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The VP1 intracapsid hook and uncoating of enteroviruses

by
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Academic Dissertation

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I envisage a world in which all viruses are free to enter cells without their motifs being questioned.

CONTENTS

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications and manuscripts, which will be referred to by their Roman numerals:

I **Airaksinen A., and Hovi T.** 1998. Modified base compositions at degenerate positions of a mutagenic oligonucleotide enhance randomness in site-saturation mutagenesis. Nucleic Acids Research 26:576-581.

II **Airaksinen A., Roivainen M., Stanway G., and Hovi T.** 1999. Site-saturation mutagenesis of the PALTAVETG motif in coxsackievirus A9 capsid protein VP1 reveals evidence of conservation of a periodic hydrophobicity profile. Journal of General Virology 80:1919-1927.

III **Airaksinen A., Roivainen M., and Hovi T.** 2000. Coxsackievirus A9 VP1 mutants with enhanced or hindered A particle formation and decreased infectivity. Submitted.

IV **Airaksinen A., Somerharju P., and Hovi T.** 2000. Variation in liposome binding among enteroviruses. Submitted.

ABBREVIATIONS

ATCC	American Type Culture Collection
ATP	adenosine triphosphate
BEV	bovine enterovirus
BFLA	bafilomycin A
bp	base pair
BSA	bovine serum albumin
CAR	coxsackievirus-adenovirus receptor
CAV	coxsackievirus A
CBV	coxsackievirus B
cDNA	complementary DNA
CMC	carboxymethylcellulose
CPE	cytopathic effect
DAF	decay-accelerating factor
E. coli	*Escherichia coli*
FCS	fetal calf serum
FMDV	foot-and-mouth disease virus
GMK	green monkey kidney cell line
GTP	guanosine triphosphate
HeLa	human tumor-derived cell line
HEp	human epithelial carcinoma cell line
HEV	human enterovirus
HRV	human rhinovirus
ICAM	intercellular adhesion molecule
IOD	integrated optical density
IRES	internal ribosome entry site
LLC Mk_2	cell line of green monkey kidney origin
MAb	monoclonal antibody
mCAR	murine coxsackievirus-adenovirus receptor
MEM	minimal essential medium

nt	nucleotide
p1, p2, p3	the three positions of a codon
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PDB	protein data bank
PV	poliovirus
PVR	poliovirus receptor
RD	human rhabdomyosarcoma cell line
RT-PCR	reverse transcription - polymerase chain reaction
SDS	sodium dodecyl sulfate
SVDV	swine vesicular disease virus
TG1	*E. coli* strain used as the M13 host
T_m	melting temperature
UTR	untranslated region
VP	viral protein
W/V	weight/volume

REVIEW OF THE LITERATURE

Enteroviruses

Enteroviruses form one of the most common groups of human pathogens, causing a wide range of diseases. The clinical features include poliomyelitis and other neurological diseases, meningitis, myocarditis, conjunctivitis, skin and mucosal eruptions, diarrhea, generalized infections of the newborn, and common cold-like illness. Many, if not most of the infections are subclinical, proceeding in a period of several weeks without significant symptoms in the host, but rare cases result in paralysis or even death. One of the peculiar features of enteroviruses is that there is both individual and temporal variation in the diseases caused by various enteroviruses. Most enterovirus serotypes, including coxsackievirus A9, may be associated with several disease patterns. Recently, prospective seroepidemiological studies have suggested a potential role of enterovirus infections in the etiology of insulin-dependent diabetes mellitus (Hyöty *et al.* 1995, Hiltunen *et al.* 1997) and atheroschlerotic heart disease (Roivainen *et al.* 1998)

The family *Picornaviridae* is formed by a large group of non-enveloped positive-stranded RNA viruses. The genus *Enterovirus* forms one of the nine genera in the family, the other eight being *Aphthovirus*, *Cardiovirus*, *Erbovirus*, *Hepatovirus*, *Kobuvirus*, *Parechovirus*, *Rhinovirus*, and *Teschovirus*. Genetically, enteroviruses cluster relatively closely with rhinoviruses, while the other genera are more distant (for example Pöyry *et al.* 1996, Hyypiä *et al.* 1997, Oberste *et al.* 1999).

