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Conformation of the 3′-end of beet necrotic yellow vein benevirus RNA 3 analysed by chemical and enzymatic probing and mutagenesis

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ABSTRACT

Secondary structure-sensitive chemical and enzymatic probes have been used to produce a model for the folding of the last 68 residues of the 3′-non-coding region of beet necrotic yellow vein benevirus RNA 3. The structure consists of two stem–loops separated by a single-stranded region. RNA 3-derived transcripts were produced containing mutations which either disrupted base pairing in the helices or maintained the helices but with alterations in the base pairing scheme. Other mutants contained substitutions in single-stranded regions (loops or bulged sequences). With a few exceptions all three types of mutation abolished RNA 3 replication *in vivo*, suggesting that both secondary structure and specific sequences are required for efficient recognition of the 3′-terminal region of RNA 3 by viral RNA-dependent RNA polymerase.

INTRODUCTION

The genome of the fungus-transmitted plant virus beet necrotic yellow vein virus (BNYVV, formerly furovirus, type member of the newly proposed benevirus group; 1) consists of five 5′-capped and 3′-polyadenylated positive strand RNAs (2). The two largest genome components, RNAs 1 and 2, encode proteins involved in replication, encapsidation and cell–cell movement and are necessary for infection of all hosts (3). RNA 3 [1773 nt plus 3′-poly(A) tail] and RNA 4 [1467 nt plus 3′-poly(A) tail] respectively encode proteins involved in proliferation of the virus in roots of *Beta vulgaris* (the natural site of infection) and virus–vector interactions. RNA 5 (1347 nt) remains functionally uncharacterized and is absent from most field isolates of the virus (4). RNAs 3–5 are not required to maintain an infection in mechanically inoculated leaves of a non-natural host like *Chenopodium quinoa*, although they readily multiply under these conditions if included in the inoculum along with RNAs 1 and 2 (3).

Because RNAs 3–5 encode no *trans*-acting functions intervening in the viral replication cycle in leaves they represent useful material for the characterization of sequences which are recognized *in cis* by the viral replication machinery to confer specificity upon the replication process. Earlier studies (5–8) with biologically active transcripts of RNAs 3 and 4 have shown that such *cis*-acting replication-essential sequences are confined to the extremities of the small viral RNAs. At the 5′-terminus *cis*-acting sequence elements are located within the first 292 residues of RNA 3 and the first ~400 residues of RNA 4 (5,6). At the 3′-terminus the essential *cis*-acting sequences fall within the ~70 residues preceding the 3′ poly(A) tail, a region that is highly conserved (80%) in all four viral RNAs and which can be folded into a computer-predicted double hairpin secondary structure which is absolutely conserved among the viral RNAs (5). The double hairpin region presumably contains the 'core' promoter sequence which is recognized by the viral replicase during initiation of minus strand RNA synthesis (5).

In this paper we have used structure-sensitive chemical and enzymatic probes to determine the secondary structure in solution of the 3′-terminal region of RNA 3. This characterization was undertaken using transcripts of both full-length RNA 3 and Rep33, an internally deleted 'replicon' consisting of the first 380 nt and the last 85 nt upstream of the poly(A) tail of RNA 3. The replicon was used in preference to full-length RNA 3 in most studies because its smaller size led to higher transcript yields. The structure of the 3′-terminal region of both full-length RNA 3 and Rep33 was found to consist of two stem–loops, α and γ, separated by a single-stranded domain, β2. Results of site-directed mutagenesis experiments revealed that disruption of both helices and most substitutions in the loops abolished Rep33 replicability. Furthermore, most compensatory substitutions which re-established the helix structures did not restore viability. Thus it appears that both secondary structure and the sequence in hairpin loops α and γ, as well as in the non-base paired region, are important for RNA 3 replication *in planta*.

MATERIALS AND METHODS

Construction of Rep33 carrying wild-type or modified 3′ promoters

DNA corresponding to the 3′-terminal region of BNYVV RNA 3 was amplified by PCR using as primers the M13 reverse primer and oligonucleotide 356, which corresponds to nt 1689–1708 of

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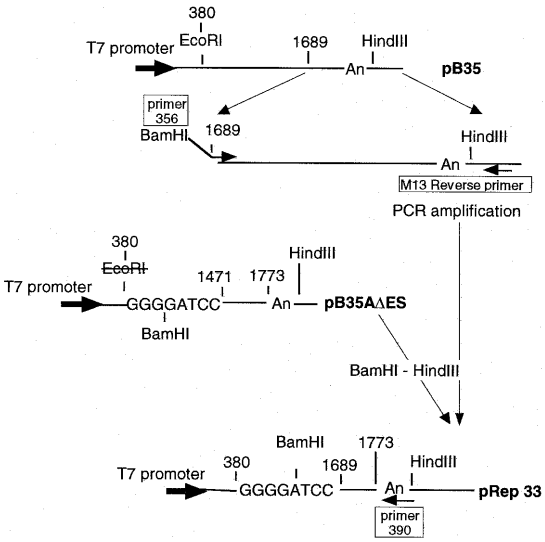

Figure 1. Cloning strategy used to produce the transcription vector pRep33.

