Cell

Supplemental Information

Structural Insights into Bunyavirus Replication and Its Regulation by the vRNA Promoter

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Supplemental Experimental Procedures

Protein Production and Crystallisation

The L protein sequence of the La Crosse virus strain LACV/mosquito/1978 (GenBank: EF485038.1, UniProt: A5HC98) was used to design a synthetic, codon-optimized gene also coding for an N-terminal His-tag followed by Tobacco Etch Virus (TEV) protease cleavage site (MG$\underline{\text{HHHHHH}}_{\text{6xHis-tag}}$DYDIPTT$\underline{\text{ENLYFQ}}_{\text{TEV}}\underline{\text{G}}$) (DNA 2.0). L protein construct 1-1750 (L_{1750}) was found by limited trypsin digestion of purified full-length L, its C-terminus also corresponding to boundaries of soluble domains identified by ESPRIT ((Yumerefendi et al., 2010)(data not shown).

L_{1750} was expressed in Hi5 insect cells using the Bac-to-Bac expression system with a pFastBac vector. Cells were collected by centrifugation, re-suspended in lysis buffer (50mM Tris-HCl, 500 mM NaCl, 20 mM imidazole 0.5 mM TCEP, pH 8) supplemented with protease inhibitors (Roche, complete mini, EDTA-free), and lysed by sonication. Cell lysate was clarified by centrifugation for 1 h at 35,000 g and further purified by precipitation with NH_4SO_4 at 0.5 g/ml lysate. The protein fraction was pelleted by 30 min centrifugation at 70,000 g and re-suspended in lysis buffer. After a final clarification step (30 min at 70,000 g), the polymerase was purified from the supernatant by sequential nickel ion affinity, heparin affinity and size exclusion chromatography (Fig. S1A).

L_{1750} protein at 5 mg/ml concentration in 20 mM Tris-HCl at pH 8, 150 mM NaCl, 2 mM TCEP, was mixed in 3:4 ratio with an equimolar mixture of nucleotides 1-16 from the 3′ vRNA end (3′-UCAUCACAUGAUGGUU-5′) and nucleotides 9-16 from the 5′ vRNA end (5′-GCUACCAA-3′), which had been pre-annealed. All oligonucleotides were purchased from Dharmacon -GE Healthcare. Brush-like clusters of needles emerged after 2-3 days from several conditions containing 15% (w/v) of PEG 4000 or 20% (w/v) of PEG 3350 as precipitant and 0.2 M of various sulphate or citrate salts. After an additive screen and pH and

precipitant/salt concentration optimization, the optimal conditions were found to be 0.1 M Hepes, 0.2 M sodium citrate tribasic dihydrate, 0.3 M ammonium sulphate, 15% PEG 3350, pH 7. Microseeding allowed production of rod-shaped single crystals suitable for data collection. Seleno-methionine derivative crystals were grown in the same conditions as native ones, using initially native seeds and then switching to seleno-methionine seeds. Heavy metal derivatives were prepared by soaking native crystals in mother liquor supplemented with either 2.5-5 mM K_2PtCl_4 for 1-2 hours, or with 5-10 mM Ta_6Br_{12} tantalum cluster (Jena Bioscience) for 1-2 days which turned the crystals green. Crystals containing the 5′ genomic end were obtained by soaking L_{1750}-vRNA crystals with 5′ nucleotides 1-8, 1-10, 1-11 and 1-12 (5′-pAGUAGUGUGCUA-3′ and shorter).

Data Collection, Structure Solution and Model Building

All datasets were collected on ID23-1 and ID29 beamlines at ESRF. Data were integrated and scaled with XDS (Kabsch, 2010).

L_{1750}-vRNA complexes crystallize in the $P2_12_12_1$ space group (a= 102.5, b=141.0, c=165.5 Å) with one complex per asymmetric unit and 60 % solvent and diffract up to 2.7 Å resolution. To obtain experimental phases optimised anomalous datasets from platinum, tantalum and selenomethionine derivatives were collected at 4.8, 4.2-4.4 and 3.5 Å resolution, respectively (Fig. S1B-D).

