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Independent evolution of tetraloop in enterovirus oriL replicative element and its putative binding partners in protein 3C

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Background. Enteroviruses are small non-enveloped viruses with (+) ssRNA genome with one open reading frame. Enterovirus protein 3C (or 3CD for some species) binds replicative element oriL to initiate replication. The replication of enteroviruses features low fidelity that allows virus to adapt to changing environment on the one hand, and requires additional mechanisms to maintain the genome stability on the other. Structural disturbances in the apical region of oriL domain d can be compensated by amino acid substitutions in positions 154 or 156 of 3C (amino acid numeration corresponds to poliovirus 3C), thus suggesting co-evolution of these interacting sequences in nature. The aim of this work was to understand co-evolution patterns of two interacting replication machinery elements in enteroviruses, the apical region of oriL domain d and its putative binding partners in the 3C protein.

Methods. To evaluate the variability of the domain d loop sequence we retrieved all available full enterovirus sequences (>6400 nucleotides) that were present in the NCBI database on February 2017 and analyzed variety and abundance of sequences in domain d of replicative element oriL and in the protein 3C.

Results. A total of 2842 full genome sequences were analyzed. Majority of domain d apical loops were tetraloops, which belonged to consensus YNHG (Y=U/C, N=any nucleotide, H=A/C/U). Putative RNA-binding tripeptide 154-156 (Enterovirus C 3C protein numeration) was less diverse than the apical domain d loop region and, in contrast to it, was species-specific.

Discussion. Despite RNA-binding tripeptide is suggested to interact with apical region of domain d, they evolve independently in nature. Together, our data indicates plastic evolution of both interplayers of 3C-oriL recognition.

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Abstract

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Results. A total of 2842 full genome sequences were analyzed. Majority of domain d apical loops were tetraloops, which belonged to consensus YNHG (Y=U/C, N=any nucleotide,

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H=A/C/U). Putative RNA-binding tripeptide 154-156 (Enterovirus C 3C protein numeration) was less diverse than the apical domain d loop region and, in contrast to it, was species-specific.

Discussion. Despite RNA-binding tripeptide is suggested to interact with apical region of domain d, they evolve independently in nature. Together, our data indicates plastic evolution of both interplayers of 3C-oriL recognition.

Introduction

Enteroviruses are small non-enveloped viruses with plus strand genome about 7500 nt, which contains one open reading frame that encodes structural (capsid) and non-structural proteins, 5' and 3' NTRs (not translated regions) and polyA on the 3' end (Palmenberg, Neubauer and Skern, 2010). (Figure 1). Most non-structural enterovirus proteins are polyfunctional. Protease 3CD is a precursor of polymerase 3D and plays the key role in the initiation of replication (Harris *et al.*, 1992; Gamarnik and Andino, 1998; Thompson and Peersen, 2004). After translation by host cell ribosomal machinery, the genome is utilized for the synthesis of the (-) strand RNA, which, in turn, serves as a matrix for synthesis of multiple daughter (+) strands. Non-translated regions of genome and a coding sequence within the genomic region encoding the viral helicase 2C contain replicative elements, which interact with

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viral and host proteins. These RNA-protein complexes regulate initiation and further steps of the replication. For poliovirus, the most clinically relevant member of the Enterovirus genus, there are at least three known RNA-protein complexes, which are formed with replicative elements oriL, oriR and oriI during replication (Figure 1).

Complex of oriL with viral protein 3CD and the host protein PCBP2 is crucial for the transcription initiation (Goodfellow *et al.*, 2000; Vogt and Andino, 2010; Chase, Daijogo and Semler, 2014). Element oriL has a cloverleaf-like secondary structure with four domains termed “a” (stem of the cloverleaf), “b”, “c” and “d” (leafs of the cloverleaf) (Trono, Andino and Baltimore, 1988; Andino, Rieckhof and Baltimore, 1990) (Figure 1). Previously, it was demonstrated *in vitro* that 3CD (or 3C) of poliovirus, coxsackievirus B3 and bovine enterovirus 1 interact with the apical loop and the flanking base pairs of hairpin d in the oriL element (Andino, Rieckhof and Baltimore, 1990; Du *et al.*, 2003; Ihle *et al.*, 2005) (Figure 1).

The apical loops of domain d in genomes belonging to several viruses of *Enterovirus* genera were shown by NMR experiments to be tetraloops with a specific spatial structure, which belongs to the UNCG structural class of stable tetraloops (Du *et al.*, 2003, 2004; Ihle *et al.*, 2005; Melchers *et al.*, 2006). There are several known structural classes of tetraloops and three of them, named according to consensus sequences, contain tetraloops of extremal stability: UNCG (where N=any nucleotide), GNRA (where R=A/G) and gCUUGc (Uhlenbeck, 1990; Cheong and Cheong, 2010). Tetraloops of UNCG and GNRA classes are the most widely represented (Woese *et al.*, 1990; Cheong and Cheong, 2010; Bottaro and Lindorff-Larsen, 2017). Previously it was shown that only tetraloops of UNCG structural class, but not tetraloops of GNRA or gCUUGc structural classes can support effective replication of poliovirus genome (Prostova *et al.*, 2015). Moreover, the exact sequence of the apical region of poliovirus domain d was of less importance for effective 3CD-oriL recognition than its spatial structure (Rieder *et al.*, 2003; Prostova *et al.*, 2015). At the same time, structural disturbance in the apical region of oriL domain d of poliovirus could be compensated by amino acid substitutions in the tripeptide 154-156 of the 3C protein (here and after amino acid numeration corresponds to poliovirus 3C protein) (Andino *et al.*, 1990; Prostova *et al.*, 2015). In addition to triplet 154-156, the conservative motif $_{82}$KFRDI$_{86}$ of the 3C protein also takes part in the oriL recognition (Andino *et al.*, 1990, 1993; Hämmerle, Molla and Wimmer, 1992; Shih, Chen and Wu, 2004).

