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C-E1 fusion protein synthesized by rubella virus DI RNAs maintained during serial passage

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Abstract

Rubella virus (RUB) replicons are derivatives of the RUB infectious cDNA clone that retain the nonstructural open reading frame (NS-ORF) that encodes the replicase proteins but not the structural protein ORF (SP-ORF) that encodes the virion proteins. RUB defective interfering (DI) RNAs contain deletions within the SP-ORF and thus resemble replicons. DI RNAs often retain the 5′ end of the capsid protein (C) gene that has been shown to modulate virus-specific RNA synthesis. However, when replicons either with or without the C gene were passaged serially in the presence of wt RUB as a source of the virion proteins, it was found that neither replicon was maintained and DI RNAs were generated. The majority DI RNA species contained in-frame deletions in the SP-ORF leading to a fusion between the 5′ end of the C gene and the 3′ end of the E1 glycoprotein gene. DI infectious cDNA clones were constructed and transcripts from these DI infectious cDNA clones were maintained during serial passage with wt RUB. The C-E1 fusion protein encoded by the DI RNAs was synthesized and was required for maintenance of the DI RNA during serial passage. This is the first report of a functional novel gene product resulting from deletion during DI RNA generation. Thus far, the role of the C-E1 fusion protein in maintenance of DI RNAs during serial passage remained elusive as it was found that the fusion protein diminished rather than enhanced DI RNA synthesis and was not incorporated into virus particles.

Introduction

Rubella virus (RUB), the etiologic agent of a disease of humans known as rubella or German measles, is the sole member of the *Rubivirus* genus in the *Togaviridae* family (for a review, see Frey, 1994). The genome of RUB is a single-stranded RNA of plus polarity, 9762 nt in length, exclusive of the 3′ poly(A) tract, that contains two long, non-overlapping open reading frames (ORFs). The 5′ proximal ORF (nonstructural protein ORF or NS-ORF), which encodes two nonstructural proteins involved in virus RNA replication, is translated from the genomic RNA, whereas the 3′ proximal ORF (structural protein ORF or SP-ORF), which encodes the three virion proteins [capsid (C) and envelope glycoproteins E1 and E2], is translated from a subgenomic (SG) RNA consisting of roughly the 3′ third of the genomic RNA. The order of the virion proteins within the SP-ORF is 5′-C-E2-E1-3′. Both RNA species are transcribed from a genome-length RNA of minus strand polarity in infected cells.

Besides protein coding regions, viral genomes contain nucleotide sequences or structures, called *cis*-acting elements, essential for recognition by the proteins involved in replication of the genomic RNA, transcription of the SG RNA, and RNA packaging. In some cases, these *cis*-acting elements overlap with the protein coding region, making them difficult to identify and study using genomic infectious cDNA vectors. A commonly used alternate approach has been to employ defective interfering (DI) RNAs (Levis et al., 1986; White et al., 1998). DI RNAs are produced by practically all viruses and contain partially deleted genomes that are spontaneously generated during viral genome replication. Due to the deletion, most DI RNAs fail to replicate autonomously and require co-infection with wt helper virus to provide replication and encapsidation proteins for propagation. DI RNAs are of interest because they maintain CAEs required for replication. Therefore, DI cDNA constructs are generally used in place of genomic infectious cDNA vectors because they are smaller and mutations can be generated without regard for coding sequences. DI RNAs containing reporter proteins have been developed that make assay procedures easier because reporter gene expression

correlates with DI RNA replication (Barclay et al., 1998; Izeta et al., 1999; Khromykh and Westaway, 1997; Levis et al., 1987; Liao et al., 1995; Molenkamp et al., 2000; Tzeng et al., 2001).

The generation of RUB DI RNAs during serial passage and persistent infection in cell culture has been characterized (Abernathy et al., 1990; Bohn and Van Alstyne, 1981; Derdeyn and Frey, 1995; Frey and Hemphill, 1988; Norval, 1979; Terry et al., 1985; Tzeng et al., 2001). DI RNAs generated during undiluted passage of RUB consistently retain the NS-ORF but contain heterogeneous deletions between the NS-ORF and the 3′ *cis*-acting elements and thus have the ability to replicate and frequently to synthesize an SG RNA. These DI RNAs are thus very similar to RUB replicons that we have developed in which the SP-ORF is replaced with a reporter gene (Tzeng et al., 2001). We recently showed that the RUB C protein can modulate replicon RNA synthesis by upregulating the synthesis of the genomic RNA relative to the SG RNA (Tzeng and Frey, 2005). The current study was initiated by the hypothesis that the residual fragment of the C gene frequently retained in RUB DI RNAs was of benefit to the DI RNA by increasing the relative level of DI genome synthesis. Unexpectedly, we found that it was actually a C-E1 fusion protein created by the deletion in the DI RNA that was of benefit and conferred upon a DI RNA the ability to be maintained during serial undiluted passage.

Results

Passaging of replicons and generation of DI RNAs

To test the hypothesis that the presence of part of the C gene conferred a selective advantage on a RUB DI RNA by upregulating the synthesis of the DI genomic RNA, the maintenance of two RUB replicons during serial passage with wt RUB was tested. The first replicon, RUBrep/GFP, expresses the reporter gene GFP and synthesizes a low level of genomic RNA relative to SG RNA (molar ratio of genome/SG ~ 0.25), whereas the second replicon, RUBrep/C-GFP, expresses a C-GFP fusion protein and synthesizes a higher level of genomic RNA relative to SG RNA (genome/SG ~ 0.75) (Tzeng and Frey, 2005; Fig. 1A). Vero cells were transfected with one of these replicons and co-infected with wt RUB and subsequently six serial undiluted passages of the transfected/infected culture fluid were made. Passage of the replicons was detected by GFP expression and additionally virus- and replicon-specific RNAs present in cells after each passage were analyzed by Northern blot. Surprisingly, very few cells exhibited GFP expression in passages of both replicons beyond the transfected/infected culture (termed P0) (data not shown) and the genomic and SG RNAs of both replicons were barely detectable in the P0 culture and essentially undetectable in passages 1–2 (P1–2). Thus, neither replicon was efficiently transmitted in the presence of wt helper virus, disproving the hypothesis. In the passage series with both replicons, at P3 an RNA band appeared that was smaller than the genomic RNA, but of different size than either replicon genomic RNA. Concomitantly, the intensity of this band increased in P4–5 but decreased in P6 whereas the

