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Sequence Analysis of the Downstream 5′ Nontranslated Region of Seven Echoviruses with Different Neurovirulence Phenotypes

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The downstream 5′ nontranslated regions of seven echoviruses with different neurovirulent phenotypes were amplified and sequenced. Neurovirulent echovirus serotypes 4, 6, 9, 11, and 30 were identical to the putative poliovirus in 18S rRNA binding sequence and the flanking conserved sequences. Less neurovirulent echoviruses, serotypes 2 and 12, exhibited variations within these regions.

The enteroviruses (EVs) comprise a genus within the family *Picornaviridae* and include the polioviruses (PVs), group A coxsackieviruses, group B coxsackieviruses (CBVs), echoviruses (ECVs), and the more recently numbered EVs. ECVs comprise the largest number of serotypes and are the most frequently isolated from clinical specimens (37). They are responsible for clinical syndromes involving almost every organ and vary in their propensity to cause meningitis and encephalitis, i.e., neurovirulence (4).

Comparison of the 5′ nontranslated regions (5′NTRs) of the sequenced EVs has disclosed multiple areas of conservation (18, 29, 32, 36). The 5′NTR of PV contains genomic elements necessary for replication (1) and translation (23, 26–28) and determinants of virulence (14, 22, 25, 42).

Picornaviruses initiate translation without the presence of a 5′ cap structure (7mGpppG). In PV, a 5′NTR *cis*-acting element is required for efficient translation of the genome (2, 5, 23, 26, 41). This element, the internal ribosome entry site (IRES), spans 450 nucleotides and contains specific sequences and/or secondary structures that may directly influence the ability of the viral genome to efficiently initiate translation (6, 8, 23, 26, 28, 30). The existence of and possible role in ECV pathogenesis of similar translation elements in the 5′NTR remain unstudied.

ECV serotype 4 (ECV4; Pesascek), ECV6 (D'Amori), ECV9 (Hill), ECV11 (Gregory), and ECV30 (Bastianni) were selected for study because of their strong association with aseptic meningitis (7, 24, 43). ECV2 (Cornelis) and ECV12 (Travis) were chosen because of their relatively avirulent phenotype as demonstrated in surveys of ECV prevalence from clinical samples and human challenge studies (24, 42, 35). All serotypes were obtained from the American Type Culture Collection (Rockville, Md.) and, except for ECV30, were grown on LLCMK2 cells and stored at −70°C. ECV30 was stored at −70°C and used directly from the American Type Culture Collection stock.

Primer JRpATG (5′-ACTTGAGCTCCCATT-3′) was designed as a consensus primer to positions −2 to +14 from the true translation initiation codon of the nine sequenced non-ECV EV serotypes (3, 9, 11, 13, 15, 16, 20, 31, 34, 39, 40) and partial sequences obtained from ECV9 (Barty) (unpublished data). The consensus sense primer JRp64 (5′-ACGGTACCT TTGTGCGCCTGTTTT-3′) was designed on the basis of the published EV sequences corresponding to nucleotides 64 to 88 of PV type 1 (Mahoney) (PV1M). Primers MD90 (5′-ATTGT CACCATAAGCAGCCA-3′) and MD91 (5′-CCTCCGGCCC CTGAATGCGGCTAAT-3′), corresponding to nucleotides 577 to 596 and 444 to 468, respectively, of PV1M, have been described previously (33). Primer JRp616 (5′-AGCTATTG GATTGGC-3′) was designed on the basis of sequences obtained from the JRpATG-MD91 amplification products of the seven ECVs studied and used for sequencing. Primers were synthesized by Research Genetics (Huntsville, Ala.).

Approximately 50 μl of virus preparation was extracted by using guanidine thiocyanate (IsoQuick; MicroProbe Corp., Garden Grove, Calif.) as instructed by the manufacturer. The nucleic acid was resuspended in 40 to 50 μl of reverse transcription mixture (33) containing primer JRpATG, divided into 10-μl aliquots, and incubated at 42°C for 90 min and then at 90°C for 10 min.

To each reverse transcription reaction tube, 39.5 μl of PCR mixture [final concentrations, 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 0.1% Triton X-100, and 200 μM each deoxynucleoside triphosphate] and 0.5 pmol each of two primers were added. The mixture was heated to 90°C for 5 min, 1 U of Vent polymerase (New England Biolabs, Beverly, Mass.) was added, and PCR was performed as follows: denaturation at 95°C for 5 min; 35 cycles of denaturation at 95°C for 2 min, annealing at 58°C for 2 min, and extension at 72°C for 2 min; and a final extension at 72°C for 9 min. Upon completion, the amplified reaction mixtures for each serotype were pooled and electrophoresed through a 3.5% agarose gel (3.0% NuSieve GTG; FMC, Rockland, Maine) and 0.5% Agarose (GIBCO BRL, Grand Island, N.Y.) containing 0.5 μg of ethidium bromide (AMRESCO, Solon, Ohio) per ml. Appropriately sized bands were identified and purified by using GeneClean II (Bio 101, La Jolla, Calif.). When multiple bands fell within the anticipated size range, the band closest in size to that predicted for PV1M was selected.

