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The NS5A protein of bovine viral diarrhoea virus interacts with the α subunit of translation elongation factor-1

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A cellular protein that interacts with the NS5A polypeptide of bovine viral diarrhoea virus (BVDV) was identified in a yeast two-hybrid screen. The NS5A interactor was identified as the α subunit of bovine translation elongation factor 1A (eEF1A). Cell-free binding studies were performed with chimeric NS5A fused to glutathione S-transferase (GST–NS5A) expressed in bacteria. GST–NS5A bound specifically to both *in vitro*-translated and mammalian cell-expressed eEF1A. Moreover, purified eEF1A bound specifically to GST–NS5A attached to a solid phase. Conservation of this interaction was then analysed using a set of NS5A proteins derived from divergent BVDV strains encompassing known biotypes and genotypes. NS5A from all BVDV strains tested so far interacted with eEF1A. The conserved association of eEF1A with virus molecules involved in genome replication and the postulated role of pestivirus and hepacivirus NS5A in replication indicate that this interaction may play a role in the replication of BVDV.

Introduction

The genome of bovine viral diarrhoea virus (BVDV) consists of a single molecule of positive-stranded RNA which is 12.5 kb in length (Collett *et al.*, 1988*a*). Sequence analysis and translation studies have shown that the BVDV genomic RNA lacks a 5' cap structure and encodes a single polyprotein of ~ 4000 amino acids, which gives rise to the mature viral proteins after processing by viral and cellular peptidases (Collett *et al.*, 1988*a, b*; Deng & Brock, 1993; Donis, 1995). It is thought that four proteins make up the enveloped virion, whereas seven mature non-structural proteins, Npro, p7, NS2-3, NS4A, NS4B, NS5A and NS5B, are potentially involved in replication (Elbers *et al.*, 1996; Rumenapf *et al.*, 1993; Tautz *et al.*, 1997; van Olphen & Donis, 1997; Xu *et al.*, 1997). These features and molecular phylogenetic studies led to the

classification of BVDV as the prototype member of the genus *Pestivirus* within the family *Flaviviridae* (Wengler *et al.*, 1995).

BVDV initiates infection by attaching to the plasma membrane, after which endocytosis and pH-dependent fusion of the envelope to the endosomal membrane occur, resulting in the delivery of the BVDV genome into the cytosol of the target cell. The genomic RNA is then translated by recruitment of translation initiation factors mediated by the IRES, which is present within the 385 nt 5' untranslated region (UTR). Newly synthesized non-structural proteins are thought to assemble into functional replicase complexes and carry out the first step of genome replication, negative-strand (antigenome) RNA synthesis. The replicase must then complete the synthesis of progeny positive-stranded RNA using the antigenomic RNA as the template. Little is known about either the molecular aspects of this process or the *cis*- and *trans*-acting factors involved. However, NS5B bears the glycine–aspartate–aspartate motif characteristic of RNA-dependent RNA polymerases and the purified protein displays polymerase activity when supplied with suitable substrates and template (Lai *et al.*, 1999; Zhong *et al.*, 1998). NS2-3 is thought to contribute its helicase activity to RNA replicase functions (Gu *et al.*, 2000). In support of this hypothesis, it has been reported that expression of NS3 lacking the NS2 region is correlated with increased levels of viral RNA accumulation (Vassilev & Donis, 2000). It

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is not known, however, if other viral or cellular proteins are involved in the activity and regulation of BVDV replicase.

Interactions among viral proteins play a central role in the assembly and regulation of the functional complexes responsible for viral RNA replication (Andino *et al.*, 1993; Lai, 1998). These replication complexes often include transient or long-lived interactions with host proteins for structural purposes or recruit regulatory and catalytic functions (Lai, 1998). It is now well established that coupling among the different sequential steps of virus replication is central to the overall infectious cycle of many RNA viruses of bacteria, plants and animals (Eigen *et al.*, 1991; Gamarnik & Andino, 1998; Janda & Ahlquist, 1998; Nguyen *et al.*, 1996; Novak & Kirkegaard, 1994; Nugent *et al.*, 1999). Identification of protein–protein interactions between viral and cellular proteins may lead to a more complete understanding of the dynamics of RNA replication, virus-mediated cellular modulation and host-range restriction.

In this report, we present the results of a yeast two-hybrid screen that describe the identification of a cellular protein that interacts with BVDV NS5A. This interaction was further analysed in a cell-free translation system and was found to be conserved among BVDV isolates of both genotypes and biotypes.

Methods

Two-hybrid interaction trap in yeast. The *Saccharomyces cerevisiae* strains EGY48 (*MAT α trp1 ura3 his3 LEU2::pLexAop6-LEU2*) and RFY206 (*mata trp1 Δ ::hisG his3 Δ 200 ura3-52 lys2 Δ 201 leu2-3*) and the plasmids pEG202, pJG4.5, pRFHM1 and pSH18-34 have been described previously (Gyuris *et al.*, 1993). The B42 Madin–Darby bovine kidney cell (B42 MDBK) cDNA library constructed with pJG4.5 was purchased from OriGene. The number of independent clones within the B42 cDNA library was 4.64×10^6 and the average size of insert was 1.1 kb. pLexANS5A was constructed by subcloning a cDNA copy of BVDV strain NADL from position 8705 to 10192, corresponding to the NS5A-coding region present in pBVSD2.3 (kindly provided by Marc Collett; Collett *et al.*, 1988a), into pEG202. pLexANS4B was obtained by subcloning a fragment of 1040 bp from pBVSD2.3 (from position 7664 to 8704 in the sequence of BVDV strain NADL). NS5A-coding sequences from BVDV strains 890, CV24 Oregon, NCP7 (GenBank accession nos U18059, AF091605 and U63479, respectively) and CP7 (Meyers *et al.*, 1996) were amplified by RT–PCR and cloned into pCRII-Topo (Invitrogen). All sequences were verified experimentally. The various NS5A cassettes were subcloned into pEG202 at the unique *EcoRI* site. Primer sequences utilized for PCR amplification and sequence analyses will be made available upon request. The DH10B strain of *Escherichia coli* was utilized for all subcloning procedures (Research Genetics). Lysates from transformed yeast harbouring each plasmid were produced by mixing clarified cultures with 250 μ l of cracking buffer [40 mM Tris–HCl pH 6.8, 0.1 mM EDTA, 5% SDS, 8 M urea, 0.05 M β -mercaptoethanol, 0.4 mg/ml bromophenol blue and a protease inhibitor cocktail containing 4-(2-aminoethyl)-benzenesulfonyl fluoride, pepstatin A, *trans*-epoxysuccinyl-L-leucylamido-(4-guanidino)butane (E-64), bestatin, leupeptin and aprotinin (Sigma)] and 300 mg of acid-washed 500 μ m diameter glass beads (Sigma, # G8772). Samples were then heated at 70 °C for 10 min, vortexed for 1 min and separated at 4 °C by