The structure was finally solved by first using a highly redundant, 5 Å resolution Ta dataset, measured at the absorption peak, from which four sites for tantalum clusters were localized in a SAD experiment using autoSHARP (Vonrhein et al., 2007). This substructure was then refined and improved using the multiple isomorphous replacement with anomalous signal (MIRAS) method with SHARP (Bricogne et al., 2003), using the same dataset at 4.2 Å resolution, another dataset measured at the Ta inflection point at 4.4 Å from the same crystal, SeMet derivative data measured at the Se absorption peak, 5 mM platinum soaked crystals at

the Pt absorption peak and native data at 2.85 Å resolution. After several rounds of refinement and sites detection by anomalous difference maps, 16 tantalum cluster, 9 platinum sites and 53 selenium sites, were found, leading to an interpretable map at 2.85 Å resolution. LACV endonuclease (PDB: 2XI5) and influenza polymerase PA and PB1 subunits (PDB: 4WSB) could be fitted into the map as a guide to model building (Fig. S1E). The rest of L_{1750}-vRNA complex model was iteratively built with COOT (Emsley and Cowtan, 2004) and refined with REFMAC (Murshudov et al., 1997), using selenomethionine positions from the anomalous difference map to help align the sequence. Autobuilding with BUCCANNEER (Cowtan, 2006) was useful to extend the model. The excellent quality of the RNA electron density allowed unambiguous building of co-crystallized vRNA and revealed that the distal short duplex stacks with its exterior blunt end on a neighbouring protein molecule, thus explaining its requirement for crystallisation.

Native L_{1750}-vRNA crystals soaked with the 5′ genomic ends diffracted to the following resolutions: 5′p1-8 –3.0 Å, 5′p1-10 –3.0 Å, 5′p1-11 –3.1 Å and 5′p1-12–2.8 Å. All showed extra 5′ RNA density (Fig. 4A) except that with 5′p1-12.

Structure figures were drawn with Pymol (DeLano, 2002).

Electron Microscopy

Purified L_{1750} was initially checked for homogeneity by negative stain electron microscopy. 4 µl of 0.02 mg/ml of sample was applied to the clear side of carbon on a carbon-mica interface and stained with 1% (wt/vol) sodium silico-tungstate pH 7.5. Images were recorded under low-dose conditions with a JEOL 1200 EX II microscope at 100 kV using a nominal magnification of 40,000× (Fig. S2A). Homogeneous samples were subsequently used for cryo-EM analysis. For cryo-EM grids preparation, 4µl of polymerase at 0.2mg/ml was applied onto a glow-discharged and pumped quantifoil grid 400 mesh 2/1 (Quantifoil Micro Tools GmbH, Germany), excess solution was blotted during 2 s with a

Vitrobot (FEI) and the grid frozen in liquid ethane. Data collection was performed on a FEI Krios microscope operated at 80 kV. CryoEM micrographs were collected on a Falcon II electron direct detector at a magnification of 138,129× and with a binning factor of 2, giving a pixel size of 2.02 Å (Fig. S2B). FEI EPU automatization software was used to collect 6129 micrographs with a defocus between 0.5 and 2 μm, an exposure time of 0.5 s and a dose of $14e^{-}/Å^{2}$. The contrast transfer function (CTF) for each micrograph was determined with CTFFIND3 (Mindell and Grigorieff, 2003). The 4646 best micrographs, based on manual inspection and CTF quality, were kept for subsequent image processing. 10,102 particles were manually picked using EMAN boxer (Ludtke et al., 1999) and used for reference-free 2D MSA classification as implemented in IMAGIC (van Heel et al., 1996). The resulting class averages were used for automated particle picking with a procedure based on the Fast Projection Matching algorithm (Estrozi and Navaza, 2008). False positives from the automated picking procedure were discarded after 2D classification in IMAGIC and RELION 1.3 (Scheres, 2012) leading to a 178,983 particle dataset. Class averages of this clean dataset allowed identification of secondary structure elements of the polymerase (Fig. S2C). An initial 3D model was generated by filtering the L_{1750}-vRNA crystal structure at 30 Å resolution and refined as implemented in RELION 1.3 autorefine using the cleaned dataset, leading to a map at 8.3 Å resolution (Fig. 2A, Figs. S2D and S2E)(Scheres and Chen, 2012). Subsequently, a similar quality map was obtained by refinement with Relion1.3 of an initial, de novo, 3D model determined with sxviper (http://sparx-em.org/sparxwiki/sxviper), using as an input twenty-three 2D class averages obtained with Relion1.3. Local resolution variations were estimated using ResMap (Fig. S2F), identifying regions with lower resolution corresponding to flexible parts of the polymerase (Kucukelbir et al., 2014). In a subsequent 3D classification run, an angular sampling of 3.75° was combined with local angular searches around the refined orientations and led to separation of the dataset into three different subsets,

