Intratypic genome variability of the coxsackievirus B1 2A protease region

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To analyse the intratypic genome variability of coxsackievirus B1, 17 coxsackievirus B1 isolates were collected over a period of 10 years. Nucleotide sequences of the 2A coding region of the various coxsackievirus B1 isolates and known sequences of other enteroviruses were compared. The maximum diversity observed within the entire group of coxsackievirus B1 isolates was 25%. Comparison of deduced amino acid sequences revealed a maximum diversity of 5%. Phylogenetic analysis demonstrates a close genetic relationship between these clinical isolates and the other different coxsackievirus B serotypes. Further analysis of nucleotide and amino acid sequences of the 2A region of known enteroviruses demonstrated that the genus enterovirus can be sub-

divided into a coxsackievirus B-like group, including the coxsackie B viruses, coxsackievirus A9, echovirus 11 and swine vesicular disease virus, and a poliovirus-like group including the polioviruses 1 to 3, and the coxsackieviruses A21 and A24. Enterovirus type 70 and bovine enterovirus represent distinct groups. The 2A coding region of coxsackievirus B1 strains cannot be distinguished from that of other members of the coxsackievirus B-like group. Although conservation of the overall amino acid sequence was almost limited to residues essential for proposed enzymatic activity, the predicted secondary structures were well conserved within the genus enterovirus.

Coxsackievirus B1 is a member of the genus enterovirus which belongs to the Picornaviridae. The genome consists of a single, positive-strand RNA molecule of approximately 7500 nucleotides, and is polyadenylated at the 3' end. The RNA molecule is translated into a single polyprotein which is cleaved by virus-encoded proteases into several capsid and non-structural proteins. The virus-encoded RNA-dependent RNA polymerase catalyses the synthesis of both minus and plus RNA strands from their complementary RNA templates (Rueckert, 1990). Owing to the absence of a proofreading mechanism during RNA synthesis, high mutation frequencies occur during replication. Although the extent of variation will be limited by biological pressure, a rapid evolution of RNA viruses is found (Holland *et al.*, 1982). Recombination events will further increase the genetic divergency (Jarvis & Kirkegaard, 1991; Lai, 1992).

The most variable region of the genome is located in the part that codes for the capsid proteins. Hypervariability of antigenic sites on the virion may provide a survival mechanism for the virus. This variability may also result in altered phenotypes with respect to tropism (Pevear *et al.*, 1988). Using oligonucleotide mapping Miyamura *et al.* (1990) estimated an evolutionary rate of coxsackievirus A24 of 0.4% per year. Nucleotide sequencing of the 5' non-translated region (NTR) and the antigenic sites of poliovirus type 3 isolated during a

poliomyelitis outbreak revealed an overall frequency of variable positions of 4.3% within the 5' NTR and 6.8% for the antigenic sites (Kinnunen *et al.*, 1991).

In enteroviruses the first proteolytic event during polyprotein processing is the cotranslational cleavage at the 1D/2A junction, mediated by the 2A protease. Further polyprotein processing is catalysed by the 3C protease or its precursor 3CD. Besides this autocatalytic activity, the enterovirus 2A protease is thought to be responsible for inhibition of protein synthesis of the infected host cell, due to its inhibitory effect on transcription and indirectly on degradation of the p220 component of eIF-4F (Davies *et al.*, 1991; Kräusslich *et al.*, 1987). Alterations within parts of the genome coding for non-structural proteins such as 2A, interfering with host cell-regulated processes, seem to be involved at the level of attenuation, adaptation and pathogenicity of the virus (Bandyopadhyay *et al.*, 1993; Emerson *et al.*, 1993; Lomax & Yin, 1989).

In order to gain more insight into the variability within a single serotype during a longer period of time, we investigated the heterogeneity of the 2A coding region of coxsackievirus B1. Results were compared with known sequences of other enteroviruses.

Several methods, such as nucleotide sequence analysis (Bae *et al.*, 1989), restriction length polymorphism of cDNA (Chumakov *et al.*, 1992) and oligonucleotide

mapping (Miyamura *et al.*, 1990), have been used to study the genomic differences between several picornavirus genera or within a population of viruses belonging to a single serotype. In this study we used enzymatic amplification with a number of oligonucleotides targeted to sequences within the P2 region followed by sequence analysis.