Table 1. NS5A specificity of interaction phenotype

S. cerevisiae EGY48 transformants bearing each of the 26 prey plasmids were mated in a pairwise fashion with RFY206 harbouring the pSH18.34 reporter plasmid and one of the following genes fused to *lexA*: a proprietary *bait-N*, human *c-max*, the *Drosophila melanogaster bicoid* gene, yeast Gal4 transcriptional activator, BVDV NS4B or NS5A or *lexA* DNA-binding domain without fused bait. Results shown as (+) and (–) depict the filter assay for β -galactosidase expression by diploid yeast cultured using either galactose [Gal (AD on)] or glucose [Glu (AD off)] media as the carbon source for a single mating. Identical results were obtained with the remaining 25 diploid yeast.

RFY206 (bait) (pSH18.34)	EGY48 (prey) pJG4.5-eEF1A nos. 1–26	
	Gal (AD on)	Glu (AD off)
<i>lexA-bait-N</i>	–	–
<i>lexA-c-max</i>	–	–
<i>lexA-bicoid</i>	–	–
<i>lexA-Gal4</i> (+)	+	+
<i>lexA-NS4B</i>	–	–
<i>lexA-NS5A</i>	+	–
<i>lexA</i>	–	–

centrifugation at 13 000 g. Supernatants were collected and an additional 50 μ l of cracking buffer were added to each mixture. These suspensions were boiled for 5 min, centrifuged as indicated above and combined with the first extract. Lysates were then stored at –80 °C until used in Western blot assays.

Strain EGY48 harbouring pLexANS5A and pSH18-34 (a reporter plasmid encoding β -galactosidase with a Gal1 promoter transcriptionally controlled by upstream LexA-binding sequences) was transformed with a pJG4.5MDBK cDNA library using the lithium–acetate method. Proceeding transformation, yeast were selected for histidine, uracil and tryptophan prototrophy (*his*⁺, *ura*⁺ and *trp*⁺), thus confirming the presence of all three plasmids within the transformants. Cells were then cultivated, induced with galactose–raffinose media and selected for leucine prototrophy, which is characteristic of a protein–protein interaction phenotype. Approximately 1×10^7 primary yeast transformants were selected for the *leu*⁺ growth phenotype on plates containing uracil, histidine, tryptophan, leucine and galactose. In order to discard false-positive colonies with the *leu*⁺ phenotype, independent of LexA–NS5A expression, colonies were also analysed for β -galactosidase activity on nitrocellulose filters using the X-Gal substrate. Plasmid DNA was extracted from leucine prototrophic library transformants expressing β -galactosidase only when growing in galactose–raffinose plates. These plasmids were introduced into *E. coli* strain KC8 cells by electroporation. Library plasmids were selected for tryptophan prototrophy on minimal media M9 agar plates that lacked tryptophan and contained 50 μ g/ml kanamycin. Yeast strain EGY48 was transformed with each candidate prey plasmid DNA from *E. coli* strain KC8 and subsequently mated with yeast strain RFY206 bearing pSH18-34 and one of the various *lexA* gene fusion baits to assess the specificity of the interaction by β -galactosidase assay (Table 1). Colonies that remained white after 3 h incubation with X-Gal were scored negative. Library plasmids that mediated transactivation, as demonstrated by leucine prototrophy and β -galactosidase activity in the presence of pLexANS5A, but not in the presence of specificity

controls, were saved for further analysis. Sequencing using the dideoxynucleotide chain termination method revealed the identity of the cDNA of each library plasmid encoding a candidate NS5A-interacting protein.

■ Expression of GST–NS5A and GST–eEF1A fusion proteins.

pGSTNS5A was obtained by subcloning a *SmaI*–*XhoI* fragment encoding NS5A from pLexANS5A into pGEX-KG (Guan & Dixon, 1991). pGSTeEF1A was constructed by subcloning the *EcoRI*–*XhoI* fragment encoding eEF1A from pJG4.5eEF1A into pGEX-KG. *E. coli* strain BL21 was transformed with either pGSTNS5A or pGSTeEF1A to express a GST fusion protein. Expression and purification of GST fusion proteins were performed as described previously, with minor modifications (Guan & Dixon, 1991). Briefly, a 1 ml overnight bacterial culture containing the appropriate plasmid was inoculated into 100 ml LB medium supplemented with 50 µg/ml ampicillin and grown for 90 min at 37 °C in a shaker incubator. Subsequently, IPTG was added to the cell culture at a final concentration of 0.5 mM and incubated for an additional 2 h. Cells were collected by centrifugation, washed once with 3 ml STE buffer (150 mM NaCl, 10 mM Tris–HCl pH 7.6 and 1 mM EDTA), resuspended in 3 ml STE containing 100 µg/ml lysozyme and 5 mM DTT and incubated on ice for 15 min. Cells were resuspended and lysed by the addition of 500 µl 10% *N*-laurylsarcosine/STE and sonication for 1 min in a cup-holder sonicator (Ultrasonics, model W-220F). Sonicated samples were clarified by centrifugation at 10 000 *g* for 5 min at 4 °C in an SS-34 rotor (Sorvall). Supernatants were adjusted to 2% Triton X-100, mixed by inversion and incubated with 350 µl of a 50% slurry of glutathione–agarose beads (Sigma, # G4510; prepared according to the manufacturer's directions) for 45 min at room temperature on a rotator. Beads were washed eight times with 10 vols PBS, resuspended in a final volume of 350 µl of storage buffer (5 mM DTT and 10% glycerol in PBS) and kept at –20 °C until used.

