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Chapter 8

Arterivirus non-structural protein 1β co-operates with cellular poly (C) binding proteins to transactivate –2/–1 programmed ribosomal frameshifting

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ABSTRACT

Porcine reproductive and respiratory syndrome virus (PRRSV) utilizes programmed ribosomal frameshifting (PRF) to direct efficient expression of a transframe protein (nsp2TF) from an alternative reading frame overlapping the viral replicase gene. This arterivirus frameshifting signal induces both –2 and –1 PRF and, unusually, lacks an obvious stimulatory RNA structure downstream of the shift site. The minimal RNA sequence required for frameshifting maps to a 34-nucleotide region that includes the slippery sequence (GG_GUU_UUU) and a downstream conserved C-rich motif (CCCAUCUCC). Unusually, efficient frameshifting is also dependent upon expression of a viral protein, non-structural protein (nsp) 1β, one of the 14 subunits produced from the PRRSV replicase polyproteins. Nsp1β is released by the combined action of two papain-like protease (PLP) domains, which reside in nsp1α and nsp1β and each cleave at their own C-terminus. Here we show that, in addition to this viral transactivator, frameshifting also requires the participation of cellular poly (C) binding proteins (PCBPs), which were previously identified as nsp1β interaction partners. In vitro translation assays demonstrated that both nsp1β and either PCBP1 or PCBP2 are required for efficient –2/–1 PRF. When cells were depleted for PCBP1 and PCBP2 by siRNA-mediated knockdown and subsequently transfected with a plasmid expressing nsp1β and nsp2, we observed a ~40% reduction of the expression of nsp2TF and nsp2N, the respective –2 and –1 PRF products. PCBP1 predominantly stimulates –2 PRF, while PCBP2 stimulates –1 PRF. We hypothesize that a complex of nsp1β and PCBP binds to the RNA signal downstream of the slippery sequence and here mimics the action of the more typical RNA pseudoknot stimulators of PRF. This unprecedented viral frameshift-stimulatory signal may provide new insights as to how the ribosomal elongation cycle can be modified by transacting protein factors. Furthermore, it broadens the repertoire of activities associated with poly (C) binding proteins and prototypes a new class of arterivirus-host interactions.
INTRODUCTION

Programmed –1 ribosomal frameshifting (–1 PRF) is a commonly used translational control strategy in which mRNA signals induce ribosomes to change reading frame at high frequency, allowing the co-ordinated expression of two or more proteins from a single mRNA (reviewed in: [323, 336, 396, 433]). In –1 PRF, the ribosome slips backwards (in the 5’ direction) by one nucleotide (nt) into an overlapping open reading frame (ORF) and continues translation, generating a fusion protein composed of the products of both upstream and downstream ORFs. First described as the mechanism by which the Gag-Pol polyprotein of the retrovirus Rous sarcoma virus (RSV) is expressed from overlapping gag and pol ORFs [434, 435], related –1 PRF signals have been documented in many other viruses of clinical, veterinary and agricultural importance [436-440]. Programmed ribosomal frameshifting has also been increasingly recognized in the expression of conventional cellular genes of both prokaryotes and eukaryotes as well as in other replicating elements, such as insertion sequences and transposons [441, 481-484].

Central to almost all examples of –1 PRF is the interaction of the ribosome with a stimulatory mRNA structure - a stem-loop (SL) or RNA pseudoknot (PK) - which acts to promote the –1 frameshift on a stretch of homopolymeric bases known as the slippery sequence. How these RNA structures act is incompletely understood, but accumulating evidence supports the view that by presenting an unusual topology [92, 336, 337, 396, 446], they confound an intrinsic unwinding activity of the ribosome with consequent effects on the elongation cycle and frame maintenance [444, 445, 485]. Indeed, kinetic analyses indicate that stimulatory RNAs can impair movements of the ribosomal small subunit (30S) head, delaying dissociation of EF-G, and the release of tRNA from the ribosome [338, 486, 487].