RNA 3 (plus a novel *Bam*HI site introduced at its 5′-terminus) and pB35 (7) DNA as template (Fig. 1). The resulting PCR fragment was purified on a low melting point agarose gel, digested with *Bam*HI and *Hin*dIII and subcloned into *Bam*HI/*Hin*dIII-digested pAΔES (5). After transformation of *Escherichia coli* Sure cells, plasmid DNA isolated from clones was subjected to restriction enzyme digestion and sequence analysis (9). Mutagenesis of the 85 nt preceding the poly(A) tail was performed by overlap extension PCR using appropriate complementary mutagenic primers, primer 356 and the M13 reverse primer (10). PCR fragments obtained were cloned into *Bam*HI/*Hin*dIII-digested Rep33 and sequenced.

Structural analysis of the RNA 3 and Rep33 3′-terminal domain by chemical and enzymatic probing *in vitro*

For structural studies non-capped full-length RNA 3 and Rep33 transcripts were prepared by bacteriophage T7 RNA polymerase transcription of *Hin*dIII-linearized pB35 (7) and pRep33. Transcripts were purified by phenol extraction and ethanol precipitation in the presence of 2 M ammonium acetate. The RNA pellets were dissolved in sterile water and purified on Sephadex G50 fine spin columns. Aliquots (1 µg) of the transcript were treated with the chemical probes dimethylsulfate (DMS; Aldrich) and 1-cyclohexyl 3-(2-morpholinoethyl)carbodiimide metho-*p*-toluene (CMCT; Merck) and the enzymatic probes RNase V1, RNase T1 and RNase U2 (Pharmacia) (11). All reactions described were performed at 37°C. For DMS modification under native conditions the reaction was carried out for 1 and 3 min in 20 µl buffer N1 (50 mM sodium cacodylate, pH 7.0, 20 mM magnesium acetate, 300 mM KCl) in the presence of 2 µl DMS freshly diluted 1/10 (v/v) in ethanol. DMS modification under semi-denaturing conditions was performed in the same manner except that the reaction was carried out in buffer D1 (50 mM sodium cacodylate, pH 7.0, 1 mM EDTA) for 1 and 2 min. CMCT modification under native conditions was in 20 µl buffer N2 (50 mM sodium borate, pH 8.0, 20 mM magnesium acetate, 300 mM KCl) in the presence of 5 µl CMCT (42 mg/ml in H_2O) for 2 and 4 min. The modification reaction under semi-denaturing conditions was carried out in buffer D2 (50 mM sodium borate, pH 8.0, 1 mM EDTA) for 1 and 3 min. RNase T1 digestion was performed on 2 µg substrate RNA in 20 µl buffer T1 (10 mM sodium cacodylate, pH 7.5, 4 mM magnesium acetate, 50 mM potassium acetate) with 2 U enzyme for 1 min. RNase U2 digestion was performed on 2 µg substrate RNA in 20 µl buffer U2 (20 mM sodium citrate, pH 5, 20 mM magnesium chloride, 250 mM potassium acetate) with 2 U enzyme for 1 min. RNase V1 digestion was performed on 2 µg substrate RNA in 20 µl buffer N1 with 0.050 U enzyme for 1 min. For each of the reactions a control was treated in parallel omitting the chemical reagent or the enzyme. All reactions were stopped by addition of 100 µl 0.3 M sodium acetate, pH 6.0, 250 µl ethanol and the RNA was allowed to precipitate at –80°C for 30 min. After centrifugation the RNA pellets were washed twice with 80% (v/v) ethanol and vacuum dried. The pellets were then dissolved in 7 ml sterile H_2O. Sites of nucleotide modification or enzymatic scission were detected by primer extension as described (12,13). The primer (390) used for extension was 5′-AGCTTTTTTTTTTTT-TTTTG-3′, which is complementary to the 3′-terminal residue (C_{1773}) of the RNA 3 sequence proper and the first 16 residues of the poly(A) tail (Fig. 1). The primer was prepared with an Applied Biosystem apparatus using the phosphoramidite method and then 5′-^{32}P-end-labelled with polynucleotide kinase (14). Primer extension products were fractionated on 9 or 12% polyacrylamide:bisacrylamide (1:20) gels containing 8 M urea. Computer-aided predictions of secondary structure for the 3′-terminal region of RNA 3 used the program Mfold (15,16) implemented on a Vax microcomputer.