corresponding to three different states of the polymerase (Fig. 2). The first subset of 90960 particles led to a 9.7 Å resolution EM map which shows only partial density for the endonuclease, depicting its flexibility (Fig. 2B). A second subset containing 43832 particles led to a 9.7 Å resolution reconstruction with improved density in vRBL (Fig. 2C). The third subset containing 44191 particles yielded a map at 9.3 Å resolution which clearly shows that the vRBL is highly flexible (Fig. 2D). Reported resolutions are based on the gold-standard FSC = 0.143 criterion and are calculated without applying any mask. Prior to visualization, all density maps were sharpened by applying a negative B-factor of 900 $Å^2$. The crystallographic model of the endonuclease (residues 1-184) and the polymerase core domain (185-1745) were fitted as two rigid bodies into the EM map using Chimera Fit-in-map module (Pettersen et al., 2004).

Polymerase-vRNA Binding Studies

For electrophoretic mobility shift assays (EMSA) (Figs. S3B and S3D) radioactively labelled RNAs were produced by *in vitro* transcription with T7 polymerase (produced in house). For each 10 µl transcription 2 µM DNA oligos were preheated and annealed in order to form double stranded T7 promoter with long overhang being the reaction template. Reactions were carried with ATP/UTP/CTP (Jena Bioscience) and [α^{32}P]-GTP (PerkinElmer). RNA products were than resolved and purified from urea TBE 15-20 % polyacrylamide gels. For each binding experiment 10 µM of L_{1750} in 10 µl buffer (100 mM Tris, 100 mM NaCl, 0.5 mM DPBA (an endonuclease inhibitor), 5 mM β-mercaptoethanol, pH 8) was mixed with radiolabelled RNA and 1 µl of non-specific poly(U) RNA (Sigma). To maintain a similar amount of radioactive RNA in each mixture the volumes added were scaled based on ImageQuant and Geiger counter measurements performed prior to the experiment. Mixtures were incubated at room temperature for several hours and resolved on native TG gels (top and bottom parts of the gel with 10 and 20% polyacrylamide

respectively). Radioactive signal from shifted bands was recorded with a Typhoon and quantified with ImageQuant. In each case the amount of bound RNA was measured with reference to the control RNA of known strong affinity to the protein.

For fluorescence anisotropy measurements (Fig. S3A), 5 nM 25-nucleotide long RNA oligos corresponding to 3′ or 5′ vRNA (IBA), labelled with fluorescein on the appropriate non-interacting end, in 150 µl buffer (100 mM Tris, 150 mM NaCl, pH 8) were titrated with L_{1750} in order to obtain 10-15 protein concentration points ranging from 3 nM to 1 µM. Fluorescence and fluorescence anisotropy measurements were carried out in quartz cuvettes, using 495 nm excitation and 515 nm emission wavelengths. KaleidaGraph (Synergy Software) was used to evaluate the data and derive dissociation constants.

