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Conserved and variable structural elements in the 5' untranslated region of two hypoviruses from the filamentous fungus *Cryphonectria parasitica*

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Abstract

Virulence-attenuating viruses (hypoviruses) of the filamentous fungus *Cryphonectria parasitica*, the causative agent of chestnut blight, have become a premier model for understanding the molecular biology of mycoviruses. However, a major gap exists in current understanding of structure and function of the untranslated regions (UTRs) of the hypovirus RNA genome, despite considerable evidence that secondary and tertiary UTR structure plays a crucial role in the control of translation and genome replication in other systems. In this study we have used structure prediction software coupled with RNase digestion studies to develop validated structural models for the 5' UTRs of the two best-characterized members of the *Hypoviridae*, CHV1-EP713 and CHV1-Euro7. These two hypovirus strains exhibit significant variation in virulence attenuation despite sharing >90 % sequence identity. Our models reveal highly structured regions in the 5' UTR of both strains, with numerous stem-loops suggestive of internal ribosome entry sites. However, considerable differences in the size and complexity of structural elements exist between the two strains. These data will guide future, mutagenesis-based studies of the structural requirements for hypovirus genome replication and translation.

Keywords

mycovirus; secondary structure; *Cryphonectria parasitica*; hypovirus; Mfold; RNA virus

First identified from mushrooms in 1962 (Hollings, 1962), mycoviruses typically comprise RNA genomes located in the cytoplasm of the host cell (Buck, 1986; Pearson et al., 2009), although a DNA mycovirus was recently identified (Yu et al., 2010). Mycoviruses are associated with all major classes of fungi (Buck, 1986; Pearson et al., 2009), but the only group of mycoviruses for which infectious cDNA clones have been developed are the hypoviruses (family *Hypoviridae*), which infect the chestnut blight fungus *Cryphonectria parasitica* (Chen et al., 1994; Choi and Nuss, 1992) and Narnavirus 23S RNA infecting *Saccharomyces cerevisiae* (Esteban and Fujimura, 2003). The family *Hypoviridae* contains four species designated CHV1, -2, -3 (Hillman, 1995) and -4 (Linder-Basso et al., 2005) and all four species can infect *C. parasitica*. However, in contrast to CHV1, -2, -3, only CHV4 has little impact on fungal virulence (Linder-Basso et al., 2005). *C. parasitica* is the fungus responsible for the near elimination of the American chestnut tree, *Castanea dentata*, from its

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natural range across the eastern United States, and understanding the biology of virulence-attenuating viruses has significant implications for their applications as biological control agents.

Two members of the *Hypoviridae* have been studied extensively, CHV1-EP713 and CHV1-Euro7. The two viruses share sequence identities of greater than 90 % at the nucleotide and amino acid levels (Chen and Nuss, 1999). CHV1-EP713 was the first hypovirus to be sequenced. The infection by CHV1-EP713 dramatically reduces fungal pigmentation, sporulation and virulence. In contrast, CHV1-Euro7 infection results in fungal phenotypes such as colony development and virulence that are intermediate between CHV1-EP713-infected and uninfected cultures.

