

## Functional features of the bovine enterovirus 5'-non-translated region

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The bovine enterovirus (BEV) serotypes exhibit unique features of the non-translated regions (NTRs) which separate them from the other enteroviruses. Their most remarkable property is an additional genome region of 110 nt located between the 5'-cloverleaf and the internal ribosome entry site (IRES). This genome region has the potential to form an additional cloverleaf structure (domain I\*) separated from the 5'-cloverleaf (domain I) by a small stem-loop (domain I\*\*). Other characteristics involve the putative IRES domains III and VI. In order to investigate the features of the 5'-NTR, several full-length coxsackievirus B3 (CVB3) cDNA plasmids with hybrid 5'-NTRs were engineered. After exchange of the CVB3 cloverleaf with the BEV1 genome region representing both cloverleaves, a viable virus chimera was generated. Deletion of domain I\*\* within the exchanged region also yielded viable virus albeit with reduced growth capacity. Deletion of sequences encoding either the first or the second BEV cloverleaf resulted in non-infectious constructs. Hybrid plasmids with exchanges of the IRES-encoding sequence or the complete 5'-NTR were non-infectious. Transfection experiments with SP6 transcripts containing 5'-NTRs fused to the luciferase message indicated that IRES-driven translation is enhanced by the presence of the CVB3 cloverleaf and both BEV1 cloverleaf structures, respectively. Deletion of either the first or the second BEV cloverleaf domain reduced but did not abolish enhanced luciferase expression. These results suggest that the substitution of two putative BEV cloverleaf structures for the putative coxsackieviral cloverleaf yields viable virus, while BEV sequences encoding the IRES fail to functionally replace CVB3 IRES-encoding sequences.

### Introduction

Viruses of the family *Picornaviridae* are presently divided into the six genera *Enterovirus*, *Rhinovirus*, *Cardiovirus*, *Aphthovirus*, *Hepatovirus* and *Parechovirus* (Pringle *et al.*, 1996). Among these viruses, the enteroviruses and the human rhinoviruses share significant molecular similarities, e.g. identical genome organization and a considerably high degree of sequence homology of viral proteins. However, the physico-chemical properties of these viruses differ significantly. The rhinoviruses have a higher buoyant density in caesium chloride and are more acid-labile and thermolabile when compared to

the enteroviruses. The virion properties of the former viruses are reflected in different optimal growth temperatures, routes of infection and pathogenicity of the respective viruses and are the main reason for the classification of different genera. Previous attempts to subclassify the numerous enterovirus and rhinovirus species by biological criteria were initially based on the observed differences of cytotoxicity and pathogenicity in man, various animal models and cultured tissue cells. Recently, the serotypes of both genera were grouped into several virus clusters by means of comparative sequence analyses of more than 90 virus strains (Horsnell *et al.*, 1995; Pöyry *et al.*, 1996; Hyypiä *et al.*, 1997; Zell & Stelzner, 1997). This phylogenetic approach disregards the previous affiliation of several serotypes to certain biological virus groups. For example, one phylogenetic cluster comprises the six coxsackieviruses of

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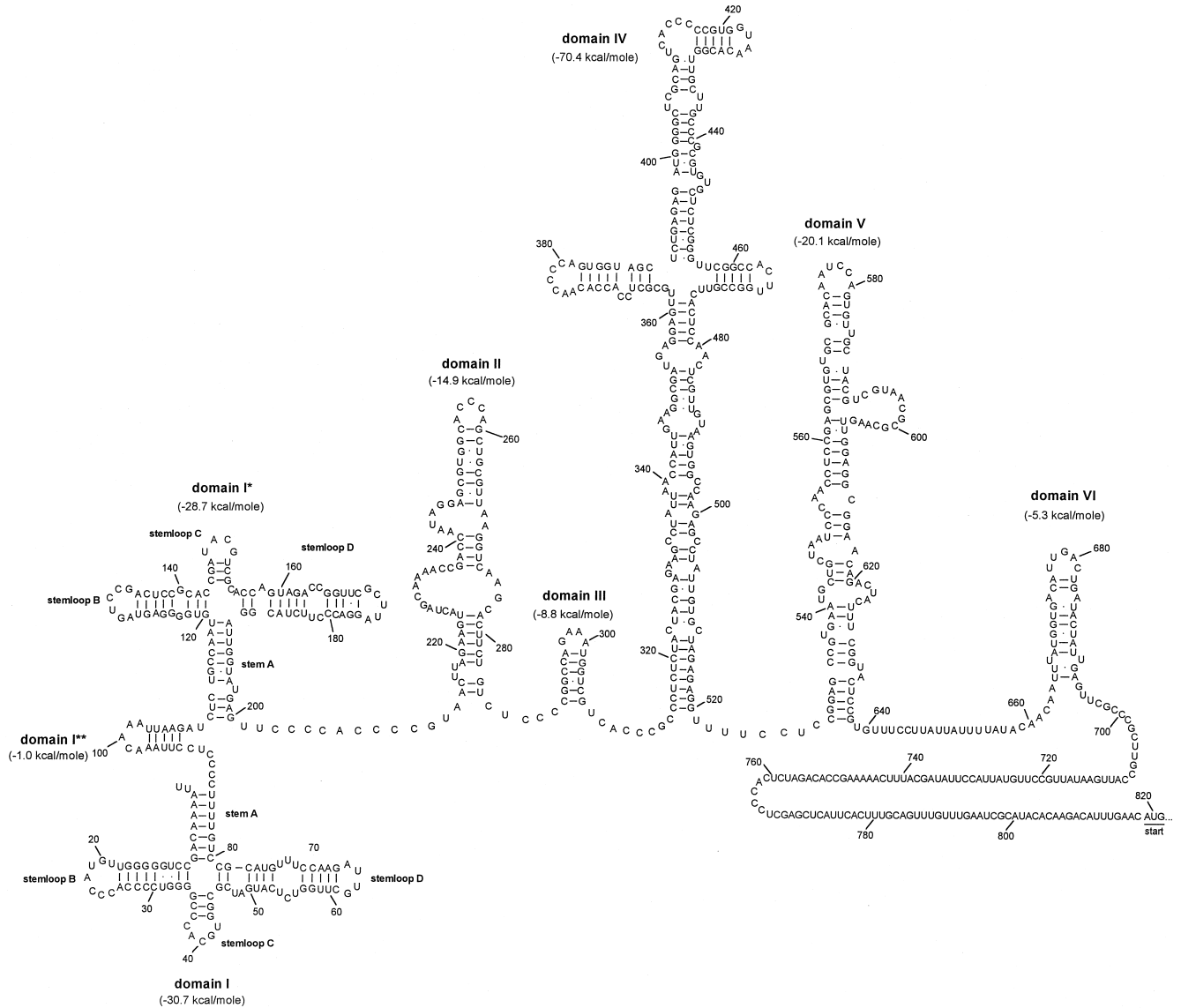


