The *cis*-acting replication signal at the 3′ end of *Flock House virus* RNA2 is RNA3-dependent

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Abstract

The nodavirus *Flock House virus* has a bipartite positive-sense RNA genome consisting of RNAs 1 and 2, which encode the viral RNA-dependent RNA polymerase (RdRp) and capsid protein precursor, respectively. The RdRp catalyzes replication of both genome segments and produces from RNA1 a subgenomic RNA (RNA3) that transactivates RNA2 replication. Here, we replaced internal sequences of RNAs 1 and 2 with a common heterologous core and were thereby able to test the RNA termini for compatibility in supporting the replication of chimeric RNAs. The results showed that the 3′ 50 nt of RNA2 contained an RNA3-dependent *cis*-acting replication signal.

Since covalent RNA dimers can direct the synthesis of monomeric replication products, the RdRp can evidently respond to *cis*-acting replication signals located internally. Accordingly, RNA templates containing the 3′ termini of both RNAs 1 and 2 in tandem generated different replication products depending on the presence or absence of RNA3.

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Introduction

*Flock House virus* (FHV) is a member of the family *Nodaviridae*, which comprises nonenveloped icosahedral viruses with bipartite, positive-sense RNA genomes (Ball and Johnson, 1998). The larger FHV genome segment, RNA1 (3107 nt), encodes protein A, the catalytic subunit of the viral RNA-dependent RNA polymerase (RdRp), while the smaller genome segment, RNA2 (1400 nt), encodes a precursor to the capsid proteins. The RdRp replicates both genomic RNAs and also transcribes RNA3 (387 nt), a nonencapsidated subgenomic RNA that is coterminous with the 3′ end of RNA1. Both genomic and subgenomic RNAs have 5′-terminal caps. Their 3′ termini have neither poly(A) tails nor identifiable tRNA-like structures (Buck, 1996; Dreher, 1999), but instead appear to be blocked either by an unusual RNA secondary structure or perhaps by a covalent modification (Dasmahapatra et al., 1985; Guarino et al., 1984).

In FHV-infected cultured *Drosophila* cells, RNA3 accumulates predominantly early during infection; at later times its synthesis is inhibited by the replication of RNA2 (Friesen and Rueckert, 1982; Guarino et al., 1984; Zhong and Rueckert, 1993). Furthermore, we demonstrated recently that RNA2 replication depends on RNA3 but not on either of its translation products (Eckerle and Ball, 2002). We proposed that this bidirectional regulation between RNA2 and subgenomic RNA3 serves as a mechanism to coordinate the replication of RNAs 1 and 2 during the viral replication cycle.

To study the regulation of FHV RNA synthesis, Lindenbach et al. (2002) used a cDNA-based RNA replication system in yeast to define a long-distance base-pairing interaction in RNA1 that was critical for the production of subgenomic RNA3. In addition, two regions in the 3′ quarter of RNA1 were identified that were important for RNA1 replication: an internal replication element and a 3′ replication element that might extend to the 3′ end at nt 3107.
However, the terminal sequence requirements for RNA1 replication are currently unknown.

Initial insights into cis-acting sequences required for FHV RNA2 replication were obtained from the characterization of spontaneous deletion mutants and from analysis of the replication of RNAs with engineered deletions (Ball, 1994; Ball and Li, 1993; Li and Ball, 1993). These studies indicated that the sequences required for efficient replication of RNA2 were minimal (3–14 nt) at the 5’ end, but more substantial (50–100 nt) at the 3’ end. An additional central region (nt 538–616) was strongly conserved in spontaneous deletions and enhanced the replication of some engineered templates, but was not strictly required for the replication of all RNA2 derivatives (Albarin et al., 2001).

In addition to positive- and negative-sense monomeric RNAs, FHV RNA replication produces dimeric RNAs of both polarities (Albarin et al., 2001). These species, which have been detected in all cell types examined, include homodimers of RNAs 1, 2, and 3, as well as heterodimers of RNA2 and RNA3. Further characterization of these RNA species by reverse transcription–polymerase chain reaction (RT-PCR) indicated that they consisted of head-to-tail covalently linked monomers whose junction sequences were characteristic of each RNA species and independent of the source of the samples: FHV-infected cultured Drosophila cells or mammalian or yeast cells supporting FHV RNA replication. Monomeric RNAs were resolved and replicated from RNA templates that consisted of complete homodimers as well as partial dimers and trimers, and also from RNA2–RNA3 heterodimers. These results indicated that the RdRp can recognize cis-acting terminal signals when they are located internally on these templates. Because short heterologous extensions at either end of an RNA template interfere strongly with the replication of FHV RNAs (Ball, 1994, 1995; Ball and Li, 1993), these results imply that the functionality of the cis-acting signals depends critically on their local sequence context. Furthermore, they raise the possibility that RNA dimers are involved in the mechanism of RNA replication.

Here we report a further investigation of the cis-acting signals required for RNA replication, focused particularly on those present at the 3’ termini of RNAs 1 and 2. We also tested the RNA termini for their compatibility in supporting the replication of chimeric RNAs. These studies mapped the RNA3-dependence of RNA2 replication to the 3’ end of RNA2. Furthermore, by testing RNAs with duplicate 3’-end signals arranged in tandem, we discovered a method to regulate which 3’ signal is active based simply on the availability of subgenomic RNA3. Finally, we present further evidence that heterodimers of RNAs 2 and 3 have template properties consistent with a role as intermediates in RNA2 replication.