For proteolysis protection experiments (Fig. S3D), L_{1750} was incubated at 1 mg/ml with 25-nucleotide long 3′ or 5′ genomic ends at 1:1 molar ratios and then digested for 1 hour at room temperature with trypsin (1:1000 w/w). Products of digestion were analyzed by various techniques including: SDS-PAGE, western-blot, ESI-TOF-MS, MALDI-TOF-MS, MALDI-TOF-MS with N-terminal acetylation, and N terminal sequencing of protein fragments by Edman degradation.

Polymerase Activity Assays

L_{1750} or full length L protein and synthetic RNA of various lengths corresponding to the 3' and 5' vRNA ends (GE Dharmacon) were used. RNAs were preheated to 90°C prior to the reaction and, depending on the approach, added either sequentially or simultaneously (as pre-annealed) to the protein sample. The replication assays were done in 10 µl aliquots mixing 0.5 µM of L-protein, 0.5 µM of 3' RNA with or without 0.5 µM 5' RNA, 0.5 mM UTP/ATP/GTP/CTP mixture (Jena Bioscience) and [α32P]-UTP (PerkinElmer), in 20 mM Tris, 150 mM NaCl, 5 mM MgCl2, pH 8, and incubated at 30°C for 2-4h. Reaction products were loaded on urea TBE 15-20 % polyacrylamide gels. The auto-radiographs showed very

weak product bands with none corresponding to the template length as would be expected for a replication product. Products only few nucleotides longer than the 3' RNA template used in the reaction were frequently observed. In most cases the control reaction containing only the radioactive UTP led to the same output as reactions containing all four NTPs, suggesting non-templated nucleotide addition. The presence of regulatory 5' RNA end affected the output of the reactions, not making however the experiment less ambiguous (data not shown). The inconclusive polymerase activity suggests that other viral (e.g. NP) or cellular factors might be essential for its proper function.

Table S1. Crystallographic Data Collection and Refinement Statistics

	Native_1	SeMet_Pk	Ta6Br12_Pk	Ta6Br12_Ip	Pt_Pk	Native_2	5′ 10 nt soak
Data collection							
Space Group	$P2_12_12_1$	$P\ 2_12_12_1$	$P\ 2_12_12_1$	$P\ 2_12_12_1$	$P\ 2_12_12_1$	$P\ 2_12_12_1$	$P\ 2_12_12_1$
Cell dimensions							
a (Å)	102.09	102.39	102.25	103.00	104.93	102.0	103.00
b (Å)	140.90	141.07	140.95	141.00	141.25	140.7	141.10
c (Å)	162.54	163.09	161.97	163.80	163.49	162.4	165.50
α (^{0})	90.00	90.00	90.00	90.00	90.00	90.0	90.00
β (^{0})	90.00	90.00	90.00	90.00	90.00	90.0	90.00
γ (^{0})	90.00	90.00	90.00	90.00	90.00	90.0	90.00
Resolution (Å)	50 – 2.8	50 - 3.49	50-4.2	50-4.4	50 – 4.8	50-2.57	50-3.0
(last shell)	(2.9-2.8)	(3.58-3.49)	(4.3-4.2)	(4.5-4.4)	(4.9-4.8)	(2.6-2.57)	(3.12-3.0)
Beamline (ESRF)	ID29	ID29	ID29	ID29	ID29	ID23_1	ID23_1
Wavelength (Å)	1.25425	0.97908	1.25438	1.25484	1.07156	0.99187	0.97924
Completeness (%)	99.1 (97.4)	96.9 (95.8)	99,8 (99,8)	98.5 (99.3)	93.2 (93.9)	94.7 (97.4)	99.5 (99.5)
Redundancy	3.35 (3.28)	6.94 (6.62)	13.91 (14.34)	6.6 (6.66)	2.3 (2.01)	3.55 (3.56)	4.32 (4.52)
R-merge	7.9 (66.5)	25.4 (74.0)	23 (90.2)	28.1 (89.9)	7.0 (57.4)	8.0 (90.6)	9.2 (82.1)
I/σI	11.37 (1.93)	7.48 (2.63)	10.8 (3.60)	6.36 (2.56)	8.11 (2.05)	11.2 (1.28)	11.91 (1.55)
No. of sites	-	53	16	13	10	-	-
Phasing power acen	-	7.547	3.407	3.076	1.712	-	-
Refinement							
Resolution (Å)						48.65-2.57	49.2–3.0
No. reflections						71088	46142
R_{work}/R_{free}						20.38/25.23	19.23/25.24
						(36.6/42.4)	(35.6/36.9)
No. residues							
Protein						1657	1666
RNA						24	34
No. atoms							
Protein						13571	13618
RNA						500	716
Water						92	-
B-factors (Å^2)							
Overall						69.6	89.5
Protein						67.2	90.7
RNA						59.2	76.9
Water						59.2	-
Ramachandran (%)							
Outliers						0.1	0.3
Favoured						96.19	95.14
RMS deviations							
Bond lengths (Å)						0.003	0.007
Bond angles (^{0})						0.634	1.148

