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Genetic analyses of the terminal protein (VPg) and of spacer II in the 5'-nontranslated region of poliovirus RNA

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Abstract of the Thesis

Genetic analyses of the terminal protein (VPg) and of spacer II in the 5'-nontranslated region of poliovirus RNA

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VPg is a small protein (20-30 amino acids long), which is present in every virus of the *Picornaviridae* family. A hydroxyl group of tyrosine at position 3 of VPg is covalently linked to the 5'-terminal UMP of genomic Poliovirus RNA via a phosphodiester bond and is crucial for virus viability. Poliovirus mutants in whom this tyrosine is substituted with a phenylalanine are quasi-infectious if threonine is present at position 4, which also has a hydroxyl group. The aim of the first part of this research project was to determine whether serine can also serve as an acceptor of UMP. An F3S4 mutant was constructed, which has a double mutation at this crucial position. This F3S4

mutant was found to be quasi-infectious and gave revertants to Y3T4 (WT) and Y3S4 after the first passage. Furthermore, mutants S3A4, S3T4 were also made and tested to determine whether serine at position 3 could also act as a substrate for uridylylation. The results of these experiments showed that both S3A4 and S3T4 are mutants with a severe replication defect, leading to a lethal growth phenotype, which suggests that serine at position 3 could not be uridylylated.

The second project refers to the spacer II region of the 5'-nontranslated region of the Poliovirus whose function is not yet known. Interestingly, this spacer contains a region (Box C), which is 12 nucleotides long and conserved among all members of *Enteroviruses*. These 12 nucleotides were deleted in order to test the viability and growth phenotype of this mutant. Furthermore, the growth kinetics of the spacer II mutant and the poliovirus wild-type were compared in different cell lines at different temperatures. The results showed no differences in either the plaque phenotype or the growth kinetics between the spacer II mutant and poliovirus wild-type, an observation suggesting that the highly conserved region is dispensable under the conditions of the experiments.

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List of abbreviations

- CNS: Central nervous system
- CPE: Cytopathic effect; rounding up of the host-cells by replication of the infective virus
- Gnd-HCl: Guanidine hydrochloride
- HeLa: Cell line from cervical cancer; The Name stands for the initials of the patient Henrietta Lacks
- hPVR: Human Poliovirus receptor
- IRES: Internal ribosome entry site
- L20B: Cell-line of mouse cells
- LB-Media: Luria Broth media; used for growth of e.coli bacteria
- MOI: Multiplicity of infection; infective particles per cell
- mRNA: Messenger RNA
- NTR: Non-translated region
- ORF: Open-reading frame
- PFU: Plaque forming units
- PV: Poliovirus
- R-Luc: Renilla-Luciferase; a fluorescent protein similar in its function as the green fluorescence protein
- RT-PCR: Reverse transcriptase polymerase chain reaction
- UMP: Uracil mono phosphate
- VPg: Viral protein genome-linked
- WHO: World health organization

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1. Historical background of Poliovirus

Poliovirus, discovered in 1909 by Karl Landsteiner, is the cause of *poliomyelitis*, a devastating neurological disease that has threatened humankind for centuries. However, the first epidemic outbreaks were not reported until improvements in hygienic standards during the process of industrialization. Usually infants had their first contact with poliovirus in their early days where they were still protected by maternal antibodies. With increased hygienic standards, this protection disappeared because either the mother was not infected with poliovirus so she could not give maternal antibodies to the child or the first contact with poliovirus occurred when protection was already gone. This led to epidemic outbreaks in the early 20th century that became a curse in industrialized countries.

PV infects via the fecal-oral-route. After infection, the virus replicates in the alimentary tract. In about 95% of infections, poliovirus does not leave the alimentary tract and infection is asymptomatic. In approximately 5% of cases, the virus spreads to other parts to replicate, e.g. in muscles. Minor symptoms like fever and headache ensue. In only 1% of cases, the virus enters the central nervous system (CNS), where it replicates in the motor neurons, leading to their destruction. This leads to temporary or permanent paralysis of the victim. In very rare cases, paralytic poliomyelitis leads to death. It is important to note that the virus does not have any known advantage to entering the CNS. Therefore the virus entering the CNS could be considered an “accident”.

Fortunately, in the 1950s two effective vaccines were developed: the inactivated vaccine by Jonas Salk and the attenuated live vaccine by Albert Sabin. In fact these vaccines were so promising that the WHO announced in 1988 a world-wide eradication campaign of poliovirus by 2000 (WHO 1988). Even if the last reported cases in the western world happened decades ago and parts of the world were declared poliovirus-free (USA 1994, Europe 2002) the challenge is

not yet won. Especially in countries of Africa and in India poliovirus still causes epidemic outbreaks. Time will tell if the goal of the WHO will succeed to eradicate poliovirus but through instable political circumstances success may move into the second half of this century.

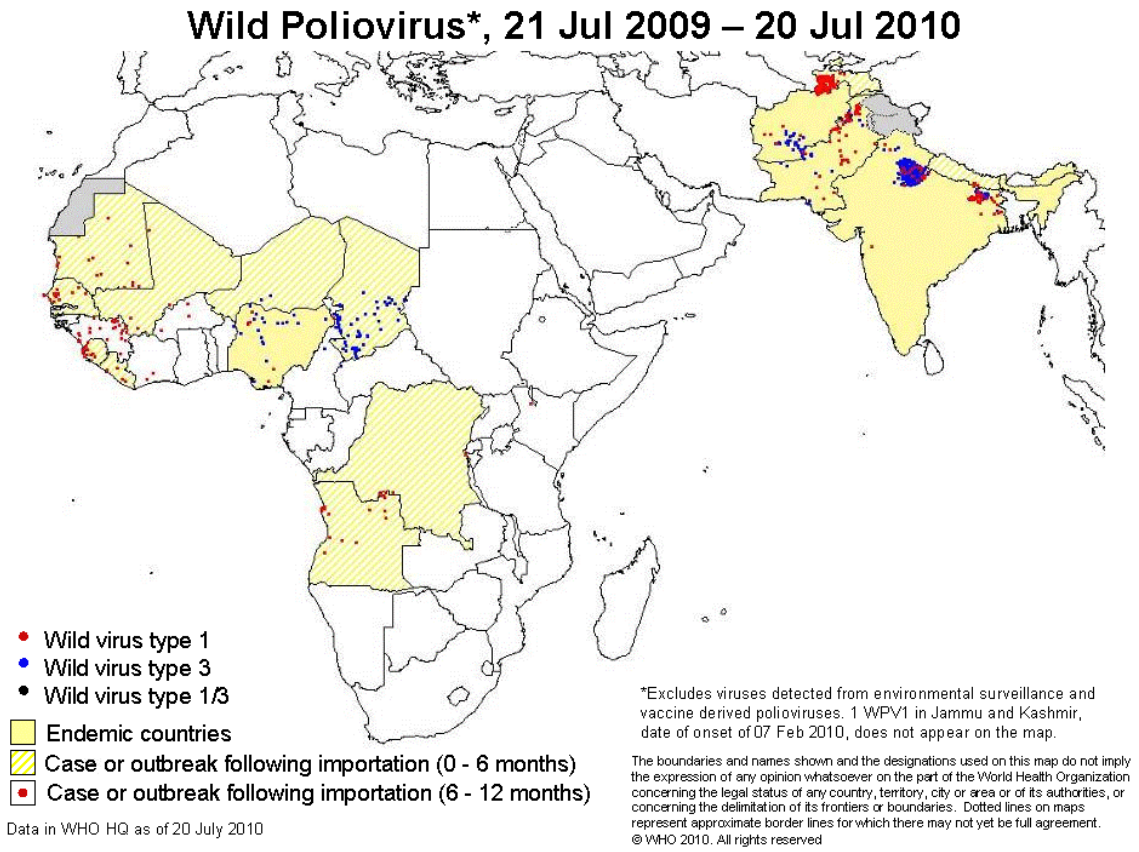

Fig. 1 – Reported cases of wild Poliovirus from 07/21/09 to 07/20/10 based on WHO statistics. Type 1 cases are indicated in red, type 3 in blue, and both types in black (WHO 2010). Epidemic countries are highlighted yellow. Picture copied with kind permission of the WHO.

Beside of the development of working vaccines a lot of important discoveries were made with poliovirus, which affected the discipline of virology as well as our understanding of special aspects of biochemistry. This work was possible especially because poliovirus can be considered one of the easiest virus systems to propagate and study. Noteworthy discoveries were:

- Development of the plaque-assay (Dulbecco and Vogt 1954)
- Confirmation of RNA as heritable information in poliovirus (Schaffer and Schwerdt 1955)
- Introduction of SDS-gel-electrophoresis for protein analysis (Jr. 1964)
- Discovery of the polyprotein (Jacobson and Baltimore 1968)
- Discovery of viral proteinases for protein cleaving (Palmenberg, Pallansch et al. 1979; Toyoda, Nicklin et al. 1986)
- Synthesis of infectious RNA from cDNA with a T7-transcriptase (van der Werf, Bradley et al. 1986)
- Discovery of the internal ribosome entry side (IRES) (Jang, Krausslich et al. 1988; Pelletier and Sonenberg 1988; Jang, Davies et al. 1989)
- Cell-free synthesis of the Poliovirus (Molla, Paul et al. 1991)
- Cell-free synthesis of the Poliovirus without a natural template (Cello, Paul et al. 2002)

2. Theoretical background

a. PV-Genome

Poliovirus is a member of the *Picornaviridae* virus family. Its genomic structure consists of a single-stranded positive sense RNA, which is approximately 7,500 nucleotides in length (Kitamura, Semler et al. 1981). The genome is divided into 3 main parts, as shown in **Fig. 2**:

1) The non-translated region (NTR) at its 5'-end containing the cloverleaf and the internal ribosome entry site (IRES)
2) A single open reading frame (ORF), which encodes a polyprotein
3) The 3'-NTR with a poly(A)-tail

The 5’-NTR is covalently bound to a small viral protein (VPg) (Lee, Nomoto et al. 1977; Rothberg, Harris et al. 1978). VPg is about 22 amino acids long and is present in all picornaviruses. This protein functions as a primer for the RNA polymerase, $3D^{pol}$, during RNA synthesis (Paul, van Boom et al. 1998). The linkage is a phosphodiester between the hydroxyl group of tyrosine in VPg and the 5’-phosphate of the terminal UMP in the viral RNA (Rothberg, Harris et al. 1978). Since this uridylylation-site is one of the subjects of this thesis it will be discussed in detail in the chapter “Motivation of this study” on page 9. The 5’-NTR itself contains 743 nucleotides, which is relatively long in comparison to the entire size of the genome. Since poliovirus does not have a proofreading enzyme, it is in the interest of the virus to keep its genome relatively short to stay under the usual error rate of replication, which lies in the range of 10^{-3} to 10^{-4}.

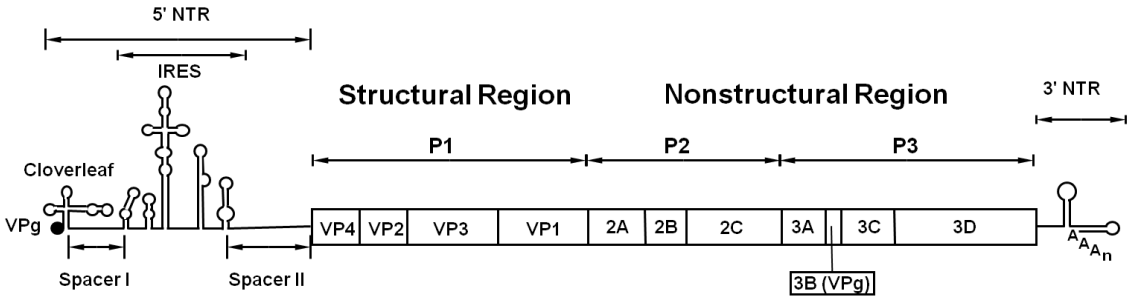

Fig. 2 – Schematic view of the Poliovirus genome (De Jesus 2007). The 5’-NTR, shown on the left, is linked to VPg. The 5’-NTR contains the cloverleaf and the IRES. The 3’-NTR with its two hairpins and the poly(A)-tail is shown on the right. Between the two NTRs, the genome contains a single open-reading frame, which encodes the polyprotein

Therefore it might be surprising that almost 10% of the viral genome is used for the 5’-NTR. However, in picornaviruses this region is needed for translation and replication. Usually mRNAs have a cap-structure at their 5’-end. This cap-structure binds to the cap-binding subunit eIF-4E of the translation initiation factor eIF-4F. Because of the bond to VPg the poliovirus genome does not have any such cap-structure. However, VPg is cleaved off the RNA by a cellular phosphodiesterase for unknown reasons prior to cellular translation. To initiate translation poliovirus evolved a region that recruits the ribosomes: the IRES. This remarkable element is a part of the 5’-NTR. The 5’-NTR is divided into 6 domains, all with complex secondary structures (Ehrenfeld and Semler 1995;

Beales, Holzenburg et al. 2003). Domain I is the cloverleaf, a structure required for RNA replication (Andino, Rieckhof et al. 1990). Domains II to VI are the building blocks of the IRES, which initiates translation and was first identified in Encephalomyocarditis virus (Jang, Krausslich et al. 1988; Jang, Davies et al. 1989). Between domains I and II there is a spacer (Spacer I) without significant secondary structure but which is an essential domain for replication. It is noteworthy that the Sabin vaccines contain attenuating mutations in the IRES. Between the IRES and the beginning of the translated region is another spacer (spacer II) whose function remains unknown. In fact, this spacer starts with a cryptic AUG-codon at position 586 and continues to the translation-initiating AUG-codon at position 743. Since the second project of this research focuses on this region it will be discussed in detail later as well.