Fig. 1. Predicted secondary structures of the 5'-NTR of BEV1 (strain VG 527). This figure shows secondary structures based on (i) predictions by the mfold program (version 3.0) of Zuker *et al.* (1999), (ii) the prediction of the PV3 5'-NTR of Skinner *et al.* (1989), and (iii) phylogenetic sequence comparisons (Zell & Stelzner, 1997). An additional genome region of about 110 nt (nt 90–200) is unique for the BEVs. This insertion has the potential to form a second cloverleaf structure (domain I\*) which is separated from the putative 5'-cloverleaf (domain I) by a small stem-loop (domain I\*\*). Domains III and VI exhibit significant differences to the human enteroviruses (see Fig. 2). Stem-loop D of both cloverleaf structures resembles the 3CD<sup>PRO</sup>-binding domain of PV. The calculated free energy of each domain is indicated. Since phylogenetic conservation of the putative secondary structures had high priority, no attempt has been made to minimize the energy of the overall structure.

group B, all echovirus serotypes (with the exception of echovirus 22 and 23, which are now classified as parechovirus 1 and 2), coxsackievirus A9 and the human enterovirus 69; likewise, the coxsackieviruses of group A are considered to belong to three distinct enterovirus clusters.

Phylogenetic studies also suggest that the 5'-non-translated region (NTR) of all enteroviruses and human rhinoviruses contains highly conserved RNA secondary structures. So far, the existence of such secondary structures was proven only for a few representatives of both genera, e.g. the polioviruses (PV)

and human rhinoviruses 2 and 14. The secondary structures of PV constitute two genetic elements; one of which serves in the initiation of positive-strand RNA synthesis while the other facilitates the cap-independent translation initiation. Of special interest is a conserved cloverleaf-like domain at the very 5'-end of the genome. This secondary structure was previously demonstrated to be part of a ribonucleoprotein complex necessary for the initiation of positive-strand RNA synthesis. It interacts with virus-encoded proteins like the 3CD proteinase (3CD<sup>PRO</sup>) and 3AB, the precursor of the VPg peptide (Andino

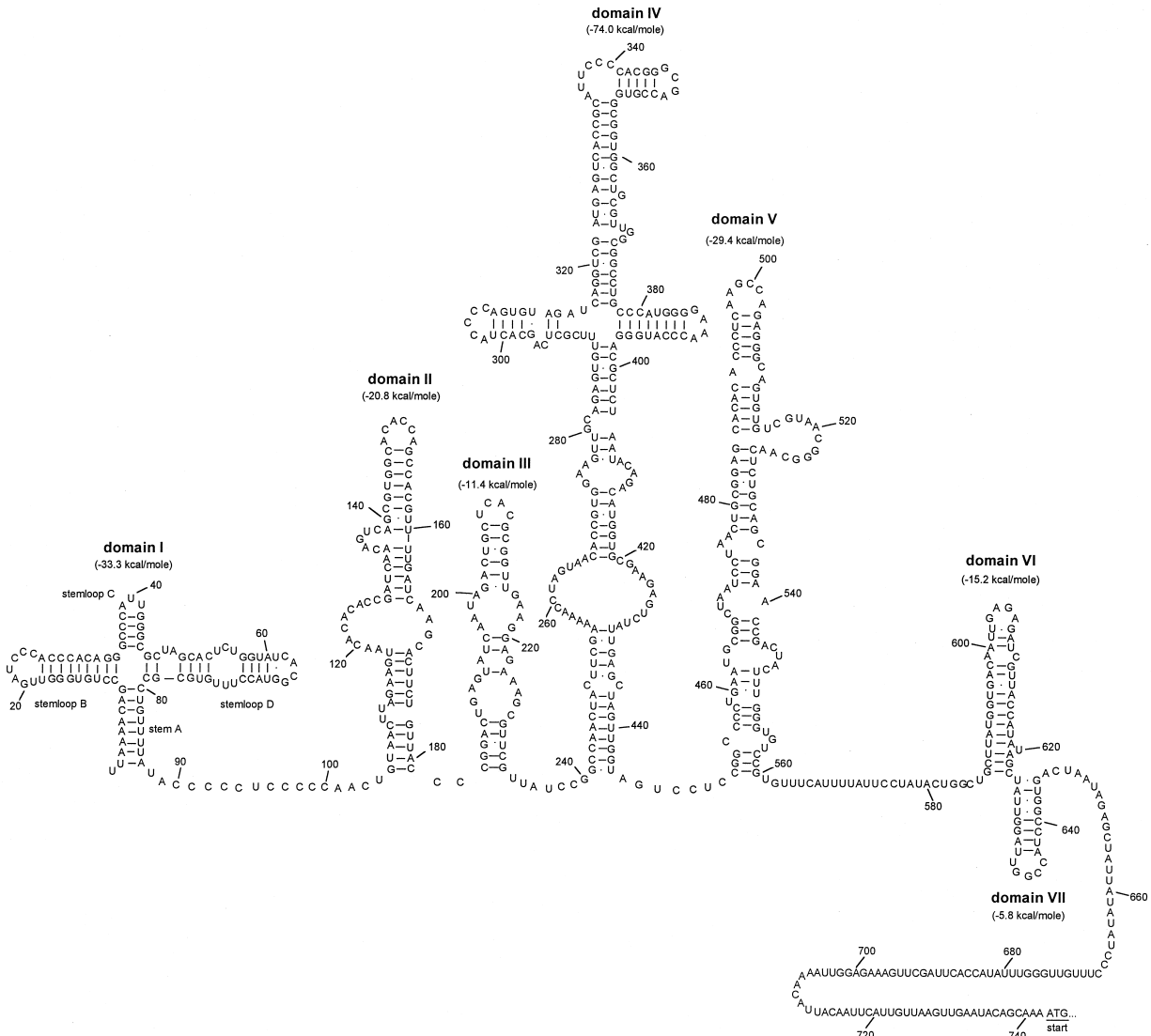


Fig. 2. Predicted secondary structures of the 5'-NTR of CVB3 (strain Nancy). This figure shows secondary structures based on (i) predictions by the mfold program (version 3.0) of Zuker *et al.* (1999), (ii) the prediction of the PV3 5'-NTR of Skinner *et al.* (1989), and (iii) phylogenetic sequence comparisons (Zell & Stelzner, 1997). Genetic evidence supporting the structure of domain III was presented by Zell *et al.* (1995). Among all sequenced human enteroviruses, the overall folding is highly conserved. The calculated free energy of each domain is indicated. Since phylogenetic conservation of the putative secondary structures had high priority, no attempt has been made to minimize the energy of the overall structure.

