ELSEVIER

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The genome of echovirus 11

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Abstract

Echoviruses are the largest enterovirus subgroup consisting of 32 serotypes. They are common human pathogens causing, for example, meningitis, encephalitis and exanthema, but in spite of their clinical importance, relatively little is known about their biology. To illuminate the molecular characteristics of echoviruses, we have completed the genomic sequence of serotype 11. The RNA genome is 7438 nucleotides in length and it codes for a 2195 amino acid long polyprotein. When compared to other sequenced enteroviruses, echovirus 11 (EV11) shows remarkable similarity with coxsackie B viruses (CBVs) and coxsackievirus A9 (CAV9). On the basis of amino acid sequence homology in the capsid region, CAV9 is the virus most closely related to EV11. These two viruses have an apparent insertion sequence located at the C-terminus of the VP1 polypeptide. EV11, however, lacks the RGD motif found in the corresponding region of CAV9. The organization of the 5′ end noncoding region resembles that of other enteroviruses, but contains a 12 nucleotides long poly-U stretch not seen in any other enterovirus sequenced to date.

Keywords: Echovirus; Genome

Echoviruses (EVs) were discovered when the first tissue-culture techniques were introduced into laboratories. Not much was known about their association with human disease at that time and they were given the name Enteric Cytopathogenic Human Orphan (ECHO) viruses. EVs are now known to be a common cause of human disease, including meningitis, rash and febrile illness

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(Grist et al., 1978). Fatal disseminated infections in newborn infants have also been described. Echoviruses can only be isolated in cell cultures and they do not cause disease in newborn mice in contrast to coxsackieviruses.

Previous studies, carried out by nucleic acid hybridization, have suggested that most of the echoviruses are closely related to coxsackie B viruses (Auvinen and Hyypiä, 1990). However, serotypes 22 and 23 are only distantly related to enteroviruses and represent a genetically distinct picornavirus group (Hyypiä et al., 1992; Stanway et al., 1994). Recently, it was shown that the integrin VLA-2 is the cellular receptor for EV1 and EV8, but not for other echovirus serotypes (Bergelson et al., 1992, 1993). Although molecular cloning of EV6 has been described (Blackburn et al., 1991), no complete sequence of any representative of the major genetic echovirus subgroup has been available. We report here the sequence of the 5′ half of echovirus 11 genome which together with the previously published sequence data (Auvinen and Hyypiä, 1990) covers the complete genome.

Echovirus 11 (strain Gregory) was obtained from the American Type Culture Collection. Propagation of plaque-purified virus in LLC-Mk_2 cells, purification and RNA preparation were as described previously (Auvinen and Hyypiä, 1990). cDNA synthesis was carried out using the RNase H method (Gubler and Hoffman, 1983; Amersham) and the dC-tailed reaction product was cloned in *Pst* I-cleaved dG-tailed pBR322. Two cDNA clones, covering the 5′ untranslated region (5′UTR) and the capsid protein coding region were selected by colony hybridization using other cloned enterovirus cDNA fragments as probes. The inserts were subcloned in a Bluescript derived vector and sequenced by the dideoxynucleotide chain termination method after generation of deletions by the *Exo* III method. Part of the sequence was obtained directly using specific oligonucleotide primers. The region between the two cDNA clones was sequenced from a cDNA fragment generated by PCR. The sequence was determined on both strands. The part of the echovirus 11 genome coding for the nonstructural proteins and the 3′ untranslated region (3′UTR) has been reported earlier (Auvinen and Hyypiä, 1990).

Comparison of selected echovirus strains by spot hybridization was carried out as described earlier (Auvinen and Hyypiä, 1990). The probes included three cloned EV11 cDNA fragments (272, 193 and 157), two EV22 cDNA fragments (57 and 48; Hyypiä et al., 1992) and a cDNA fragment representing the 5′ untranslated region of the EV23 genome (188; Stanway et al., 1994).

The overall structure of the EV11 genome resembles that of other known enteroviruses (Fig. 1). The 5′ untranslated region (UTR) was predicted to be 751 nucleotides in length. The open reading frame codes for a 2195 amino acids long polyprotein and the predicted 3′ UTR is 102 nucleotides long (Auvinen and Hyypiä, 1990). The nucleotide composition of EV11 genome without the 3′ poly-A tail is A 28.1%, C 23.8%, G 24.4% and U 23.7%. Typically for enteroviruses A-residues slightly predominate.

