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Functional Replacement of a Domain in the Rubella Virus P150 Replicase Protein by the Virus Capsid Protein▽

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The rubella virus (RUBV) capsid (C) protein rescues mutants with a lethal deletion between two in-frame NotI sites in the P150 replicase gene, a deletion encompassing nucleotides 1685 to 2192 of the RUBV genome and amino acids (aa) 548 to 717 of P150 (which has a total length of 1,301 aa). The complete domain rescuable by the C protein was mapped to aa 497 to 803 of P150. Introduction of aa 1 to 277 of the C protein (lacking the C-terminal E2 signal sequence) between the NotI sites in the P150 gene in a replicon construct yielded a viable construct that synthesized viral RNA with wild-type kinetics, indicating that C and this region of P150 share a common function. Further genetic analysis revealed that an arginine-rich motif between aa 60 and 68 of the C protein was necessary for the rescue of ΔNotI deletion mutants and substituted for an arginine-rich motif between aa 731 and 735 of the P150 protein when the C protein was introduced into P150. Possible common functions shared by these arginine-rich motifs include RNA binding and interaction with cell proteins.

Rubella virus (RUBV) (family *Togaviridae*, genus *Rubivirus*) is a plus-strand RNA virus with a 10-kb genome containing two open reading frames (ORFs): the 5′ ORF encodes the P150 and P90 replicase proteins, and the 3′ ORF encodes the virion structural proteins, the capsid (C) and envelope glycoproteins E2 and E1. After translation of P150 and P90 from the genome RNA, the genome RNA is used as a template to produce a genome-length minus-strand RNA, which in turn is used as a template for the plus-strand genomic RNA (gRNA) and a subgenomic RNA (sgRNA), from which the structural proteins are translated (7).

In addition to being structural components of the virion, capsid proteins play roles in the virus replication cycle distinct from virion assembly (1, 4, 6, 11, 14, 15, 18, 20, 27). RUBV C participates in RNA synthesis in an apparent variety of ways. C accelerates the time course of RNA synthesis by replicons (virus constructs in which the structural protein ORF is replaced by a reporter gene) (26), as well as rescues two distinct types of mutants: (i) virus and replicon mutants with a deletion between two in-frame NotI sites (nucleotides [nt] 1685 and 2192 of the RUBV genome) in the P150 gene (23) and (ii) replicon mutants with a variety of deletions in the 5′ and 3′ *cis*-acting elements (CAEs) of the genome (5, 26). Rescue is at an early step in the replication cycle, before accumulation of detectable RNA. Finally, C participates at a later stage in RNA synthesis by modulating the ratio of gRNA and sgRNA (25).

In a recent study (26), we found C associated with P150, leading us to propose a working model of C-mediated mutant rescue in which, by virtue of its binding to P150, C is included in the replication complexes in which virus RNA synthesis occurs. We hypothesize that C shares a common function with the NotI region of the P150 gene, allowing it to rescue NotI deletion mutants. The rescue of CAE mutants suggests that the role played by C is in the recruitment of RNA to the replication complex and/or in serving as a chaperone to modulate the secondary structure of the RNA to facilitate replication, a role played by capsid or nucleocapsid proteins of other viruses (1, 6, 8, 9, 13, 15, 29).

In previous studies, we utilized constructs with deletions between the two in-frame NotI sites (at nt 1685 and 2192 of the RUBV genome) (22, 23, 26). We began the present study by mapping the complete region of the P150 gene that can be rescued by C and found it to extend both upstream and downstream from the region delineated by the two NotI sites. We termed this expanded region the "Q" domain of P150. We then tested one hypothesis from our working model, namely, that C and the Q domain of P150 share a common function, by substituting the C gene between the NotI sites in the P150 gene in a replicon construct, with the result that the construct was viable and exhibited wild-type kinetics of replicon-specific RNA synthesis. Further genetic analysis revealed that an arginine-rich motif within the C gene was necessary for C-mediated rescue of ΔNotI replicons and substituted for an arginine-rich motif in the Q domain of P150 when C was introduced into P150.

MATERIALS AND METHODS

Constructs. The replicons RUBrep/GFP, RUBrep/C-GFP, and RUBrep/GFP-ΔNotI, the latter of which contained a deletion between nt 1685 and 2192 of the RUBV genome, were initially described elsewhere (23). A series of deletions which expanded the NotI deletion both 5′ terminal to nt 1685 and 3′ terminal to nt 2172 were made using strategies described previously (24). Progressive N-terminal deletions of C were made by PCR using primers with NotI sites for ready introduction at the deletion site in RUBrep/GFP-Δ548-802. A PCR amplicon encoding aa 1 to 277 of C was also introduced into the pcDNA expression vector (Invitrogen). The substitution mutageneses of two arginine-rich motifs in C (R1 between aa 35 and 43, in which RRPRPPRQR was changed to AAPAPPAQA, and R2 between aa 60 and 68, in which RRRRGNRGR was changed to AAAAGNAGA) and an arginine-rich motif in P150 (R_O between aa 731 and 735, in which RRARR was changed to AAAAA, QQAQQ, or KKAKK) were performed using strategies described previously (23, 26). The specific details of any of these mutageneses described are available upon request.