Synthesis of wild-type and mutant transcripts of Rep33 and inoculation of *Chenopodium quinoa*

Capped wild-type and mutant Rep33 transcripts were synthesized and template DNA was eliminated as described (5,6,8). Wild-type or mutant Rep33 transcript plus BNYVV RNA of an isolate (Stras 12) containing only RNAs 1 and 2 (17) was inoculated into leaves of *C.quinoa*. The viral progeny RNA in RNA extracted from the infected leaves 8 days post-inoculation was detected by Northern hybridization using ^{32}P-labelled antisense RNA probes (18). The Rep33-specific probe was complementary to nt 1–380 of RNA 3. RNA 1 and RNA 2 antisense probes were as described (19). The accumulation of wild-type or mutated Rep33 relative to RNA 2 was quantified with a Fuji MAS 1000 BioAnalyser.

Preparation and inoculation of *C.quinoa* protoplasts

Chenopodium quinoa protoplasts were prepared as described (20). About 200 000 freshly prepared protoplasts were electroporated with wild-type or mutated Rep 3 transcripts (2–4 µg) alone for stability studies (21) or together with 2 µg Stras 12 RNA for replication studies (19). Extraction of total RNA from the inoculated protoplasts at various times post-inoculation and detection of residual transcript RNA by dot blot or RNA replication by Northern hybridization were as described (18–20,22).

Characterization of viral RNA progeny

To study the stability of mutations introduced within the 3′ promoter of replicable transcripts RNA purified from infected

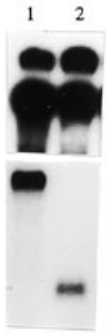

Figure 2. Northern blot hybridization to detect progeny RNA in *C.quinoa* inoculated with BNYVV RNAs 1 and 2 plus the full-length RNA 3 transcript t35 (lane 1) or tRep33 (lane 2). The upper portion of the blot was hybridized with a mixture of ^{32}P-labelled antisense RNA probes specific for RNAs 1 and 2 and the lower portion of the blot was hybridized with an antisense probe specific for RNA 3.

leaves was treated with RNase-free DNase (Promega) and subjected to reverse transcription using oligo(dT) as primer followed by PCR amplification with specific primers (RT-PCR). The PCR fragments were then characterized by sequencing the entire 3′ promoter region with an Applied Biosystems 373 DNA sequencer.

RESULTS AND DISCUSSION

Construction of a short RNA 3-based replicon by deletion of replication-dispensable sequences

To facilitate structural study of the 3′-terminal region of RNA 3 most of the replication-dispensable internal region (nt 381–1688) of the full-length RNA 3 transcription vector pB35 was replaced by a *Bam*HI site (Fig. 1). The resulting construct, pRep33, contains the 5′-terminal 380 residues and the 3′-terminal 84 residues plus a 16 residue 3′ poly(A) tail. Presumably because of its reduced size, pRep33 is transcribed *in vitro* to ~5-fold higher molar yield than was pB35, which is an advantage for structural analysis. The Rep33 transcripts were productively replicated in *C.quinoa* leaves when co-inoculated with RNAs 1 and 2. The level of accumulation of the Rep33 progeny, however, was about five times lower than observed with full-length RNA 3 transcripts (Fig. 2), illustrating that sequences within the deleted internal portion of the RNA, although not strictly essential, can markedly stimulate productive replication rates. Transcripts of a construct in which the 3′-terminal domain had been extended upsteam to nt 1471 accumulated *in planta* to levels similar to full-length RNA 3 (data not shown), indicating that the putative replication enhancer sequences map just upstream of the 3′-terminal *cis*-essential core domain of 84 residues.