*et al.*, 1990, 1993; Leong *et al.*, 1993; Harris *et al.*, 1994; Walker *et al.*, 1995; Xiang *et al.*, 1995). Although the binding site of the 3CD<sup>pro</sup> was not defined precisely, some evidence indicates that it may map to stem-loop D of the cloverleaf. Interaction was also demonstrated with the cellular protein *p36*, which was identified as a processed form of the eukaryotic elongation factor 1 $\alpha$  (Andino *et al.*, 1993; Harris *et al.*, 1994; Roehl & Semler, 1995; Roehl *et al.*, 1997). Binding of the poly(rC) binding proteins 1 and 2 (PCBP1, PCBP2) to the PV cloverleaf seems to up-regulate translation initiation (Gamarnik & Andino, 1997, 1998). Cap-independent translation initiation, the other function of the 5'-NTR, is facilitated by a type I

internal ribosome entry site (IRES). The IRES region was demonstrated to be a *cis*-acting element that directs *in vivo* the binding of ribosomal subunits and several cellular protein factors to the viral RNA in order to accomplish internal translation initiation (for recent reviews, see Jackson & Kaminski, 1995; Belsham & Sonenberg, 1996). Although some experiments support the idea of two genetic elements which are physically separated and function independently (e.g. Rohll *et al.*, 1994), more recent data indicate that RNA synthesis of PV depends on sequences of both elements (Borman *et al.*, 1994), suggesting a dual role of the IRES sequences. Due to the high degree of sequence homology of the 5'-NTR, it is

believed that the similar mechanisms of replication initiation and translation initiation apply to the other enteroviruses and rhinoviruses.

Although the general RNA folding pattern appears to be very similar, the 5'-NTRs of the bovine enteroviruses (BEVs; see Fig. 1), the human enteroviruses (see Fig. 2) and the human rhinoviruses differ significantly from each other. Unique features of the BEVs are (i) the presence of sequences encoding a second putative cloverleaf-like secondary structure (domain I\*) separated from the 5'-cloverleaf (domain I) by a small stem-loop structure (domain I\*\*), (ii) the size and shape of the putative domains III and VI of the IRES region, and (iii) the characteristic nucleotide sequence of the 3'-NTR (Zell & Stelzner, 1997). With the exception of stem-loop D, domain I\* of the BEV 5'-NTR exhibits only little homology to the cloverleaf domains of the other enteroviruses and rhinoviruses (compare Figs 1 and 2). The function of domains I\* and I\*\* is still unclear.

Construction of hybrid cDNAs was done to demonstrate that the exchange of large regions within the 5'-NTRs of PV, coxsackie B viruses (CVB) and human rhinoviruses may yield viable cDNA-generated virus chimeras (Semler *et al.*, 1986; Johnson & Semler, 1988; Rohll *et al.*, 1994; Xiang *et al.*, 1995; Zell *et al.*, 1995; Todd *et al.*, 1997). These experiments indicate that the putative secondary structures necessary for replication initiation and translation initiation can be substituted. Since virus chimeras are only viable when they are able to run through a complete cycle of replication, artificial chimeric constructs are an attractive genetic system to demonstrate functional substitution, which may be explained by *cis*- and *trans*-complementation of RNA-RNA and RNA-protein interactions *in vivo*. Moreover, hybrid virus construction is a method suited to dissecting functional genetic elements of picornaviruses and analysing their function independently from each other in different genetic backgrounds.

## Methods

■ **Construction of full-length cDNA clones with hybrid 5'-NTRs.** A unique *Sna*BI restriction site was introduced into the full-length cDNA clone pCVB3-M2 (Lindberg *et al.*, 1992) using the synthetic oligonucleotides CVB3SnaBI (5' TCACGGTACCTTTGTGCGCCTGTTTATACCCCTCCCCCTACGTAAGTACTAGAAGTAACACACACC 3'; *Kpn*I and *Sna*BI restriction sites are in bold and underlined) and CVBx (5' CAGTTAGGATTAGCCGCATTC 3') to amplify a DNA fragment with a length of approximately 420 nt. This DNA fragment had a unique *Sna*BI restriction site 3' to genome position 104 of CVB3 (Fig. 2) and was digested with *Kpn*I and *Cel*III to enable an exchange with the corresponding *Kpn*I-*Cel*III fragment of pCVB3-M2. Construction of full-length cDNA plasmids with mutated or hybrid 5'-NTRs was also achieved by PCR mutagenesis. Using the plasmids pGEM-3Z(BEV1), which has an insert of approximately 900 nt representing the 5'-end of the BEV1 genome, and pCVB3-M2 as template DNA, the following synthetic oligodeoxyribonucleotides were employed to amplify different genome regions of the BEV1 and CVB3 5'-NTR, respectively (restriction sites are in bold and underlined): BEV1Mlu1-1 (5' CATCACGGTTAAAACAGCCTGGGGTGT 3'); BEV1Mlu1-2 (5' GTGATC-

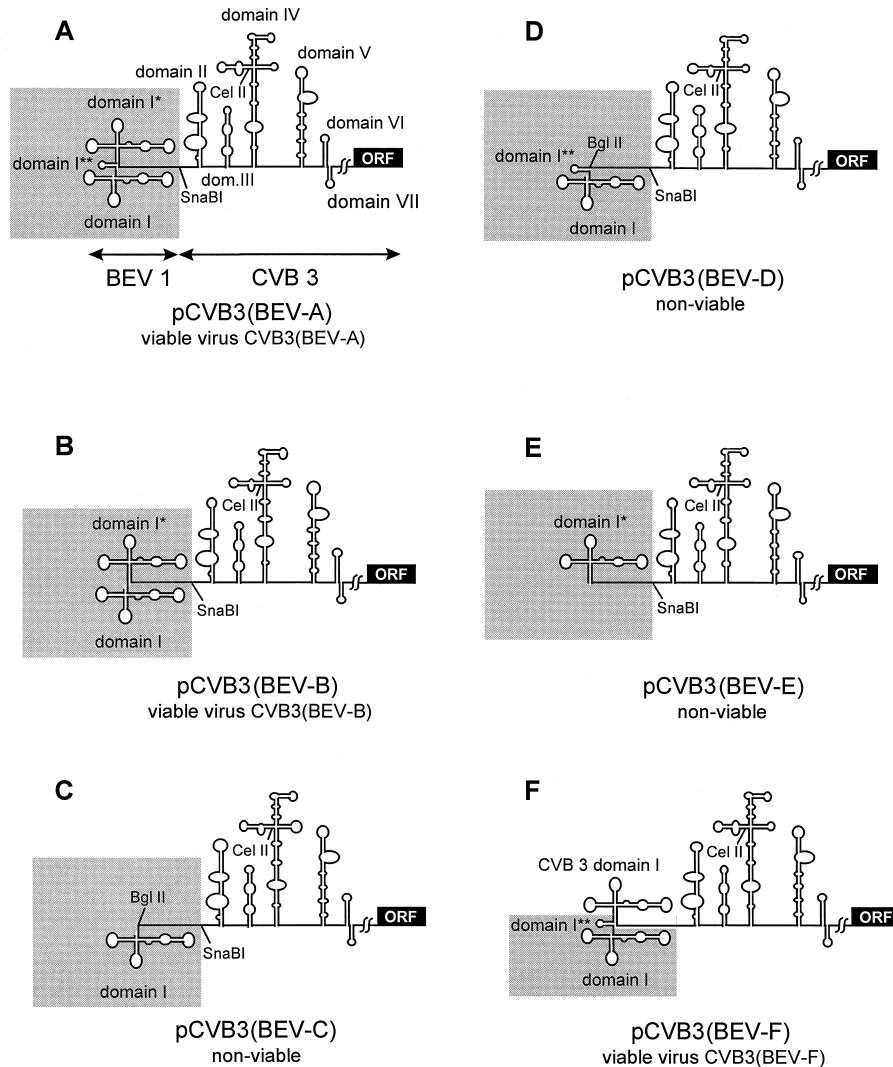
ACGCGTTTAACTCTGCCAATGTGGGGAGTAGTCCGA 3'); BEV1SnaBI-sense (5' CACCCCTACGTAAGTACTAGCAAAAACCG 3'); BEV1SnaBI-antisense (5' AGTTACGTAGGGGTGGGAACCTACATACCAA 3'); BEV1Bgl2-sense (5' GTGATCAGATCTTCCCCACCCCTACGTAAGTACTAG 3'); BEV1Bgl2-antisense (5' GTGATCAGATCTAAAACAGCCGTACGGGGTTCTAAC 3'); BEV1Sac1 (5' ACTTGAGCTCCCATGTTCAAATGTCTTGATGCGATTCAAACAACTGCAAAGTGAATGAGTTTCGAGGGTG 3'); BEV4 (5' GTTAGGATTAGCCGCATTC 3'); CVB3Mlu1 (5' GACATACGCGTTTAAAACAGCCTGTGGG 3'); and CVB3Bgl2 (5' GAGATCAGATCTGGGTAAAACAGCCTGTGGG 3').