Recently, we identified a novel, highly efficient –2/–1 PRF event that functions without a recognizable stimulatory RNA structure [442, 488]. This signal operates during translation of the genome of porcine reproductive and respiratory syndrome virus (PRRSV), a member of the arterivirus family in the order Nidovirales [442, 488]. The PRRSV genome (Figure 1A) is a ~15 kb positive-sense RNA molecule, and its 5’-proximal ORF (the large replicase ORF1a) in fact harbors two consecutive PRF signals. A “canonical” –1 PRF signal, conserved in all nidoviruses, is located in the short overlap region of ORF1a and the downstream replicase ORF1b [361]. It facilitates expression of the ORF1b-encoded sequence as a fusion with the ORF1a product and defines the ratio of the synthesis of the two viral replicase polyproteins, pp1a and pp1ab, produced by nidovirus genome translation. The second signal, which stimulates both –2 PRF and –1 PRF, is only found in (most) arterivirus genomes, where it is located within the region of ORF1a that encodes a large, multifunctional replicase subunit, nonstructural protein 2 (nsp2). Here, about 20% of ribosomes translating nsp2 frameshift into the –2 reading frame to generate a
Figure 1: (A) Overview of the PRRSV genome organization and localization of ribosomal frameshifting signals. The long 5′ ORFs 1a and 1b encode nonstructural polyproteins, and at least eight shorter 3′ ORFs (2a-7) encode structural proteins. The 3′ ORFs are translated from a nested set of subgenomic mRNAs, two of which are bicistronic. ORF1a and ORF1b are translated from the genomic RNA, with translation of ORF1b depending on −1 PRF at the end of ORF1a. The TF ORF overlaps the central ORF1a region in the −2 reading frame and is accessed via −2 PRF. A −1 frameshift at the same site generates the nsp2N product. The vertical red line indicates the location of the RG_GUU_UUU shift site (R = A or G, in different arteriviruses). Domains in nsp2/nsp2TF: C, Cys-rich domain; HVR, hypervariable region; PLP2, papain-like proteinase; TM/TM’, (putative) transmembrane domains. Below the genome organization, the sequence of the SD01-08 RNA in the region of the −2/−1 PRF signal is shown, with the slippery sequence (red) and C-rich motif (blue) highlighted. The −1 reading frame stop codon is underlined and codons for each of the reading frames are indicated. Figure reproduced from [488]. (B) PRRSV nsp1β sequence and structure. Amino acid sequence alignment of the PLP1β domains from selected arterivirus nsp1β proteins. Secondary structure elements (based on the published crystal structure from type 2 PRRSV isolate XH-GD) [453] are shown above the alignment and are color matched to the nsp1β structure shown below. Conserved basic residues in PLP1β helix α4 are boxed in orange. #, residues mutated in RBD- mutant. *, residue mutated in PR- mutant. The PRRSV sequence is numbered (black) from the nsp1α/nsp1β cleavage site, whereas the two other sequences are numbered (gray) starting from the N terminus of the pp1a polyprotein. The names of specific isolates used are indicated. GenBank accession nos. of sequences used are as follows: DQ489311 (PRRSV SD01-08), NC_001639 (LDV P), NC_003092 (SHFV LVR). In the cartoon representation of the nsp1β dimer structure, the N-terminal domains are colored purple, whereas the PLP1β domains and the C-terminal extensions (leading up to the nsp1β/nsp2 site cleaved by PLP1β) are colored green and red, respectively. Helix α4 of PLP1β, containing the conserved GKYLQRLQ motif, is colored orange with basic residues represented as sticks. Figure modified from [488].
transframe fusion protein (nsp2TF) comprising the N-terminal two-thirds of nsp2 and the product encoded by a conserved alternative ORF (transframe; TF) in the –2 reading frame. An estimated further 7% of ribosomes shift into the –1 reading frame where they immediately encounter a stop codon resulting in the synthesis of a truncated version of nsp2 termed nsp2N [442]. As depicted in Fig. 1A, the RNA downstream of the slippery sequence (GG_GUU_UUU) used for –2/–1 PRF in PRRSV does not harbor an obvious higher-order structure compatible with canonical RNA-structure-stimulated PRF. However, mutations within a conserved CCCAUUCUC motif located 11 nt downstream of the shift site reduce or inhibit frameshifting, consistent with the presence of a 3’ stimulatory element of some form [442]. A further novelty of the PRRSV –2/–1 PRF signal is a requirement for the presence of viral protein, nsp1β, which functions as a transactivator of both –2 and –1 PRF [488]. The 205-amino acid nsp1β contains a papain-like protease domain (PLP1β) that cleaves the nsp1β/nsp2 junction in the arterivirus nonstructural polyprotein [363]. How nsp1β acts to stimulate frameshifting is unclear, although basic residues in a highly conserved putative RNA-binding motif (GKYLQRRLQ) (Figure 1B), integrated into the structure of nsp1β’s papain-like autoprotease, were found to be critical for the stimulation of frameshifting [488].

In PRRSV-infected cells, in addition to its role in PRF control and the PLP1β–mediated cleavage of the nsp1β-nsp2 junction, nsp1β has also been implicated in arteriviral innate immune evasion (reviewed by [361, 489]). Part of nsp1β can be detected in the nucleus of infected cells and ectopic expression of nsp1β can suppress the induction of innate immune responses [452, 490, 491], possibly by inhibiting downstream interferon-induced signaling pathways [452, 478]. The highly conserved nsp1β motif that was implicated in the control of PRF was also linked to innate immune evasion [447], as mutagenesis of its conserved basic residues yielded viable recombinant viruses inducing increased expression of IFN-α, IFN-β, and ISG15. Whether this effect should be attributed directly to nsp1β’s role in mediating the expression of nsp2TF/nsp2N or may (in part) be linked to the protein’s other functions remains to be investigated in more detail.

We previously determined the –2 and –1 PRF frameshifting efficiencies in different cell culture settings. In PRRSV SD01-08-infected MARC-145 cells the –2 PRF efficiency was around 20%, while in RK13 cells transiently expressing PRRSV ORF1a a much higher –2 PRF efficiency of about 50% was observed [442]. These large differences led us to hypothesize that frameshifting may be modulated by host factors expressed at different levels in these cells. Given its involvement in PRF outlined above, cellular proteins interacting with nsp1β were obvious candidates for such a role. During a previous study that used GST-tagged nsp1β as bait and aimed to discover cellular factors involved in PRRSV replication, a number of nsp1β interaction partners was identified, including the poly(C) binding proteins 1 and 2 (PCBP1 and PCBP2) [463]. PCBP1 and PCBP2 belong to a family of proteins that is characterized by three nucleic acid-binding hnRNP K
homology (KH) domains. The KH1 and KH2 domains are grouped near the N-terminus and KH3 is located at the C-terminus, separated from KH1 and KH2 by a sequence of variable length [492]. The other members of this family are PCBP3, PCBP4 and hnRNPK [492]. Yeast two-hybrid binding assays revealed that the interaction between nsp1β and PCBP2 requires minimally the PLP1β and C-terminal extension (CTE) domains of nsp1β and the KH2 domain of PCBP2 [493]. Interestingly, the conserved C-rich motif required for –2/–1 PRF in PRRSV shares some similarities with previously established PCBP-binding consensus sequences, although these sequences commonly have three (or more) C-triplets, each potentially binding one KH domain. Thus, PCBPs could bind to the PRRSV mRNA either directly or indirectly, by virtue of an association with nsp1β. In PRRSV-infected cells, overexpressed recombinant PCBP1 and PCBP2 were reported to partially localize to the region of the cell in which viral RNA synthesis is presumed to occur [463]. Endogenous PCBP2 was shown to be predominantly present in the nucleus of mock-infected cells, but to translocate to the cytoplasm in PRRSV-infected cells, after which it partially co-localized with nsp1β [493]. Furthermore, recombinant PCBPs were found to bind RNA transcripts representing the 5’ untranslated region (5’-UTR) of the PRRSV genome [463]. Finally, siRNA-mediated knockdown of the two PCBPs moderately inhibited PRRSV replication. In these PCBP-depleted cells, viral genome translation was claimed to be not affected, but this was concluded on the basis of measuring nsp1α and nsp1β expression levels only (the occurrence of –2 and –1 PRF in the nsp2-coding region was unknown at the time; [463]). In another study in which PCBP2 was depleted, both viral replication and nsp1β expression were decreased during PRRSV infection [493]. Beura et al. postulated that PCBP1 and PCBP2 are important host cell factors for PRRSV RNA synthesis [463], but our subsequent discovery of nsp1β-mediated PRF, at a signal containing a conserved poly(C)-containing RNA motif, prompted us to revisit this hypothesis. If indeed PCBP1 and PCBP2 are interaction partners of nsp1β and have affinity for poly(C)-containing RNA sequences, their presence or absence may (also) affect the occurrence of –2/–1 PRF. As previously described, the inactivation of –2/–1 PRF in itself suffices to severely cripple PRRSV replication [442, 488], a defect that should also be reflected in viral RNA accumulation levels, but is in fact determined at the translational level.