intensity of the virus genomic RNA decreased in P4–5 and recovered in P6, indicating the generation of *de novo* DI RNAs. To evaluate these *de novo* DI RNAs, RT-PCR of the total cell RNA extracted from these passages was employed using primers spanning the SP-ORF, the region in which deletions in RUB DI RNAs characteristically occur (Fig. 1C). Heterodisperse RT-PCR products were produced from P4–6 RNA of both replicons that were smaller than the RT-PCR product produced from the parent replicon. Despite the heterodisperse nature of the RT-PCR products, a predominant band of ~ 1300 bp was produced from P5–6 of both replicons. The RT-PCR products from P5 of both passage series were cloned and several clones were sequenced to determine the deletions present. As shown in Table 1, the population of deletions was heterogeneous, similar to our previous study (Derdeyn and Frey, 1995). Comparing the deletions in the replicons with which the serial passage series were initiated and the deletions in the resulting DI clones, most of the DIs were generated from wt RUB and relatively few could have been generated from either wt RUB or the replicon (Table 1). The sequence from the downstream deletion break point to the 3′ end of the clone, a region containing the 3′ *cis*-acting elements with the exception of the 3′ eight nts (which were present in the primer used for RT-PCR amplification) were identical in all of the DI clones to the corresponding sequence in wt RUB and both replicons (data not shown).

Despite the heterogeneity of the RT-PCR sequences, 5/14 clones from the wt RUB + RUBrep/GFP passage series had an identical deletion which would yield an RT-PCR product of ~ 1000 nts and 5/15 clones from the wt RUB + RUBrep/C-GFP passage series had an identical deletion which would yield an RT-PCR product of ~ 1200 nts (both of these DI clones were generated from wt RUB). This is the first study of RUB DI-RNAs in which a majority sequence has been identified. Intriguingly, the deletion in the majority DI RNA from both passage series was in-frame, resulting in potential expression of a C-E1 fusion protein, as diagrammed in Fig. 2A.

DI infectious cDNA constructs expressing a C-E1 fusion protein

To test whether expression of a C-E1 fusion protein conferred a selective advantage on a DI-RNA, two DI constructs based on the majority DI RNA sequence from the wt RUB + RUBrep/GFP passage series (designated RUB DI-1) were generated that were tagged at the deletion site with a FLAG epitope tag (Fig. 2B). Because the breakpoints of the deletion in this DI-RNA were in the middle of codons, creating a novel proline codon (CCC) in the C-E1 fusion protein, in one of these constructs (DI-1.1F) the FLAG epitope was placed upstream of this codon and in the second (DI-1.2F) the FLAG epitope was placed downstream of this codon. When cells transfected with these DI constructs were co-infected with wt RUB followed by five serial undiluted passages, DI genomic and SG RNAs were maintained throughout the five passages (Fig. 2C). To prove that the input DI RNA was preserved through five passages, RT-PCR was performed on total cell RNA extracted after P5 with primers spanning the SP-

ORF. With both DIs, a single band was obtained that when subjected directly to sequencing (without cloning) yielded the sequence of the input DI-RNA (data not shown).

To determine if the C-epitope-E1 fusion protein was synthesized in DI-infected cells, Western blot probed with anti-FLAG antiserum was employed. As shown in Fig. 2B, this species was synthesized and its abundance was maintained (DI-1.2F) or increased (DI-1.1F) through the passage series. When translation of the fusion protein was blocked by incorporating into the DI-1.1F and DI-1.2F constructs a mutation that replaced the two in-frame AUGs at the 5′ end of the C gene (these are separated by seven codons) with AUA
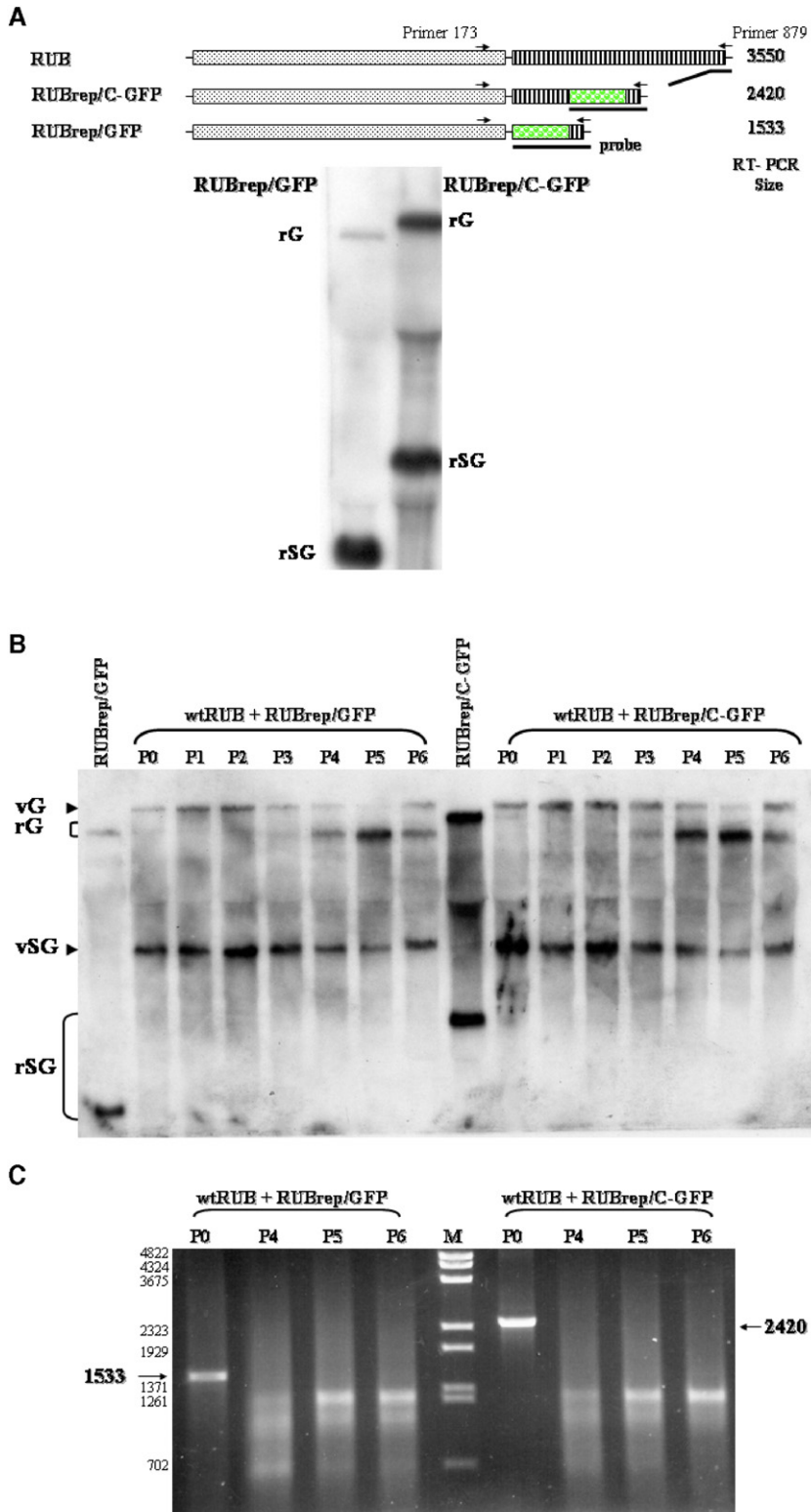
Table 1
Summary of SP-ORF deletions detected in DI-RNAs