The replication of enterovirus is a low fidelity process, generating on average one mutation per genome (Sanjuán *et al.*, 2010; Acevedo, Brodsky and Andino, 2013). The high probability of mutation allows virus to adapt to constantly changing environment on the one hand, but requires additional mechanisms to maintain the genome stability on the other (Wagner and Stadler, 1999; Lauring, Frydman and Andino, 2013). The aim of the present study was to understand co-evolution patterns of two interacting replication machinery elements in enteroviruses, the apical domain d of oriL and the 3C protein.

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Materials and methods

Formation and filtration of sets of full genomes

Genomes of species of *Enterovirus* genus with length 8000>n>6800 were extracted from the NCBI database. For every species, a multiple sequence alignment was done with Clustal (Larkin *et al.*, 2007). Sequences that contained more than 50 “N” characters in succession and sequences that were annotated as "Modified_Microbial_Nucleic_Acid", were deleted from alignments. In order to reduce the bias towards particular loop sequences present in a large set of closely related genomes, which, for example, belonged to one outbreak, all sequences that differed from any other sequence in the dataset by less than 1% of the nucleotide sequence were omitted.

Analysis of tetraloop and amino acid variety in the sets of genomes

For analysis of domain d sequence variety, the multiple sequence alignments were used. The relevant region of multiple sequence alignment and respective names of sequences were analyzed in Microsoft Excel. To analyze correlation of domain d loop and tripeptide of 3C sequences the same alignments were translated in the protein 3C coding region. The resulting amino acid sequences that corresponded to tripeptides 154-156 (poliovirus 3C numerations) were analyzed using Microsoft Excel. Amino acid frequency plot was formed with WebLogo server using the set of filtered genomes for every species (Crooks *et al.*, 2004). To do this the multiple

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sequence alignment of filtered genomes of every species was translated in the region that codes protein 3C, and positions 71-89 and 147-160 were saved in separate MAS files, which were then used to produce Logo.

Domain d secondary structure

Domain d secondary structure was folded with Vienna RNA Websuit server with subsequent manual editing (Gruber *et al.*, 2008; Lorenz *et al.*, 2011).

Results

Sample characteristics

To evaluate the variability of the domain d loop sequence we retrieved all available complete genome (8000>n>6800 nucleotides) enterovirus (EV) sequences that were present in the NCBI database on February 2017. A total of 1173, 414, 773, 462, 12, 13, 15, 3, 7, 202, 76 and 51 sequences were analyzed for species *Enterovirus A, Enterovirus B, Enterovirus C, Enterovirus D, Enterovirus E, Enterovirus F, Enterovirus G, Enterovirus H, Enterovirus J, Rhinovirus A, Rhinovirus B* and *Rhinovirus C*, respectively. As expected, genomes of epidemiologically significant viruses were the most represented in database. For example, 66% of *Enterovirus A* species genomes belonged to EV71 type, the causative agent of hand, foot and mouth disease (Solomon *et al.*, 2010); most *Enterovirus C* species sequences (78%) belonged to

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poliovirus; most *Enterovirus D* species sequences (98.7%) represented EV68, an etiological agent of severe respiratory illness (Oermann *et al.*, 2015). The number of genome sequences of each species that contained the oriL region is shown in Table 1.

Sequences of apical regions in oriL domain d and the amino acids involved in RNA recognition in 3C protein were analyzed. In genomes of *Enterovirus E* and *Enterovirus F* species that have two oriLs (Pilipenko, Blinov and Agol, 1990; Zell *et al.*, 1999) sequences of both oriLs were analyzed (Table 1). To reduce the bias towards particular loop sequences present in a large set of closely related genomes, which, for example, belonged to one outbreak, all sequences that differed from any other sequence in the dataset by less than 1% of the nucleotide sequence were omitted. After curation, the sizes of the largest data sets decreased dramatically, but the number of unique loop sequences in every set did not change significantly (Table 1). Unique tetraloop variants were lost for *Enterovirus A* (tetraloop UGUG), Enterovirus C (tetraloops CCCG, CAUG and UGUG) and *Enterovirus D* (tetraloop UUGG). This indicates that even among closely related genomes tetraloop sequence can vary. Indeed, in several outbreaks caused by EV71 or PV1 closely related genomes contained different apical domain d sequences (not shown). It should be noted that filtration of the dataset using a 95% sequence identity threshold resulted in a dramatic loss of unique tetraloop variants (data not shown).

Variability of oriL domain d apical loop sequence

The secondary structure of domain d was conservative in all species, except *Enterovirus*

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G, which has elongated domain d (Figure 2) (Krumbholz *et al.*, 2002).

Variety and occurrence of various loops in apical region of domain d in all species of Enterovirus genera were analyzed in filtered sets of full genome sequences. Most of domain d apical loops were tetraloops (i.e. consisted of four nucleotides) (Table 2). However, triloops (3-nucleotide loop) could be found in genomes of *Enterovirus C* and *Rhinovirus A* and *B* species, whereas pentaloops (5-nucleotide loop) were detected in genomes of *Enterovirus E* species (Table 2).

The most common loop sequences belonged to consensuses YNMG (Y=C/U, N=any, M=A/C; tetraloops with UNCG class spatial structure belong to this consensus) and YNUG (tetraloops with UNCG class and gCUUGc class spatial structures belong to this consensus) (Table 2). Consensus YNMG and consensus YNUG together corresponded to 24 unique sequence variants. Interestingly, in our dataset of 2842 full genomes, four tetraloops out of 24 possible variants have never been found in the domain d apical region: UUAG, UCAG, CUAG and CCAG (Table 2). Thus, dinucleotides UA and CA are likely to be avoided at positions 2-3 of tetraloop in enterovirus genomes.