Results and discussion

Replication of RNA1 and 2 derivatives with a common central core

To generate a series of comparable RNA molecules derived from both FHV RNAs 1 and 2, we constructed a set of cDNA clones based on two transcription plasmids: FHV1(1, 0) and FHV2(0, 0) (Ball, 1995; Ball and Li, 1993). These plasmids contain a T7 promoter to drive transcription of the viral cDNA, which is followed by cDNA encoding the hepatitis delta virus antigenomic ribozyme upstream of the T7 terminator. This arrangement serves to generate an authentic viral 3’ end after ribozyme-mediated cleavage of the primary transcript. To make derivatives of RNA1 and RNA2 that were of similar size and sequence composition, we replaced the cores of RNA1 and RNA2 with a central sequence of nonviral origin: 728 nt derived from the enhanced yellow fluorescent protein (eYFP) gene in the antisense orientation with respect to the flanking FHV RNA termini.

Replacing the centers of RNAs 1 and 2 with the same heterologous sequence created a family of RNA molecules that differed only at their termini. This approach yielded three significant benefits: the ease of constructing chimeric cDNAs, a uniform assay for the different replicons (by Northern blot hybridization using a single probe to the central core), and most importantly, a collection of comparable RNA replicons whose different properties could be confidently attributed to their termini. Earlier experiments had shown that simple chimeras of FHV RNAs 1 and 2 that lacked a common core sequence replicated unpredictably and gave results that defied systematic interpretation.

To map the replication signals at the 3’ ends, we introduced progressive deletions in the viral sequences downstream of the eYFP central core. The schematic structures and replication of different RNA1 and 2 derivatives are shown in Fig. 1. RNA1 replicons contained 297 nt (nt 1–297) from the 5’ end of RNA1 followed by the eYFP central core and 306 (nt 2802–3107), 192 (nt 2916–3107), 108 (nt 3000–3107), or 48 nt (nt 3060–3107) of the RNA1 3’ end in FlcF1306, FlcF1192, FlcF1108, and FlcF148, respectively. These RNA1 replicons lack at least 81 nt downstream of the RNA3 start site (G2721), and therefore, cannot serve as templates for RNA3 synthesis. RNA2 replicons had a similar structure (Fig. 1): 184 nt (nt 1–184) from the 5’ end of RNA2 followed by the same eYFP central core, and then 172 (nt 1229–1400), 103 (nt 1298–1400), or 50 nt (nt 1351–1400) of the RNA2 3’ end in F2eF2172, F2eF2103, and F2eF250, respectively.

To support RNA replication, the viral RdRp was supplied from plasmid pTMFA (Eckerle and Ball, 2002), which is transcribed by T7 RNA polymerase to generate mRNA for the translation of FHV protein A from an internal ribosome entry site. This RNA does not itself constitute a template for replication because it lacks both the 5’ and the
Infection and total RNAs were extracted, resolved in 1% agarose gels. BSR-T7/5 cells were transfected with the indicated replicons provided (Fig. 1, lanes 5–7 and 12–14). These results confirmed our previous observation that replication of wild-type RNA2 was transactivated by RNA3 (Eckerle and Ball, 2002). Since F2eF2172, F2eF2103, and F2eF250 replicated equally well, the 3’ signal for RNA2 replication evidently resides within the last 50 nt of RNA2, which lie entirely within the 3’ UTR. A previous study mapped the 3’-end signal to the last 100 nt of RNA2 (Ball and Li, 1993), but our current results indicate that only the 3’ half of this region is necessary.

For all the replicons, minor RNA species were observed that migrated more slowly than the major replication products. These species, which are especially prominent in Fig. 1, lanes 1–4 and 8–11, but RNA2 derivatives replicated only when RNA3 was provided (Fig. 1, lanes 5–7 and 12–14). These results confirmed our previous observation that replication of wild-type RNA2 was transactivated by RNA3 (Eckerle and Ball, 2002). Since F2eF2172, F2eF2103, and F2eF250 replicated equally well, the 3’ signal for RNA2 replication evidently resides within the last 50 nt of RNA2, which lie entirely within the 3’ UTR. A previous study mapped the 3’-end signal to the last 100 nt of RNA2 (Ball and Li, 1993), but our current results indicate that only the 3’ half of this region is necessary.

For all the replicons, minor RNA species were observed that migrated more slowly than the major replication products. These species, which are especially prominent in Fig. 1, lanes 12–14, had electrophoretic mobilities appropriate for covalently linked RNA homodimers analogous to those described previously (Albariño et al., 2001). Such homodimers were detected routinely in the experiments reported here and can be seen in most of the figures that
In an attempt to map the RNA3-dependence of replication to a particular region of RNA2, we generated chimeric replicons containing 5’ ends from RNA1 and 3’ ends from RNA2 or vice versa. The schematic structures and replication of such RNA chimeras and their parental replicons are shown in Fig. 2. The RNA2 replicon F2eF2\textsubscript{172} was modified by replacing its 3’ end with the 3’-terminal 306 or 108 nt of RNA1, generating the chimeras F2eF1\textsubscript{306} and F2eF1\textsubscript{108}, respectively. Similar replacement of the 3’ terminus of the RNA1 replicon F1eF1\textsubscript{306} resulted in the chimeras F1eF2\textsubscript{172} and F1eF2\textsubscript{103}.

