Short Communication

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New insights into processing of bovine viral diarrhea virus glycoproteins E^{rns} and E1

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Bovine viral diarrhea virus (BVDV) is a member of the genus *Pestivirus* within the family *Flaviviridae*. Its single-stranded RNA encodes a polyprotein that is cleaved co- and post-translationally by viral and cellular proteases. However, the cleavage between the envelope proteins E^{rns} and E1 is still unexplained. In this study, an E^{rns}–E1 protein could be identified and characterized with a new E1-specific antiserum. With bicistronic constructs bearing a deletion in the E^{rns}-encoding region and expressing E^{rns} or the E^{rns}–E1 protein, it could be shown that this protein is not essential for virus replication. Furthermore, two putative cleavage sites were mutated in eukaryotic expression plasmids, as well as in full-length cDNA constructs. The mutation of position P3 of a potential signal peptide peptidase site abolished cleavage completely and no infectious virus progeny could be observed, indicating that cleavage of the E^{rns}–E1 protein is indispensable for virus growth.

Bovine viral diarrhea virus (BVDV), the causative agent of an economically important disease of cattle worldwide, belongs, like classical swine fever virus and border disease virus, to the genus *Pestivirus* within the family *Flaviviridae* (Collett *et al.*, 1988a; Fauquet *et al.*, 2005; Houe, 1999; Korn, 1977; Pringle, 1998). The genome is a single-stranded RNA of positive polarity with a size of about 12.3 kb. One large open reading frame (ORF) encodes a polyprotein that is cleaved co- and post-translationally by viral and cellular proteases (Collett *et al.*, 1988b; Rümenapf *et al.*, 1993). The ORF is flanked by 5′ and 3′ untranslated regions (UTRs) that are important for virus replication (Collett *et al.*, 1988a; Yu *et al.*, 1999, 2000). An internal ribosomal entry site (IRES) within the 5′ UTR allows cap-independent translation of the virus polyprotein (Pestova & Hellen, 1999; Poole *et al.*, 1995; Yu *et al.*, 2000). The virions consist of four structural proteins (C, E^{rns}, E1 and E2). The basic protein C was believed to form a capsid that surrounds the virus genome, but recent studies by Rümenapf *et al.* (2008) have shown that it is dispensable for virus assembly (Gray & Nettleton, 1987; Thiel *et al.*, 1991). The three glycosylated proteins E^{rns}, E1 and E2 are located in the lipid membrane of cellular origin (Chu & Zee, 1984; Coria *et al.*, 1983; Fetzer *et al.*, 2005). The envelope protein E^{rns}, which lacks a typical membrane anchor, seems to be important for first cell contact by binding to glycosaminoglycans (Fetzer *et al.*, 2005; Iqbal *et al.*, 2000). The cellular receptor for BVDV is the bovine CD46 molecule and it has recently been shown that E1–E2 heterodimers are essential for virus entry (Maurer *et al.*, 2004; Ronecker *et al.*, 2008). The envelope proteins E^{rns}, E1 and E2 are processed from the polyprotein in a hierarchical way, starting with the translocation of the C-terminal signal peptide downstream of the capsid protein into the endoplasmic reticulum (ER), followed by the release of the capsid and E2 protein (Heimann *et al.*, 2006; Rümenapf *et al.*, 1993). The cleavage between the proteins E^{rns} and E1 is the last event in the processing of the envelope proteins, and the responsible protease as well as the compartment have remained undefined until now (Rümenapf *et al.*, 1993).

In this study, we report the identification and characterization of an E^{rns}–E1 protein of BVDV by means of a new E1-specific antiserum. For the generation of E1-specific antibodies that were not yet available, we immunized 8–10-week-old rabbits with the KLH-conjugated synthetic peptide PRNTKIIGPGRFDTC (EMC Microcollections GmbH) in combination with Polygen (MVP Laboratories, Inc.) as adjuvant. For further studies, the genomic regions encoding the structural proteins of BVDV type I strain CP7 were cloned into the pCITE-2a(+) vector (Novagen). The plasmids pCITE_SgE^{rns}, pCITE_SgE^{rns}-E1, pCITE_SgE^{rns}-E2, pCITE_C-E^{rns}, pCITE_C-E1 and pCITE_C-E2 were constructed by amplification of PCR fragments of the infectious cDNA clone pA/BVDV (Meyers *et al.*, 1996) using the Expand High Fidelity PCR system (Roche) with primers corresponding to the signal sequence within C and the terminal sequences of C, E^{rns}, E1 and E2, respectively. The primer sequences are available on request. The PCR fragments were digested with *Nco*I and *Xba*I and ligated into the vector pCITE-2a(+) digested with *Nco*I and *Xba*I (Fig. 1a). BSR-T7 cells, i.e. T7 RNA-polymerase expressing cells [RIE583; Collection of Cell Lines in Veterinary Medicine (CCLV), Friedrich-Loeffler-Institut],