In enterovirus A species 17 out of 24 possible unique tetraloop sequences were identified (Table 2, Table S2). Twelve unique loops of *Enterovirus A* belonged to consensus YNMG, while 5 others belonged to consensus YNUG. Frequency of particular tetraloop sequences varied significantly (Table 2, Table S2). One tetraloop (UGUG) was lost upon filtration and manually added to the final data set (Table 2). Interestingly, the diversity of tetraloops among EV71 serotype was similar to the diversity of tetraloops in the whole *Enterovirus A* species (Table S1).

In *EV-B* genomes 18 unique tetraloops out of 24 possible were found. Twelve of these tetraloops belonged to consensus YNMG and six to consensus YNUG (Table 2, Table S3). The most abundant tetraloops were CACG (98 genomes), UACG (51 genomes) and UGCG (31 genomes), that also were present among the most abundant tetraloops of species Enterovirus A.

In genomes of *Enterovirus C* species 9 unique tetraloops belonged to YNMG consensus and 4 to YNUG consensus. Three unique tetraloops were lost upon filtration and added to final data set (CCCG, UGUG, CAUG) (Table 2, Table S4). Two genomes annotated at the NCBI data base as Human coxsackievirus A21 strain Coe (accession number D00538) and Human coxsackievirus A21 strain BAN00-10467 (accession number EF015031) contained triloops CAG and CCG, respectively. The most abundant tetraloops in *EV-C* species were UACG (106), CACG (101), UGCG (43) (Table 2, Table S4), which corresponds to the Sabin vaccine strains of poliovirus serotypes 2, 3 and 1, respectively. To evaluate bias caused by redundant number of vaccine strain sequences in the data set, we subtracted genomes of vaccine/vaccine derived poliovirus strains from the analyzed set. As a result, tetraloops UACG, CACG and UGCG were still the most frequent variants (Table S1).

Just 57 *EV-D* genomes out of 419 were left after 1%-identity filtration. Fifty genomes belonged to Human enterovirus 68, the etiological agent of respiratory illness. All genomes of

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this type contained loop UUCG in the domain d apical region. Other tetraloops were UUUG (1), CUCG (2), CCCG (1), CUUG (2) and CACG (1) (Table 2, Table S5). One tetraloop (UUGG) was lost upon filtration and manually added to the final data set.

Species *Enterovirus E* and *F* have 2 oriLs in the 5'NTR, with, in general, similar sequences in apical region of domain d (Pilipenko, Blinov and Agol, 1990; Zell *et al.*, 1999) (Table S6). Due to this, we united sequences from the first and the second oriL of these viruses in heat map (Table 2). Domain d loops in 10 genomes of *EV-F* were tetraloops, while in 10 *EV-E* genomes there were both tetraloops (first oriL) and pentaloops (the first and the second oriL) (Table 2, Table S6). There were four diverse tetraloop sequences in oriLs of Enterovirus E and F with no obvious preference between these species. These sequences were GCUA, GUUA, GCCA, AUUA (Table 2, Table S6). Tetraloop AUUA was found once in the first oriL domain d of EV-F (strain PS87/Belfast, accession number DQ092794) (Table 2, Table S6). There were six diverse pentaloop sequences in domain d of Enterovirus E genomes – GCUUA, GUUUA, GCCUA, GCGUA, GAUUA, GUCUA (Table 2, Table S6).

All domain d loops in genomes of Enterovirus G, H and J species were tetraloops, and all except one tetraloop variant belonged to consensus YNMG (Table 2, Table S7). One Enterovirus G representative had GUUA tetraloop sequence (strain LP 54, accession number AF363455), similar to loops of Enterovirus E and F species (Table 2). This genome had only one oriL with the same domain d length as of Enterovirus G genomes (Krumbholz *et al.*, 2002).

All except one (isolate V38_URT-6.3m, accession number JF285329) full genomes of Rhinoviruses A species and all full genomes of *Rhinovirus C* species had tetraloops in apical regions of domain d (Table 2). Tetraloops of these viruses in almost all cases belonged to consensus YNMG, with one exception in *Rhinovirus C* (tetraloop CUUC, isolate JAL-1, accession number JX291115) (Table 2, Table S8). All loops in the *Rhinovirus B* domain d apical region were triloops (Table 2, Table S8).

Thus, the secondary structure of domain d was very similar among species of the genus *Enterovirus,* with the exception of *Enterovirus G* species (Figure 2). Apical region of domain d has high diversity of sequences, however in species *Enterovirus A, B, C, D, G, H, J* and *Rhinovirus A* and *C* it mostly can correspond to the same consensus YNHG (Y=C/U, N=any, H=A/C/U).

Variety of RNA-recognition tripeptide of 3C

Two motifs of protein 3C are involved in RNA recognition and interact with oriL: the conservative motif KFRDI (positions 82-86 of poliovirus 3C) and putative RNA-binding tripeptide (positions 154-156 in poliovirus 3C) (Andino *et al.*, 1990, 1993; Hämmerle, Hellen and Wimmer, 1991; Shih, Chen and Wu, 2004). Substitutions in putative RNA-binding tripeptide are known to compensate the disturbance in apical region of domain d, and therefore the RNA-binding tripeptide is a putative candidate to co-evolve with domain d loop (Andino *et al.*, 1990; Prostova *et al.*, 2015). There are other amino acids that are shown to affect oriL-3CD interaction, but tripeptide 154-156 (*Enterovirus C* 3C protein numbering here and below) is the only one that was proven to compensate structural disturbance in domain d apical region (Andino *et al.*, 1990, 1993). To evaluate the possible co-evolution between domain d tetraloop and its putative interaction partners in the protein 3C, relevant sequences in the filtered full genome data sets were analyzed.

Motif $_{82}$KFRDI$_{86}$ was conserved in all species, as well as amino acids Glu 71 and Cys 147 of the protease catalytic triade (Figure 3). Second position of the putative RNA-binding tripeptide (position 155) was invariantly Gly.