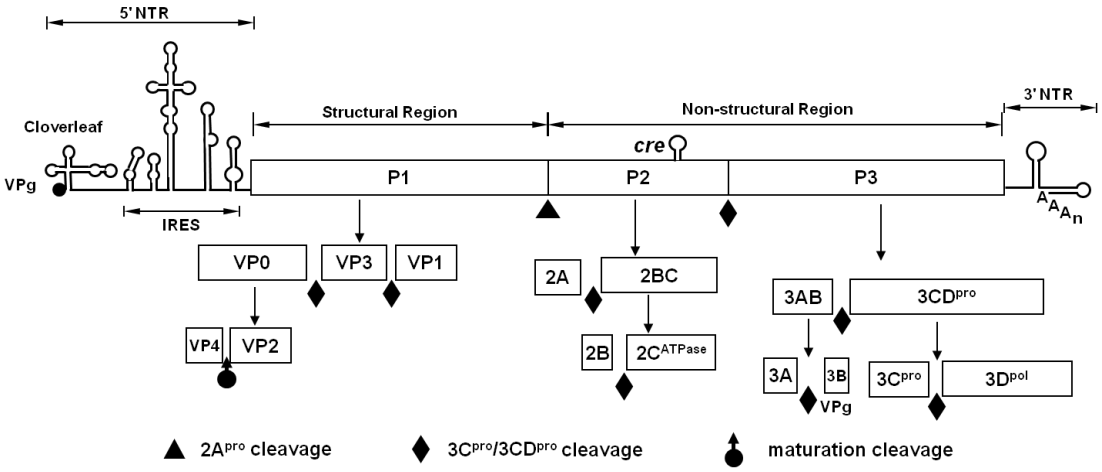

Fig. 3 – Genome structure of Poliovirus and processing of the Poliovirus polyprotein: The first cleavage occurs by $2A^{pro}$ between the structural (P1) and the non-structural regions (P2 and P3). Afterwards P2/P2 is separated into regions P2 and P3 by $3CD^{pro}$. Domains P1, P2 and P3 are cleaved afterwards by $3C^{pro}/3CD^{pro}$ into precursors and mature proteins. The last cleavage, which is the maturation process, occurs by an unknown mechanism where VP0 is cleaved into VP2 and VP4

The middle part of the PV genome is the translated region. This region contains a single ORF encoding a polyprotein of approximately 247 kDa (Jacobson and Baltimore 1968; Summers and Maizel 1968). This polyprotein is processed into precursor and mature proteins as shown in **Fig. 3**. It is noteworthy that specific amino acid sequences for proteolytic cleavage were found through mutations,

which led to the conclusion that there are even more cleavage sites than actually used. It is believed that some cleavage sites remain inactive because of steric effects (Krausslich and Wimmer 1988; Wimmer, Hellen et al. 1993).

The last part of the poliovirus genome is the 3'-NTR. Only little is known about this region. Its end is polyadenylated with approximately 60 to 120 adenine residues (Yogo and Wimmer 1972). It has been shown that this 3'-NTR region plays an important role also in viral RNA translation and replication (Pierangeli, Bucci et al. 1995; Meredith, Rohll et al. 1999; Brown, Cornell et al. 2005). In addition it has been shown that deletion of this region has a highly detrimental effect for virus viability in HeLa-cells but an even more dramatic effect is observed with neuronal cells both *in vivo* and *in vitro* (Brown, Kauder et al. 2004).

b. Life cycle

The cellular life cycle of poliovirus is schematically illustrated in **Fig. 4**. It begins with the interaction of the infectious particle with the cell surface receptor CD155 (1), also called the human PV receptor (hPVR) (Mendelsohn, Wimmer et al. 1989; Selinka, Zibert et al. 1991). It is believed that the receptor is binding to the canyon of the capsid, which causes a destabilization of the entire capsid (Hogle 2002) resulting in RNA release into the cell's cytoplasm (2). After entry VPg is cleaved off the RNA by a yet unknown cellular phosphodiesterase. After this step translation is initiated by host cell ribosomes (3), first generating the polyprotein. This polyprotein is proteolytically cleaved into the 3 precursors P1, P2 and P3, which are subsequently cleaved into precursor and mature proteins (4). Processing occurs mainly through the viral proteinases $2A^{pro}$ and $3C^{pro}/3CD^{pro}$. In the end of this maturation process eleven proteins are obtained. It is noteworthy that the virus uses both mature and precursor proteins so it can keep its genome

short. These viral proteins form a replication complex together with cellular proteins in which the positive-sense RNA first serves as a template to synthesize a complementary negative-sense RNA yielding a double-stranded RNA (5). This double-stranded RNA is called replicative form (RF). Using the negative-strand RNA in the RF as a template many positive-strand copies are synthesized (6), which are called replicative intermediates (RIs). Several RIs are synthesized at the same time overlapping on the template as illustrated in (6). The final product is a plus strand progeny RNA. This RNA now has the possibility of acting as a template for translation as in step 3 (7) or could be encapsidated (8) to form infectious particles, which in the end are released by cell lysis (9). Each cell produces approximately 25.000 to 50.000 new infectious particles.

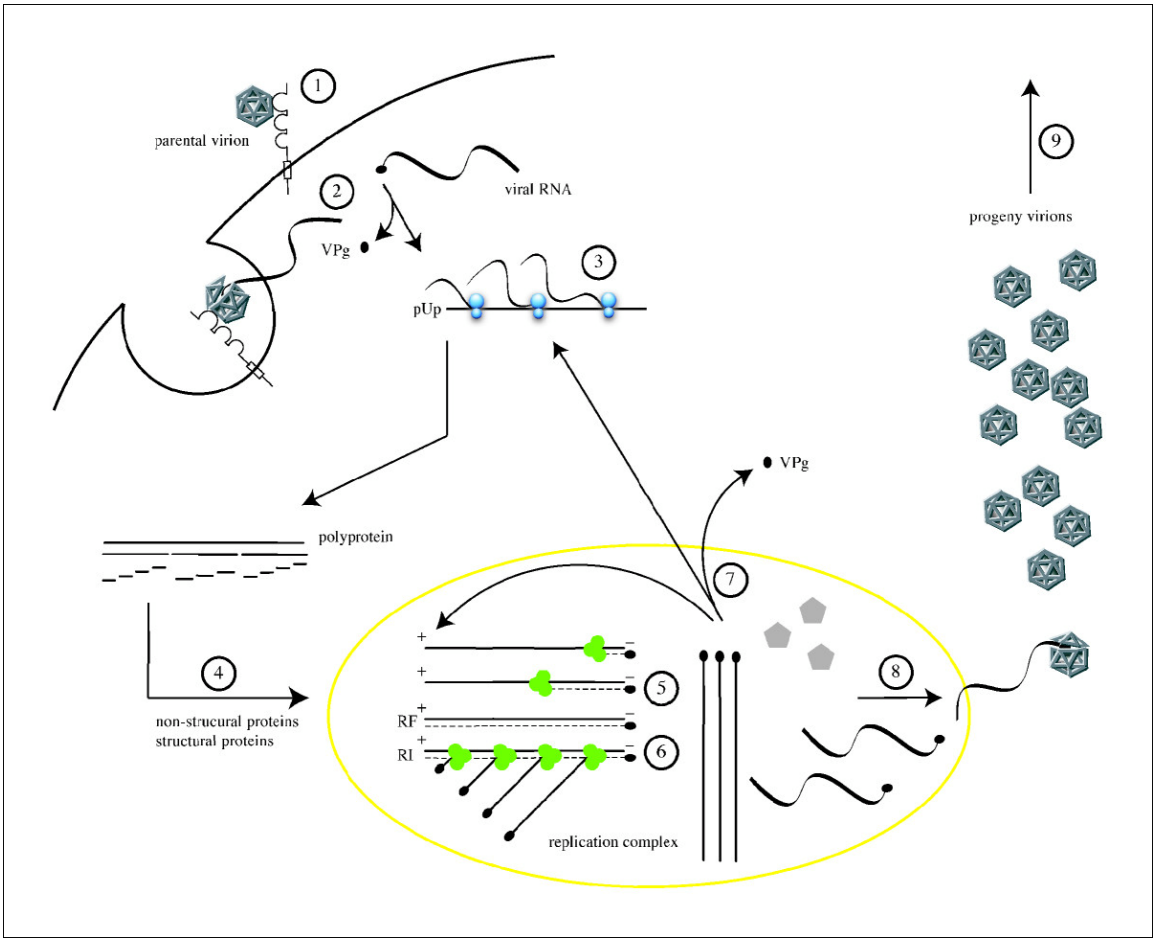

Fig. 4 – The cellular life cycle of Poliovirus (De Jesus 2007). The Poliovirus life cycle is illustrated from entry into the cell until the release of newly synthesized infectious particles when the cell lyses. All steps are described in detail in the text.

For the process of encapsidation a few notes have to be given since it involves several steps. The first step of encapsidation is the cleavage of the capsid protein precursor P1 by $3CD^{pro}$. This cleavage yields the capsid proteins VP0, VP1 and VP3. They assemble to a protomer (Wetz 1987); five of these protomers arrange to form a pentamer (Phillips and Fennell 1973). Afterwards twelve of these pentamers form together a procapsid (Jacobson and Baltimore 1968). It is believed that the pentamers interact with protein 2C in the replication complex and that they assemble around the replicated RNA. In this model the procapsid would be an unused byproduct. The last step is the cleavage of VP0 to VP2 and VP4, which may happen by auto-catalysis. This last step finishes the maturation process and leads to infective particles.

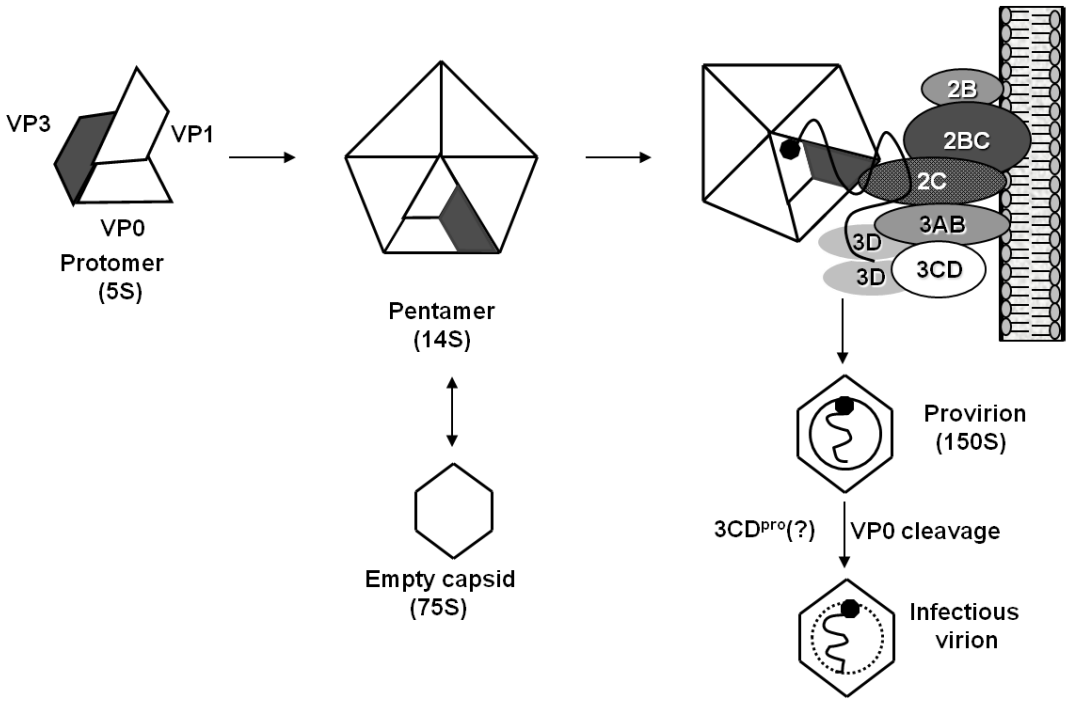

Fig. 5 – Process of RNA encapsidation. First the protomer (5S) is formed from VP0, VP1 and VP3. 5 protomers form a pentamer (14S), which can assemble to the empty capsid (75S), or encapsidate the replicated RNA by interaction with 2C to form the Provirion (150S). VP0 cleavage is the maturation process, which yields in the infectious virion. The mechanism of this maturation process is not fully understood.