Enterovirus taxonomy has recently been changed in accordance with the increasing genetic data (King *et al.* 1999). The current classification recognizes 87 serotypes in the genus *Enterovirus*, 64 of these being human viruses. The human enterovirus serotypes are divided into five species: the poliovirus species (PV), and the human enteroviruses A to D (HEV-A to HEV-D). Enteroviruses have been isolated from other animals as well, and some of these viruses form additional species.

The enterovirus mainly studied in this thesis is coxsackievirus A9 (CAV9) which is one of the most frequently isolated enteroviruses in clinical diseases (Hovi *et al.* 1996), and belongs to the species human enterovirus B (HEV-B) (King *et al.* 1999). In addition to CAV9, this species consists of all coxsackievirus B serotypes (1-6), all echoviruses (28 serotypes), and enterovirus 69. CAV9 is thus the only CAV serotype in this species, the 22 other ones belonging to species HEV-A (11 serotypes) and HEV-C (11 serotypes) (King *et al.* 1999, Oberste *et al.* 1998, 1999). The controversy between classification and nomenclature reflects the initially used primitive classification system that was based on replication in cell cultures and pathogenicity in experimental animals.

Table 1. Classification of human enteroviruses according to King *et al.* (1999).

Species	**Serotypes in the species**
Poliovirus	Human poliovirus 1-3
Human enterovirus A	Human coxsackievirus A 2-8, 10, 12, 14, 16[a] Human enterovirus 71
Human enterovirus B	Human coxsackievirus A 9 Human coxsackievirus B 1-6 Human echovirus 1-7, 9, 11-21, 24-27, 29-33 Human enterovirus 69
Human enterovirus C	Human coxsackievirus A 1, 11, 13, 15, 17-22, 24
Human enterovirus D	Human enterovirus 68, 70

[a] CAV4 and CAV6 were unassigned in this work, but according to Oberste *et al.* (1998, 1999), they belong to the HEV-A species.

The knowledge about enterovirus biology comes mostly from studies concerning poliovirus, information on other enteroviruses being much more limited, and rather sporadic. In discussing the biology of enteroviruses, the focus is therefore in poliovirus. Today, there is rather abundant genetic, and still, relatively narrow experimental data giving assistance in educated guesses concerning other enteroviruses.

Enterovirus structure

Enteroviruses are non-enveloped particles of about 30 nm diameter, composed of sixty copies of each of the four structural proteins VP1 to VP4, that surround a positive-stranded RNA genome of approximately 7500 nucleotides. The icosahedral capsid contains 12 pentagon-shaped pentamers of 5 protomers, each protomer being formed by one copy of each of the structural proteins. The basic organization of the capsid is shared by all picornaviruses.

To date, atomic-resolution structures of six enteroviruses have been resolved by x-ray crystallography. These structures include poliovirus 1 (PV1) (Hogle *et al.* 1985), PV3 (Filman *et al.* 1989), coxsackievirus B3 (CBV3) (Muckelbauer *et al.* 1995), bovine enterovirus (BEV) (Smyth *et al.* 1995), PV2 (Lentz *et al.* 1997), and coxsackievirus A9 (CAV9) (Hendry *et al.* 1999). In addition, structures of human rhinovirus 14 (HRV14; Rossmann *et al.* 1985, Arnold & Rossmann 1988), HRV1A (Kim *et al.* 1989), HRV3 (Zhao *et al.* 1996), and HRV16 (Hadfield *et al.* 1997) have been resolved. All these structures share a remarkable degree of overall similarity, while variation is seen on the surface and on the inner structures of the capsid. The major capsid proteins VP1 to VP3 are each folded into eight-stranded antiparallel β-sheets with a jelly-roll topology. These β-barrels of five copies of VP1 are located around the fivefold axis, while VP2 and VP3 are around the threefold axis. VP4 is much smaller than the other structural proteins, having a less

ordered structure, and being located on the inner surface of the capsid, facing the RNA.