No mutual dependence between loop sequences and tripeptide sequences was found within enterovirus genomes of the same species. For example, *Enterovirus A* genomes contained 17 unique variants of tetraloop sequence, whereas the predominant fraction of 3C sequences (548 out of 564) contained the conservative tripeptide VGK at positions 154-156 (Figure 3, Table S9). Noteworthy, this tripeptide was found not only in genomes of EV71 serotype, although genomes of this serotype prevailed in the dataset. Other *EV-A* genomes contained tripeptides VGR (7 out of 564), TGK (4 out of 564), IGK (3 out of 564), VGE (1 out of 564) and SRK (1 out of 564) (Figure 3, Table S9). Genomes with tripeptides other than VGK did not contain any peculiarities of domain d loop sequence (Table S9). This observation confirms that the specific loop sequence is likely not the main subject for recognition by the RNA-binding tripeptide. Similarly, all or almost all genomes of *Enterovirus B* (242 out of 244), *Enterovirus C* (272 out of 274), *Enterovirus D* (all), *Enterovirus G* (7 out of 8), *Enterovirus H* (a total of 2 genomes – one with TGK, one with TGR), *Enterovirus J* (all) and *Rhinovirus B* (36 out of 37) species contained tripeptide TGK at position 154-156 of 3C protein (Figure 3, Table S7, S9, S10). Alternative tripeptides were TGR in two genomes of *Enterovirus B* and one genome of *Enterovirus H*; IGK in one genome of *Enterovirus C* species and in one genome of *Rhinovirus B* species; PGK in one genome of *Enterovirus C* species; MGK in one genome of *Enterovirus G* species (Table S7, S9, S10).

Genomes of species *Enterovirus E* and *F* contained two oriLs with tetraloops in domain d mostly of consensus GYYA or pentaloops of consensus GHBUA, where H = A/C/U, and B = U/C/G. All genomes contained tripeptide MGK at positions 154-156 of the protein 3C (Figure 3, Table S7). Interestingly, a similar loop-tripeptide pair was found in one genome of *Enterovirus G* species (strain LP 54, accession number AF363455). It contained tetraloop GUUA in the domain d of its single oriL and tripeptide MGK in 3C. Unlike this unique genome, other genomes of *Enterovirus G* species contained tetraloops of YNMG consensus and tripeptide TGK in the protein 3C.

Rhinovirus genomes contained tetraloops mostly of consensus YNMG (species *Rhinovirus A* and *C*) or triloops (*Rhinovirus A* and *B*) (Table 2). *Rhinovirus A* genomes with tetraloops in domain d contained tripeptides in 3C with positively charged amino acid before the tripeptide, but not at the last position of it, as in genomes of *Enterovirus A-C* species (Figure 3, Table S11, S12). Sequence of tripeptides did not depend on tetraloop sequence and was, in descending order, IGQ (the most abundant, 65 genomes out of 119), IGL (20 genomes out of 119), IGS, VGS, IGN, VGQ, IGV, VGH (Table S11). In the case of *Rhinovirus A* genome with triloop UCU in domain d (isolate V38_URT-6.3m, accession number JF285329), protein 3C contained tripeptide TGK without positive charged amino acid before it (Table S11). All genomes of *Rhinovirus B* species contained triloops in the domain d and all, except one with IGK, contained tripeptide TGK in 3C. Genomes of Rhinovirus C contained tetraloops mostly of consensus YHCG (H=all but G) and tripeptides in 3C without a positively charged amino acid at the last position (TGN, VGN, TGH) or outside of the tripeptide (Table S12). One genome contained tetraloop CUUC paired with most abundant tripeptide TGN (23 out of 37 genomes)(Table S12).

Thus, dependence between apical domain d sequences and tripeptides in protein 3C within a species was not detected (Figure 3). We can state that the tripeptide and motif KFRDI are almost not variable within a species compared to domain d loop sequence, but there is a specific preferred tripeptide sequence for each species. Hence, tripeptide sequences are species-specific, while the domain d loop sequences are almost universal among species *Enterovirus A, B, C, D* and *Rhinovirus A* and *C*.

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Discussion

Most of the domain d apical loops in enterovirus genomes were represented by tetraloops. The most common variants of tetraloop sequences corresponded to consensuses YNHG (Y=C/U, N=any, H=A/C/U) (Table 2). Similar results were obtained in our previous experimental work, where eight apical nucleotides of domain d of poliovirus genome were randomized, viable variants were selected *in vitro* and the majority of selected tetraloops belonged to consensus YNHG (Prostova *et al.*, 2015). Some tetraloops of consensus YNHG were found in genomes in the NCBI database, but not among the variants selected *in vitro*, namely tetraloops CACG, CUCG, UAAG, UGAG, CAAG, CGAG, UGUG, CUUG (Prostova *et al.*, 2015). Tetraloops UGAG, UGUG and CUUG were reconstructed with U****G flanking base pair in context of poliovirus genome strain Mahoney and supported effective virus replication (Prostova *et al.*, 2015).

Vice versa, tetraloops UUAG, UCAG, CCAG were found in domain d of selected *in vitro* viable poliovirus variants and were able to support virus reproduction, but were not found in naturally circulating viruses (Prostova *et al.*, 2015). One tetraloop of YNHG consensus (CUAG) was not found neither in genomes from the NCBI database (n=2842), nor in the randomized poliovirus genomes selected *in vitro* (n=62) (Table 2). Thermodynamic stability is unlikely to be the reason why this and other tetraloops were unrepresented, as the melting temperature of stem loops with avoided tetraloops is within range of the melting temperature of YNHG tetraloops

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that supported replication (Proctor *et al.*, 2002). Moreover, tetraloops UUAG and UCAG are common in rRNA (Woese *et al.*, 1990). Sample insufficiency cannot be excluded for both database and *in vitro* selected sets of genomes, but it is safe to conclude that these tetraloop variants are at least extremely rare. In any case, the fact that incidence of these tetraloops is much less, than of other tetraloops points out that such variants are possibly less fit.

