A novel cGUUA\text{Ag} tetraloop structure with a conserved yYNMG\text{Gg}-type backbone conformation from cloverleaf 1 of bovine enterovirus 1 RNA

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ABSTRACT

The 5\textsuperscript{-}terminal cloverleaf (CL)-like RNA structures are essential for the initiation of positive- and negative-strand RNA synthesis of entero- and rhinoviruses. SLD is the cognate RNA ligand of the viral proteinase 3C (3C\textsuperscript{pro}), which is an indispensable component of the viral replication initiation complex. The structure of an 18mer RNA representing the apical stem and the cGUUA\text{Ag} D-loop of SLD from the first 5\textsuperscript{-}CL of BEV1 was determined in solution to a root-mean-square deviation (r.m.s.d.) (all heavy atoms) of 0.59 Å (PDB 1Z30). The first (an\textit{ti}G) and last (\textit{syn}A) nucleotide of the D-loop forms a novel ‘pseudo base pair’ without direct hydrogen bonds. The backbone conformation and the base-stacking pattern of the cGUUA\text{Ag}-loop, however, are highly similar to that of the coxsackieviral uCAC\text{Gg} D-loop (PDB 1RFR) and of the stable cUUC\text{Gg} tetraloop (PDB 1F7Y) but surprisingly dissimilar to the structure of a cGUUA\text{Ag} stable tetraloop (PDB 1MSY), even though the cGUUA\text{Ag} BEV D-loop and the cGUUA\text{Ag} tetraloop differ by 1 nt only. Together with the presented binding data, these findings provide independent experimental evidence for our model (O. Ohlenschläger, J. Wöhnert, E. Bucci, S. Seitz, S. Häfner, R. Ramachandran, R. Zell and M. Görlach (2004) \textit{Structure}, 12, 237–248) that the proteinase 3C\textsuperscript{pro} recognizes structure rather than sequence.

INTRODUCTION

The enteroviruses consist of at least 8 species comprising more than 90 serotypes and belong to the family of \textit{Picornaviridae} (1). They are the causative agents of a number of diseases including neurological diseases and myocarditis. Enteroviruses are positive-strand RNA viruses. Their genome consists of a 7.5 kb RNA encoding a polyprotein of \textasciitilde2200 amino acids, which is processed post-translationally by the virus-encoded proteinases 2A\textsuperscript{pro}, 3C\textsuperscript{pro} and 3CD\textsuperscript{pro}. The open reading frame is flanked on both sides by non-translated regions (NTRs). The 5\textsuperscript{-}NTR contains important signals necessary for the initiation of translation and replication (2,3). Viral and cellular proteins bind to the 5\textsuperscript{-}cloverleaf (CL), a structure at the 5\textsuperscript{-}end of the positive-strand RNA, to form a functional ribonucleoprotein complex essential for the initiation of viral RNA replication. This 5\textsuperscript{-}CL is highly conserved among all enteroviruses. It consists of \textasciitilde90 nt and contains four joint subdomains: stem A and the stem–loops B, C and D (Figure 1). While stem–loop B is the ligand of the human poly(rC)-binding protein 2 (PCBP2), stem–loop D (SLD) binds the viral 3C\textsuperscript{pro} and 3CD\textsuperscript{pro}, respectively (4–8). A number of \textit{in vivo} and \textit{in vitro} studies, also including chimeric enteroviral constructs, indicated that the SLD:3C\textsuperscript{pro} interaction is not serotype-specific (9–13). Instead, a degenerate consensus sequence residing in the apical tetraloop of SLD, the D-loop, was shown to be important for the RNA:3C\textsuperscript{pro} interaction (7). Structural analysis of the SLD of coxsackievirus B3 (CVB3) indicated that its apical D-loop (uCAC\text{Gg}) forms a tetraloop (8,14) highly similar in structure to stable cUNC\text{Gg}-type tetraloops, thereby extending the consensus sequence for this tetraloop family (cYNMG\text{Gg}) (15) to yYNMG\text{Gg}. Analysis

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in vivo and by NMR spectroscopy revealed that, in addition to 2 nt adjacent to a triple pyrimidine mismatch of the SLD stem, this D-loop structure constitutes an essential element for the specific 3C pro:SLD interaction (8). The D-loop sequence of CVB3 (uCA CGg) is prototypical for the human enteroviruses, and based upon our structural and binding studies, we proposed that the common structural features rather than the sequence of individual D-loops determine the specificity of RNA:protein interaction in this system (7,8).

