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Trans-complementation of a genetic defect in the coxsackie B3 virus 2B protein

Frank J. M. van Kuppeveld, Patrick J. J. C. van den Hurk, Ina W. J. Schrama, Jochem M. D. Galama and Willem J. G. Melchers

Department of Medical Microbiology, Nijmegen Center for Molecular Life Sciences, University Medical Center Nijmegen, PO Box 9101, 6500 HB Nijmegen, The Netherlands

The enterovirus 2B protein contains a putative amphipathic α-helix that includes three positively charged and one negatively charged residue. Previously, we observed that replacement of the glutamic acid-40 residue with a lysine residue (mutation 2B-E[40]K) in the amphipathic α-helix of the coxsackie B3 virus 2B protein resulted in a quasi-infectious phenotype. On one occasion, however, transfection of 2B-E[40]K RNA transcripts gave rise to a virus stock in which the mutation was retained. This study was aimed at elucidating the molecular mechanism underlying this observation. Sequence analysis of the viral RNA provided no evidence for a second-site suppression mutation that rescued the defect of the 2B-E[40]K mutation in *cis*. Therefore, the possibility was considered that the defect caused by the 2B-E[40]K mutation was complemented in *trans* by viable revertants that had emerged in the virus population. The transfection-derived virus stock indeed contained a small fraction of (pseudo)revertant viruses, carrying the original glutamic acid-40, threonine-40 or asparagine-40, rather than the introduced lysine-40. Consistent with the idea that the 2B-E[40]K virus is unable to grow without the aid of *trans*-acting wild-type(-like) proteins, only the (pseudo)revertant viruses were able to produce individual plaques. Further support for the idea of *trans*-rescue was obtained using a genetic complementation assay, which revealed the occurrence of a low level of *trans*-complementation of the 2B-E[40]K mutation by wild-type virus. This is the first report that provides evidence that a genetic defect in the enterovirus 2B protein can be complemented in *trans*.

Introduction

The genus *Enterovirus*, a member of the family *Picornaviridae*, includes poliovirus, coxsackievirus, echovirus and several unnamed enteroviruses. Enteroviruses are small, cytolytic RNA viruses that contain a 7·5 kb RNA molecule of positive polarity as their genome (Fig. 1). The genomic RNA harbours one large open reading frame that encodes the viral polyprotein. This polyprotein is processed by virally encoded proteinases to yield both the individual P1 capsid proteins and the nonstructural P2 and P3 region proteins ($2A^{pro}$, 2B, 2C, 3A, 3B, 3C, $3D^{pol}$), as well as the relatively stable precursor proteins, 2BC, 3AB and $3CD^{pro}$, whose functions differ from those of their cleavage products (reviewed in Wimmer *et al.*, 1993). Replication of the viral RNA (vRNA) takes place in replication complexes localized at virus-induced membrane vesicles which proliferate and accumulate in the cytoplasm of the infected cell, and which are most likely induced by the 2BC protein (Bienz *et al.*, 1983; Cho *et al.*, 1994; Barco & Carrasco, 1995).

The nonstructural proteins function directly in vRNA replication or indirectly by inducing specific biochemical and morphological alterations in the host cell to facilitate vRNA replication. Genetic complementation experiments have provided insight into the functions of the viral proteins and have helped to dissect the multiple functions of a given protein. Several approaches have been used to investigate the *in vivo* complementation efficiency. Complementation experiments have been performed with viable poliovirus mutants in mixed infection experiments (Bernstein *et al.*, 1986; Li & Baltimore, 1998; Charini *et al.*, 1991; Johnson & Sarnow, 1991; Giachetti *et al.*, 1992; Tolskaya *et al.*, 1994). Other approaches used to investigate complementation of (lethal) lesions include cotrans-

Author for correspondence: Frank van Kuppeveld.
Fax +31 24 3540216. e-mail f.vankuppeveld@ncmls.kun.nl

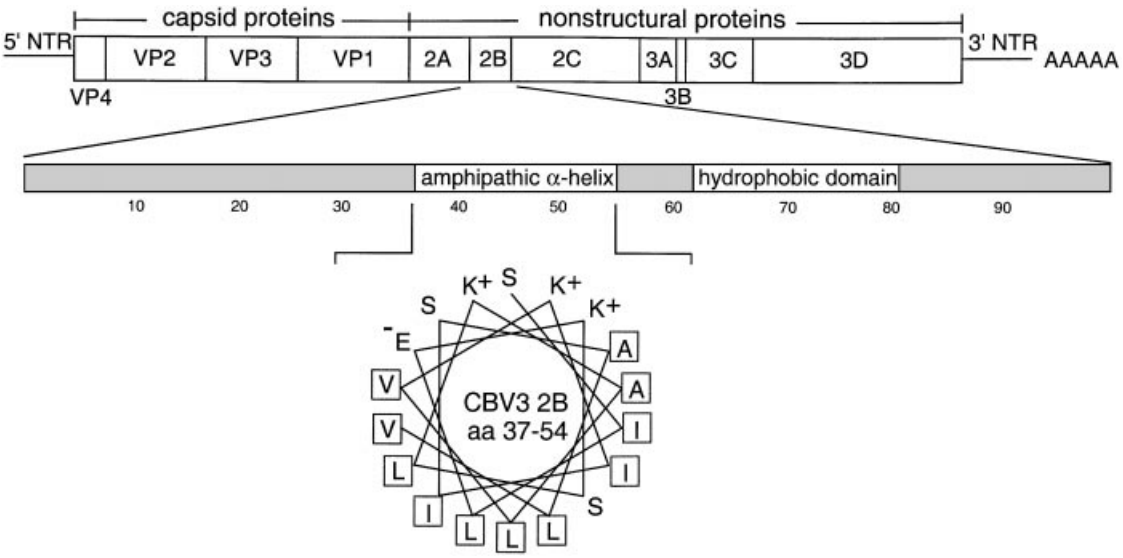

Fig. 1. Schematic representation of the 7·5 kb enterovirus RNA genome. The polyprotein-encoding region (boxed) is flanked by the 5′ nontranslated region (NTR) and the 3′ NTR, which contains a polyadenylate tract at its end. The P1 coding region of the RNA genome encodes the structural capsid proteins. The P2 and the P3 coding regions encode the nonstructural proteins involved in RNA replication. The 2B protein (99 aa) is enlarged and the positions of the putative cationic amphipathic α-helix and the hydrophobic domain are indicated. A helical wheel representation of the putative cationic amphipathic α-helix (aa 37–54) of the 2B protein of CBV3 is shown. Charged residues are indicated. Boxed residues represent hydrophobic residues.

fection of mutant and helper RNA (Collis *et al*., 1992), dicistronic RNAs (Cao & Wimmer, 1995), amber-suppressing cell lines (Novak & Kirkegaard, 1994), transient expression of nonstructural proteins (Teterina *et al*., 1995) and the HeLa cell-free replication assay (Towner *et al*., 1998). Collectively, these studies indicated that most viral proteins have multiple functions, some of which can be complemented in *trans* by wild-type virus proteins and some of which are *cis*-dominant (i.e. noncomplementable).