In this paper, through reconstitution of the PRRSV –2/–1 PRF mechanism in vitro and studying the PRF-specific effects of PCBP knockdown in an expression system, we have established that efficient frameshifting indeed depends on the presence of cellular PCBPs, and that their presence is essentially equally important as that of the viral transactivator nsp1β. Through in vitro translation assays, we demonstrate that the combined presence of nsp1β and PCBP1 or PCBP2 mimics the action of the more typical RNA pseudoknot stimulators of programmed frameshifting. PCBP depletion in a cell culture-based expression system resulted in a significant reduction of nsp2TF and nsp2N expression.
This unprecedented arteriviral frameshift-stimulatory signal may provide new insights as to how the ribosomal elongation cycle can be modified by transacting protein factors. Furthermore, our study confirms and extends the important role of PCBPs as pro-viral host factors during PRRSV replication and prototypes a new class of arterivirus-host interactions.

MATERIALS AND METHODS

Viruses and cells
MARC-145 and RK-13 cells were cultured in DMEM (Lonza) containing 8% fetal calf serum (FCS; PAA), 100 IU/ml penicillin and 100 μg/ml streptomycin at 37 °C and 5% CO₂. Recombinant vaccinia virus vTF7-3 [429] was propagated in RK-13 cells.

Protein expression and purification
Full-length, N-terminally hexa-histidine tagged nsp1β, PCBP1 and PCBP2 were expressed in Escherichia coli Rosetta2(DE3)pLysS (Novagen). Bacteria were grown in 2× TY medium to an A₆₀₀ of 0.8 at 37 °C and cooled to 22 °C, and protein expression induced by the addition of 0.2 mM isopropyl β-d-thiogalactopyranoside. After 16 h, cells were harvested by centrifugation at 5000 × g for 15 min at 4 °C, and the pellet was stored at −20 °C until required.

Cells were thawed and resuspended in 20 mM Tris, 500 mM NaCl, 30 mM imidazole, 1.4 mM β-mercaptoethanol, 0.05% Tween 20, pH 7.5, supplemented with 400 units of bovine DNase I (Sigma-Aldrich) and 200 μl of EDTA-free protease inhibitor mixture (Sigma-Aldrich) before lysis at 165.5 MPa using a TS series cell disruptor (Constant Systems) and centrifugation at 40,000 × g for 30 min at 4 °C. Cleared lysate was incubated with Ni²⁺-NTA-agarose (Qiagen) for 1 h at 4 °C, the beads were washed, and the bound protein eluted in 20 mM Tris, 500 mM NaCl, 250 mM imidazole, pH 7.5, before dialysis against 20 mM Tris, pH 7.6, 200 mM NaCl, 2 mM DTT. Purified proteins were concentrated, snap-frozen in liquid nitrogen, and stored at −80 °C until required.

Frameshift reporter plasmids and in vitro translation
Dual luciferase reporter plasmids were prepared by inserting a 79-nt sequence from PRRSV isolate SD01-08 containing the GG_GUU_UUU shift site, 5 upstream nucleotides and 66 downstream nucleotides between the Renilla and firefly luciferase genes in pDluc [448, 449] to create pDluc-PRRSV/wt so that −2PRF is required for firefly luciferase expression. To create pDuc-PRRSV/stop, the stop codon (UGA) in the −1 frame was changed to UUA, extending the −1 reading frame by a further 56 codons, whilst terminating the Rluc gene slightly earlier. In vitro transcribed RNA from the pDluc constructs was translated in
rabbit reticulocyte lysates (RRL) and wheat germ extracts (WG) as previously described [407] with or without recombinant nsp1β, PCBP1 and PCBP2 present. Translation products were separated by SDS-PAGE and visualized by WB using an antibody recognizing Renilla luciferase which is common to all pDluc translation products.