Amplified DNA fragments were digested with the appropriate enzymes to generate compatible ends and ligated to the corresponding vector fragments of pCVB3-M2 derivatives. The following full-length cDNA plasmids were constructed (Fig. 3): pCVB3(BEV-A) (BEV domains I-I\*\* in CVB3 background), pCVB3(BEV-B) (BEV domains I-I\* in CVB3 background), pCVB3(BEV-C) (BEV domain I in CVB3 background), pCVB3(BEV-D) (BEV domains I-I\*\* in CVB3 background), pCVB3(BEV-E) (BEV domain I\* in CVB3 background), pCVB3(BEV-F) (BEV domains I-I\*\* and CVB3 domain I in CVB3 background), pCVB3(BEV 5'-NTR) (BEV 5'-NTR in CVB3 background), and pCVB3(BEV-G) (BEV IRES in CVB3 background). The structural intactness of all plasmid clones was verified by DNA sequencing.

■ **Cell lines and viruses.** African green monkey kidney (GMK) cells (courtesy of Norbert Beuscher; Schaper und Brümmer) were maintained in Dulbecco's modified Eagle minimal medium (DMEM) supplemented with 10% foetal bovine serum.

Viruses were usually propagated in GMK cells. Virus titres were determined by TCID<sub>50</sub> assays according to Reed & Muench (1938). Plaque morphology was determined by plaque assays. CVB3 (Nancy strain), CVB3(SnaBI) and the hybrid viruses CVB3(BEV-A), CVB3(BEV-B) and CVB3(BEV-F) were generated upon transfection of GMK cells with pCVB3-M2 and mutated full-length cDNA constructs, respectively. For transfection, GMK monolayers were incubated with 15 µg plasmid DNA per 6 cm Petri dish together with 15 µl Lipofectin (Gibco BRL Life Technologies) as liposome reagent in 2 ml DMEM for 3 h according to the manufacturer's instructions. Using wild-type cDNA, virus-induced cytopathic effect (CPE) was visible within 48 h post-transfection. If no CPE was visible, cells were passaged at day 3 and, if necessary, again at days 6 and 9. Cells were observed for at least 14 days. All viable chimeric viruses of this study appeared after passage at day 3. Hybrid cDNA constructs were considered to be non-infectious when at least three transfection experiments did not yield viable virus within 14 days at 37 °C and 33 °C, respectively. Mutations and the hybrid 5'-NTRs of virus chimeras were verified by reverse transcription followed by PCR and DNA sequencing.

■ **Construction of plasmids supporting IRES-driven translation and *in vivo* translation experiments.** Starting from *Hind*III-linearized plasmid pD6 (Niepmann *et al.*, 1997), amplified DNA fragments of the 5'-NTRs of PV1 (template DNA pT7-XL), CVB3 (template DNA pCVB3-M2), BEV1 (template DNA pGEM-3Z-BEV1) and the various hybrid constructs, respectively, were cloned 3' to the chloramphenicol acetyltransferase (CAT) gene and 5' to the firefly luciferase reporter gene. The following synthetic oligodeoxyribonucleotides were used for the amplification: pD6-BEV1 (5' GCAGGAAAGCTTGTAAGTACTAGCAA 3'); pD6-BEV2 (5' GCAGGAAAGCTTTCAAATGTCTGTGTRATGCTG 3'); pD6-PV1 (5' GCAGGAAAGCTTGTAAGTACTAGACGCACAAAACC 3'); pD6-PV2 (5' GCAGGAAAGCTTTATGATACAATTGTCTGATTG 3'); pD6-CVB1 (5' GCAGGAAAGCTTGTAAGTACTAGTAAGTAACACAC 3'); pD6-CVB2 (5' GCAGGAAAGCTTTGTCTGTATTCAACTTAACAA 3'); pD6-BEV-CL (5'



**Fig. 3.** Schematic illustration of chimeric full-length plasmid constructs. Shaded boxes indicate BEV1-derived genome regions substituting for the putative CVB3 cloverleaf. A unique *Sna*BI restriction site was introduced to facilitate the exchange of DNA fragments. The BEV1 genome region which is thought to represent the intact replication initiation signal contains two putative cloverleaf-like secondary structures separated by a small stem-loop (A).

GCAGGAAAGCTTTTAAAACAGCCTGGGGGTTG 3'); pD6-BEV-CL2 (5' GCAGGAAAGCTTCTCTACCAATGTGGGGAGTAG 3'); and pD6-CVB-CL (5' GCAGGAAAGCTTTTAAAACAGCCTGTGGGTTG 3').

The following pD6 derivatives were constructed (see also Figs 5 and 6): pD6(CVB IRES), pD6(PV IRES), pD6(CVB 5'-NTR), pD6(BEV 5'-NTR), pD6(BEV-A) (BEV domains I-I\*\* followed by a CVB IRES), pD6(BEV-B) (BEV domains I-I\* followed by a CVB IRES), pD6(BEV-C) (BEV domain I followed by a CVB IRES), pD6(BEV-D) (BEV domains I-I\*\* followed by a CVB IRES), pD6(BEV-E) (BEV domain I\* followed by a CVB IRES), pD6(BEV-F) (BEV domains I-I\*\* followed by CVB domains I-VII) and pD6(BEV-G) (CVB cloverleaf followed by a BEV IRES). The correct DNA sequence of the inserts was verified prior to translation experiments.