Viability assays. After mutagenesis or other described manipulations, plasmids containing each replicon construct were isolated from a minimum of four

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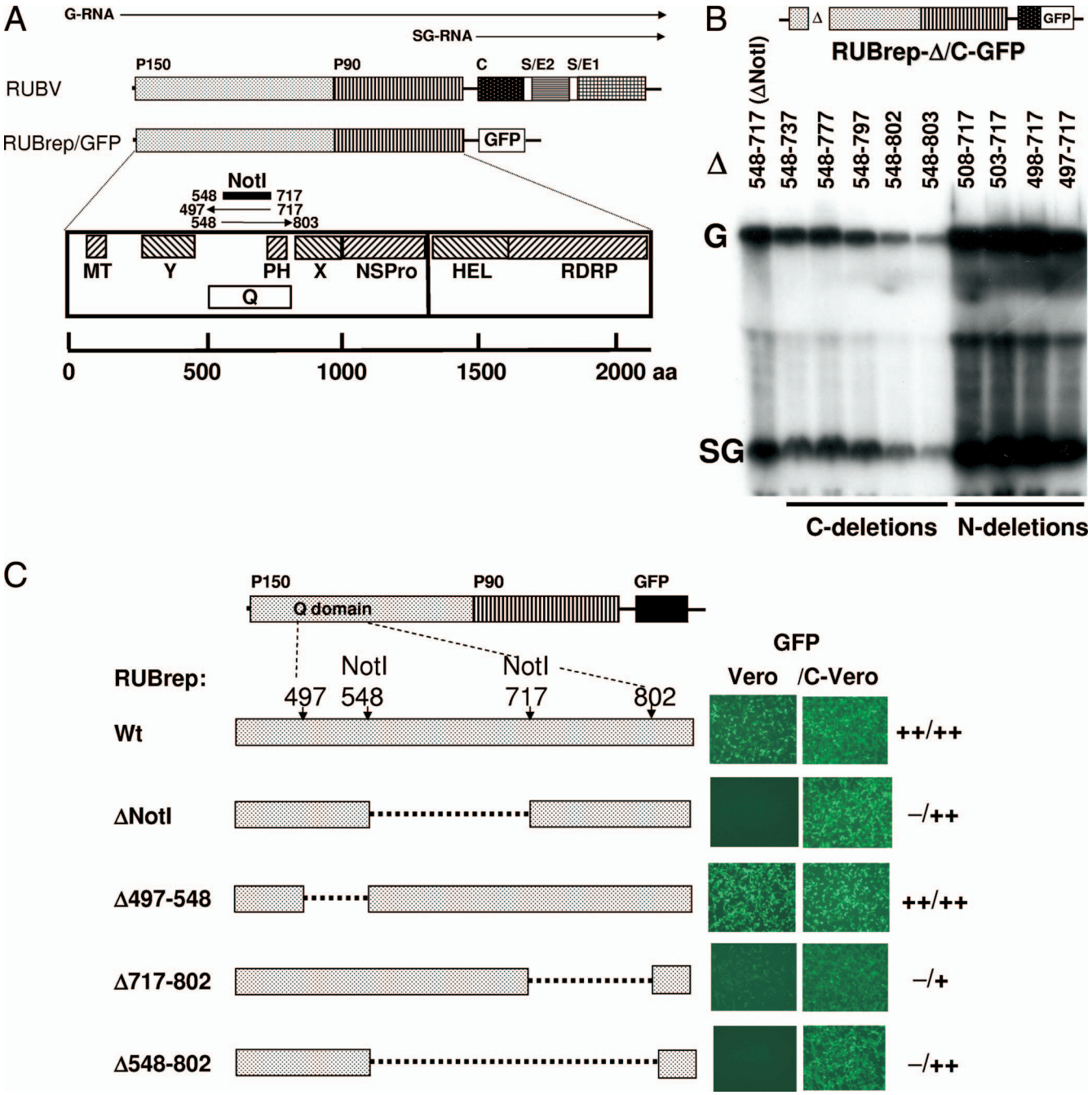

