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 coterminal 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.

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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 (Albariño et al., 2001).

In addition to positive- and negative-sense monomeric RNAs, FHV RNA replication produces dimeric RNAs of both polarities (Albariño 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 $F1eF1_{306}$, $F1eF1_{192}$, $F1eF1_{108}$, and $F1eF1_{48}$, 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 $F2eF2_{172}$, $F2eF2_{103}$, and $F2eF2_{50}$, 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
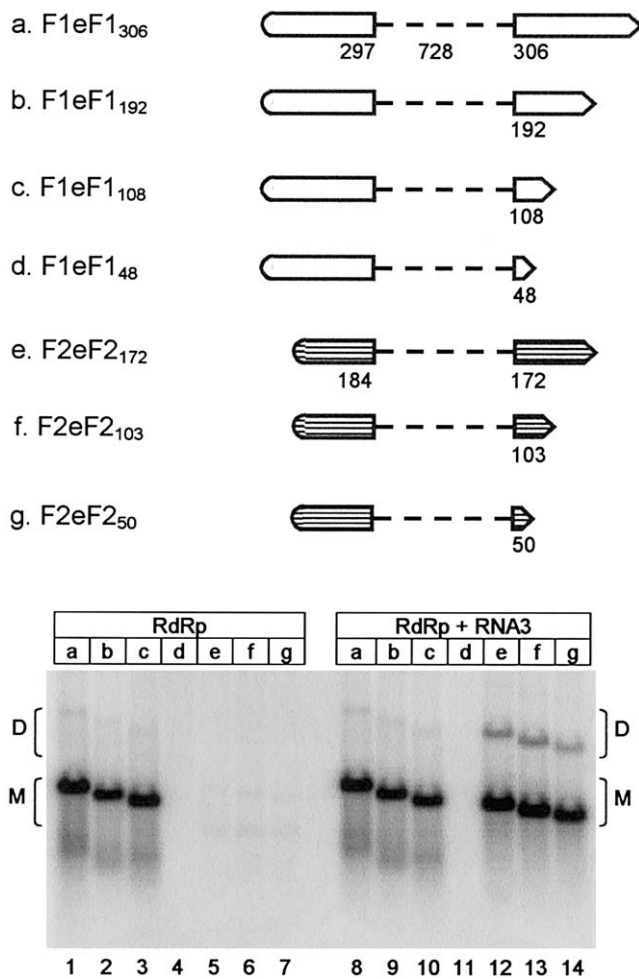
Fig. 1. Replication of FHV RNA1 and RNA2 derivatives that share a common heterologous central core. FHV RNA1- and RNA2-derived replicons were expressed from T7 transcription plasmids. Schematics of each replicon are shown at the top, with rounded and pointed boxes representing the 5′ and 3′ termini, respectively. The sources of the terminal sequences are indicated in the name of each replicon (F1 = RNA1; F2 = RNA2) and schematically by open boxes for RNA1 sequences or striped boxes for RNA2 sequences. The central core (dashed line) contains 728 nt derived from the eYFP gene in the antisense orientation with respect to the flanking sequences. Numbers below each segment indicate its length in nucleotides. The length of the 3′-terminal segments are also indicated as subscripts in the name of each replicon. In this and all subsequent figures, each lane of the Northern blot is labeled with a letter to indicate the corresponding replicon. Monomeric (M) and dimeric (D) RNA species are indicated with brackets. BSR-T7/5 cells were transfected with the indicated replicons (a–g), together with pTMFA either without (RdRp, lanes 1–7) or with FHV3(1, 0) (RdRp + RNA3, lanes 8–14) to supply the viral RdRp and the subgenomic RNA3, respectively. Cells were harvested 3 days posttransfection and total RNAs were extracted, resolved in 1% agarose–formaldehyde denaturing gels, and transferred to nylon membranes. Positive-sense replication products were detected by hybridizing to an RNA probe complementary to the sequence of the central core.

3′ untranslated regions (UTRs) of RNA1. Because replication of RNA2 depends on subgenomic RNA3, it was provided in trans by cotransfection of plasmid FHV3(1, 0) (Eckerle and Ball, 2002). Transcription plasmids were transfected into BSR-T7/5 cells (Buchholz et al., 1999), a

BHK-derived cell line that constitutively expresses T7 RNA polymerase (Albariño et al., 2001). Cells were harvested 3 days posttransfection and total RNA was extracted and resolved in 1% agarose–formaldehyde denaturing gels. Positive-sense RNAs were detected by Northern blotting using an RNA probe complementary to the antisense eYFP sequence in the replicons.