Sixteen coxsackievirus B1 isolates, and a myotropic coxsackievirus B1 variant (Tucson strain), were used to determine the variability of the 2A protease region at both nucleotide and amino acid level. Clinical isolates of coxsackievirus B1 were obtained from the National Institute of Public Health and Environmental Protection, Bilthoven, The Netherlands. The myotropic variant of coxsackievirus B1 was obtained from the University of Arizona, Tucson, U.S.A. (Ray *et al.*, 1979). Viruses were propagated in Buffalo green monkey kidney cells. After reaching complete cytopathic effect the cultures were frozen and thawed three times. Debris was removed by centrifugation at 1000 *g* for 15 min at room temperature. RNA was extracted by SDS-proteinase K treatment followed by phenol-chloroform extraction and ethanol precipitation (Sambrook *et al.*, 1989). Purified RNA was collected in 100 µl diethylpyrocarbonate-treated distilled water which contained 0.4 units (U)/µl human placenta ribonuclease inhibitor (RNasin, Promega).

Oligonucleotide sequences were selected from the P2 region according to the published sequence of coxsackievirus B1 (Iizuka *et al.*, 1987). Oligonucleotides were synthesized in a DNA synthesizer (380A; Applied Biosystems) by the methoxyphosphoramidite method.

The complete 2A gene (450 bp) was amplified by PCR. cDNA was synthesized and amplified as previously described (Zoll *et al.*, 1992) in a 20 µl reaction mixture containing 75 mM-KCl, 50 mM-Tris-HCl pH 8.3, 3 mM-MgCl₂, 10 mM-DTT, 0.2 mM (each) dNTPs (Boehringer Mannheim), 50 pmol of the downstream primer (5' CGGGCTCCATTGCATCATCTTC 3', nucleotide positions 3715 to 3736), 5 U of avian myeloblastosis virus reverse transcriptase (Promega), and RNA. After incubation at 37 °C for 60 min, 80 µl of the PCR mixture was added. This contained 50 mM-KCl, 10 mM-Tris-HCl pH 8.9, 3.6 mM-MgCl₂, 0.2 mM (each) dNTPs, 100 µg/ml BSA, 80 pmol of the downstream primer, 40 pmol of the upstream primer (5' GGCGCTTTTGGACAACAGTC-AGGGGC 3', nucleotide positions 3286 to 3311), and 0.2 U of SuperTaq DNA polymerase (HT Biotechnology). RNA-cDNA hybrids were denatured at 94 °C for 5 min. The amplification was performed in 30 cycles consisting of denaturation for 1 min at 94 °C, primer annealing for 1 min at 42 °C and elongation for 2 min at 72 °C. Amplification products were analysed by electrophoresis in 1.5% agarose gels. For the purpose of

sequencing, the amplification products were purified from low melting point agarose with the Prep-a-Gene purification system (Bio-Rad) according to the instructions of the manufacturer. DNA sequencing was performed by the dideoxynucleotide method (Sanger *et al.*, 1977). Annealing of a primer to the template was performed as follows. Double-stranded PCR products were heat-denatured in the presence of a primer and immediately frozen in a CO₂-cooled ethanol bath. The 5' and 3' oligonucleotides that were used for reverse transcription and PCR respectively served as sequencing primers. The sequences obtained with these primers were aligned in order to select an additional set of primers which were used for sequence analysis of the 2A encoding region at the 5' (5' CTCGATGCACAGAAGTACAC 3', nucleotide positions 3469 to 3488) and 3' (5' TACTAT-CCCAAGAGATACCA 3', nucleotide positions 3550 to 3569) ends.

Sequence analysis and comparisons were made using the UWGCG sequence analysis software package on a VAX computer. Phylogenetic studies were performed on enterovirus sequences available in the EMBL database. The nucleotide sequences of the 450 bp PCR fragment which were obtained from the various clinical isolates were subjected to pairwise comparison using the UPGMA program.