FIG. 1. Mapping the "Q" domain domain of P150. (A) At the top of the panel is a schematic diagram of the genomes of RUBV and the RUBrep/GFP replicon showing untranslated regions as lines, the two ORFs as boxes (with the extent of the individual genes demarcated), plus the two RNAs from which the ORFs are translated (G-RNA, gRNA; SG-RNA, sgRNA). In the RUBV genomic diagram, the white regions between C-E2 and E2-E1 are the hydrophobic signal sequences (S) of E2 and E1, respectively, which remain attached to C and E2, respectively, after cleavage. Below the genomes is an expanded diagram of the nonstructural protein ORF with the location of the NotI region and previously defined or predicted domains and motifs: MT, methyl/guanylyl-transferase; PH, proline hinge; NSPro, nonstructural protease; HEL, helicase; RDRP, RNA-dependent-RNA-polymerase. The "Y" domain, of unknown function, is also found in HEV and BNYVV, while the "X" domain has ADP-ribose-1″-phosphatase activity (19) and is also found in HEV, BNYVV, alphaviruses, and some coronaviruses. To map the domain of P150 that can be rescued by C protein, progressive deletions were made in RUBrep-ΔNotI/GFP, and the ability of the replicon containing each deletion to be rescued was determined by the presence or absence of GFP expression in Vero cells that had been transfected with the replicon and coinfected with RUBV (as a source of C protein). Deletions first proceeded in a 5′ direction from the upstream NotI site and then 3′ from the downstream NotI site. The largest deletions that could be rescued in each direction are shown as single-headed arrows. The newly defined "Q" (as in "res-Q") domain is entered on the domain map. (B) Subsequently, deletions that could be rescued were introduced into RUBrep/C-GFP (rescue is mediated by the C-GFP fusion proteins; a schematic genomic diagram of this replicon is shown), and replicon RNA synthesis was assayed in transfected Vero cells 3 days posttransfection by Northern hybridization using GFP gene as a probe (23, 26). (C) To test whether the regions of the Q domain upstream and downstream of the NotI region were necessary for viability, RUBrep/GFP derivatives were constructed with deletions in the Q domain between the N terminus of the domain and the upstream NotI site (aa 497 to 548), between the downstream NotI site

to six bacterial colonies and sequenced to ascertain that the desired modification had been made, and then the resident replicon was tested for viability. Synthesis of in vitro replicon transcripts and subsequent transfection of Vero cells and C-Vero cells, a line of Vero cells that constitutively expresses aa 1 to 277 of C, were done as described previously (26). Cells were examined microscopically for green fluorescent protein (GFP) expression 2 to 3 days posttransfection by using a Zeiss Axioplan microscope with epifluorescence capacity, and images were made using a ×10 objective lens. Replicons were considered nonviable only if all four to six clones screened were negative for GFP. Lysis of transfected cells and processing for replicon RNA analysis by Northern blotting or protein analysis by Western blotting was performed as previously described (26).

RESULTS

Mapping the boundaries of the region within P150 that can be rescued by C. To determine the extent of the region of P150 that can be deleted but rescued by C, progressive expansions of the NotI deletion were first made 5′ from the upstream NotI site (nt 1685 of the genome, which encodes aa 548 of P150) in the replicon RUBrep/GFP-ΔNotI. Vero cells transfected with these constructs were coinfected with RUBV (as a source of C), and the rescue of replicon replication was monitored by GFP expression (GFP expression can only occur if replicon replication occurs, successfully leading to the production of the sgRNA from which GFP is translated). In the first set of progressive expansions, it was found that a deletion beginning at aa 508 of P150 could be rescued, but a deletion beginning at aa 488 could not. Finer mapping between these sites showed that a deletion beginning at aa 497 was rescuable, but one beginning at aa 496 was not, defining the N-terminal end of rescuable region as aa 497. Similarly, when progressive expansion of the NotI deletion was done 3′ to the downstream NotI site (nt 2192 of the RUBV genome encoding aa 717 of P150), coarse mapping showed that deletion to aa 800 could be rescued by C, but deletion to aa 821 could not, and finer mapping defined the C-terminal boundary as being between aa 803 (which could be rescued) and aa 804 (which could not).

All of the expanded NotI deletions in both directions that could be rescued by C provided by RUBV were also viable when introduced into RUBrep/C-GFP-ΔNotI (in this replicon, C is provided as a C-GFP fusion protein). Analysis of RNA synthesized by these two series of replicons is shown in Fig. 1B. Interestingly, replicons with the NotI deletion expanded in the N-terminal direction synthesized as much or more of both gRNA and sgRNA than did RUBrep/C-GFP-ΔNotI, whereas replicons with the NotI deletion expanded in the C-terminal direction synthesized less of both RNA species with increasing size of the deletion. In addition, when rescuable, expanded deletions in both directions were introduced into the infectious cDNA virus clone, Robo502 (which expresses C as part of the structural protein ORF), all of the resulting constructs were viable (data not shown).