Replication of RNA1 derivatives $F1eF1_{306}$, $F1eF1_{192}$, and $F1eF1_{108}$ indicated that the foreign eYFP core was tolerated by the FHV RdRp and that the 3′ end of RNA1 could be deleted to 108 nt without impairing replication (Fig. 1, lanes 1–3). A further shortening of the RNA1 3′-end sequence to 48 nt yielded a replication-defective molecule (Fig. 1, lanes 4 and 11). Notice that in the absence of replication, the RNA produced by DNA-templated primary transcription was undetectable at this level of sensitivity; the same applies to almost all the replicons described in this work. These results indicate that the 3′ signal necessary for RNA1 replication lies between the 3′ terminus and 48–108 nt upstream, and therefore, includes most or all of the 49-nt 3′ UTR.

Lindenbach et al. (2002) identified elements encompassing RNA1 nt 2322–2501 and 2735–3011 that were necessary for RNA1 replication. However, none of the RNA1 replicons shown in Fig. 1 contained the first of these elements and each lacked part of the second, yet they all clearly replicated. These results indicate that the signals for replication of RNA1 can be trimmed significantly beyond the limits reported by Lindenbach et al. (2002). This may be due to the use of different RNA1 replicons and/or different host cells (yeast versus mammalian) in the two studies. Sindbis virus provides a precedent for the latter possibility since the sindbis virus RNA sequences required for 3′-end recognition appear to vary between different host cells (Kuhn et al., 1990).

Replication of RNA1 derivatives was unaffected by the absence or presence of RNA3 (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 $F2eF2_{172}$, $F2eF2_{103}$, and $F2eF2_{50}$ 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
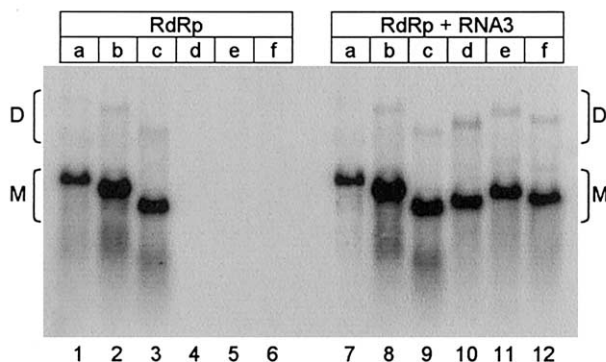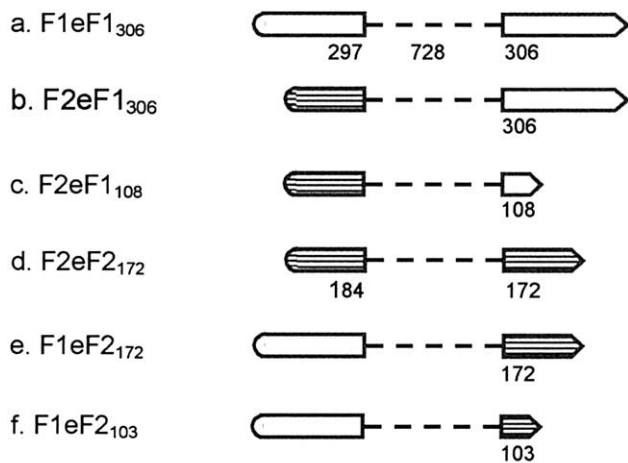
a. F1eF1₃₀₆

b. F2eF1₃₀₆

c. F2eF1₁₀₈

d. F2eF2₁₇₂

e. F1eF2₁₇₂

f. F1eF2₁₀₃

Fig. 2. Replication of chimeric RNAs. Chimeric replicons were constructed by exchanging the viral 3′-end sequences from the parental replicons F1eF1₃₀₆ and F2eF2₁₇₂, which resulted in RNA molecules where the central core was flanked by termini from different RNA segments. 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–f), together with pTMFA either without (RdRp, lanes 1–6) or with FHV3(1, 0) (RdRp + RNA3, lanes 7–12).

follow. Junction sequences from some of these dimers were further characterized by RT-PCR and DNA sequencing of the junction products (see below).

Chimeric RNAs

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 F2eF2₁₇₂ was modified by replacing its 3′ end with the 3′-terminal 306 or 108 nt of RNA1, generating the chimeras F2eF1₃₀₆ and F2eF1₁₀₈, respectively. Similar replacement of the 3′ terminus of the RNA1 replicon F1eF1₃₀₆ resulted in the chimeras F1eF2₁₇₂ and F1eF2₁₀₃.