■ Expression of myc- and haemagglutinin (HA)-tagged proteins.

pmycNS5A was obtained by subcloning NS5A as a *SmaI*–*XhoI* fragment from pLexANS5A into pcDNAMyc (D. R. Perez, unpublished data). pmycNS5A-890, -CV24 Oregon, -CP7 and -NCP7 were generated by subcloning an *EcoRI* fragment encoding NS5A from pCRII-Topo into pcDNAMyc. pmycNS4B was generated by subcloning an *EcoRI*–*XhoI* fragment from pLexANS4B into pcDNAMyc. pHAeEF1A was generated by subcloning eEF1A as an *EcoRI*–*XhoI* fragment from pJG4.5eEF1A into pcDNAHA (D. R. Perez, unpublished data). To produce myc–NS5A and HA–eEF1A fusion proteins in mammalian cells, 5×10^5 African green monkey kidney cells (CV-1) in 6-well plates were infected with recombinant vaccinia virus vTF7-3 for 45 min at an m.o.i. of 5. After washing twice with minimal essential medium (MEM), cells were subsequently transfected for 4 h with either pmycNS5A or pHAeEF1A using a mixture containing 2 µg of plasmid DNA, 6 µl lipofectamine (Gibco BRL) and 1 ml MEM. At the end of the transfection period, the transfection mixture was removed and cells were maintained in 2 ml MEM supplemented with 10% foetal bovine serum for approximately 12 h. Cells were harvested and lysed by sonication at 4 °C for 45 s in 700 µl of lysis/binding buffer (20 mM Tris–HCl pH 7.6, 150 mM NaCl, 1 mM EDTA, 1 mM Na₃VO₄, 2 mM Na₃P₂O₇, 30 mM NaF, 0.05% Triton X-100 and a protease inhibitor cocktail, as described previously). Lysates were clarified by centrifugation at 10 000 *g* and the resulting supernatants were used for *in vitro* binding assays. *In vitro* expression of HA–eEF1A was performed using a Coupled Wheat Germ T7 Transcription/Translation system (Promega). *In vitro*-translated HA–eEF1A was diluted in lysis/binding buffer and used during *in vitro* binding assays, as explained below.

■ ***In vitro* binding assays.** GST fusion proteins were subjected to 10% SDS–PAGE, fixed and stained with Coomassie blue to estimate the

amount of protein needed for use during *in vitro* binding assays. In all cases, an equal concentration of GST fusion protein was used by adjusting the amount with additional glutathione–agarose beads to keep the volume constant. A volume of 20 µl of bead slurry was incubated with 200 µl of CV-1 protein lysates for 2 h at room temperature with rotation. Unbound proteins were removed by washing the beads six times with lysis/binding buffer in a cycle consisting of ten 1 min washes and 5 s centrifugation for bead collection. Finally, beads were resuspended in 1 × SDS sample buffer and boiled for 2 min (Ausubel *et al.*, 1989). To assay the binding of pure eEF1A to GST–NS5A, 5 µg eEF1A, purified as described previously, was diluted in 200 µl lysis/binding buffer and treated as explained above (Carvalho *et al.*, 1984; Cavallius *et al.*, 1997).

■ **Western blot assays.** Cell lysates or protein samples dissolved in sample buffer were separated by 10% SDS–PAGE and electrotransferred onto Hybond-C nitrocellulose filters (Amersham) using a semi-dry electroblotter (Bio-Rad). Prestained molecular mass standards for electrophoresis were purchased from Sigma (# SDS7B). LexA fusion proteins were detected using an anti-LexA monoclonal antibody (MAb) at a concentration of 20 ng/ml (Clontech, # 5397-1). To detect HA epitope-tagged proteins, anti-HA MAb 12C5 was used in a 1:50 dilution in PBS supplemented with 0.05% Tween 20. Detection of eEF1A was performed with an anti-eEF1A MAb at a concentration of 1 µg/ml (Upstate Biotechnology). Myc epitope-tagged proteins were detected using a 1:400 dilution of an anti-c-myc MAb (Chemicon). Incubation with the primary antibody at room temperature (22–24 °C) for 1 h was followed by three washes. Samples were then incubated under the same conditions with a secondary goat anti-mouse IgG MAb conjugated to horseradish peroxidase at a dilution of 1:500 (Sigma, # A5278). Blots were subjected to enhanced chemiluminescence (ECL, Amersham), according to the manufacturer's instructions.

■ **Sequence identity, translation and alignments.** Amino acid sequence alignments were produced using the PileUp program within the Wisconsin Package, version 9.1 (Altschul *et al.*, 1990). Electronic translation of the nucleotide sequence of bovine eEF1A was accomplished using the Translate program within the same software package. Initial sequences were compared with the NCBI database using the BLAST program and the similarity among the proteins was analysed as described previously (Feng & Doolittle, 1996).

Results

Interaction of BVDV NS5A with a host cell protein

We utilized an *S. cerevisiae* two-hybrid approach to screen the mRNA population of bovine cells for expression of individual proteins that interact with the BVDV NS5A non-structural protein. To this end, NS5A, expressed as a chimera with the bacterial LexA DNA-binding domain, was used as bait to screen a bovine cDNA library (B42 MDBK). Oligo(dT)-primed cDNA from poly(A)⁺ mRNA cloned at the C terminus of the prokaryotic B42 activation domain gave rise to the prey library used in the screen. A GalI inducible promoter mediated transcriptional control of the B42 MDBK fusion proteins. By using yeast host cells with a chromosomal *LEU2* gene under the transcriptional control of LexA operators, a specific interaction between the bait and the prey confers leucine prototrophy. Alternatively, using a similar strategy, interactions can activate expression of β-galactosidase in the yeast

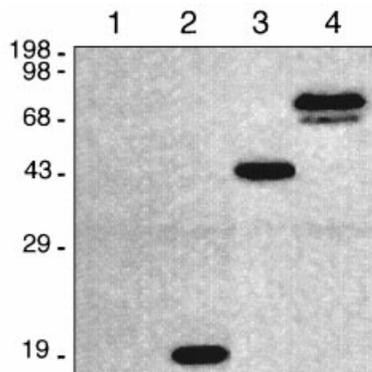


Fig. 1. Expression of LexA–NS5A fusion proteins in yeast. *S. cerevisiae* strain EGY48 (lane 1) harbouring either pEG202, encoding LexA (lane 2), pLexA–NS4B (lane 3) or pLexA–NS5A (lane 4) were grown in media lacking histidine and containing glucose as the carbon source. Yeast lysates were separated by 10% SDS–PAGE, electroblotted onto a membrane and probed with an anti-LexA MAb. Molecular mass size markers are indicated on the left.