3. Motivation of this study
    a. Mutations in VPg

It was mentioned before that at the 5'-end of the poliovirus genome the viral RNA is bound to a small protein, called VPg. This protein occurs in every virus of the family *Picornaviridae*. It is noteworthy that all picornaviral VPgs are between 20-23 amino acids long but their amino acid sequences are not well conserved. An amino acid, which is conserved in every VPg, is tyrosine at position 3 where binding of the RNA occurs. The structure of poliovirus VPg was solved recently (Schein, Oezguen et al. 2006; Schein, Volk et al. 2006; Schein, Oezguen et al. 2010) and is presented in **Fig. 6**.

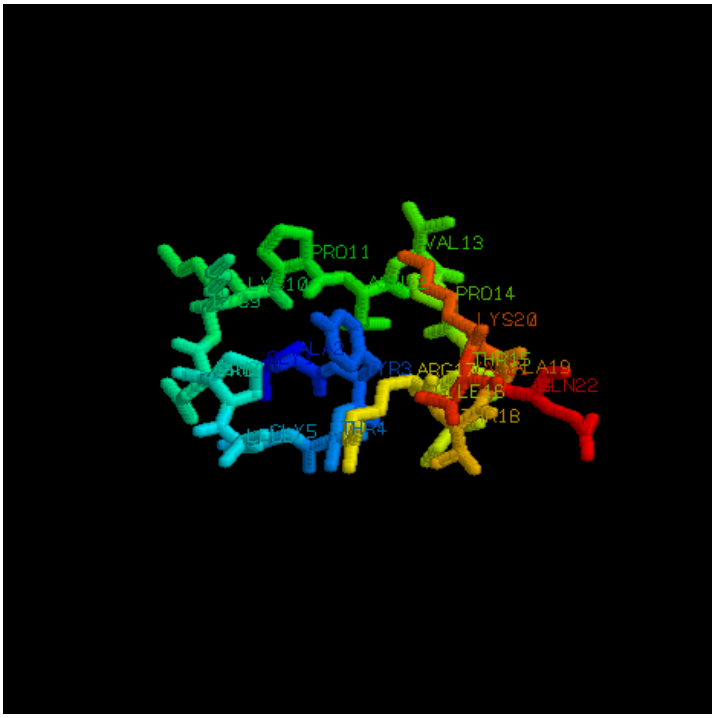

Fig. 6 – NMR structure of VPg. The structure of VPg was measured in a stabilizing solvent (Schein, Oezguen et al. 2006; Schein, Volk et al. 2006; Schein, Oezguen et al. 2010). Tyrosine at position 3 in the middle of the protein is shown in blue.

Several mutations have been done in the VPg of poliovirus. Some of them had almost no (K10R (Kuhn, Tada et al. 1988)) or just little effect on virus-growth

(T4A. N8A, K10A, P11A (Schein, Volk et al. 2006)) while some others grew very poorly (P7A, K20E (Schein, Volk et al. 2006)) or were reported to be dead (Y3T/T4Y, Y3S, G5P, K9A/K10A (Reuer, Kuhn et al. 1990; Schein, Volk et al. 2006)). The growth phenotypes of these VPg mutations were clearly related to their ability to serve as substrates for uridylylation in vitro (Paul, Peters et al. 2003). It also has been shown that VPgs of other viruses could be inserted into the PV-genome to form chimeras, which could yield viable virus (Reuer, Kuhn et al. 1990).

Table 1 – Sequences of VPgs of selected Enteroviruses

Virus	VPg amino-acid sequence
Poliovirus 1	**G**A**Y**T**G**L**P**NKKPNV**P**TI**R**TAKV**Q**
Poliovirus 2	**G**A**Y**T**G**L**P**NKKPNV**P**TI**R**TAKV**Q**
Poliovirus 3	**G**A**Y**T**G**L**P**NKRPNV**P**TI**R**AAKV**Q**
Enterovirus 70	**G**P**Y**T**G**L**P**NQKPKV**P**TL**R**TAKV**Q**
Enterovirus 71	**G**A**Y**S**G**A**P**NQVLKK**P**VL**R**TATV**Q**
CAV9	**G**A**Y**T**G**I**P**NQKPKV**P**TL**R**QAKV**Q**
CAV16	**G**A**Y**S**G**A**P**KQTLKK**P**IL**R**TATV**Q**
CBV1	**G**A**Y**T**G**M**P**NQKPKV**P**TL**R**QAKV**Q**
CBV5	**G**A**Y**T**G**M**P**NQKPKV**P**TL**R**QAKV**Q**
Rhino1A	**G**P**Y**S**G**E**P**KPKTKV**P**E.**R**RIVA**Q**
Rhino2	**G**P**Y**S**G**E**P**KPKTKI**P**E.**R**RVVT**Q**

For this project by far the most interesting mutation is Y3F, which led to the mutant F3T4. This mutation was shown to be quasi-infectious (Cao and Wimmer 1995) while mutant F3A4 was dead. This finding led to the idea that if a tyrosine at position 3 is not available the hydroxyl group at position 4 could act as an acceptor for the UMP. This suggestion was supported by the finding that Y3A4 is viable.

Recently a mechanism was proposed which includes a nucleophilic attack of the hydroxyl group of tyrosine 3 on the α-phosphate of UTP. It is possible that if the hydroxyl group of tyrosine is not available (due to a mutation) then the hydroxyl group at position 4 (threonine) takes its place to bind UMP.

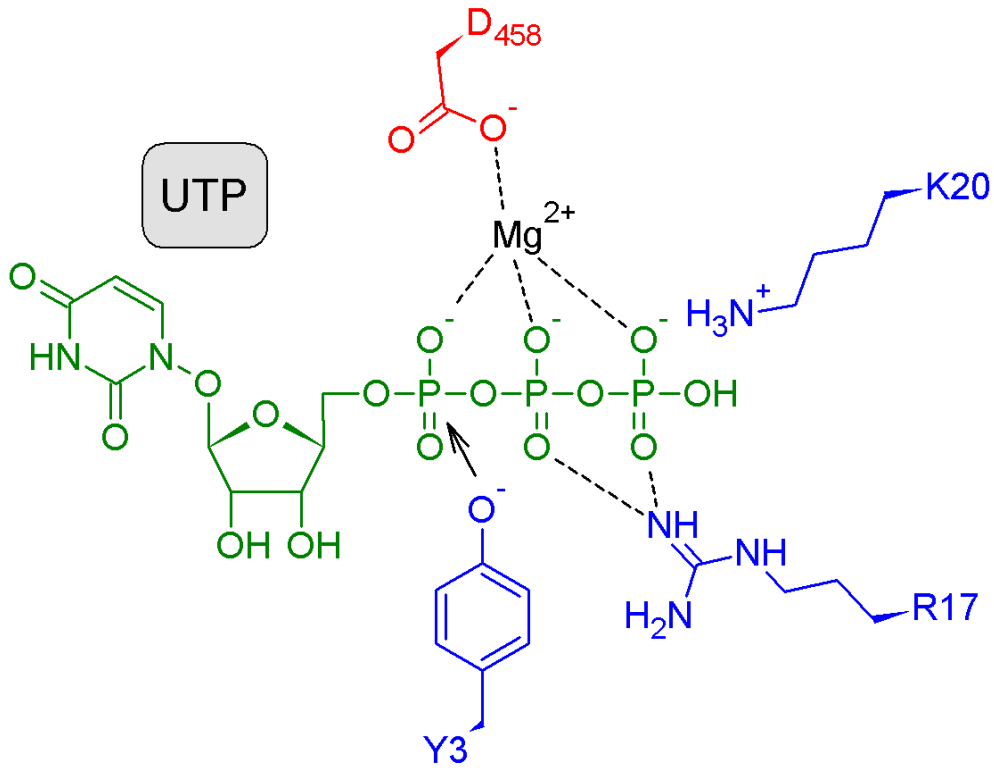

Fig. 7 – Proposed mechanism of VPg uridylylation. VPg uridylylation is proposed to occur by nucleophilic attack of tyrosine at position 3 at the α-phosphate of UTP **(Schein, Volk et al. 2006)**; The γ-phosphate might interact with the arginine at position 17 or lysine at position 20 of VPg. Aspartic acid at position 458 of $3D^{pol}$ forms a complex with magnesia.

To confirm these given results the construct F3S4 was tested containing a double mutation. The hope was that this mutant would show the same phenotype as F3T4, which would prove the theory of a UMP-acceptor at position 4 of VPg. This possibility is supported by the fact that in the plant cowpea mosaic virus, which is closely related to the poliovirus in its genomic structure, the VPg is uridylylated at a serine residue (Drygin, Sapotsky et al. 1987).

b. The mystery of Box C at Spacer II

As mentioned before, poliovirus aims to keep its genome as short as possible since it has no ability of proof-reading during replication. Therefore it is surprising that between a cryptic AUG-codon in domain VI of the IRES and the AUG-codon, which initiates translation, there is another spacer (Spacer II), which has no known function yet. Furthermore, Spacer II consists of highly conserved parts, e.g. one at the cryptic AUG-codon, which is called Box B and one called Box C (**Fig. 8**). Box C is the most notable region in Spacer II. This box consists of 12 nucleotides (^{621}UUGGAUUGGCCA), which are conserved in their sequence among almost all *Enteroviruses*. Moreover, the length of spacer II (154 nucleotides) is, surprisingly, also conserved in all three type polioviruses.

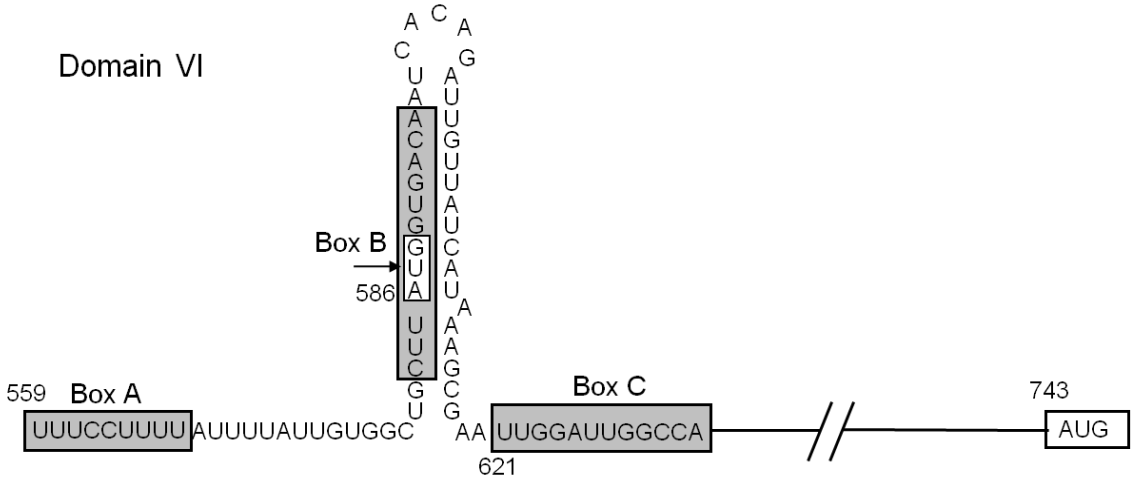

Fig. 8 – Enlarged view of Spacer II in the 5'-NTR of Poliovirus. Spacer II, which begins with the cryptic AUG-codon at position 586, is shown in detail. The cryptic ^{586}AUG is surrounded by a highly conserved region, which is called Box B and highlighted gray. The 12 nucleotides of Box C, which are important for this project, are beginning at position 621 and are highlighted with a gray box. The nucleotide sequence between box C and the initiating AUG-codon is not shown.

To test whether or not Box C has a specific function I wanted to test the viability of a mutant which has this entire Box C deleted. It has already been shown that mutants are viable where spacer II was modified by insertions of large fragments, even including one AUG-codon or deletions (Iizuka, Kohara et al. 1989; Gmyl, Pilipenko et al. 1993; Hellen, Pestova et al. 1994; Slobodskaya, Gmyl et al.

1996). However, so far not a single mutation has been targeted to box C, whose function is not known in any of the *Enteroviruses*.