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.

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

Table 1
Junction sequences of homo- and heterodimers of FHV RNAs 1, 2, and 3

RdRp + RNA3[a]	RNA junction	Positive-sense sequence[b]
F1eF1[c]	RNA1 3′	. . .AGAGGU
	RNA1-RNA1	. . .**AGAGGTGTTTTC**. . .
	5′ RNA1	GUUUUC. . .
	RNA3 3′	. . .AGAGGU
	RNA3-RNA3	. . .**AGAGGTGTTACC**. . .[g]
	5′ RNA3	GUUACC. . .
	RNA1 3′	. . .AGAGGU
	RNA1-RNA3	. . .**AGAGGTGTTACC**. . .
	5′ RNA3	GUUACC. . .
	RNA3 3′	. . .AGAGGU
	RNA3-RNA1	. . .**AGAGGTGTTTTC**. . .
	5′ RNA1	GUUUUC. . .
F2eF2[d]	RNA2 3′	. . .UAAGGU
	RNA2-RNA2	. . .**TAAGGTAAAC**. . .[h]
	5′ RNA2	GUAAAC. . .
	RNA3 3′	. . .AGAGGU
	RNA3-RNA2	. . .**AGAGGTAAAC**. . .[h]
	5′ RNA2	GUAAAC. . .
	RNA2 3′	. . .UAAGGU
	RNA2-RNA3	. . .**TAAGGTGTTACC**. . .[g]
	5′ RNA3	GUUACC. . .
F1eF2[e]	RNA2 3′	. . .UAAGGU
	RNA2-RNA1	. . .**TAAGGTGTTTTC**. . .
	5′ RNA1	GUUUUC. . .
F2eF1[f]	RNA1 3′	. . .AGAGGU
	RNA1-RNA2	. . .**AGAGGTAAAC**. . .[h]
	5′ RNA2	GUAAAC. . .

[a] BSR-T7/5 cells were transfected with the indicated replicons and pTMFA and FHV3(1, 0), which supplied the RdRp and RNA3, respectively.

[b] RNA samples were extracted at 3 days posttransfection and aliquots were used in divergent RT-PCR reactions, designed to amplify the junction from negative-sense RNA dimers generated during replication. Junction sequences were determined from bulk (uncloned) RT-PCR products.

[c] $F1eF1_{306}$ and $F1eF1_{108}$ yielded the same junction sequence.

[d] $F2eF2_{172}$ and $F2eF2_{103}$ yielded the same junction sequence.

[e] $F1eF2_{172}$ and $F1eF2_{103}$ yielded the same junction sequence.

[f] $F2eF1_{306}$ and $F2eF1_{108}$ yielded the same junction sequence.

[g] Samples harvested at 2 days posttransfection occasionally yielded an additional G at the junction, corresponding to the 5′ nonviral residue in the primary transcript from FHV3(1, 0).

[h] Dimeric junctions containing the complete sequence were detected as minor species (see text).