We designated this expanded domain that can be rescued by C as the "Q" domain. In the mapping studies described above, all of the expanded deletions included the region between the NotI sites. As shown in Fig. 1C, when the region between the mapped N terminus of the Q domain and the upstream NotI site (aa 497 to 548) was deleted in RUBrep/GFP, the resulting construct was viable; however, when the region between the downstream NotI site and the mapped C terminus of the Q domain (aa 717 to 802) was similarly deleted, a viable construct was not recovered (this construct was rescued in C-Vero cells, a cell line that constitutively expresses C). Although it was unexpected that the region between the N terminus of the Q domain and the upstream NotI site was dispensable for viability, this finding appeared consistent with the analysis of RNA synthesis shown in Fig. 1B that showed that expansion of the NotI deletion in the N-terminal direction had no effect on replicon RNA synthesis (in the presence of the C protein), while expansion in the C-terminal direction progressively reduced RNA synthesis. As expected, a construct with a deletion from the upstream NotI site through the C terminus of the Q domain (RUBrep/GFP-Δ548-802) was also lethal in Vero cells but was rescued in C-Vero cells.

Introduction of C into P150. To test our hypothesis that C and the Q domain of P150 share a common function, we first substituted the complete C gene (300 aa) for the region between the NotI sites in the P150 gene in the replicon, RUBrep/GFP, but the resulting replicon (RUBrep-1220/GFP) was not viable (Fig. 2A). However, when residues 1 to 277 of C were used (i.e., lacking the C-terminal 23 aa that function as a signal sequence for E2; see the map of C in Fig. 2B), the resulting construct (RUBrep-1221/GFP) expressed GFP, although with reduced intensity compared to the wild-type replicon, thus proving our hypothesis. We reasoned that RUBrep-1220/GFP was not viable due to interference with proper targeting of P150 by the E2 signal sequence. As controls, two reporter genes, CAT and GFP, and C(1-300) and C(1-277) in the reverse orientation were substituted for the NotI region, with the result that none of these constructs was viable (data not shown). The time course of RNA accumulation by RUBrep-1221/GFP was similar to that of wild-type RUBrep/GFP (Fig. 2B). The wild-type replicon synthesized more sgRNA than did RUBrep-1221/GFP, likely explaining the relatively lower level of expression of GFP by the mutant replicon; however, the mutant replicon synthesized more gRNA than did the wild-type replicon. P150 with the C(1-277) substitution was larger than wild-type P150, and this size was maintained throughout the standard transfection time course (i.e., through 4 days posttransfection) (Fig. 2C), indicating that the C(1-277) substitution was retained by the mutant replicon. Subsequently, we found that when C(1-277) was substituted for the expanded deletion from the upstream NotI site through the C terminus

and C terminus of the domain (aa 717 to 802), and between the upstream NotI site and the C terminus of the domain (aa 548 to 802). (It should be noted that in these constructs, aa 802 was used as the C terminus of the Q domain because of the restricted amount of RNA synthesis in deletions extending to aa 803, as shown in panel B.) In vitro transcripts of these derivatives were transfected into both Vero cells and C-Vero cells, a line of Vero cells constitutively expressing C, and the ability to replicate was determined by GFP expression. Micrographs were taken on day 2 (C-Vero cells) or day 3 (Vero cells) posttransfection. The relative GFP intensity in each cell line transfected with wild-type (Wt) RUBrep/GFP was scored as "++."