cell. Before performing the screen, we analysed the expression of the predicted LexA–NS5A bait protein, with a molecular mass of ~ 78 kDa, within transformed yeast cells by immunoblot with a MAb against LexA (Fig. 1, lane 4). The expression levels of LexA alone were similar to those of the chimeras between LexA and each of the different BVDV proteins, suggesting that the chimeric construct had no adverse effect on expression (Fig. 1, lanes 2 and 3). The absence of degradation fragments was taken as an indication that the stability of the chimeric proteins within the yeast cytosol was comparable to that of the wild-type (Fig. 1, lanes 2 and 3). Yeast cells expressing LexA–NS5A were sequentially transformed with the β -galactosidase reporter plasmid (pSH18-34) and the B42 MDBK cDNA library by selecting for uracil and tryptophan prototrophs, respectively. Yeast bearing the three plasmid markers were induced to express the prey chimeras by growth on galactose–raffinose medium and then plated on medium selecting for interaction-dependent leucine prototrophy. A total of 26 yeast colonies showed a strict dependence of their leu^+ and β -gal $^+$ phenotypes on the expression of LexA–NS5A fusion proteins; these characteristics were not observed when any of five other prey clones bearing LexA fusions to diverse proteins were checked for transactivation activity by a mating assay (Table 1).

Nucleotide sequence analysis revealed that all cDNA clones encoded independent partial or full-length copies of the same NS5A-interacting protein. Comparison of these prey clones with sequences deposited in databases revealed a large region of identity to the bovine eEF1A mRNA sequence. This sequence (GenBank accession no. AF013213) is a partial entry whose 5' end corresponds to nt 428 of the bovine eEF1A-coding sequence. Our results indicate that the bovine eEF1A mRNA is 1661 bp in length [not including the poly(A) stretch] comprising an open reading frame (ORF) of 1389 nt flanked by

a 7 nt 5' UTR and a 265 nt 3' UTR followed by the poly(A) tail. Of the 26 clones recovered from the screen, the predominant sequence contained the 7 nt 5' UTR, a conserved ORF and a 3' UTR of 265 nt. Clones departing from this consensus sequence included some that were missing the 5' UTR or sequences extending into the first 9 nt of the ORF. One clone had a 939 nt 3' UTR (data not shown). The complete coding sequence for the bovine eEF1A mRNA has been deposited into EMBL under the accession number AJ238405.1.

NS5A binds to eEF1A *in vitro*

Yeast two-hybrid screens are excellent tools to identify interacting proteins *in vivo*. However, by virtue of the complexity of the nuclear environment of live yeast, it is not possible to equate reporter gene expression unequivocally with biologically relevant interactions. One approach to determine the significance of the interaction between NS5A and eEF1A revealed in the two-hybrid assay is to study the interaction *in vitro*. To this end, we performed pull-down assays with GST fusion proteins and epitope-tagged interaction partners. NS5A and bovine eEF1A were subcloned under the control of a T7 promoter and in-frame with N-terminal myc and HA epitope tags, respectively, yielding myc–NS5A and HA–eEF1A fusion proteins. Expression of these proteins from plasmids transfected into CV-1 cells was achieved by infection with the recombinant vaccinia virus expressing T7 RNA polymerase (vTF7-3). Expression of myc–NS5A and HA–eEF1A in CV-1 cell lysates was demonstrated by probing immunoblots with specific anti-HA and anti-c-myc MAbs (Fig. 2A). NS5A and eEF1A were also expressed in *E. coli* as GST fusion proteins (GST–NS5A and GST–eEF1A) and purified using glutathione–agarose beads (data not shown). Mammalian cell lysates containing HA–eEF1A were incubated with GST–NS5A or GST alone, produced in the prokaryotic system, and bound to agarose beads. Likewise, lysates containing myc–NS5A were incubated in the presence of either GST–eEF1A or GST alone. As shown in Fig. 2(B), Western blot analysis revealed that HA–eEF1A (~ 54 kDa) was retained by binding to GST–NS5A on the agarose beads. The reverse was also true, as GST–eEF1A interacted with the ~ 60 kDa myc–NS5A protein expressed in CV-1 cells (Fig. 2B). These interactions were specific, as GST alone was incapable of binding to either HA–eEF1A or myc–NS5A. We also noted that GST–NS5A bound the endogenous ~ 54 kDa eEF1A present in CV-1 lysates (Fig. 2C); this is expected given the absolute amino acid sequence identity between bovine and primate eEF1A.

We also examined whether additional factors were required for the interaction between NS5A and eEF1A. For this purpose, purified eEF1A (from rabbit reticulocytes) was incubated with either GST–NS5A or GST–NS4B coupled to glutathione–agarose beads (see Methods). The interaction between GST–