Structural analysis of the 3′-terminal region of RNA 3 and Rep 33

Full-length transcripts of BNYVV RNA 3 and Rep33 were subjected to the chemical structure-sensitive probes DMS and CMCT or to the enzymatic probes RNase T1, U2 and V1, followed by primer extension analysis to locate sites of preferential chemical modification or nuclease cleavage within the last 68 nt (13,23). Adenosine (N1-A) and cytosine (N3-C) were tested for accessibility of their Watson–Crick positions to DMS and guanine (N1-G) and uridine (N3-U) to CMCT. Advantages and limitations of such probing approaches have been discussed elsewhere (11). To estimate the degree of stability of the helices, chemical probing was carried out in the presence (native conditions) or absence (semi-denaturing conditions) of magnesium ions. Additional information was obtained by probing the transcripts with RNase T1, specific for unpaired G residues, RNase U2, specific for unpaired A residues, and RNase V1, specific for double-stranded or stacked regions (11,24). Proton-catalysed artifactual hydrolysis (25) and artifactual stops or pauses of the reverse transcriptase were detected in parallel control incubations (11). Such stops or pauses result in loss of information for the position affected. Typical autoradiographs obtained after primer extension are shown in Figure 3. Full-length RNA 3 and the Rep33 transcripts showed similar accessibility to chemical and enzymatic probes within the common 3′ sequence (compare the nuclease T1 digestion profiles, the CMCT accessibility of nt $_{1746}$UUGU$_{1749}$ and the DMS accessibility of nt $_{1732}$AAA$_{1734}$ or A$_{1741}$, for example, in Fig. 3A) and present the same pattern of artifactual stops (for example C$_{1740}$, G$_{1742}$ U$_{1743}$ and C$_{1744}$ in Fig. 3A). We conclude that deletion of nt 381–1688 from RNA 3 does not alter the folding of the 3′-terminal core promoter domain. Therefore, further analysis was performed on Rep33 transcripts. Accessibility of the residues based on the gels shown in Figure 3 and many other gels (not shown) are summarized on the secondary structure model proposed in Figure 4.

The structure model in Figure 4 is based upon computer predictions using the Zuker algorithm on the last 84 nt of RNA 3 (15,16) and our experimental data. In the structure the RNA chain folds into two distinct stem–loop structures (α and γ) and two single-stranded regions, β1 and β2, forming the β domain. These three regions will be discussed separately. Note that the folding in Figure 4 is similar but not identical to our earlier model based on computer predictions and phylogenetic considerations (5). In particular, in the earlier model $_{1711}$AAGA$_{1714}$ was predicted to base pair with $_{1755}$UCUU$_{1758}$ because of co-variation, which conserved the base pairing potential in this region of the three other BNYVV RNAs. Our chemical and enzymatic probing experiments, however, indicate that this region does not participate in stable base pairing, at least *in vitro*.

Stem–loop α

Helix α (1721–1729/1737–1744) in Figure 4 is predicted to be structured based on the lack of reactivity of many nucleotides and the presence of RNase V1 cuts in the helix. The information on nt 1740–1744 is poor due to the presence of a compression in the sequence gels and reverse transcriptase pauses in this region, resulting in a loss of data. However, the lack of reactivity of bases on the opposite strand indicates a quite stable structure. The basal part of the helix is well stabilized by four G-C base pairs which are only cleaved by double-strand-specific RNase V1. The presence of a marginally non-reactive bulged G$_{1727}$ may be explained either by a stacked nucleotide within the helix or by a possible equilibrium for base pairing between the G residues at positions 1725–1727 and C residues 1739–1740. This bulge may also explain the behaviour of the neighbouring U$_{1728}$-A$_{1738}$ base pair, which is weakly reactive under native conditions, although the reactivity of U$_{1728}$ is increased in the absence of magnesium. The stem is closed by two non-canonical base pairs: G$_{1729}$-U$_{1737}$