The 2B protein is a small hydrophobic membrane-active protein that is involved in an early step of vRNA replication (Johnson & Sarnow, 1991; van Kuppeveld *et al*., 1995, 1996), but its exact function is as yet unknown. So far, all genetic defects in the 2B protein have been found to be noncomplementable in *trans*. Poliovirus mutants carrying linker insertions at amino acid positions 28 and 34 of the 2B protein caused primary defects in vRNA replication that could not be *trans*-complemented by wild-type virus (Bernstein *et al*., 1986; Li & Baltimore, 1988; Johnson & Sarnow, 1991). Instead, the mutants exhibited a dosage-dependent *trans*-dominance over wild-type poliovirus (Johnson & Sarnow, 1991). Noncomplementability of a lesion in the 2B protein was also observed by Collis *et al*. (1992), who studied *trans*-complementation of subgenomic RNA transcripts carrying in-frame deletions by cotransfecting helper RNA.

Here, we report the first evidence of *trans*-complementation, albeit with low efficiency, of a replication-defective mutation in the enterovirus 2B protein. The genetic defect was the E[40]K mutation in the putative cationic amphipathic α-helix in the 2B protein of coxsackie B3 virus (CVB3) (van Kuppeveld *et al*., 1996). The 2B-E[40]K mutation caused a quasi-infectious (*qi*) phenotype, a definition introduced by V. I. Agol and his colleagues (Gmyl *et al*., 1993) to indicate that the mutation disrupts vRNA replication to such an extent that (pseudo)reversion mutations can arise but that no virus progeny can be observed harbouring the original mutation. Remarkably, on one occasion, we observed that RNA transfection gave rise to a virus stock in which the introduced 2B-E[40]K mutation was retained. Here, we show that this is not due to a compensating second-site mutation elsewhere in the RNA genome. Instead, we present evidence that the growth of viruses carrying the 2B-E[40]K mutation was due to *trans*-complementation of the defective function by viable (pseudo)revertant viruses that had emerged in the transfection-derived virus population.

Methods

■ **Cells.** All experiments were performed with Buffalo Green Monkey (BGM) cells. Cells were grown in minimal essential medium (Gibco) supplemented with 10% foetal bovine serum (FBS), 100 units penicillin/ml and 25 μg gentamycin/ml. Cells were grown at 36 °C in a 5% CO_2 incubator. After transfection, cells were supplied with medium containing 10% FBS. After infection, cells were supplied with medium containing 3% FBS.

■ **Viruses and virus titrations.** All viruses used in this study are recombinant CB3 viruses obtained by transfection of RNA run-off transcripts from plasmid pCB3/T7 (Klump *et al*., 1990), which contains a full-length cDNA of CBV3 strain Nancy behind a T7 RNA polymerase

promoter. The recovery of the virus stock containing mutation 2B-E[40]K has been described previously (van Kuppeveld *et al.*, 1996). Virus yields were determined by endpoint titration as described previously (van Kuppeveld *et al.*, 1995). Virus titres were calculated and expressed in 50% tissue culture infective dose ($TCID_{50}$) values (Reed & Muench, 1938).

■ **Sequence analysis of viral RNA.** RNA was isolated from virus suspensions using guanidium thiocyanate–phenol–chloroform (Chomczynski & Sacchi, 1987). RNA was reverse transcribed into cDNA using Superscript reverse transcriptase (RT) according to the recommendation of the manufacturer (Gibco). Amplification by PCR was performed with Super*Taq* DNA polymerase (HT Biotechnology). Dideoxy chain termination sequence analysis was performed according to standard procedures.

For the sequence analysis of the RNA genome of the 2B-E[40]K mutant virus, the following RT–PCR products were generated. Nucleotides 1–780 were amplified by RT–PCR using forward primer 5′ TTAAAACAGCCTGTGGGT 3′ and reverse primer 5′ ATGTGCCCCAGTCTTTTG 3′. Nucleotides 3280–5295 were amplified by RT–PCR using forward primer 5′ ACAATGACAAATACGGGCGCA 3′ and reverse primer 5′ TTGAAAACCCGCAAAGAGCTT 3′. Nucleotides 4933–7420 were amplified by RT–PCR using forward primer 5′ CAGGTAAGATACTCTCTAGACATG 3′ and reverse primer 5′ GGGGGGGTCGACTTTTTTTTTTTTTTTTTTTC 3′.

For the verification of the 2A-N[14]S mutation, RT–PCR was performed with forward primer 5′ AACGTGAACTTCCAACCCAGCGGA 3′ (nt 3235–258) and reverse primer 5′ CTGTTCCATTGCATCATCTTC 3′ (nt 3724–3744). Sequence analysis was performed with reverse primer 5′ TGTGGTCGTGCTCAATAAGAG 3′ (nt 3412–3432).

For the sequence analysis of the 2B coding region of the 2B-E[40]K and 2B-E[40]T mutant viruses, RT–PCR was performed with forward primer 5′ TGGTGTCATTGGCATTGTGACCATGGGGGG 3′ (nt 3648–3677) and reverse primer 5′ TTGGGATGGCGCGCTCTGCTC 3′ (nt 4231– 4251). Sequence analysis was performed with reverse primer 5′ CCATTCAATGAATTTCTG 3′ (nt 4117–4134).

■ **Site-directed mutagenesis.** *In vitro* mutagenesis was performed with single-stranded DNA generated from a subgenomic pALTER phagemid construct that contained the *Hin*dIII (nt 2080)–*Xba*I (nt 4947) fragment of CBV3, using the Altered Sites *in vitro* Mutagenesis System according to the recommendations of the manufacturer (Promega). Synthetic oligonucleotides were used to introduce site-specific mutations. The nucleotide sequence of the oligonucleotides were: 5′ ATTTACCACCCTGTAGCTCCCTACGTACACTGCCCCTGATTG 3′ (mutation 2A-N[14]S); 5′ TTTTAGAGATTTCTTTAAAATGGAGTCTTGACC 3′ (mutation 2B-E[40]K); 5′ GGCTTTTAGAGATTTCGTTAAGATGGAGTCTTG 3′ (mutation 2B-E[40]T). The nucleotide sequence of the mutant pALTER clones was verified by sequence analysis. The 2A mutation was introduced into the pCB3/T7 plasmid using the unique *Psh*AI (nt 2803) and *Spe*I (nt 3837) sites. The 2B mutations were introduced into the pCB3/T7 plasmid using the unique *Spe*I (nt 3837) and *Bss*HII (nt 4238) sites.