In the presence of RNA3, each of the chimeras replicated at least as robustly as the parental replicons, indicating that the 5’- and 3’-end sequences from the different genomic RNAs are compatible in the same template (Fig. 2, lanes 7–12), despite the fact that the 3’ ends of RNA1 and RNA2 lack extensive recognizable similarity in either primary sequence or predicted secondary structure. All replicons that contained the 3’ end of RNA1 replicated in both the absence and the presence of RNA3 (Fig. 2, lanes 1–3 and 7–9). In contrast, all replicons that contained the 3’ end of RNA2 replicated only in the presence of RNA3 (Fig. 2, lanes 4–6 and 10–12). We conclude from these results that the dependence on RNA3 is associated with the 3’ end of RNA2, and that this property can be transplanted together with this 3’-end sequence into replicons containing the 5’ end of RNA1.

**Dimer junction sequences**

We previously reported that dimeric RNAs are generated during FHV RNA replication, probably through a template-switching process similar to RNA recombination. Such dimers were found in cells infected with wild-type FHV and in cells replicating RNAs transcribed from FHV cDNA plasmids (Albariño et al., 2001). These species were detected by Northern blot hybridization of RNAs resolved in denaturing agarose–formaldehyde gels, and unique junction sequences were recovered by RT-PCR from homodimers of RNAs 1, 2, and 3, and from both isomers of heterodimers of RNAs 2 and 3: 5’-RNA2–RNA3–3’ and 5’-RNA3–RNA2–3’ (Albariño et al., 2001).

The RNAs shown in Fig. 2 provided the opportunity to analyze the junction sequences of RNA dimers generated by the replication of RNA1–RNA2 chimeras. We used RT-PCR to amplify the junctions of negative-sense dimeric molecules in samples of total RNA extracted from BSR-T7/5 cells supporting replication of the parental or chimeric replicons. The RT-PCR products obtained in this way were shown previously (Albariño et al., 2001) to correspond to RNA dimers created by the viral RdRp switching templates during RNA replication. They were sequenced without cloning to focus on the major dimeric species. For clarity, the sequences shown in Table 1 are presented as positive-sense, with the recovered junctions in bold and with the terminal sequences of their monomeric RNA templates above and below.

**Chimeric RNAs**

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As in our previous work, we identified distinct types of dimer junctions. One type (complete junctions) contained the complete 3’ end of one RNA monomer linked to the 5’ end of the other monomer (e.g., RNA1–RNA1 junctions). The other type (deleted junctions; e.g., RNA2–RNA2 junctions) lacked one copy of the terminal dinucleotide (GU), which is terminally redundant in FHV RNAs 1, 2, and 3. Junctions from homodimers of RNAs 1 and 3 and from the RNA2–RNA3 heterodimers were complete. Although the major species of primary transcripts of F1eF1 and FHV3(1, 0) will contain an extra nonviral G residue at the 5’ end
porting RNA1 replication (Albarin et al., 2001). In some experiments in which RNAs were harvested 2 rather than 3 days posttransfection, the extra G residue was detected as during FHV infection), at least some of the RNA1–RNA3 heterodimers contained complete junctions (Table 1). We would predict that when RNA3 is templated by RNA1 (such as during FHV infection), at least some of the RNA1–RNA3 junctions will contain an extra C residue corresponding to nt 2720.

In agreement with our previous reports (Albariño et al., 2001; Eckerle and Ball, 2002), the majority junction sequences recovered from RNA2 homodimers or RNA3–RNA2 heterodimers lacked one copy of the GU terminal redundancy, although minor species containing the complete junction (both GU copies) were also recovered (Table 1). Similar major and minor junction sequences were recovered from F2eF1 homodimers. The recovery of complete junctions from the majority of RNA3 homodimers and RNA2–RNA3 heterodimers, and of GU- deleted junctions from the majority of RNA2 homodimers and RNA3–RNA2 heterodimers, suggested that the missing GU dinucleotide was lost specifically from the 5'-end of RNA2. To test this hypothesis, we analyzed the junctions of homodimers generated from chimeric RNAs (Table 1). The RNA2–RNA1 junctions generated during replication of F1eF2 were complete, but the RNA1–RNA2 junctions generated during replication of F2eF1 lacked one copy of the GU dinucleotide. These results confirmed that the GU dinucleotide missing from deleted junctions corresponded specifically to the 5'-end of RNA2.

(Ball, 1995; Ball and Li, 1993), this residue was not detected in the corresponding homodimer junctions. This result indicated that the extra G residue was corrected, as previously reported for RNA1 dimer junctions (Albariño et al., 2001). In some experiments in which RNAs were harvested 2 rather than 3 days posttransfection, the extra G residue from FHV3(1, 0) was detected in RNA3 dimer junctions. Nonviral residues from the 5'-end of RNA polymerase II transcripts of FHV RNA1 have previously been detected in RNA1 dimer junctions from yeast supporting RNA1 replication (Albariño et al., 2001). The presence of extra nucleotides in RNA dimer junctions indicates that the primary transcript or an uncorrected replication product can participate in template-switching by the RdRp.

We previously reported the presence of an extra C residue in junctions from RNA3 homodimers and RNA2–RNA3 heterodimers and showed by mutagenesis that this residue was templated by C2720 of RNA1 (Albariño et al., 2001). However, this nucleotide was absent from plasmid FHV3(1, 0), which contained only nt 2721–3107 of RNA1 preceded by a nonviral G. The absence of the extra C residue from dimer junctions when RNA3 was supplied from FHV3(1, 0) was therefore consistent with our earlier identification of C2720 as its template and showed that this residue was not essential for the formation of homo- or heterodimers involving RNA3.