**siRNA-mediated knockdown**

To determine frameshifting efficiencies after siRNA-mediated knockdown of PCBP expression, 6x10⁴ MARC-145 or 4.8x10⁴ RK-13 cells were seeded per well in 12-well clusters in DMEM containing 8% FCS. MARC-145 cultures were transfected with siGENOME Human siRNA SMARTpools targeting PCBP1, PCBP2, PCBP3, PCBP4, or hnRNPK (final concentration 10 nM) using 2 ul of Dharmafect1 lipofection reagent (Dharmacon) per well. RK-13 cultures were transfected with siRNA pools targeting PCBP1 or PCBP2 (final concentration 25 nM) using 3 ul Lipofectamine 2000 (LifeTechnologies) per well. A non-targeting pool (NTP) of “scrambled” siRNAs (Dharmacon) was used as a negative control. At 24 h post transfection (p.t.), the transfection medium was replaced with DMEM containing 8% FCS. Possible cytotoxic effects of siRNA transfection were monitored at 48 h p.t., using the CellTiter 96® AQueous Non-Radioactive Cell Proliferation Assay (Promega). After 120 min, the reaction was stopped by the addition of 25 µl of 10% SDS and absorbance at 490 nm (A490) was measured using a 96-well plate reader (Berthold). At 48 h p.t., the cells were infected with a T7 RNA polymerase-expressing recombinant vaccinia virus [429] and 1 h later transfected with plasmid pL-nsp1β-2 [363], which encodes a self-cleaving nsp1β-nsp2 polyprotein under the control of a T7 promoter. Four hours later, the cells were starved for 30 min in methionine- and cysteine-free DMEM (Gibco) containing 2% FCS prior to a 45- (MARC-145) or 30-min (RK-13) metabolic labeling with 500 µCi/mL of a [35S]Met/Cys mixture (EXPRE 35S35S Protein Labeling Mix; Perkin-Elmer). Subsequently, cells were washed twice with PBS and harvested in lysis buffer (20 mM Tris, pH 7.6, 150 mM NaCl, 1% v/v NP-40, 0.1% DOC, 0.1% SDS and Complete protease inhibitor (Roche)).

The human sequences targeted by the siRNA pools were compared with the sequences of the African green monkey (Chlorocebus sabaueus) genes encoding PCBP1 (Genbank accession XM_007970341), PCBP2 (Genbank accession XM_008003436), PCBP3 (Genbank accession XM_007970531), PCBP4 (Genbank accession XM_007984435) and hnRNPK (Genbank accession XM_007969613) and the rabbit (Oryctolagus cuniculus) PCBP1 and PCBP2 genes (Genbank accession NM_001082124 and XM_002711018, respectively).

**Immunoassays**

Following siRNA pool transfection, MARC-145 and RK-13 cells were harvested at 48 h p.t. or 72 h p.t./24 h p.i. by first washing with PBS and then lysing in 4× Laemmlli sample buffer (100 mM Tris-HCl, pH 6.8, 40% glycerol, 8% SDS, 40 mM DTT, 0.04 mg/
ml bromophenol blue). Proteins were visualized by Western blot analysis as described previously [208] using primary antibodies mAb-nsp2 Eu58-46, mAb-nsp1beta Eu22-28 [403], rabbit polyclonal anti-hnRNP E1 (C-terminal) (PCBP1) (Sigma-Aldrich), mouse monoclonal anti-PCBP2 (M07) (Abgent), rabbit polyclonal anti-human MGC10 (PCBP4) (PromoKine), mouse monoclonal anti-hnRNPK (Abcam), or mouse monoclonal antibody H68.4 against the transferrin receptor (Invitrogen) diluted in PBST containing 1% casein. Biotin-conjugated swine-a-rabbit (DAKO) or goat-a-mouse (DAKO), and Cy3-conjugated mouse-a-biotin (Jackson, Pennsylvania, USA) diluted in PBST containing 0.5% casein were used for fluorescent detection with a Typhoon-9410 imager (GE Healthcare, UK).

Lysates from [35S]Met/Cys-labelled cells were used to immunoprecipitate nsp2, nsp2TF and nsp2N using mAb-nsp2 Eu58-46 [403], which recognizes the common N-terminal domain of the three products. Proteins were separated on a 6% SDS-PAGE gel and imaged as described previously [403]. Band intensities of nsp2, nsp2′, nsp2TF, nsp2TF′, nsp2N and nsp2N′ (nsp2′, nsp2TF′ and nsp2N′ are faster migrating forms of the three products; [442]) were quantified using ImageQuant TL (GE Healthcare) and normalized using the Met+Cys content of the respective products, while assuming that [35S]Met and [35S]Cys are incorporated with an efficiency ratio of 73:22 (the Met:Cys ratio in the mixture according to the manufacturer’s documentation). Using these values, –2 PRF efficiencies were calculated as (nsp2TF + nsp2TF′)/(nsp2 + nsp2′ + nsp2TF + nsp2TF′ + nsp2N + nsp2N′) and –1 PRF efficiencies were calculated as (nsp2N + nsp2N′)/(nsp2 + nsp2′ + nsp2TF + nsp2TF′ + nsp2N + nsp2N′). Quantification was performed in triplicate.