Table 2 – Sequences of parts of spacer II of Poliovirus types 1-3 and of other selected Enteroviruses.*

Virus	Sequence
Poliovirus type 1	^{616}GCGAAUUGGAUUGGCCAUCCGG
Poliovirus type 2	^{621}GCAAAUUGGAUUGGCCAUCCGG
Poliovirus type 3	^{619}GCGAGUUGGAUUGGCCAUCCAG
CAV 2	^{621}AGCUAUUGGAUUGGCCAUCCGG
CAV 3	^{621}AGCUAUUGGAUUGGCCAUCCGG
CAV 4	^{626}AGCUAUUGGAUUGGCCAUCCGG
CAV 5	^{625}AGCUAUUGGAUUGGCCAUCCGG
CAV 6	^{626}AGCUAUUGGAUUGGCCAUCCGG
CAV 7	^{621}AGCUAUUGGAUUGGCCAUCCGG
CAV 8	^{622}AGCUAUUGGAUUGGCCAUCCGG
CAV 9	^{622}AGCUAUUGGAUUGGCCAUCCGG
CAV 10	^{622}AGCUAUUGGAUUGGCCAUCCGG
CAV 12	^{624}AGCUAUUGGAUUGGCCAUCCGG
CAV 13	^{619}GCGAAUUGGAUUGGCCAUCCGG
CAV 19	^{616}AGCUUUUGGAUUGGCCAUCCGG
CAV 22	^{617}AGCUUUUGGAUUGGCCAUCCGG
CBV 1	^{620}AGUUAUUGGAUUGGCCAUCCGG
CBV 2	^{621}AGCUAUUGGAUUGGCCAUCCGG
CBV 6	^{621}AGCUAUUGGAUUGGCCAUCCGG
Enterovirus 2	^{620}AGUUAUUGGAUUGGCCAUCCAG
Enterovirus 3	^{621}AGCUAUUGGAUUGGCCAUCCGG
Enterovirus 4	^{624}AGCUAUUGGAUUGGCCAUCCGG
Enterovirus 5	^{625}UUGGAUUGGAUUGGCCAUCCAG
Enterovirus 7	^{621}AGCUAUUGGAUUGGCCAUCCGG
Enterovirus 71	^{618}AGCUAUUGGAUUGGCCAUCCGG

* CAV: coxsackie A virus; CBV: coxsackie B virus

4. Results

a. Mutations at the uridylylation site of VPg

As discussed before, the initial experiment was to confirm that the tyrosine is crucial at position 3 of VPg for viral viability. In addition, the aim was to show that the serine at position 4 can act as an acceptor of UMP if the hydroxyl group in position 3 is not available. Serine would then play the role of a threonine just as in mutant F3T4.

Table 3 – Overview of VPg-mutants and their sequences used in this study. Changes in nucleotide- and amino-acid-sequences are highlighted.

Construct	Name	Nucleotide seq.*	Amino-acid seq.
PV-VPg-WT	PV-WT	^{5375}GCA TAC ACT GGT	G A Y T G ..
PV-VPg(Y3F/T4S)	F3S4	^{5375}GCA TTC AGT GGT	G A F S G ..
PV-VPg(Y3S/T4A)	S3A4	^{5375}GCA TCC ACG GGT	G A S A G ..
PV-VPg(Y3S)	S3T4	^{5375}GCA TCC ACG GGT	G A S T G ..
PV-VPg(T4S)	Y3S4	^{5375}GCA TAC AGT GGT	G A Y S G ..

* Changes in nucleotide- and amino-acid-sequences are highlighted

To test the phenotype of the F3S4 mutant, HeLa-cells were transfected in 3 independent series with 3 different amounts of transcript RNAs (5, 10 and 20 µl of the transcription-reaction [TrRNA]). The results are presented below (Table 4). As expected, the F3S4 construct did not show cytopathic effects (CPE) after the initial transfection. Surprisingly, already after the first passaging 6 out of 9 plates clearly exhibited CPE (1a-1c, 2b, 2c and 3a), another sample gave CPE after the third passage (2a). Sequencing the cDNA obtained from RT-PCR, prepared from viral RNA in the transfected cells, it was proven what was expected: the tyrosine is crucial for uridylylation. Two mutants reverted completely to PV-WT (1b and 2b) but two gave an almost 1:1 mixture of the PV-WT and the mutant Y3S4 (1a and 1c). One mutant actually reverted only the phenylalanine to tyrosine but kept the serine-mutation at position 4 (2c). For 3a no RT-PCR was possible, 3b and 3c did not give CPE even after the 10th passage. These results not only confirm

again that tyrosine is crucial at position 3 of VPg but also demonstrate that F3S4 is quasi-infectious. This means that the mutant is able to replicate but needs to revert to yield detectable virus. In fact F3S4 seems to give revertants earlier than F3T4, which was previously reported to show CPE in HeLa-cells only after the 7th passage. In addition, these results show that the mutant Y3S4 is viable but has a defective growth phenotype compared to the WT.

Table 4 – Results of passaging-experiments with F3S4-mutants and identification of revertants

F3S4		Passage						Revertants*
		0	1	2	3	4-9	10	
Sample	TrRNA(µl)	CPE?						AA-seq.
1a	5	no	yes					Y3T4 / Y3S4
1b	10	no	yes					Y3T4
1c	20	no	yes					Y3T4 / Y3S4
2a	5	no	no	no	yes			no RT possible
2b	10	no	yes					Y3T4
2c	20	no	yes					Y3S4
3a	5	no	yes					no RT possible
3b	10	no	no	no	no	no	no	no CPE
3c	20	no	no	no	no	no	no	no CPE

* Sequences were determined by cDNA obtained from RT-PCR as described in materials and methods

After these results the mutants S3A4 and S3T4 were constructed to determine whether serine at position 3 would also serve as an UMP acceptor.

To test the growth phenotypes of mutants S3A4 and S3T4, the same passaging-experiment was done as with F3S4 (Table 5). In contrast to F3S4, no CPE was visible at least up to the 5th passage for each mutant. This led to the conclusion that both S3A4 and S3T4 are lethal mutants (which was actually published for S3T4 previously (Reuer, Kuhn et al. 1990)) or extremely sick mutants. These results suggest that serine at position 3 is not a functional substrate for VPg uridylylation. Similar results were obtained for a T3Y4 mutant, which was also dead, showing that tyrosine at position 4 cannot act as a substrate for VPg uridylylation.

Table 5 – Results of passaging-experiments with the S3A4/S3T4-mutants

Passage:	0	1	2	3	4	5
Mutant	CPE?					
S3A4 1	No	no	no	no	no	no
S3A4 2	No	no	no	no	no	no
S3T4 1	No	no	no	no	no	no
S3T4 2	No	no	no	no	no	no

In view of these results it was of interest to determine the reason for the defective growth phenotypes of S3A4 and S3T4. To test for a possible defect in translation, an *in-vitro*-translation reaction was performed (**Fig. 9**). The results showed that both transcript RNAs of mutants S3A4 and S3T4 translate and process the polyprotein similar to WT RNA. That means the ORF does not contain any defect for an explanation of the genotypes and a translation defect can be ruled out.

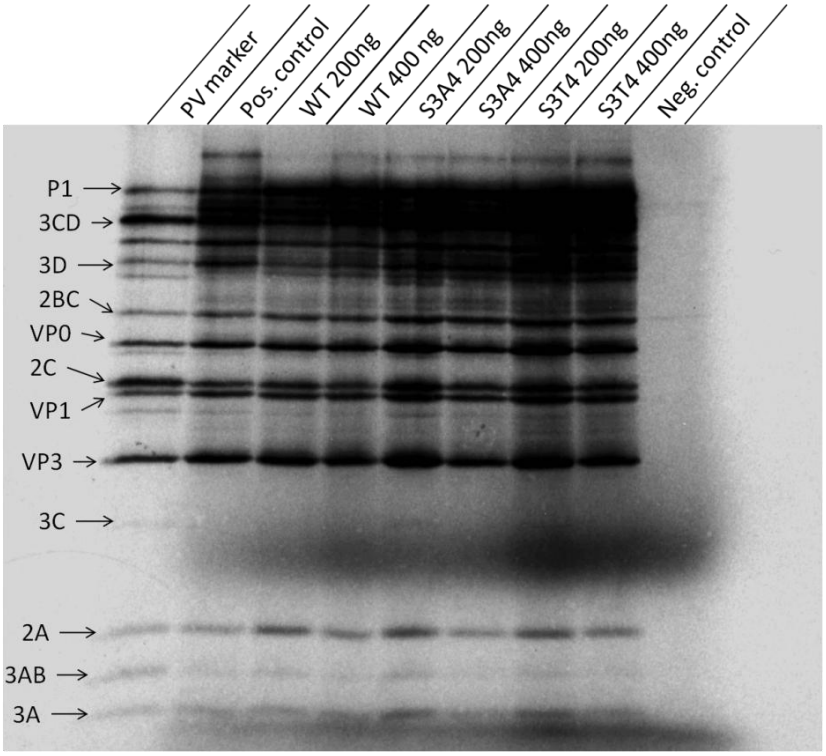

Fig. 9 – In-vitro translation of lethal VPg-mutants. The in-vitro translation reactions were done in cell-free HeLa-extracts with lethal VPg mutants S4A4 and S3T4 as well as with PV-WT in the presence of ^{35}S methionine. The samples, as well as their amount of transcript RNA is indicated on top of the gel, proteins are identified on the left. The bands were separated on a 10% arcylamide-SDS-gel for 3 hours. The bands were visualized on a XRP-film, as described in Materials and Methods. All samples translated and processed well.

To test a possible replication-defect, R-Luciferase reporter viruses of S3A4 and S3T4 (S3A4-R-Luc/S3T4-R-Luc) and of F3S4 (F3S4-R-Luc) were constructed. These R-Luc-constructs consist of a R-Luciferase coding sequence, which is inserted in the PV-genome between the 5'-NRT and the P1 region of the polyprotein (**Fig. 10**). Since a $3CD^{pro}$ cleavage site has been introduced between the R-Luciferase protein and P1 it will be cleaved off after translation. This construct can distinguish between defects in translation, replication and encapsidation. After transfection, the incoming RNA will first be translated. After translation the transfected RNA will initiate the cycle of genome synthesis if the mutant does not contain a replication defect. If the Luciferase signal is similar to that of the WT transfected RNA then the Luciferase signal will steadily increase. However if the transfected mutant RNA has lost the ability for encapsidation, no infectious particles will be formed. In contrast to WT Luciferase-containing transcript the mutant RNA has then reached a "dead-end" causing the loss of the Luciferase signal after passage on fresh cells. If there would be a different signal than the WT the mutant contains either a translation of replication defect. All experiments were carried out with the addition of guanidine-hydrochloride (Gnd-HCl) (+Gnd-HCl, 2mM) and without (-Gnd-HCl) since Gnd-HCl is known to inhibit replication (Barton and Flanegan 1997). If the Luciferase signals of the sample with and without addition of Gnd-HCl are the same, it could be concluded that in both cases only the translation signal was measured, and the investigated construct is defective in replication. If the signal without Gnd-HCl is significantly higher than the one without, it would mean that the construct replicates well. If no significantly different signal than the background is measured, it would mean that the sample may have a translation defect. It is noteworthy that due to protein degradation the peak of the Luciferase signal is reached around 8 hours post-transfection, and it is lower by 20 hours.

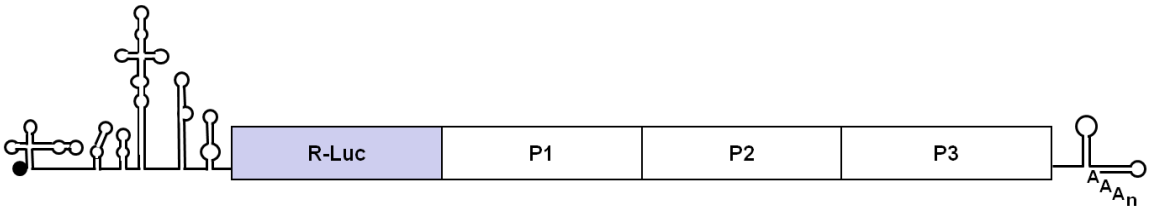

Fig. 10 – Schematic view of the Poliovirus R-Luciferase construct. The coding sequence of the R-Luciferase protein is inserted between the 5'-NTR and the P1 region. A cleavage site for $3CD^{pro}$ was inserted to cleave the R-Luciferase protein off the P1 region.