wt RUB+RUBrep/GFP P5			wt RUB+RUBrep/C-GFP P5		
Clone [a]	Deletion [a, b]	Amplicon size [a]	Clone [a]	Deletion [a,b]	Amplicon size [a]
1	6392–9359 [b]	583	1	6530–6750, 6840–9209	958
2	6414–9226	737	2 [+]	6892–9237	1204
3	6404–9382 [b]	571	3	6537–8963	1123
4	6414–9364 [b]	599	4 [+]	6892–9237	1204
5*	6895–9456	988	5	6430–9359 [b]	620
6*	6895–9456	988	6	6935–9367 [b]	1117
7*	6895–9456	988	7	6890–9220	1219
8	6893–9229	1213	8	6886–9372 [b]	1063
9*	6895–9456	988	9 [+]	6892–9237	1204
10	6273–9097	725	10	6566–9653 [b]	462
11	6394–9409 [b]	534	11	6899–9235	1213
12	6388–8878	1059	12 [+]	6892–9237	1204
13*	6895–9456	988	13 [+]	6892–9237	1204
14	6899–9235	1213	14	6898–9228	1219
15	6429–9101	877			
RUBrep/GFP	(6512–9334) [b]	1533	RUBrep/C-GFP	(7411–9334) [b]	2420

[a] RT-PCR products amplified from total cell RNA extracted after P5 of wtRUB+RUBrep/GFP and wtRUB+RUBrep/C-GFP were cloned and fifteen clones were sequenced. The deletion in the SP-ORF found in each clone is tabulated, numbered according to the RUB genomic sequence, as is the expected size of the RT-PCR amplicon containing the deletion. The sequences deleted in both replicons are also given. Clones with identical deletions are marked with * and + for the wtRUB+RUBrep/GFP and wtRUB+RUBrep/C-GFP passages, respectively.

[b] Based on the deletion in the replicon, only the DI clones indicated could have been generated from either the replicon or wt RUB. The remainder of the DI clones contain sequences not present in the replicon and therefore were generated from wt RUB.

and GTC (Tzeng and Frey, 2005; see diagram in Fig 2B), neither DI was maintained past P0 in a serial undiluted passage series with wt RUB (data not shown) and thus synthesis of the fusion protein is necessary for DI maintenance during serial undiluted passage. To more rapidly screen for DI RNA maintenance during a passage series, two further DI constructs were generated that placed GFP at the deletion point either upstream (DI-1.1G) or downstream (DI-1.2G) from the proline codon created by the deletion (Fig. 2B). As shown in Figs. 3A and B, both of these DIs were maintained through a serial undiluted passage series in the presence of wt RUB as determined by either fluorescence microscopy of the cells or by Northern blot of RNA extracted from the cells using a GFP probe. To determine if the presence of the C-terminus of E1 as part of the fusion protein was necessary for DI maintenance during serial undiluted passage in the presence of wt RUB, a stop codon was introduced between the GFP and E1 in the DI-1.1G and DI-1.2G constructs (producing DI-1.1GZ and DI-1.2GZ, respectively). As shown in Figs. 2D and E, neither of these constructs was efficiently transmitted past P0 in a passage series with wt RUB and thus the inclusion of the C-

terminal E1 domain in the fusion was necessary for DI maintenance during serial passage.