■ **Transfection of cells with RNA transcripts.** Plasmids were linearized with *Sal*I, purified, and transcribed *in vitro* by T7 RNA polymerase as described previously (van Kuppeveld *et al.*, 1995). BGM monolayers cells grown in 25 cm^2 flasks to 75% confluency were transfected with 2·5 μg of RNA transcripts using the DEAE-dextran method as described previously (van Kuppeveld *et al.*, 1995). After transfection, cells were grown at 36 °C. When virus growth was observed, the cultures were incubated until cytopathic effect (CPE) was complete. The cultures were then subjected to three cycles of freezing and thawing and the viruses were aliquoted. If no CPE was observed after 5 days, the cultures were subjected to three cycles of freezing and thawing, and passaged to fresh BGM monolayer cells, which were grown for another 5 days.

■ **Single cycle-growth analysis.** 100% confluent BGM monolayer cells were infected with virus at an m.o.i. of 1 $TCID_{50}$ per cell for 30 min at room temperature. The cells were washed three times with PBS, supplied with medium, and grown at 36 °C. At the indicated times post-infection, cells were disrupted by three cycles of freezing and thawing. Virus titres were determined by endpoint titration.

■ **Plaque assay.** Plaque assays were performed with 100% confluent BGM cell monolayers grown on 10 cm^2 dishes in six-well plates. Cells were infected with different virus dilutions for 30 min at room temperature. Cells were washed three times with PBS and overlaid with culture medium containing 1% plaque agarose (Gibco) and 25 mM $MgCl_2$. The cells were grown at 36 °C. After 4 days, individual plaques were picked and inoculated to fresh BGM cell monolayers. These cells were grown at 36 °C until CPE was complete.

■ **Analysis of viral RNA synthesis.** BGM cell monolayers were transfected with 1 μg of T7 RNA polymerase-generated RNA transcripts of *Sal*I-linearized pCB3/T7-LUC plasmids as described above. At the indicated times post-transfection, the cells were lysed and the luciferase production was measured as described previously (van Kuppeveld *et al.*, 1995).

Results

Background

Previously, we described the construction and analysis of mutations in the cationic amphipathic α-helix of CVB3 protein 2B (Fig. 1). It was found that both the amphipathic character of the domain and the presence of cationic residues in the hydrophilic face of the α-helix are required for vRNA replication and virus growth (van Kuppeveld *et al.*, 1996). In that study, we also tested two mutations that altered the negatively charged glutamic acid-40 residue, which is well-conserved among all enterovirus 2B proteins (van Kuppeveld *et al.*, 1995). Mutation 2B-E[40]D yielded viruses with wild-type growth characteristics. Mutation 2B-E[40]K caused a *qi* phenotype due to a defect in vRNA replication (summarized in Fig. 2A). Seven out of the eight transfected cell cultures failed to produce CPE. Virus growth was observed in one culture due to a reversion of the introduced lysine-40 (AAG) to the wild-type glutamic acid residue (GAG) by a single point mutation. Upon passage of the seven CPE-negative cell cultures, virus growth was observed in one culture. Sequence analysis of the viral RNA isolated from this (non-plaque purified) virus stock showed that the introduced lysine-40 was retained and that no compensating second-site suppression mutation in the 2B coding region had occurred (van Kuppeveld *et al.*, 1996). RT–PCR failed to detect any viral RNA in the six passaged cultures that did not reveal virus growth, demonstrating that the detection of viral RNA carrying mutation 2B-E[40]K in the culture that revealed virus growth was not merely due to passage of the transfected RNA (data not shown). Another

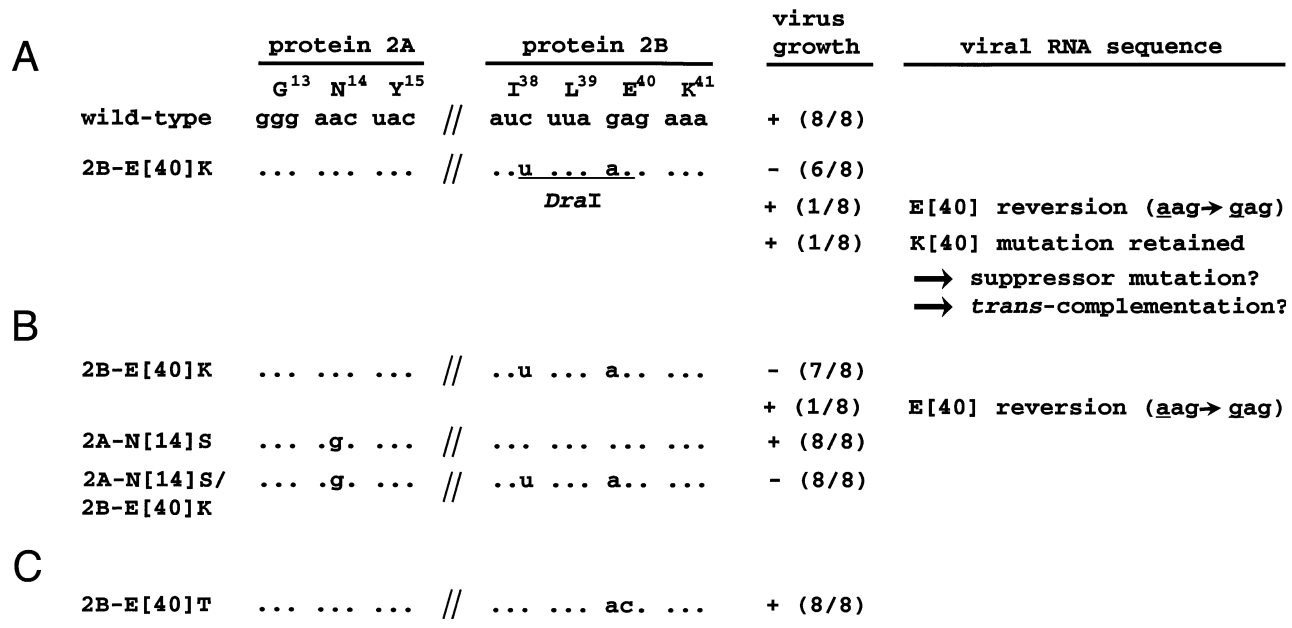

Fig. 2. Partial amino acid and nucleotide sequence of the 2B-E[40]K mutant (A), the 2A-N[14]S mutant and the 2A-N[14]S/2B-E[40]K double mutant (B), the 2B-E[40]T mutant (C) and a summary of the effects of these mutations on virus growth. The data shown in (A) were described previously (van Kuppeveld *et al*., 1996). The data shown in (B) and (C) represent new results obtained in this study. The number of transfections performed with the mutant RNAs is given in parentheses. Note that in the 2B-E[40]K mutant, the isoleucine-38 codon is altered in order to create a *Dra*I restriction site (underlined).