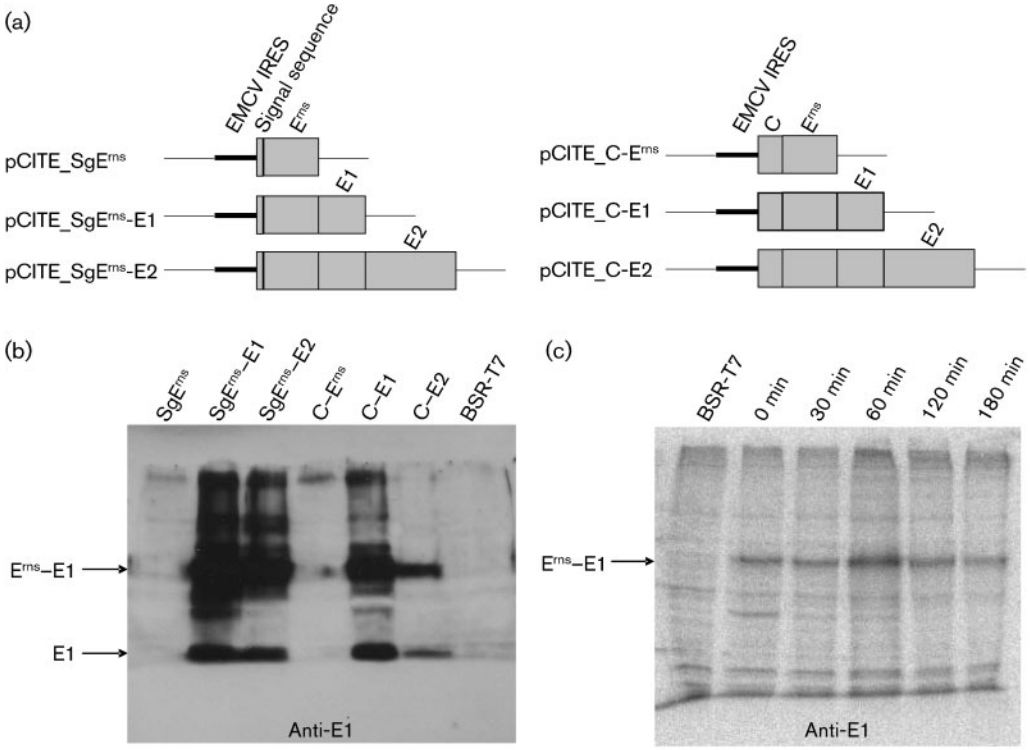

Fig. 1. (a) Schematic representation of the generated pCITE-2a(+) constructs that were used for expression of the structural proteins C, E^{rns}, E1 and E2 of BVDV type I strain CP7. (b) Western blot of BSR-T7 cells transfected with the pCITE-2a(+) constructs with the E1-specific rabbit serum. (c) Radioimmunoprecipitation of BSR-T7 cells transfected with the plasmid pCITE_SgE^{rns}-E1 with the E1-specific antiserum.

were transfected with the recombinant plasmids by using SuperFect transfection reagent (Qiagen) and lysed for Western blot experiments 24 h post-transfection (p.t.). The proteins were separated by SDS-PAGE under reducing conditions and transferred to nitrocellulose membranes. Subsequently, the blot was incubated with the E1-specific antiserum (diluted 1 : 3000 in 2.5 % low-fat milk) followed by incubation with horseradish peroxidase-conjugated anti-rabbit antibody (Dianova) [diluted 1 : 20 000 in Tris-buffered saline with 0.1 % Tween 20 (TBS-T)].