RNA synthesis by DI constructs

Because the hypothesis that initiated this study was that retention of part of the C gene conferred a selective advantage on DI RNAs during serial passage with wt RUB through enhanced DI genomic RNA synthesis, DI RNA synthesis in comparison to replicon RNA synthesis in transfected cells was characterized. As shown in Fig. 4, through day 3 post-transfection, RUBrep/C-GFP exhibited a more rapid accumulation of both genomic and subgenomic RNA and a greater accumulation of genomic RNA relative to subgenomic RNA than did RUBrep/GFP, illustrating the C protein-mediated enhancement of replicon RNA synthesis and modulation of genomic/SG RNA ratio that we reported previously (Tzeng and Frey, 2005; Tzeng et al., 2006). Surprisingly, both DIs accumulated substantially less of both RNA species and thus enhancement of RNA synthesis played no role in the maintenance of these DI RNAs during serial passage with wt

Fig. 1. Passaging of replicons in presence of wt RUB. (A) At the top of the panel are genomic diagrams of RUB (Robo502) and both replicons. The relative positions of the RT-PCR primers are shown and the amplicon size expected from each genome is given on the right margin. Replicon-specific genomic (rG) and subgenomic (rSG) RNAs were assayed by Northern blot in extracts of cells transfected with transcripts from RUBrep/GFP or RUBrep/C-GFP constructs (lower panel). The probe used was pGEM-GFP, a plasmid containing GFP gene. (B) Cells were infected with wt RUB (MOI=1) and 24 h later were transfected with RUBrep/GFP or RUBrep/C-GFP transcripts and the culture fluid was harvested 2 days post-transfection and one tenth of the culture was used to infect fresh cells. This passaging procedure was repeated for six passages and the medium was harvested 2–3 