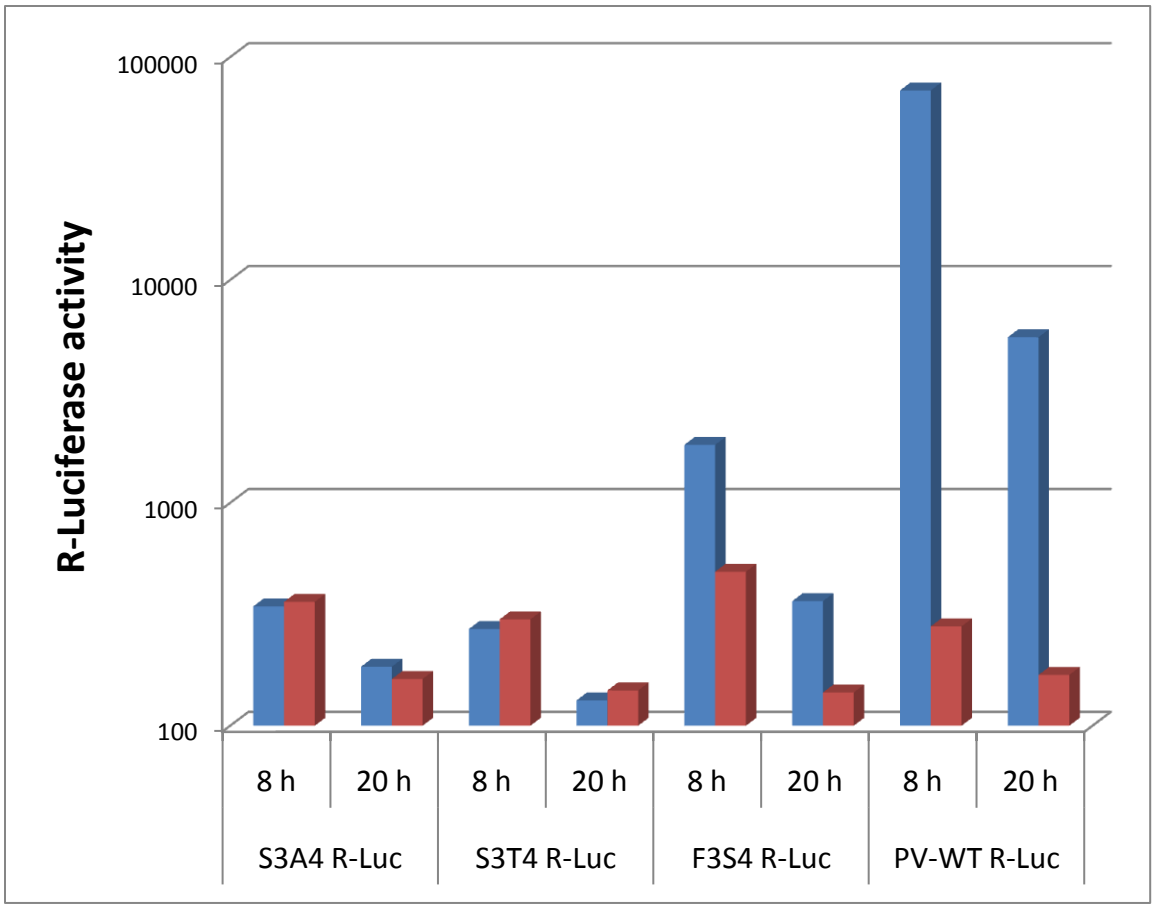

Fig. 11 – The VPg mutants are defective in RNA replication. R-Luciferase reporter transcript RNAs were transfected into HeLa cells with addition of Gnd-HCl (2mM) and without. The cells were stored at 37°C for 8 and 20 hours. After these times the R-Luciferase signals were measured. For S3A4 and S3T4 no difference in the Luciferase signal is measured with addition of Gnd-HCl (red columns) or without addition of Gnd-HCl (blue columns) for both times indicating a replication defect for these mutants. For F3S4 the signal without addition of Gnd-HCl is significantly higher than in the presence of Gnd-HCl at both times but less than PV-WT which shows that F3S4 is sick but replicates.

These data show clearly that mutants S3A4 and S3T4 only translate but do not replicate at all, since there is no difference in the measurement with or without

the addition of Gnd-HCl. Mutant F3S4 exhibited a weak signal in comparison to PV-WT. This leads to the conclusion that mutants S3A4 and S3T4 have a severe replication defect; F3S4 is very sick but is able to replicate. These results altogether show clearly that mutants S3A4 and S3T4 have a lethal replication defect but are able to translate unaffected by the mutations.

To better characterize the viable Y3S4 mutant an experiment to test the temperature sensitivity was carried out. The supernatant from sample 2c (Table 4) was used in this experiment. After infection, the cells were incubated at 35, 37 and 39.5°C for 24 hours. After this time the supernatants were collected and the titers of all the samples were determined using a standard plaque assay (**Fig. 12**). Titers for all temperatures were as high as or even higher than those of PV-WT. The sequencing of cDNA obtained from RT-PCR showed no revertants for any of the Y3S4 samples, which means that this mutation is stable even at high temperatures. The plaque phenotype of Y3S4 also showed no difference compared to PV-WT.

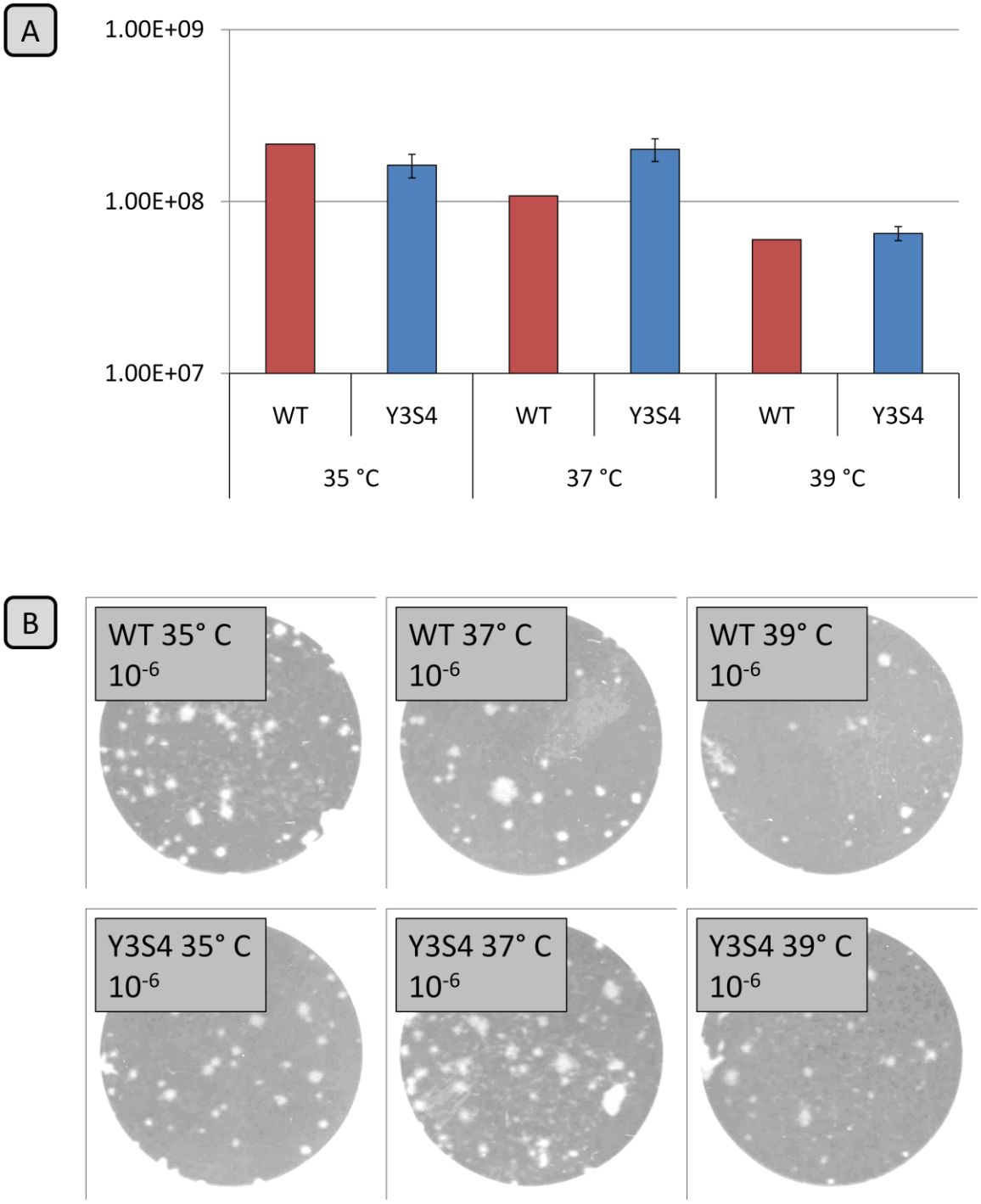

Fig. 12 – Growth phenotype of the Y3S4 mutant at different temperatures. (A) Growth phenotypes of Y3S4 and PV-WT at different temperatures. HeLa cells were infected with WT and Y3S4 mutant at an MOI of 10. The plates were incubated at different temperatures. The virus titers were determined by plaque assay, as described in Materials and Methods. Titers of the temperature-sensitive experiment of mutant Y3S4 show no difference than those of PV-WT in HeLa-cells at 35, 37 and 39.5°C. **(B) Plaque phenotypes of Y3S4 and PV-WT at different temperatures**. The plaque phenotypes of Y3S4 and PV-WT show no difference at any temperature. Plaque assays were carried out in HeLa-cells at 37°C for 48 hours, as described in materials and methods.

b. Spacer II

In order to test the importance of Spacer II, first all the conserved 12 nucleotides from Spacer II Box C were deleted (Table 6).

Table 6 – Nucleotide sequence of Spacer II Box C deletion-mutant

Construct	Nucleotide-seq.
PV-WT	^{616}GCGAATTGGATTGGCCATCCGG
PV-SII-BoxC-Deletion	^{616}GCGAA----------TCCGG

RNAs from 3 independent transcription reactions were transfected into HeLa-cells. Surprisingly, with all 6 plates CPE was observed approximately at the same time as CPE with PV-WT. The mock transfected control did not give CPE, so contaminations of the cells can be ruled out. This was confirmed by sequencing of cDNA obtained after RT-PCR. The sequences showed that the resulting virus still contains the entire Spacer II deletion (Table 7).

Table 7 – Growth phenotypes of Box C deletion mutants

Infection cycle: 1			Progeny virus*	
Mutant	TrRNA(µl)	CPE?	Revertants?	NT-seq.
Del1-12 #1	10 µl	yes	No	Box C deleted
	20 µl	yes	No	Box C deleted
Del1-12 #2	10 µl	yes	No	Box C deleted
	20 µl	yes	No	Box C deleted
Del1-12 #3	10 µl	yes	No	Box C deleted
	20 µl	yes	No RT available	

* Sequences were determined from the cDNA obtained by RT-PCR, as described in materials and methods

After this surprising result, the growth phenotype of two selected Spacer II mutants were determined by plaque assay. No significant difference between the mutant and PV-WT was observed, either in the titer or the plaque-phenotype in HeLa-cells (**Fig. 13**).

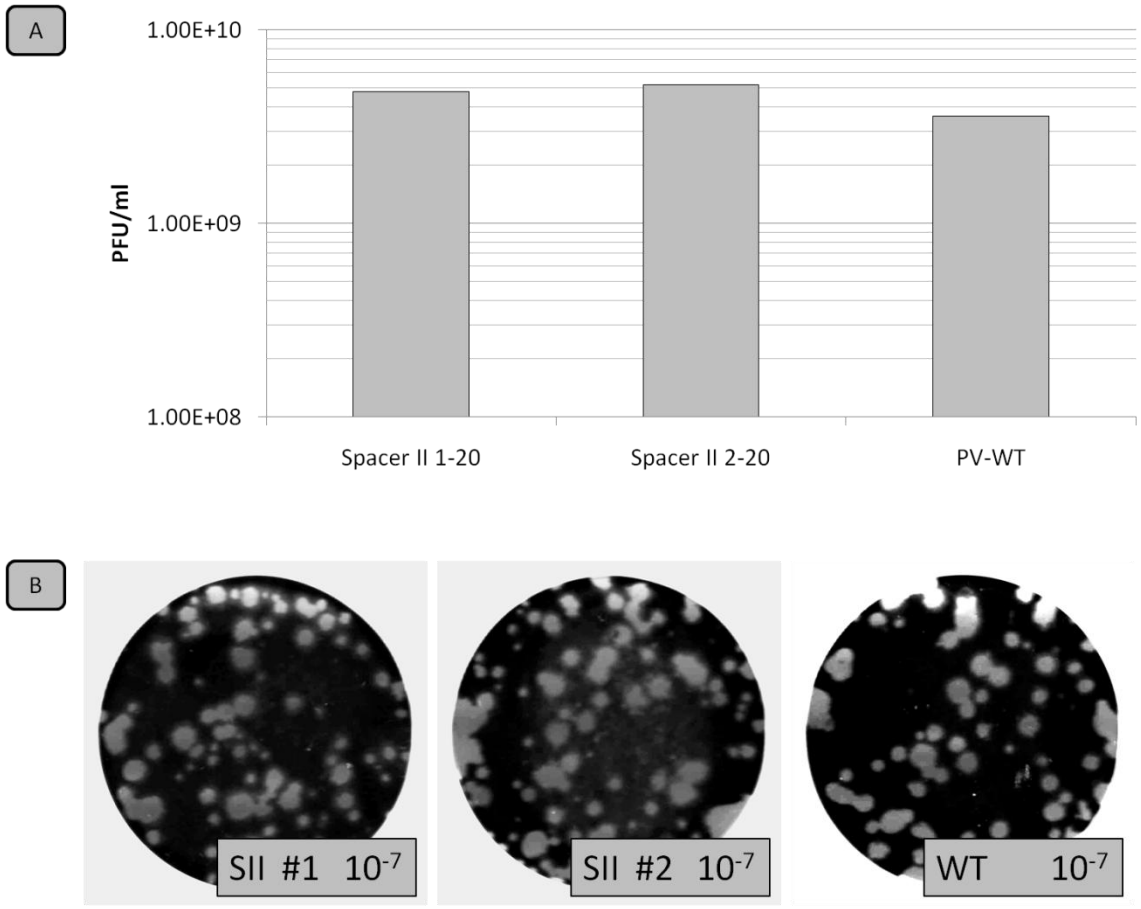

Fig. 13 – Growth phenotype of Box C deletion mutants. (A) Titers of selected Box C deletion samples and PV-WT. The virus titers of the Box C deletion mutant and PV-WT were determined by a standard plaque assay, as described in Materials and Methods. The growth phenotype did not show any difference. **(B) Plaque phenotypes of selected Box C deletion samples.** The plaque phenotype of the Box C deletion mutants did not show any difference to those from the PV-WT.