Primer annealing for direct ^{35}S sequencing was performed with 20 pmol of primer and approximately 0.5 pmol of DNA in 10 μl of buffer (U.S. Biochemical, Cleveland, Ohio). The mixture was heated to 100°C for 5 min, frozen on dry ice for 2 to 3 min, and transferred to 2 to 4°C. While the mixture was at 2 to 4°C, 3.5 μl of cold labeling mixture, 1 μl of cold 100 mM $MgCl_2$ (U.S. Biochemical), and 3.25 U of Sequenase enzyme were added, and the mixture was incubated at 4°C for 3 to 5 min. Extension/termination reactions were performed as recommended except that the reaction temperature was increased

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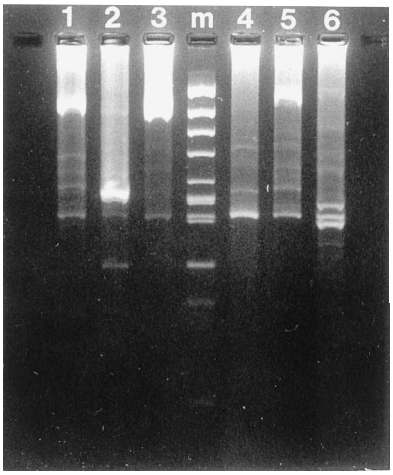

FIG. 1. Amplification products generated by the JRpATG-MD91 primer pair. Ethidium bromide-stained 3.5% agarose gel. Lanes: 1, ECV2; 2, ECV4; 3, ECV6; m, *Hin*cIII digest of bacteriophage ϕX174 DNA; 4, ECV9; 5, ECV11; 6, ECV12.

to 45°C. Samples were electrophoresed through 5 to 6% Hydrolink gels (J. T. Baker, Phillipsburg, N.J.) and processed according to standard protocols. Sequence alignment was performed by using MacVector version 4.1.1 (Kodak/IBI, Rochester, N.Y.).

Figure 1 shows the products of ECV reversion transcription-based PCR using primers JRpATG and MD91 (except for ECV30). The JRpATG-MD91 product encompasses the 3′ end of the IRES (stem-loop structures VI and VII and the intervening oligopyrimidine tract (OPT)), the 5′NTR variable region, and the true initiation codon of translation. The corresponding region in PV1M spans nucleotides 444 to 756. All serotypes produced at least one band within the expected size range (284 to 319 bp). For ECV12, three bands migrating near or within the expected size range are seen.

The sequence of the entire fragment was unambiguously determined for all serotypes except ECV30, for which the number of thymidines comprising a tract within the OPT could not be determined (Fig. 2). The sequence of the MD91 target site was determined by sequencing the extreme 3′ end of the product generated by the MD90-JRp64 primer pair (not shown). The 3′-terminal 15 nucleotides of each sequence represent those of the JRpATG primer.

Alignment of the ECV sequences demonstrates multiple areas of absolute or nearly absolute identity (Fig. 2). These regions correspond to similar sequences described for the sequenced human picornaviruses (23, 29, 32, 36). Comparisons between ECV sequences and previously determined EV sequences suggest that the ECVs are more closely related to the CBVs in the sequenced region (data available on request).