The most abundant tetraloops in the domain apical region of genomes from NCBI database and variants selected *in vitro* could be compiled into consensuses UNCG and CNCG (Table 2). At the same time, these tetraloops are the most abundant in rRNA, and with certain closing base pairs are among the most thermodynamically stable tetraloops (Woese *et al.*, 1990; Proctor *et al.*, 2002). Tetraloops of these consensuses and some other found tetraloops of YNHG consensus form specific spatial structure of UNCG structural class of stable tetraloops (Cheong, Varani and Tinoco, 1990; Varani, Cheong and Tinoco, 1991; Du *et al.*, 2003, 2004).

Another set of tetraloops, which correspond to GNYA consensus, was found both in genomes of *Enterovirus E* and *F* and in genomes of viable polioviruses selected *in vitro* (Prostova *et al.*, 2015). Tetraloop GCUA was able to support effective replication of poliovirus and, together with tetraloop GUUA, are known to assume UNCG fold (Ihle *et al.*, 2005; Melchers *et al.*, 2006; Prostova *et al.*, 2015).

Together, this data indicates that the spatial structure, rather than the exact sequence, is the main subject for recognition by virus protein 3C, and, together with literature data, let us assume that the sequence-structure degeneracy is an universal way RNA tetraloops are used in nature (Lebars *et al.*, 2001; Wu *et al.*, 2004; Ihle *et al.*, 2005; D'Ascenzo *et al.*, 2016, 2017; Bottaro and Lindorff-Larsen, 2017).

It can be speculated that pentaloops in domain d of *Enterovirus E* genome and triloops of domain d of rhinoviruses have a potential to have the same UNCG fold as some YNHG and GNYA tetraloops do. For HRV14 domain d it was shown that its triloop resembles the structure of the first and two last nucleotides of UNCG structural class tetraloops (Headey *et al.*, 2007). There are pentaloops with four nucleotides that belong to consensuses UNCG, GNRA or gCUUGc and are able to form spatial structures of corresponding structural classes with the fifth nucleotide bulged (Cai *et al.*, 1998; Schärpf *et al.*, 2000; Theimer, Finger and Feigon, 2003; Oberstrass *et al.*, 2006; Liu *et al.*, 2009). It is possible that four nucleotides of pentaloops in domain d of Enterovirus E species have UNCG fold with one bulged nucleotide.

Tetraloops that didn't belong to YNHG or GNYA consensuses were found in both sets of natural and *in vitro* selected genomes. However, in an experiment such variants evolved towards YNHG or GNYA consensuses (Prostova *et al.*, 2015). Apparently, tetraloops that don't belong to YNHG or GNYA consensuses are less fit in most settings and in experimental conditions. However, as these variants still could be found in few naturally circulating viruses (consequently, they have emerged and have been fixed), we speculate that they may be beneficial under specific replication conditions.

Similar structure of domain d and its apical region suggests free exchange of this region between genomes of the same and of different species of Enterovirus genera. Indeed, viable intra and inter species recombinants for this region could be obtained *in vitro* (Muslin *et al.*, 2015; Bessaud *et al.*, 2016). To evaluate relative impact of the high mutation rate and recombination on domain d apical loop variability, sequences of EV71 C4 genotype viruses were analyzed. Natural recombination in EV71 genotype C4 is much less frequent than other EV-A types (Lukashev *et al.*, 2014), and only one recombinant genome (accession number HQ423143) was detected in our data set. Therefore variability of its domain d loop sequence reflects changes that were accumulated via mutations only. Diversity of domain d loop sequence of EV-71 C4 viruses was far less than among EV-A genomes and was represented only by 5 tetraloop sequence variants (Table S1). The most recent common ancestor of EV71 genotype C4 dates about 20 years back (McWilliam Leitch *et al.*, 2012), therefore this diversity, although limited, emerged very recently. On the other hand, high sequence variability of domain d apical region in all enterovirus genomes was possibly assisted by inter- and intraspecies recombination events.

Interestingly, in contrast to similar structure of domain d and very similar distribution of its apical sequences in genomes of different enterovirus species, its putative RNA-recognition tripeptide of 3C is diverse (Figure 3). Most of *Enterovirus A* genomes contain tripeptide VGK in 3C, while genomes of *Enterovirus B, C* and *D* species prevalently have TGK tripeptide (Figure 3). Genomes of *Rhinovirus A* and *C* also contain common enterovirus tetraloops in the domain d apical region, but in the 3C they, unlike other species, contain tripeptides without positively charged amino acids (Figure 3, Table S11, Table S12). Positively charged amino acids are often involved into interaction with RNA, in particular with phosphates of the RNA backbone, thus being of importance for RNA-protein recognition (Jones *et al.*, 2001; Bahadur, Zacharias and Janin, 2008). In *Rhinovirus A* genomes positively charged amino acid “jumped” from the last position of tripeptide (position 156) to the position that precedes tripeptide (position 153) (Figure 3, showed by arrow). Residue at position 153 starts and residue at position 156 ends reverse turn between beta strands dII and eII of the protein 3C (Mosimann *et al.*, 1997; Matthews *et al.*, 1999; Cui *et al.*, 2011). In a crystal structure of Rhinovirus A2 protein 3C side chain of Lys153 (preceding tripeptide) is positioned in the similar region, as side chain of Lys156 (at the last position of tripeptide) in crystal structure of Enterovirus 71 and Poliovirus 1 proteins 3C (Mosimann *et al.*, 1997; Matthews *et al.*, 1999; Cui *et al.*, 2011). Thus, Lys at position 153 of 3C has almost the same potential to interact with RNA-ligand as Lys at position 156 (Mosimann *et al.*, 1997; Matthews *et al.*, 1999; Cui *et al.*, 2011). Genomes of *Rhinovirus C* species do not contain a positively charged amino acid neither inside the tripeptide of 3C protein, nor in the neighboring positions, possibly indicating that tripeptide 154-156 in protein 3C of *Rhinovirus C* genome does not interact directly with RNA. Thereby, 3C is able to recognize domain d of oriL with tripeptides of different sequence. In contrast to domain d structure and its apical sequence, tripeptide is species-specific. Diversity of the tripeptide that is expected to recognize domain d has several compatible explanations. The residue 154 of tripeptide possibly does not interact with domain d directly. The tripeptide may be involved into a species-specific cooperative amino acid network (amino acid “epistasis”). Moreover, different tripeptides might reflect slightly different molecular mechanisms for domain d recognition.