RESULTS

Reconstitution of the PRRSV –2/–1 PRF signal in vitro

Transactivation of the PRRSV –2/–1 PRF signal by nsp1β was previously demonstrated by co-expression of nsp1β and nsp2 in cultured cells and by site-directed mutagenesis of the viral genome to inactivate the critical RNA and protein signals described above [488]. To study the phenomenon in vitro, a 79-bp cDNA fragment encompassing the slippery sequence and C-rich region was subcloned between the Renilla and firefly genes of the frameshift-reporter plasmid pDluc [448] such that expression of the downstream cistron (fluc) was dependent upon a –2 PRF within the inserted PRRSV sequences (Figure 2A). Upon in vitro translation in the rabbit reticulocyte system (RRL), mRNAs transcribed from pDluc/PRRSV/wt specified the synthesis of only the product of the 5′ cistron of the reporter mRNA (Rluc) and no frameshifting was evident (Figure 2B). However, in translation reactions supplemented with recombinant, purified His6-tagged nsp1β, two additional bands were observed, the most abundant corresponding to the product of –2 ribosomal frameshifting. The second band migrated more rapidly than that of the
The previously reported crystal structure of nsp1β reveals an overall elliptical structure consisting of six α-helices and seven β-strands [453]. The protein is composed of two
major domains, a 48-amino acid N-terminal domain (NTD), which adopts an architecture similar to that of several metal ion-dependent nucleases, and a C-terminal papain-like cysteine protease (PLP1β) domain [453]. Within the latter domain, we previously identified a conserved sequence motif GK_{124}YLQR_{128}RLQ as a potential RNA binding domain (RBD) (Figure 1B). This sequence forms one of three α helices in the region between active site C_{96} and H_{165} residues of PLP1β [488]. To investigate the importance of protease or RNA binding activities to PRF transactivation, amino acid substitutions were introduced into the protease active site (C96S; mutant PR-) or within the putative nsp1β RBD (K124A/R128A; mutant RBD-, previously described as mutant 1βKO [488]) of the recombinant His_{6}-tagged nsp1β used to transactivate frameshifting in vitro (Figure 1B). These variants were expressed and tested in the RRL-based PRF assay (Figure 2C). In this experiment, the protease-defective variant retained full activity, ruling out the involvement of PLP1β’s protease activity in the stimulation of PRF by nsp1β. The RBD- mutation, on the other hand, inactivated nsp1β’s potential to induce frameshifting, supporting a role for RNA binding by nsp1β.

A requirement for poly (C) binding proteins in PRRSV –2/–1 PRF in vitro

To explore the potential role of PCBPs in PRRSV PRF, we translated the pDLuc/PRRSV/wt mRNA in RRL reactions supplemented with nsp1β, His_{6}-tagged PCBP2 or both proteins (Figure 3A). We found that PCBP2 alone did not stimulate PRF, but when added together with nsp1β, a substantial increase in the synthesis of the –1 PRF product was observed. To account for this observation, we reasoned that if the “active” transacting stimulator of PRRSV PRF is indeed a complex of nsp1β and PCBPs, then the RRL system must already contain an abundant form of PCBPs, but one which would, in complex with nsp1β, preferentially lead to –2 PRF. In this scenario, supplementation with PCBP2 may have generated nsp1β-PCBP2 complexes that could preferentially promote –1 PRF. We therefore translated the PRRSV frameshift reporter mRNA in the wheat germ (WG) in vitro translation system in the hope that this lysate would contain fewer endogenous PCBPs, or PCBPs of sufficient evolutionary diversity to preclude any interactions with nsp1β. Consistent with this expectation, ribosomal frameshifting at the PRRSV signal in WG was found to be completely dependent upon the simultaneous addition of both nsp1β and PCBP2, with neither protein alone having any frameshift-stimulatory activity in this system (Figure 3B). In further support of the hypothesis above, supplementation of WG translations with PCBP2 led preferentially to a –1 PRF, whereas with PCPB1, the –2 PRF was most evident (Figure 4), suggesting that the abundant form in RRL is PCBP1.
Figure 3: PCBP1 and PCBP2 can reconstitute –2/–1 PRF in WG. In vitro translation reactions with RRL or WG and in vitro transcribed PRRSV/wt RNA were supplemented with nsp1β and his-tagged PCBP1 or his-tagged PCBP2 or both PCBP1 and PCBP2.

Figure 4: PCBP1 and PCBP2 can reconstitute –2/–1 PRF in WG. In vitro translation reactions with RRL or WG and in vitro transcribed PRRSV/wt RNA were supplemented with nsp1β and his-tagged PCBP1 or his-tagged PCBP2 or both PCBP1 and PCBP2.
Knockdown of poly (C) binding proteins reduces frameshifting efficiency in cultured cells

To explore the potential involvement of PCBPs on –2/–1PRF in living cells, siRNA-mediated knockdown of PCBP1, PCBP2, PCBP3, PCBP4, and hnRNPK in MARC-145 cells was combined with transient expression of nsp1β and nsp2 using the recombinant vaccinia virus/T7 polymerase expression system (Figure 5ABC). For PCBP1 and PCBP2 the findings from MARC-145 cells were further confirmed in RK-13 cells (Figure 5DEF).

The siRNA pools that were used target human sequences, but most of these pools are expected to also mediate knockdown in MARC-145 (African green monkey) and RK-13 (rabbit) cells. For example, the four sequences targeted by the siRNA pool against PCBP1 are fully conserved in African green monkey, and only one of these has a 1-nt mismatch with the rabbit PCBP1 sequence. Likewise, only one of the four sequences targeted by the siRNA pool against PCBP2 has a 1-nt mismatch in African green monkey, while two of these sequences have a 1-nt mismatch in rabbit. The four sequences targeted by the siRNA pools for PCBP3 and hnRNPK are fully conserved in African green monkey, but one of the sequences targeted by the siRNA pool against PCBP4 contains a 1-nt mismatch and one sequence contains three 1-nt mismatches. In MARC-145 cells, knockdown of PCBP1, PCBP2, PCBP4 and hnRNPK was successful, as established by Western blot analysis (Figure 5A). Unfortunately, a suitable antibody to monitor PCBP3 knockdown was not available. Also in RK-13 cells knockdown of PCBP1 and PCBP2 was successful (Figure 5D). When assessed via an MTS-based cell viability assay, knockdown of none of these targets was toxic to uninfected cells. However, knockdown of PCBP3 in MARC-145 cells resulted in accelerated cell death during infection with recombinant vaccinia virus vT7-3, resulting in lower band intensities for the three nsp2 products upon immunoprecipitation compared to the other conditions (Figure 5B).