For translation experiments, pD6 derivatives were linearized with *Hpa*I and *in vitro*-transcribed using SP6 polymerase. The efficiency of RNA synthesis was analysed with agarose gel electrophoresis and

photometrically quantified. Transfection of GMK cells was performed with 1 µg RNA per 6 cm Petri dish mixed with 1.5 ml serum-free DMEM and 15 µl Lipofectin transfection reagent. Petri dishes were incubated for 8 h at 37 °C. After transfection, cells were lysed and extracted with 25 mM Tris pH 7.8 (with H<sub>3</sub>PO<sub>4</sub>), 2 mM CDTA, 2 mM DTT, 10% glycerol, 1% Triton X-100. The protein concentration of the lysates was determined and adjusted to 0.5 mg/ml. The luciferase activity of a 2 µl aliquot (corresponding to 1 µg total protein) of the extracts was determined employing the luciferase assay system of Promega.

## Results

### Construction of full-length CVB3 cDNA clones with substitutions of the cloverleaf structure

In the first set of experiments, we aimed to demonstrate the functional necessity of two putative RNA secondary structures

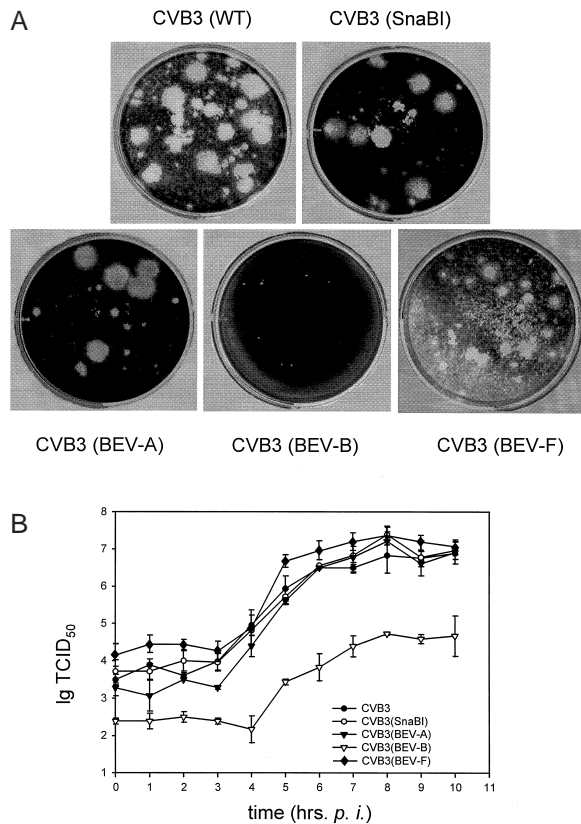


Fig. 4. Plaque phenotype (A) and one-step growth curve (B) of CVB3 and BEV-CVB chimeras. (A) For demonstration of the plaque phenotype, GMK cells were infected with  $10^{2.8}$  TCID<sub>50</sub> CVB3 (wild-type virus),  $10^{2.5}$  TCID<sub>50</sub> CVB3(SnaBI),  $10^{2.2}$  TCID<sub>50</sub> CVB3(BEV-A),  $10^{2.2}$  TCID<sub>50</sub> CVB3(BEV-B) and  $10^{2.17}$  TCID<sub>50</sub> CVB3(BEV-F). Cells were fixed 72 h post-infection and stained with crystal violet. (B) For estimation of the one-step growth curve, Petri dishes of GMK cells were infected with virus. After 1 h, the virus was washed off and Petri dishes were frozen at the time-points indicated to release virus. The curves are the mean of two independent experiments, with each value estimated three times. The standard deviation is indicated by bars.

located between the 5'-cloverleaf and the putative IRES region of BEV1. For this purpose, full-length CVB3 cDNA clones with hybrid 5'-NTRs were constructed using PCR mutagenesis (Fig. 3). To identify the genome region best suited to serve as a linker joining the BEV sequences and the CVB IRES region, the previous nucleotide sequence alignment of all sequenced human enterovirus, human rhinovirus and BEV strains was taken as a basis (Zell & Stelzner, 1997). This alignment revealed a short oligopyrimidine tract separating the cloverleaf domain from the well-conserved domain II of the IRES region (Figs 1 and 2). To facilitate the subsequent exchange of the genome regions, the oligopyrimidine tract was chosen to introduce a unique *Sna*BI restriction enzyme recognition site into the genome of CVB3. After PCR mutagenesis, viable virus CVB3(SnaBI) was generated upon transfection of GMK cells and found to have identical properties to the wild-type CVB3 (Fig. 4). For replacement of the CVB3 cloverleaf, the 5'-