Within the IRES, the OPT is a crucial element for efficient cap-independent initiation of translation (23, 26–28). The OPT of the sequenced non-ECV EV has demonstrated extremely tight size constraints, measuring 19 to 20 nucleotides. It has been proposed that efficient initiation of translation requires a critical distance between an OPT 5′ pyrimidine motif (see

```
                 *         *         *        40        *         *         *        80
ECV2      TCTCCGGCCCCTGAATGCGGCTAATCCTAACTGCGGAGCAGGTACCCACGAGCCAGTGGGCAGCCTGTCGTAATGGCAA
ECV4      TCTCCGGCCCCTGAATGCGGCTAATCCTAACTGCGGAGCACACGCTCACAAGCCAGTGAGTGGTGTGTCGTAATGGTAA
ECV6      TCTCCGGCCCCTGAATGCGGATAATCTTAACTGCGGAGCAGGTGCTCACAATCCAGTGGGTGGCCTGTCGTAACGGGCAA
ECV9      TCTCCGGCCCCTGAATGCGGCTAATCCTAACTGTGGAGCACATGCCCCTAATCCAAGGGGTAGTGTGTCGTAACGGGCAA
ECV11     TCTCCGGCCCCTGAATGCGGTTAATCCTAACTGCGGAGCACATACCCCTAATCCAAGGGGCAGTGTGTCGTAACGGGCAA
ECV12     TCTCCGGCCCCTGAATGCGGCTAATCCTAACTGTGGAGCAAGTGCCCACAACCCAGTGGGTGGCTTGTCGTAATGGCAA
ECV30     TCTCCGGCCCCTGAATGCGGCTAATCCTAACTGCGGAGCAGGTGCTCACAGACCAGTGAGTAGCCTGTCGTAACGGGCAA
Consensus TCTCCGGCCCCTGAATGCGG-TAATCcTAACTG-GGAGCA--t-C-C--aa-CCA--G-G--G--TGTCGTAA-GGGcAA

                 *         *         *       120        *         *         *       160
ECV2      CTCTGCAGCGGAACCGACTACTTTGGGTGTCCGTGTTTCCATTTACTCTTATACTGGCTGCTTATGGTGACAATCACGGA
ECV4      CTCCGCAGCGGAACCGACTACTTTGGGTGTCCGTGTTTCCTTTTAACTTCATTTTGGCTGCTTATGGTGACAATTAAGAA
ECV6      CTCTGCAGCGGAACCGACTACTTTGGGTGTCCGTGTTTCCTTTTATCTCTTTGTTGGCTGCTTATGGTGACAATTGAACG
ECV9      CTCCGCAGCGGAACCGACTACTTTGGGTGTCCGTGTTTCCTTTTATTCTTACATTGGCTGCTTATGGTGACAATTGAGAG
ECV11     CTCTGCAGCGGAACCGACTACTTTGGGTGTCCGTGTTTCCTTTTATTTTTATACTGGCTGCTTATGGTGACAATCTCAGA
ECV12     CTCTGCAGCGGAACCGACTACTTTGGGTGACCGTGTTTCTCTTTATTCTTATATTGGCTGCTTATGGTGACAATCTCAGA
ECV30     CTCTGCAGCGGAACCGACTACTTTGGGTGTCCGTGTTTCCT(T_n)CTCTTATATTGGCTGCTTATGGTGACAATTAAAGA
Consensus CTC-GCAGCGGAACCGACTACTTTGGGTGTCCGTGTTTCc-TTTa---ttat--TGGCTGCTTATGGTGACAAT------

                 *         *         *       200        *         *         *       240
ECV2      ATTGTTACCATATAGCTATTGGATTGGCCATCCAGTGACTAACAGAGCGATTATATTCCTTTTTGTTGGTTTTGTACCAC
ECV4      ATTGTTACCATATAGCTATTGGATTGGCCATCCGGTGACTAGTAGAGCTATTATATACTTGTTTGTTGGCTTTGTACCAC
ECV6      ATTGTTGCCATATAGCTATTGGATTGGCCATCCGGTGAGCAGCAGATCCATTATTTACCAGTTTGTTGGATTTGTACCAC
ECV9      ATTGTTGCCATATAGCTATTGGATTGGCCATCCGGTGTCTAATAGAGCGATAATCTACCAGTTTGTTGGATTTGTACCAC
ECV11     GTTGTTACCATATAGCTATTGGATTGGCCATCCGGTGAGCAACAGAGCTGTCATTTATCAGTTTGTTGGCTTTATACCTC
ECV12     GTTGTTACCATATAGCTATTGGTTTGGCCAACCAGTGACTAACAGAGCAATTATATATCTTTTCATTGGATTTATACCTA
ECV30     ATTGTTACCATATAGCTATTGGATTGGCCATCCGGTGACGAGCAGAGCCATTGTTTACCTCTTTGTTGGATTTGTACCTT
Consensus -TTGTT-CCATATAGCTATTGGaTTGGCCAtCCgGTGaC-A--AGAgC-aTaaT-Ta-c--TTtgTTGG-TTT-TACC--

                 *         *         *         *       290
ECV2      TCACCGTCACGAGCTTTACAACGCTAAACTACATTTTGTCATTGAACACGGC......AAAATGGGAGCTCAAGT(309)
ECV4      TAAACTATAAAGTCCTTAGAACTCTTGATTTTATACTAATTTTGAATAAGGC......AAAATGGGAGCTCAAGT(309)
ECV6      TTGATCACATAGTCTTTAGAACACTTGTCTTTATTCTAAATCTTAACAAGGC......AAAATGGGAGCTCAAGT(309)
ECV9      TCAACTTTTCAACACTCAAGACACTCAACTACATACTAGCACTCAACACAGT......AAAATGGGAGCTCAAGT(309)
ECV11     TAAATCACACGGTTTTTGGAACGCTTGTATTCATCTTAACCCTCAATAAGGC......AAAATGGGAGCTCAAGT(309)
