respectively; (mutated) P2 with mRNA and protein tagged with 3 copies of the 21 nt target of shRNA #2 (si2) and HA peptide, respectively. The virus is rescued and amplified in si2 cells (middle) to ensure successful recovery of a wt-like virus thanks to the selective expression of the Pwt gene. This virus can then be used to infect, parental (Ø, expressing no shRNA, left), si2 (middle) and si1 (right) cells to allow the expression and functional analysis of [Flag-P1 + HA-P2], Flag-P1 and HA-P2, respectively. The grey and black colour lettering code for Flag-P1 and HA-P2 illustrates absence or presence of the protein in the cells, respectively. (B) Efficiency of selective silencing of P1 and P2 from recombinant bi-P MeV in si1 and si2 cells after infection with biP MeV (MOI 1) revealed by western blot analysis at 24 hpi.

**Figure 7.** Impact of the D497 and A497 substitutions on P function in the viral context. [Pwt + P-D497] and [Pwt + P-A497] bi-P viruses were rescued and used to infect (MOI 1) parental (Ø, A left panel and B shaded columns), si2 (A middle panel and B white columns) and si1 (A right panel and B black columns) cells. Expression (in % of wt/wt virus as estimated by western blot with cl55 anti-N and 49.21 anti-P) of N (yellow columns) and P (blue columns) at 24 hpi (A), percentage of (F-expressing) infected cells at 24 hpi (B) and virus production after MOI 0.01 infection (C). For cytometry analysis of
F expression, cell-cell fusion was prevented by adding fusion inhibitor peptide z-fFG after MeV infection. In panel (C), virus production of si1 cells infected by the [Pwt + P-A497] virus was measured after one (p1) and two (p2) successive passages. Error bars are standard deviations based on three experimental replicates. (D,E) Virions produced from si2 cells infected by bi-Pwt, [P1wt + P2-D497] and [P1wt + P2-A497] viruses, have similar high Flag-P1 and low HA-P2 protein contents (D) while infection of parental Vero cells by these viruses resulted in the expression of both Flag-P1/HA-P2 in similar ratio (E).

Figure 8. (A) Analysis of biP virus stocks produced in Vero cells for their contamination with deletion (del-DI) and copyback (cb-DI). DI as detected by RT-PCR. Heavy DI contents (identified by sequencing) of a Moraten MeV stock is shown as DI positive controls (right lanes). Note the similar intensity of the genomic amplicons (gen) indicative of a similar genomic RNA load for the three viruses. Kinetics of N mRNA (B) and genome (C) accumulation after infection with biP wt/wt and wt/D497 viruses produced in parental Vero cells in which protein is expressed from both P genes (see Figure 7E). Error bars are standard deviations based on three experimental replicates. Slopes correspond to the transcript accumulation rate (according to (7)). Genome inputs at earlier times (not shown) for both viruses were of similar range with 83 ± 41 (wt/wt) and 62 ± 18 (wt/D497) copies/μg of RNA (Student t, p>0.15). (B) Inset: Pwt and P-D497 make homo- and hetero-oligomers with similar efficiencies as determined by PCA.

Figure 9. Kinetics of N transcript (A,B) and genome (C,D) accumulation in si1 cells after infection with either wt/wt (A,C) or wt/D497 (B,D) biP viruses in the absence (full symbols, full lines) or presence of 20 μg/ml cycloheximide (empty symbols and dotted lines). The linear accumulation of N transcripts in the presence of cycloheximide in the 2.5 to 12.5 hour time interval (A, B) and in the absence of cycloheximide (B) is shown by the straight lines, equations and correlation r factor. Note that at the latest time point (18.5 hpi), the N mRNA (isolated symbols) amount dropped near the levels detected at 2.5 hpi (A, B) in agreement with the genome contents drop (C & D) in these three experimental settings, while upon infection with wt/wt virus in the absence of cycloheximide, the N mRNA accumulation sharply increased to reach a level well out of the graphic scale (A) and genome accumulation started to rise (C). The P1wt gene is indicated in grey to reflect its silencing.