Table S2. Table of Protein-vRNA Hydrogen Bonding Contacts

3′ vRNA

RNA base		Atom		Residue		Atom		Distance (Å)	
G	14B	OP2	...	Lys	862A	NZ	...	2.59	***
G	10B	O2'	...	Lys	381A	N	...	2.85	***
U	9B	OP1	...	Leu	383A	N	...	2.94	***
			...	Arg	526A	NH2	...	2.88	***
U	9B	O4	...	Lys	870A	NZ	...	3.13	***
A	8B	OP1	...	Arg	526A	NH2	...	3.06	***
A	8B	O2'	...	Ile	378A	N	...	3.22	***
A	8B	N7	...	Lys	381A	O	...	3.42	*
A	8B	N6	...	Lys	381A	O	...	2.79	***
C	7B	OP1	...	Lys	1516A	NZ	...	2.39	***
C	7B	O2'	...	Trp	395A	NE1	...	3.25	***
C	7B	N4	...	Gln	398A	NE2	...	3.11	***
C	7B	N3	...	Arg	531A	NH1	...	2.86	***
C	7B	O2	...	Arg	531A	NH2	...	3.42	*
			...	Arg	531A	NH1	...	3.06	***
C	7B	OP2	...	Tyr	524A	OH	...	2.67	***
A	6B	OP1	...	Arg	372A	NE	...	3.07	***
			...	Arg	372A	NH2	...	3.41	*
A	6B	OP2	...	Arg	372A	NH2	...	2.86	***
A	6B	O5'	...	Arg	372A	NH2	...	3.49	*
A	6B	O3'	...	Asn	1308A	ND2	...	3.11	***
A	6B	O2'	...	Asn	1308A	OD1	...	3.52	*
			...	Asn	1308A	ND2	...	3.46	*
A	6B	N3	...	Ser	520A	OG	...	3.51	*
A	6B	N6	...	Gln	397A	OE1	...	3.60	*
C	5B	OP1	...	His	1515A	NE2	...	3.35	*
C	5B	N4	...	Glu	396A	OE1	...	3.26	***
			...	Gln	397A	NE2	...	3.17	***
C	5B	N3	...	Gln	397A	NE2	...	3.58	*
C	5B	O2	...	Asn	517A	ND2	...	2.91	***
U	4B	O4	...	Lys	368A	NZ	...	3.49	*
A	3B	O2'	...	Asn	318A	ND2	...	3.03	***
A	3B	N7	...	Lys	323A	NZ	...	3.03	***
A	3B	N6	...	Cys	535A	N	...	3.29	***
C	2B	N4	...	Lys	472A	O	...	2.83	***
C	2B	N3	...	Lys	472A	N	...	3.41	*
			...	Lys	472A	O	...	3.42	*
U	1B	O3'	...	Pro	314A	N	...	3.45	*
			...	Asn	538A	ND2	...	2.71	***
			...	Asn	313A	O	...	3.18	***
U	1B	O2'	...	Asn	318A	OD1	...	3.53	*
			...	Asn	538A	N	...	2.99	***
			...	Asn	538A	ND2	...	2.85	***
			...	Ala	536A	O	...	2.75	***
U	1B	N1	...	Ala	536A	O	...	3.19	***
U	1B	N3	...	Ala	536A	O	...	3.57	*
U	1B	O2	...	Ala	536A	O	...	3.55	*
			...	His	312A	ND1	...	3.56	*
			...	Gln	506A	NE2	...	3.03	***
U	1B	OP2	...	Asn	318A	ND2	...	3.40	*