ECV12     TAAACAACACCAGTTATACCACTTTGTGTTACATTATTGGCTTGAACTCGAA......AAGATGGGAGCTCAAGT(309)
ECV30     TGAACCACAAAGTCTTGAATACCATTCATCTCATTTTAAAGTTCAACTCAGCTAAAAGAAAATGGGAGCTCAAGT(315+)
Consensus T-aa----a------t-a--AC--T----t--AT--T-----T-AA----g-------AAaATGGG
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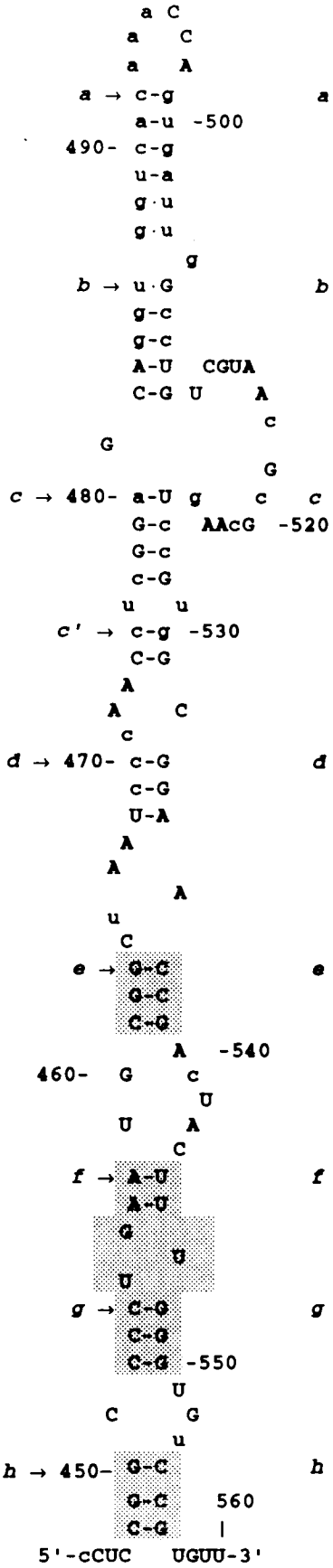
FIG. 2. Sequence alignment of the ECV downstream 5′NTR. The consensus sequence for the ECVs is shown in boldface below each grouping of sequences. Nucleotides conserved 100, 85, and <85% among the ECVs are designated by uppercase letters, lowercase letters, and -, respectively. Initiation codons (ATG) are designated in boldface within each EV sequence.



Nt	ECV2	ECV4	ECV6	ECV9	ECV11	ECV12	ECV30
a → 49-56				U-A	U-A		
48-57			C-G	C-G			
47-58							
46-59	C-G		U·G	C-G	C-G	C-G	U-A
45-60	C-G	C-G	C-G	C-G	C-G	C-G	C-G
44-61	a c				a c		
b → 43-63		C-G					
42-64		A-U		A-U	A-U		
41-65		C-G		C-G	C-G	A-U	
40-66							
39-67							
c → 37-82							
36-83				U·G		U·G	
35-84	G·U		G·U		G·U	G·U	
34-85				U·G		U·G	
33-86	G-C	G-C	G-C	G-C	G-C	G-C	G-C
32-87	U-A	U-A	U-A	U-A	U-A	U-A	U-A
31-88							
d → 27-90			U·G				
26-91							
25-92							
e → 20-94							
19-95							
18-96							
f → 15-102							
14-103							
g → 11-105							
10-106							
9-107							
h → 7-111							
6-112							
5-113							

FIG. 3. Comparison of the folding pattern of stem-loop VI of the ECVs with PV1M nucleotides 448 to 556. Absolutely conserved EV nucleotides (Nt) are designated in uppercase letters. Structures immutable with respect to sequence are designated in shaded areas. EV base pairings differing from PV1M are designated in each serotype's boxed area. Noncanonical base pairings are designated by dots. Base pairings not formed for a given ECV serotype are designated by a bold box outline. The folding pattern is as published by Haller and Semler (8).

below) and a cryptic AUG located within the conserved downstream flanking sequences (30).

The upstream half of the OPT (5′-UUUCCUUUUA-3′), particularly the extreme 5′ tetra- or pentapyrimidine sequence, 5′-UUUCC-3′, is crucial for efficient initiation of translation. This domain may base pair with complementary sequences within the host 18S rRNA (10, 18, 26, 30). In PV constructs, point mutations or deletions within the tetrapyrimidine sequence resulted in total or nearly total ablation of internal initiation. Mutations downstream of the tetrapyrimidine sequence, but still within the 5′ half of the OPT, led to variable degrees of reduction in the translational efficiency (8, 23, 26, 30).

Nucleotides 85 to 154 contain the OPT (nucleotides 116 to 135) and bilateral EV conserved flanking sequences. For the six ECV serotypes unambiguously sequenced, the OPT measured 20 nucleotides, supporting the premise of a critical distance required for efficient translation initiation. The 5′ tetrapyrimidine sequence was conserved among all seven ECVs, attesting to its importance in translation (Fig. 2). The five ECVs possessing neurovirulent phenotypes, i.e., frequently causing infections of the central nervous system, possessed the upstream OPT sequence 5′-TTTCCTTTT-3′. An identical motif is seen in the highly neurovirulent PV. The less neurovirulent serotypes, ECV2 and -12, demonstrated changes at position 121 and positions 120 and 121, T to A and CT to TC, respectively. Identical upstream OPT deviations have not previously been described for any of the picornaviruses.