Although a putative RNA1/RNA3 heterodimer can be detected by Northern blot hybridization (Albariño et al., 2001; Eckerle and Ball, 2002), our earlier experiments did not differentiate between the RNA1–RNA3 heterodimer junction and that of the RNA3 homodimer, due to the presence of the complete RNA3 sequence at the 3'-end of RNA1. For the same reason, we did not differentiate between the junction sequence of the RNA3–RNA1 heterodimer and that of the RNA1 homodimer (Albariño et al., 2001; Eckerle and Ball, 2002). However, the nonviral eYFP core present in the replicons used in the current study let us amplify such RNA1–RNA3 and RNA3–RNA1 junctions selectively. Sequence analysis showed that both these heterodimers contained complete junctions (Table 1). We predicted that when RNA3 is templated by RNA1 (such as during FHV infection), at least some of the RNA1–RNA3 junctions will contain an extra C residue corresponding to nt 2720.

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Fig. 3. Replication of RNAs containing mixed sequences from the 3' ends of RNAs 1 and 2. The viral 3' sequences from parental replicons F1eF1<sub>108</sub> and F2eF2<sub>103</sub> were subdivided into two overlapping segments and exchanged between RNAs 1 and 2 to generate replicons F1eF1<sub>108</sub>, F1eF2<sub>64,74</sub>F2<sub>50</sub>, F1eF2<sub>48,64</sub>F1<sub>48</sub>, F2eF1<sub>103</sub>, F2eF1<sub>74,48</sub>F2<sub>50</sub>, F2eF2<sub>64,103</sub>F1<sub>108</sub>, and F2eF2<sub>64,48</sub>F1<sub>108</sub>. The lack of replication of F1eF2<sub>64,48</sub>F1<sub>108</sub> and F2eF2<sub>64,48</sub>F1<sub>108</sub> was compensated by extending the RNA1 terminal signal in replicons F1eF2<sub>64,48,F1</sub><sub>108</sub> and F2eF2<sub>64,48,F1</sub><sub>108</sub>. Schematics of each replicon are shown at the top, using the same conventions as in Fig. 1. RNA replication was analyzed as in Fig. 1. Monomeric and dimeric RNA species are indicated as in Fig. 1. BSR-T7/5 cells were transfected with the indicated replicons (a–h), together with pTMFA either without (RdRp, lanes 1–6 and 13–14) or with FHV3(1, 0) (RdRp + RNA3, lanes 7–12).

RNA templates containing mixed sequences from the 3' ends of RNAs 1 and 2

The demonstration that RNA3-dependence was associated with the 3' end of RNA2 and that this property could be transferred to other RNAs led us to further characterize the 3'-end signals. Guided by the secondary structures predicted for the RNA termini (data not shown), we subdivided the 3'-terminal regions from the parental replicons into smaller, overlapping segments and exchanged them between RNA1- and RNA2-derived replicons. This approach generated RNAs in which either the internal or the terminal 3' segment was heterologous with respect to the 5' region (Fig. 3). Replacing the 3'-terminal 34 nt in F1eF1<sub>108</sub> with the 3'-terminal 50 nt from RNA2 resulted in a new replicon designated F1eF1<sub>74,48,F2</sub><sub>50</sub>. Conversely, F1eF2<sub>64,48,F1</sub><sub>108</sub> was generated by replacing the internal 60 nt in F1eF1<sub>108</sub> with the internal 64 nt from RNA2. Similar manipulation of F2eF1<sub>103</sub> resulted in replicons F2eF1<sub>74,48,F2</sub><sub>50</sub> and F2eF2<sub>64,48,F1</sub><sub>108</sub>. As before, replication was supported by the RdRp in the absence or presence of RNA3 transcribed from FHV3(1, 0) (Fig. 3).

F1eF1<sub>74,48,F2</sub><sub>50</sub> replicated exclusively in the presence of RNA3 (Fig. 3, lanes 2 and 8), indicating that the 5' 297 nt of RNA1 and the internal 74 nt remaining from the 3' end of RNA1 were not sufficient to confer independence of RNA3. In view of this result, it was predictable that the replication of F2eF1<sub>74,48,F2</sub><sub>50</sub> was also RNA3-dependent (lanes 5 and 11), further demonstrating that the internal transplanted region of RNA1 was not sufficient to confer RNA3-independent replication.

F1eF2<sub>64,48,F1</sub><sub>108</sub> and F2eF2<sub>64,48,F1</sub><sub>108</sub> failed to replicate in the presence or absence of RNA3 (Fig. 3, lanes 3, 6, 9, and 12). Because the 5' segments of each of these molecules were active in other replicons, this result likely indicates that the internal segment from RNA2 and the 3'-terminal segment from RNA1 were not compatible to form an active replication signal. However, the lack of replication observed in F1eF2<sub>64,48,F1</sub><sub>108</sub> and F2eF2<sub>64,48,F1</sub><sub>108</sub> could be overcome by extending the 3'-terminal RNA1 sequence to 108 nt as in F1eF2<sub>64,48,F1</sub><sub>108</sub> and F2eF2<sub>64,48,F1</sub><sub>108</sub> (Fig. 3, lanes 13 and 14). Both of these RNA molecules replicated in the absence of RNA3, indicating that the internal 64 nt from RNA2 did not confer RNA3-dependence.