Historically referred to as having ds RNA genomes, it has been suggested that the genomic form found in the fungal mycelium may represent a replication intermediate of what is in fact a positive sense, ss genome (Nuss, 2005), a contention supported by the closest relatives in the supergroup I RNA virus category, which are members of the positive stranded *Potyviridae* family (Ghabrial and Suzuki, 2009; Nuss, 1993; Suzuki et al., 1999). The genome organization of CHV1-EP713 has been well characterized and consists of a 12.7 kb RNA molecule. This includes, at the 5' end, a 495 nucleotide UTR and, at the 3' end, a 851 nucleotide UTR (Shapira et al., 1991). CHV1-Euro7 is believed to be similarly organized, although the 5' UTR in this case is 494 nucleotides (nts) in length (Chen and Nuss, 1999). Interestingly, the less well-characterized members of the *Hypoviridae* do not share striking sequence or organizational similarity in the 5' regions. CHV4 has a relatively short 5' UTR compared to CHV1, -2 and -3, of only about 193 nts long. The length range of 5' UTRs of other species generally ranges from 369 nts to 487 nts (Hillman and Suzuki, 2004). It is well known that the UTRs of many positive-strand RNA viruses fold into complex structures that may regulate translation and RNA replication (Jang, 2006; Li et al., 2010; Polacek et al., 2009; Romero et al., 2006; Watts, 2009). Furthermore, naturally-occurring variation in UTR structure has been shown to effect variation in virulence in Theiler's murine encephalomyelitis virus (Lipton, 1991; Stein et al., 1992) and carmovirus (Truniger et al., 2008). However, little is currently known about the structure or function of *Hypoviridae* UTRs. To gain insight into such functions, we have analyzed and compared the structural elements in the 5' UTRs of two hypoviruses, CHV1-EP713 and CHV1-Euro7, known to differ in virulence.

Mfold version 2.3 (<http://mfold.rna.albany.edu/>) was initially used to predict the secondary structure of the positive sense strand 5' UTR regions of both CHV1-EP713 and CHV1-Euro7 using publicly available sequence information (Genbank accession numbers NC_001492.1 and AF082191, respectively). Since the growth of *C. parasitica* under laboratory conditions occurs in the range of 20 – 25 °C the structures were predicted for each extreme of this range. No differences were noted in the lowest free energy structures at either temperature; therefore all subsequent predictions were performed assuming a temperature of 25 °C. The length of nucleotides to be analyzed was determined by progressively expanding the length of the tested fragment. Initial predictions with only portions of the 5' UTR returned markedly different structures from those predicted by the entire 5' UTR (data not shown). Furthermore, the predicted structure of the UTR was also influenced by the presence of proximal portions of the translated region. The smallest combination of UTR and translated region, beyond which increasing the size of modeled fragment did not change the predicted structure of the UTR, was found to be 675 nucleotides in both cases. Initial structures predicted by Mfold in the absence of any constraints were prepared using these parameters for both hypovirus sequences and are available as electronic supplementary Fig. S1 and S2. Common to both predictions are large, compound, domains of approximately 100 to 300 nucleotides.

In order to refine these structural models, we used RNase mapping. Positive sense RNA transcripts representing the complete CHV1-EP713 and CHV1-Euro7 genomes were generated from the hypovirus clones pLDST and pTE7 kindly provided by D.L. Nuss (University of Maryland) that contain full-length cDNA copies of the positive sense strand of CHV1-EP713 (Choi and Nuss, 1992) or CHV1-Euro7, respectively. Detailed protocols for RNA isolation and analysis are described in detail elsewhere (Dawe et al., 2011). Briefly, 1 µg pLDST and pTE7 were linearized with SpeI (New England Biolabs) and transcribed with T7 RNA polymerase (AmpliCap™ T7 and SP6 kits, Epicenter Technologies) respectively, then purified using an RNeasy Mini Kit (QIAGEN Inc.). 1 µg of RNA products were then treated in individual reactions with 0.1U of RNase T1, 0.02U of RNase A, 4U of RNase I or 0.01U of RNase V1 respectively in a total volume of 11 µl. RNase T1 cleaves unpaired guanines; RNase A cleaves inter-nucleotide bonds 3' of unpaired uridines or cytosines, but also has some affinity for double-stranded regions (Christiansen et al., 1990); RNase I specifically cleaves single-stranded RNA, while RNase V1 specifically cleaves double-stranded RNA.