There was no variation among the ECVs in the downstream sequences (nucleotides 135 to 154). Only ECV12 demonstrated variation (T to A, position 110) within the 17-nucleotide region universally conserved among the sequenced picornaviruses, immediately upstream of the OPT (18). Although not as well studied as the OPT, this region may be also be important for efficient initiation of translation. A T-to-C mutation at the same relative location within CBV1 (CBV1 nucleotide 557) resulted in dramatically reduced in vitro translation (12). In PV1, a G-to-A change at position 549, corresponding to position 106 in this report, reduced the relative transcriptional efficiency of the primary transcript to approximately 10% of wild-type levels (8).

Numerous lines of evidence support the existence of secondary structures within the IRES (19, 29, 32, 34). The overall proposed base-pairing patterns for stem-loop VI (nucleotides 5 to 113) are conserved in all seven ECVs studied (Fig. 3). The predicted pairings comprising stem-loop VII (nucleotides 118 to 183) appear to be generally conserved among all ECVs except ECV12 (Fig. 4). In ECV12, stem structure c may be substantially less stable, if it forms at all, than in any of the ECVs or previously sequenced EVs. The noncanonical U · G pairing between nucleotides 120 and 181 coupled with a U-

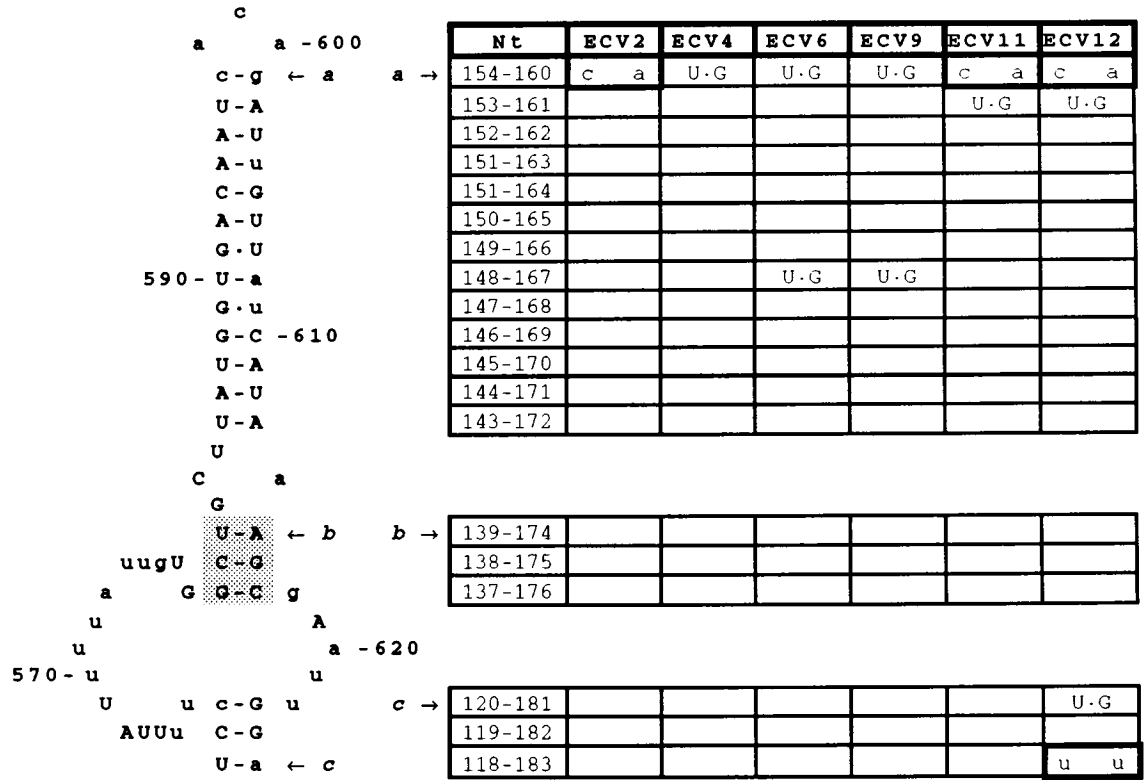


FIG. 4. Comparison of the folding pattern of stem-loop VII of the ECVs with PV1M nucleotides 561 to 625. See the legend to Fig. 3 for explanation.

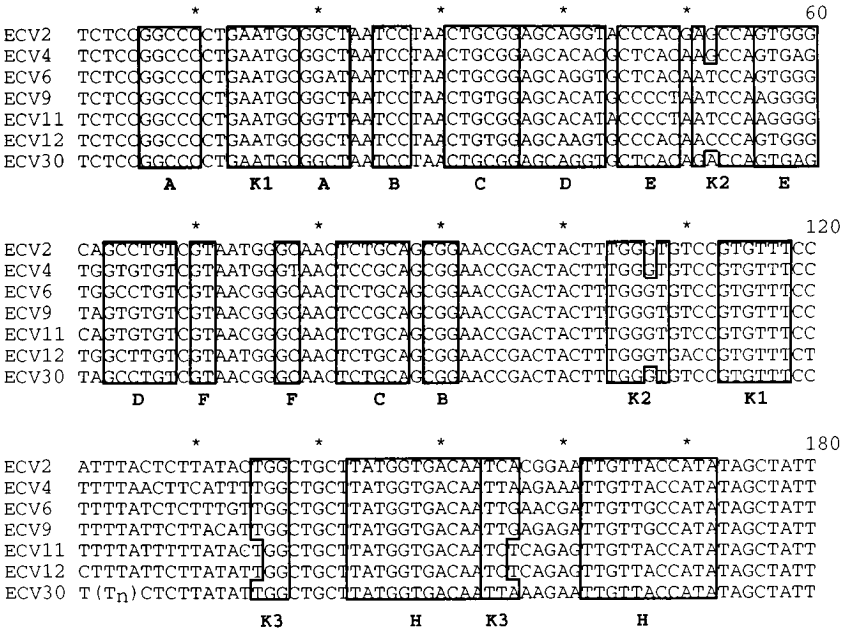


FIG. 5. Tertiary structural elements within the ECV 5′NTR. Proposed interactions are designated in boxed areas. A to H, base pairing resulting in secondary structures; K1 to K3, tertiary-level base pairing resulting in the formation of pseudoknots. Alignments and nomenclature are those proposed by Le et al. (18).

to-U mismatch between nucleotides 118 and 183 may not permit a structure of sufficient thermodynamic stability to exist.

A new model of proposed RNA tertiary interactions for the EV 5′NTR has been reported (18). Alignment of the ECV sequences according to that folding pattern would yield the predicted pseudoknots K1 and K2 (Fig. 5). As in the case of the CBVs (18), the ECV sequences contributing to the formation of K3 pseudoknot indicated that the formation of this pseudoknot was variable among the ECVs sequenced. Our sequences appear to be in accord with and further substantiate the tertiary folding model.

The contribution of the downstream IRES to the clinical phenotype of the PVs has been demonstrated. Lower viral titers observed when a Leon-Lansing PV recombinant containing a C-to-U change at position 472 was grown on a neuroblastoma cell line may have been due to a reduction in protein synthesis (17). Using the PV3 Sabin strain, the same mutation decreased the efficiency of in vitro translation in Krebs-2 cell extracts (38). Two reports have shown that the ability of the PV2 and PV3 Sabin strains to grow at elevated temperatures on BGM cells implies a correlation between attenuation and predicted secondary structure (21, 22).