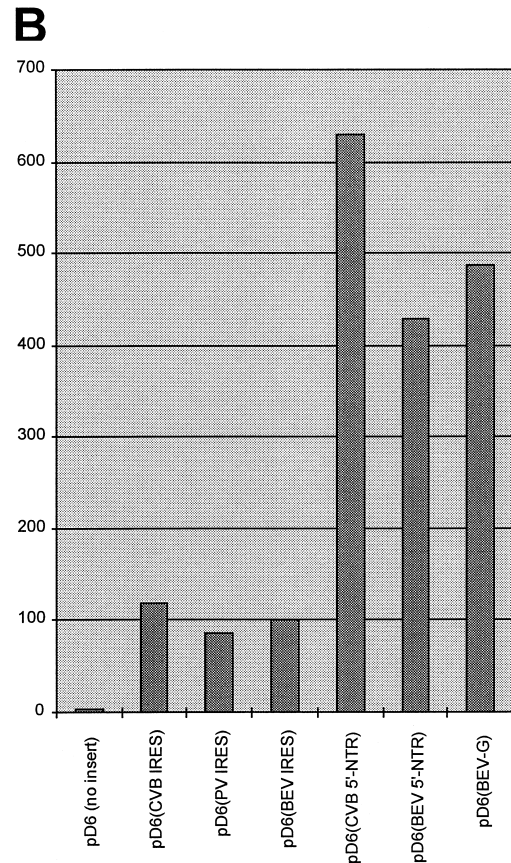
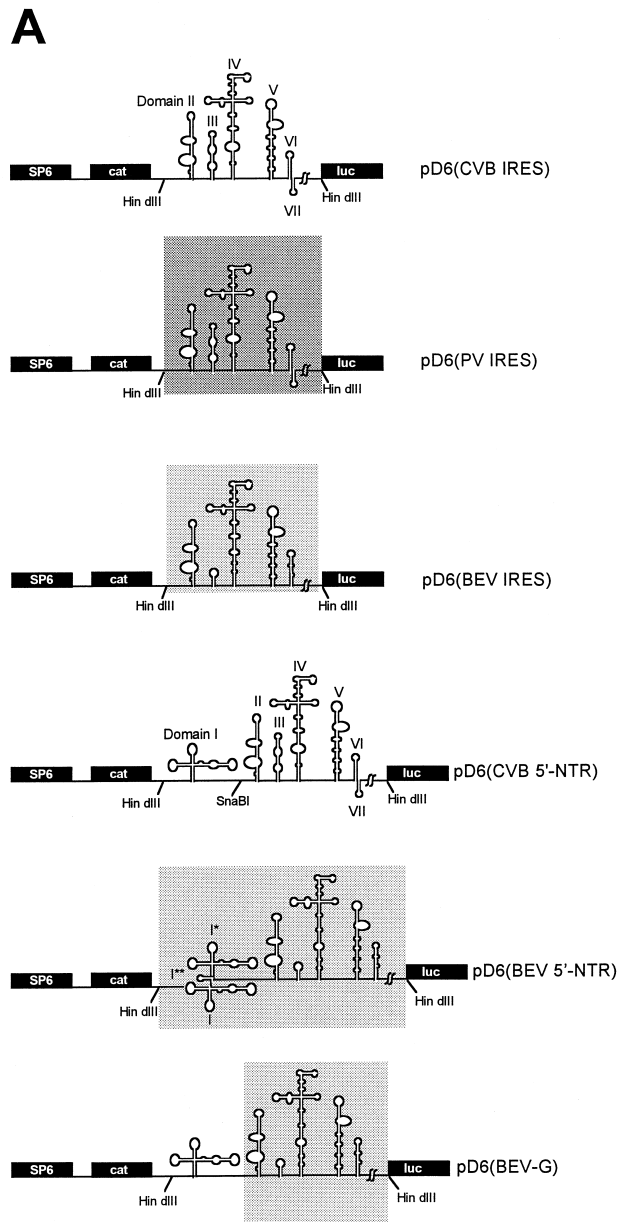
terminal 100 nt of plasmid pCVB3(SnaBI) encoding the cloverleaf and the short oligopyrimidine tract were exchanged with a 215 bp fragment of BEV1 generated by PCR with pGEM-3Z(BEV1) as template DNA. The resulting plasmid pCVB3(BEV-A) has a BEV-derived insert that contains the putative cloverleaf domains I and I\* and a small stem-loop (domain I\*\*) separating the two cloverleaves (Fig. 3A). After transfection of GMK cells, this plasmid construct readily yielded viable virus, which was designated CVB3(BEV-A).

Several deletions of DNA fragments encoding putative RNA domains were introduced into the chimeric viral genome in order to estimate the minimal genome region which is able to functionally replace the CVB3 cloverleaf (Fig. 3B-E). Transfection experiments using these cDNA constructs yielded viable virus in the case of plasmid pCVB3(BEV-B) to give the chimeric virus CVB3(BEV-B). The other plasmids [pCVB3(BEV-C), pCVB3(BEV-D), pCVB3(BEV-E)] were non-infectious.

The relevant genome region of the hybrid 5'-NTR of both cDNA-generated virus chimeras was sequenced after reverse transcription and amplification of a 450 nt DNA fragment. As a result, no sequence deviation from the expected sequence was observed (data not shown). For further characterization, the plaque phenotypes and the one-step growth curves at 37 °C were determined (Fig. 4). The chimeric virus CVB3(BEV-A) replicated normally, as estimated from the large-plaque phenotype and the one-step growth curve. In contrast, the other chimera CVB3(BEV-B) had a pinpoint plaque phenotype. The one-step growth curve revealed slow growth and a replication efficiency of about 10% when compared to the wild-type virus. Since both virus chimeras replicated in different cell lines of human and simian origin (data not shown), the cell type specificity seems to be unchanged in comparison to the CVB3 wild-type virus.

#### The hybrid BEV-CVB double cloverleaf is also functional in CVB3

Construction of hybrid plasmids with a deletion of either the first or the second putative cloverleaf of BEV1 resulted in non-infectious DNAs, indicating the necessity of a genome region encoding the putative double cloverleaf. To test the hypothesis of whether the function of the second BEV cloverleaf can also be performed by the CVB3 cloverleaf, a plasmid with a hybrid double cloverleaf was constructed. Starting from the non-infectious plasmid pCVB3(BEV-D), which has a deletion of the second BEV cloverleaf, the genome region representing the CVB3 cloverleaf was inserted 3' to the BEV domain I\*\*. This construct contained BEV domains I and I\*\* followed by the CVB domain I and the remnant of the CVB3 genome (Fig. 3F). Transfection experiments with this plasmid yielded viable virus [designated CVB3(BEV-F)], which was further characterized (Fig. 4). The chimera had a normal plaque phenotype and replicated like wild-type CVB3.



**Fig. 5.** (A) Schematic illustration of pD6 derivatives designed to assay the IRES-driven translation initiation of the firefly luciferase message. The 5'-NTR sequences were derived from CVB3. Shaded boxes indicate genome regions derived from BEV1 (light grey) and PV1 (dark grey), respectively. Relevant restriction sites used to exchange DNA fragments are indicated. (B) Efficiency of IRES-driven translation initiation of the firefly luciferase message 8 h post-transfection. The luciferase activity of the respective constructs was related to the efficiency of translation initiation driven by the BEV IRES (nt 215–821 of the BEV1 genome). Each value represents the mean of six measurements with each measurement determined three times. The standard deviation (not indicated) ranged from 5 to 15%.

Sequencing of viral RNA revealed that the nucleotide sequence was as expected (data not shown).