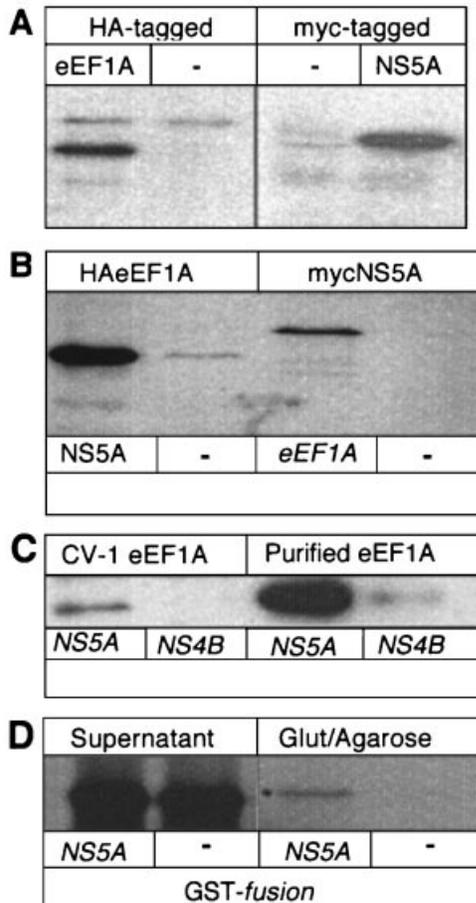


Fig. 2. *In vitro* interaction between NS5A and eEF1A. (A) Expression of HA-tagged eEF1A in CV-1 cells infected previously with T7 vaccinia virus and transfected with either pcDNAHAeEF1A (HA-tagged/eEF1A) or the empty pcDNAHA vector (HA-tagged/-) was detected in Western blots by probing with an anti-HA epitope MAb. Identical expression and detection approaches with the anti-c-myc MAb were used to visualize myc-NS5A as a band corresponding to a protein with a molecular mass of ~ 60 kDa in cells transfected with pcDNAmycNS5A (myc-tagged/NS5A), but not in the control cells transfected with the empty pcDNAmyc vector (myc-tagged/-). (B) Proteins bound to GST alone (-) or to GST fusions with either interactor partner (NS5A or eEF1A) were detected by Western blot analysis. HA-eEF1A and myc-NS5A present in CV-1 lysates were retained by the cognate interacting protein. (C) Binding of GST-NS5A to eEF1A present in CV-1 lysates or purified from rabbit reticulocytes. BVDV NS4B fused to GST was used to monitor interaction specificity. (D) Binding of *in vitro*-translated, ³⁵S-labelled eEF1A to GST-NS5A. The total bound (Glut/agarose) and unbound (Supernatant) eEF1A after incubation with GST-NS5A (NS5A) or GST alone (-) was resolved by 10% SDS-PAGE and analysed by autoradiography.

NS5A and eEF1A was readily observed (Fig. 2C). Since binding took place in the absence of any mammalian protein other than eEF1A, we postulate that the interaction between these two factors is direct; i.e. it does not require additional host proteins. In addition, and consistent with these observations, *in vitro*-translated eEF1A interacted specifically with GST-NS5A, but not with GST expressed alone (Fig. 2D). Moreover, GST-NS5A bound to purified eEF1A more

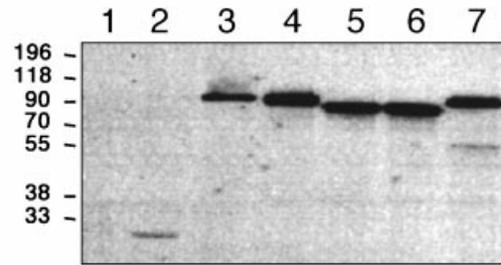


Fig. 3. Expression of NS5A proteins from divergent BVDV strains. EGY48 yeast (lane 1) bearing plasmids encoding LexA alone (lane 2) or LexA fused to NS5A (lanes 3–7) grown in auxotrophic selection media and harvested for protein electrophoresis and immunoblotting. Lanes 3, BVDV NADL strain (LexANADL-NS5A); 4, strain 890 (LexA890-NS5A); 5, strain CP7 (LexACP7-NS5A); 6, strain NCP7 (LexANCP7-NS5A); 7, strain CV24 Oregon (LexAOregon-NS5A). Total cell lysates were analysed by 10% SDS-PAGE, blotted and probed with an anti-LexA MAb and a goat anti-mouse IgG horseradish peroxidase conjugate. The migration of molecular mass standards is indicated on the left. The LexA protein migrates as a 19 kDa polypeptide (lane 2).

efficiently than endogenous eEF1A, which is present in CV-1 lysates at similar concentrations (data not shown).

eEF1A binds to NS5A from divergent BVDV strains

NS5A is the most variable non-structural protein among divergent BVDV isolates (Deng & Brock, 1992). Consequently, it was important to establish whether binding to eEF1A is conserved among different strains of BVDV. The ability of eEF1A to bind NS5A from several strains of BVDV, including non-cytopathic and cytopathic viruses from genotypes II and I, was analysed in the two-hybrid assay. We chose BVDV strains NADL, NCP7, CP7, CV24 Oregon and 890 to amplify NS5A-coding regions by RT-PCR and cloned the resulting amplicons as LexA chimeras. Subsequently, expression of polypeptides of the expected size by yeast transformed with plasmids encoding each of the LexA-NS5A chimeras was demonstrated by Western blot analysis (Fig. 3). To assess their capacity to interact with eEF1A, two-hybrid assays were performed by mating EGY48 and RFY206 yeast strains to yield diploid progeny expressing B42-eEF1A and LexA-NS5A chimeras. All the BVDV NS5A proteins mediated transactivation of β -galactosidase activity and leucine prototrophy, indicating the ability of these NS5A fusions to interact with eEF1A. To extend the significance of the *in vivo* interactions of the NS5A proteins from divergent isolates with eEF1A, we performed GST pull-down assays. For this purpose, we expressed the viral proteins in CV-1 cells as N-terminal myc-tagged proteins and examined their retention by the GST-eEF1A chimera or by GST expressed alone. No major differences in the levels of expression of the BVDV NS5A proteins were observed, although we noted significant differences in the electrophoretic mobility of all NS5A proteins expressed (Figs 3 and 4A). Interestingly, we found that all the

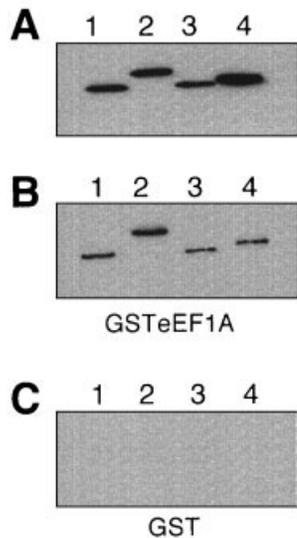


Fig. 4. Conservation of NS5A binding of eEF1A. (A) Expression and electrophoretic mobility differences among BVDV NS5A chimeras. myc-tagged NS5A of different strains of BVDV expressed in mammalian cells analysed by Western blot. Lanes 1, strain CP7 (cytopathic, genotype I); 2, strain 890 (non-cytopathic, genotype II); 3, strain NCP7 (non-cytopathic, genotype I); 4, strain CV24 Oregon (cytopathic, genotype I). (B) Binding of myc-tagged NS5A proteins to GST–eEF1A. (C) Binding of proteins shown in (A) and (B) to GST expressed without fused domains. Conditions for GST-binding assays and analysis by Western blot are as described in Fig. 2 and in Methods.

NS5A proteins bound eEF1A as efficiently as the prototype NS5A from the NADL strain (Fig. 4B). LexA–NS5A and myc–NS5A proteins from each isolate displayed electrophoretic mobility shifts relative to the prototype NS5A from the NADL strain. Because these proteins are only between 67 and 89% identical, we postulate that amino acid composition and/or post-translational modifications are probably responsible for this effect. NS5A is a phosphoprotein and sequence divergence can result in different patterns of phosphorylation, which could, at least in part, explain the altered migration among these proteins. Nevertheless, our results suggest that, whatever the reasons for the mobility shifts in NS5A, they do not alter binding to eEF1A significantly.