In cells transfected with the constructs pCITE_SgE^{rns}-E1, pCITE_SgE^{rns}-E2, pCITE_C-E1 and pCITE_C-E2, we could specifically identify an E^{rns}–E1 protein with a size of 60–65 kDa, as well as free E1 protein with a size of about 25 kDa (Fig. 1b). In order to analyse the kinetics of the E^{rns}–E1 protein, we performed a pulse–chase experiment with BSR-T7 cells transfected with the plasmid pCITE_SgE^{rns}-E1 and pulse-labelled for 1 h with 100 µCi (3.7 MBq) [^{35}S]methionine/[^{35}S]cysteine ml^{-1} (Hartmann Analytic GmbH) 24 h p.t. After 0, 30, 60, 120 and 180 min, the cells were chased with unlabelled medium. The proteins were precipitated with the E1 antiserum (final dilution 1 : 50) and separated by SDS-PAGE under reducing conditions. We could demonstrate that the E^{rns}–E1 protein remained stable for at least 3 h (Fig. 1c). To investigate the role of this protein for the generation of infectious virus progeny, we generated bicistronic constructs with a deletion of the main part of the E^{rns}-encoding region (Δ nt 1179–1794) expressing E^{rns} or the E^{rns}–E1 protein under control of an encephalomyocarditis virus (EMCV) IRES. Briefly, the plasmid pCDNA_C-E2mod (Reimann *et al.*, 2003), encoding the structural proteins of BVDV type I strain PT810 (Wolfmeyer *et al.*, 1997) as a synthetic ORF, was used to generate the plasmid pCITE_SgE^{rns}-E2mod. By using the heterologous strain PT810, recombination of sequences from the first and second cistrons should be prevented. In the next step, PCR fragments were amplified with primers corresponding to the terminal sequences of IRES, E^{rns} and E1, digested with *Mlu*I and ligated into the plasmid CP7ΔE^{rns}_1179_*Mlu*I, derived from the infectious cDNA clone pA/BVDV digested with *Mlu*I^{11570} (Fig. 2a). The primer sequences are available on request. *In vitro* transcription of the generated cDNA constructs CP7ΔE^{rns}_SgE^{rns}mod and CP7ΔE^{rns}_SgE^{rns}-E1mod was performed by using the T7 RiboMax Large Scale RNA production system (Promega). KOP-R cells, a diploid bovine oesophageal cell line (RIE244; CCLV, Friedrich-Loeffler-Institut), were electroporated with the *in vitro*-synthesized RNAs by using a Gene Pulser Xcell electroporation system (Bio-Rad). Immunofluorescence (IF) staining with the pan-pestivirus NS3-specific mAb C16