The genetic relationships between the clinical isolates is shown in Fig. 1. As can be seen in the dendrogram, a number of closely related clinical isolates can be distinguished. Isolates obtained within a period of 12 months (from September 1983 to August 1984) differ at most by 4%. Two closely related isolates were obtained from surface water (6 July 1981) and, 4 months later, from a clinical specimen (13 November 1981) respectively. Two isolates were from immigrant children from Israel (23 July 1978) and Colombia (1 April 1986). These isolates and the myotropic coxsackievirus B1 strain Tucson (Ray *et al.*, 1979) contained the most divergent sequences. A variability at nucleotide level of up to 25% was observed between these isolates.

A maximum diversity of 5% was calculated from the various deduced amino acid sequences of the clinical isolates, including the coxsackievirus B1 Tucson strain and the reference sequence according to Iizuka *et al.* (1987). Obviously, most of the observed nucleotide mutations are silent. It has been shown for foot-and-mouth disease virus populations that the evolution of the capsid genes within a virus population is also associated with an accumulation of silent mutations. Variability at the amino acid level, however, was restricted to a limited combination of amino acid residues (Martínez *et al.*, 1992).

Since the correct genetic relationship between the various clinical isolates is unknown, it is not possible to

Fig. 1. Dendrogram composed by UPGMA, based on nucleotide sequences of the 2A coding region of coxsackievirus B1 isolates and other known coxsackie-B-like enteroviruses. The branches of the various coxsackievirus B1 isolates are indicated by the date of isolation. CAV9: coxsackievirus A9 (Chang *et al.*, 1989); CBV3: coxsackievirus B3 (Klump *et al.*, 1990); CBV4: coxsackievirus B4 (Jenkins *et al.*, 1987); CBV5: coxsackievirus B5 (Zhang *et al.*, 1993); ECHO11: echovirus type 11 (Auvinen & Hyypiä, 1990); SVDV: swine vesicular disease virus (Inoue *et al.*, 1989).

Fig. 2. Dendrogram based on nucleotide sequences of enterovirus serotypes. PV1 to -3: poliovirus types 1, 2 and 3 (Toyoda *et al.*, 1984); CAV21: coxsackievirus A21 (Hughes *et al.*, 1989); CAV24: coxsackievirus A24 (Supanaranond *et al.*, 1992); BEV: bovine enterovirus (Earle *et al.*, 1988); EV70: enterovirus type 70 (Ryan *et al.*, 1990). Other enterovirus sequences are the same as specified in Fig. 1.

Fig. 3. Dendrogram based on deduced enterovirus 2A amino acid sequences as shown in Fig. 4. Abbreviations as in Fig. 1 and 2.

make a reliable estimation of the mutational rate for the 2A coding region of coxsackievirus B1 from these data. Hughes *et al.* (1993) found a variability of, at most, 14% within a group of coxsackievirus B4 isolates from aquatic samples and clinical specimens collected in Ireland within a period of 3 years. Kinnunen *et al.* (1990) studied the molecular evolution of poliovirus type 3 during infection in individual hosts. Differences of 0.8% were determined between isolates from one person, whereas differences of up to 2% were observed between isolates from different persons during the same poliovirus outbreak in 1984 in Finland. The observations of Kinnunen *et al.* (1990) are in accordance with our results obtained with the related clinical isolates from 1983 and 1984. At a molecular

evolutionary rate of 2 to 4% per year, the maximum diversity of 25% observed in our study will be reached within 10 years.

The nucleotide sequences of the various clinical isolates and those of the coxsackievirus B-like group have also been compared as indicated in Fig. 1. Fourteen clinical isolates are more related to the coxsackievirus B1 reference strain (Iizuka *et al.*, 1987) than to the other coxsackievirus B serotypes. The overall variability between the reference strain and these 14 isolates is 22%. Echovirus type 11 (Auvinen & Hyypiä, 1990) however, appeared to be more related to the coxsackievirus B1 reference strain than to any of the collected clinical isolates. Although all isolates are serotyped as coxsackie-