In line with the observations made during the in vitro translation experiments outlined above (Figure 4), PCBP1 knockdown mostly reduced the efficiency of the –2 shift, while PCBP2 knockdown mostly impaired the –1 shift (Figure 5C and F). Whereas knockdown of a single PCBP reduced –1 or –2 PRF by about 40%, simultaneous knockdown of PCBP1 and PCBP2 reduced the total amount of –2/–1 PRF by ~50% in MARC-145 cells (Figure 5C) and ~70% in RK13 cells (Figure 5F). In multiple independent experiments, PCBP1 depletion, while decreasing –2 PRF, at the same time increased the –1 PRF efficiency in RK-13 cells, but not in MARC-145 cells. Interestingly, PCBP2 knockdown also induced knockdown of PCBP1 in RK-13 cells (Fig. 5D) and as a result the –2 shift was also reduced in these cells when only siRNAs targeting PCBP2 were used (Figure 5F). This might be caused by siRNA off-target effects because the PCBP1 and PCBP2 sequences are very similar and one of the siRNAs targeting PCBP2 has only a single mismatch with rabbit PCBP1, while there are two mismatches with African green monkey PCBP1. The mismatch is located at position 14 of the siRNA and siRNAs with a mismatch at this posi-
Figure 5: siRNA knockdown of PCBP1 and PCBP2 decreases –2/–1 frameshifting efficiency in cell culture. (A & D) MARC-145 (A) and RK-13 cells (D) were transfected with siRNA pools targeting PCBP1, PCBP2, PCBP3, PCBP4, hnRNPK. Knockdown of proteins targeted by siRNAs was assessed using WB analysis. A suitable antibody for PCBP3 was not available. (B & E) MARC-145 (B) and RK-13 cells (E) were transfected with siRNA pools targeting PCBP1, PCBP2, PCBP3, PCBP4, hnRNPK. The recombinant vaccinia virus–T7 RNA polymerase expression system was used to express pLnsplβ-2. After metabolic labeling, expression products were immunoprecipitated with mAb α-EU-nsp2, this antibody recognizes the common N-terminal domain of nsp2, nsp2TF, and nsp2N. Immunoprecipitated proteins were separated by SDS/PAGE and visualized by autoradiography. (C & F) Protein bands were quantified (triplicates) using ImageQuant TL software for MARC-145 cells (C) and RK-13 cells (F). The total intensity for all protein bands (nsp2, nsp2', nsp2TF, nsp2TF', nsp2N and nsp2N') was set as 100%. Efficiencies were corrected for the methionine and cysteine content of each protein product. nsp2', nsp2TF' and nsp2N' are precursors.
tion are often still able to induce knockdown [494]. Knockdown of PCBP3, PCBP4 and hnRNPK in MARC-145 cells increased PCBP1 expression, most likely due to some kind of a compensatory mechanism at the level of genome expression (Figure 5A). Knockdown of PCBP3, PCBP4 and hnRNPK had little or no effect on frameshifting (Figure 5BC), but such effects may have been masked by the increased PCBP1 levels in these cells (Figure 5A). If this is the case, these proteins are more likely to affect –2 PRF than –1 PRF.

DISCUSSION

In this paper we describe the discovery that –2/–1 PRF in arteriviruses does not only depend on the presence of viral protein nsp1β, but both frameshifts also require PCBPs as additional, host cell-encoded protein transactivators.

RNA-protein complex

In mammalian cells, two PCBP subsets have been described, hnRNPs K/J [495] and the αCP proteins αCP1 and αCP2, commonly referred to as PCBP1 and PCBP2 [496, 497]. The latter group also includes the more recently described isoforms PCBP3 and PCBP4 [498]. Being members of the KH domain superfamily of nucleic acid binding proteins, PCBPs have been implicated in a wide spectrum of biological activities, including the regulation of RNA splicing, the stabilization of cellular and viral mRNAs, transcriptional activation and inhibition, and translational silencing and enhancement (reviewed in: [492, 499]. The PCBPs are ubiquitously expressed across many tissues, but the level of expression and isoform(s) predominantly expressed varies per cell type [496, 500-502]. This probably explains why we observed different frameshifting efficiencies when expressing pLnsp1β-2 in MARC-145 and RK-13 cells. Likewise, the levels of PCBP1 and PCBP2 in a PRRSV-infected cell will most likely determine the relative efficiencies of the two PRF events, which must to a certain extent be connected because they are mutually exclusive translational events. In the siRNA-based knockdown experiments, PCBP1 depletion mainly reduced –2 PRF, while PCBP2 knockdown mainly affected –1 PRF. In the in vitro translation assays PCBP1 and PCBP2 were both able to induce –2 as well as –1 PRF, although PCBP1 was concluded to be more efficient at promoting –2 PRF, whereas PCBP2 more efficiently induced –1 PRF. In an artificial experimental system in which alternative shifts at a single slippery sequence could be observed, the spacing between slippery sequence and downstream stimulatory RNA structure was found to determine whether –1 or –2 PRF was more prominent. The optimal distance for –2 PRF was 1-2 nt shorter than the optimal distance for –1 PRF [407]. This suggests that the RNA-protein complexes containing PCBP1 might have a slightly different orientation or conformation than those containing PCBP2, resulting in more frequent induction of –2 PRF. For –1
PRF, it has been shown that roadblocks, such as RNA pseudoknots downstream of the slippery site, stall ribosomes in a metastable conformational state. Slippage into the –1 frame accelerates completion of translocation [337, 338, 486, 487, 503]. The complexes containing PCBP1 might induce more tension on a paused ribosome, thus favoring slippage into the –2 rather than the –1 frame to allow completion of translocation. The exact composition of the RNA-protein complex will likely have to be determined by structural biology techniques to understand exactly how the proteins interact with each other and the viral RNA and whether the complexes formed by PCBP1 have a different conformation than the ones formed by PCBP2. Another explanation for the different effects that PCBP1 and PCBP2 have on inducing PRF would be that the affinity of the KH domains of PCBP1 for the C-rich motif is different than the affinity of those present in PCBP2, which could also result in variable degrees of tension on the ribosome. We postulate that PCBPs interact with the C-rich motif through KH1 and KH3 and that KH2 interacts with nsp1β since it has previously been shown that this domain is required for nsp1β binding [504]. For PCBP1 it has been determined that for optimal binding to its KH1 domain, cytosine is preferred in all four positions in the oligonucleotide binding cleft and that a C-tetrad binds KH1 with 10 times higher affinity than a C-triplet [505]. The PRRSV C-rich motif, the evolution of which may have been restricted by its presence within overlapping ORFs, only contains two sets of three consecutive cytosines and this probably does not result in a high enough affinity of PCBPs for the RNA to induce frameshifting on their own, but requires the additional interaction with nsp1β.