The 5′ UTR structure of EV11 was similar to those of other enteroviruses. The degree of identity is highest when EV11 is compared to CBV4 (Fig. 2A). Highly conserved regions are observed throughout the 5′ UTR and a common stem-loop formation, with 9 nucleotides in the stem, is found near the 5′ terminus (Skinner et

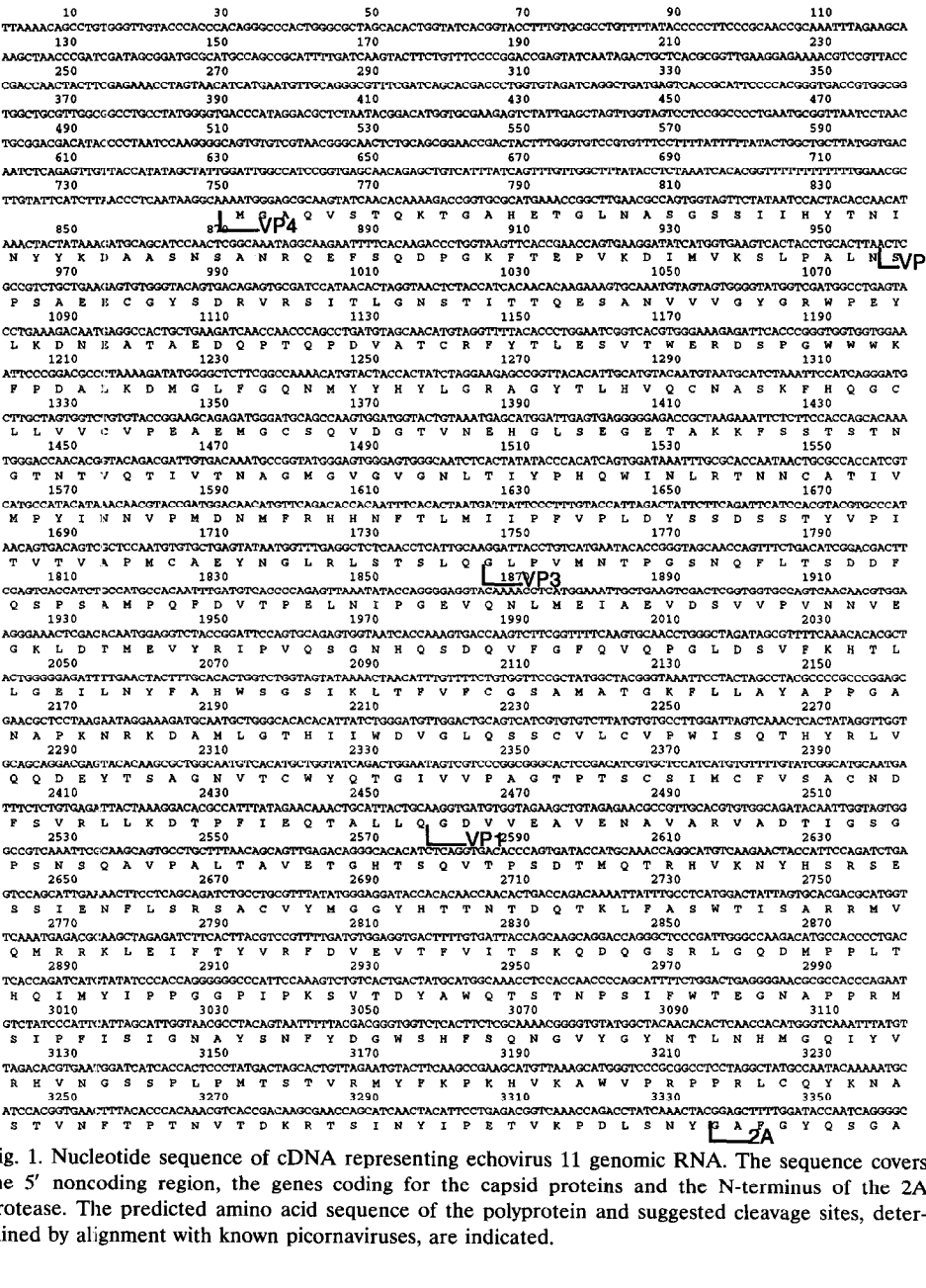

Fig. 1. Nucleotide sequence of cDNA representing echovirus 11 genomic RNA. The sequence covers the 5′ noncoding region, the genes coding for the capsid proteins and the N-terminus of the 2A protease. The predicted amino acid sequence of the polyprotein and suggested cleavage sites, determined by alignment with known picornaviruses, are indicated.