The complexity of tripeptide’s role in domain d recognition can be shown in several examples. The 3C protein of different species with the same RNA-binding tripeptide is not guaranteed to bind the same-structured domain d. Genomes of *Rhinovirus B* contain triloops in the apical region of domain d that are paired with tripeptide TGK in 3C, common for genomes with tetraloops. In contrast, protein 3C of Coxsackie virus B3 (*Enterovirus B* species, contain tripeptide TGK) cannot recognize sufficiently well oriL with domain d capped with a triloop (Zell *et al.*, 2002). This indicates that sequence of RNA-binding tripeptide probably is not the exclusive participant in oriL-3C recognition. In other words, different molecular mechanisms of oriL-3C recognition evolved in every enterovirus species independently. For example, it was shown for Rhinovirus 14 (*Rhinovirus B* species) that protein 3C recognizes stem region of domain d, rather than its apical loop (Leong *et al.*, 1993). Another oriL-3C recognition mechanism apparently is used by *Enterovirus E* and *F* species, two oriLs of which play the same role as the single oriL in genomes of other enteroviruses (Pilipenko, Blinov and Agol, 1990; Zell *et al.*, 1999). Apical loop of their domain d is tetra- or pentaloop with sequence that differs from the loop consensuses of other enteroviruses. RNA-binding tripeptide in the 3C is species specific as well, and is always MGK (Table S6). Interestingly, one genome of *Enterovirus G* species had the same pair domain d loop – tripeptide of 3C, i.e. GUUA – MGK. Domain d of *Enterovirus G* species is prolonged in comparison to length of domain d in genomes of other species (Krumbholz *et al.*, 2002) (Figure 2). The tripeptide MGK in 3C of *Enterovirus E, F* and *G* possibly indicates another molecular mechanism of oriL-3C recognition (Krumbholz *et al.*, 2002). Therefore, we assume that though putative RNA-binding tripeptide in most cases possibly interacts with the domain d apical region (since amino acid substitutions in it are known to compensate for structural disturbance in domain d), this interaction is not the only one that determines the evolution oriL-3C interaction. Altogether, data demonstrates independent evolution of putative RNA-binding tripeptide of 3C and domain d of oriL.

Conclusions

We performed analysis of variety and occurrence of replication element oriL functional loop and its protein ligand virus protease 3C. RNA-binding motifs of 3C are species-specific in contrast to domain d loop sequences: domain d loop sequence variety is almost the same for species *Enterovirus A, B, C, D* and *Rhinovirus A* and *C*, whereas tripeptide sequence variety differ.

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Figure 1

Schematic representation of poliovirus genome and detailed representation of secondary structure of poliovirus replicative element oriL.

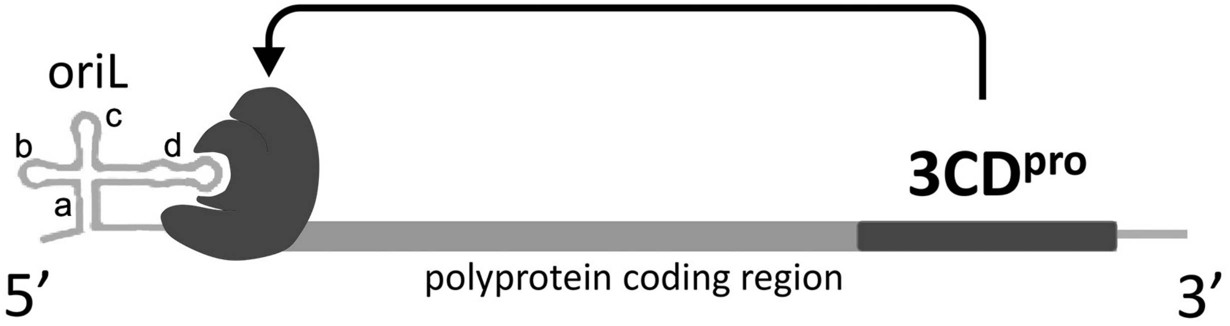

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Figure 2

Secondary structure of oriL domain d of distinct enterovirus species.

For *Enterovirus E* and *F* domain d of the first oriL is shown. Secondary structure of domain d of Porcine enterovirus 9 strain UKG/410/73 was folded with use as reference Krumbholtz et al., 2002.