argument that argues against the possibility that the detected 2B-E[40]K RNA represents non-replicated RNA is that only replicating RNAs can be encapsidated and thereby infect fresh cells (Nugent *et al*., 1999).

The present study was aimed at elucidating the molecular mechanism that enabled the growth of mutant viruses carrying mutation 2B-E[40]K. We hypothesized that the growth of this virus was made possible either through (i) a *cis*-acting second-site suppression mutation outside the 2B coding region, or (ii) the complementation of the defective 2B-E[40]K mutant protein *in trans* by viable revertants that had emerged in the virus population. Both possibilities were considered in this investigation.

Sequence analysis of the viral RNA genome

To search for the possible presence of a second-site suppression mutation outside the 2B coding region, the RNA genome of the mutant virus stock was sequenced. For this purpose, three large RT–PCR products were generated. These RT–PCR products covered the 5′ NTR, the P2 coding region, and the P3 protein coding region plus the 3′ UTR, respectively. The P1 capsid coding region was not analysed because it has been shown previously that this region is dispensable for vRNA replication (van Kuppeveld *et al*., 1995), making the presence of suppressing mutations in this region very unlikely.

Sequence analysis confirmed the presence of the lysine-40 residue (AAG) in the 2B protein. Furthermore, the replacement of adenine-3335 with guanine, causing the mutation of the 2A residue asparagine-14 (AAC) into a serine residue (AGC), was noted. This mutation was observed upon sequencing in both directions, and was found consistently in three independently generated RT–PCR products. Sequence analysis of wild-type CBV3 and the parental pCB3/T7 plasmid confirmed the presence of adenine-3335, indicating that the observed guanine-3335 is not due to a mistake in the published sequence. We also noted the insertion of a guanine at nt position 33. This mutation, however, was also observed in the wild-type CBV3 and the pCB3/T7 plasmid, indicating an error in the published pCB3/T7 sequence (Klump *et al*., 1990). No other mutations were observed.

No evidence for a second-site mutation that rescues the 2B-E[40]K mutation

To investigate whether the 2A-N[14]S mutation could compensate for the defect caused by mutation 2B-E[40]K, *in vitro* mutagenesis was performed to construct the single mutants pCB3/T7-2A-N[14]S and pCB3/T7-2B-E[40]K, and the double mutant pCB3/T7-2A-N[14]S/2B-E[40]K. For each mutation, two independently generated clones were constructed. Each clone was transfected in quadruplicate. The outcome of the transfections is summarized in Fig. 2(B). Transfection of BGM cells with RNA transcripts derived from the wild-type pCB3/T7 plasmid and the 2A-N[14]S mutant resulted in complete CPE within 3 days in all eight cultures.

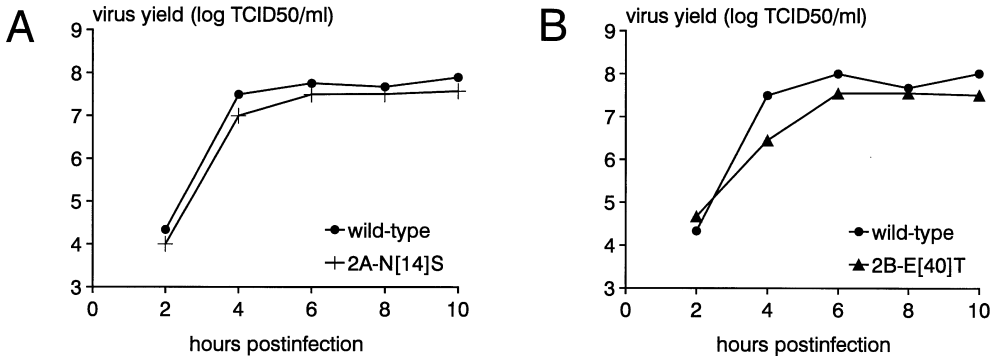

Fig. 3. Single-cycle growth curves of the wild-type CVB3 virus and the mutant viruses carrying mutation 2A-N[14]S (A) or 2B-E[40]T (B). BGM cells were infected at an m.o.i. of 1 and incubated at 36 °C. At 2, 4, 6, 8 or 10 h post-infection, viruses were released from the infected cells by three cycles of freezing and thawing. The virus titres were determined by titration on BGM cells at 36 °C. The virus titres are expressed in $TCID_{50}$ values.

Amplification and sequence analysis of the 2A coding region of the obtained virus demonstrated that the introduced mutation was retained in the viral genome. No CPE was observed in cells transfected with RNA from the 2A-N[14]S/2B-E[40]K mutant. Passage of cytoplasmic extracts to fresh BGM cells also failed to reveal virus growth. Upon transfection of cells with RNA from the 2B-E[40]K mutant, CPE was observed in one of the eight cell cultures. Amplification and sequence analysis of the 2B coding region of the obtained virus revealed the reversion of the introduced lysine-40 (AAG) to the original glutamic acid (GAG). This wild-type revertant virus still contained the introduced AUU codon at isoleucine-38 (Fig. 2B), indicating that this obtained virus is a true revertant and not merely a wild-type virus contamination. No CPE was observed in the remaining seven cultures. Passage of cytoplasmic extracts to fresh BGM cells also failed to reveal virus growth.

These results clearly demonstrate that the N[14]S mutation in the 2A protein does not suppress the defect caused by the 2B-E[40]K mutation. Viruses carrying mutation 2A-N[14]S mutation exhibited wild-type growth characteristics in single-cycle growth experiments (Fig. 3A). The non-deleterious effect of the 2A-N[14]S mutation is in agreement with the occurrence of serine-14 in the 2A protein of some enteroviruses (e.g. enterovirus type 70 and bovine enterovirus type 1). Most likely, the observed 2A-N[14]S mutation is an accidental mutation that arose early in the RNA replication process and that survived the genetic selection pressure because of the wild-type activity of the 2A-N[14]S mutant protein.