(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 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 major 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.
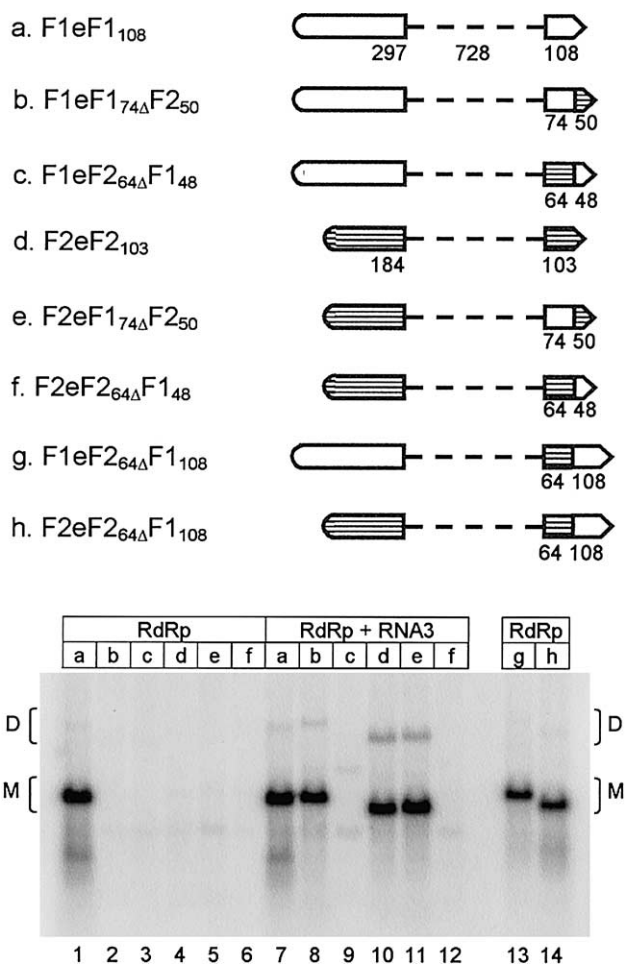
Fig. 3. Replication of RNAs containing mixed sequences from the 3′ ends of RNAs 1 and 2. The viral 3′ sequences from parental replicons $F1eF1_{108}$ and $F2eF2_{103}$ were subdivided into two overlapping segments and exchanged between RNAs 1 and 2 to generate replicons $F1eF1_{74\Delta}F2_{50}$, $F1eF2_{64\Delta}F1_{48}$, $F2eF1_{74\Delta}F2_{50}$, and $F2eF2_{64\Delta}F1_{48}$. The lack of replication of $F1eF2_{64\Delta}F1_{48}$ and $F2eF2_{64\Delta}F1_{48}$ was compensated by extending the RNA1 terminal signal in replicons $F1eF2_{64\Delta}F1_{108}$ and $F2eF2_{64\Delta}F1_{108}$. 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 $F1eF1_{108}$ with the 3′-terminal 50 nt from RNA2 resulted in a new replicon designated $F1eF1_{74\Delta}F2_{50}$. Conversely, $F1eF2_{64\Delta}F1_{48}$ was generated by replacing the internal 60 nt in $F1eF1_{108}$ with the internal 64 nt from RNA2. Similar manipulation of $F2eF2_{103}$ resulted in replicons $F2eF1_{74\Delta}F2_{50}$ and $F2eF2_{64\Delta}F1_{48}$. As before, replication was supported by the RdRp in the absence or presence of RNA3 transcribed from FHV3(1, 0) (Fig. 3).

$F1eF1_{74\Delta}F2_{50}$ 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 $F2eF1_{74\Delta}F2_{50}$ 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.

$F1eF2_{64\Delta}F1_{48}$ and $F2eF2_{64\Delta}F1_{48}$ 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 $F1eF2_{64\Delta}F1_{48}$ and $F2eF2_{64\Delta}F1_{48}$ could be overcome by extending the 3′-terminal RNA1 sequence to 108 nt as in $F1eF2_{64\Delta}F1_{108}$ and $F2eF2_{64\Delta}F1_{108}$ (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 $F1eF1_{108}$ was modified by the 3′ addition of the 3′-terminal 103 or 172 nt of RNA2 to generate $F1eF1_{108}F2_{103}$ and $F1eF1_{108}F2_{172}$, respectively. Similarly, $F2eF2_{103}F1_{108}$ and $F2eF2_{50}F1_{306}$ were derived from the parental clones $F2eF2_{103}$ and $F2eF2_{50}$ 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).
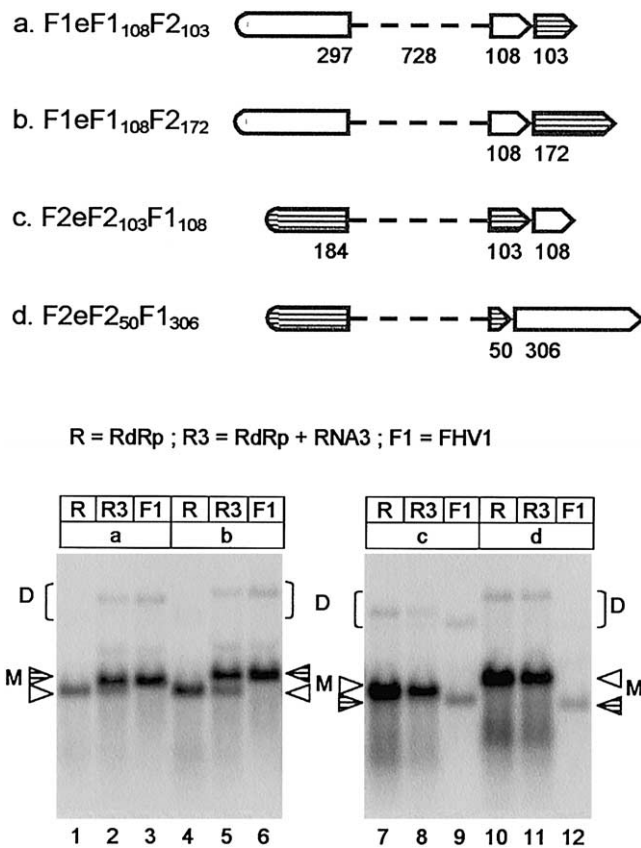
In the presence of the RdRp alone, replicons