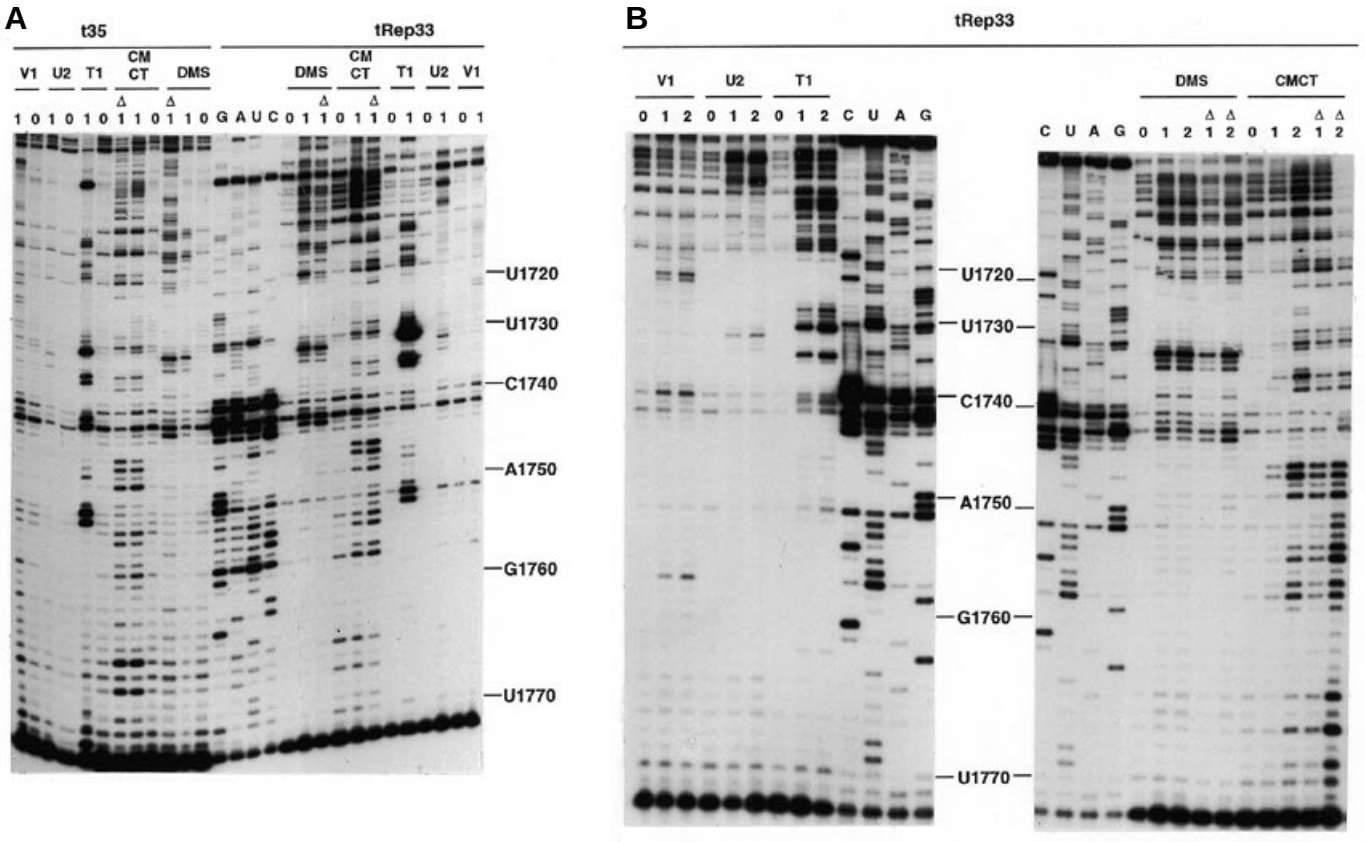

Figure 3. Polyacrylamide gel fractionation of cDNA products obtained after primer extension using as template t35 (**A**) or Rep33 (**A** and **B**) transcripts which had undergone treatment with DMS or CMCT or with RNase T1, U2 or V1. Chemical and enzymatic reactions were carried out at 37°C in the appropriate buffer (see Materials and Methods). Native conditions: (0) incubation control; (1) DMS 1 min, CMCT 2 min, RNase T1 30 s, RNase U2 30 s, RNase V1 30 s; (2) DMS 3 min, CMCT 4 min, RNase T1 1 min, RNase U2 1 min, RNase V1 1 min. Semi-denaturing conditions: (Δ1) DMS 1 min, CMCT 1 min; (Δ2) DMS 2 min, CMCT 3 min. Lanes G, A, U and C are conventional sequence ladders generated by primer extension in the presence of ddGTP, ddATP, ddTTP and ddCTP respectively. The 5′-^{32}P-labelled primer (see Materials and Methods) used to generate the extension products and the electrophoresis time were chosen for optimal resolution of the sequence of interest on the gel. For additional information concerning the probing method see Ehresmann *et al.* (11), Lempereur *et al.* (12) and Baudin *et al.* (13). Reactivity differences between Rep33 and t35 near the top of (A) are due to the fact that the sequences differ in this region because of the deletion present in Rep33 RNA. Differences of intensities for RNase T1 digestions between (A) and (B) are due to use of different batches of enzyme.

and U_{1730}-G_{1736}. U_{1730} and G_{1729} are only reactive in the absence of magnesium. G_{1736}, like U_{1737}, is exposed to the solvent, as shown by RNase T1 cleavage and its reactivity under native conditions. The sensitivities of these nucleotides to structural probes may reflect low stability of the upper part of the stem, possible breathing of the loop or the existence of long distance base pairing. Non-canonical interactions have already been described for other RNAs and tRNAs and the resulting irregular structures may correspond to protein binding sites (for a review see 26). The α hairpin is closed by loop $_{1731}$GAAAU$_{1735}$, whose nucleotides are fully reactive and highly sensitive to RNases T1 and U2 under native conditions.

Single-stranded domain β

Based upon their full accessibility to RNases T1 and U2 and to chemical structural probes, nucleotides $_{1705}$AAGGUUAAGAU-GUAC$_{1719}$ of the β1 region preceeding helix α are predicted to be in single-stranded conformation. Its poor reactivity under native conditions suggests that U_{1720} is stacked on helix α. The other part of the β domain consists of nt $_{1745}$CUUGUAGG-GUUCUU$_{1758}$, where the strong reactivity with all probes indicates that the β2 region is also more likely to be single-stranded rather than base paired.