In contrast to other enteroviruses, a characteristic feature of the bovine enteroviral 5'-NTR is the presence of two 5'-CLs in tandem. The second CL enhances the translational activity of the internal ribosome entry site (13,16), carries a pentanucleotide (cGCUUAg) D-loop and does not bind CVB3 3C pro in vitro (7). In contrast, the first CL binds CVB3 3C pro in vitro and carries a cGUUAg D-loop (7), which does not fit to the abovementioned yYNMGg consensus for 3C pro binding rendering the structural basis for this interaction less clear. However, the viability of CVB3 chimeras, where the cognate CL was replaced by the cloverleaves of BEV1 (13), suggested that this in vitro observation carries biological relevance.

In addition, one of the three porcine enteroviruses carries a D-loop matching the BEV1 cGUUAg sequence (17). To address the structural basis for the SLD:3C pro interaction in these viruses, the solution structure of the apical part of the SLD from CL1 of BEV1 (Figure 1, right panel, BEVSLDap) was determined. This construct carries the cGUUAg D-loop representing the most significant deviation in sequence as compared with other highly conserved enteroviral SLDs binding to CVB3 3C pro (7,8).

The D-loop of BEV1 presented here constitutes a novel tetraloop structure. However, its backbone adopts a conformation similar to the cUNCGg-like conformations of the yYNMGg sequence family (8,14,15,18). In addition, an analysis of the interaction between an RNA representing the SLD from CL1 of BEV1 (Figure 1, right panel, BEVSLD) with 3C pro is reported. Together, this analysis and the structural findings strongly support our model (8) of a structure-specific recognition of enteroviral SLDs by 3C pro.

**MATERIALS AND METHODS**

**Synthesis, purification and preparation of RNA and 3C pro**

Labelled nucleotides and the apical portion of SLD RNA (BEVSLDap, Figure 1, nucleotides 58–69) from bovine enterovirus 1 cloverleaf 1 (BEV1 CL1) were prepared and purified in uniformly 15N-labelled and uniformly 15N,13C-labelled form by in vitro transcription with T7 RNA polymerase as described previously (7,19). The NMR samples had final concentrations of 0.9 mM U-[13C,15N]-BEVSLDap in H2O and D2O, respectively, and 1.0 mM U-[15N]-BEVSLDap. The NMR buffer contained 10 mM KH2PO4/K2HPO4, pH 6.2 with 40 mM KCl, 0.2 mM EDTA and 10% v/v 2H2O or 99.99% v/v 2H2O, respectively. For binding experiments with 3C pro, a 34mer (BEVSLD, Figure 1) representing nucleotides 50–77 of the BEV1 CL1 was prepared in uniformly 15N-labelled and uniformly 15N,13C-labelled form by in vitro transcription with T7 RNA polymerase as described previously (7,19). The NMR samples had final concentrations of 0.9 mM U-[13C,15N]-BEVSLDap in H2O and D2O, respectively, and 1.0 mM U-[15N]-BEVSLDap. The NMR buffer contained 10 mM KH2PO4/K2HPO4, pH 6.2 with 40 mM KCl, 0.2 mM EDTA and 10% v/v 2H2O or 99.99% v/v 2H2O, respectively. For binding experiments with 3C pro, a 34mer (BEVSLD, Figure 1) representing nucleotides 50–77 of the BEV1 CL1 was prepared in uniformly 15N-labelled and uniformly 15N,13C-labelled form. The binding experiments were performed at 60 μM U-[13C,15N]-BEVSLD and 170 μM 3C pro in 20 mM KOAc, pH 5.5, 300 mM KCl. Overexpression and purification of 3C pro was carried out as described previously (7). After exchange into NMR buffer (20 mM KOAc, pH 5.5, 300 mM KCl) by
dialysis, the protein was concentrated to 180 μM. The BEVSLD U64A mutant RNA was purchased in unlabelled form from Dharmaco (Lafayette), deprotected as suggested by the manufacturer and purified via high-performance liquid chromatography as detailed for the labelled RNAs above.