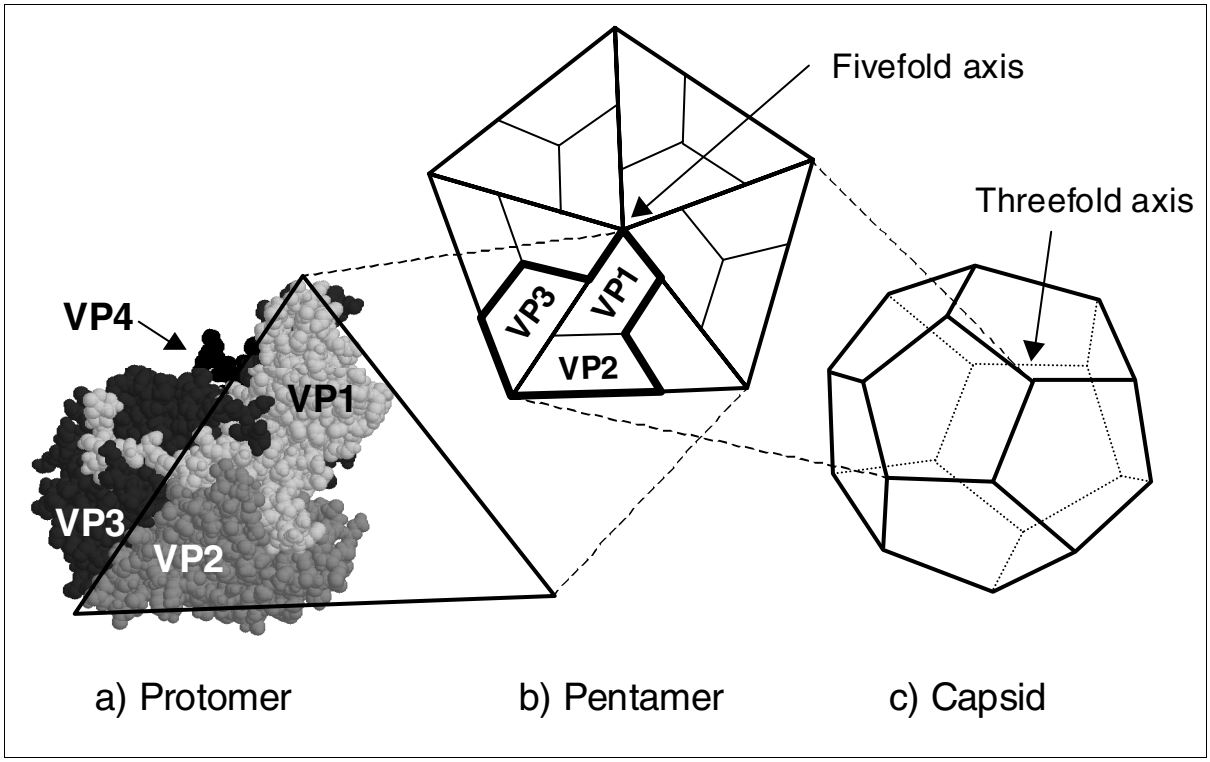


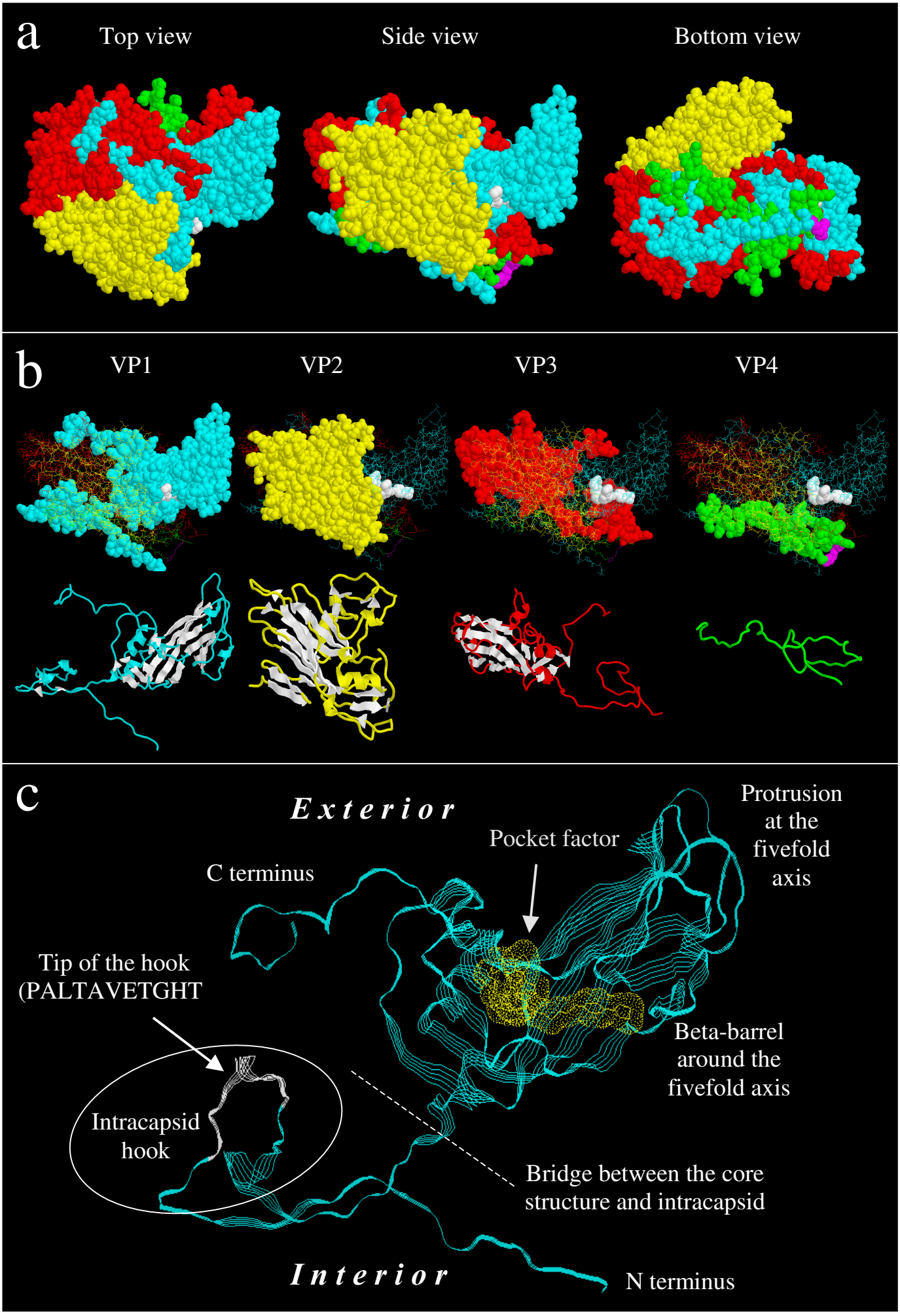
Figure 1. Structural organization of an enterovirus.
a) Each protomer is formed by the proteolytic cleavage products of the P1 polyprotein, the capsid proteins VP1 to VP4. The surface of the virion is formed by VP1, VP2 and VP3, while VP4 is internal.
b) Five protomers assemble into a pentamer. The fivefold axis of the pentamer is shown.
c) An icosahedral capsid is formed by twelve pentamers. One of the twenty threefold axes is marked. The capsid figure (1c) is adapted from Rueckert (1996).

One of the most striking structural features in picornaviruses is the circular canyon around the fivefold axis, that was first seen in the structure of human rhinovirus 14 (HRV14) (Rossmann *et al.* 1985). The canyon hypothesis (Rossmann *et al.* 1985, Rossmann 1989) suggests that one strategy to escape the immune surveillance of the host organism would be to hide the receptor attachment site in a surface depression. This would sterically protect the attachment site from antibodies, still allowing recognition by the cell surface receptor that would be narrower than an

antibody. The canyon is located roughly between VP1 on the 'north' side (the side closer to the fivefold axis), and VP2 and VP3 on the 'south' side. This organization leaves five copies of VP1 as a protrusion at the fivefold axis. The general topology of the canyon is variable, appearing more like five distinct depressions than a single canyon in CAV9 (Hendry *et al.* 1999).