Even though these results suggested that the Spacer II mutant is viable under standard conditions on HeLa-cells at 37°C, they do not rule out the possibility that the mutant differs from the WT under different growth conditions. To examine these possibilities one-step* growth-curves in different cell lines were performed. The first cell line was HeLa-cells and the temperature dependence was determined. After that neuroblastomia (Human neuroblastomia cell line) cells and L20B-mouse (mouse cell line (L-cells), genetically engineered to express the human poliovirus receptor) cells were used. All plaque assays were still

* One-step means that the cells were infected with a high MOI to make sure statistically all cells are infected with the virus

performed in HeLa-cells at 37°C. For all cell lines CPE occurred at roughly the same time as with PV-WT at all temperatures tested.

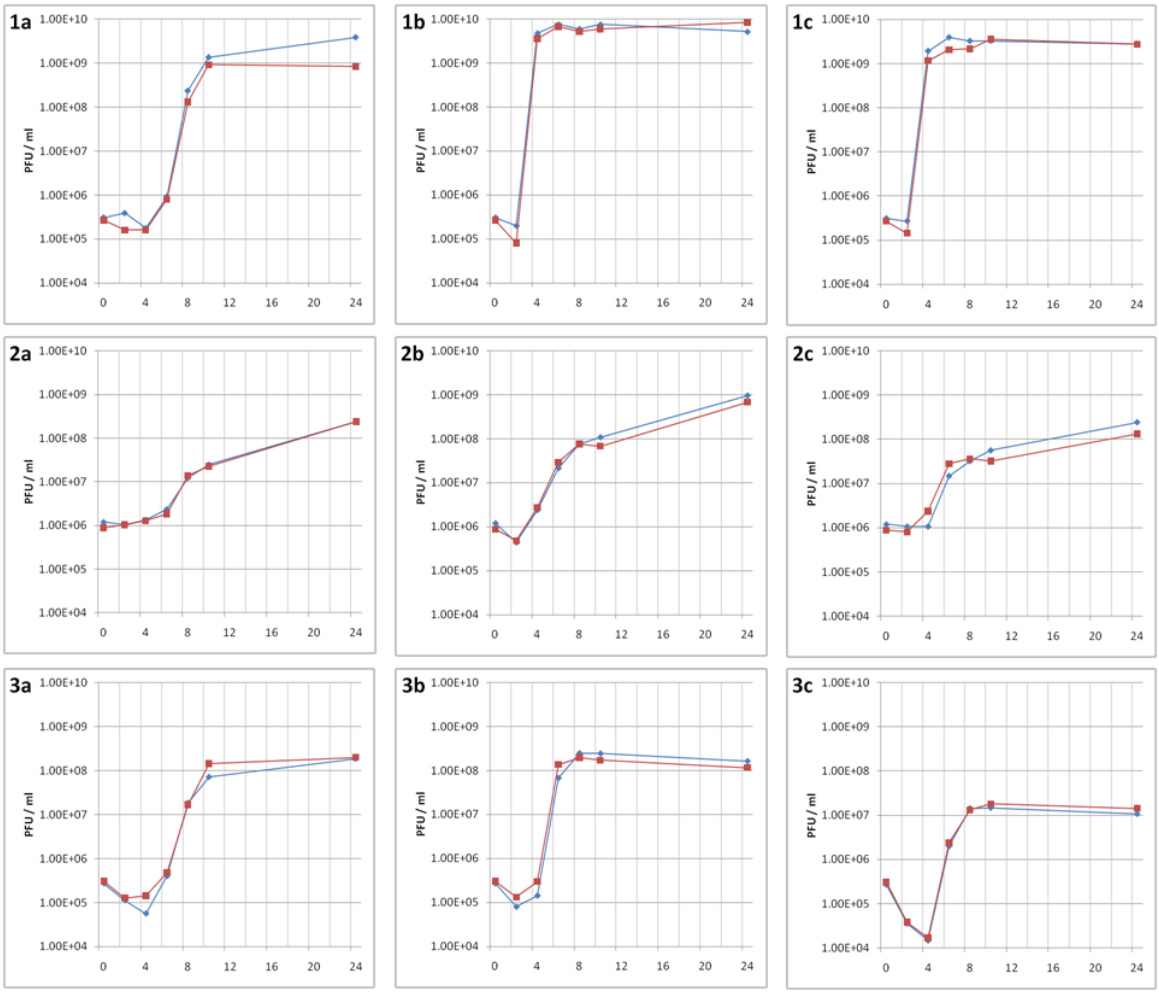

Fig. 14 – One-step growth curves of PV-WT and box C deletion mutant. One-step growth curves were measured for PV-WT (blue) and the Box C deletion mutant (red) as described in materials and methods. The one-step growth curves were carried out in HeLa-cells (1), SK neuroblastomia (2) and L20B mouse cells (3) at 35 (a), 37 (b) and 39°C (c). None of the nine one-step growth curves show any significant difference between the Box C deletion mutant and the PV-WT. This shows that the growth phenotype of the Box C deletion mutant is comparable to that of the PV-WT under all the conditions tested.

The results of one-step-growth curves do not show any significant differences between the PV-WT and the SII mutant. For nearly every time-point taken the measured titers are almost identical. This suggests that the deletion of spacer II box C does not affect the virus viability for the different growth conditions measured.

5. Discussion, conclusions and outlook

For the VPg mutants the experiments described here show that serine can act as an uridylylation substrate if it is in position 4 (F3S4) but not at position 3 (S3T4, S3A4), which leads to a dead virus. This result corresponds with earlier studies, which showed that a T3Y4-mutant is dead. It can only be assumed that the reason for these results is a different pattern of protein folding of the small VPg. The steric demand as well as the hydrophobicity of a benzene ring as it appears in tyrosine and phenylalanine requires a totally different environment than the tiny serine or threonine. To verify this it would be interesting to determine the structures of the VPg-mutants S3A4, S3T4 and even T3Y4 to see how much the structures change. In addition, it would be interesting to compare the structure of the Poliovirus VPg with the cowpea mosaic plant virus, discussed before, where serine could be uridylylated. Another very interesting experiment would be to do an in-vitro uridylylation assay with these mutagenic VPg peptides. The results could not only show that those mutations cause a dead virus because uridylylation is not possible; it could also confirm again the strong dependence between virus viability and VPg uridylylation as done before in a easy 1-day experiment using purified VPg and $3D^{pol}$ (Paul, van Boom et al. 1998).

The deletion of Box C at spacer II did not result in any significant growth defect under the tested conditions. This surprising result continues the mystery of the entire spacer II region. These experiments did not uncover any function of either box C or spacer II in general; all that was shown is that the virus is viable without these 12 nucleotides. Still, it would be very interesting to test this construct in mice to determine whether or not there will be an effect on pathogenesis. Another interesting experiment would be to make the spacer II mutant to compete with the PV-WT over several passages. With such an experiment, the ratio of WT to mutant after passaging would indicate whether or not there is an advantage with one of them.

6. Materials and methods
    a. Chemicals and enzymes

All chemicals which have been used were an A.C.S. grade, and were used without further purification. Chloroform was purchased from J. T. Baker, glacial acetic acid, isopropyl alcohol and 100% ethanol were purchased from Pharmco-AAPER. For RNA work and other sensitive procedures, purified water from a Barnstead Nanopure ultrapure water purification system (Thermo Scientific) was used.

    b. Solutions and buffers

Buffers for Midi-Prep and Gel-purification: All buffers for gel purification and midi-preps were purchased from Quiagen. For buffer P1, RNAse had to be added. For buffer PE, pure ethanol was added. The amounts were different related to the volume of developed buffer. All other buffers were used as delivered without any additional steps of purification.

Media: All media for cell-work like HeBSS, PBS, DME and Modified eagle media were purchased from Invitrogen. To avoid contaminations of bacteria 5ml pen strep was added after opening.

Buffers for Maxi-Prep which were prepared prior to usage:

- **Solution I:** 50mM glucose, 25mM Tris-HCl (pH 8.0) and 10mM EDTA (pH 8.0)
- **Solution II:** 2ml 5N NaOH, 5ml 10% SDS, 43ml water
- **Solution III:** 60ml 5M potassium acetate, 11.5ml glacial acetic acid, 28.5 ml water

c. Methods

i. Genetic engineering of mutant poliovirus

1st PCR: Mutagenic primers and outside oligos for the desired regions have been ordered from EUROFINS MWG|OPERON. The nucleotide fragments had been dissolved in a specified volume of TE-buffer in order to get a 100µM stock solution. This stock solution is diluted to a 20µM solution for PCR usage. As a template for the initial PCR a cDNA of WT poliovirus pT7PVM[†] was used.

For the PCR a 50µl solution was prepared containing 5µl of the 20µM solutions of each primer, approximately 400ng of the pT7PVM-plasmid as a template and 4µl of a 5mM solution of dNTPs. For VPg-mutants the TAQ-polymerase enzyme (Roche) had been used, for Spacer II mutants *Vent*® Polymerase (New England Biolabs). For each PCR sample 1,5µl of the enzyme and 5µl of the corresponding 5x concentrated buffer were given to the reaction mixture. Water was added for the final volume of 50µl.

For the initial PCR reaction the following protocol was used:

Table 8 – Protocol of 1st PCR

	Temperature	Time	Action
25 cycles	94 °C	1 min	Melt
	42 °C	2 min	Anneal
	72 °C	2 min	Polymerize

For VPg mutants S3A4 and S3T4 a lot of side-products were obtained. Increasing the annealing temperature gave a slightly better result so for those the annealing temperature was raised to 57 °C.

[†] pT7PVM stands for plasmid including the T7 promoter with the PV type 1 (Mahoney) sequence in a pBR322 vector

Agarose-gel purification: PCR-fragments had been separated on 0.8% agarose-gel. Therefore 0.4g of ultrapure Agarose purchased from Invitrogen were dissolved in 50ml of 1x concentrated TBE-buffer and heated for 1 minute in a microwave oven. 5µl of a 10mg/ml solution Ethidium bromide was added to the warm gel in order to visualize the fragments. Various DNA markers were used so the sizes of the fragments could be determined. The gel was visualized under UV-light and the desired fragments had been cut out from the gel for further purifications via ethanol-precipitation or PCR purification using a Quiagen purification kit. The ethanol precipitation resulted in a slightly better yield.

Purification by ethanol-precipitation: The cut out gel pieces were placed in a Quiagen gel purification spin column and 200µl of water were added. The mixture was allowed to stand at -20°C for at least 30 minutes. The gel was warmed to 37°C in a water bath and centrifuged at 13.200rpm at room temperature. The filtered solution containing the desired fragment was mixed with 1ml of pure ethanol and placed in a -20°C freezer for at least 30 minutes. After that the mixture was centrifuged at 13.200rpm at 4°C for 30 minutes. The supernatant was aspirated off and the visible pellet washed with 500ml of cold 70% ethanol. The samples were placed at -20°C for 15 minutes and centrifuged at 13.200rpm at 4°C for 10 minutes. The supernatant was discarded and the clean pellet was allowed to dry on air. The dry pellet was dissolved in 20µl of TE-buffer.