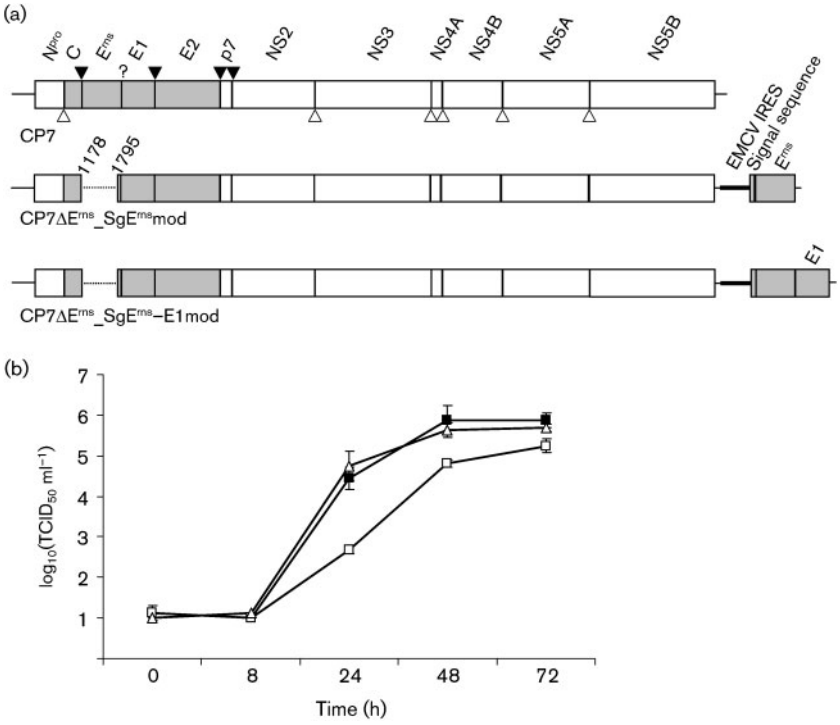

Fig. 2. (a) Overview of the genome structure of BVDV type I strain CP7 and of the generated bicistronic constructs with deletion of the main part of the E^{rns}-encoding region (Δ nt 1179–1794) in the first cistron and an expression cassette for E^{rns} or the E^{rns}–E1 protein encoded by a synthetic ORF in the second cistron. ▼, Cleavage site of cellular proteases; △, cleavage site of viral proteases. (b) Growth-kinetics experiment for comparison of the recombinant bicistronic viruses CP7ΔE^{rns}_SgE^{rns}mod (□), CP7ΔE^{rns}_SgE^{rns}-E1mod (△) and the parental virus CP7 (■). KOP-R cells and an m.o.i. of 0.1 were used.

(Edwards *et al.*, 1988; Peters *et al.*, 1986), kindly provided by Irene Greiser-Wilke (Tierärztliche Hochschule, Hannover, Germany), revealed that both constructs were able to replicate and to produce infectious virus progeny (Fig. 2b). In conclusion, the expression of E^{rns} alone was sufficient for the generation of infectious virus and the E^{rns}–E1 protein was not essential for virus replication, confirming previous studies by Frey *et al.* (2006) and Widjojoatmodjo *et al.* (2000) in which E^{rns} deletions could be *trans*-complemented with E^{rns}-expressing cell lines.

In order to investigate whether the recombinant viruses differ in growth efficiency, growth-kinetics experiments were performed. KOP-R cells were inoculated with an m.o.i. of 0.1 with CP7ΔE^{rns}_SgE^{rns}mod, CP7ΔE^{rns}_SgE^{rns}–E1mod or CP7. The parental virus CP7 was derived from the infectious cDNA clone pA/BVDV. The titres were examined as $TCID_{50}$ ml^{-1} by IF staining with mAb C16 at 72 h post-infection (p.i.). The final titres of the virus CP7ΔE^{rns}_SgE^{rns}–E1mod that expressed the E^{rns}–E1 protein from the second cistron were comparable to the titres of the parental virus CP7 (Fig. 2b). In contrast, the virus CP7ΔE^{rns}_SgE^{rns}mod, which expressed E^{rns} alone from the second cistron, showed a clearly reduced growth efficiency, indicating that it is beneficial for the generation of infectious virus progeny when E^{rns} is generated from the E^{rns}–E1 protein by a cleavage event (Fig. 2b). In a further step, two potential cleavage sites between E^{rns} and E1 were mutated. First, a potential subtilisin kexin isozyme-1/site-1 protease (SKI-1/S1P) site in the N-terminal region of E1 was mutated (Fig. 3a). This subtilase is responsible for e.g. the processing of Crimean–Congo hemorrhagic fever virus and lymphocytic choriomeningitis virus glycoprotein and requires an arginine at position P4 (Bergeron *et al.*, 2007; Beyer *et al.*, 2003). Second, a potential signal peptide peptidase (SPP) site in the C-terminal region of E^{rns} was mutated (Fig. 3a). This SPP is responsible for e.g. the cleavage of the pestiviral capsid protein at its C terminus and excludes an aromatic, charged or large polar amino acid at position P3 (Heimann *et al.*, 2006; von Heijne, 1986). The plasmid pCITE_SgE^{rns}-E2 was used as a target for site-directed mutagenesis using a Quik Change II XL Site-Directed Mutagenesis kit (Stratagene) to substitute aa R506 with A and aa A495 with R. BSR-T7 cells were transfected with the recombinant plasmids and lysed for Western blot experiments 24 h p.t. In cells transfected with the construct pCITE_SgE^{rns}-E2MutA495R, only the E^{rns}–E1 protein and no free E1 protein could be detected (Fig. 3b). In contrast, cells transfected with the construct pCITE_SgE^{rns}-E2MutR506A showed the E^{rns}–E1 protein as well as free E1 protein (Fig. 3b). In addition, in Western blot experiments with the E^{rns}-specific mAb 0103, kindly provided by Christian Schelp (IDEXX, Bern, Switzerland), no free E^{rns} protein could be detected in cells transfected with the construct pCITE_SgE^{rns}-E2MutA495R (Fig. 3b). Probably, this antibody recognizes a conformation-dependent epitope and cannot detect the E^{rns}–E1 protein. This blot was incubated with the E^{rns}-specific antibody (diluted 1 : 1000 in TBS-T) followed by incubation with horseradish peroxidase-conjugated anti-mouse antibody (Dianova) (diluted 1 : 20000 in TBS-T). Therefore, mutation of position P3 of the potential SPP site into an aromatic arginine completely abolished cleavage of the E^{rns}–E1 protein in the pCITE-2a(+) constructs.