Discussion

Identification of cellular factors that interact with non-structural proteins of BVDV will provide a better understanding of its replication and perhaps help to elucidate the mechanisms of virus-mediated alterations of cellular homeostasis. A cDNA library from bovine kidney cells susceptible to BVDV infection was screened in a yeast two-hybrid interaction assay, leading to the identification of only one interactor of the NS5A protein, bovine eEF1A (Table 1). The homogeneity of the prey clone population identified as NS5A interactors (26/26 were eEF1A) is postulated to be a consequence of the extreme abundance of this mRNA in the cell transcriptome

(usually ~ 1% in most cell types). Cell-free binding assays between purified GST–NS5A and either an epitope-tagged bovine eEF1A, endogenous monkey eEF1A or purified eEF1A from rabbit reticulocytes provided confirmation of this interaction *in vitro*. Independently, we showed that a reciprocal chimerization strategy using purified GST–eEF1A to bind myc–NS5A expressed in mammalian cells yielded results consistent with our previous observations (Fig. 2A). Moreover, the binding of purified eEF1A from rabbit reticulocytes to GST–NS5A suggested that other bovine proteins are not required for this interaction (Fig. 2). However, we cannot rule out the presence of trace amounts of contaminant RNA or other small molecules that could act as a bridge between these two proteins. Interestingly, GST–NS5A showed greater capacity to bind purified eEF1A than endogenous eEF1A present in cell lysates (Fig. 2C). Since the amount of eEF1A substrate was similar within these assays (4 µg purified eEF1A and ~ 4% composition of eEF1A in cell lysates; 100 µg total protein), this may reflect competition between NS5A and host proteins for eEF1A-binding sites.

The conserved genomic localization of NS5A and the generation of similar processing intermediates among both hepaciviruses and pestiviruses suggest a common and essential role of NS5A in the virus life cycle. However, we were unable to detect an interaction between hepatitis C virus NS5A and bovine eEF1A, whose amino acid sequence is identical to primate eEF1A. Nevertheless, the interaction described herein is conserved among divergent cytopathic and non-cytopathic BVDV isolates as well as in isolates belonging to genotypes I and II. Conservation of the eEF1A–NS5A interaction among divergent BVDV strains does not constitute evidence of the essential nature of the binding interface, but it suggests strongly that this character may at least confer some selective advantage to BVDV.

An overwhelming body of data demonstrates the potential roles for the components of the host translation machinery in virus life cycles. Such viral–host interactions in the context of translation factors were demonstrated first within the bacteriophage Q β RNA-dependent RNA polymerase or replicase. The active enzyme was found to exist as a heterotetramer consisting of a virus-encoded subunit plus three host proteins: ribosomal protein S1 and elongation factors Tu and Ts (Blumenthal *et al.*, 1972). More recently, it has been shown that two of these subunits, S1 and EF-Tu, provide the differential template recognition of positive- and negative-strand RNA present during virus replication (Brown & Gold, 1996). This model demonstrates the direct role of host proteins in the mechanics of virus replication.

Binding of eEF1 α , - β and - γ components to the viral RNA-dependent RNA polymerase of vesicular stomatitis virus is required for its replicase activity *in vitro* (Das *et al.*, 1998). Binding of eEF1A to viral RNA, directly or in association with viral proteins, has long been observed among bacterial, plant and animal viruses. These interactions have been demonstrated

with poliovirus (Harris *et al.*, 1994), West Nile virus (Blackwell & Brinton, 1997), brome mosaic virus (Bastin & Hall, 1976), furoviruses (Goodwin & Dreher, 1998) and bacteriophage Q β with EF-Tu, as mentioned previously. Viral RNA–eEF1A interactions generally occur within the UTRs of viral genomes at domains containing conserved secondary structures.

Interestingly, the NS5A proteins of two highly divergent strains of BVDV analysed, 890 (genotype II) and NADL (genotype I), are only 77% similar, yet the ability to bind eEF1A is conserved. NS5A is hydrophilic, relatively stable within infected cells and phosphorylated at serine and threonine residues. Phosphorylation is conserved among all NS5A and NS5 proteins within members of the family *Flaviviridae*, suggesting its importance in the flavivirus life cycle (Reed *et al.*, 1997, 1998). The electrophoretic mobility variability of the LexA–NS5A fusion proteins represented in Figs 3 and 4(A) may reflect the different phosphorylation states of each polypeptide. However, differential processing events by an exopeptidase, for example, cannot be ruled out. Interestingly, electrophoretic mobility shifts were observed when comparing NS5A expressed in yeast and mammalian cells. This is probably a reflection of different post-translational modification events occurring in these cells. There was no consistent trend towards increased or decreased mobility that could be correlated with the type of host cell. Collectively, changes mediating gel mobility alterations did not abrogate NS5A binding to eEF1A, although they may well modulate binding affinities.

Nucleotide sequence analysis revealed the conserved identity of all the clones obtained in the MDBK cDNA screen as the highly conserved eEF1A. Comparison of the bovine eEF1A amino acid sequence revealed 100% identity to all known mammalian counterparts, with 81% identity to yeast eEF1A and 79% identity to barley eEF1A. eEF1A shows 56% amino acid similarity and conserved function to prokaryotic EF-Tu (Negrutskaa & El'skaya, 1998; Sprinzl, 1994). eEF1A constitutes 1–4% of all soluble proteins within active cells, being second only to actin with regard to protein abundance (Slobin, 1980). eEF1A is essential for cell viability by virtue of its role in the formation of every peptide bond during protein translation. In addition to this primary role, eEF1A has secondary functions, including the binding and bundling of actin (Condeelis, 1995; Murray *et al.*, 1996; Yang *et al.*, 1990), microtubule severing (Shiina *et al.*, 1994), protein degradation mediated through ubiquitin-dependent pathways (Gonen *et al.*, 1994) and association with ribonucleoprotein complexes (Kruse *et al.*, 1998). Taken together, these roles implicate eEF1A in the global regulation of mRNA translation, stability of expressed proteins and cytoskeletal organization. The astounding versatility of this highly abundant and conserved cellular protein renders it attractive for recruitment by the virus replication machinery. Further insight into the role of this interaction in virus replication may be provided by its manipulation in cell-free BVDV replication systems. Ulti-

mately, functional evidence of the significance of the interaction would be provided by mapping the critical residues of NS5A for the interaction with eEF1A, followed by phenotypic analyses of viruses bearing mutations in these residues obtained through reverse genetics.