a. $F1eF1_{108}F2_{103}$ 297   728   108   103

b. $F1eF1_{108}F2_{172}$ 108   172

c. $F2eF2_{103}F1_{108}$ 184   103   108

d. $F2eF2_{50}F1_{306}$ 50   306

R = RdRp ; R3 = RdRp + RNA3 ; F1 = FHV1

Fig. 4. Replication of RNAs containing tandem 3′ ends. Chimeric replicons were constructed by placing the 3′-end sequences from RNAs 1 and 2 in tandem. Schematics of each replicon are shown at the top, using the same conventions as in Fig. 1. RNA replication was supported by the viral RdRp expressed from plasmid pTMFA in the absence (R; lanes 1, 4, 7, and 10) or presence of RNA3 (R3; lanes 2, 5, 8, and 11), or in the presence of autonomously replicating RNA1 expressed from plasmid FHV1(1, 0), which supplied both RdRp and RNA3 (F1; lanes 3, 6, 9, and 12). Replication products were analyzed by Northern blot hybridization as in Fig. 1. Monomeric RNAs generated by each replicon are indicated by open or striped triangles when they contain 3′-terminal sequences from RNA1 or RNA2, respectively. Dimeric RNA species are indicated as in Fig. 1.

$F1eF1_{108}F2_{103}$ and $F1eF1_{108}F2_{172}$, which had the 3′-end sequence of RNA1 located internally and that of RNA2 located at the 3′ terminus, each yielded a single replication product with the mobility expected for molecules lacking the respective RNA2 region (Fig. 4, lanes 1 and 4). In contrast, when RNA3 was supplied from plasmid FHV3(1, 0), two major replication products were observed from each of these replicons: a smaller and less abundant product that comigrated with the species detected in the absence of RNA3, and a larger predominant product with the mobility expected for the complete replicon (Fig. 4, lanes 2 and 5). These results indicated that in the absence of RNA3, the RNA1 internal signal was recognized exclusively, whereas in the presence of RNA3, the RNA2 terminal signal was also functional. Furthermore, when both RdRp and RNA3 were provided by the autonomous replication of RNA1 from plasmid FHV1(1, 0), only the products corresponding

to the complete replicons were detected (Fig. 4, lanes 3 and 6). We attribute the absence of the smaller products to competition between the autonomously replicating RNA1 (which produces abundant RNA3) and the $F1eF1_{108}$ portions of these replicons. Such competition between full-length RNA1 and other RNA1-derived replicons or chimeric replicons that contained RNA1 3′ ends was routinely observed in other experiments (data not shown).

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
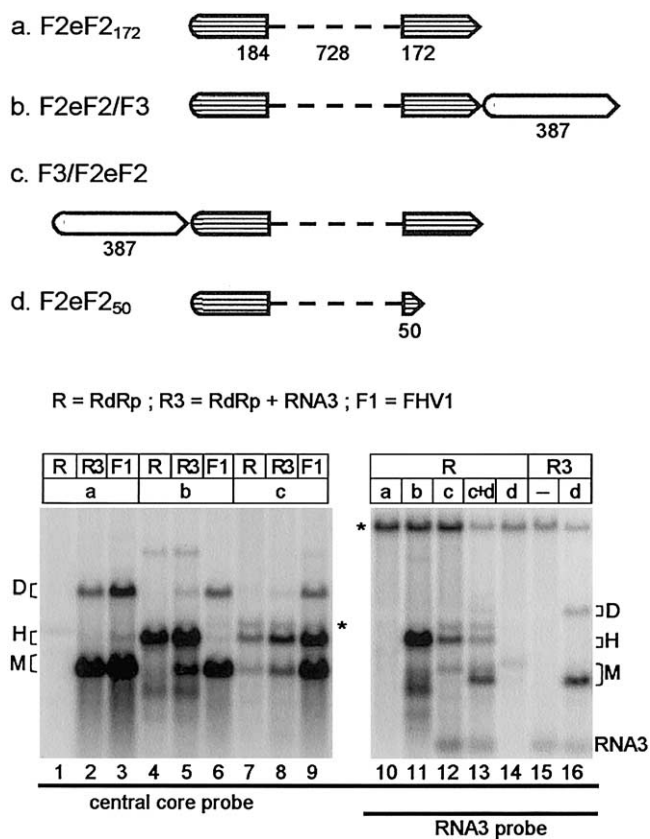
Fig. 5. Replication of heterodimers of RNA2 and 3. Heterodimeric repli-cons F2eF2/F3 and F3/F2eF2 were constructed by adding the complete subgenomic RNA3 sequence to an RNA2 replicon at the 3′ or 5′ end, respectively. Schematics of each replicon are shown at the top, using the same conventions as in Fig. 1. RNA replication was supported in three different ways as in Fig. 4. Positive-sense replication products were ana-lyzed 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 F2eF2$_{50}$ (lane 16). One-fifth of the amount of RNA was loaded in lane 16 compared to the other lanes.