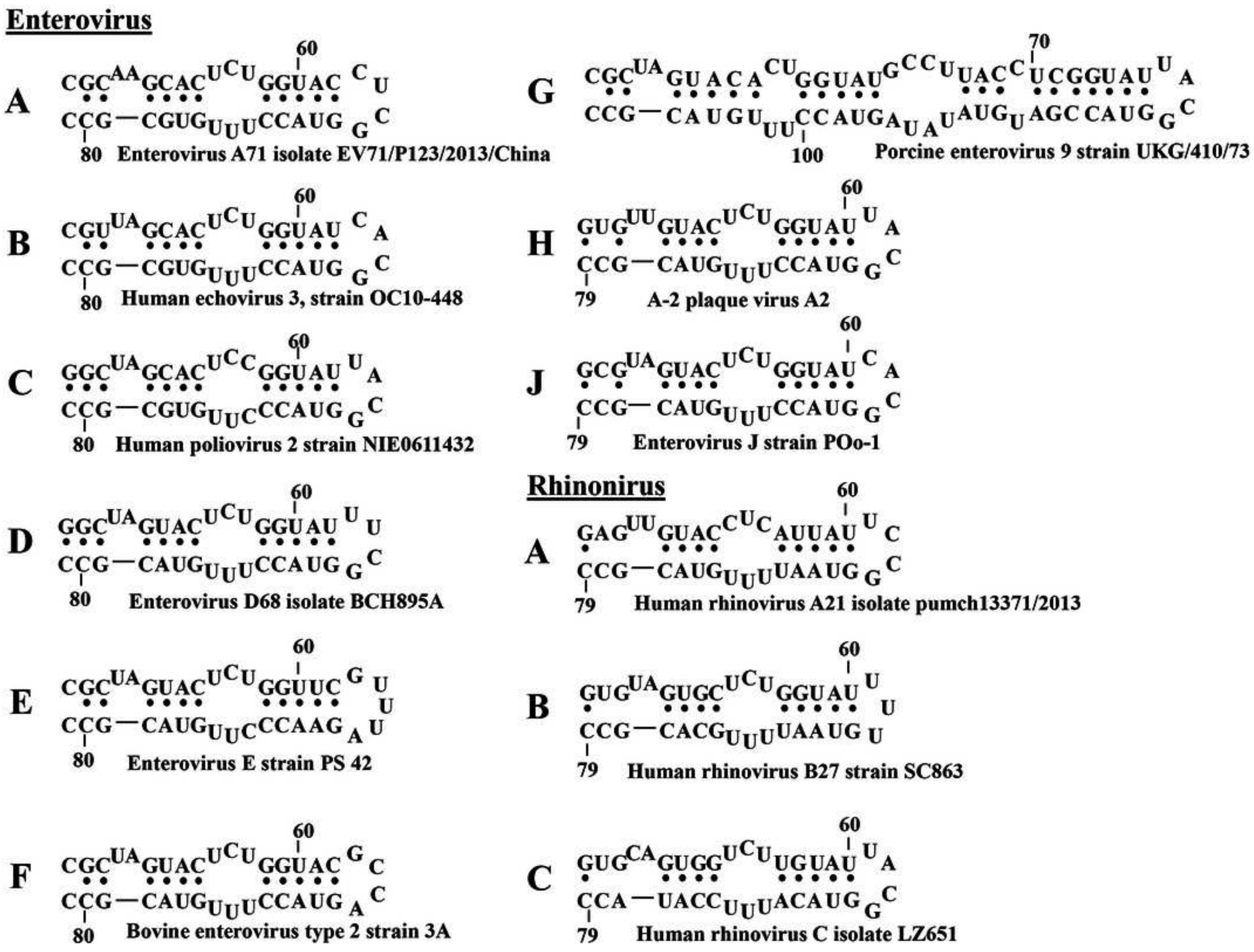

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Figure 3

Distribituion of domain d loop sequence and amino acid motifs in the 3C protein.

A - Distribution of domain d loop sequences. The regions corresponding to tetraloop consensuses, triloops and pentaloops are shown. Number of genomes cut off at 15 for clear view of sequence distribution. **B -** The frequency plot of amino acid sequence of 3C in species of genus *Enterovirus*. The amino acid sequence logo was done with WebLogo server (Crooks *et al.*, 2004). Arrows indicate amino acids of the proteolytic triade (Glu71 and Cys 147), the first and the last amino acids of motif $_{82}$KFRDI$_{86}$, the putative RNA-binding tripeptide 154-156 of 3C and Lys153 in the protein 3C of *Rhinovirus A*.