Purification using a QIAquick Gel extraction kit: The cut-out gel was weighed in a 1.5ml Eppendorf tube and 3 volumes of QC buffer were added to the tube (each mg of gel means 3µl QG buffer). The mixture was warmed up in a 37°C water bath until the gel was completely dissolved. After that 1 gel-equivalent of isopropanol was added to the mixture (each mg of gel means 1µl of isopropanol) and everything was mixed by brief vortexing.

To purify the fragment, the solution was placed into a QIAquick spin column in a 2ml collection tube and it was centrifuged for 1 minute at 13.200rpm. During this step the DNA was bound to the column. After discarding the flow-through the column was washed with 500µl of QG buffer and after that with 750µl buffer PE and centrifuged afterwards. It is noteworthy that in order to get rid of the entire buffer PE it is necessary to centrifuge twice and to discard the flow-through after the first centrifugation. After the washing was completed the DNA was eluted from the column with 20µl of buffer EB into a 1,5ml Eppendorf tube by centrifugation at 13.200 rpm for 1 minute.

2nd PCR: The plus- and minus-strand PCR fragments from the PCR were combined in a second PCR. Therefore a PCR mixture containing 7,5µl of each fragment and 5µl of each outside oligo was prepared. 4µl of a 5mM solution of dNTPs was added, also the polymerase enzyme, fitting to the desired mutant, as mentioned before. The mixture was filled up with water to a final volume of 50µl.

The protocol for the second PCR is shown below:

Table 9 – Protocol of 2nd PCR

	Temperature	Time	Action
25 cycles	94 °C	1 min	Melt
	42 °C	2 min	Anneal
	72 °C	2 min	Polymerize

After PCR the fragments were purified by Agarose-gel purification as described before.

Digestion: For digestion of the PCR-fragments obtained from the 2nd PCR and the vector plasmid solutions were prepared containing all of the PCR-fragment or, in case of the plasmid, approximately 10µg. 1,5µl of each digestion enzyme and 3µl of the corresponding buffer as mentioned in Table 10 were given to the

solution and the reaction mixture was filled up to 30µl with water. The reaction were stored at 37°C and allowed to proceed for 2 hours. After that time 1µl of the solution was checked on an Agarose-gel to determine the completeness of the digestion. After digestion the enzyme-activity was stopped by freezing at -20°C for a short time. Afterwards the fragments and the vector were purified as mentioned before.

Table 10 – Restriction enzymes used, the corresponding buffers and producers

Enzyme	Mutant	Cleaving side	Producer	Buffer
Hpa I	VPg-mutants	GTTAAC CAATTG	Roche	Roche A
Blg II	VPg-mutants	AGATCT TCTAGA	Roche	Roche A
Age I	Spacer II	ACCGGT TGGCCA	New England Biolabs	N.E.B. 1
Nhe I	Spacer II	GCTAGC CGATCG	New England Biolabs	N.E.B. 1

Ligation: For ligation of the cut insert into the plasmid-vector a solution was prepared containing 10nmol of the vector and 30nmol of the insert. 1µl T4-DNA-ligase enzyme (Roche) with 2µl of the corresponding buffer was added and the mixture was filled up to 20µl with water. The reaction was allowed to proceed for 30 minutes at room temperature, in case of the Spacer II mutants because only sticky ends were involved. Incubation was overnight in case of the VPg-mutants because here a blunt end was involved. In both cases, the reaction proceeded at room temperature.

Transformation: The plasmids obtained from the ligation were transformed into competent cells to check and select the mutagenic plasmids. Therefore 3,5µl of the ligation mixture was added to approximately 40µl of competent cells which had been stored at -80°C. The mixture was allowed to stand on ice for 30 minutes. After that time the mixture was threaten by a heat shock in a 42°C water bath for no longer than 60 seconds. After that the mixture was stored on ice for 5 minutes, 200µl of LB medium was added afterwards and the cells were incubated for 45-60 minutes in a 37°C water bath. After incubation time the mixture was

well spread on an agar plate containing LB + 50µg/ml ampicillin. The plates were incubated at 37°C over night. In case of a successful ligation and transformation colonies were obtained on the plate the next day.

Growth of plasmid-containing bacteria: To select the correct clones, single colonies were picked and placed into 4ml of LB-media containing 50µg/ml ampicillin. The mixture was incubated in a shaker at 37°C for 24 hours. To grow larger volumes of bacteria 200ml LB + 50µg/ml ampicillin was incubated with the overnight culture. In this case purification was done via a maxi-prep. In case not so much plasmid was needed the cells were centrifuged down in order to continue with a midi-prep.

Midi-prep: The cells obtained from a 4ml culture were centrifuged down in a 2ml Eppendorf tube for 1 minute at 13.2000 rpm and stored at -20°C overnight after the supernatant was removed. The cells were resuspended in 250µl buffer P1 containing RNase A. For lysis 250µl of buffer P2 was added to the suspension and the tube was inverted 6 times in order to mix the solution. The lysis reaction was allowed to proceed for 5 minutes at room temperature. After this time 350µl neutralization buffer N3 was added to the mixture and mixed well in order to inhibit the lysis reaction. The solution usually became cloudy after this step. The mixtures were centrifuged for 10 minutes at 13.200rpm. The supernatant was removed and transferred into a QIAprep spin column, which was centrifuged for 1 minute at 13.200rpm. The column was washed with 500µl buffer PB and centrifuged down for 1 minute at 13.200 rpm afterwards. Another washing step was done with 750µl buffer PE where it was again necessary to centrifuge twice in order to remove every residual washing reagent. The desired amplified DNA was obtained from the column by elution with 30µl buffer EB and centrifugation at 13.200rpm for 2 minutes.

Maxi-prep: The amplified cells from a 200ml culture were centrifuged down for 30 minutes at 3.000 rpm and stored at -20°C overnight. Afterwards the cells were suspended in 5.5ml of solution I. 1ml of lysozyme (10mg/ml) in a solution of 10mM Tris-HCl with pH 8.0 was added to the suspension and mixed well. 12ml of solution 2 was added to the mixture and mixed well. The reaction was allowed to stand at room temperature for 5 minutes. After that 9ml of ice-cold solution 3 was added and mixed by shaking. While standing on ice for 10 minutes a white pellet formed. The mixture was centrifuged down at 14.000 rpm for 15 minutes at 4°C.

The supernatant was transferred into another centrifuge tube and 0.6 volumes of isopropanol was added, mixed well and allowed to stand at room temperature for 10 minutes. The DNA was recovered by centrifugation at 10.000 rpm for 15 minutes at 4°C. The supernatant was decanted or aspirated and the dry pellet was carefully washed once with 1ml of 70% ethanol. The tube was opened and allowed to stand inverted in a hood until the last visible traces of ethanol was evaporated.

The pellet was dissolved in 500µl of TE and transferred into a 1,5ml Eppendorf tube. 1µl of DNAse-free pancreatic RNAse (20µg/ml) was added. The reaction proceeded for 30 minutes at room temperature. After that 500µl of a solution containing 1,6M NaCl and 13% PEG were added and mixed well. The plasmid was recovered by centrifugation at 12.000 rpm for 10 minutes at 4°C and the supernatant was discarded.

The pellet was again dissolved in 400µl TE and extracted once with 400µl phenol, after that with 400µl of a 1:1 mixture of phenol and chloroform and in the end by addition of 400µl pure chloroform. After every step the upper aqueous phase containing the DNA was transferred into a new 1,5ml Eppendorf tube. The phases were separated by centrifugation at 13.200 rpm, in case of pure phenol for 10 minutes, for the 1:1 mixture for 5 minutes and with pure chloroform at 1

minute. The extraction was necessary in order to remove the pancreatic RNAse completely. After extraction 100µl of 10M ammonium acetate and 1ml 100% ethanol were added, mixed well and stored for 10 minutes at room temperature. The mixture was centrifuged at 12.000 rpm for 10 minutes at 4°C and the DNA recovered in the pellet by aspiration of the supernatant. For washing 1ml of 70% ethanol was added and mixed carefully, afterwards centrifuged for 2 minutes at 13.200 rpm at 4°C. The pellet was dissolved in 50µl TE. The concentration and purity (OD_{280}/OD_{260}) of the DNA was measured using a Nanodrop.

Linearization: 10µl of the purified plasmid was mixed with 1µl of digestion enzyme EroR1 (Roche) and 2µl of the corresponding EcoR1-buffer (Roche). The mixture was filled up with water to 20µl and stored at 37°C for 2 hours. The progress of the reaction was checked on an agarose gel. After the reaction was complete the linear fragment was purified on an agarose gel, as described before.

Transcription: 10µl of the linearized plasmid was mixed with 10µl of 5x concentrated transcription buffer, 10µl of a 5mM rNTPs solution, 1µl of RNAse-inhibitor and 3µl of T7-RNA-polymerase. The reaction was filled up to 50µl with water and incubated at 37°C for 2 hours. The success of the transcription was checked on a 0.8% RNA-agarose gel using Tris-Acetate buffer (4ml 2M Tris base, 200µl glacial acetic acid in 200ml H_20).

ii. Other experimental procedures

Transfection of viral RNA: For Transfection, 20µl of the transcription mixture (approx. 200ng of transcript RNA) were mixed with 80µl of HeBSS-buffer and 100µl of 1mg/ml DEAE-Dextran in HeBSS. The mixture was incubated on ice for 30 minutes. During the incubation the cells needed for the transfection were washed 2 times with 2ml of pure DME. After incubation the entire DEAE mixture

containing transcript RNA was given to the cells. The reaction was allowed to incubate at room temperature for another 30 minutes. Afterwards the DEAE mixture was aspirated off and 2ml DME containing 5% BCS was added to the cells and the plates were stored in an incubator at 37°C if not mentioned differently.

Passaging: In order to transfer virus which was grown into new cells for a new infection (e.g. for a plaque assay) the cells containing the virus were freeze-thawed 3 times from -50°C to room temperature. By this treatment the membranes of the cells were broken up and the virus released in the media solution. After 3 cycles the supernatant was taken into 2 ml Eppendorf tubes to centrifuge down insoluble cell organelles for 5 minutes at 5.000rpm. The supernatant was stored at -20°C or used directly without further purification needed.

For the next infection cycle the cells were washed 2 times with pure. After washing 500µl of the supernatant was added to the cells and the mixture was incubated at room temperature for 30 minutes while gently rotating. After 30 minutes the supernatant was aspirated off and 2ml of DME + 2% BCS was added. The cells were then stored in an incubator at 37°C.

Virus titer by plaque assays: The virus titer which states the concentration of the virus was determined by a plaque assay. Therefore the supernatant, which was obtained from freeze-thawing as described above was taken and diluted. For this purpose 50µl of the supernatants were mixed with 450µl DME in a 1,5ml Eppendorf tube to obtain a dilution by the factor 10. Afterwards 50µl of this solution were taken and mixed in anther 450µl of DME in order to obtain a dilution by the factor 100 relative to the initial supernatant. This procedure was followed 9 times to get the final dilution. After all dilutions were prepared 250µl of each dilution was put on HeLa-cells which incubated with the virus for 30

minutes. After this 2ml of the Plaque-media were placed on the cells and the plates were stored at 37°C for 48 hours. After this given time the media was thrown away and the plates were dyed with crystal violet, which will only stain the intact cells. Infected cells which were broken up due to CPE did not give color which results in little blank dots, the plaques. Each plaque stands for one infective particle so in relation to the dilution and the used amount of solution the virus titer could be calculated with the unit PFU/ml.

Growth curves: For growth curves in a specific cell line the cells were infected with virus at a MOI of 10. After 30 minutes of incubating the cells at room temperature were washed and stored in incubators with the different desired temperatures, e.g. 35, 37 and 39.5°C. One sample was frozen before incubation which is the sample for time-point zero. After this time one sample from each incubator is frozen after a specific time. After all the infections were done the supernatants from the samples were used for plaque assays in order to determine the PFU/ml of the samples. By this a plot of PFU/ml over time is obtained which shows the growth kinetics.

RNA extraction: The viral RNA was extracted from the supernatants after infection using the Phenol-based chemical Trizol® purchased from Invitrogen‡. 200µl of the supernatant were given into a 2ml Eppendorf tube and 800µl of Trizol® was added. The solutions were shaken for 15 seconds yielding a cloudy pink mixture, which was allowed to incubate at room temperature for 5 minutes. After this time the mixture became clear. For extraction 300µl of Chloroform was given to the mixture and shaken again for 10 seconds. The milky mixture was allowed to stand at room temperature for 3 minutes and was centrifuged afterwards at 4°C and 11.000rpm for 15 minutes. After centrifugation the mixture was separated into 2 phases where the upper, aqueous phase contained the viral and cellular RNA. The aqueous phase was transferred into a 1.5ml

‡ With this method not only viral RNA but also cellular RNA was extracted which did no harm to the use of viral RNA for RT-PCR.