NMR spectroscopy

NMR spectra were recorded at temperatures of 283 K (exchangeable protons) and 298 K (exchangeable and non-exchangeable protons) on Varian UNITY INOVA 600 MHz or UNITY INOVA 750 MHz four channel NMR spectrometers equipped with pulse field gradient accessories and triple resonance probes with actively shielded Z gradient coils. The NMR spectra were processed with VNMRS (Varian Assoc., Palo Alto) and analysed using the program XEASY (20). Chemical shifts were referenced as described previously (21). Resonance assignment of the exchangeable and non-exchangeable protons of BEVSLDap was performed using (1H,1H)-HSQC (22), (1H,15N)-CPMG-NOESY (23), (1H,2H)-H5(C5C4N)H(CU) (24), (1H,1H)-HCCCN-HOCSY (G) (25), (1H,1H)-HCCCN-TOCSY (A) (26), (1H,1H)-HCCCN-COSY (C,U) (27), (1H,1N)-2bond-HSQC (28), (1H,13C)-H(N)CO (29), (1H,13C)-HSQC, 3D (1H,1H,13C)-HCCH-COSY (sugar), 3D (1H,1H,13C)-HCCCN-TOCSY (sugar) (27) and (1H,13N)-HCN (30). For sequential assignments 3D (1H,1H)-NOESY-HSQC (31) and (1H,15P)-HCP-CCH-TOCSY (32) spectra were acquired. For computational assignments 3D (1H,1H)-NOESY-HSQC, (1H,1H)-NOESY, (1H,13C)-HSQC, H5(C5C4N)H, (1H,1H)-HCCCN-TOCSY, (1H,1H)-HCCCN-COSY and (1H,1H)-HCCCN-COSY experiments on a 700 MHz INOVA-750 MHz four channel NMR spectrometer using the Sander module of the Amber8 (Assisted Model Building with Energy Restrains) package (42) starting from the average NMR structure. The simulations were performed in a periodic box including ~3000 TIP3 water molecules (43), sodium counter ions and using the force field described previously (40). A 9 Å cut-off for the short-range non-bonded interactions was used in combination with the particle mesh Ewald option (44) using a grid spacing of ~0.9 Å to account for long-range electrostatic interactions. Following initial energy minimization, the system was gradually heated from 50 to 300 K with positional restraints on the RNA atoms over a period of 0.1 ns. During another 0.1 ns simulation time at 300 K, the positional restraining force constant was gradually reduced from 50 kcal mol⁻¹ Å⁻² to zero. The simulations were continued for a total simulation time of 4 ns. Conformations of the final 2 ns were used for comparison with the experimentally determined NMR structures.

Molecular dynamics

Molecular dynamics (MD) simulations were performed on the tetraloop segment (5'-CGUUCGUUAGAACG) using the Sander module of the Amber8 (Assisted Model Building with Energy Restrains) package (42) starting from the average NMR structure. The simulations were performed in a periodic box including ~3000 TIP3 water molecules (43), sodium counter ions and using the force field described previously (40). A 9 Å cut-off for the short-range non-bonded interactions was used in combination with the particle mesh Ewald option (44) using a grid spacing of ~0.9 Å to account for long-range electrostatic interactions. Following initial energy minimization, the system was gradually heated from 50 to 300 K with positional restraints on the RNA atoms over a period of 0.1 ns. During another 0.1 ns simulation time at 300 K, the positional restraining force constant was gradually reduced from 50 kcal mol⁻¹ Å⁻² to zero. The simulations were continued for a total simulation time of 4 ns. Conformations of the final 2 ns were used for comparison with the experimentally determined NMR structures.