5′ vRNA

RNA base		Atom		Residue		Atom		Distance (Å)	
A	1C	OP2	...	Lys	423A	NZ	...	3.20	***
A	1C	O2'	...	Ala	593A	N	...	3.09	***
			...	Ala	593A	O	...	2.63	***
A	1C	N3	...	Cys	419A	O	...	3.51	*
A	1C	N1	...	Cys	419A	O	...	3.21	***
			...	Arg	595A	NE	...	2.95	***
G	2C	OP1	...	Arg	592A	NH1	...	2.69	***
G	2C	O2'	...	Arg	595A	N	...	2.65	***
			...	Arg	595A	O	...	2.78	***
G	2C	O4'	...	Ala	593A	O	...	3.14	***
G	2C	OP2	...	Lys	302A	NZ	...	3.34	*
U	3C	O3'	...	Arg	600A	NH2	...	3.02	***
			...	Thr	642A	OG1	...	3.16	***
U	3C	O4'	...	Arg	595A	O	...	3.48	*
A	4C	OP1	...	Arg	600A	NH2	...	2.82	***
			...	Thr	642A	OG1	...	3.00	***
			...	Lys	643A	N	...	2.96	***
A	4C	OP2	...	Thr	642A	OG1	...	3.13	***
A	4C	O2'	...	His	761A	ND1	...	2.68	***
G	5C	OP1	...	Lys	643A	NZ	...	2.86	***
G	5C	O5'	...	Tyr	677A	OH	...	3.14	***
G	5C	O4'	...	His	761A	NE2	...	3.49	*
G	5C	N7	...	Tyr	677A	OH	...	2.94	***
			...	Lys	679A	NZ	...	2.83	***
G	5C	N2	...	Ser	438A	O	...	3.53	*
G	5C	OP2	...	Tyr	677A	OH	...	2.72	***
U	6C	O5'	...	Arg	292A	NH2	...	3.37	*
U	6C	O4'	...	Gln	291A	O	...	3.23	***
U	6C	N1	...	Gln	291A	O	...	3.60	*
U	6C	OP2	...	Arg	292A	NH1	...	3.49	*
			...	Arg	292A	NH2	...	3.26	***
G	7C	N7	...	Tyr	1120A	OH	...	3.36	*
			...	Lys	768A	NZ	...	3.16	***
G	7C	N3	...	Gln	1116A	NE2	...	3.02	***
G	7C	N2	...	Gln	1116A	OE1	...	3.10	***
			...	Gln	1116A	NE2	...	3.44	*
			...	Asp	1123A	OD2	...	2.79	***
G	7C	N1	...	Asp	1123A	OD2	...	3.46	*
			...	Asp	1123A	OD1	...	3.22	***
G	7C	OP2	...	Tyr	1120A	OH	...	2.92	***
U	8C	OP1	...	His	761A	NE2	...	3.07	***
U	8C	N3	...	Val	1118A	O	...	2.55	***
U	8C	O2	...	Lys	1117A	N	...	3.01	***
			...	Val	1118A	N	...	3.00	***
			...	Val	1118A	O	...	3.26	***
U	8C	O4	...	Val	1118A	O	...	3.47	*
			...	Tyr	1120A	N	...	3.21	***
U	8C	OP2	...	His	760A	NE2	...	2.61	***
G	9C	O4'	...	His	760A	NE2	...	3.13	***

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