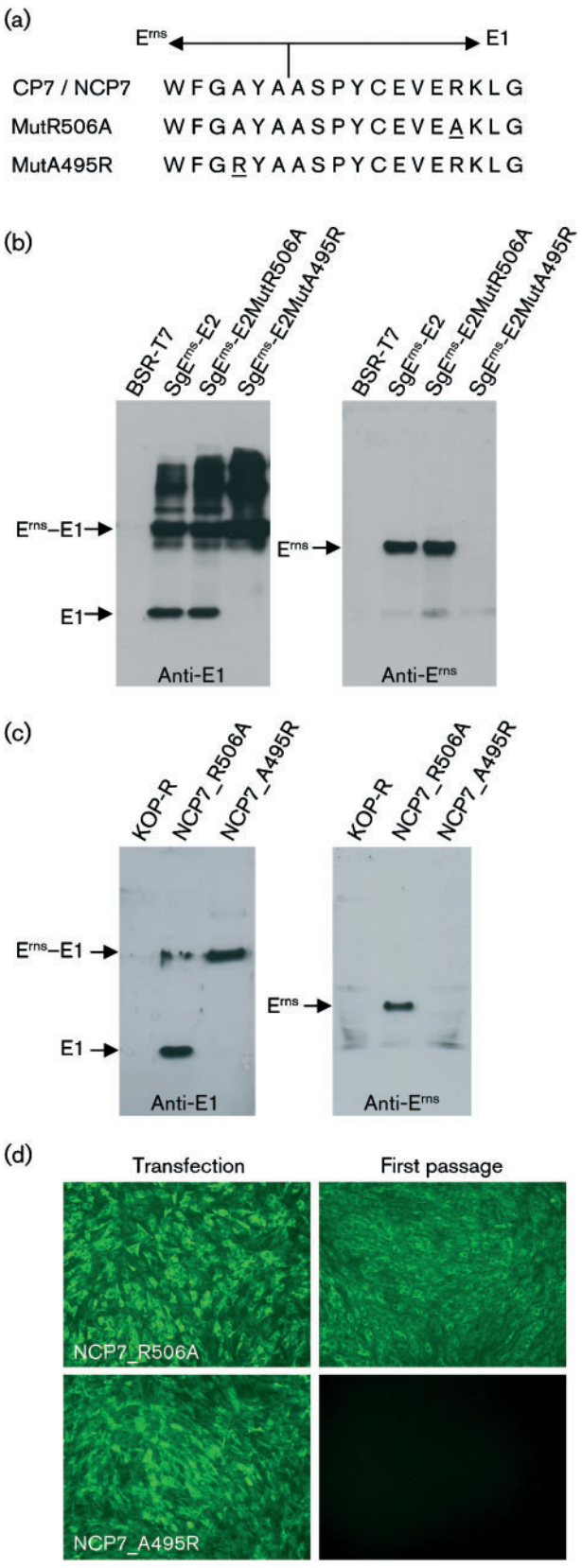

Fig. 3. (a) Amino acid sequence around the protease-cleavage region between E^{rns} and E1 of BVDV type I strain CP7/NCP7 and overview of the mutated putative protease-cleavage sites. Substituted amino acids are underlined. (b) Western blots of BSR-T7 cells transfected with the plasmids pCITE_SgE^{rns}-E2, pCITE_SgE^{rns}-E2MutR506A and pCITE_SgE^{rns}-E2MutA495R with the E1-specific rabbit serum and the E^{rns}-specific mAb 0103. (c) Western blots of KOP-R cells transfected with the full-length cDNA constructs NCP7_R506A and NCP7_A495R with the E1-specific rabbit serum and the E^{rns}-specific mAb 0103. (d) IF staining with mAb C16 of KOP-R cells transfected with the plasmids NCP7_R506A and NCP7_A495R or inoculated with the appropriate transfection supernatants.