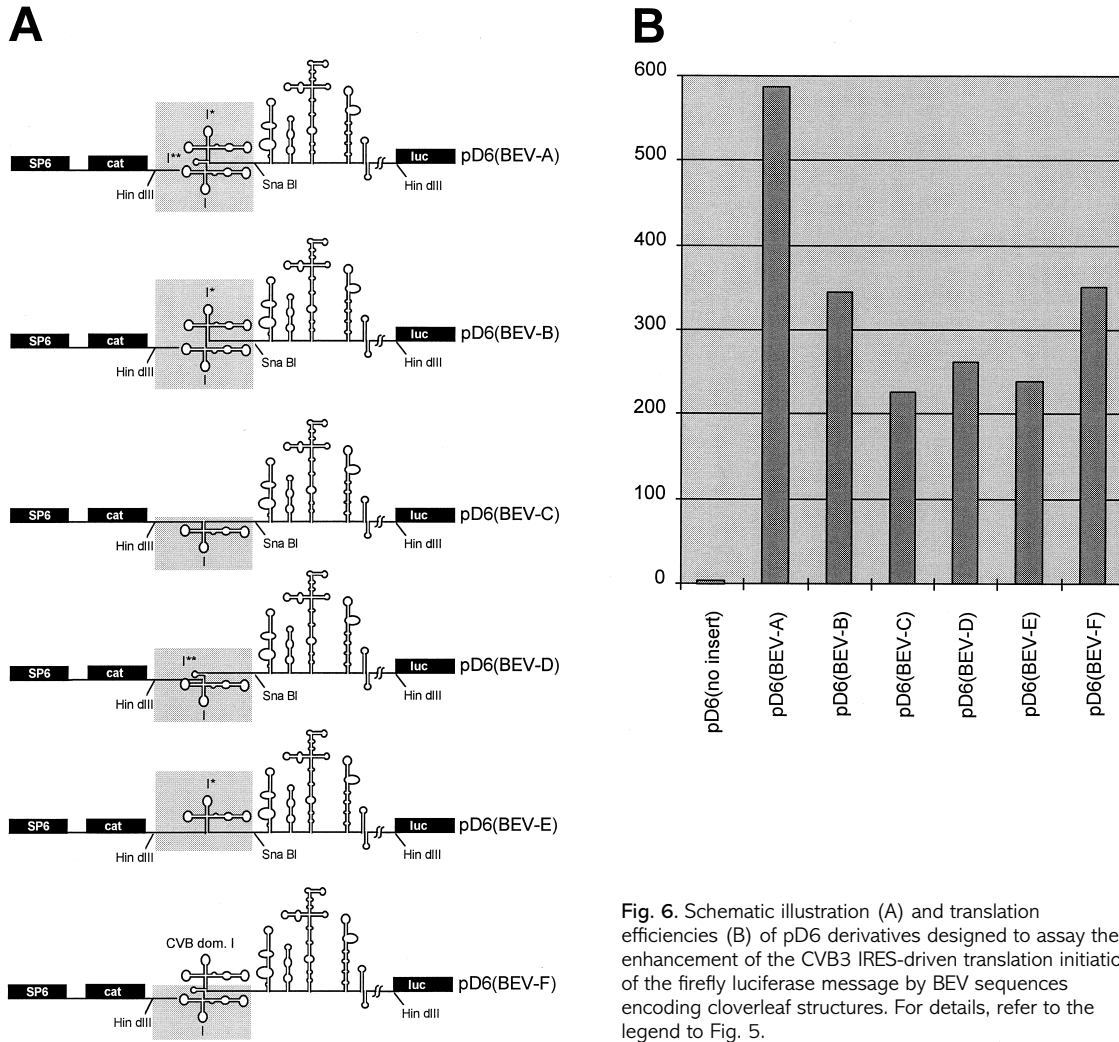
### Exchange of the IRES region

As depicted in Fig. 1, the putative BEV IRES (nt 215–700) exhibits significant differences in domains III and VI, which may result in altered properties of the IRES region. In order to investigate the capability of the BEV1 IRES region to substitute for the CVB3 IRES, domains II–VII of CVB3 were exchanged with the corresponding BEV1 domains II–VI. This construct [pCVB3(BEV-G)] contained the complete putative BEV IRES and the remnant of the BEV 5'-NTR up to the start codon (nt 215–821; Fig. 1). Although the exchanged region of this

plasmid construct showed no deviation from the published sequence, it was not possible to rescue viable virus. Since a functional BEV IRES may require specific interactions with other parts of the 5'-NTR, the exchanged region was expanded to the complete 5'-NTR of BEV1 [pCVB3(BEV 5'-NTR), nt 1–821]. This plasmid construct was also non-infectious.

### IRES-driven luciferase expression in GMK cells

Translation experiments were designed to define the BEV1 and CVB3 IRES, respectively, and to assay the translation efficiency of hybrid NTRs leading to a null-phenotype of full-length cDNA constructs. For this purpose, several pD6 plasmids allowing the *in vitro* transcription of bicistronic RNAs



**Fig. 6.** Schematic illustration (A) and translation efficiencies (B) of pD6 derivatives designed to assay the enhancement of the CVB3 IRES-driven translation initiation of the firefly luciferase message by BEV sequences encoding cloverleaf structures. For details, refer to the legend to Fig. 5.

were constructed and used to transfect GMK cells. The pD6 plasmids contain two reporter genes separated from each other by a DNA fragment derived from the 5'-NTR of either BEV1, PV1, CVB3 or the hybrid constructs. These DNA fragments were cloned 3' to the CAT reporter gene and 5' to the luciferase reporter gene (Figs 5 and 6).

The first experiment was intended to demonstrate the ability of the putative IRES regions of BEV1 and CVB3 to direct cap-independent translation of the firefly luciferase message *in vivo* (Fig. 5). GMK cells were transfected with 1 µg *in vitro*-transcribed uncapped RNA per Petri dish. After incubation at 37 °C for 8 h, the cells were lysed and the cell extracts were assayed for CAT and luciferase activity. Since uncapped RNA was used for transfection, no CAT expression was traceable, neither with a CAT activity assay nor with a CAT-specific ELISA. Therefore, read-through activity was considered to be negligible. However, specific luciferase activities were detectable when the luciferase messenger was under control of IRES-encoding BEV1 sequences (nt 215–821)

and CVB3 sequences (nt 110–743). Mutated or inverted IRES sequences cloned into the pD6 vector did not promote translation initiation (data not shown). Comparison of the CVB3 IRES with the PV IRES (as positive control) and the BEV IRES revealed slightly superior efficiency of the former element.

Recent experiments of Gamarnik & Andino (1997, 1998) have indicated that binding of PCBP to the cloverleaf region of PV seems to up-regulate translation initiation. Since sequences of the putative BEV double cloverleaf may exhibit a similar effect on IRES-driven translation, a pD6 construct with the BEV 5'-NTR was tested and compared to the translation efficiency of the CVB3 5'-NTR. The translation initiated by the complete 5'-NTRs of both viruses was significantly enhanced, as demonstrated in Fig. 5(B). Since a full-length cDNA construct containing a replacement of the CVB IRES with the BEV IRES was non-infectious, the possibility could not be excluded that translation initiation of this construct was severely affected. Therefore, the translation efficiency of the corresponding pD6 derivatives was also assayed. Fig. 5(B)

indicates that translation driven by the BEV IRES was enhanced by the presence of the CVB3 cloverleaf.

Translation experiments employing artificial bicistronic plasmids with BEV sequences fused to CVB3 IRES sequences were constructed to determine whether the putative BEV cloverleaf domains enhance CVB3 IRES-driven translation initiation. The results of these experiments are presented in Fig. 6. The translation efficiency of the CVB3 IRES is significantly enhanced in the presence of the BEV double cloverleaf. Since the non-viability of certain full-length constructs (Fig. 3 C–E) may be due to a lack of translation initiation, their efficacy of translation initiation was also assayed. As shown in Fig. 6, all constructs stimulated IRES-driven translation, albeit in various amounts. A possible correlation was observed between the non-viability of the full-length plasmids pCVB3(BEV-C), pCVB3(BEV-D) and pCVB3(BEV-E) (Fig. 3) and little luciferase expression induced by the respective pD6 constructs.