The N terminal glycine of VP4 has a myristate, a saturated tetradecanoic fatty acid, covalently attached to it (Chow *et al.* 1987). In site-directed mutagenesis studies, myristoylation and myristate-protein contacts were found to be necessary in pentamer formation, RNA encapsidation (Moscufo *et al.* 1991), and for the stability of the virion (Moscufo & Chow 1992). It was also shown that the VP4 protein is necessary in uncoating or cell entry, as a nonviable VP4 mutant was capable of forming the structural intermediates thought to be necessary for infection, and when transfected to cells, the cDNA was capable of initiating infection that resulted in apparently mature virions (Moscufo *et al.* 1993).

Figure 2. Molecular structure of coxsackievirus A9.
a) Spacefill model of a protomer seen at three different angles. VP1, cyan; VP2, yellow; VP3, red; VP4, green; pocket factor, white; myristate, magenta.
b) Structures of individual capsid proteins (side view). In the upper panel, one protein is shown as a spacefill model, while others are shown as wireframes. In the lower panel, the individual proteins are shown as cartoon models with secondary structures highlighted, and β-sheets shown in white.
c) Ribbon model of VP1 and the pocket factor. Wide strands show the positions of α-helices and β-sheets, while narrow strands are turns and random coil.
Figures were generated by the RasMol 2.6 Molecular Visualisation Program by Roger Sayle, Glaxo Wellcome Research and Development, Stevenage, Hertfordshire, U.K. The CAV9 coordinates (Hendry *et al.* 1999) were from the Protein Data Bank (PDB) of the Research Collaboratory for Structural Bioinformatics (RCSB) at http://www.rcsb.org/pdb/.


a
Top view
Side view
Bottom view
b
VP1
VP2
VP3
VP4
c
Exterior
Protrusion at the fivefold axis
Pocket factor
C terminus
Tip of the hook (PALTAVETGHT
Beta-barrel around the fivefold axis
Intracapsid hook
Bridge between the core structure and intracapsid
Interior
N terminus

All the known enterovirus and rhinovirus structures contain a hydrophobic pocket at the base of the canyon, covered by loops of VP1 (Hendry *et al.* 1999). This space is assumed to be normally filled by a natural pocket factor, a fatty acid with an aliphatic chain of variable length. These pocket factors, including sphingosine (PV1, PV3), palmitate (CBV3), and myristic acid (BEV), are believed to be present in the virion upon release from the infected cell. The pocket factors are seen as stabilizing agents in the capsid, and their presence is thought to prevent uncoating. It is therefore necessary that these molecules should leave the pocket when infection is initiated. This pocket is the site of action of several antiviral drugs and these are thought to bind to the pocket more tightly than does a natural pocket factor, thereby hindering uncoating (McSharry *et al.* 1979, Caliguiri *et al.* 1980, McKinlay 1985).

Enterovirus genome

Enteroviruses have a single molecule of positive-stranded RNA as their genome. The RNA is about 7500 nucleotides long, and there is a small virus-encoded protein VPg (or 3B) attached to the 5' end of the molecule. At both the 5' end and 3' end of the genome, there is an untranslated region (UTR). The 5' UTR makes up approximately 10 % of the genome. This 5' UTR is involved in the initiation of translation, directing ribosomes into the IRES, the internal ribosome entry site (Jang *et al.* 1988, Pelletier and Sonenberg 1988). The 3' UTR is shorter, only 70-100 nt in length, and it is followed by a poly-A tail. The 3' UTR is used in initiating the synthesis of negative-strand RNA (Rohll *et al.* 1995), but even deletion mutants lacking the 3' UTR have been shown to be infectious in cell culture (Todd *et al.* 1997).