days post-infection. Following medium harvest after each passage, total cell RNA was extracted from the cell monolayer. Northern blot was used to assay for RUB- and replicon-specific RNAs in each of the passages using pGEM-GFP-3′CSE probe, a plasmid containing the GFP gene and the 3′ 300 nt of the RUB genome (the RUB 3′ cis-acting element or 3′CAE; extracts from RUBrep/GFP and RUBrep/C-GFP-transfected cells were included as controls. (C) Total cell RNA from P0 and P4–6 was used as a template for RT-PCR using primers spanning the SP-ORF. An aliquot of each RT-PCR reaction was analyzed by agarose gel electrophoresis and ethidium bromide staining. The size of the standard markers (M) is given on the left margin (in bp).

RUB (the genomic/SG RNA ratio exhibited by the DIs resembled that of RUBrep/GFP, i.e., no relative increase in the amount of genomic RNA synthesized despite the presence of the N-terminus of the C protein in the C-E1 fusion). The DI-1.1GZ and DI-1.2GZ mutants exhibited RNA accumulation intermediate between the replicons, indicating that the E1 portion of the C-E1 fusion protein was important in down-regulating DI RNA synthesis in comparison to replicons.
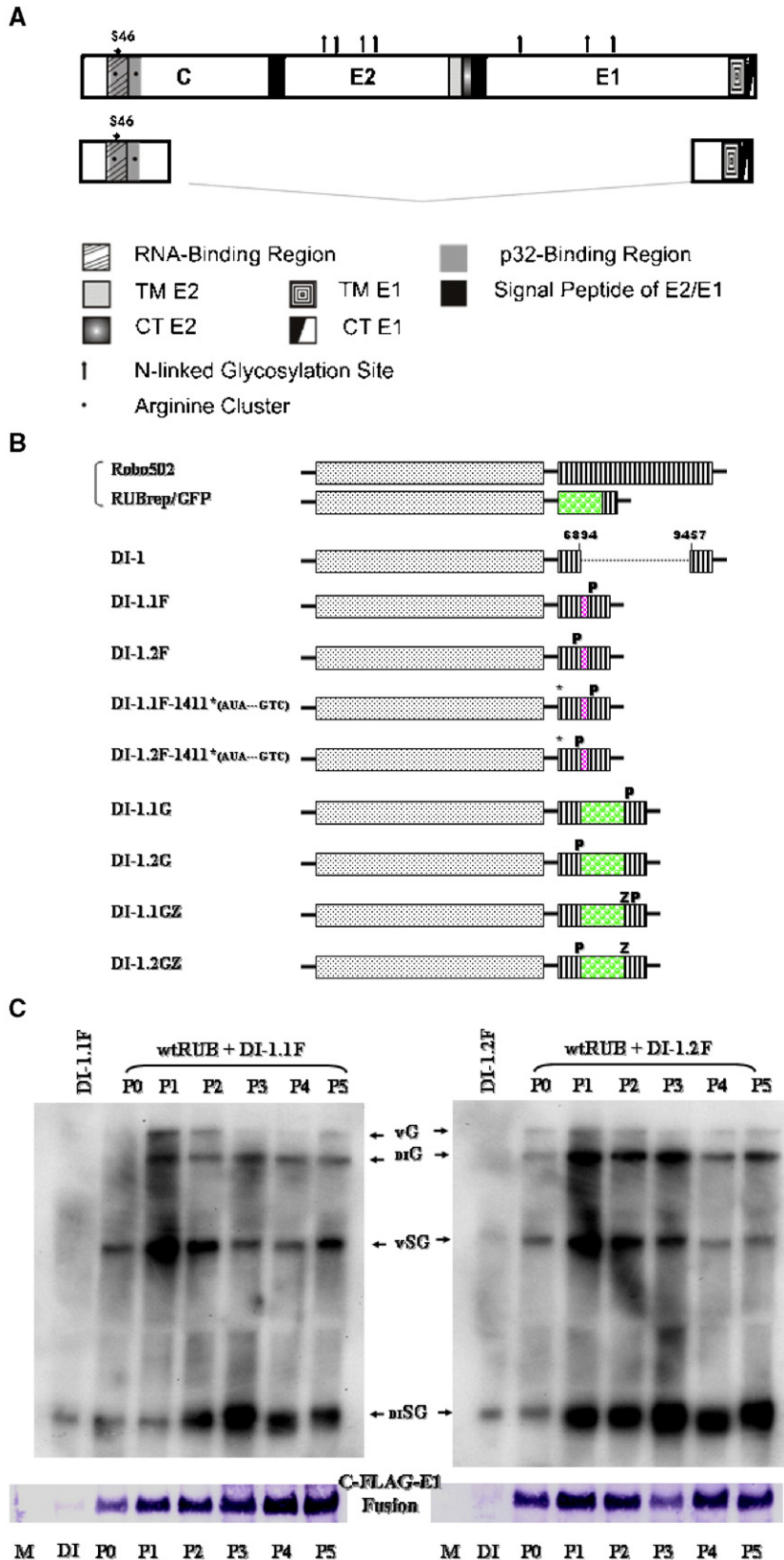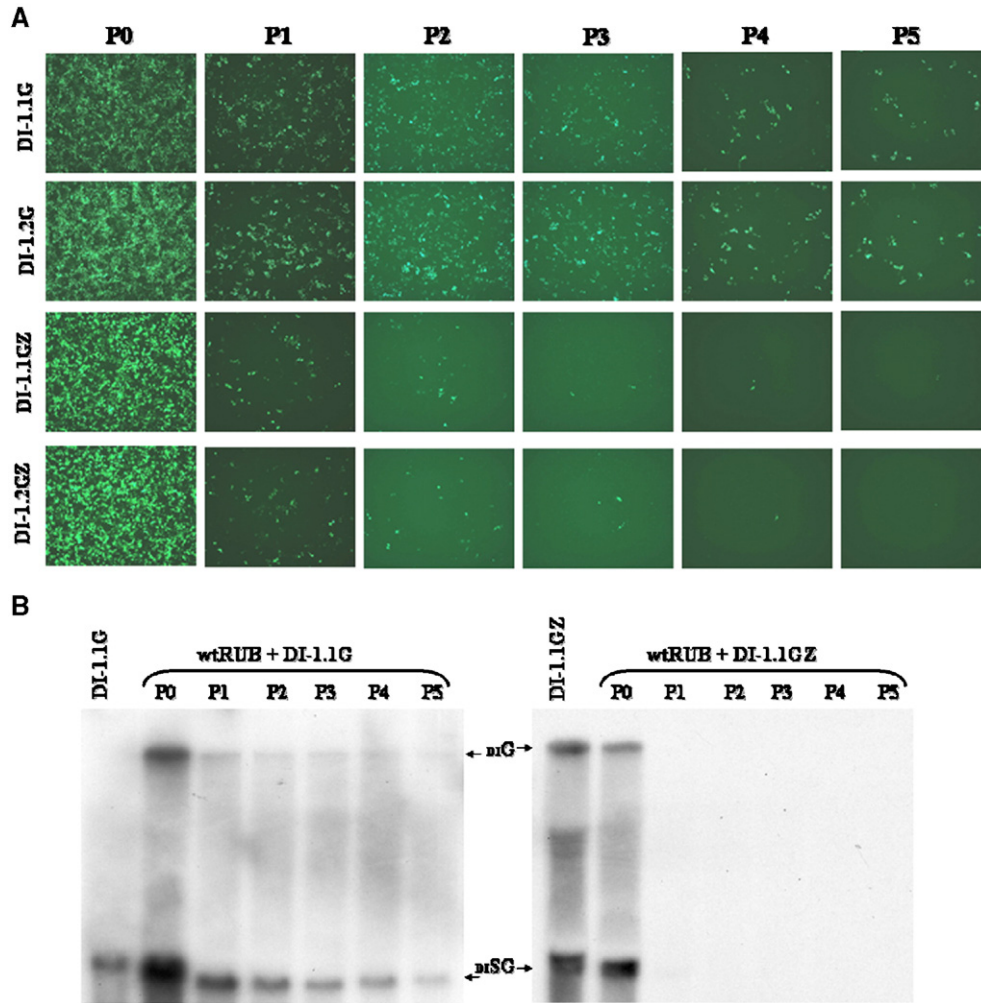
Fig. 3. GFP as a marker for DI RNA maintenance during serial passage. Wt RUB transfected cells were co-transfected with DI-1.1G, DI-1.2G, DI-1.1GZ, or DI-1.2GZ and five serial passages were performed. Following each passage, the cultures were examined for GFP expression by fluorescence microscopy at low magnification (10× objective) (panel A) and subsequently total cellular RNA was extracted and assayed for DI-specific RNA species by Northern blot using pGEM-GFP as a probe (only the DI RNA species are detected using this probe).