Following RNase digestion, sites of RNA cleavage were detected using reverse transcription primer extension and compared to simultaneously analyzed DNA sequence of the corresponding plasmid sequenced using the same primer. Undigested template was used as a control to detect intrinsic stops that resulted from premature transcriptional termination; these were observed at nucleotide (nt) 46, 65, 142, 400 and 410 in CHV1-EP713 and nt 47, 142, 208, 210 and 400 in CHV1-Euro7. Reverse transcription of the digested RNA used the IR-700 labeled primer mrep713seq-1 5'-CCACTGTAGTAGGATCAAC-3' complementary to nts 562 of the CHV1-EP713 genome (synthesized by Li-Cor Biosciences) and Superscript III reverse transcriptase (Invitrogen). The products were analyzed by a Li-Cor Biosciences 4200 DNA sequencer as described by Romero et al. (2006). Resolution was possible up to nt 520 for both hypovirus templates, thus covering the entire 5' UTR. The representative gels in Fig. 1 and 2 were each confirmed with two additional experiments (data not shown). However, due to the size of the analysis, the images have been divided for clarity into three panels (Fig. 1 and 2). Complete gel images are available as Supplementary Fig. S3 and S4. We did not attempt to make qualitative judgments about the relative intensity of bands, but scored as positive any band that was detectable on the original images.

These data were then used to identify unpaired nucleotides and subsequently constrain Mfold predictions. Constraints were limited to those identified cuts produced by the RNase T1 and RNase I enzymes. Although RNase mapping information can identify paired nucleotides as well as unpaired, no information is provided as to the partner nucleotide and therefore these constraints cannot be applied. Cuts by RNase A, while generally 3' to unpaired U or C nucleotides, may also occur at paired nucleotides as well (Christiansen et al., 1990). For this reason, the annotated cleavages at U211, U213, U259, U362, G482 (in CHV1-EP713) and U260, U301, U415, U451 and C499 in CHV1-Euro7 were not included. For CHV1-EP713, 67 constraints were applied, while for CHV1-Euro7 73 constraints were applied; specific details of the constraints employed are provided in the Supplementary Information. Additionally, ten dynamic cleavages, revealed by the generation of bands by both enzymes that cut double-stranded and enzymes that cut single-stranded nucleotides, were detected for CHV1-EP713 within the 520 nucleotides analyzed (Fig. 1). Similarly, five such cleavages were noted for CHV1-Euro7 (Fig. 2). Although such cleavages cannot be used to constrain the structure, they can indicate ambiguous regions that might be involved in formation of transient pseudoknots or other long-range associations.

Fig. 3 and 4 show the revised structure models for CHV1-EP713 and CHV1-Euro7; both generally resolve into smaller structures than the unconstrained models. Each model was extended for 675 nucleotides in order to account for the influence of proximal regions of

ORF A, as noted above. However, for clarity, each model is truncated following prediction to show only up to the last structural feature of the UTR (nt 540 for CHV1-EP713 and nt 520 for CHV1-Euro7). The output of Mfold was then manually adjusted to separate overlapping structures. Complete, unmodified, predictions are available as Supplementary Fig. S5 and S6. Color-coding of the individual nucleotides was performed by Mfold according to the *P-num* value, a measure of how well determined the prediction is for each nucleotide. This determination is then expressed as a color where the red end of the spectrum is expected to be paired and blue-black represents a more likely unpaired state (Zuker and Jacobson, 1998). The consistency between predicted structures and RNase maps was increased from 40 % (unconstrained) to 75 % (constrained) for CHV1-EP713 (i.e. of the total cuts identified, 75 % of the nucleotides were in the correct paired or unpaired state). Similarly, for CHV1-Euro7, the unconstrained value of 49 % increased to 71 %. These figures are consistent with previously published studies of other predicted RNA structures (Baird et al., 2006; Favaretto et al., 2005; Mertz et al., 2009; Romero et al., 2006) and are also broadly supported by *P-num* (Zuker and Jacobson, 1998).