It is tempting to speculate that the variations observed in the sequences of the downstream portion of the 5′NTR (IRES) of ECV2 and, in particular, ECV12 affect their ability to cause central nervous system disease. The observed genomic differences in ECV2 and ECV12 may decrease the ability of these serotypes to efficiently initiate translation, offering the host immune system an advantage in limiting viral spread from the portal of entry to the central nervous system.

The ECV sequences described in this report substantiate models of potential higher-order structures within the downstream portion of the EV 5′NTR. These structures may play critical roles in pathogenesis and clinical disease.

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REFERENCES

1. **Andino, R., G. E. Rieckhof, P. L. Achacoso, and D. Baltimore.** 1993. Poliovirus RNA synthesis utilizes a RNP complex formed around the 5′-end of viral RNA. EMBO J. **12:**3587–3589.
2. **Bienkowska-Szweczyk, K., and E. Ehrenfeld.** 1988. An internal 5′-noncoding region required for translation of poliovirus RNA in vitro. J. Virol. **62:**3068–3072.
3. **Chang, K. H., P. Auviene, T. Hyypiä, and G. Stanway.** 1989. The nucleotide sequence of coxsackievirus A9; implications for receptor binding and enterovirus classification. J. Gen. Virol. **70:**3269–3280.
4. **Cherry, J. D.** 1992. Enteroviruses: polioviruses (poliomyelitis), coxsackieviruses, echoviruses, and enteroviruses, p. 1705–1752. *In* R. D. Feigin and J. D. Cherry (ed.), Textbook of pediatric infectious diseases, 3rd ed. The W. B. Saunders Co., Philadelphia.
5. **Dildine, S. L., K. R. Stark, A. A. Haller, and B. L. Semler.** 1991. Poliovirus translation initiation: differential effects of directed and selected mutations in the 5′ noncoding region of viral RNAs. Virology **181:**742–752.
6. **Gebhard, J. R., and E. Ehrenfeld.** 1992. Specific interactions of HeLa cell proteins with proposed translation domains of the poliovirus 5′ noncoding region. J. Virol. **66:**3101–3109.
7. **Grist, N. R., and E. J. Bell.** 1984. Paralytic poliomyelitis and nonpolio enteroviruses: studies in Scotland. Rev. Infect. Dis. **6:**S385–S386.
8. **Haller, A. A., and B. L. Semler.** 1992. Linker scanning mutagenesis of the internal ribosome entry site of poliovirus RNA. J. Virol. **66:**5075–5086.
9. **Hughes, P. J., C. North, P. D. Minor, and G. Stanway.** 1989. The complete nucleotide sequence of coxsackievirus A21. J. Gen. Virol. **70:**2943–2952.
10. **Iizuka, N., M. Kohara, K. Hagino-Yamigushi, S. Abe, T. Komatsu, K. Tago, M. Arita, and A. Nomoto.** 1989. Construction of less neurovirulent polioviruses by introducing deletions into the 5′ noncoding sequence of the genome. J. Virol. **63:**5354–5363.
11. **Iizuka, N., S. Kuge, and A. Nomoto.** 1987. Complete nucleotide sequence of the genome of coxsackievirus B1. Virology **156:**64–73.
12. **Iizuka, N., H. Yonekawa, and A. Nomoto.** 1991. Nucleotide sequences important for translation initiation of enterovirus RNA. J. Virol. **65:**4867–4873.
13. **Jenkins, O., J. D. Booth, P. D. Minor, and J. W. Almond.** 1987. The complete nucleotide sequence of coxsackie B4 and its comparison to other members of the picornaviridae. J. Gen. Virol. **68:**1835–1848.