al., 1989). Several stretches are almost identical to the corresponding regions of CBV4. Another stem-loop structure, which may be involved in initiation of translation, is located between positions 592 and 621. Interestingly, the 5′ UTR of

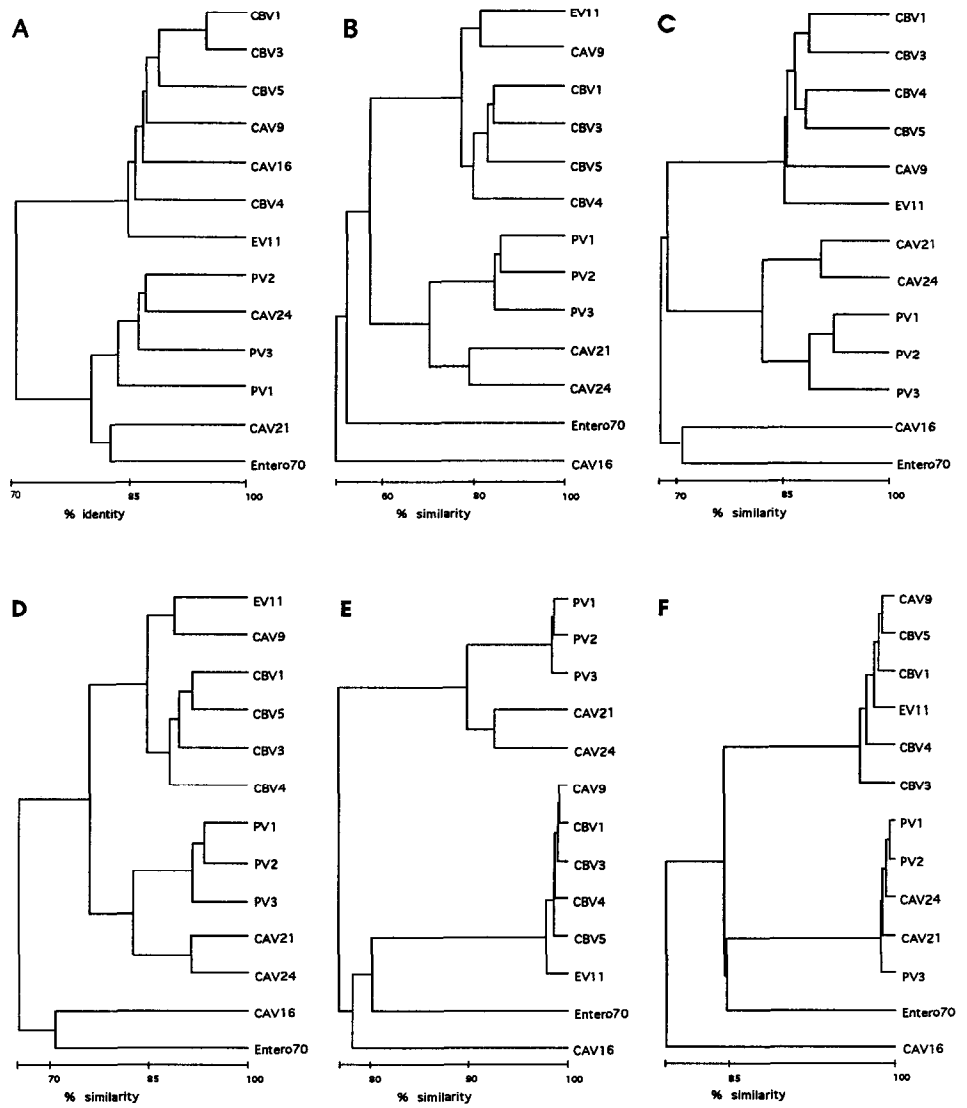

Fig. 2. Dendrograms illustrating relationships of echovirus 11 (EV11) with other sequenced enteroviruses. cDNA sequence of the 5′ untranslated region (A), and predicted amino acid sequences of VP1 (B), VP2 (C), VP3 (D), 2C (E) and 3D (F) are shown. CAV = coxsackie A virus; CBV = coxsackie B virus; PV = poliovirus; entero70 = enterovirus 70.