The mechanism of translation initiation employed by hypoviruses is not well understood. Rae et al. (1989) found that a small portion of 5' noncoding sequence could inhibit hypovirus' translation *in vitro*, which indicated a possible function for the 5' UTR. Subsequently, Suzuki and colleagues (2000) provided additional support to suggest that the 5' UTR might function to facilitate an IRES-mediated translation mechanism together with the N-terminal portion of the first ORF. It has also not been empirically determined whether hypoviruses have a 5' cap structure to facilitate translation, although uncapped RNA produced *in vitro* is capable of initiating infection (Chen et al., 1994). As positive strand RNA viruses, hypoviruses may be candidates for a 5' cap-independent translation mechanism similar to picornaviruses and hepatitis C virus, which have been well documented (Jang, 2006). For an instance, poliovirus, a member of picornaviridae, has a 750 nt long 5' UTR with seven or eight upstream AUGs (Pelletier et al., 1988). Hepatitis C virus contains a ~340 nt long 5' UTR with three or four upstream AUGs (Tsukiyamakohara et al., 1992). In this study, CHV1 group has ~495 nt with seven AUG triplets present upstream of the authentic start codon at nts 5, 28, 109, 138, 319, 332 and 376. The similarity of the length and structural complexity observed in this study provides further support that translation in the CHV1 group is mediated by an internal ribosome entry site (IRES).

Unfortunately, it is not possible to identify an IRES based solely on secondary RNA structure (Baird et al., 2006). Future studies should investigate the role of elements conserved in both CHV1-EP713 and CHV1-Euro7, for instance domains I and II, in translation. It is quite striking, however, that the structures in the region between nt 220 to the end of the UTRs vary significantly between the two virus strains despite the high degree of sequence identity. CHV1-Euro7 is predicted to contain two large domains (IV and VIII), both of which include large loop structures (numbered 17 and 30, approximately nt 250 – 500) while the equivalent region of CHV1-EP713 appears to contain much smaller and more defined structural elements. Although we cannot exclude the possibility that this is an artifact of the analysis, it should be noted that 20 of the 37 nucleotide differences between the two UTRs are clustered in this section. Furthermore, the gel results from approximately nt 250 to 350 are generally fainter than those found at other stretches of the sequence. As noted above, we scored as positive any band that was detectable on the original images. The fainter bands may indicate that some of these structural features are transient in nature. This is also suggested by the observation of dynamic cleavages in this region, such as C476 and U496 of CHV1-Euro7.

The presence of double-stranded structures in the UTR is also supported by the previous observation of Zhang et al. that the RNA silencing mechanisms of *C. parasitica* produce an

abundance of viral-derived small RNAs, or vsRNAs, that target this region (Zhang et al., 2008). Of particular note, the vsRNAs from the 5' UTR sequence of CHV1-EP713 (vsRNA information is not available for CHV1-Euro7), are derived exclusively from the positive strand and are, in two cases, complementary to regions on the structure that we predict to be predominantly double-stranded (nt 118 – 144 and 198 – 214; Fig. 5A). Such correspondence is consistent with the response of RNA silencing mechanisms to dsRNA structures (Hammond, 2005). However, the third vsRNA sequence corresponds to a region that is predicted to contain a stretch of single-stranded nucleotides (nt 451 – 471, Fig. 5B). There are extensive cuts with RNase VI that span nt 443 – 520 (Fig. 1). In fact, 79 % (27 out of 34) of the RNase VI cuts identified in the entire UTR, including eight of nine identified dynamic cleavages, occurred in this region. Interestingly, there were fewer RNase VI cuts detected in the CHV1-Euro7 analysis, although they were still clustered in this region surrounding the AUG codon. These data indicate that this zone of CHV1-EP713, which includes the translational start site at nt 496 – 498, may contain more double-stranded structure than is predicted by the current methods, either due to binding with proximal nucleotides or, perhaps more likely, participation in long-range tertiary structures. The observation of asymmetric distribution of siRNAs derived from viral sequences noted with *Cymbidium ringspot tomosvirus* (Molnar et al., 2005) and, in the current system, by Zhang et al (2008), taken together with our information, suggests that the location of vsRNAs can be used to better infer secondary structure of the single stranded genome. With this in mind, and given the observation of a cluster of vsRNA's targeting the 3' UTR of CHV1 (Zhang et al., 2008), it is likely that further study of secondary structural features in the 3' UTR would be fruitful.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