14. **Kawamura, N., M. Kohara, S. Abe, T. Komatsu, K. Tago, M. Arita, and A. Nomoto.** 1989. Determinants in the 5′ noncoding region of poliovirus Sabin 1 RNA that influence the attenuation phenotype. J. Virol. **63:**1302–1309.
15. **Kitimura, N., B. Semler, P. G. Rothberg, G. R. Larsen, C. J. Adler, A. J.**

Dorner, E. A. Emini, R. Hanecak, J. J. Lee, S. van der Werf, C. W. Andersen, and E. Wimmer. 1981. Primary structure, gene organization and polypeptide expression of poliovirus RNA. Nature (London) **291:**547–553.

16. **Klump, W. M., I. Bergmann, B. C. Muller, D. Ameis, and R. Kandolf.** 1990. Complete nucleotide sequence of infectious coxsackievirus B3 cDNA: two initial 5′ uridine residues are regained during plus-strand RNA synthesis. J. Virol. **64:**1573–1583.
17. **La Monica, N., and V. R. Racaniello.** 1989. Differences in replication of attenuated and neurovirulent polioviruses in human neuroblastoma cell line SH-SY5Y. J. Virol. **63:**2357–2360.
18. **Le, S. Y., J. H. Chen, N. Sonenberg, and J. V. Maizel.** 1992. Conserved tertiary structure elements in the 5′ untranslated region of human enteroviruses and rhinoviruses. Virology **191:**858–866.
19. **Le, S. Y., and M. Zuker.** 1990. Common structures of the 5′ non-coding RNA in enteroviruses and rhinoviruses. Thermodynamic stability and statistical significance. J. Mol. Biol. **216:**720–741.
20. **Lindberg, A. M., P. O. K. Stalhandske, and U. Petterson.** 1987. Genome of coxsackievirus B3. Virology **156:**50–63.
21. **Macadam, A. J., G. Ferguson, J. Burlison, D. Stone, R. Skuce, J. W. Almond, and P. D. Minor.** 1992. Correlation of RNA secondary structure and attenuation of Sabin vaccine strains of poliovirus in tissue culture. Virology **189:**415–422.
22. **Macadam, A. J., S. R. Pollard, G. Ferguson, G. Dunn, R. Skuce, J. W. Almond, and P. D. Minor.** 1991. The 5′ non-coding region of the type 2 poliovirus vaccine contains determinants of attenuation and temperature-sensitivity. Virology **181:**451–458.
23. **Meerovitch, K., R. Nicholson, and N. Sonenberg.** 1991. In vitro mutational analysis of *cis*-acting RNA translational elements within the poliovirus type 2 5′ untranslated region. J. Virol. **65:**5895–5901.
24. **Moore, M.** 1982. Enteroviral disease in the United States, 1970–1979. J. Infect. Dis. **146:**103–108.
25. **Moss, E. G., R. E. O'Neill, and V. R. Racaniello.** 1989. Mapping of attenuating sequences of an avirulent poliovirus type 2 strain. J. Virol. **63:**1884–1890.
26. **Nicholson, R., J. Pelletier, S. Y. Le, and N. Sonenberg.** 1991. Structural and functional analysis of the ribosome landing pad of poliovirus type 2: in vivo translation studies. J. Virol. **65:**5886–5894.
27. **Pelletier, J., G. Kaplan, V. R. Racaniello, and N. Sonenberg.** 1988. Cap-independent translation of the poliovirus mRNA is conferred by sequence elements within the 5′ noncoding region. Mol. Cell. Biol. **8:**1103–112.
28. **Pestova, T. V., C. U. T. Hellen, and E. Wimmer.** 1991. Translation of poliovirus RNA: role of an essential *cis*-acting oligopyrimidine element within the 5′ nontranslated region and involvement of a cellular 57-kilodalton protein. J. Virol. **65:**6194–6204.
29. **Pilipenko, E. V., V. M. Blinov, L. I. Romanova, A. N. Sinyakov, S. V. Maslova, and V. I. Agol.** 1989. Conserved structural domains in the 5′-untranslated region of picornaviral genomes: an analysis of the segment controlling translation and neurovirulence. Virology **168:**201–209.