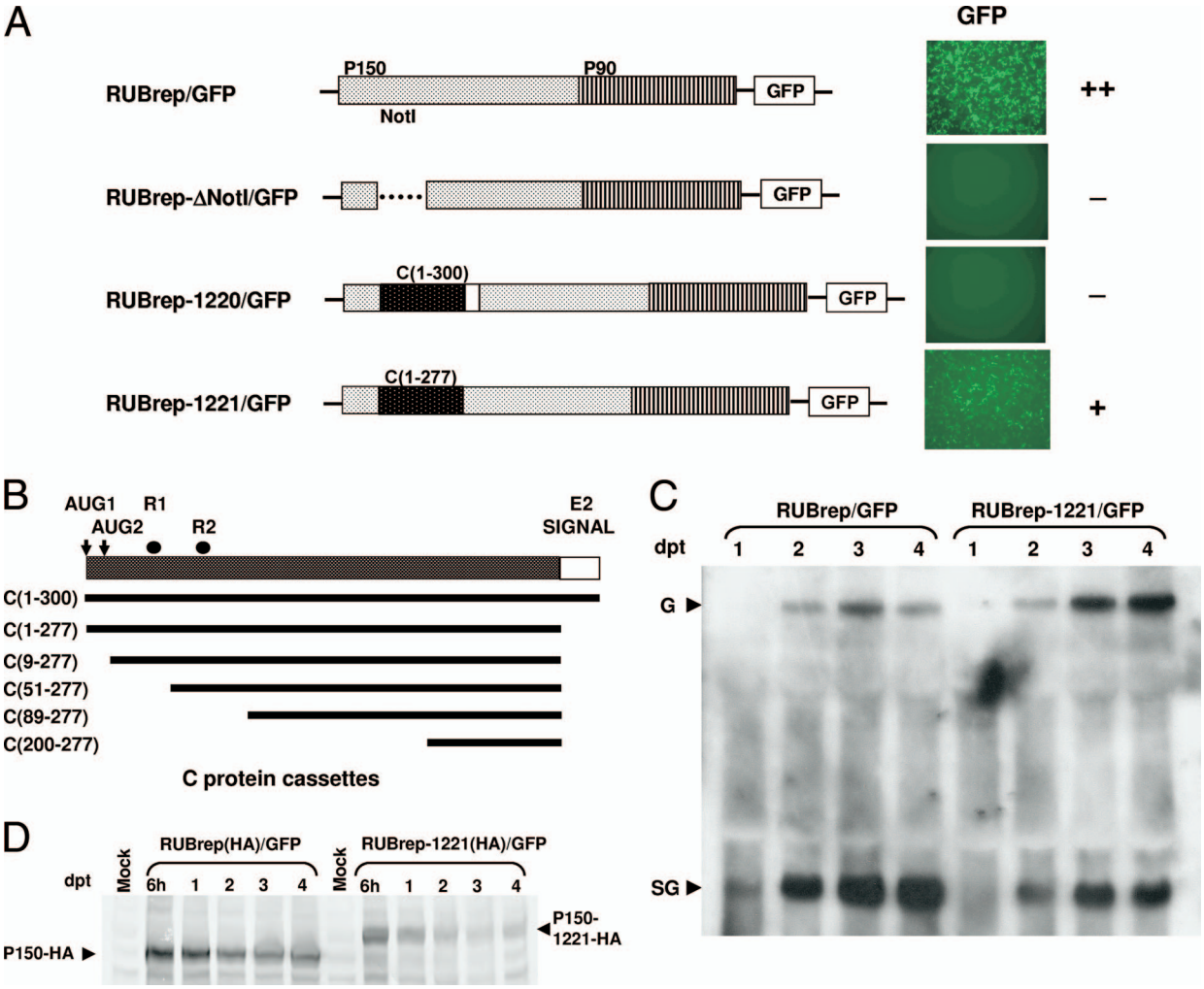

FIG. 2. Substitution of C for the NotI region of P150. (A) Genomic diagrams are shown of RUBrep/GFP, RUBrep/GFP-ΔNotI, and derivative replicons in which the NotI region of P150 was replaced with C(1-300), the complete C gene encoding all 300 aa (RUBrep-1220/GFP), or C(1-277), the C gene lacking the 23-aa signal sequence for E2 (RUBrep-1221/GFP). Vero cells were transfected with in vitro transcripts of these replicon constructs and ability of the construct to replicate was determined by the expression of the GFP reporter gene. The micrographs were taken on day 3 posttransfection. The relative GFP intensity in wild-type RUBrep/GFP-transfected cells was scored as "++." (B) Map of landmarks within C and N- and C-terminally deleted cassettes of C introduced into the Q domain of P150. Landmarks shown include the in-frame AUGs at aa 1 and 9; the two arginine-rich clusters, R1 (aa 35 to 43) and R2 (aa 60 to 68); and the C-terminal signal sequence for E2 (aa 278 to 300). (C) Vero cells were transfected with RUBrep/GFP and RUBrep-1221/GFP transcripts and, on the indicated days posttransfection (dpt), total intracellular RNA was extracted, and the accumulation of gRNA and sgRNA was detected by Northern hybridization using a GFP gene probe (23, 26). (D) Vero cells were transfected with transcripts from RUBrep-HA/GFP or RUBrep-1221-HA/GFP, constructs with an HA epitope tag inserted immediately upstream from the 5′ NotI site (26). At the indicated day posttransfection (dpt), transfected cells were lysed, and the HA-tagged P150 was detected by Western blotting probed with anti-HA monoclonal antibody. Because the C insert (277 aa) is larger than the NotI region (169 aa), the P150 from the RUBrep-1221-HA/GFP replicon is larger than wild-type P150-HA.

of the Q domain (aa 548 to 802, see Fig. 1C), a viable replicon was recovered (data not shown).