nt	nucleotide
UTR	untranslated region

References

- Baird SD, Turcotte M, Korneluk RG, Holcik M. Searching for IRES. *RNA*. 2006; 12:1755–1785. [PubMed: 16957278]
- Buck, KW. Fungal virology. CRC Press; Boca Raton, Fla: 1986. p. 305
- Chen B, Choi GH, Nuss DL. Attenuation of fungal virulence by synthetic infectious hypovirus transcripts. *Sci*. 1994; 264:1762–1764.
- Chen B, Nuss DL. Infectious cDNA clone of hypovirus CHV1-Euro7: a comparative virology approach to investigate virus-mediated hypovirulence of the chestnut blight fungus *Cryphonectria parasitica*. *J Virol*. 1999; 73:985–992. [PubMed: 9882299]
- Choi GH, Nuss DL. Hypovirulence of chestnut blight fungus conferred by an infectious viral cDNA. *Sci*. 1992; 257:800–803.

- Christiansen, J.; Egebjerg, J.; Larsen, N.; Garrett, RA.; Spedding, G., editors. Ribosomes and protein synthesis: A practical approach. IRL Press; Oxford, UK: 1990. Analysis of rRNA structure: Experimental and theoretical considerations; p. 229-252.
- Dawe, AL.; Mu, R.; Rivera, G.; Salamon, JA. Molecular methods for studying the *Cryphonectria parasitica* hypovirus experimental system. In: Xu, J-R.; Bluhm, BH., editors. Methods in Molecular Biology. Vol. 722. 2011. (Fungal Genomics). In press
- Esteban R, Fujimura T. Launching the yeast 23S RNA Narnavirus shows 5' and 3' cis-acting signals for replication. Proceedings of the National Academy of Sciences of the United States of America. 2003; 100:2568–2573. [PubMed: 12591948]
- Favaretto P, Bhutkar A, Smith TF. Constraining ribosomal RNA conformational space. Nucleic Acids Res. 2005; 33:5106–5111. [PubMed: 16155182]
- Ghabrial SA, Suzuki N. Viruses of Plant Pathogenic Fungi. Annu Rev Phytopathology. 2009; 47:353–384.
- Hammond SM. Dicing and slicing: the core machinery of the RNA interference pathway. FEBS Lett. 2005; 579:5822–5829. [PubMed: 16214139]
- Hillman BI, Suzuki N. Viruses of the chestnut blight fungus, *Cryphonectria parasitica*. Adv Virus Res. 2004; 63:423–72. [PubMed: 15530566]
- Hillman, BIF.; DW; Nuss, DL.; VanAlfen, NK.; Murphy, FAFCM.; Bishop, DHL.; Ghabrial, SA.; Jarvis, AW.; Martelli, GP.; Mayo, MA.; Summer, MP., editors. Viruses. 6. New York: 1995. Hypoviridae.
- Hollings M. Viruses Associated with a Die-Back Disease of Cultivated Mushroom. Nature. 1962; 196(4858):962–965.
- Jang SK. Internal initiation: IRES elements of picornaviruses and hepatitis c virus. Virus Res. 2006; 119:2–15. [PubMed: 16377015]
- Li W, Manktelow E, von Kirchbach JC, Gog JR, Desselberger U, Lever AM. Genomic analysis of codon, sequence and structural conservation with selective biochemical-structure mapping reveals highly conserved and dynamic structures in rotavirus RNAs with potential cis-acting functions. Nucleic Acids Res. 2010; 38:7718–7735. [PubMed: 20671030]
- Linder-Basso D, Dynek JN, Hillman BI. Genome analysis of *Cryphonectria* hypovirus 4, the most common hypovirus species in North America. Virol. 2005; 337:192–203.
- Lipton HL, Calenoff M, Bandyopadhyay P, Miller SD, Dalcanto MC, Gerety S, Jensen K. The 5' noncoding Sequences from a Less Virulent Theilers Virus Dramatically Attenuate Gdvii Neurovirulence. J Virol. 1991; 65:4370–4377. [PubMed: 2072455]
- Mertz JA, Chadee AB, Byun H, Russell R, Dudley JP. Mapping of the functional boundaries and secondary structure of the mouse mammary tumor virus Rem-responsive element. J Biol Chem. 2009; 284:25642–25652. [PubMed: 19632991]
- Molnar A, Csorba T, Lakatos L, Varallyay E, Lacomme C, Burgyan J. Plant virus-derived small interfering RNAs originate predominantly from highly structured single-stranded viral RNAs. J Virol. 2005; 79:7812–7818. [PubMed: 15919934]
- Nuss, DL. Similarities between hypoviruses of the chestnut blight fungus, plant potyviruses and barley yellow mosaic virus. Proceedings of the Second Symposium of the International Working Group on Plant Viruses with Fungal Vectors; 25–27 July, 1993; Montreal, Canada: McGill University; 1993.
- Nuss DL. Hypovirulence: Mycoviruses at the fungal-plant interface. Nat Rev Microbiol. 2005; 3:632–642. [PubMed: 16064055]
- Pearson MN, Beever RE, Boine B, Arthur K. Mycoviruses of filamentous fungi and their relevance to plant pathology. Mol Plant Pathol. 2009; 10:115–28. [PubMed: 19161358]
- Pelletier J, Flynn ME, Kaplan G, Racaniello V, Sonenberg N. Mutational analysis of upstream AUG codons of poliovirus RNA. J Virol. 1988; 62:4486–4492. [PubMed: 2846865]
- Polacek C, Foley JE, Harris E. Conformational changes in the solution structure of the dengue virus 5' end in the presence and absence of the 3' untranslated region. J Virol. 2009; 83:1161–1166. [PubMed: 19004957]