Lack of incorporation of C-E1 fusion protein into virus particles

Because packaging is critical to DI maintenance during serial passaging in the presence of wt virus, it was of interest to determine if the C-E1 fusion protein was incorporated into virus particles. We could detect no C-GFP-E1 fusion protein in gradient purified virus particles harvested from P1 of a DI-1.2G+wt RUB passage series (data not shown).

Discussion

The unexpected finding of this study was that deletions in the SP-ORF of DI RNAs of RUB generated during serial passage in the presence of wt RUB create a C-E1 fusion protein that is both expressed and necessary for efficient maintenance of the DI RNA during serial passage. Thus, RUB DI RNAs generated during serial passage require the expression of two ORFs, the

Fig. 2. C-E1 fusion protein and passaging of DI infectious cDNA constructs expressing a C-E1 fusion protein in the presence of wt RUB. (A) Topology of C-E1 fusion protein produced by the majority DI RNA species generated during the wt RUB+RUBrep/GFP passage series (designated as DI-1). Shown are schematic diagrams of the RUB SP-ORF and the C-E1 fusion protein with the location of transmembrane domains (TM), cytoplasmic tails (CT), signal sequences, and N-linked glycosylation sites within the glycoproteins and the RNA and p32 binding domains, which including two arginine clusters, and the S46 phosphorylation control site within panel C. (B) DI infectious cDNA constructs. Under the genomic diagrams of Robo 502, RUBrep/GFP, and DI-1 are a series of DI constructs that incorporate either a FLAG epitope ("F" constructs) or GFP ("G" constructs) at the break point in DI-1. The deletion in DI-1 created a novel proline residue (P) that was placed at the C terminus of the FLAG or GFP insert in DI-1.1 constructs and at the N-terminus of the FLAG or GFP insert in DI-1.2 constructs. In DI-1.1F-1411* and DI-1.2F-1411*, the two in-frame AUGs at the beginning of the C gene were changed to AUA and GTC to eliminate translation of the fusion protein. In the DI-1.1GZ and DI-1.2GZ constructs, a termination codon (Z) was placed at the 3′ end of the GFP insert. (C) Passaging of DI-1.1F and DI-1.2F in the presence of wt RUB. Transfection of wt RUB-infected cells with DI-1.1F and DI-1.2F transcripts, serial passaging, and detection of intracellular RUB-specific RNA species were done as described in the legend to Fig. 1; intracellular RNA from DI-1.1F and DI-1.2F-transfected cells were included as controls. Virus (v) and DI genomic (G) and subgenomic (SG) RNAs are denoted. In addition, Western blots of cell lysates at each passage were probed for production of the C-FLAG-E1 fusion protein using an anti-FLAG monoclonal antibody (M=lysate from mock-infected cells). Only the region from the Western blot containing the fusion protein (∼23.9 kDa) is shown.
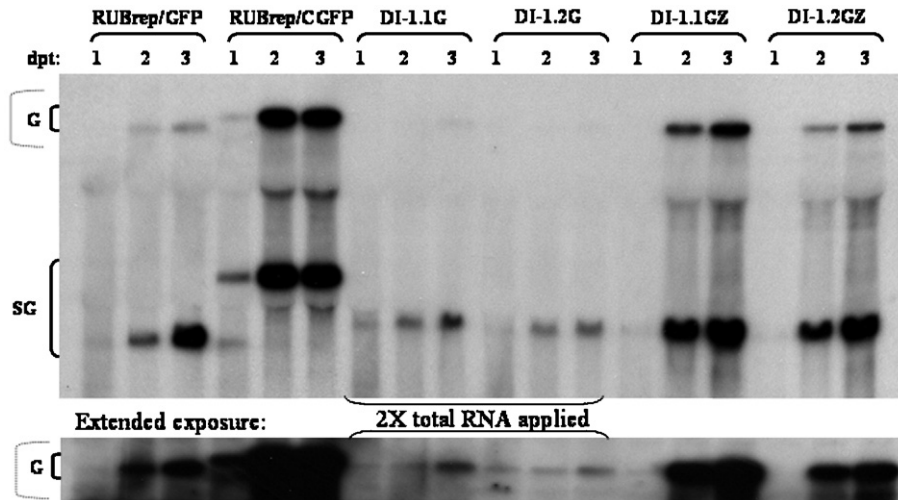
Fig. 4. RNA synthesis in DI-1 vs. replicon transfected cells. Total RNA was extracted from cells transfected with RUBrep/GFP, RUBrep/C-GFP, DI-1.1G, DI-1.2G, DI-1.1GZ, or DI-1.2GZ on 1, 2, and 3 days post-transfection (dpt) and virus-specific RNAs were assayed by Northern blot probed with pGEM-GFP. Equal amounts of total cell RNA were applied to the replicon and DI-GZ lanes whereas twice as much total cell RNA was applied to the DI lanes. Because the genome band was faint in some of the lanes, an extended exposure of the genome bands is shown below the complete blot.

NS-ORF and the SP-ORF with a deletion leading to production of the C-E1 fusion protein. DI RNAs of many other virus genera and families, e.g., Sindbis virus and Semliki Forest virus of the alphavirus genus of the Togavirus family, are reduced to ∼25% or less of the genome, retain only the 5′ and 3′ *cis*-acting elements, and encode no proteins (Lehtovaara et al., 1982; Levis et al., 1986; White et al., 1998). In contrast, DI RNAs of both picornaviruses and flaviviruses contain in-frame deletions in the structural protein genes at the 5′ end of the single ORF contained by the genomes of these viruses, both of which require translation of a genomic or DI RNA prior to its use as a template for replication (Hagino-Yamagishi and Nomoto, 1989; Lancaster et al., 1998). The maintenance of the NS-ORF by RUB DI-RNAs is also mandated by a strong *cis* preference by replicase components for replicating the genome from which they were encoded, rendering replication of a DI RNA by replicase components supplied in *trans* by helper wt RUB a low probability occurrence (Adams et al., 2003; Liang and Gillam, 2001; Tzeng et al., 2001). Interestingly, it was recently found that DI RNAs of the alphavirus, Venezuelan equine encephalitis virus (VEE), generated during serial passage retain proteins nsP1, nsP2, and nsP3 from the NS-ORF and it was shown that expression of these proteins in *cis* was required for packaging of VEE replicons into virus particles (Volkova et al., 2006).

The C-E1 fusion protein created by the deletions in RUB DI RNAs appears to be the only DI-specific protein thus far reported in the literature that is functional in DI maintenance. Deletions in flavivirus DI RNAs lead to synthesis of a truncated NS1 gene product that is present during persistent infection (Blitvich et al., 1999; Chen et al., 1996; Lancaster et al., 1998; Yoon et al., 2006); however, the functionality of this protein has not been demonstrated. DI RNAs of coronaviruses and arteriviruses contain in-frame deletions within ORF1 that encodes the replicase (de Groot et al., 1992; Makino et al., 1990; Molenkamp et al., 2000). Whereas one study showed that the fused ORF1 gene product was translated in vitro (Makino et

al., 1990), two other studies that mutationally disrupted the fused ORF1 yielded conflicting results as to whether these mutations had an effect on DI RNA survival (de Groot et al., 1992; Liao and Lai, 1995). A fusion between the nonstructural and coat protein ORFs was created by deletions in DI RNAs of clover yellow mosaic virus, but the predicted fusion protein was only detected by in vitro translation of the DI RNA template (White et al., 1991).