Chimeric tandem 3' end signals

The results shown in Figs. 2 and 3 indicated that the replication phenotypes of RNA1 and RNA2 could be changed by replacing the 3'-terminal regions with sequences as short as 50 nt from RNA2 or 108 nt from RNA1. Consequently, we sought to examine the replication properties of RNA molecules that contained two complete but different 3'-end signals placed in tandem. The schematic structures and replication of these dual 3'-end chimeras are shown in Fig. 4.

The parental clone F1eF1<sub>108</sub> was modified by the 3' addition of the 3'-terminal 103 or 172 nt of RNA2 to generate F1eF1<sub>108,F2</sub><sub>103</sub> and F1eF1<sub>108,F2</sub><sub>172</sub>, respectively. Similarly, F2eF2<sub>103,F1</sub><sub>108</sub> and F2eF2<sub>48,306</sub>F1<sub>306</sub> were derived from the parental clones F2eF2<sub>103</sub> and F2eF2<sub>48,40</sub> by the 3' addition of the 3'-terminal 108 or 306 nt of RNA1, respectively. Replication of these chimeras was examined under three different conditions: supported by the RdRp alone (Fig. 4, lanes 1, 4, 7, and 10); by the RdRp and RNA3 together (Fig. 4, lanes 2, 5, 8, and 11); and by autonomously replicating wild-type RNA1 transcribed from plasmid FHV1(1, 0) (Fig. 4, lanes 3, 6, 9, and 12).
a. F1eF1_{108}F2_{103}

b. F1eF1_{108}F2_{172}

c. F2eF2_{103}F1_{108}

d. F2eF2_{50}F1_{306}

Replicons that had the 3' end sequence of RNA2 located internally and that of RNA1 at the 3' terminus yielded somewhat different results. In the presence of the RdRp with or without RNA3, F2eF2_{103}F1_{108} and F2eF2_{50}F1_{306} each yielded a single predominant replication product with the mobility of the complete replicon (Fig. 4, lanes 7, 8, 10, and 11). In the presence of autonomously replicating RNA1, only a smaller product corresponding to the F2eF2 portion of each of these replicons was detected (Fig. 4, lanes 9 and 12). We again attribute the absence under these conditions of the full-length, RNA1-terminated replicons to suppression by competition with the autonomous replication of full-length RNA1. Replication of the F2eF2 portion of these replicons showed that under the appropriate conditions, the RdRp could access the internal RNA2 3' end signal (Fig. 4, lanes 9 and 12). However, these smaller products were undetectable when RNA3 was transcribed from FHV3(1, 0) (Fig. 4, lanes 8 and 11), suggesting that RNA3 expressed in this manner was less efficient in promoting access to the internal RNA2 3' end signal.

Together, the results from these dual 3' end chimeras implied that the replication signals placed at the 3' ends were recognized as independent units. The terminal position was apparently more favorable for both RNA1 and RNA2 signals, since in each case replication products generated from a terminal signal were more abundant than those generated by the same 3' sequence when it was located internally (Fig. 4, compare lane 1 with 7 and lane 3 with 9). Nonetheless, the detection of the shorter replication product from each chimera confirms our previous demonstration that the FHV RdRp can recognize internal copies of cis-acting RNA signals present within dimeric RNAs (Albariño et al., 2001). Moreover, the combination of internal recognition and RNA3-mediated transactivation of the RNA2 3' end signals enabled us to use RNA3 as a molecular switch to generate different RNA replication products from the same starting template.

Heterodimers of RNAs 2 and 3

Heterodimers that contain covalently linked RNAs 2 and 3 are produced during FHV infection of Drosophila cells and thus constitute naturally occurring RNA molecules that combine the 3' end sequences of both RNAs 1 and 2 in the same molecule (Albariño et al., 2001). To examine the replication properties of these heterodimers, we generated positive-sense replicons that contained the eYFP central core flanked by the termini of RNA2, either followed or
ERODIMERS: 5 sponded to the two naturally occurring isomers of het-
F2eF2/F3 and F3/F2eF2, respectively, and they corre-
preceded by the entire 387-nt sequence of RNA3 (referred
to as F3 in the replicon names). These replicons were called
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sponded to the two naturally occurring isomers of hetero-
dimers: 5'-RNA2–RNA3–3' and 5'-RNA3–RNA2–3'.
Because the junctions of 5'-RNA2–RNA3–3' heterodimers
contain an extra C residue and those of 5'-RNA3–RNA2–3'
lack one copy of the terminally redundant GU dinucleotide
(Albariño et al., 2001), we reproduced these natural junction
sequences in the corresponding cDNA clones. The sche-
matic structures and replication of these RNA2/3 het-
erodimers are compared with those of the parental RNA2
replicon F2eF2172 in Fig. 5.

As expected, F2eF2172 failed to replicate in the absence
of RNA3 (Fig. 5, lanes 1 and 10), but F2eF2 monomers and
homodimers were detected either when RdRp and RNA3
were supplied or when autonomously replicating RNA1 was
used to support replication (Fig. 5, lanes 2 and 3). In addition,
an F2eF2/F3 heterodimer accumulated detectably un-
der the latter conditions (Fig. 5, lane 3, band H). When
presented to the RdRp as a DNA-templated primary tran-
script, the F2eF2/F3 heterodimer replicated in its entirety,
consistent with the RNA1 sequence at its 3' end. It was not
resolved into its component monomers in the absence of
exogenous RNA3 (Fig. 5, lane 4). Even the higher sensi-
tivity afforded by RT-PCR failed to detect the F2eF2 ho-
omodimer that would have indicated resolution and replica-
tion of F2eF2 monomers (data not shown).