preceded by the entire 387-nt sequence of RNA3 (referred to as F3 in the replicon names). These replicons were called F2eF2/F3 and F3/F2eF2, respectively, and they corre-sponded to the two naturally occurring isomers of het-erodimers: 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 F2eF2$_{172}$ in Fig. 5.

As expected, F2eF2$_{172}$ 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 addi-tion, 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-modimer 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

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 F2eF2$_{50}$. In the presence of RdRp alone, only the uncleaved primary transcript of F2eF2$_{50}$ was detected (Fig. 5, lane 14), but addition of F3/F2eF2 transactivated F2eF2$_{50}$ replication, as predicted (Fig. 5, lane 13). As shown before (Fig. 1), RNA3 itself also transactivated F2eF2$_{50}$ 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 F2eF2$_{103}$F1$_{108}$ and F2eF2$_{50}$F1$_{306}$ (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-derived replicon with the last 50 nt of RNA2 (to create F1eF1$_{74Δ}$F2$_{50}$) 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 Schneemann, 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. . .TAA]GAATTC) containing the entire ORF of the enhanced yellow fluorescent protein (pIRES-EYFP, Clontech) in the antisense orientation with respect to the T7 promoter and flanking FHV 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, $F1eF1_{306}$ and $F2eF2_{172}$ were modified to generate the chimeras $F2eF1_{306}$ and $F1eF2_{172}$, respectively. Moreover, according to specific features observed in the secondary structure predicted for these regions by MFOLD version 3.0 (Zuker et al., 1999) and to maintain the predicted stem-loops intact, we subdivided the 3′-end signals from RNAs 1 and 2 into smaller overlapping regions and exchanged them. For example, exchanging the 3′ termini of $F1eF1_{108}$ and $F2eF2_{103}$ generated $F1eF1_{74\Delta}F2_{50}$ ($F1_{74\Delta}F2_{50}$ = RNA1: 3000–3073 and RNA2: 1351–1400) and $F2eF2_{64\Delta}F1_{48}$ ($F2_{64\Delta}F1_{48}$ = RNA2: 1298–1361 and RNA1: 3060–3107). Similarly, exchanging the internal regions from the same parental clones generated $F1eF2_{64\Delta}F1_{48}$ and $F2eF1_{74\Delta}F2_{50}$.

We also constructed replicons that contained two different 3′-end signals in tandem. For example, the 3′ ends from the replicons $F1eF1_{108}$ and $F2eF2_{103}$ were used to generate $F1eF1_{108}F2_{103}$ ($F1_{108}F2_{103}$ = RNA1: 3000–3107 and RNA2: 1298–1400) and $F2eF2_{103}F1_{108}$ ($F2_{103}F1_{108}$ = 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 $F2eF2_{172}$, 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% CO_2 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 ^{32}P-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 2×10^6 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′ TCGCCGTCCAGCTCGAC-CAG 3′) and performed PCR amplification with the same oligodeoxynucleotide and eYFP+2558 (5′ CATGGTCCT-GCTGGAGTTCGTG 3′). To detect (−)sense dimers of RNA3, we primed first-strand cDNA synthesis with oligodeoxynucleotide FHV1+3060 (5′ TGGCTCTTAGGAG-CACCC 3′) and performed PCR amplification using this oligodeoxynucleotide and FHV1−3015 (5′ TCACTTCCG-GTTGTTGGAAGGC 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.

Acknowledgments

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