Binding assays

RNA:protein interactions in vitro were studied by employing electrophoretic mobility shift assays (EMSAs) as detailed previously (7). Interactions in vivo were analysed in the yeast three-hybrid system using the RNA–protein Hybrid HunterTM Kit (Invitrogen) according to the manufacturer’s instructions. The gene regions of CBV3 3C<sup>pro</sup> and 3CD<sup>pro</sup> were cloned into the pYESTrp3 vector. The catalytic cysteine was substituted by a glycine to inactivate the proteinases. DNA encoding the desired RNAs was cloned into the vector pRH5<sup>+</sup> (45). Saccharomyces cerevisiae strain L40-ura3 was used for transformation with the protein prey plasmids, the RNA bait plasmids containing SLD encoding BEV1 sequences and pHybLex/Zeo-M52. Transformed colonies of S.cerevisiae strain L40-ura3 were grown on selective agar plates, transferred to filter paper, lysed and assayed for β-galactosidase activity 3–6 days after transformation. For documentation, colonies were grown overnight in supplemented minimal salt medium and spotted onto selective x-gal (40 mg/l).
phosphate-buffered (pH 7.0) minimal salt plates. Blue yeast colonies were scored after 5–6 days of incubation at 30°C.

RESULTS AND DISCUSSION
Structure determination
Recently, we demonstrated that the CVB3 proteinase 3C (3Cpro) interacts in vitro and in vivo with CLs from a number of entero- and rhinoviruses and that this binding occurs to the SLD subdomain of the CL (7,8). We solved the structure of SLD from CVB3 and mapped the interaction of 3Cpro to the apical D-loop and to residues adjacent to the internal triple pyrimidine mismatch region of SLD. The apical D-loop of SLD was shown to adopt a structure highly similar to the canonical cUNCGg-type tetraloop in spite of its deviating sequence uCACGg. Together with the binding data, we proposed that the specific SLD:3Cpro interaction critically depends upon the cUNCGg-like backbone conformation common to D-loops with the rather degenerate consensus yNYNgg (7,8). The observed interaction of the CL1 of the BEV1 5'-NTR (Figure 1) with 3Cpro (7) and the viability of CVB3 chimeras, where the cognate CL was replaced by the CLs of BEV1 (13), posed the intriguing question whether the SLD of the BEV1 CL1 (BEVSLD, Figure 1) is similar in structure to CVBSLD (Figure 1) as the D-loop sequence (cGUUAg) of BEVSLD much more closely resembles that of the GNRA-tetraloop family (46–48) than that of the UNCG-like family consensus (yYNMGg) (14,15,18). Although the remainder of the two SLDs is highly homologous, the most significant difference between BEVSLD and the CVBSLD lies in the apical D-loop (Figure 1). Hence, we determined the structure of an RNA (BEVSLDap, Figure 1) representing the apical stem and the D-loop of BEVSLD in order to study the structural basis of the RNA:3Cpro interaction further.

For structure determination, BEVSLDap was produced in [15N] and [13N],[13C]-labelled form by in vitro transcription. Virtually complete resonance assignments were achieved by standard triple resonance experiments. An extremely broadened resonance typical for an amino group was observed at 6.81 p.p.m./80.8 p.p.m. which could not be assigned. A defined resonance typical for an amino group was observed at 10.44 p.p.m./147.8 p.p.m. was broadened even at 10°C/C14

Figure 2. 1H,13C]-HSQC spectra of the BEV (left) and the CVB3 stem-loop D (right) RNAs. Characteristic cross peaks originating from the tetraloops and their closing base pairs are coloured (loop nucleotide 2, red; nucleotide 3, yellow; nucleotide 4, blue; and G of closing base pair, green).