In contrast, when exogenous RNA3 was added or when
autonomously replicating RNA1 was used to support RNA
replication, F2eF2 monomers and homodimers were de-
tected (Fig. 5, lanes 5 and 6). Whereas the heterodimer was
the major product when RNA3 was transcribed from
FHV3(1, 0) (Fig. 5, lane 5), it was barely detectable when
RNA replication was supported by RNA1. Instead, F2eF2
was the major product of replication under these conditions
(Fig. 5, lane 6). We attribute these quantitative differences
to a combination of two effects: the supply of RNA3 and
replicative competition between the F2eF2/F3 heterodimer
and autonomously replicating RNA1.

Unlike most other templates having RNA2-derived 3'
ends, the other isomer of heterodimeric RNA (F3/F2eF2)
was replicated by the RdRp alone and yielded a more
complex pattern of products (Fig. 5, lane 7). The slowest
migrating RNA (identified with an asterisk) corresponded
to the primary transcript, most of which remained uncleaved
by the HDV ribozyme. This RNA was equally abundant in
the absence of replication and can be attributed to the
stronger T7 promoter context created by the sequence at the
5' end of RNA3 (data not shown). The two smaller RNAs
(labeled H and M) were detected only in the presence of
the RdRp and corresponded to the complete F3/F2eF2 het-
erodimer and the F2eF2 monomer, respectively (Fig. 5,
lanes 7, 8, and 9). The same two species were detected when
RNA3 was transcribed from FHV3(1, 0), and they were
particularly abundant when replication was supported by
RNA1 (Fig. 5, lanes 8 and 9). The F3/F2eF2 heterodimer
replicated less abundantly than the F2eF2/F3 isomer (Fig. 5,
compare lanes 4 and 5 with lanes 7 and 8), except when
replication was supported by autonomous RNA1 (Fig. 5,
compare lanes 6 and 9). This result may be attributed to
reduced competition between the F3/F2eF2 heterodimer and
RNA1 due to their different 3' ends.

The replication properties of the heterodimers described
above mirrored those of the corresponding full-length
RNAs that contained the complete sequences of RNAs 2
and 3 instead of the heterologous core (data not shown).
Moreover, this was true for primary transcripts of either
polarity (data not shown). As shown in Fig. 5, lane 6, F2eF2

Fig. 5. Replication of heterodimers of RNA2 and 3. Heterodimeric replic-
ons F2eF2/F3 and F3/F2eF2 were constructed by adding the complete
subgenomic RNA3 sequence to an RNA2 replicon at the 3' or 5' end,
respectively. Schematics of each replicon are shown at the top, using the
same conventions as in Fig. 1. RNA replication was supported in three
different ways as in Fig. 4. Positive-sense replication products were ana-
alyzed by Northern blot hybridization using an RNA probe complementary
to the sequences of the central core in both panels (lanes 1 to 16), while an
additional RNA probe complementary to (+) sense RNA3 was also used in
the right panel (lanes 10–16). RNA2 monomers (M), dimers (D), and
RNA2/RNA3 heterodimers (H) are indicated with brackets, while the
primary transcripts from pTMFA (top) and F3/F2eF2 (bottom) are indi-
cated with asterisks. To generate size markers of RNA2 and 3 with similar
intensities to those in lane 13, cells were transfected with pTMFA and
FHV3 (0, 0) (lane 15) (Eckerle and Ball, 2002) or with pTMFA, FHV3 (1, 0)
and F2eF250 (lane 16). One-fifth of the amount of RNA was loaded in
lane 16 compared to the other lanes.
monomers and homodimers were generated from F2eF2/F3 when RNA replication was supported by RNA1, and analogous full-length RNA2 species were replicated from the RNA2–RNA3 heterodimer expressed in the negative-sense (Albariño et al., 2001). Furthermore, the template properties of full-length RNA2–RNA3 heterodimers were unaffected by the presence of the extra C residue at the junction, and those of full-length RNA3–RNA2 heterodimers were indistinguishable whether they contained one or two copies of the terminal GU dinucleotide at the junction (data not shown). Together, these results indicate that natural junction sequences are not critical for the replication or resolution of heterodimers.

The context of the RNA3 sequence influences transactivation

When supported by the RdRp alone, the F3/F2eF2 heterodimer replicated as a full-length RNA and was also resolved to yield monomeric F2eF2 (Fig. 5, lane 7). Both these results were unexpected since RNA3 was not provided in trans. To examine the possibility that the F3/F2eF2 primary transcript directed the synthesis of RNA3, we analyzed the replication products templated by F2eF2/F3 and F3/F2eF2 heterodimers by Northern blot hybridization using a probe for RNA3 in addition to the probe for the central eYFP core. The RNA3 probe also detected the mRNA for the RdRp that was transcribed from plasmid pTMFA (marked with an asterisk in Fig. 5, lanes 10–16). Monomeric RNA3 was templated by the F3/F2eF2 template (Fig. 5, lane 12), and the presence of the RNA3 homodimer was confirmed by RT-PCR and sequencing of the RNA3–RNA3 junctions (data not shown). In contrast, the F2eF2/F3 heterodimer showed no evidence of RNA3 synthesis by Northern blot analysis (Fig. 5, lane 11) nor of RNA3 dimers by RT-PCR (data not shown).