EV11 contains a 12 nucleotides long poly-U stretch not seen in any other sequenced enterovirus. The possible function of this structure located in the hypervariable region (positions 702–713, 39 nt from the initiation codon) is yet unknown. Pyrimidine-rich sequences close to the AUG have been observed in

several picornaviruses and there is some evidence that these regions are involved in the initiation of translation (Jang and Wimmer, 1990).

In poliovirus 1 all the polyprotein cleavage sites have been established by amino acid sequencing (Larsen et al., 1982; Pallansch et al., 1984). Alignment of amino acid sequences thus allows prediction of the cleavage sites of EV11. The enterovirus-encoded 3CD protease performs cleavages usually between Q/G amino acids in the capsid region. The predicted VP2/VP3 and VP3/VP1 boundaries of EV11 exhibit high conservation with other enteroviruses and these cleavages are suggested to take place at such sites (Fig. 1). The cleavage of VP0 is thought to occur at an N/S dipeptide by analogy with poliovirus. On the basis of alignment with poliovirus 1, the VP1/2A cleavage occurs at a Y/G. Another possible site is the adjacent F/G, located 3 amino acids downstream.

When the capsid region is compared with the corresponding regions of other enteroviruses, CAV9 appears to be the closest relative of EV11. The dendrograms in Fig. 2 show the relationships between capsid proteins of different human enteroviruses. In the cases of VP1, VP2 and VP3, EV11 is tightly clustered together with CAV9 and CBVs. The amino acid identity in this group is higher than 65%. The nonstructural proteins of EV11 are much more conserved than the structural proteins when compared with other enteroviruses (Fig. 2).

Translation starts at the 10th AUG codon (nt 752–754) which is in favourable Kozak context (AXXAUGG). The internal VP4 polypeptide is highly conserved. 54–94% of the amino acids in VP4 are identical with other sequenced enteroviruses. The VP1–3 proteins, which form the outer capsid, are more diverse. VP1 differs particularly in amino acid sequence, and contains an approximately 8 amino acids long insertion sequence at the C-terminus. This insertion, however, does not contain the arginine-glycine-aspartic acid tripeptide (RGD) located in a similar insertion in CAV9 and known to be responsible for receptor binding (Chang et al., 1989; Roivanen et al., 1991). The EV11 insertion sequence shows no homology with other sequenced enteroviruses.

EV11 and CAV9 are the only enteroviruses known to have an apparent insertion to the C-terminus of the VP1 polypeptide. It has also been reported that these two viruses compete for cellular receptor (Mbida et al., 1991, 1992). This cannot be explained by identical molecular mechanisms since the EV11 VP1 insertion does not contain an RGD motif known to be crucial for CAV9 receptor interaction (Roivanen et al., 1994). It is known, however, that trypsin treatment cleaves the CAV9 VP1 terminus but the virus still retains infectivity, suggesting that an alternative receptor may also exist. This could be a molecule which can act as a receptor for both EV11 and CAV9.

When the SwissProt database was searched for protein sequences similar to that of the EV11 insertion (VKPDLSNY) a four amino acids long identical motif (DLSN; Fig. 3) was found in measles virus hemagglutinin, a protein responsible for attaching the virus to the cell receptor and for initiating the infection. Studies on the functional significance of this motif are underway.