Mapping of the C insertion in P150 and of the Q domain. RUBrep/GFP-Δ548-802, which contains the deletion between the upstream NotI site and the C terminus of the Q domain, was used as a scaffold for deletion mutagenesis of C to examine which regions of C when introduced into this deletion in P150 were necessary for viability. As shown in Table 1, when C(1-277), as well as corresponding cassettes with deletions of the N-terminal 9 and 51 aa of C, were introduced, viable replicons were recovered. Interestingly, the replicon with aa 51 to 277 of C inserted into P150 exhibited enhanced GFP expression. However, the constructs with cassettes with N-terminal deletions of 89 and 200 aa of C introduced into P150 were not viable. These results were consistent with findings from our previous study in which we mapped the region of C necessary for ΔNotI replicon rescue in *trans* to aa 1 to 88 (23) and, taken together, indicate that the functional moiety within C responsible for the viability of some of these constructs with C introduced into P150 lies between residues 51 and 88. A notable landmark within this region is an arginine-rich motif between residues 60 and 68; this motif is designated "R2" since there is another R-rich motif (R1) upstream between residues 35 and 43 (Fig. 2B). When C(51-277) with the arginines in the R2

TABLE 1. Wild-type or RUBrep construct substitutions and GFP intensities[a]

Wild type or RUBrep construct	Substitution for aa 548 to 802 of P150	GFP intensity[b]
Wild type		++
Δ548-802	Δ	−
Δ548-802:C	C(1-277)	+
Δ548-802:CΔ9	C(9-277)	+
Δ548-802:CΔ51	C(51-277)	+++
Δ548-802:CΔ51*[c]	C(51-277/R2A)	−
Δ548-802:CΔ89	C(89-277)	−
Δ548-802:CΔ200	C(200-277)	−

[a] Derivatives of RUBrep/GFP were engineered with a deletion between aa 548 and 802 of P150 (see Fig. 1C) or with this region replaced with the progressive N-terminal deletion series of the C protein (see Fig. 2B).

[b] In vitro transcripts of each construct were used to transfect Vero cells, and GFP expression was analyzed by fluorescence microscopy at 3 days posttransfection. The relative GFP intensity in wild-type RUBrep/GFP-transfected cells was scored as "++."

[c] One construct, RUBrep/GFP-ΔNotI:CΔ51*, also contained a substitution mutation in which members of the R2 arginine-rich motif were changed to alanine.

motif mutated to alanine (R2A) was inserted in the deletion in RUBrep/GFP-Δ548-802, the construct was not viable, indicating that the R2 motif was necessary for viability (Table 1) (this construct was rescued in C-Vero cells [data not shown]).

Identification of an arginine-rich motif in the Q domain necessary for viability. Notably, there is an arginine-rich motif in the Q domain at aa 731 to 735 between the downstream NotI site and the C terminus of the domain, which was termed R_Q. To test whether these residues were critical for viability, three mutations were constructed in RUBrep/GFP, one in which the arginine residues were mutated to alanine (R_QA), a second in which the arginine residues were mutated to glutamine (R_QQ), and a third in which the arginine residues were mutated to lysine (R_QK). All three mutant replicons were nonviable in Vero cells, but all three were rescued in C-Vero cells (Table 2). When C(1-277) was inserted between the NotI sites in RUBrep-R_QA, viability was recovered (Fig. 3). If the C(1-277) insert contained a mutation of the first R motif to alanine (R1A), the replicon was still viable (and GFP expression was higher than with either RUBrep-R_QA/GFP or RUBrep/GFP); however, if both arginine motifs were mutated to alanine (R1A/R2A), viability was lost (Fig. 3).

TABLE 2. Wild-type or RUBrep construct R_Q sequence and GFP Vero/C-Vero expression[a]

Wild type or RUBrep construct	R_Q sequence	GFP Vero/C-Vero expression
Wild type	RRARR	++/++
R_QA	AAAAA	−/++
R_QQ	QQAQQ	−/++
R_QK	KKAKK	−/++

[a] RUBrep/GFP was mutagenized to replace the members of the arginine-rich motif in the Q domain (R_Q, aa 730 to 734 of P150) with alanine, glutamine, or lysine. In vitro transcripts of each replicon construct were used to transfect Vero cells and C-Vero cells, and GFP expression was analyzed by fluorescence microscopy 2 days (C-Vero cells) or 3 days (Vero cells) posttransfection. The relative GFP intensity in wild-type RUBrep/GFP transfected cells was scored as "++."

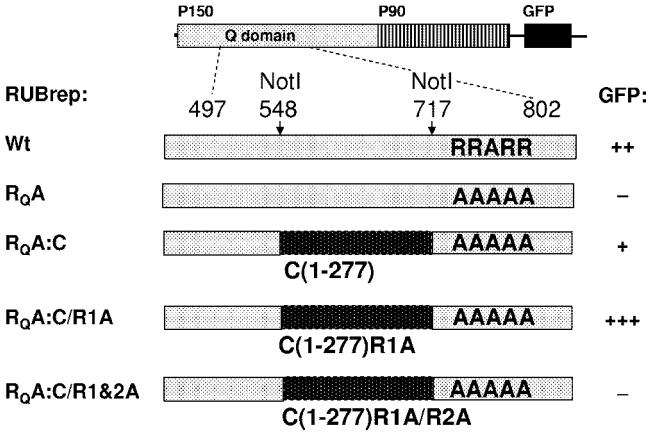

FIG. 3. Viability of replicons with a mutated R_Q motif in the presence of the C gene. To determine whether one or both of the arginine-rich motifs in C could compensate for mutation of the R_Q motif in P150, the NotI region in RUBrep-R_QA was replaced with C(1-277) containing the wild-type (Wt) sequence or with either the R1 motif mutated to alanine (R1A) or both the R1 and the R2 motifs mutated to alanine (R1A/R2A). The resulting replicons were used to transfect Vero cells, and the ability to replicate was determined by GFP expression on 2 days posttransfection. The relative GFP intensity in wild-type RUBrep/GFP-transfected cells was scored as "++."