The ability of F3/F2eF2 to template the synthesis of RNA3 (Fig. 5, lane 12) provided an explanation for the resolution and replication of the F2eF2 portion of the heterodimer by the RdRp alone (Fig. 5, lane 7), reactions shown above to be dependent on RNA3 (see Fig. 1, lanes 5–7 and 12–14). This explanation further predicted that the F3/F2eF2 heterodimer should transactivate the replication of other RNA2 derivatives such as F2eF250. In the presence of RdRp alone, only the uncleaved primary transcript of F2eF250 was detected (Fig. 5, lane 14), but addition of F3/F2eF2 transactivated F2eF250 replication, as predicted (Fig. 5, lane 13). As shown before (Fig. 1), RNA3 itself also transactivated F2eF250 replication (Fig. 5, lane 16). These results showed that one heterodimeric isomer (F3/F2eF2) could template the synthesis of RNA3, which in turn transactivated the replication of RNA2 replicons, including F3/F2eF2 itself.

The results reported above show that the context of the RNA3 sequence affects transactivation of RNA2 replication. Both isomers of the heterodimer (F2eF2/F3 and F3/F2eF2) contained the entire, contiguous RNA3 sequence, but only the latter replicon templated the synthesis of monomeric RNA3 and transactivated the replication of RNA2. This difference was not directly due to the different positions of the RNA2 sequence in the two heterodimers, because in the presence of RNA3 the 3′ end of RNA2 can be recognized whether located terminally or internally, as in RNA2 dimers (Albariño et al., 2001) or in the dual 3′-end chimeras F2eF210:F110 and F2eF245:F130 (Fig. 4). Rather, the difference must be attributed to the different locations of the RNA3 sequence in the heterodimers. The inability of F2eF2/F3 to produce F2eF2 indicates that a free 3′ terminus of RNA3 is insufficient for transactivation. Furthermore, production of monomeric RNA3 from F3/F2eF2 and the concomitant replication of F2eF2 suggest that RNA3 may require both its termini to be free for transactivation. However, we cannot exclude the possibility that the free 5′ end of the RNA3 sequence in the context of the F3/F2eF2 molecule was itself sufficient to promote transactivation of RNA2.

Summary and conclusions

By comparing the replication of RNAs that contained a common core sequence, we were able to examine systematically the replication properties of the RNA termini. The results presented above showed that the minimal cis-acting replication signal at the 3′ end of RNA1 was fully contained within the 3′ 108 nt but not within the 3′ 48 nt, while that of RNA2 was fully contained in the last 50 nt (Fig. 1). The observation that these 3′ signals were compatible with the 5′ terminus of either RNA segment let us map the RNA3-dependence of RNA2 replication to the 3′ end of RNA2 (Fig. 2). Interestingly, replacing only the last 34 nt of an RNA1-derivied replicon with the last 50 nt of RNA2 (to create F1eF1745:F250) rendered a previously independent replicon absolutely dependent on RNA3 (Fig. 3). A consequence of these regulatory interactions was that in RNA templates that contained complete 3′ signals of both RNAs 1 and 2 in tandem, such as the heterodimers of full-length RNAs 2 and 3 that occur naturally during FHV infection, the choice of signal depended on the presence or absence of RNA3 (Figs. 4 and 5).

We have interpreted the RNA3-dependence of RNA2 replication as a mechanism by which the synthesis of the two viral genome segments is coordinated during RNA replication (Eckerle and Ball, 2002). Since nodavirus RNAs are copackaged during virus assembly (Krishna and Schneeemann, 1999; Selling and Rueckert, 1984), it is likely that optimum virus yield requires equimolar synthesis of the two genome segments. However, the mechanism of transactivation remains obscure. The naturally occurring heterodimers that link RNAs 2 and 3 are candidates for possible intermediate templates which might serve to recruit RNA2 into a replication complex. Their template properties are consistent with such a role (Fig. 5), and their occurrence correlates
precisely with RNA2 replication. However, further studies are required to establish whether these heterodimers are replicative intermediates or merely by-products of replication, and to determine how RNA3 permits the RdRp to access an otherwise silent RNA2 replication signal.

Materials and methods

Transcription plasmids

We used standard methods of DNA manipulation (Sambrook and Russell, 2001) to construct a series of T7 transcription plasmids that expressed positive-sense derivatives of FHV RNA1 and RNA2. Plasmids FHV1(1, 0) and FHV2(0, 0), which were called FHV1:3107(1, 0) and FHV2:1400(0, 0), respectively, in our earlier publication (Albariño et al., 2001), contained cDNAs corresponding to the full-length genomic RNAs of FHV (Ball, 1995; Ball and Li, 1993). To satisfy the promoter requirements of T7 RNA polymerase, all RNA1-derived expression plasmids contained one nonviral G residue between the T7 promoter and the FHV cDNA.

We replaced the central sequences of RNAs 1 and 2 with a central core consisting of 728 nt (CC[ATG]...eYFP ORF...TAAG[GAATTC]) containing the entire ORF of the enhanced yellow fluorescent protein (pRES-EYFP, Clontech) in the antisense orientation with respect to the T7 promoter and flanking sequences. Plasmids expressing RNA1 replicons contained 297 nt (RNA1: 1–297) from the 5’ end of RNA1 followed by the eYFP central core and 306 (RNA1: 2802–3107), 192 (RNA1: 2916–3107), 108 (RNA1: 3000–3107) or 48 nt (RNA1: 3060–3107) from the 3’ end of RNA1. RNA2 replicons had a similar structure and contained 184 nt (RNA2: 1–184) from the 5’ end of RNA2 followed by the same eYFP central core and 172 (RNA2: 1229–1400), 103 (RNA2: 1298–1400), or 50 nt (RNA2: 1351–1400) from the 3’ end of RNA2. Plasmids designed to transcribe RNA chimeras were made by exchanges between different parental clones. For example, F1eF1108 and F2eF2172 were modified to generate the chimeras F2eF1306 and F1eF2172, respectively. More example, F1eF1108 and F2eF2172 were made by exchanges between different parental clones. For example, exchanging the 3’ termini of F1eF1108 and F2eF2172 generated F1eF1745F250 (F1745F250 = RNA1: 3000–3073 and RNA2: 1351–1400) and F2eF2643F148 (F2643F148 = RNA2: 1298–1361 and RNA1: 3060–3107). Similarly, exchanging the internal regions from the same parental clones generated F1eF2643F148 and F2eF1745F250.