with respect to the C2 resonances of the U residues involved in canonical AU base pairs. Together with the observed strong cross peaks in a (1H,1H)-NOESY spectrum between the imino protons of both residues this indicated a standard GU wobble pair. During the process of assignment, we noticed characteristic chemical shifts for ribose carbon and hydrogen nuclei of the BEV D-loop, e.g., H5/H5/C5' of U63, U64 and A65, H4/C4' of U64, H3/C3' of A65, which were reminiscent of the corresponding shifts found for the CVB3 D-loop (8), thus exhibiting a similar ‘fingerprint’ for the respective nuclei in the (1H,13C)-HSQC spectra (Figure 2). The structure of BEVSLDap was calculated from 518 NOE constraints derived from (1H,1H)-NOEESY, (1H,13C)-CPMG-NOESY and 1H- and 13C-edited 3D-NOESY-HSQC spectra. 36 hydrogen bond constraints deduced from the HNN-COSY and the H(N)CO experiments, 17 torsion angle constraints for angle 0 and 0 ζ as derived from 31P chemical shift analysis and 17 3JH1'H1' coupling constants extracted from a HCHC.-COSY defining the torsion angle 0.

BEVSLDap is capped by a novel tetraloop structure
The structure of BEVSLDap represented by the ensemble of 15 energy minimized structures with the lowest target function is shown in Figure 3A. The structural statistics is given in Table 1. The structure consists of an A-helix capped by a tetraloop. The helical portion of the molecule involves residues 58–61 and 66–69 of the BEVSLD (see also Figure 1) and includes the residues at the base of the stem added for in vitro transcription purposes (Figure 1, residues in italics). The entire stem of BEVSLDap adopts a standard A-form conformation and will not be discussed any further.

The apical cGUUAag D-loop represents a well-defined tetraloop structure (Figure 3C) resting upon the canonical C61:G66 slightly buckled closing base pair. Three of its bases are engaged in stacking interactions (Figure 3B). This tetraloop exhibits several novel properties (Figure 3B and C). G62 and A65 are found in an unusual staggered juxtaposition, which apparently does not allow for the formation of direct
hydrogen bonds between their base moieties. This conformation contrasts the situation in UNCG-type (50,51) and GNRA-tetraloops where the first and the fourth loop residue are paired via hydrogen bonds. However, the calculated structure could accommodate a hydrogen bond of the amino group of G62 and the O4\(^\cdot\)ribose oxygen of U64 (Figure 3B). Likewise, hydrogen bonds between 2\(^\cdot\)OH of U64 and a non-bridging oxygen of A65 as well as the 2\(^\cdot\)OH of A65 and the non-bridging oxygen of G66 (Figure 3B) would be consistent with the calculated structure. G62 adopts A-form conformation, including a glycosidic angle in anti conformation and a canonical C3\(^\cdot\)-endo sugar pucker, similar to the first residue in the two tetraloop families mentioned above. The ribose moiety of A65 is shown as hatched ellipses to indicate a possible C2\(^\cdot\)-endo, C3\(^\cdot\)-endo equilibrium (see text). (C) Stereoview (side-by-side) of the local superimposition (heavy atoms) of the apical tetraloop (G62–A65) and its closing base pair (C61–G66) of BEVSLDap. View into the major groove.

Table 1. Structural statistics for the BEVSLDap RNA

| Number of experimental constraints | Distance constraints | 518 |
|-----------------------------------|----------------------|-----|
| Torsion angle constraints         | 156                  |
| Number of hydrogen bond constraints| 36                   |
| CYANA target function before energy minimization (Å\(^2\)) | 0.0463 ± 0.0022 |
| AMBER energies (kcal/mol) after energy minimization: | Physical energy | -159.28 ± 8.4 |
| | Van der Waals energy | -206.19 ± 3.2 |
| | Electrostatic energy | -370.24 ± 6.2 |
| Number of violations | NOE violation >0.2 Å | 0 |
| | Torsion angle restraints violation >2.5\(^\circ\) | 0 |
| Mean deviation from idealized covalent geometry | Bond length (Å) | 0.00452 ± 0.000086 |
| | Bond angles (\(^\circ\)) | 1.34654 ± 0.001190 |
| Heavy atom r.m.s.d. from mean structure (Å) | All | 0.509 |
| | Best to mean | 0.419 |
| | Tetraloop (C61–G66) | 0.295 |