Analysis of the arginine-rich motifs in C on rescue in *trans*. The results to this point show that the arginine-rich motif at the C terminus of the Q domain of P150 is necessary for infectivity but can be compensated for by arginine-rich motifs in C when C is substituted in the Q domain. Mutations of the arginine-rich motif in P150 can be rescued, as well, by the presence of C in *trans* in C-Vero cells. We next examined the role of the arginine-rich motifs in C on *trans* rescue. C(1-277) constructs containing the wild-type sequence or the R1A, R2A, or R1A/R2A mutations were cotransfected into Vero cells with RUBrep/GFP-ΔNotI replicon transcripts. As shown in Fig. 4, wild-type C and C with the R1A mutation rescued the ΔNotI replicon, whereas C with either the R2A or the R1A/R2A

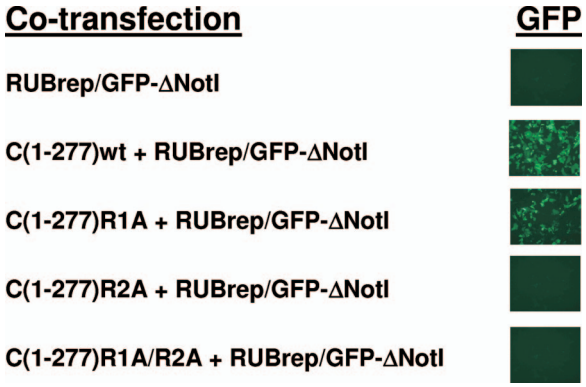

FIG. 4. RUBrep/GFP-ΔNotI rescue by C with mutations in the arginine-rich motifs. Vero cells transfected with RUBrep/GFP-ΔNotI were cotransfected with plasmids expressing wild-type C(1-277) or C(1-277) with either the first arginine-rich motif (R1A), a second arginine-rich motif (R2A), or both arginine-rich motifs (R1A/R2A) mutated to alanine. Rescue of RUBrep/GFP-ΔNotI was determined by GFP expression. Micrographs were taken on day 2 posttransfection.

mutation did not. Thus, the arginine-rich R2 motif in C is specifically necessary for rescue in *trans*.

DISCUSSION

We have been studying the unexpected finding that a lethal deletion in one of the RUBV replicase proteins, P150, is rescued by the virus C protein (22, 23). This deletion was created between two in-frame NotI sites at nt 1685 to 2192 of the RUBV genome which encode aa 548 to 717 of P150, but we have now mapped the complete domain that can be rescued by C as being between aa 497 and 803 of P150. As can be seen in the RUBV nonstructural protein domain map in Fig. 1A, this new domain, which we term the "Q" domain, fills the space between two domains, the Y and X domains, previously identified by computer-assisted homology searching (12). The Y domain, of unknown function, also exists in the replicase proteins of hepatitis E virus (HEV) and beet necrotic yellow vein virus (BNYVV) but, interestingly, not the alphaviruses, the other genus in the *Togaviridae* family. The X domain is also found in HEV and BNYVV, as well as the alphaviruses and some coronaviruses. The X domain shares homology with enzymes with ADP-ribose-1″-phosphatase activity and the domain from coronaviruses can bind poly-ADP-ribose and therefore may be involved in binding poly-ADP-ribosylated proteins in infected cells (19, 21, 28). The N-terminal two-thirds of the Q domain shares no homology with other viruses; however, the C-terminal third contains a previously recognized "proline hinge" that also exists in HEV (12).

Capsid-mediated rescue of mutant replicons with deletions in the Q domain occurs at an early step in the replication cycle since RUBrep/GFP-ΔNotI accumulates no detectable RNA in Vero cells (23). Our working model is that C and the Q domain share a common function that C can exert in the replication process by virtue of its ability to bind to P150 (26). The goal of this project was to test a hypothesis based on this model, namely, that C and the Q domain share a common function, by substituting C directly into the Q domain. When the complete C gene was inserted between the NotI sites, a viable replicon was not recovered, likely because the C-terminal 23 aa of C, which function as the signal sequence for E2 in the structural protein precursor, interfered with targeting of P150. Indeed, when the C gene lacking the E2 signal sequence was used, a viable replicon was recovered, a finding consistent with the hypothesis. Insertion of irrelevant sequence between the NotI sites, such as CAT or GFP, did not yield viable constructs, indicating that a specific function was involved and that the C gene was not serving as a spacer. Although these experiments were done by making substitutions between the NotI sites as a matter of convenience, we also found that the C gene lacking the E2 signal sequence could also be used to functionally replace the larger deletion from the upstream NotI site through the C terminus of the Q domain, and this construct was used for mapping of the C sequences necessary for such functional replacement.