	1	75
CBV1	GAFGQQSGAVYVGNYRVVNRELATREDWQRCVWEDYNRDLLVSTTTAAGCDIIARCQCTTGUVYFCASRNKHYPVS	
Tucson	F.....HT..LN.....T.....A.....S.....	
23 July 1978	I.....HN..N.....T.....K.....IT	
6 July 1981	I.....HS..N.....T.....	
13 November 1981	I.....HS..N..G..H.....G.....T.....	
4 January 1982	I.....HS..N.....I..B..QV..HT.....K.....A	
18 September 1983	L..Y.....R..L.....T	
16 December 1983	L..Y.....R..L.....T	
24 April 1984	NSL..Y.....Q..L.....Y.....T	
1 June 1984	NSL..Y.....Q..F.....T	
5 June 1984	L..Y.....Q..L.....T	
24 June 1984	L..Y.....Q..L.....T	
9 July 1984	L..Y.....Q..L.....L.....T	
20 July 1984	L..Y.....I..N..Q..L.....T	
29 July 1984	L..Y.....Q..L.....T	
6 August 1984	L..Y.....Q..L.....T	
1 April 1986	HS..N.....S.....K.....V	
5 December 1988	I.....T.....R..N.....Q.....T	
CAV9	I.....HT..N.....V.....K.....	
CBV3	SA..N..S.....K.....I.	
CBV4	..PY..H.....K.....HV..N.....T.....KS.....	
ECHO11	...Y.....V.....HT..N.....I.....V...R..S...Y..Q..KG...N	
SVDV	A..N.....D.....T.....D..A.....T	
EV70	-----GAF..S.KII.Y...D.EKE.S.YV.WQS.V..T.VA...KHQ...R..N...Y.KHK.RS..C	
BEV	..P...Q..A...S.KIL.....YA..ENE..QS.Q...TRVD...T...N.RS.I.Y.K.TA...IV	
PV1	...H.NK...TAG.KIC.Y...Q..L.NA.NVMW.....TESR.Q.T.S...N.NA...Y.E..R.Y...	
PV2	...H.NK...TAG.KIC.Y...Q..L.NA.NIMWI.....VESK.Q.I.S...N.H...Y.E..R.Y...	
PV3	...H.NK...TAG.KIC.Y...K..L.NT.SIMW.....VESK.Q.T.S...N.NA...Y.E..R.Y...	
CAV21	...H.NK...AG.KIC.Y...PS.HLNAISVLWD...M.VESR.Q.T.T...S.RC...Y.E..R.Y..L.T	
CAV24	...H.NM...AG.KIC.Y...P..HDNA.RVLW...MIVSSR.Q.S.T...N.R...Y.K.MK.Y...T	
	76	150
CBV1	FEFGPLVEVQSEYYPKRYQSEVLLAAGFSEPGDCGGILRCEHGVVGIIVTMGGEGVVGAFADVRDLLWLWLEDDAMEQ	
Tucson	I..L.....A..I.....	
23 July 1978	S.....V..Q..I.....	
6 July 1981	C.....I..V.....	
13 November 1981	N.....I..V.....	
4 January 1982	I.....	
18 September 1983	...M.....I.....E.....	
16 December 1983	...M.....I.....E.....	
24 April 1984	...M.....C.....I.....E.....	
1 June 1984	...M.....R.....C.....I.....E.....	
5 June 1984	...M.....D.....I.....E.....	
24 June 1984	..D...M...E.....K.....I.....E.....	
9 July 1984	...M.....I.....E.....	
20 July 1984	...M.....I.....E.....	
29 July 1984	...M.....TAG.KIC.Y...S.....I.....E.....	
6 August 1984	...I..R.....I.....R..P...E.....	
1 April 1986	...A.....C..I.....LS..I.....	
5 December 1988	...M.....L..T.....I.....	
CAV9	I.....	
CBV3	R.....I.....I.....	
CBV4	T.....I..L.....	
ECHO11	I.....	
SVDV	KH.....A.....Q..I.....	
EV70	...IQWIN..D...A...TNT...M.PCQ...L.V.S...I..L..A...I..A..T..I..N...	
BEV	VTP.SIYKIEAND...E.M.T.I..GI..A.....L.....M..L.V..GDH.....I.....	
PV1	..V...TFQYMEANN...A...M.IGH..AS.....H..I..I..A...L..A..T..I...YAY.EE...	
PV2	..T...TFQYMEAN...A...M.IGH..AS.....Q..I..I..A...L..A..S..I...YAY.EE...	
PV3	..V...TFQYMEAND...A...M.IGH..AS.....Q..I..I..A...L..A..S..I...YAY.EE...	
CAV21	..T...TFRPFMEAND...A...M.IGC..A.....T...I..I..A...I..A...I...WVY.EE...	
CAV24	VTE.TFQYMEAND...A...T.M..GH..A.....N..M...A..N..I..A...I...WVY.EE...	