Figure 3. The NMR structure of BEVSLDap. (A) Superimposition (all heavy atoms) of 15 energy minimized conformers of 100 calculated structures. Residues of the A-helical stem are shown in black. The tetraloop residues are coloured (G62, green; U63, blue; U64, yellow; A65, red). (B) Schematic representation of stacking interactions and possible hydrogen bonding consistent with the calculated BEVSLDap structure: (a) G62H22–U64O4\(^\cdot\), (b) U64H2O\(^\cdot\)–A65OP2 and (c) A65H2O\(^\cdot\)–G12OP1. Filled ellipses represent residues with a C2\(^\cdot\)-endo, empty ellipses a C3\(^\cdot\)-endo sugar pucker, respectively. The ribose moiety of A65 is shown as hatched ellipses to indicate a possible C2\(^\cdot\)-endo, C3\(^\cdot\)-endo equilibrium (see text). (C) Stereoview (side-by-side) of the local superimposition (heavy atoms) of the apical tetraloop (G62–A65) and its closing base pair (C61–G66) of BEVSLDap. View into the major groove.
constants of 3.40 and 3.49 Hz, respectively (53). The magnitude of the $^3J_{H1'H2'}$ coupling constant (4 Hz) for the ribose of A65 is different from the respective values observed in BEVSLDap for residues in C3'-endo (0.0–2.5 Hz) or C2'-endo conformation (7 Hz), suggesting an equilibrium between C3'-endo and C2'-endo conformation (54). The base of A65 stacks onto G66 of the closing base pair at the 3' side of the loop (Figure 3B and C). Prompted by the unusual conformation of the experimentally determined Gs:ynA 'pseudo base pair' (Figure 4A), an MD simulation for a total of 4 ns and in the presence of explicit solvent and counter ions was performed. The MD trajectory shows a stable hydrogen bonding between the amino group of G62 and the N1 of A65, as well as the 2'OH of G62. However, the base-base alignment needed for this Gs:ynA 'pseudo base pair' (Figure 4A), an MD simulation for a total of 4 ns and in the presence of explicit solvent and counter ions was performed. The MD trajectory shows a stable hydrogen bonding between the amino group of G62 and the N1 of A65, as well as the 2'OH of G62. Moreover, the observation of several NOEs, i.e. H1'/H2' of G62 to H2 of A65, H3' of U64 to H8 of A65, H1' of U63 to H5' of U64 and H2' of G62 to H2 of A65, would not be expected for the MD-derived loop structure (Figure 4B).

The second and third nucleotide of the loop, U63 and U64, projects their bases into the minor or the major groove, respectively. Both sugar moieties adopt a C2'-endo conformation. The base of U63 is not involved in interactions with any part of the tetraloop. The base of U64 stacks onto G62, which in turn is stacking on C61 of the closing base pair (Figure 3B and C). The calculated backbone conformation indicates a reversal of direction between the second and third loop nucleotide, i.e. U63 and U64, resulting in an 'S'-shaped overall appearance (Figure 4C). The observed chemical shift signature (see above), the stacking pattern, the projection of the second and third loop base into opposite helical grooves and the overall backbone conformation are very similar (Figure 4C) to the D-loop of CVBSLD (r.m.s.d. 0.87 Å) (8) and a cUUCGg stable tetraloop (r.m.s.d. 0.66 Å) (51). In contrast, in the cGUAAg tetraloop (46), which deviates from BEV D-loop by one residue only and superimposes with an r.m.s.d. of 1.66 Å (Figure 4D), the reversal of backbone direction occurs between the first and second loop nucleotide which in turn results in a continuous stacking of the bases of loop nucleotides 2–4 on the major groove side of the loop. Thus, a difference in 1 nt only causes a transformation between an UNCG-type and a GNRA-type tetraloop structure.