The virus stock contains a small fraction of viable (pseudo)revertants

We investigated the possibility that the growth of the viruses carrying mutation 2B-E[40]K was due to *trans*-rescue of the defective 2B function by viable revertants present in the virus population. To test this hypothesis, the 2B coding region (nucleotides 3745–4042) of the virus stock was amplified by RT–PCR and cut with *Dra*I (nt 3860) (see Fig. 2A). Fig. 4(A) (lane 2) shows that a small fraction of the 297 bp PCR product was resistant to *Dra*I cleavage. Even after prolonged incubation times and using an excess of restriction enzyme, this fraction failed to be cut by *Dra*I. Under the same conditions, we always observed complete digestion of a PCR product amplified from the pCB3/T7-2B-E[40]K plasmid (data not shown). The 297 bp RT–PCR product (Fig. 4A, lane 1) was cloned in a TA-cloning vector. Twenty colonies were tested for the presence of the additional *Dra*I site (nt 3860). Seventeen colonies contained the additional *Dra*I site and sequence analysis confirmed the presence of the introduced lysine-40 (AAG). Three of the twenty colonies (i.e. 15%) lacked the additional *Dra*I site. Sequence analysis revealed that one of them contained the original glutamic acid-40 (GAG) residue, whereas the other two contained a threonine-40 (ACG) residue. These results are summarized in Fig. 4(B).

The observation of the threonine-40 residue prompted us to investigate the phenotype of viruses carrying this pseudo-reversion mutation. *In vitro* mutagenesis was performed to introduce mutation 2B-E[40]T in the pCB3/T7 plasmid. Two independently generated clones were constructed, and each clone was transfected in quadruplicate. Transfection of BGM cells with RNA transcripts carrying mutation 2B-E[40]T resulted in CPE in all transfected cultures (Fig. 2C). Amplification and sequence analysis of the 2B coding region of the obtained viruses revealed that the introduced mutation was retained and that no other mutations had occurred. The 2B-E[40]T mutation caused only a minor delay in development of CPE and single-cycle virus growth (Fig. 3B). The same phenotype was observed with the double mutation 2A-N[14]S/2B-E[40]T (data not shown), again demonstrating that the 2A-N[14]S mutation cannot complement a (minor) defect caused by mutation of the glutamic acid-40 residue of the 2B protein.

Taken together, these results support the idea that the virus stock contains a small fraction of viable revertant viruses and pseudorevertant viruses.

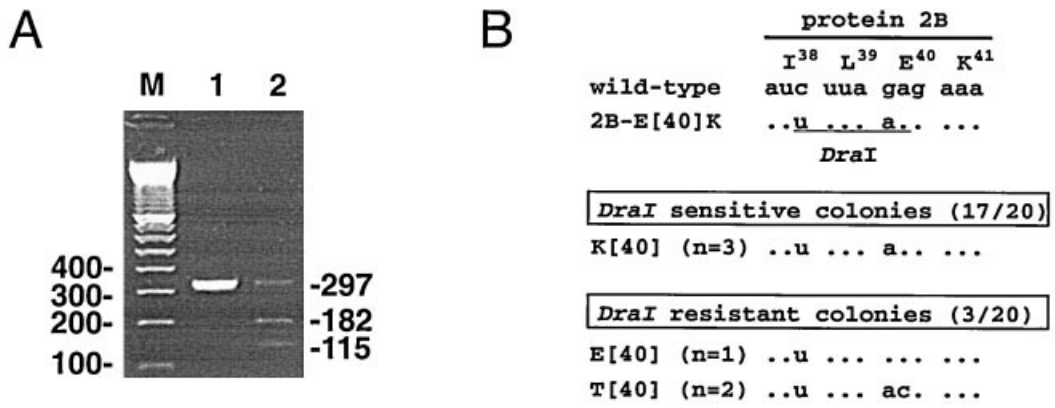

Fig. 4. The virus stock contains revertant and pseudorevertant viruses. (A) The RT–PCR product encompassing the 2B coding region (nt 3745–4042) generated from the virus stock is partially resistant to *Dra*I. Lane M, 100 bp molecular mass marker. Lane 1, the uncut RT–PCR product. Lane 2, the RT–PCR product cut with *Dra*I. The positions of the 297 bp PCR product and its 182 bp and 115 bp *Dra*I cleavage products are indicated. (B) Sequence analysis of the *Dra*I-resistant PCR strands. The 297 bp RT–PCR product was cloned in a TA cloning vector and 20 colonies were cut with *Dra*I. Seventeen colonies contained a 2B insert with a *Dra*I site. Sequence analysis of three of these colonies revealed the presence of the 2B-E[40]K mutation. Three colonies contained a 2B insert that lacked the *Dra*I site. Sequence analysis of these three colonies revealed the occurrence of a reversion mutation to the original glutamic acid-40 residue ($n = 1$) and the occurrence of a pseudoreversion mutation to yield threonine-40 ($n = 2$).

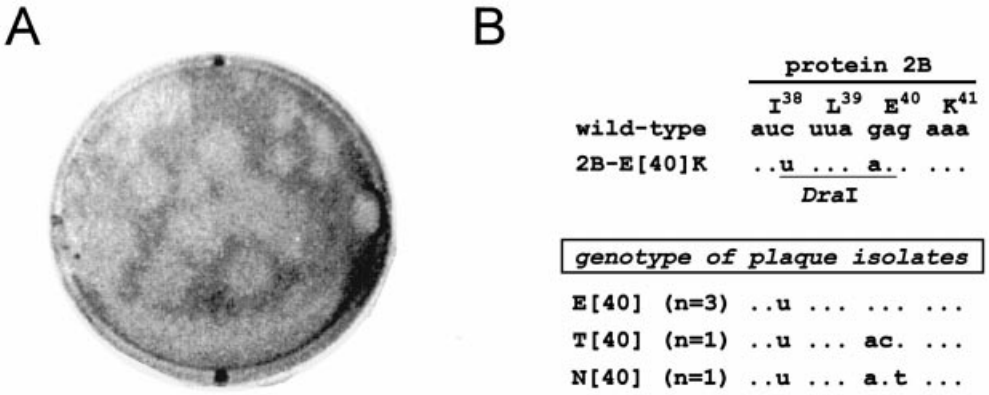

Fig. 5. (A) Plaque phenotypes produced by the viruses present in the virus stock. Plaque assay was performed on BGM cells as described in Methods. (B) Genotype of five plaque isolates. These five plaque isolates were randomly picked from the plate shown in (A) and grown on BGM cells. The 2B coding region was amplified by RT–PCR and sequenced. Sequence analysis revealed the occurrence of both revertant viruses, containing the original glutamic acid-40 residue, and pseudorevertant viruses, carrying either a threonine-40 or asparagine-40 residue. No plaque isolates that contained the introduced 2B-E[40]K mutation were observed.