This study was initiated by the hypothesis that RUB DI RNAs maintain the 5′ end of the C gene because of its role in enhancement and modulation of RNA synthesis. However, although this part of the C protein was included in the C-E1 fusion protein (Fig. 2A), RNA synthesis by DI constructs was substantially reduced in comparison to replicons or DI mutants with a stop codon preventing translation of the E1 part of the fusion protein. Thus, C-mediated enhancement and modulation of RNA synthesis plays no role in DI RNA maintenance during serial passage. We have now done two studies of DI populations generated during serial passage (Derdeyn and Frey, 1995; and this study), each of which included two passages, and whereas DI RNAs with deletions in the SP-ORF were identified in all four of the passages, in two of the passages (P12 in the earlier study and RUBrep/GFP + wt RUB in this study as summarized in Table 2), the 5′ breakpoint of the deletion in up to half or more of the DI RNAs was upstream of the SG RNA start site, rendering the DI RNA incapable of synthesizing either an SG RNA or a C-E1 fusion protein. Whereas this finding potentially indicates that DI RNAs without a C-E1 fusion protein can be successfully passaged, it was not determinable if such DI RNAs had been generated *de novo* during the passage that was sampled or had been generated in previous passages (and thus successfully passaged). It is possible that the C-E1 fusion protein is capable of functioning in *trans* leading to maintenance of DI RNAs that do not synthesize a C-E1 fusion protein during serial passage as long as DI RNAs that synthesize such a protein are also present in the population. In

Table 2
Characteristics of DI RNAs

DI-RNAs with [a]	wtRUB + RUBrep/GFP P5	wtRUB + RUBrep/C-GFP P5
No SG RNA	1,2,3,4,10[b],11,12[b],15	5
SG RNA, deletion in-frame	(5,6,7,9,13)[c], 8	(2,4,9,12,13)[c], 1,3,6,7,8,10, 11, 14
SG RNA, deletion out-of-frame	14	None

[a] Based on the deletion breakpoints of the RT-PCR clones summarized in Table 1, the DI RNAs were characterized as unable to synthesize a subgenomic RNA (because the upstream breakpoint was between the 3′ end of the NS-ORF and the SG start site) or able to synthesize an SG RNA containing an in-frame (creating a C-E1 fusion protein) or out-of-frame deletion in the SP-ORF.

[b] The upstream breakpoint of clones 10 and 12 of the RUBrep/GFP passage series were upstream of the 3′ end of the NS-ORF. In clone 10, an in-frame fusion between the NS-ORF and E1 was produced whereas in clone 12 the NS-ORF and E1 were out of frame.

[c] The majority DI RNAs, i.e., with the same deletion, are grouped by parentheses.

this regard, the C-E1 fusion protein could also exert an effect in *trans* on helper wt RUB replication.

The function of C-E1 fusion protein synthesized by RUB DI RNAs is unknown. The topology of the C-E1 fusion protein synthesized by DI-1, the DI construct analyzed in the study, is shown in Fig. 2A. Within the C part of the fusion protein are the RNA binding domain, the phosphorylated peptide including the serine 46 residue that controls phosphorylation, the region involved in enhancement and modulation of RNA synthesis, and the mitochondrial p32 protein binding site whereas within the E1 part of the fusion protein are the transmembrane domain and the cytoplasmic tail. The presence of the E1 transmembrane domain suggests that the C-E1 fusion protein is membrane associated. Interestingly, C itself is membrane associated via the E2 signal sequence that is retained at its C-terminus (the C-GFP fusion protein synthesized by the RUBrep/C-GFP replicon used in this study also retains the E2 signal sequence). However, the C-E1 fusion protein may have a different intracellular localization than does C and possibly the binding of DI RNA by the C part of the C-E1 fusion protein and its alternate intracellular localization dictated by the E1 part of the C-E1 fusion protein could explain the downregulation of DI RNA synthesis that the C-E1 fusion protein mediates. Despite the putative association of the C-E1 fusion protein with membranes, it was not incorporated into virus particles and thus does not appear to be involved in packaging of DI RNAs into virus particles. Consistent with this observation, we recently found that the packaging efficiency of DI and RUBrep/GFP replicon transcripts was similar in cells co-infected with wt RUB or a line of BHK cells that constitutively express the SP-ORF (C. Claus, unpublished observations). Resolution of the function of the C-E1 fusion protein in DI maintenance during serial passaging thus awaits further experimentation.

Materials and methods

Replicon and DI infectious cDNA clones

The following constructs were described previously: the RUB infectious genomic cDNA clone Robo502 (Tzeng and Frey, 2002); the replicons RUBrep/GFP (Tzeng et al., 2001), RUBrep/C-GFP (Tzeng and Frey, 2003), RUBrep/C(AUA, GTC)-GFP (Tzeng and Frey, 2005). In the initial passage series, Vero cells were infected with wt RUB (F-Therien strain, MOI = 1 pfu/cell) and 24 h later transfected with either RUBrep/GFP or RUBrep/C-GFP transcripts (initial infection or P0). The culture fluid was harvested 2 days later and 1/10 of it was passaged to fresh Vero cells and culture fluid was harvested 2–3 days post passage (passage 1 or P1). This process was repeated for up to six passages. To sample the deletions present in DI RNAs in P4–6 of both passage series, total cell RNA was extracted after culture fluid harvest (Tri-Reagent, Molecular Research Center) and RT-PCR was performed using oligo 879 [5′-CTAGGAATTCACTAGTTTTTTTTTTTTTTTTTTTTTTC-TATACAG-3′; EcoRI and SpeI sites (underlined) followed by T$_{20}$ and a sequence complementary to nts 9755 to 9762 of the genome] to prime reverse transcription and oligos 879 and 173 [5′-CCGGAATTCCGACTACAGCGCGGAGC-3′; EcoRI site (underlined) followed by nts 6244 to 6260 of the genome] for PCR amplification. The amplified products from P5 were restricted with SpeI and EcoRI and subcloned into XbaI and EcoRI-restricted pGEM-3Z vector (Progema). SP6 and T7 primers were used for sequencing as described previously (Tzeng and Frey, 2002). The clones generated were designated by the nts of the RUB genome at which the deletion breakpoint occurred (e.g., the majority deletion in the wt RUB + RUBrep/GFP passage series was designated as pGEM 6895-9456).