BEVSLD:3Cpro interaction

To address the functional significance of our structural observations, the binding of BEVSLD to CVB3 3Cpro was investigated. In addition to binding to CL1 of BEV (7), 3Cpro binds to BEVSLD in vivo in the yeast three-hybrid system shown in Figure 5A and in vitro in an EMSA (Figure 5D and data not...
shown). Deleting 2 nt from the D-loop of BEVSLD renders the RNA non-binding (Figure 5A). Also, the SLD derived from the second CL of BEV1 did not bind in yeast to CVB3 3Cpro (Figure 5A), matching the in vitro results obtained with the entire CL2 earlier (7). To analyse the interaction of BEVSLD and 3Cpro further (1H,13C)-HSQC spectra for aromatic resonances were recorded for the free BEVSLD and for the BEVSLD:3Cpro complex under identical conditions. Owing to the limited solubility of 3Cpro, those spectra had to be taken at elevated ionic strength and on dilute samples of 60 μM RNA and 170 μM 3Cpro (RNA:protein molar ratio 1:3). In the (1H,13C)-HSQC spectra of the complex (Figure 5B) resonances of the aromatic groups of C53, G62, U63, U64 and G66 are missing from their original positions. However, for the newly appearing resonances, the solubility-limited concentration of the complex precluded assignments. In addition, the resonances of C61, A65 and C69 are significantly broadened. This observation is consistent with binding at the intermediate to slow NMR timescale and with a $K_D$ of 3.7 μM for the interaction of 3Cpro with the first 50-CL of BEV1 (7). Assuming a bimolecular binding reaction, this $K_D$ corresponds to a 97% saturation of the RNA with 3Cpro in the NMR experiment described above. Importantly, the affected residues are located in the D-loop of BEVSLD and adjacent to the internal triple pyrimidine mismatch and are equivalent to the residues of the CVBSLD showing a similar line broadening upon complex formation with 3Cpro (8). For BEVSLD, we also observed the expected line broadening for the imino resonances of G62 and G66 in (1H,15N)-HSQC spectra (data not shown). Additional residues (U59 and U60) showed such an imino line broadening, suggesting that they also might contribute to 3Cpro binding. This effect, however, was not reflected in the signals of their H6 base protons in the corresponding (1H,13C)-HSQC spectrum (Figure 5B).

The residues of BEVSLD implied in 3Cpro binding here are essentially the same as in CVBSLD (8) and indicated in Figure 5C. The most significant deviation in sequence between BEVSLD (cGUUAg) and CVBSLD (uCACGg), however, resides in their D-loops which, nevertheless, share the UNCG-type backbone conformation of the yYNMGg sequence family. Hence, the novel tetraloop structure presented here sheds new light on the sequence plasticity for such tetraloops. The conserved structural motif rather than a limited sequence family explains their specific interaction with 3Cpro, which is essential for the initiation of enteroviral replication. On the other hand, a mutation of the D-loop sequence (cGUUAg) into cGUAAg would cause a transformation from an UNCG-type to a GNRA-type tetraloop structure. This transformation, however, would result in significantly...
different D-loop surface properties thereby interfering with the 3C\textsuperscript{pro}-RNA interaction. Consistent with this, a dramatically reduced binding of 3C\textsuperscript{pro} to the cGUAAg-mutant of BEVSLD under NMR buffer conditions is observed in an EMSA (Figure 5D). No binding was detected for the unrelated stem–loop B from CVB3 (data not shown). Based on our findings, one could speculate that such a mutation in vivo would lead to non-viable virus mutants. This notion is supported by the fact that D-loop sequences accommodating a GNRA-type structure have not been reported in the NCBI GenBank (www.ncbi.nlm.nih.gov/Genbank) or the Picornavirus Sequence Database (www.iah.bbsrc.ac.uk/virus/Picornaviridae/SequenceDatabase/Index.html) to date.

The coordinates, distance and angle constraints as well as the chemical shifts of the BEVSLD\textsubscript{ap} RNA have been deposited in the PDB (accession code 1Z30) and the BioMagResBank (accession code 6562), respectively.

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