Eppendorf tube and mixed well with 500µl of isopropanol. The mixture incubated at room temperature for 20 minutes and was centrifuged at 4°C and 13.200rpm for 40 minutes. The supernatant was aspirated off and a white pellet was obtained on the bottom of the tube. The pellet was washed with 300µl of 70% ethanol and centrifuged again at 4°C and 13.200rpm for 10 minutes. The supernatant was again aspirated off and the pellet was air-dried until all ethanol was visibly evaporated. The pellet was dissolved in 10µl of RNase-free water for further usage.

RT-PCR: The reverse transcriptase PCR is in general a normal PCR with modified enzymes. 10 µl of the viral RNA was mixed with 1µ of 10mM dNTPs, 2,5µl of each primer and 1µl RNAse-inhibitor. Other chemicals were used from a "Titan one-tube RT-PCR" kit developed by Roche. 10µl of buffer, 2,5µl of a 100mM solution DTT and 1µl of the titan enzyme were mixed with the solution. Afterwards water was added to obtain a final volume of 50µl.

The RT-PCR protocol was as followed:

Table 11 – Protocol of RT-PCR

	Temperature	Time	Action
	50 °C	35 min	Transcription to cDNA
	94 °C	2 min	Melt
30 cycles	94 °C	45 sec	Melt
	45 °C	1 min	Anneal
	68 °C	2 min	Polymerize
	68 °C	20 min	Polymerize
	22 °C	5 min	Cool down

For further usage the obtained PCR-products were purified using a 0.8% agarose-gel as described before.

In-vitro translation: For in-vitro translation a specific amount of viral RNA (e.g. 200 or 400ng) in 4.5µl of water was mixed with 8µl of a translation master mix. This master mix contained of 37.5µl micrococcal nuclease treated HeLa-cell extract, 12.5µl translation mix which is described later, 5.5µl KAc (2M, 0.1M final), 0.75µl MgAc (50mM, 0.35mM final), 4µl $MgCl_2$ (320mM, 20mM final) and 6.25µl of ^{35}S-labeled methionine. The composition of the translation mix was as followed:

Table 12 – Composition of translation mixture used for *in-vitro* translation

40µl	ATP 100mM
6µl	GTP 40mM
40µl	Creatine phosphate 1M
10µl	CPK (Sigma) 10mg/ml, 10mM buffer
76µl	K-Hepes pH 7.6 1M
8µl	DTT 1M
10µl	Calf liver t-RNA (Boehringer) 10mg/ml
50µl	Amino acid mixture without methionine 1mM
10µl	Spermidine 100mM
250µl	Water to final volume of 500µl

The mixture of viral RNA and translation master mix was incubating over night in a 30°C water bath. Afterwards 20µl of 2x SDS buffer containing 2-mercaptoethanol were added. The mixture was stored in boiling water for 3 minutes. The entire volume was then added on a 12%-SDS gel which ran for 3 hours. The gel was fixed and dried and the picture taken on a XRP-film over night at -80°C.

Literature cited

Andino, R., G. E. Rieckhof, et al. (1990). "A functional ribonucleoprotein complex forms around the 5' end of poliovirus RNA." Cell **63**(2): 369-380.

Barton, D. and J. Flanegan (1997). "Synchronous replication of poliovirus RNA: initiation of negative- strand RNA synthesis requires the guanidine-inhibited activity of protein 2C." J. Virol. **71**(11): 8482-8489.

Beales, L. P., A. Holzenburg, et al. (2003). "Viral internal ribosome entry site structures segregate into two distinct morphologies." J. Virol. **77**(11): 6574-6579.

Brown, D. M., C. T. Cornell, et al. (2005). "An authentic 3' noncoding region is necessary for efficient poliovirus replication." J Virol **79**(18): 11962-11973.

Brown, D. M., S. E. Kauder, et al. (2004). "Cell-Dependent Role for the Poliovirus 3' Noncoding Region in Positive-Strand RNA Synthesis." J. Virol. **78**(3): 1344-1351.

Cao, X. and E. Wimmer (1995). "Intragenomic complementation of a 3AB mutant in dicistronic polioviruses." Virology **209**(2): 315-326.

Cello, J., A. V. Paul, et al. (2002). "Chemical synthesis of poliovirus cDNA: Generation of infectious virus in the absence of natural template." Science **297**(5583): 1016-1018.

De Jesus, N. (2007). "Epidemics to eradication: the modern history of poliomyelitis." Virology Journal **4**(1): 70.

Drygin, Y. F., M. V. Sapotsky, et al. (1987). "Radish mosaic virus VPg characteristics and linkage with virion RNAs." FEBS Letters **215**(2): 247-251.

Dulbecco, R. and M. Vogt (1954). "Plaque formation and isolation of pure lines with poliomyelitis viruses." The Journal of Experimental Medicine **99**(2): 167-182.

Ehrenfeld, E. and B. L. Semler (1995). "Anatomy of the poliovirus internal ribosome entry site." Curr Top Microbiol Immunol **203**: 65-83.

Gmyl, A. P., E. V. Pilipenko, et al. (1993). "Functional and genetic plasticities of the poliovirus genome: quasi-infectious RNAs modified in the 5'-untranslated region yield a variety of pseudorevertants." J. Virol. **67**(10): 6309-6316.

Hellen, C. U., T. V. Pestova, et al. (1994). "Effect of mutations downstream of the internal ribosome entry site on initiation of poliovirus protein synthesis." J. Virol. **68**(10): 6312-6322.

Hogle, J. M. (2002). "Poliovirus cell entry: common structural themes in viral cell entry pathways." Annu Rev Microbiol **56**: 677-702.

Iizuka, N., M. Kohara, et al. (1989). "Construction of less neurovirulent polioviruses by introducing deletions into the 5' noncoding sequence of the genome." J. Virol. **63**(12): 5354-5363.

Jacobson, M. F. and D. Baltimore (1968). "Morphogenesis of poliovirus: I. Association of the viral RNA with coat protein." Journal of Molecular Biology **33**(2): 369-378.

Jacobson, M. F. and D. Baltimore (1968). "Polypeptide cleavages in the formation of poliovirus proteins." Proceedings of the National Academy of Sciences of the United States of America **61**(1): 77-84.

Jang, S. K., M. V. Davies, et al. (1989). "Initiation of protein synthesis by internal entry of ribosomes into the 5' nontranslated region of encephalomyocarditis virus RNA in vivo." J. Virol. **63**(4): 1651-1660.

Jang, S. K., H. G. Krausslich, et al. (1988). "A segment of the 5' nontranslated region of encephalomyocarditis virus RNA directs internal entry of ribosomes during in vitro translation." J. Virol. **62**(8): 2636-2643.

Jr., J. V. M. (1964). "Preparative electrophoresis of proteins in acrylamide gels*." Annals of the New York Academy of Sciences **121**(Gel Electrophoresis): 382-390.

Kitamura, N., B. L. Semler, et al. (1981). "Primary structure, gene organization and polypeptide expression of poliovirus RNA." Nature **291**(5816): 547-553.

Krausslich, H. and E. Wimmer (1988). "Viral proteinases." Annual Review of Biochemistry **57**(1): 701-754.

Kuhn, R. J., H. Tada, et al. (1988). "Mutational analysis of the genome-linked protein VPg of poliovirus." J. Virol. **62**(11): 4207-4215.

Lee, Y. F., A. Nomoto, et al. (1977). "A protein covalently linked to poliovirus genome RNA." Proceedings of the National Academy of Sciences of the United States of America **74**(1): 59-63.

Mendelsohn, C. L., E. Wimmer, et al. (1989). "Cellular receptor for poliovirus: molecular cloning, nucleotide sequence, and expression of a new member of the immunoglobulin superfamily." Cell **56**(5): 855-865.

Meredith, J. M., J. B. Rohll, et al. (1999). "Similar interactions of the poliovirus and rhinovirus 3D polymerases with the 3' untranslated region of rhinovirus 14." J. Virol. **73**(12): 9952-9958.

Molla, A., A. Paul, et al. (1991). "Cell-free, de novo synthesis of poliovirus." Science **254**(5038): 1647-1651.

Palmenberg, A. C., M. A. Pallansch, et al. (1979). "Protease required for processing picornaviral coat protein resides in the viral replicase gene." J. Virol. **32**(3): 770-778.

Paul, A. V., J. Peters, et al. (2003). "Biochemical and genetic studies of the VPg uridylylation reaction catalyzed by the RNA polymerase of poliovirus." J. Virol. **77**(2): 891-904.

Paul, A. V., J. H. van Boom, et al. (1998). "Protein-primed RNA synthesis by purified poliovirus RNA polymerase." Nature **393**(6682): 280-284.

Pelletier, J. and N. Sonenberg (1988). "Internal initiation of translation of eukaryotic mRNA directed by a sequence derived from poliovirus RNA." Nature **334**(6180): 320-325.

Peng-Nien Huang, J.-Y. L., Shin-Ru Shin (2010). Identification of the functional roles of cellular proteins that associate with 5' untranslated region of enterovirus 71. American Society for Virology, 29th Annual Meeting, Scientific Program & Abstracts. Bozeman, Montana**:** 190.

Phillips, B. A. and R. Fennell (1973). "Polypeptide composition of poliovirions, naturally occurring empty capsids, and 14S precursor particles." J Virol **12**(2): 291-299.

Pierangeli, A., M. Bucci, et al. (1995). "Mutational analysis of the 3'-terminal extra-cistronic region of poliovirus RNA: secondary structure is not the only requirement for minus strand RNA replication." FEBS Letters **374**(3): 327-332.

Reuer, Q., R. J. Kuhn, et al. (1990). "Characterization of poliovirus clones containing lethal and nonlethal mutations in the genome-linked protein VPg." J. Virol. **64**(6): 2967-2975.

Rothberg, P. G., T. J. Harris, et al. (1978). "O4-(5'-uridylyl)tyrosine is the bond between the genome-linked protein and the RNA of poliovirus." Proceedings of the National Academy of Sciences of the United States of America **75**(10): 4868-4872.

Schaffer, F. L. and C. E. Schwerdt (1955). "Crystallization of purified MEF-1 poliomyelitis virus particles." Proceedings of the National Academy of Sciences of the United States of America **41**(12): 1020-1023.

Schein, C. H., N. Oezguen, et al. (2010). "NMR solution structure of poliovirus uridylyated peptide linked to the genome (VPgpU)." Peptides **31**(8): 1441-1448.

Schein, C. H., N. Oezguen, et al. (2006). "NMR structure of the viral peptide linked to the genome (VPg) of poliovirus." Peptides **27**(7): 1676-1684.

Schein, C. H., D. E. Volk, et al. (2006). "Novel, structure-based mechanism for uridylylation of the genome-linked peptide (VPg) of picornaviruses." Proteins: Structure, Function, and Bioinformatics **63**(4): 719-726.

Selinka, H. C., A. Zibert, et al. (1991). "Poliovirus can enter and infect mammalian cells by way of an intercellular adhesion molecule 1 pathway." Proceedings of the National Academy of Sciences of the United States of America **88**(9): 3598-3602.

Slobodskaya, O. R., A. P. Gmyl, et al. (1996). "Poliovirus neurovirulence correlates with the presence of a cryptic AUG upstream of the initiator codon." Virology **221**(1): 141-150.

Summers, D. F. and J. V. Maizel (1968). "Evidence for large precursor proteins in poliovirus synthesis." Proceedings of the National Academy of Sciences of the United States of America **59**(3): 966-971.

Toyoda, H., M. J. H. Nicklin, et al. (1986). "A second virus-encoded proteinase involved in proteolytic processing of poliovirus polyprotein." Cell **45**(5): 761-770.

van der Werf, S., J. Bradley, et al. (1986). "Synthesis of infectious poliovirus RNA by purified T7 RNA polymerase." Proceedings of the National Academy of Sciences of the United States of America **83**(8): 2330-2334.

Wetz, K. (1987). "Cross-linking of poliovirus with bifunctional reagents: biochemical and immunological identification of protein neighbourhoods." Journal of Virological Methods **18**(2-3): 143-151.

WHO. (1988). "Global eradication of poliomyelitis by the year 2000." from http://www.polioeradication.org/content/WHA.Resolutions.and.Decisions.pdf.

WHO. (2010). "Wild poliovirus, 21 Jul 2009 - 20 Jul 2010." from http://www.polioeradication.org/content/general/casemap.gif.

Wimmer, E., C. U. T. Hellen, et al. (1993). "Genetics of poliovirus." Annual Review of Genetics **27**(1): 353-436.

Yogo, Y. and E. Wimmer (1972). "Polyadenylic acid at the 3'-terminus of poliovirus RNA." Proc Natl Acad Sci U S A **69**(7): 1877-1882.