The findings that a viable replicon was recovered when C(1-277), C(9-277), or C(51-277), but not C(89-277) or C(200-277), was substituted for the Q domain indicated that neither the eight N-terminal amino acids between the in-frame AUGs at the start of the C gene nor the R1 arginine-rich motif was necessary for compensation of Q domain function and that the R2 arginine-rich motif was. The N-terminal 8 aa are necessary for rescue of ΔNotI replicons in *trans* by C (22), but we previously showed that these residues were needed for correct intracellular targeting of C (26) and thus would be dispensable in replicons in which C was inserted into the Q domain. Considering that the R2 motif was necessary for compensation of the Q domain and that our hypothesis was that C and the Q domain shared a common function, we inspected the sequence in the Q domain and found an arginine-rich motif (which we termed R_Q). Substitution of alanine, glutamine, or lysine for the arginine residues in the R_Q motif resulted in a nonviable replicon that was rescuable by C in *trans*. Viability was also recovered when C(1-277) or C(1-277) with the R1 motif mutated to A was inserted between the NotI sites of the R_QA mutant, but not when C(1-277) with both R1A and R2A mutations was similarly inserted, indicating that the R2 motif was sufficient to compensate for the R_Q motif. Because of the presence of inconvenient restriction sites, we were not able to insert C(1-277) with the individual R2A mutation into the NotI region of P150, but when C with individual R1A and R2A mutations was used to rescue RUBrep/GFP-ΔNotI, the former exhibited rescue, while the latter did not, showing that the R2 motif was specifically involved. Interestingly, the intensity of GFP expression increased dramatically in constructs with C insertions into the Q domain in which the R1 motif was deleted or altered by mutagenesis, but the R2 domain was retained (Fig. 3 and Table 1), suggesting that the R1 motif interferes with R2 function when C is in an integral context within P150.

Thus, in the present study we have proven our hypothesis that the RUBV C protein and Q domain of the P150 replicase protein share a common function. Through genetic mapping, we found that this function centers on the R2 arginine-rich motif in C and an arginine-rich motif, R_Q, at the C-terminal end of the Q domain. While nothing has been deduced about this latter motif prior to the present study, the R2 motif in C has been shown to be involved in RNA binding (2). Thus, a potential function shared by C and the Q domain of P150 is RNA binding. Both the N-terminal region of C and the Q domain contain a significant amount of predicted disordered structure (www.pondr.com). Since disordered structure is a hallmark of RNA chaperones and the capsid proteins of other RNA viruses have been shown to possess chaperone activity (1, 6, 8, 9, 13, 15, 29), chaperone activity may also be a component of the common function. This is also consistent with the ability of C to rescue replicons with mutations in CAEs at both ends of the genome However, the R_Q motif lies within the proline-rich region (12). Although originally identified as a "hinge" region, it also contains several SH3 adaptor motifs and thus could be important in binding cell proteins (17). Of interest, C has been shown to bind to cell proteins, in particular mitochondrial p32 (2, 3, 10, 16). Both of the arginine-rich motifs in C were shown to be necessary for binding this cell protein (2), and thus it would not appear to be a common primary pairing partner of both C and the Q domain since only the R2 cluster is involved in compensation for Q domain function by C. However, binding of other cell proteins is an alternative common function that merits exploration.

Finally, it is curious that RUBV evolved a domain within one of its replicase proteins that then appears to have been

recapitulated in its capsid protein. We speculate that the redundant function is necessary in a very early stage of the replication cycle and thus can be carried out initially by the capsid protein in the incoming virion and subsequently by the P150 Q domain after the replicase proteins are translated. Interestingly, we previously showed that virus with the NotI deletion was viable in cell culture due to the presence of C encoded by the SP-ORF (22). We also have constructed a virus in which C(1-277) was inserted between the NotI sites in P150 (corresponding to the RUBrep-1221/GFP replicon) but found that the insertion was rapidly deleted (data not shown). Thus, the C sequences do not appear to be tolerated in P150, either because their duplication leads to lethal recombination during replication or because the amino acids are deleterious to P150 function. In ether case, the deletions that are generated can survive because of C provided in the SP-ORF. However, although the Q domain appears dispensable to RUBV in cell culture, its preservation indicates that it is essential in nature, possibly because RUBV replicates to lower levels in its normal target cells without producing enough C to routinely compensate for P150 Q domain function.

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