The protein coding region encodes a single polyprotein, which is proteolytically cleaved into precursor proteins P1, P2 and P3, and thereafter into the structural proteins VP1 to VP4 (P1), and seven nonstructural proteins (P2 and P3). Many of the intermediate cleavage products are functional as well. Proteins 2A, 3C and 3CD are proteases, 3D is the RNA-dependent RNA polymerase, 2C is apparently a

helicase, and has a function in encapsidation of RNA (Vance *et al.* 1997), and 2B, 2BC, 3A and 3AB have been associated with various functions in the replication of viral RNA.

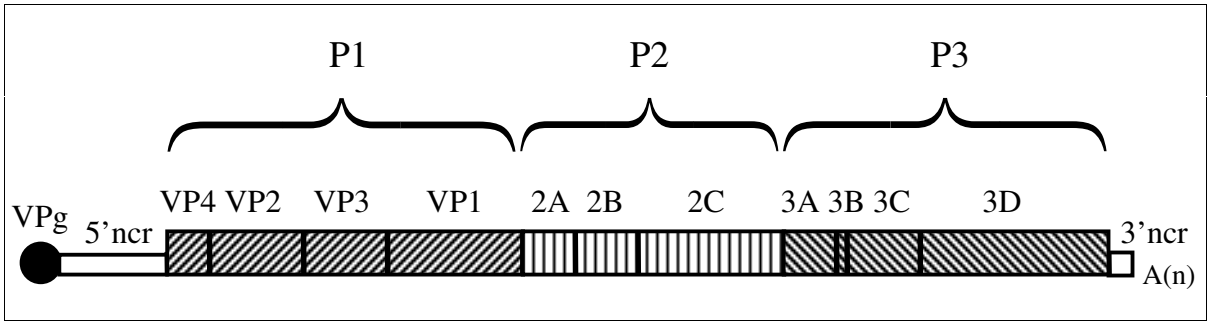


Figure 3. The enterovirus genome.
P1, P2, and P3 are the products of the initial proteolytic cleavages of the viral polyprotein.

Overview of enterovirus replication cycle

In order to initiate an infectious cycle, the virus must attach to a cell surface receptor on a susceptible cell. Upon binding to its receptor, an enterovirus undergoes structural rearrangements resulting in a particle that has lost the majority of the internal capsid protein VP4, has externalized the N terminus of VP1, and has a different sedimentation coefficient (135S versus 160S of the native virion), altered antigenic properties, and increased protease sensitivity. Some altered poliovirus particles are found in endosomes soon after infection (Fenwick & Wall 1973), but the entry site of a productive infection is still not known.

Before synthesizing complementary negative-stranded copies of the genome, the genomic RNA acts as a template for protein synthesis. The viral proteins need to be synthesized first to obtain the RNA-dependent RNA polymerase that the cellular machinery lacks. Translation is initiated from the IRES present in the 5' UTR (Jang *et al.* 1988, Pelletier and Sonenberg 1988). The complete viral protein coding region is translated as a single polyprotein, which is co-translationally cleaved, by

virus-encoded proteases, into three precursor proteins P1, P2 and P3 (Kitamura *et al.* 1981). One of these, the capsid protein precursor P1, is co-translationally myristoylated. The P2 and P3 proteins are cleaved into seven nonstructural proteins, in addition to several forms of functional intermediates, acting in RNA replication and protein processing. The P3 cleavage products include the proteases 3C and 3CD, that are needed in the cleavage of other virus proteins from the precursors.

The same RNA molecule that has been used in translation, is then used as a template for synthesis of negative-stranded RNA molecules by the virus-encoded polymerase 3D. These strands are then used in synthesizing large amounts of positive-stranded copies that are used in translation of viral proteins, as well as being encapsidated into the assembling virions. The small VPg protein is linked to the 5' end of all transcripts (Flanegan *et al.* 1977).

The infection process is enhanced by cleavage of the eukaryotic initiation factor 4GII (eIF4GII) (Gradi *et al.* 1998, Svitkin *et al.* 1999) and apparently the poly(A)-binding protein as well (Kerekatte *et al.* 1999), by a mechanism that involves the protease 2A (Hellen *et al.* 1991, Wyckoff *et al.* 1992). These cleavages are among the known alterations in the host cell, resulting in cessation of cellular (cap-dependent) protein synthesis by poorly understood mechanisms. Enteroviruses use a different (cap-independent) method of translation initiation (Jang *et al.* 1988, Pelletier and Sonenberg 1988), being therefore still capable of performing protein synthesis in the infected cell.