Fig. 4. Deduced amino acid sequences of the 2A region of the various isolates and other enteroviruses. The sequences of the various isolates are indicated by the date of isolation. Other enteroviruses are abbreviated as in Fig. 1 and 2. The complete amino acid sequence of the reference coxsackievirus B1 sequence (Iizuka *et al.*, 1987) is given. Amino acid residues in the sequences of the isolates and other enteroviruses are indicated by '.' for corresponding residues; mutated residues are indicated by their corresponding letter; deletions are depicted '-'.

virus B1 strains, the 2A coding sequences of the most divergent coxsackievirus B1 strains were not more similar to those of the coxsackieviruses B3, B4 and A9 than to the reference coxsackievirus B1. Hence, by sequencing the 2A coding region it will not be possible to distinguish between the various coxsackievirus B-like serotypes.

The genetic relationship of the 2A coding regions of other enteroviruses is shown in Fig. 2. Four enterovirus subclasses can be distinguished, namely a coxsackievirus B-like group, a poliovirus-like group, and two distinct enteroviruses: enterovirus type 70 and bovine enterovirus. The relationship between the enteroviruses was also calculated for the known sequences of the 2C and 3D coding regions and revealed the same grouping of the enteroviruses (data not shown). Calculated genetic relationships based on the deduced 2A amino acid sequences (Fig. 3) demonstrate that an almost identical classification can be achieved. Variability at the amino acid level between the various members of the coxsackievirus B-like group does not exceed 5%, which is equal to the variability found within the group of coxsackievirus B1 isolates.

Recently, Zhang *et al.* (1993) described the sequence of coxsackievirus B5 and its relationship to other enteroviruses. They also indicated that, based on available sequences, enteroviruses can be subdivided into these four groups.

The 2A proteases of enteroviruses and rhinoviruses can be classified as cysteine proteases, based on their active site residues His₂₁, Asp₃₉ and Cys₁₁₀ (positions refer to the coxsackievirus B1 deduced amino acid sequence). In spite of the presence of the Cys residue in the active site of the enzyme, alignment studies revealed similarities between the poliovirus 2A protease and small trypsin-like serine proteases of bacterial origin (Bazan & Fletterick, 1988). Because of the similarities in function and amino acid sequences, it is proposed that the viral Cys proteases and the small trypsin-like proteases will have comparable secondary and tertiary structures (Bazan & Fletterick, 1988; Neurath, 1984; Yu & Lloyd, 1990). As a consequence, the secondary structure of the 2A protein is assumed to be conserved among all members of the genus enterovirus. Fig. 4 demonstrates that the amino acid residues His₂₁, Asp₃₉ and Cys₁₁₀ involved in catalysis are indeed conserved. Conservation of secondary structures of the 2A proteins was determined using the program PHD (Rost & Sander, 1992) with representatives from each enterovirus subgroup (data not shown). Comparison of the predicted secondary structures of the 2A proteins from coxsackievirus B1, poliovirus type 1, enterovirus type 70 and bovine enterovirus reveals the conservation among the genus enterovirus of all β -strands and the C-terminal α -helix, as described by Bazan & Fletterick (1988).

In conclusion, the 2A coding region of coxsackievirus B1 strains cannot be distinguished from other members of the coxsackievirus B-like group. The intratypic variability of coxsackievirus B1 is not reflected in the amino acid sequence. Use of intratypic nucleotide variability determination can be valuable for molecular epidemiological studies.

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