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References

- Altschul, S. F., Gish, W., Miller, W., Myers, E. W. & Lipman, D. J. (1990).** Basic local alignment search tool. *Journal of Molecular Biology* **215**, 403–410.
- Andino, R., Rieckhof, G. E., Achacoso, P. L. & Baltimore, D. (1993).** Poliovirus RNA synthesis utilizes an RNP complex formed around the 5'-end of viral RNA. *EMBO Journal* **12**, 3587–3598.
- Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A. & Struhl, K. (1989).** *Current Protocols in Molecular Biology*, 2nd edn, vol. 1–2. Edited by F. M. Ausubel, R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith & K. Struhl. New York: Greene Publishing Associates and Wiley Interscience.
- Bastin, M. & Hall, T. C. (1976).** Interaction of elongation factor 1 with aminoacylated brome mosaic virus and tRNAs. *Journal of Virology* **20**, 117–122.
- Blackwell, J. L. & Brinton, M. A. (1997).** Translation elongation factor-1 α interacts with the 3' stem-loop region of West Nile virus genomic RNA. *Journal of Virology* **71**, 6433–6444.
- Blumenthal, T., Landers, T. A. & Weber, K. (1972).** Bacteriophage Q replicase contains the protein biosynthesis elongation factors EF Tu and EF Ts. *Proceedings of the National Academy of Sciences, USA* **69**, 1313–1317.
- Brown, D. & Gold, L. (1996).** RNA replication by Q β replicase: a working model. *Proceedings of the National Academy of Sciences, USA* **93**, 11558–11562.
- Carvalho, J. F., Carvalho, M. D. & Merrick, W. C. (1984).** Purification of various forms of elongation factor 1 from rabbit reticulocytes. *Archives of Biochemistry and Biophysics* **234**, 591–602.
- Cavallius, J., Popkie, A. P. & Merrick, W. C. (1997).** Site-directed mutants of post-translationally modified sites of yeast eEF1A using a shuttle vector containing a chromogenic switch. *Biochimica et Biophysica Acta* **1350**, 345–358.
- Collett, M. S., Larson, R., Gold, C., Strick, D., Anderson, D. K. & Purchio, A. F. (1988a).** Molecular cloning and nucleotide sequence of the pestivirus bovine viral diarrhoea virus. *Virology* **165**, 191–199.
- Collett, M. S., Larson, R., Belzer, S. K. & Retzel, E. (1988b).** Proteins encoded by bovine viral diarrhoea virus: the genomic organization of a pestivirus. *Virology* **165**, 200–208.
- Condeelis, J. (1995).** Elongation factor 1 α , translation and the cytoskeleton. *Trends in Biochemical Sciences* **20**, 169–170.
- Das, T., Mathur, M., Gupta, A. K., Janssen, G. M. & Banerjee, A. K. (1998).** RNA polymerase of vesicular stomatitis virus specifically associates with translation elongation factor-1 $\alpha\beta\gamma$ for its activity. *Proceedings of the National Academy of Sciences, USA* **95**, 1449–1454.