- Rae BP, Hillman BI, Tartaglia J, Nuss DL. Characterization of double-stranded RNA genetic elements associated with biological control of chestnut blight: organization of terminal domains and identification of gene products. *EMBO J.* 1989; 8:657–663. [PubMed: 2721496]
- Romero TA, Tumban E, Jun J, Lott WB, Hanley KA. Secondary structure of dengue virus type 4 3' untranslated region: impact of deletion and substitution mutations. *J Gen Virol.* 2006; 87:3291–3296. [PubMed: 17030863]
- Shapira R, Choi GH, Nuss DL. Virus-like genetic organization and expression strategy for a double-stranded RNA genetic element associated with biological control of chestnut blight. *EMBO J.* 1991; 10:731–739. [PubMed: 2009854]
- Stein SB, Zhang L, Roos RP. Influence of Theiler's murine encephalomyelitis virus 5' untranslated region on translation and neurovirulence. *J Virol.* 1992; 66:4508–4517. [PubMed: 1602556]
- Suzuki N, Chen BS, Nuss DL. Mapping of a hypovirus p29 protease symptom determinant domain with sequence similarity to potyvirus HC-Pro protease. *J Virol.* 1999; 73:9478–9484. [PubMed: 10516056]
- Suzuki N, Geletka LM, Nuss DL. Essential and dispensable virus-encoded replication elements revealed by efforts to develop hypoviruses as gene expression vectors. *J Virol.* 2000; 74:7568–7577. [PubMed: 10906211]
- Truniger V, Nieto C, Gonzalez-Ibeas D, Aranda M. Mechanism of plant eIF4E-mediated resistance against a Carmovirus (Tombusviridae): cap-independent translation of a viral RNA controlled in cis by an (a)virulence determinant. *Plant J.* 2008; 56:716–727. [PubMed: 18643998]
- Tsukiyamakohara K, Iizuka N, Kohara M, Nomoto A. Internal ribosome entry site within Hepatitis C Virus RNA. *J Virol.* 1992; 66:1476–1483. [PubMed: 1310759]
- Watts JM, Dang KK, Gorelick RJ, Leonard CW, Bess JW, Swanstrom R, Burch CL, Weeks KM. Architecture and secondary structure of an entire HIV-1 RNA genome. *Nat.* 2009; 460:711–716.
- Yu X, Li B, Fu YP, Jiang DH, Ghabrial SA, Li GQ, Peng YL, Xie JT, Cheng JS, Huang JB, Yi XH. A geminivirus-related DNA mycovirus that confers hypovirulence to a plant pathogenic fungus. *Proc Natl Acad Sci USA.* 2010; 107:8387–8392. [PubMed: 20404139]
- Zhang X, Segers GC, Sun Q, Deng F, Nuss DL. Characterization of hypovirus-derived small RNAs generated in the chestnut blight fungus by an inducible DCL-2-dependent pathway. *J Virol.* 2008; 82:2613–2619. [PubMed: 18199652]
- Zuker M, Jacobson AB. Using reliability information to annotate RNA secondary structures. *RNA.* 1998; 4:669–679. [PubMed: 9622126]