Subsequently, the role of this mutation for the generation of infectious virus progeny was investigated with the full-length constructs NCP7_R506A and NCP7_A495R, carrying identical amino acid substitutions. These constructs were generated on the basis of the infectious cDNA clone pA/BVDV/Ins− (Meyers *et al.*, 1996). Further information concerning the cloning procedures of all described constructs is available on request. KOP-R cells were transfected by electroporation with the *in vitro*-synthesized RNAs and lysed for Western blot experiments 48 h p.t. In cells transfected with the construct NCP7_A495R, also only the E^{rns}–E1 protein and no free E1 or E^{rns} protein could be detected (Fig. 3c). The construct NCP7_R506A allowed detection of the E^{rns}–E1 protein, as well as free E1 and E^{rns} protein (Fig. 3c). In contrast to the pCITE-2a(+) system, where the structural proteins were overexpressed, the E^{rns}–E1 protein was visible only as a faint band when virus-infected cells were tested. Obviously, mutation of position P3 of the potential SPP site fully abolished cleavage of the E^{rns}–E1 protein also in full-length constructs. IF staining with mAb C16 revealed that both constructs showed autonomous RNA replication in bovine cells, but only the construct with the amino acid substitution R506 to A produced infectious virus progeny, indicating that cleavage of the E^{rns}–E1 protein is indispensable for the virus (Fig. 3d). In growth-kinetics and Western blot experiments, the virus NCP7_R506A behaved very similarly to the parental virus NCP7 (data not shown). To exclude accidental site mutations in the construct NCP7_A495R, the genomic region C–p7 was sequenced.

Taken together, we could identify a stable E^{rns}–E1 protein with a unique E1-specific antiserum generated by using a synthetic peptide for immunization of rabbits. The detailed reasons for the stability of this protein, at least for 3 h, are still unexplained. Maybe its cleavage takes place at a later time point during infection not in the ER, but in another compartment, such as the Golgi apparatus. This possibility is supported by the as-yet-undefined protease, which is able to cleave the protein independently of a hydrophobic signal peptide. Another explanation is that cleavage of the E^{rns}–E1 protein is very efficient and that detection of this protein is only possible when the protease fails to cleave, e.g. in the

case of overexpression. The estimated molecular mass of 60–65 kDa is contradictory to the theoretical molecular mass of about 75 kDa. Probably, the protein contains immature E^{rns} and E1 proteins that acquire their glycosylation residues later after cleavage. In agreement with previous studies, it could be shown that the protein itself is not essential, but it is beneficial when E^{rns} is generated from the E^{rns}–E1 protein. Maybe cleavage of E^{rns} and E1 generated from the second cistron of the CP7ΔE^{rns}_SgE^{rns}–E1mod virus is more efficient than cleavage of the residual amino acids of E^{rns} and E1 generated from the first cistron of the CP7ΔE^{rns}_SgE^{rns}mod virus. Furthermore, it could be demonstrated that mutation of position P3 of a potential SPP site into an aromatic arginine completely abolished cleavage of the E^{rns}–E1 protein and no infectious virus progeny could be observed. These findings indicate that cleavage of this protein is indispensable for virus replication.

In conclusion, our study showed the existence of an E^{rns}–E1 protein that is not essential for virus growth. Nevertheless, the detailed function of this protein is still unclear. Maybe it has some regulatory function by retaining E1 and releasing it at a late time point of infection so that E1 is the limiting factor for generation of E1–E2 heterodimers. Further experiments must be done to identify the place of cleavage as well as the responsible protease, and to highlight the function of the E^{rns}–E1 protein during virus assembly.

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