30. **Pilipenko, E. V., A. P. Glyml, S. V. Maslova, Y. V. Svitkin, A. N. Sinyakov, and V. I. Agol.** 1992. Prokaryotic-like cis elements in the cap-independent internal initiation of translation on picornavirus RNA. Cell **68:**119–131.
31. **Racaniello, V. R., and D. Baltimore.** 1981. Molecular cloning of poliovirus cDNA and determination of the complete nucleotide sequence of the viral genome. Proc. Natl. Acad. Sci. USA **78:**4887–4891.
32. **Rivera, V. M., J. D. Welsh, and J. V. Maizel.** 1988. Comparative sequence analysis of the 5′ noncoding region of the enteroviruses and rhinoviruses. Virology **165:**42–50.
33. **Rotbart, H. A.** 1990. Enzymatic RNA amplification of the enteroviruses. J. Clin. Microbiol. **28:**438–442.
34. **Ryan, M. D., O. Jenkins, P. J. Hughes, A. Brown, N. J. Knowles, D. Booth, P. D. Minor, and J. W. Almond.** 1990. The complete nucleotide sequence of enterovirus type 70: relationships with other members of the picornaviridae. J. Gen. Virol. **71:**2291–2299.
35. **Schiff, G. M., G. M. Stefanovic, E. C. Young, D. S. Sander, J. K. Pennekamp, and R. L. Ward.** 1984. Studies of echovirus-12 in volunteers: determination of minimal infectious dose and the effect of previous infection on infectious dose. J. Infect. Dis. **150:**858–866.
36. **Skinner, M. A., V. R. Racaniello, G. Dunn, J. Cooper, P. D. Minor, and J. W. Almond.** 1989. New model for the secondary structure of the 5′ non-coding RNA of poliovirus is supported by biochemical and genetic data that also show that RNA secondary structure is important in neurovirulence. J. Mol. Biol. **207:**379–392.
37. **Strikas, R. A., L. J. Anderson, and R. A. Parker.** 1986. Temporal and geographic patterns of isolates of nonpolio enterovirus in the United States, 1970–1983. J. Infect. Dis. **153:**346–351.
38. **Svitkin, Y. V., N. Cammack, P. D. Minor, and J. W. Almond.** 1990. Translation deficiency of the Sabin type 3 poliovirus genome: association with an attenuating mutation $C_{472} \rightarrow U$. Virology **17:**5103–109.
39. **Takeda, N.** 1989. Complete nucleotide and amino acid sequences of enterovirus 70, p. 419–424. *In* K. Ishii, Y. Uchida, K. Miyamara, S. Yamazaki (ed.), Acute hemorrhagic conjunctivitis etiology, epidemiology, and clinical manifestations. University of Tokyo Press, Tokyo.
40. **Toyoda, H., M. Kohara, Y. Kataoka, T. Suganuma, T. Omata, N. Imura, and A. Nomoto.** 1984. Complete nucleotide sequences of all three poliovirus serotype genomes: implication for genetic relationship, gene function and antigenic determinants. J. Mol. Biol. **174:**561–585.
41. **Trono, D., R. Andino, and D. Baltimore.** 1988. An RNA sequence of hundreds of nucleotides at the 5′ end of poliovirus RNA is involved in allowing viral protein synthesis. J. Virol. **62:**2291–2299.
42. **Westrop, G. D., K. A. Wareham, D. M. A. Evans, G. Dunn, P. D. Minor, D. I. Magrath, F. Taffs, S. Marsden, M. A. Skinner, G. C. Schild, and J. W. Almond.** 1898. Genetic basis of attenuation of the Sabin type 3 oral poliovirus vaccine. J. Virol. **63:**1338–1344.
43. **Yamashita, K., K. Miyamura, S. Yamadera, N. Kato, M. Akatsuka, S. Inouye, and S. Yamazaki.** 1992. Enteroviral aseptic meningitis in Japan, 1981–1991. Jpn. J. Med. Sci. Biol. **45:**151–161.