Only (pseudo)revertant viruses produce plaques

We reasoned that if viruses carrying mutation 2B-E[40]K can only grow in cells coinfected with (pseudo)revertant viruses, then only these latter viruses will be able to produce individual plaques. A plaque assay on BGM cells was performed to test this hypothesis. Plaques of more or less homogeneous size were observed (Fig. 5A). Five individual plaques were picked from the plates and the viruses were grown on BGM cells. Sequence analysis revealed that three isolates contained the original glutamic acid-40 (GAG). One isolate was found to contain threonine-40 (ACG). The fifth isolate revealed the occurrence of asparagine-40 (AAU), another pseudoreversion mutation. All (pseudo)revertant viruses contained the introduced AUU codon at isoleucine-38 (Fig. 2B), confirming that these viruses had originated from the 2B-E[40]K mutant (Fig. 5B).

Thus, none of the isolates contained the introduced lysine-40 (AAG), although the vast majority ($\sim 85\%$) of the viruses present in the population contained this mutation. This finding supports the idea that viruses carrying mutation 2B-E[40]K are unable to grow without the aid of *trans*-rescuing proteins of viable viruses. Moreover, this finding provides further evidence against the occurrence of a second-site suppression mutation. If viruses carrying mutation 2B-E[40]K would be able to grow due to the occurrence of a second-site suppression mutation, then we should have obtained plaques containing these viruses. The possibility that the plates did contain plaques of viruses carrying a second-site suppression mutation

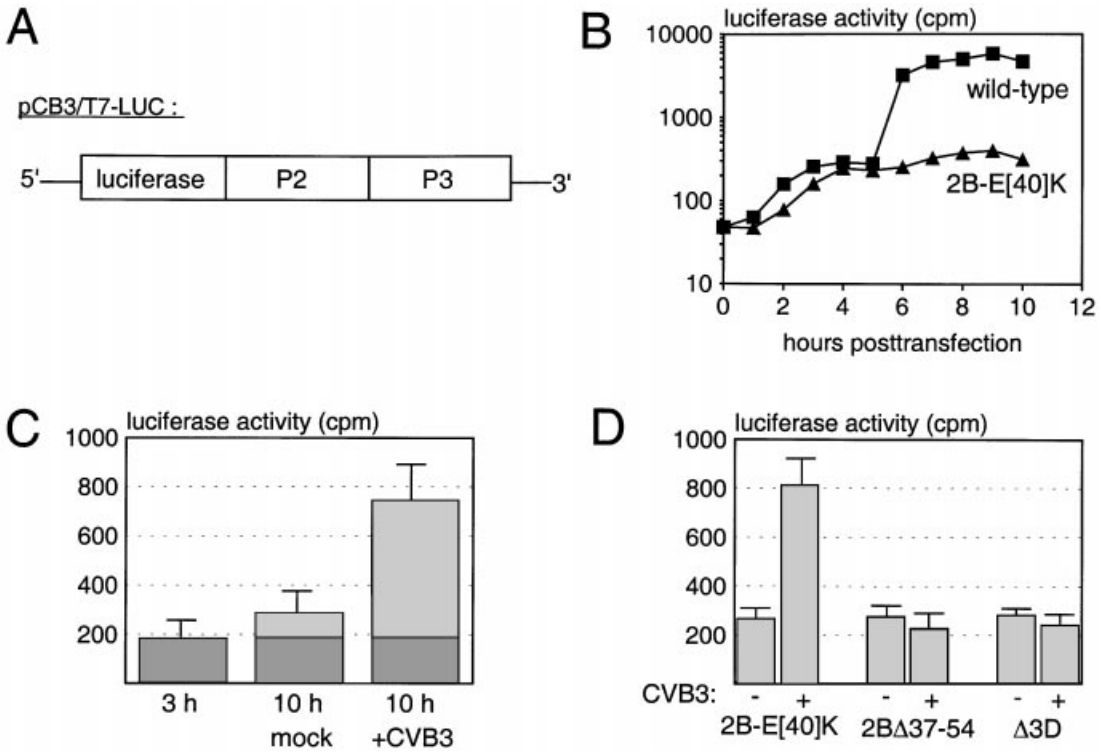

Fig. 6. (A) Schematic structure of plasmid pCB3/T7-LUC, a subgenomic replicon that contains the luciferase gene in place of the capsid coding P1 region. (B) Effect of the 2B-E[40]K mutation on RNA replication. BGM cells were transfected with copy RNA transcripts of the wild-type and the mutant replicon and the luciferase activity was measured at the indicated times post-transfection. (C) Genetic complementation assay to investigate *trans*-complementation of the defect in RNA replication caused by mutation 2B-E[40]K. BGM cells were transfected with RNA of the luciferase replicon carrying mutation 2B-E[40]K and either mock-infected or virus-infected with CBV3 wild-type virus (m.o.i. 25) at 2 h post-transfection. Luciferase activities were determined at 3 h post--transfection (a time-point at which luciferase activity reflects the translation of the input replicon RNA) and at 10 h post-transfection. Data are means ± standard error of triplicate transfected cultures. In cells infected with CBV3, a fourfold increase in RNA replication (indicated in light grey) above the RNA translation level (indicated in dark grey) was observed relative to mock-infected cells. One of four representative experiments is shown. (D) Genetic complementation assay with replicons RNAs carrying mutation 2B-E[40]K, deletion 2BΔ37–54 (deletion of the amphipathic α-helix in 2B) or deletion Δ3D (large in-frame deletion of the 3D polymerase gene). BGM cells were transfected with RNA of the luciferase replicons and either mock-infected or virus-infected with CBV3 wild-type virus (m.o.i. 25) at 2 h post-transfection. Luciferase activities were determined at 10 h post-transfection. Data are means ± standard error of triplicate transfected cultures. One of two representative experiments is shown.

and that, by sheer accident, we picked only plaques of (pseudo)revertant viruses seems unlikely [this chance is less than 0·01% when (pseudo)revertant viruses represent only 15% of the population].