Stem–loop γ

Stem–loop γ consists of a short helix closed by a four base loop ($_{1765}$UAUA$_{1769}$). Structural data on helix γ (1759–1763/1770–1733+A) could be determined only for positions upstream of nt 1770, due to the proximity of the 3′-end of the primer binding site at nt 1773. Helix γ seems to be highly structured, as shown by the low reactivity of nt $_{1760}$GUCAG$_{1764}$ and the presence of RNase V1 cuts in this region. The reactivity of U_{1759} is characteristic of an A-U pair at the end of a helix (13). The γ loop is closed by a non-canonical interaction between U_{1769} and G_{1764}. U_{1769} is reactive under semi-denaturing conditions, indicating that magnesium is required to stabilize this interaction. Nucleotides $_{1765}$UAUA$_{1768}$ are only weakly reactive under native conditions, suggesting the possible existence of an intrinsic structure of the loop or of long distance base pairing with another part of the RNA. Such a long distance interaction could occur with complementary nucleotides $_{1731}$GUGUG$_{1727}$ but not be sufficiently stable to persist *in vitro*. In some cases such long

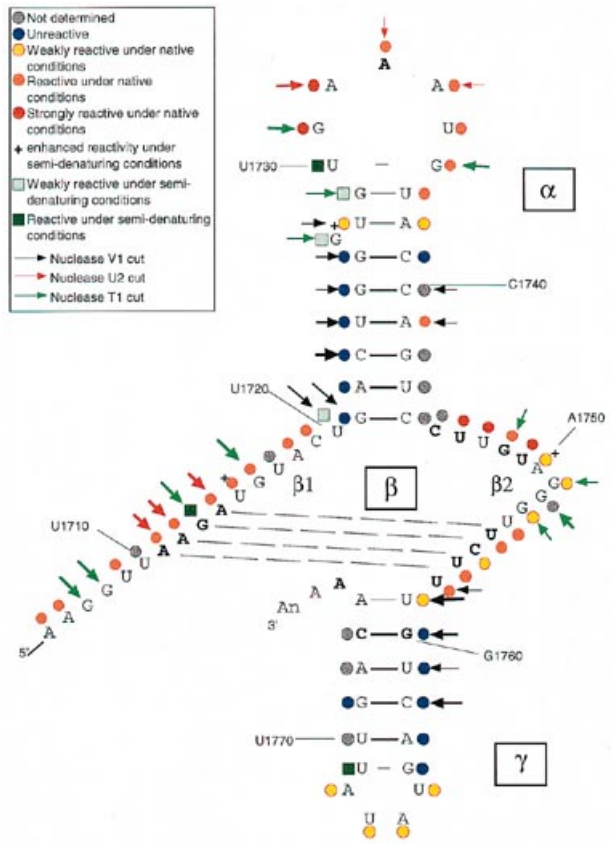

Figure 4. Reactivity with DMS and CMCT and sensitivity to RNAses T1, U2 and V1 of the 3′-terminal 68 residues of RNA 3. The results are displayed on the deduced secondary structure. Reactivities towards the chemical probes at the Watson–Crick positions were as indicated in the key at the upper left of the figure, with bold arrows designating major cuts and thin arrows moderate cuts. Dashed lines represent the previously described base pairing deduced by base compensatory variations (bold nucleotides) between the 3′ promoters of different BNYVV RNAs (5). The two different helices and the single-stranded region referred to in the text are designated α, γ and β respectively.

distance interactions require the assistance of an RNA chaperone to properly fold the RNA (for a review see 27).

Mutagenesis of the 3′ core promoter region

Replication of a viral single-stranded positive sense RNA is accomplished by a virus-encoded RNA-dependent RNA polymerase (RdRp), probably assisted by host proteins (28). First, a complementary, or minus strand, RNA is synthetized using the positive sense RNA as a template. The minus strand RNA is then used as template for production of plus strand RNAs. The promoter sequence for minus strand RNA synthesis recognized by RdRp is generally located at or near the 3′-terminus of the viral plus sense RNA. To correlate the fine structure of the Rep33 3′-terminal region to its function in replication, a series of mutations were designed to disrupt or stabilize hairpins α and γ and to probe for possible interactions within the putative single-stranded regions. In order to investigate the effects of the different mutations, mutant Rep33 transcripts were inoculated along with RNA 1 and 2 into *C.quinoa* leaves and tested for accumulation of the progeny Rep RNAs relative to 'wild-type' Rep33. For those mutants which replicated *in planta* the persistence of the mutations in the progeny RNA was routinely characterized by RT-PCR and sequence analysis. No reversions or pseudo-reversions were identified for any of the mutants tested below (data not shown).