Potential interaction of other PCBP family members

The involvement of PCBP1 and PCBP2 as protein transactivators of PRF is clear, but for the other members of the PCBP family their involvement is still uncertain. The study of separate PCBPs in living cells was complicated by an apparently compensatory mechanism that resulted in an increase in PCBP1 levels when PCBP3, PCBP4 or hnRNPK were knocked down in MARC-145 cells. It seems likely that PCBP3, PCBP4 and hnRNPK are also capable of stimulating frameshifting, but additional studies are required to determine their potential role in more detail. Knockdown of these targets in combination with knockdown of PCBP1 could perhaps clarify their involvement. For PCBP3 we will, however, need to use another system since knockdown of this factor appeared to influence vaccinia virus replication resulting in accelerated cell death. Supplementation of the WG in vitro translation assay with these proteins could also help to assess their (potential) involvement.

The involvement of PCBPs in frameshifting in infected cells

Experiments to determine the impact of PCBP knockdown on –2/–1 PRF in PRRSV-infected cells are currently ongoing. However, combining efficient siRNA-mediated...
knockdown with a single-cycle PRRSV infection is a technical challenge. During the primary phase of PRRSV infection in MARC-145 cells only a subset of cells is permissive to infection, even when an amount of virus is used that should result in a high MOI. The majority of the cells become infected in subsequent rounds of infection, by cell-to-cell transmission to clusters of neighboring cells [506]. We hope to be able to overcome this issue by efficient transfection of \textit{in vitro} transcribed, full-length viral RNA into siRNA-treated cells. An additional complicating factor is the reported possible interaction of PCBP1 and PCBP2 with sequences in the 5'-UTR of the PRRSV genome, which might influence viral replication and transcription, even though this interaction did not appear to be very strong and remains to be corroborated in infected cells [463]. Beura \textit{et al}, hypothesized that PCBP1 and PCBP2 play a role in regulating PRRSV RNA synthesis because knockdown of both proteins resulted in a reduction of genomic and subgenomic RNA synthesis [463]. However, this effect might also result from the reduced expression of nsp2TF and nsp2N when PCBP1 and PCBP2 are depleted. We previously showed that PRRSV mutants lacking a functional –2/–1 PRF mechanism are seriously crippled in their replication [442, 488]. The siRNA-mediated depletion of PCBP1 and PCBP2 was reported to result in a \~{}0.5-1 log reduction in viral progeny titers [463, 493] which, for example, is slightly less than what was observed with a mutant virus (KO2) in which \~{}2/-1 PRF is completely knocked out by mutating the RNA signals involved (1.5 log reduction) [442]. This difference may be explained by the incomplete depletion of PCBPs when performing siRNA-mediated knockdown. In our transient expression system, we were able to reduce the total amount of frameshifting by only \~{}50-70\% when PCBP1 and PCBP2 were depleted simultaneously. The small amount of PCBP1 and PCBP2 that remains present in the cell after knockdown, or the presence of PCBP3, PCBP4 and hnRNPK if these proteins are also capable of transactivating PRF, most likely still suffices to induce a reasonable level of frameshifting and, consequently, expression of nsp2TF and nsp2N during PRRSV infection. We may be able to distinguish between the role PCBPs play in PRF transactivation and additional functions during PRRSV infection by using this KO2 mutant virus. During a KO2 infection, any effects of PCBP knockdown on viral RNA or protein synthesis should be explained as an involvement of PCBPs in other processes than PRF transactivation, since \~{}2/–1 PRF is already completely knocked out in this mutant virus.

There have been some reports that PCBP1 and PCBP2 co-localize with nsps during PRRSV infection. Overexpressed recombinant PCBP1 and PCBP2 were reported to partially co-localize with nsp1β and nsp2/3 during PRRSV infection [463] and endogenous PCBP2 was shown to translocate from the nucleus to the cytosol upon PRRSV infection where it co-localized with nsp1β [493]. A proportion of nsp1β localizes to the nucleus during infection [403] and we hypothesize that PCBP1 and PCBP2 are recruited to the cytosol through their interaction with nsp1β. If this were true, most of the PCBPs that remain after siRNA-mediated knockdown might still be recruited to the site of viral
genome translation and stimulate frameshifting. This would explain the relatively high level of frameshifting observed even after efficient siRNA-mediated knockdown of PCBPs. It will be interesting to determine whether PCBP1 and PCBP2 are still recruited to the cytosol during infection with the virus mutant containing the nsp1β RBD- mutations, since this mutant is no longer able to transactivate –2/–1 PRF. This could be because it can no longer interact with the PRRSV RNA, but might also be because its interaction with PCBPs is abolished.