Echoviruses represent the largest enterovirus subgroup consisting of 30 serotypes in the major genetic group, while there are only 3 poliovirus and 6 coxsackie B

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                                                                VP1/2A
CBV1  KAWVPRPPRLCQYEKQKNVNFNPTGVTTTRSNIT...TT.................GAFGQQSGAVYVGNY
CBV3  KAWIPRPPRLCQYEKAKNVNFQPSGVTTTRQSITTMTNT................GAIWTTIRGSVCGDY
CBV4  KAYVPRPPRLCQYKKAKSVNFDVEAVTAERASLI...TT.................GPYGHQSGAVYVGNY
CBV5  KTWVPRPPRLCQYQKAGNVNFEPTGVTESRTEITAMQTT................GVLGQQTGAICIGNY
CAV9  RAWVPRPPRLCQYKKAFSVDFTPTPITDTRKDINTV.TTVAQSRRRGDMSTLNTHGAFGQQSGAVYVGNY
EV11  KAWVPRPPRLCQYKNASTVNFTPTNVTDKRTSINYIPETVKPDLSNY.........GAFGYQSGAVYVVNY
MV                                    HMTNYFEQPVSNDLSNCMV
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Fig. 3. Alignment of the VP1/2A polypeptide boundary with the closest relatives of echovirus 11 (EV11). The functional RGD in CAV9 and the identical amino acids between EV11 and measles virus (MV) hemagglutinin are shown in bold. CAV = coxsackie A virus; CBV = coxsackie B virus.

virus serotypes. The explanation for this diversity could be structural differences in the antigenically important regions. Alignment of the capsid proteins with other enteroviruses did not, however, reveal any clear explanation for this phenomenon because the primary structure of the EV11 polypeptides closely resembles that of CAV9 and CBVs. Therefore localization of the epitopes in echoviruses and detailed immunological studies will be needed to elucidate the mechanisms of high antigenic diversity of EVs.

To analyse genetic relationships with selected members of the echovirus group, a hybridization analysis using cDNA probes from different regions of the EV11, EV22 and EV23 genomes was carried out (Fig. 4). A probe including the conserved 5′UTR of EV11 (EV11/272) gave a signal with serotypes 1, 6, 9, 11, 17, 24 and 30, but not with EV22 and EV23. Only a weak signal was observed with EV16. On the other hand, probes representing the capsid region (EV11/193) and the

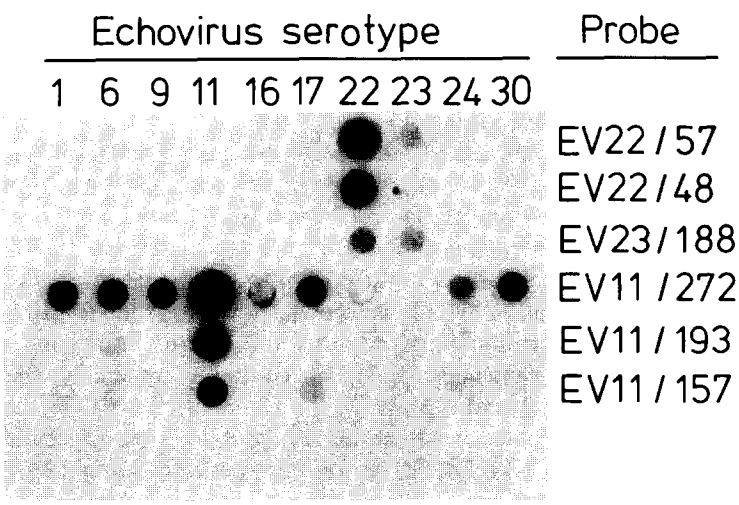

Fig. 4. Hybridization analysis of selected echovirus serotypes with a collection of cDNA probes. Probe 57 represents the 5′ half and 48 the 3′ half of the echovirus 22 genome, while 188 is from the 5′ untranslated (5′ UTR) region of the echovirus 23 genome. Echovirus 11 probe 272 covers the 5′ UTR and most of the capsid region, 193 part of the capsid region and 157 part of the nonstructural region in the genome.

nonstructural region (EV11/157) of echovirus 11 were more selective in their reactivity among the major EV group. The EV22/57 probe (the 5′ half of the genome) detected serotypes 22 and 23, while the EV22/48 probe (3′ half of the genome) reacted only with the homologous virus. Cross-reactivity between EV22 and EV23 was seen when a probe covering the 5′ UTR of EV23 (EV23/188) was used.

In conclusion, our results support the idea that a vast majority of echoviruses, possibly the whole genetic group consisting of more than 30 members, are close relatives to CBVs and CAV9. However, the replication and pathogenic properties of these virus groups differ in many important aspects, including receptor binding and antigenic diversity. Further studies on all these important determinants will be needed to elucidate the detailed characteristics of the echovirus group.