- Deng, R. & Brock, K. V. (1992).** Molecular cloning and nucleotide sequence of a pestivirus genome, noncytopathic bovine viral diarrhoea virus strain SD-1. *Virology* **191**, 867–869.
- Deng, R. & Brock, K. V. (1993).** 5' and 3' untranslated regions of pestivirus genome: primary and secondary structure analyses. *Nucleic Acids Research* **21**, 1949–1957.
- Donis, R. O. (1995).** Molecular biology of bovine viral diarrhoea virus and its interactions with the host. *Veterinary Clinics of North America. Food Animal Practice* **11**, 393–423.
- Eigen, M., Biebricher, C. K., Gebinoga, M. & Gardiner, W. C. (1991).** The hypercycle. Coupling of RNA and protein biosynthesis in the infection cycle of an RNA bacteriophage. *Biochemistry* **30**, 11005–11018.
- Elbers, K., Tautz, N., Becher, P., Stoll, D., Rumenapf, T. & Thiel, H. J. (1996).** Processing in the pestivirus E2–NS2 region: identification of proteins p7 and E2p7. *Journal of Virology* **70**, 4131–4135.
- Feng, D. F. & Doolittle, R. F. (1996).** Progressive alignment of amino acid sequences and construction of phylogenetic trees from them. *Methods in Enzymology* **266**, 368–382.
- Gamarnik, A. V. & Andino, R. (1998).** Switch from translation to RNA replication in a positive-stranded RNA virus. *Genes & Development* **12**, 2293–2304.
- Gonen, H., Smith, C. E., Siegel, N. R., Kahana, C., Merrick, W. C., Chakraborty, K., Schwartz, A. L. & Ciechanover, A. (1994).** Protein synthesis elongation factor EF-1 α is essential for ubiquitin-dependent degradation of certain N α -acetylated proteins and may be substituted for by the bacterial elongation factor EF-Tu. *Proceedings of the National Academy of Sciences, USA* **91**, 7648–7652.
- Goodwin, J. B. & Dreher, T. W. (1998).** Transfer RNA mimicry in a new group of positive-strand RNA plant viruses, the furoviruses: differential aminoacylation between the RNA components of one genome. *Virology* **246**, 170–178.
- Gu, B., Liu, C., Lin-Goerke, J., Maley, D. R., Gutshall, L. L., Feltenberger, C. A. & Del Vecchio, A. M. (2000).** The RNA helicase and nucleotide triphosphatase activities of the bovine viral diarrhoea virus NS3 protein are essential for viral replication. *Journal of Virology* **74**, 1794–1800.
- Guan, K. L. & Dixon, J. E. (1991).** Eukaryotic proteins expressed in *Escherichia coli*: an improved thrombin cleavage and purification procedure of fusion proteins with glutathione S-transferase. *Analytical Biochemistry* **192**, 262–267.
- Gyuris, J., Golemis, E., Chertkov, H. & Brent, R. (1993).** Cdi1, a human G1 and S phase protein phosphatase that associates with Cdk2. *Cell* **75**, 791–803.
- Harris, K. S., Xiang, W., Alexander, L., Lane, W. S., Paul, A. V. & Wimmer, E. (1994).** Interaction of poliovirus polypeptide 3CD^{pro} with the 5' and 3' termini of the poliovirus genome. Identification of viral and cellular cofactors needed for efficient binding. *Journal of Biological Chemistry* **269**, 27004–27014.
- Janda, M. & Ahlquist, P. (1998).** Brome mosaic virus RNA replication protein Ia dramatically increases *in vivo* stability but not translation of viral genomic RNA3. *Proceedings of the National Academy of Sciences, USA* **95**, 2227–2232.
- Kruse, C., Grunweller, A., Willkomm, D. K., Pfeiffer, T., Hartmann, R. K. & Muller, P. K. (1998).** tRNA is entrapped in similar, but distinct, nuclear and cytoplasmic ribonucleoprotein complexes, both of which contain vigilin and elongation factor 1 α . *Biochemical Journal* **329**, 615–621.
- Lai, M. M. (1998).** Cellular factors in the transcription and replication of viral RNA genomes: a parallel to DNA-dependent RNA transcription. *Virology* **244**, 1–12.
- Lai, V. C., Kao, C. C., Ferrari, E., Park, J., Uss, A. S., Wright-Minogue, J., Hong, Z. & Lau, J. Y. (1999).** Mutational analysis of bovine viral diarrhoea virus RNA-dependent RNA polymerase. *Journal of Virology* **73**, 10129–10136.
- Meyers, G., Tautz, N., Becher, P., Thiel, H. J. & Kummerer, B. M. (1996).** Recovery of cytopathogenic and noncytopathogenic bovine viral diarrhoea viruses from cDNA constructs. *Journal of Virology* **70**, 8606–8613.
- Murray, J. W., Edmonds, B. T., Liu, G. & Condeelis, J. (1996).** Bundling of actin filaments by elongation factor 1 α inhibits polymerization at filament ends. *Journal of Cell Biology* **135**, 1309–1321.
- Negrutskii, B. S. & El'skaya, A. V. (1998).** Eukaryotic translation elongation factor 1 α : structure, expression, functions, and possible role in aminoacyl-tRNA channeling. *Progress in Nucleic Acid Research and Molecular Biology* **60**, 47–78.
- Nguyen, L., Lucas, W. J., Ding, B. & Zaitlin, M. (1996).** Viral RNA trafficking is inhibited in replicase-mediated resistant transgenic tobacco plants. *Proceedings of the National Academy of Sciences, USA* **93**, 12643–12647.
- Novak, J. E. & Kirkegaard, K. (1994).** Coupling between genome translation and replication in an RNA virus. *Genes & Development* **8**, 1726–1737.
- Nugent, C. I., Johnson, K. L., Sarnow, P. & Kirkegaard, K. (1999).** Functional coupling between replication and packaging of poliovirus replicon RNA. *Journal of Virology* **73**, 427–435.
- Reed, K. E., Xu, J. & Rice, C. M. (1997).** Phosphorylation of the hepatitis C virus NS5A protein *in vitro* and *in vivo*: properties of the NS5A-associated kinase. *Journal of Virology* **71**, 7187–7197.
- Reed, K. E., Gorbalenya, A. E. & Rice, C. M. (1998).** The NS5A/NS5 proteins of viruses from three genera of the family *Flaviviridae* are phosphorylated by associated serine/threonine kinases. *Journal of Virology* **72**, 6199–6206.
- Rumenapf, T., Unger, G., Strauss, J. H. & Thiel, H. J. (1993).** Processing of the envelope glycoproteins of pestiviruses. *Journal of Virology* **67**, 3288–3294.
- Shiina, N., Gotoh, Y., Kubomura, N., Iwamatsu, A. & Nishida, E. (1994).** Microtubule severing by elongation factor 1 α . *Science* **266**, 282–285.
- Slobin, L. I. (1980).** The role of eucaryotic factor Tu in protein synthesis. The measurement of the elongation factor Tu content of rabbit reticulocytes and other mammalian cells by a sensitive radioimmunoassay. *European Journal of Biochemistry* **110**, 555–563.
- Sprinzl, M. (1994).** Elongation factor Tu: a regulatory GTPase with an integrated effector. *Trends in Biochemical Sciences* **19**, 245–250.
- Tautz, N., Elbers, K., Stoll, D., Meyers, G. & Thiel, H. J. (1997).** Serine protease of pestiviruses: determination of cleavage sites. *Journal of Virology* **71**, 5415–5422.
- van Olphen, A. L. & Donis, R. O. (1997).** Identification of bovine viral diarrhoea virus nonstructural polypeptide NS4B/P38. *Virus Research* **51**, 197–201.
- Vassilev, V. B. & Donis, R. O. (2000).** Bovine viral diarrhoea virus induced apoptosis correlates with increased intracellular viral RNA accumulation. *Virus Research* **69**, 95–107.
- Wengler, G., Bradley, D. W., Collett, M. S., Heinz, F. X., Schlesinger, R. W. & Strauss, J. H. (1995).** The *Flaviviridae*. In *Virus Taxonomy. Sixth Report of the International Committee on Taxonomy of Viruses*, pp. 415–427. Edited by F. A. Murphy, C. M. Fauquet, D. H. L. Bishop, S. A. Ghabrial, A. W. Jarvis, G. P. Martelli, M. A. Mayo & M. D. Summers. Vienna: Springer-Verlag.
- Xu, J., Mendez, E., Caron, P. R., Lin, C., Murcko, M. A., Collett, M. S. &**

Rice, C. M. (1997). Bovine viral diarrhea virus NS3 serine proteinase: polyprotein cleavage sites, cofactor requirements, and molecular model of an enzyme essential for pestivirus replication. *Journal of Virology* **71**, 5312–5322.

Yang, F., Demma, M., Warren, V., Dharmawardhane, S. & Condeelis, J. (1990). Identification of an actin-binding protein from Dictyostelium as elongation factor 1 α . *Nature* **347**, 494–496.

Zhong, W., Gutshall, L. L. & Del Vecchio, A. M. (1998). Identification and characterization of an RNA-dependent RNA polymerase activity within the nonstructural protein 5B region of bovine viral diarrhea virus. *Journal of Virology* **72**, 9365–9369.

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