## Discussion

### Substitution of the CVB3 cloverleaf function

Functional secondary structures of the PV 5'-NTR are necessary to accomplish the initiation of positive-strand RNA synthesis and cap-independent translation. Since the nucleotide sequences of the 5'-NTRs of enterovirus and rhinovirus clusters are highly conserved, it is believed that similar molecular mechanisms apply to other enteroviruses and rhinoviruses including the BEVs. Previously, it was proposed that an additional genome region of the BEVs may form a second cloverleaf-like secondary structure at the 5'-end of the viral genome (Zell & Stelzner, 1997). This proposal raises the question of whether the additional sequences also play a functional role in the virus life-cycle. One hypothesis suggests that both cloverleaves form a functional unit which is involved in BEV replication initiation. This hypothesis is supported by the high degree of phylogenetic conservation of domain I and the observation that stem-loop D sequences of both cloverleaves have a striking similarity to the 3CD<sup>pro</sup>-binding domain of PV and rhinovirus. To demonstrate the functional necessity of this genome region, chimeric full-length cDNA plasmids were constructed and used for subsequent transfection experiments. Dissection of the putative double cloverleaf and the BEV IRES region allows us to study both genome regions separately in the genetic background of CVB3. In general, viable cDNA-generated virus chimeras with exchanges of essential genome regions provide strong evidence for functional substitution, which can be explained by functional interactions of the substituting genome region with viral RNA and/or proteins. After exchange of the CVB3 cloverleaf with a BEV1 genome region encoding two putative cloverleaf-like secondary structures, the inserted genome region was able to substitute for the CVB3 cloverleaf, as indicated by the viability of the virus chimera CVB3(BEV-A). It was expected that successive deletions of the transferred sequences would help to

define the minimal BEV region which can functionally substitute for the putative CVB3 cloverleaf. After deletion of the small stem-loop (domain I\*\*), the viable chimera CVB3(BEV-B) was rescued and had a clearly impaired growth capacity (Fig. 4). A reduced translation efficiency of this hybrid NTR could explain this finding (Fig. 6B). Sequences encoding either the first or the second BEV cloverleaf are not sufficient for substitution (Fig. 3 C–E). The failure of the former construct is of interest, since the first BEV cloverleaf and the CVB3 cloverleaf have a nucleotide identity of 79%. For comparison, the nucleotide identity of the PV1 and CVB3 cloverleaves is very similar (77%) and the viability of CVB3 chimeras with a PV1 cloverleaf was demonstrated (Zell *et al.*, 1995; R. Zell, unpublished). The putative second cloverleaf exhibits only a low homology (with the exception of stem-loop D; Figs 1 and 2). Our results demonstrate that a functional substitution of the CVB3 cloverleaf requires a BEV sequence encoding a putative double cloverleaf. Whether this sequence comprises the BEV genome region necessary for replication initiation has to be investigated. The viability of chimera CVB3(BEV-F) is interesting since the significance of the presence of BEV domains I–I\*\* in this chimera is unclear. A 110 nt deletion at its 5'-end would re-establish a 'wild-type' CVB3, but genetic instability (i.e. deletions or other genomic rearrangements) was not observed (data not shown).

### Enhancement of reporter gene translation

Phylogenetic studies as well as translation experiments described in this study suggest the presence of functional IRES elements within the BEV1 and CVB3 5'-NTR, respectively. While the latter is very similar to the well-studied PV 5'-NTR, significant differences in the former 5'-NTR were described which may affect functional properties of the IRES element. However, translation efficiencies of both elements are comparable (Fig. 5 B). In our *in vivo* system, the BEV sequence from nt 215 to 821 and the CVB3 sequence from nt 105 to 743 are sufficient to drive translation of the luciferase message (Fig. 5 B). Since the IRES-encoding sequences were dissected from the virus background and fused to the heterologous firefly luciferase gene, efficient translation initiation seems not to require essential sequences far distant from the 5'-NTR. Significant enhancement of the luciferase activity was observed when the IRES element was fused to the CVB3 cloverleaf or the putative BEV double cloverleaf. The intact 5'-NTRs of both viruses as well as hybrid 5'-NTRs with reciprocal exchanges of cloverleaf-encoding sequences yield translation efficiencies which are four to six times higher than the respective IRES regions (Figs 5 and 6). This translation stimulation was significantly reduced in the case of hybrid NTRs with deletions of either the first or second BEV cloverleaf. The low values of luciferase activity correlated with the non-viability of the respective full-length cDNA plasmids (Fig. 3 C–E). Therefore, one may assume that reduced translation efficiency is the cause of non-viability. However,

disturbed replication could also explain the null-phenotype. To address this question, experiments with suitable bicistronic full-length cDNA clones are presently being performed. It has to be investigated whether the enhancement of translation is based on PCBP-binding to the cloverleaf. In PV, Gamarnik & Andino (1997, 1998) have observed an up-regulation of translation after binding of these proteins to the cloverleaf.

### Non-complementation of the IRES function

Phylogenetic analyses as well as the translation assays described in this study indicate that in analogy to the PV IRES, the BEV IRES spans a genome region ranging approximately from nt 215 to 696 followed by the downstream AUG start codon at nt 819 (Fig. 1). The putative domains III and VI of the BEV IRES differ significantly in size and shape from the corresponding domains of the enteroviruses and rhinoviruses. Also, the BEV IRES has no putative domain VII, which is conserved in all the other enteroviruses (Zell & Stelzner, 1997). Therefore, it may be considered as a third specimen of the so-called type I IRES. Whereas previous experiments proved that type I and type II IRES regions of certain picornaviruses can substitute for the IRES function of other related viruses (e.g. Johnson & Semler, 1988; Rohll *et al.*, 1994; Xiang *et al.*, 1995; Todd *et al.*, 1997), there is still a lack of information as to whether the BEV IRES also has this ability. In this study, rescue of viable chimeric virus was not successful after transfer of the IRES region (nt 215–821) and the complete 5'-NTR (nt 1–821). The translation efficiency of both constructs was not affected. The null-phenotype may indicate that the BEV IRES fails to functionally substitute for the coxsackieviral IRES. However, non-viability of both full-length constructs could also be a result of disturbed replication. Borman *et al.* (1994) have demonstrated that sequences of domains IV and V are essential for RNA synthesis of PV. This may also be the case for BEV1 and CVB3. The significance of this observation for other enteroviruses is unknown. A third explanation could be a failure of other steps of the virus life-cycle (e.g. encapsidation). At present, one cannot exclude the removal of unknown functional sequences of CVB3 located 3' to the IRES element. Experiments addressing the replication efficiency of the BEV–CVB chimeric constructs are currently being performed to resolve these questions.

We thank Nina Böddeker, Andrea Gamarnik, Esteban Domingo and Bert Semler for helpful discussion. We also thank Eckard Wimmer for the generous gift of plasmid pT7-XL and Michael Niepmann for plasmid pD6. The technical assistance of M. Müller, S. Wachsmuth, V. Güntzschel and C. Beck is appreciated.

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Received 19 February 1999; Accepted 17 May 1999