We also constructed replicons that contained two different 3’-end signals in tandem. For example, the 3’ ends from the replicons F1eF1108 and F2eF2103 were used to generate F1eF1108F2103 (F1108F2103 = RNA1: 3000–3107 and RNA2: 1298–1400) and F2eF2103F1108 (F2103F1108 = RNA2: 1298–1400 and RNA1: 3000–3107). Heterodimers of FHV RNA2 and RNA3 were made by adding the entire sequence of RNA3 (RNA1: 2721–3107) upstream or downstream of parental F2eF2172, thereby generating the two isomers F3/F2eF2 and F2eF2/F3. RNA replication was supported by RdRp expressed from the plasmid pTMFA, which supplied FHV protein A, in the absence or presence of plasmid FHV3(1, 0) to provide RNA3 (Eckerle and Ball, 2002). Alternatively, plasmid FHV1(1, 0) was used to initiate autonomous RNA1 replication and thus supply both the viral RdRp and the RNA3 (Ball, 1995).

Transfection of mammalian cells

BSR-T7/5 cells are derived from BHK-21 cells and constitutively express T7 RNA polymerase in the cytoplasm (Buchholz et al., 1999). These cells were routinely maintained at 37°C in DMEM supplemented with 5% newborn calf serum and 5% fetal bovine serum in a 5% CO2 atmosphere. Geneticin (Invitrogen Life Technologies) was added to a final concentration of 0.6 mg/ml in alternate passages. Confluent monolayers of BSR-T7/5 cells growing in 35-mm plates were transfected with 1 μg pTMFA, 2 μg of the replicon-expressing plasmids, and 15 μl Lipofectamine 2000 according to the manufacturer’s directions (Invitrogen Life Technologies). Four micrograms of plasmid FHV3(1, 0) were cotransfected where indicated. Alternatively, plasmids pTMFA and FHV3(1, 0) were replaced by 4 μg of plasmid FHV1(1, 0) where indicated. Transfected cells were incubated for 24 h at 28°C in DMEM without serum and for an additional 48 h in DMEM supplemented with 2.5% fetal bovine serum.

RNA analysis by Northern blotting

Cells were harvested 72 h posttransfection and total RNAs were extracted using the RNeasy MiniKit (Qiagen) and treated with RNase-free DNase I (Qiagen) at room temperature for 30 min. Purified RNAs were resolved by electrophoresis on 1% agarose-formaldehyde denaturing gels (Lehrach et al., 1977) and transferred to Nytran nylon membranes (Schleicher and Schuell). In vitro transcription of strand-specific 32P-labeled RNA probes and hybridization were performed as described previously (Price et al., 2000).

Positive-sense replication products were detected using RNA probes that corresponded to the entire eYFP ORF (Figs. 1–5). Positive-sense RNA3 was detected using an RNA probe complementary to nt 2749 to 3107 at the 3’ end of FHV RNA1 (Fig. 5, lanes 10–16). RNA probes were applied at 2 × 106 cpm per membrane, and each blot was exposed for 24 h and imaged using a Molecular Dynamics Storm digital radioactivity imaging system.
RT-PCR and sequencing

The unique junction sequences from head-to-tail covalent RNA dimers were amplified by RT-PCR using a modified protocol from the OneStep RT-PCR kit (Qiagen) on total RNAs from transfected cells. First-strand cDNA synthesis was performed in the presence of only one primer, and the second primer was added for amplification by PCR. To detect (−)sense dimers of eYFP-containing replicons, we primed first-strand cDNA synthesis with oligodeoxynucleotide eYFP-1950 (5′ TCGCCGTCCAGCTGAC-CAG 3′) and performed PCR amplification with the same oligodeoxynucleotide and eYFP+2558 (5′ CATGGTCCT-GCTGGAGTTGGT 3′). To detect (+)sense dimers of RNA3, we primed first-strand cDNA synthesis with oligodeoxynucleotide FHV1+3060 (5′ TGGCTCTTAGGAC-CACCC 3′) and performed PCR amplification using this oligodeoxynucleotide and FHV1−3015 (5′ TACATCCGG-GTGTGGAGAACG 3′). RNA1–RNA3 and RNA2–RNA3 heterodimers were detected using oligodeoxynucleotide eYFP-1950 for first-strand cDNA synthesis, followed by PCR amplification using the same oligodeoxynucleotide and FHV1−3015. RNA3–RNA1 and RNA3–RNA2 heterodimers were detected using FHV1+3060 for first-strand cDNA synthesis, followed by PCR amplification using this oligodeoxynucleotide and eYFP+2558. RT-PCR products were resolved by agarose gel electrophoresis, isolated using the Qiaquick Gel Extraction kit (Qiagen) and sequenced using fluorescent dideoxynucleotides in an automated DNA sequencer.

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References