Compensatory base variations in $_{1711}$AAGA$_{1714}$ and $_{1754}$UCUU$_{1758}$ do not enable efficient replication

As related above, phylogenetic arguments (5) suggested that base pairing might exist between $_{1711}$AAGA$_{1714}$ and $_{1754}$UCUU$_{1750}$ (Fig. 5), although we could find no evidence in this study for base pairing of these regions *in vitro*. To test whether such an interaction is important for replication, the potential for base pairing was disrupted by introducing A/C mismatches in mutant B (Fig. 5). As shown in Figure 6, mutant B still replicated *in planta*, although with lower efficiency (56% of the level of Rep33). Mutants A and A-B (Fig. 5) were constructed to alter the sequence but maintain the base pairing by using G-U and G-C base pairs respectively. These mutants were also able to replicate *in planta* (Fig. 6), indicating that none of the targeted sequences are essential for efficient replication. Mutant A accumulated to a level similar to wild-type Rep 33, but the double mutant A-B (four G-C base pairs) accumulated about twice as efficiently *in planta*. The more efficient accumulation of A-B might be related to extra stability conferred on the transcript or its progeny by the extended helix in the mutant. We have tested the stability of A-B by electroporating the transcript alone into *C.quinoa* protoplasts and monitoring its degradation at various times post-inoculation. The half-life of mutant A-B transcripts following electroporation into protoplasts proved to be about twice that of Rep33 (data not shown; similar tests with mutant A and B transcripts revealed that they were similar in stability to Rep33). Secondary structure probing of mutants A, B and A-B did not show any reactivity variations from the wild-type sequence except for the mutated nucleotides, which were sites of artifactual stops of the reverse transcriptase due to high contents of cytosine and/or guanine (data not shown).

Other mutants were designed to replace the single-stranded region in domain β by an extended helix (Fig. 5). This was achieved by replacing $_{1715}$UGUACU$_{1720}$ by ACCCUA (mutant C) or ACCCUACAA (mutant D). The half-lives of transcripts C and D following electroporation into protoplasts were similar to that of Rep33. Mutant C was able to replicate when co-inoculated along with RNAs 1 and 2 into *C.quinoa* leaves (Fig. 6), indicating that the exact sequence *per se* between nt 1715 and 1720 is dispensable and that some extra base pairing in this region is tolerated. In contrast, mutant D was not replicated (Fig. 6). Possibly, the extended helix created in domain β by this mutation locks the 3′ promoter domain into a conformation which is not recognized by RdRp. These findings may indicate that some flexibility in the promoter region is required for replication to occur. Alternatively, replication may initiate but stall once chain extension attains the highly structured portion of Mutant D.

Sequence disruptions in loops abolish replication

Possible 'kissing interactions' between loop γ and hairpin α ($_{1765}$UAUAU$_{1769}$ with $_{1731}$GUGUG$_{1727}$) within the 3′ promoter have been studied using mutants Q, R, S, T, U, Q-S and R-S (Fig. 5). Mutants Q, S, T and R-S destabilize possible interactions between the two above-mentioned domains, whereas mutants R, U and Q-S were designed to maintain such interactions. None of