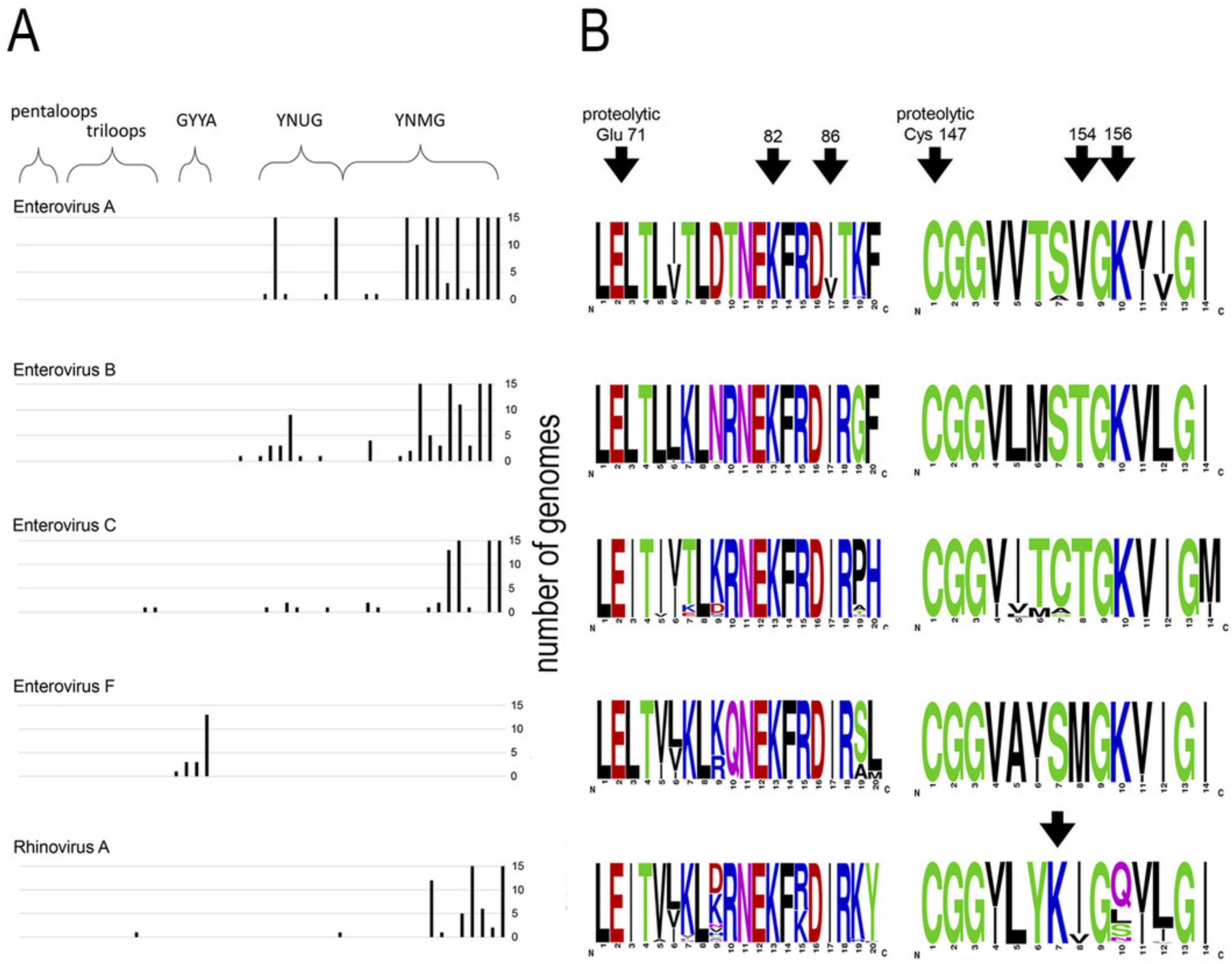

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Table 1(on next page)

Number of full genome sequences that contained oriL region and number of unique domain d sequences before and after filtration.

For *Enterovirus E* and *F* number of unique tetraloops is shown separately for first and the second oriL.

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<table>
<tr><th>Species</th><th>Number of full genome sequences</th><th>Number of full genome sequences after 1% nucleic identity filtration</th><th colspan="2">Number of unique tetraloops before filtration</th><th colspan="2">Number of unique tetraloops after filtration</th></tr>
<tr><td>Enterovirus A</td><td>1052</td><td>564</td><td colspan="2">17</td><td colspan="2">16</td></tr>
<tr><td>Enterovirus B</td><td>339</td><td>244</td><td colspan="2">18</td><td colspan="2">18</td></tr>
<tr><td>Enterovirus C</td><td>747</td><td>274</td><td colspan="2">15</td><td colspan="2">12</td></tr>
<tr><td>Enterovirus D</td><td>419</td><td>57</td><td colspan="2">7</td><td colspan="2">6</td></tr>
<tr><td>Enterovirus E</td><td>12</td><td>10</td><td>6</td><td>5</td><td>6</td><td>5</td></tr>
<tr><td>Enterovirus F</td><td>13</td><td>10</td><td>4</td><td>3</td><td>4</td><td>3</td></tr>
<tr><td>Enterovirus G</td><td>10</td><td>8</td><td colspan="2">6</td><td colspan="2">6</td></tr>
<tr><td>Enterovirus H</td><td>3</td><td>2</td><td colspan="2">2</td><td colspan="2">2</td></tr>
<tr><td>Enterovirus J</td><td>8</td><td>5</td><td colspan="2">3</td><td colspan="2">3</td></tr>
<tr><td>Rhinovirus A</td><td>151</td><td>118</td><td colspan="2">8</td><td colspan="2">8</td></tr>
<tr><td>Rhinovirus B</td><td>50</td><td>37</td><td colspan="2">7</td><td colspan="2">7</td></tr>
<tr><td>Rhinovirus C</td><td>38</td><td>37</td><td colspan="2">6</td><td colspan="2">6</td></tr>
</table>

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Table 2(on next page)

Occurrence of domain d apical sequences in filtered sets of full genomes of different enterovirus species.

Tetraloops CCCG, UGUG, CAUG and UUGG that were unique for species *Enterovirus A, C* and *D* and were lost upon filtration, were added and are shown in blue. The gradient coloring from red to green represents abundancy heat map for the genomes with different domain d sequence.

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Loop sequence	Enterovirus									Rhinovirus		
	A	B	C	D	E	F	G	H	J	A	B	C
Triloops												
CCG			1									
CAG			1									
UCU										1	5	
UUU											17	
UAU											8	
AUU											4	
UGU											1	
UUC											1	
GAU											1	
YNMG[a] Tetraloops												
UACG	85	51	106				3	1	2	38		15
UGCG	114	31	43				2	1		2		
UUCG	16	3		50						6		6
UCCG	2	11	1							53		10
CACG	48	98	101	1			1		2	5		
CGCG	3	3	13				1					
CUCG	132	5	2	2						1		3
CCCG	40	16	1	1						12		2
UAAG	10	2										
UGAG	22	1										
UUAG												
UCAG												
CAAG	1	4	1						1			
CGAG	1		2									
CUAG												
CCAG												
YACG		1										
YNUG Tetraloops												
UAUG	54											
UGUG	1	1	1									
UUUG				1								
UCUG		1										
CAUG		9	1									
CGUG	1	3	2									
CUUG	34	3		2								
CCUG	1	1	1									
GYYA Tetraloops												
GCUA					2	13						
GCCA						3						
GUUA					2	3	1					
Other tetraloops												
UUGG				1								
CUUC												1
AUUA						1						
Pentaloops												
GCUUA					7							
GUUUA					2							
GCCUA					4							
GCGUA					1							
GAUUA					1							
GUCUA					1							

1
2
11
31
51
132

[a] – here and after Y=C/U, N=any nucleotide, M=A,C