To create DI-1.1F and DI-1.2F in which the FLAG epitope gene was introduced into the breakpoint of the deletion in the majority DI RNA species generated during the wt RUB + RUBrep/GFP passage series, a three-round, asymmetric PCR strategy was employed (Tzeng and Frey, 2002). In the first round, the mutagenic oligos 5′-CTGGGGCCACCGACCAACGACTATAAGGAC-GACGACGACAAGCCCTGCGGGGGGCGAGAGC-3′ and 5′-GGGCCACCGACCAACCCCGACTATAAGGACGACGAC-GACAAGTGCGGGGGGCGAGAGCGATC-3′ (FLAG gene underlined), respectively, were used to prime asymmetric amplification on EcoRI-restricted pGEM-6895-9456. In the second round, asymmetric amplification on the first round PCR product as template was primed with oilgo 879 [5′-CTAGGAATT-CACTAGTTTTTTTTTTTTTTTTTTTTTTCTATACAG-3′; EcoRI and SpeI sites (underlined) followed by T$_{20}$ and a sequence complementary to nts 9755 to 9762 of the genome]. In the third round, the second round PCR product and oligo 1047 [5′-TAAGATATCCATGTCGCAGTCGCGCCGGCC-3′; XbaI site (underlined) followed by nts 6605 to 6621 of the genome] was used to prime PCR amplification on EcoRI-linearized pGEM-6895-9456 template. The NotI–EcoI-digested PCR amplification product was included in a three-fragment ligation with the BglII–NotI fragment from RUBrep/GFP (a fragment from nts 5355 to 6622 of the genome) and BglII–EcoRI fragment of RUBrep/GFP (containing the plasmid backbone and the 5′ end of RUBrep/GFP through the BglII site at nt 5355).

To generate the DI-1.1F-1411 and DI-1.2F-1411 in which the AUG1 and AUG2 of the C gene were replaced with AUA and GTC, a three-fragment ligation strategy was employed with the BglII–NotI fragment from RUBrep/C(AUA,GTC)-GFP (a

fragment from nts 5355 to 6622 of the genome), the *Not*I–*Eco*RI fragment from DI-1.1F or DI-1.2F (nt 6622 through the 3′ end of the replicon sequences), and *Bgl*II–*Eco*RI fragment of RUBrep/GFP.

To create DI-1.1G, DI-1.2G, DI-1.1GZ, and DI-1.2GZ, versions of DI-1.1 and DI-1.2 were first generated in which an *Xba*I–*Nsi*I double restriction site was placed at the deletion site. To do so, a three-round, asymmetric PCR strategy was employed. The oligos 5′-GGGGCCACCGACCAACCCCTC-TAGAATGCATTGCGGGGGGCGAGAGCGATC-3′ or 5′-GCTGGGGCCACCGACCAACTCTAGAATGCATCCCT-GCGGGGGGCGAGAGCG-3′ (*Xba*I–*Nsi*I underlined) with pGEM-6895-9456 as templates were used in the first round. Oligo 879 with the first round PCR product as template were used in the second round (asymmetric amplification), and oligo 1047 and the second round PCR product with pGEM-6895-9456 as template were used in the third round. The *Not*I–*Eco*I-digested PCR amplification product was included in a three-fragment ligation with the *Bgl*II–*Not*I fragment from RUBrep/GFP (a fragment from nts 5355 to 6622 of the genome) and *Bgl*II–*Eco*RI fragment of RUBrep/GFP (containing the plasmid backbone and the 5′ end of RUBrep/GFP through the *Bgl*II site at nt 5355). To create DI-1.1G and DI-1.2G, PCR was done using oligo 5′-GTACTCTAGAATGGTGAGCAAGGGC-3′ (*Xba*I site underlined) and oligo 5′-GACTATGCATCTTGTA-CAGCTCGTCCATGCC-3′ (*Nsi*I site was underlined) with pGEM-GFP as template, the amplified product was restricted with *Xba*I and *Nsi*I and ligated with *Xba*I–*Nsi*I-restricted DI-1.1 and DI-1.2, respectively. To create DI-1.1GZ and DI-1.2GZ, the *Xba*I–*Nsi*I fragment of RUBrep/GFP (containing the GFP gene with a stop codon) was ligated with *Xba*I–*Nsi*I-restricted DI-1.1 and DI-1.2, respectively.

In vitro transcription, transfection, and detection of viral RNAs and C-E1 fusion proteins

All plasmids were purified on CsCl isopycnic density gradients prior to use. RUBrep/GFP and RUBrep/C-GFP were linearized with *Eco*RI, whereas the DI constructs were linearized with *Spe*I prior to in vitro transcription, which was carried out as previously described (Tzeng and Frey, 2002). The in vitro transcription reaction mixtures were used directly for transfection without DNase treatment or phenol-chloroform extraction. Vero cells were transfected with Lipofectamine 2000 (Invitrogen) as previously described (Tzeng and Frey, 2002). For low magnification fluorescence microscopy (10 or 20× objective), cells were examined directly without fixation using a Zeiss Axioplan 2 microscope with epifluorescence capacity. Total cell RNA was extracted and virus-, replicon-, and DI-specific RNA species present were detected by Northern blot (Tzeng and Frey, 2002) using a NorthernMax-Gly Kit (Ambion; Houston, TX) and nick-translated, ^{32}P-labeled pGEM-GFP-3′ CAE or pGEM-GFP as a probe. To detect intracellular synthesis of the C-E1 fusion, Western blot analysis of cell lysates (Tzeng et al., 2006) was performed using anti-FLAG monoclonal antibodies (Sigma). To detect the C-E1 fusion protein in virus particles, cells were infected with wt RUB or infected with wt

RUB and co-transfected with DI-1.2G, the medium was passaged once (P1), and the P1 medium was collected three days post-passage. Following purification of virus particles on discontinuous potassium tartrate gradients, resolution of proteins by SDS–PAGE and Western blotting were performed using either anti-E1, E2, and C monoclonal antibodies (viral antigens) or antibodies against GFP (Clonetech) as previously described (Zheng et al., 2003).

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