**Binding partners of nsp1β and PCBPs**

It might be possible that other binding partners of either nsp1β or PCBPs are also part of the frameshift stimulatory complex that presumably interacts with the translating ribosome to induce frameshifting. Nsp1β has been reported to interact with rpS14 [463], which is located adjacent to rpS3 of the ribosomal helicase [466]. Cytosolic PCBP1 has been shown to interact with RACK1 [467], a protein that interacts with the head region of the 40S ribosome close to the mRNA exit channel [507]. It was recently shown that ribosomes lacking Asc1, a yeast homolog of RACK1, frameshift more often at CGA codon repeats [508], suggesting that an interaction of PCBP1 with RACK1 could potentially modulate frameshifting frequency on the PRRSV mRNA as well.

**PCBP interactions with viral genomes**

The interaction of PCBPs with the PRRSV (C-rich) mRNA is not unique, as interactions with other viral RNAs have been described previously and such interactions often modulate viral protein translation and RNA replication. PCBP1 and PCBP2 interact with the terminal clover leaf structure of the poliovirus (PV) genome and that of other picornaviruses, forming a ribonucleoprotein complex with viral protein 3CD [509-511]. This interaction promotes mRNA stability by protecting it from degradation by 5’ exo- nucleases [512, 513]. The interaction of PCBPs with the clover leaf structure also plays a role in circularization of the viral genome through an RNA-protein-protein-RNA bridge that is required for the initiation of negative strand RNA synthesis [514]. PCPB2 also interacts with stem-loop IV of the type 1 picornavirus IRES to mediate translation initiation [515, 516]. During mid-to-late phase of PV infection, PCBP1 and PCBP2 are cleaved by viral proteinases 3C/3CD resulting in a truncated protein lacking the KH3 domain [517]. The same has been reported for PCBP2 and the 3C protease of hepatitis A virus, an atypical picornavirus [518]. Cleaved PCBP2 can no longer function in translation initiation but retains its activity in viral RNA replication. It was suggested that this cleavage mediates the shift from viral translation to RNA replication [517, 518].

Binding of PCBPs to 5’ UTRs has also been reported for hepatitis C virus (HCV) and Norwalk virus [519, 520]. For HCV, PCBP2 is also required for circularization of the viral
genome [521]. Finally, PCBP1 has been reported to interact with N\textsuperscript{pro}, a cysteine-like autoprotease, of classical swine fever (CSFV) and promotes virus growth [522].

**Protein-dependent recoding**

This is the first time that cellular proteins have been shown to stimulate PRF. Previously, Annexin A2 was shown to inhibit –1 PRF in the coronavirus infectious bronchitis virus by destabilizing the stimulatory pseudoknot [457]. In a very artificial system the –1 HIV-1 PRF signal could be stimulated by replacing the RNA structure with the iron-responsive-element (IRE) from ferritin mRNA. Binding of iron regulatory proteins to the IRE did increase frameshifting efficiency about 2-3 fold, but efficiency was still less than 10% [461]. While this was a very unnatural example, it did show that RNA-protein interactions can stimulate frameshifting.

Only one other example of protein-dependent recoding where a protein interacts directly with the mRNA has been described. The vascular endothelial growth factor A (VEGFA) mRNA undergoes stop codon readthrough which generates an isoform containing a unique 22-amino acid C terminus extension called VEGF-Ax. This readthrough is transactivated by a high affinity interaction of hnRNPA2/B1 with a near-consensus hnRNPA2/B1 recognition element (A2RE) in a 63-nt long Ax element located in between the two stop codons in the VEGFA 3’ UTR RNA sequence. The A2RE starts 11 nt downstream from the stop codon and the authors suggest that bound hnRNPA2/B1 interacts with ribosomal proteins to induce translational pausing allowing the incorporation of serine at the stop codon position [523]. Like hnRNPK, hnRNPA2/B1 belongs to the heterogeneous ribonucleoproteins, but RNA recognition of hnRNPA2/B1 goes through two RNA recognition motifs (RRMs) and an arginine/glycine-rich (RGG) box instead of KH domains [524].

Over 1500 RNA binding proteins have been described [525] and it does seem likely that more of these proteins might interact with RNA sequences downstream from slippery sequences forming RNA-protein complexes that could potentially block translating ribosomes.

**CONCLUSION**

The requirement of a host protein in the RNA-protein complex that induces ribosomal frameshifting in this, so far, unique arterivirus mechanism was a surprise. This mechanism thus constitutes a novel type of arterivirus-host interaction and shows that protein complexes can replace RNA structures to stimulate frameshifting with high efficiency. It raises the question whether protein-mediated frameshifting might be more common, since many different RNA binding proteins that interact with RNA through various RNA
binding domains could potentially interact with RNA motifs downstream of slippery sequences to induce shifting on their own (or as a protein-complex) or interact with (viral) RNA structures to modulate the shifting frequency.

The WG in vitro translation assay allowed us to study the effects of the protein factors of the PRRSV mechanism separately. For other eukaryotic frameshifting sequences this might also be a good system to test whether these are also modulated by cellular or viral protein factors. This unique frameshift-stimulatory signal may also provide novel insights into how transacting protein factors can modify the ribosomal elongation cycle.

The siRNA-mediated knockdown experiments clearly showed that the expression level of individual PCBPs determines the direction and efficiency of frameshifting in cells. PCBP1 predominantly stimulated −2 PRF and PCBP2 stimulated −1 PRF, but other PCBP family members may be able to stimulate −2/−1 PRF as well. This study adds yet another function to the already broad repertoire of cellular PCBP activities.

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Conflict of interest statement: The authors have filed a patent application that relates to some aspects of this work.