Fig. 1. Nuclease map of the 5' UTR of CHV1-EP713 (nt 1 – 520 shown). Lanes 1, 3, 5 and 12 represent the reactions with RNase T1, A, I and V1, respectively; lanes 2, 4, 6 and 11 are primer-extension controls. Nuclease cleavages were compared with the DNA sequence (lanes 7–10). Dots beside nucleotide numbers on right side indicate dynamic cleavages (see text).

Fig. 2. Nuclease map of the 5' UTR of CHV1-Euro7 (nt 1 – 520 shown). Lanes 1, 3, 5 and 12 represent the reactions with RNase T1, A, I and V1, respectively; lanes 2, 4, 6 and 11 are primer-extension controls. Nuclease cleavages were compared with the DNA sequence (lanes 7–10). Dots beside nucleotide numbers on right side indicate dynamic cleavages (see text).

Fig. 3.

Mfold constrained predicted structure for 5' UTR of CHV1-EP713. The prediction was generated using the first 675 nucleotides of the CHV1-EP713 genome (nt 1 – 540 shown). The structure was annotated based on *P-num* and each base is colored according to its *P-num* value (Zuker and Jacobson, 1998). Colors are ranged from red to black representing unusually well-determined to poorly determined. The arrow indicates the start codon of ORF A. Each stem-loop structure was numbered sequentially; structures containing more than one stem-loop were defined as domains and are labeled with Roman numerals.

Fig. 4.

Mfold constrained predicted structure for 5' UTR of CHV1-Euro7. The prediction was generated using the first 675 nucleotides of the CHV1-Euro7 genome (nt 1 – 520 shown). The structure was annotated based on *P-num* and each base is colored according to its *P-num* value (Zuker and Jacobson, 1998). Colors are ranged from red to black representing unusually well-determined to poorly determined. The arrow indicates the start codon of ORF A. Each stem-loop structure was numbered sequentially; structures containing more than one stem-loop were defined as domains and are labeled with Roman numerals.

Fig. 5. Selected portions of the Mfold-constrained predicted structure of the 5' UTR of CHV1-EP713. Black lines adjacent to the structure show the locations at which complementary vsRNAs were identified by Zhang et al (2008). All of these vsRNAs were complementary to the positive sense strand; no vsRNAs complementary to the negative sense were detected (Zhang et al., 2008). Each stem-loop and domain has been numbered according to Fig. 3. Panel A: nt 45 – 225 encompassing domains I and II. Panel B: nt 445 – 540 encompassing loops 22 and 23 plus domain IV. Arrow = AUG codon at position 496.