The sequence data have been submitted to the EMBL database with the accession number: x80059 (EV11 complete genome).

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References

Auvinen, P. and Hyypiä, T. (1990) Echoviruses include genetically distinct serotypes. J. Gen. Virol. 71, 2133–2139.

Bergelson, J.M., Shepley, M.P., Chan, B.M.C., Hemler, M.E. and Finberg, R.W. (1992) Identification of the integrin VLA-2 as a receptor for echovirus 1. Science 255, 1718–1720.

Bergelson, J.M., St.John, N., Kawaguchi, S., Chan, M., Stubdal, H., Modlin, J. and Finberg, R.W. (1993) Infection by echoviruses 1 and 8 depends on the α_2 subunit of human VLA-2. J. Virol. 67, 6847–6852.

Blackburn, R.V., Racaniello, V.R. and Righthand, V.F. (1991) Construction of an infectious cDNA clone of echovirus 6. Virus Res. 22, 71–78.

Chang, K.H., Auvinen, P., Hyypiä, T. and Stanway, G. (1989) The nucleotide sequence of coxsackievirus A9—implications for receptor binding and enterovirus classification. J. Gen. Virol. 70, 3269–3280.

Grist, N.R., Bell, E.J. and Assaad, F. (1978) Enteroviruses in human disease. Prog. Med. Virol. 24, 114–157.

Gubler, U. and Hoffman, B.J. (1983) A simple and very efficient method for generating cDNA libraries. Gene 25, 263–269.

Hyypiä, T., Horsnell, C., Maaronen, M., Khan, M., Kalkkinen, N., Auvinen, P., Kinnunen, L. and Stanway, G. (1992) A distinct picornavirus group identified by sequence analysis. Proc. Natl. Acad. Sci. USA 89, 8847–8851.

Jang, S.K. and Wimmer, E. (1990) Cap-independent translation of encephalomyocarditis virus RNA: structural elements of the internal ribosomal entry site and involvement of a cellular 57-kD RNA-binding protein. Genes Dev. 4, 1560–1572.

Larsen, G.R., Anderson, C.W., Dorner, A.J., Semler, B.L. and Wimmer, E. (1982) Cleavage sites within the poliovirus capsid protein precursors. J. Virol. 41, 340–344.

Mbida, A.D., Pozzetto, B., Grattard, F. and Gaudin, O.G. (1992) Protective effect of a monoclonal antibody specific for an echovirus cellular receptor in human fibroblast and simian kidney cell lines. Res. Virol. 143, 397–400.

Mbida, A.D., Pozzetto, B., Sabido, O., Akono, Y., Grattard, F., Habib, M. and Gaudin, O.G. (1991) Competition binding studies with biotinylated echovirus 11 in cytofluorimetry analysis. J. Virol. Meth. 35, 169–176.

Pallansch, M.A., Kew, O.M., Semler, B.L., Omilianowski, D.R., Anderson, C.W., Wimmer, E. and Rueckert, R.R. (1984) Protein processing map of poliovirus. J. Virol. 49, 873–880.

Roivainen, M., Hyypiä, T., Piirainen, L., Kalkkinen, N., Stanway, G. and Hovi, T. (1991) RGD-dependent entry of coxsackievirus A9 to host cells and its bypass after cleavage of VP1 by intestinal proteases. J. Virol. 65, 4735–4740.

Roivanen, M., Piirainen, L., Hovi, T., Virtanen, I., Riikonen, T., Heino, J. and Hyypiä, T. (1994) Entry of coxsackievirus A9 into host cells: specific interactions with $\alpha_v\beta_3$ integrin, the vitronectin receptor. Virology 203, 357–365.

Skinner, M.A., Racaniello, V.R., Dunn, G., Cooper, J., Minor, P.D. and Almond, J.W. (1989) New model for the secondary structure of the 5′ noncoding 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.

Stanway, G., Kalkkinen, N., Roivainen, M., Ghazi, F., Khan, M., Smyth, M., Meurman, O. and Hyypiä, T. (1994) Molecular and biological characteristics of echovirus 22, a representative of a new picornavirus group. J. Virol. 68, 8232–8238.