Low level of *trans*-complementation of the 2B-E[40]K mutation by wild-type virus

To obtain further evidence that the defect caused by mutation 2B-E[40]K could be rescued in *trans*, a genetic complementation assay was performed. For this, the 2B-E[40]K mutation was introduced in the plasmid pCB3/T7-LUC (Fig. 6A), a subgenomic replicon that contains the luciferase gene in place of the P1 capsid coding region (van Kuppeveld *et al.*, 1995). First, the effects of the 2B-E[40]K mutation on viral plus-strand RNA replication was studied. BGM cells were transfected with RNA transcripts of the wild-type and mutant pCB3/T7-LUC constructs. Fig. 6(B) shows that the 2B-E[40]K mutation did not affect the initial increase in luciferase activity (between 1 and 4 h post-transfection) that reflects translation of the input RNA, but that the mutation interfered with the second increase in luciferase activity, which occurs from the fifth hour and reflects the replication of the input RNA and subsequent translation of the newly synthesized RNA strands (van Kuppeveld *et al.*, 1995).

To investigate the ability of wild-type virus to complement in *trans* the defect caused by the 2B-E[40]K mutation, BGM cells were transfected with transcripts carrying the 2B-E[40]K mutation and either mock-infected or virus-infected with wild-type CBV3 at 2 h post-transfection. Luciferase activities were assayed at 3 and 10 h post-transfection (i.e. at 1 and 8 h post-infection). A total of four experiments was performed. In all experiments, a threefold to fourfold increase in luciferase level was observed at 10 h post-transfection in virus-infected cells relative to mock-infected cells. Fig. 6(C) shows the results of

one representative experiment: there was roughly a fourfold increase in the level of luciferase produced in CVB3-infected cells compared to mock-infected cells (after subtraction of the level of luciferase produced by translation of the input replicon RNA). The observed increase in luciferase activity in virus-infected cells relative to that in mock-infected cells is indicative of *trans*-complementation of the defect in viral RNA replication caused by mutation 2B-E[40]K. The amount of luciferase produced in cells transfected with pCB3/T7-LUC-2B-E[40]K transcripts and subsequently superinfected with wild-type virus, however, is roughly only 10% of the amount produced in cells transfected with pCB3/T7-LUC wild-type transcripts. These data suggest that *trans*-complementation occurs but that it is rather inefficient.

To exclude the possibility that the increase in the luciferase level upon CVB3 infection was due to *trans*-activation of the CVB3 IRES in the pCB3/T7-LUC-2B-E[40]K replicon by the superinfecting virus or to increased translational efficiency due to the viral infection, we also tested two mutant pCB3/T7-LUC replicons harbouring coding region deletions that rendered the viral infectious RNA nonviable. One of these replicons contains a 2B protein with an in-frame deletion of the amphipathic α-helix (aa 37–54). The other replicon, pCB3/T7-LUC-Δ3D, contains an almost complete in-frame deletion of the 3D polymerase (van Kuppeveld *et al.*, 1995). BGM cells were transfected with RNA transcripts from pCB3/T7-LUC-2B-E[40]K mutation, pCB3/T7-LUC-2BΔ37–54, or pCB3/T7-LUC-Δ3D and either mock-infected or infected with wild-type CBV3 at 2 h post-transfection. Luciferase activities were assayed at 10 h post-transfection (i.e. at 8 h post-infection). Again, the luciferase production by replicon pCB3/T7-LUC-2B-E[40]K was increased in the virus-infected cells. No increases in luciferase production were observed upon infection of cells transfected with replicon RNAs carrying either the 2BΔ37–54 or the Δ3D deletion (Fig. 6D). These data suggest that the observed increase in luciferase production by replicon pCB3/T7-2B-E[40]K upon CBV3 infection truly reflects a low level of *trans*-complementation.

We reasoned that if *trans*-complementation of the defect in the 2B-E[40]K protein is rather inefficient, it will be unlikely that viruses carrying the 2B-E[40]K mutation will be stably maintained in the virus population upon several passages. To investigate this, the virus stock was passaged three times on BGM cells. The 2B coding region was amplified by RT–PCR and cut with *Dra*I. Fig. 7 shows that viruses carrying the 2B-E[40]K mutation were gradually deleted from the virus population upon serial passage. After one passage, the amount of viruses carrying the mutation 2B-E[40]K mutation was already decreased. After the second passage, only a very small proportion of the virus population contained the 2B-E[40]K mutation. The RT–PCR product obtained after the third passage was completely resistant to *Dra*I cleavage, indicating that viruses carrying the 2B-E[40]K mutation were deleted from the virus population. Sequence analysis of the RT–PCR product obtained after the third passage showed the presence of the original glutamic acid-40 (GAG).

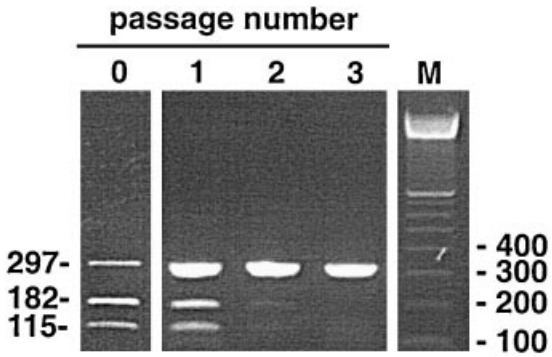

Fig. 7. Viruses carrying mutation 2B-E[40]K are deleted from the virus population upon serial passage. The virus stock was subjected to three serial passages (m.o.i. 10) on BGM cells. RNA was extracted from each passage and the 2B coding region (297 bp) was amplified by RT–PCR. The RT–PCR products were cut with *Dra*I and analysed by agarose gel electrophoresis. Lane M, 100 bp molecular mass marker. The positions of the 297 bp PCR product and its 182 bp and 115 bp *Dra*I cleavage products are indicated.

Discussion

The data presented in this study provide the first evidence for *trans*-complementation of a mutation in the enterovirus 2B protein. The mutation that was rescued in *trans* is the 2B-E[40]K mutation in the CBV3 protein 2B. This mutation caused a *qi* phenotype due to a primary defect in vRNA replication (van Kuppeveld *et al.*, 1996). On one occasion, however, RNA transfection gave rise to viruses that had retained the 2B-E[40]K mutation. In this paper, we showed that virus growth was not due to a second-site suppression mutation elsewhere in the RNA genome. Instead, we found evidence that virus growth was most likely due to *trans*-complementation of the genetic defect caused by the 2B-E[40]K mutation by the emergence of replication-competent revertant and pseudo-revertant viruses in the transfection-derived virus stock. We found that only the (pseudo)revertant viruses were able to produce individual plaques. Furthermore, a genetic complementation assay demonstrated a low level of *trans*-complementation of the debilitated function of the 2B-E[40]K mutant protein by wild-type virus proteins. Taken together, these findings are consistent with the idea that following transfection of RNA carrying mutation 2B-E[40]K, reversion mutation and pseudoreversion mutations did arise that restored a *trans*-acting function of the 2B protein (or its precursor protein 2BC) and, thereby, rescued the replication defect caused by mutation 2B-E[40]K.