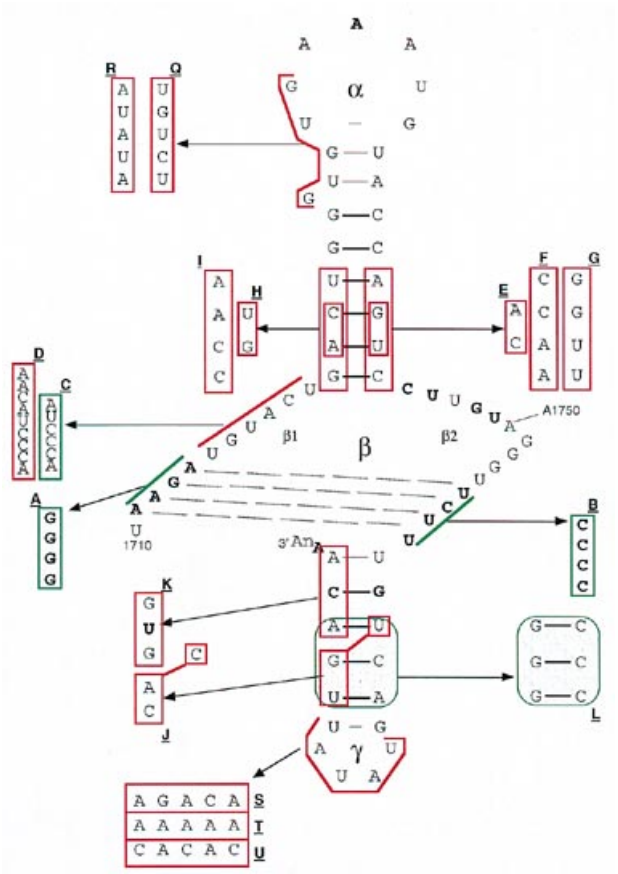

Figure 5. Mutations introduced in the 3′-terminus of Rep33. The names of the different mutants tested in this study are underlined. Sequence replacements which abolished replicon replication are boxed in red (mutants D, E, F, G, H, I, J, K, E-H, G-H, F-I, Q, R, S, T, U, Q-S and R-S). Green boxes indicate mutations that were not lethal for RNA accumulation (mutants A, B, C, L and A-B). Nomenclature of the figure is as in Figure 4.

the aforesaid mutants were productively replicated *in vivo* (Fig. 6). Thus no conclusion can be drawn concerning the existence of the putative kissing interactions, although the looped sequences *per se* are apparently important for specific interactions with the replicase.

Destabilization of helix α or γ interferes with replication

In view of the fact that the sequences of loops α and γ are important for replication, we have tested whether helices α and γ are likewise important. Mutants (Fig. 5) of helices α and γ were designed to abolish two or more base pairs (mutants E, F, I, J and F-I), alter the base pairing scheme (mutants G, H, K and L) or restore base pairs by compensatory mutations (mutants E-H and G-H). Of this series of mutants only L was replicated and only with ~10% of the efficiency of wild-type Rep33 (Fig. 6). In the case of mutant E-H the sequence of the two base pairs in stem α was inverted with respect to the positions of purines and pyrimidines in the wild-type sequence. This change is calculated to decrease the stability of the mutant helix α by 1.6 kcal and we cannot strictly rule out the possibility that the non-replicability of this mutant is related to the decrease in helix stability rather than the sequence changes. Nevertheless, taken as a whole our results

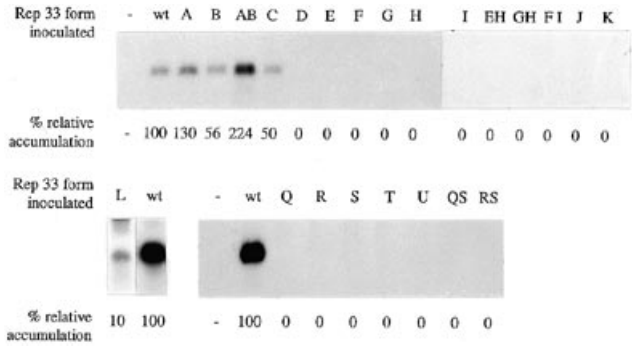

Figure 6. Effects of mutations in the 3′-terminal core promoter on Rep33 replication *in planta*. Replicon transcripts carrying the mutations shown in Figure 5 were inoculated along with RNA 1 and 2 into *C.quinoa* leaves and the progeny viral RNA contents were analysed by Northern blot hybridization 8 days post-inoculation. The blot was first probed with a specific antisense RNA 3 riboprobe and the amount of radioactivity (mean of two experiments) in the band corresponding to each mutant replicon was calculated relative to the radioactivity in wild-type Rep33 (100%). Then the blot was probed with riboprobes specific for RNAs 1 and 2 (not shown) to ensure that similar levels of RNAs 1 and 2 were present in each lane.

indicate that both secondary structure and the sequence itself in helices α and γ are important for efficient replication of the viral molecule.

In view of the high degree of sequence conservation in the 3′-terminal region of the four BNYVV RNAs we have constructed chimeric versions of Rep33 carrying the 3′-terminal core promoter sequence of RNA 1, RNA 2 or RNA 4 instead of that of RNA 3. Structural probing of such chimeric transcripts showed similar reactivities against chemical and enzymatic probes (data not shown), confirming the presence of a similar structural motif at the 3′ extremity of the four RNAs.

The overall configuration of the BNYVV 3′ core promoter is somewhat similar to the double hairpin structure of the poliovirus *ori*R 3′ promoter (29). In the latter case long distance interactions between the loops of two domains (X and Y) were found to be critical for efficient recognition by the replication machinery. Poliovirus transcripts carrying mutations within the loop sequences were either not replicated or their replication was accompanied by reversion or pseudo-reversion of the mutated sequences. Long distance interactions, such as those observed in the poliovirus *ori*R promoter, were not detected by our study.

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