Genetic complementation of mutants mapping to the 2B protein has not been described before. The defects in vRNA synthesis caused by linker insertions at amino acid positions 28 and 34 of the poliovirus 2B protein were found to be noncomplementable (Bernstein *et al.*, 1986; Li & Baltimore,

1988; Johnson & Sarnow, 1991). Mutations in a hydrophobic domain (aa 63–80) of the CBV3 protein 2B were also found to be *cis*-dominant (van Kuppeveld *et al*., 1995). It should be emphasized that the level of *trans*-complementation observed in this study was very low. Such a low level of *trans*-complementation may also be considered as inefficient and interpreted as evidence for a preference for the protein to function in *cis*. Notwithstanding this fact, the low level of *trans*-complementation was relevant in supporting the growth of viruses carrying the 2B-E[40]K mutation.

Efficient *trans*-complementation of genetic defects in the nonstructural proteins has thus far been observed only with mutations that render the RNA genome replication competent, albeit in a defective or conditional-lethal manner. Efficient *trans*-complementation of non-self-replicating genomes is rare. The only direct example of *trans*-complementation of a lethal mutation has been reported by Cao & Wimmer (1995), who were able to rescue a lethal mutation in the poliovirus 3AB protein using an intragenomic complementation procedure. This intragenomic complementation was very inefficient and the intragenomic recombinants displayed only a *qi* phenotype. Teterina *et al*. (1995) examined rescue of lethal 2C mutations in poliovirus and observed up to a tenfold increase of synthesis of the mutated RNA upon cotransfection with wild-type helper RNA. However, the maximal levels of the mutated RNA represented still only a fraction of the level of the wild-type virus RNA. Therefore, they concluded that *trans*-complementation did occur, but was very inefficient, and that the RNA shows a marked preference for the 2C protein provided in *cis*. Giachetti *et al*. (1992) observed low levels of *trans*-complementation of nonviable mutations targeted to the hydrophobic domain of the poliovirus 3A protein and concluded that the 3A protein cannot be provided in *trans*. Unfortunately, in these latter two studies it was not examined whether this low level of *trans*-complementation was sufficient to enable the emergence of mutant viruses. In this study, we observed that even a low level of *trans*-complementation of a *qi* mutation in the 2B protein was sufficient to enable virus growth. Taken together, these observations indicate that although some functions of the enterovirus 2B, 2C (or their precursor 2BC) and 3A proteins exhibit a clear *cis*-preference, these functions are not absolutely *cis*-dominant.

The identification of both *cis*-acting and *trans*-acting functions of the enterovirus 2B protein most likely reflects the multifunctional nature of this protein (or its precursor 2BC). The identity of these *cis*-acting and *trans*-acting functions remains to be established. It is as yet unclear whether the 2B-E[40]K mutation exerts its effect at the level of the precursor 2BC or at the level of the mature cleavage product 2B. The *qi* phenotype of this mutation and the emergence of (pseudo) revertant viruses argue that this mutation does not abolish the ability of the 2BC protein to induce the proliferation of the membrane vesicles that build the virus replication complex. At present, the function of the 2B protein in the enteroviral life-cycle is unknown. Individual expression of the 2B protein results in alterations in host cell membrane permeability (Doedens & Kirkegaard, 1995; Aldabe *et al*., 1996; van Kuppeveld *et al*., 1997a, b), the disassembly of the Golgi complex (Sandoval & Carrasco, 1997) and, possibly as a consequence, the inhibition of protein secretion (Doedens & Kirkegaard, 1995; van Kuppeveld *et al*., 1997b). Through the individual expression of mutant 2B proteins, we found that the activities of protein 2B to modify plasma membrane permeability and to inhibit protein secretion may represent two different functions, rather than that one effect is the consequence of the other (van Kuppeveld *et al*., 1997b). Individual expression of the 2B-E[40]K protein revealed that this protein exhibited a wild-type activity in permeabilizing the plasma membrane to hygromycin B, but a reduced activity (about 60% relative to the wild-type 2B protein) in inhibiting protein secretion (F. J. M. van Kuppeveld, W. J. G. Melchers, K. Kirkegaard & J. R. Doedens, unpublished data). The glutamic acid-40 residue is located in the hydrophilic part of a predicted cationic amphipathic α-helix, a well-conserved structural element in the enterovirus 2B protein (van Kuppeveld *et al*., 1996). In all enterovirus 2B proteins, this hydrophilic part is formed by three positively charged residues (most often lysines), one glutamic acid residue and a number of polar residues. Cationic amphipathic α-helical peptides have been implicated in the permeabilization and destabilization of membranes (Bernheimer & Rudy, 1986; Segrest *et al*., 1990). Previously, we demonstrated that both the cationic character and the amphipathic character of the α-helix are required for the membrane permeabilization function as well as for the secretion inhibition function of the 2B protein (van Kuppeveld *et al*., 1997b). That the E[40]K mutation does not disrupt the membrane permeabilizing function of the 2B protein is not surprising, because this mutation disrupts neither the cationic nature nor the amphipathy of the α-helical domain. The finding that the E[40]K mutation specifically interfered with the ability of the 2B protein to inhibit protein secretion is remarkable and points to an important role of the aa-40 residue in this function. It is tempting to speculate that the secretion inhibition function represents the *trans*-acting function of the 2B protein. However, this suggestion must be taken with care, as it cannot be excluded that another, yet unidentified, function, which may represent the *trans*-acting function, is affected by the E[40]K mutation as well.

In summary, we have provided the first evidence for *trans*-complementation of a genetic defect in the enterovirus 2B protein. The identity of the *trans*-acting function of the 2B protein may be the secretion inhibition function, but this awaits further investigation. Understanding the *cis*-acting and *trans*-acting functions of the 2B protein requires the elucidation of how the activities of the 2B protein to modify membrane permeability and to manipulate the protein secretion machinery contribute to the process of vRNA replication and/or other steps in the virus life-cycle.

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