The assembly of a new virion begins as the P1 precursor protein is cleaved into capsid proteins VP0, VP1 and VP3, and these proteins form a heterotrimer (Korant 1973). Thereafter, five of these heterotrimers associate into a pentameric structure, which is actually a 15-mer of five copies of each of the procapsid proteins VP0, VP1 and VP3 (Phillips *et al.* 1968). Again, 12 of these pentamers then associate together to form procapsids ($VP013_{60}$) that are filled with a copy of the positive-stranded genome. It is not known whether empty procapsids are first formed and

then filled with the RNA molecule, or the procapsids associate around the RNA molecule. Poliovirus and foot-and-mouth disease virus (FMDV) particles have been reported to contain nonstructural proteins 2C, 3CD, 3C, and 3D, and although there is no evidence of their usefulness, it has been suggested that their presence in the virions might reflect a functional role in initiating replication in an infected cell (Newman & Brown 1997). During encapsidation of the RNA, the majority of the capsid protein VP0 is cleaved into VP2 and VP4. This is called the maturation cleavage, which is thought to lock the assembled capsid into a stable, mature virion. Poliovirus mutants that are unable to undergo this cleavage, are defective in assembly and RNA encapsidation (Compton *et al.* 1990, Ansardi & Morrow 1995), or can accumulate 150S particles that appear like procapsids filled with RNA, but are highly unstable (Hindiyeh *et al.* 1999). Maturation cleavage is known to be absent in parechoviruses 1 and 2, and in Aichi virus, demonstrating that this cleavage is not an absolute requirement for all picornaviruses (Hyypiä *et al.* 1992, Stanway *et al.* 1994, Yamashita *et al.* 1998). Empty capsids are also produced in natural infection, and these particles have intact VP0 proteins instead of VP2 and VP4, thus having the protein composition $VP0_{13_{60}}$ (Holland & Kiehn 1968, Jacobson *et al.* 1970). All the assembly intermediates described above can be demonstrated in an infected cell by sedimentation analysis: the heterotrimeric protomer (5S), the pentamers (14S), the empty capsid (approximately 75S), and the encapsidated mature virion (160S).

A single infected cell can typically produce 10^4 to 10^5 poliovirus particles, that are released by lysis of the cell. The lysis has been proposed to occur due to increased permeability of the cell membranes (Carrasco 1977), which results from increasing concentrations of nonstructural proteins 3A and 3AB (Lama & Carrasco 1992a, Lama & Carrasco 1992b), 2 BC (Aldabe *et al.* 1997), and 2B (Lama & Carrasco 1992b, van Kuppeveld *et al.* 1997). Recent data suggests that apoptosis might also be a significant factor in the symptoms that arise from persistent infections of the central nervous system (Girard *et al.* 1999), and that poliovirus 3C induces apoptosis by a caspase-dependent mechanism (Agol *et al.* 1998, Barco *et al.* 2000).

Cellular receptors for enteroviruses

Initiation of viral infection is dependent on attachment to a cellular receptor molecule. Attachment to these molecules is specific, and during evolution, viruses have adapted to use a variety of receptors. Several such receptors are known for various enteroviruses (Table 2). There are several examples of closely related viruses using different receptors for entry. On the other hand, some evolutionarily distant viruses can use the same receptor, as shown by the coxsackievirus-adenovirus receptor (CAR) (Bergelson *et al.* 1997a). The murine homologue of this molecule (mCAR) is also capable of functioning as a receptor for both coxsackie- and adenoviruses (Bergelson *et al.* 1998). Moreover, there are viruses that have been shown to use at least two different receptors. An example of such viruses is CAV9 (Roivainen *et al.* 1991a, Hughes *et al.* 1995, Roivainen *et al.* 1996), the virus mainly studied in this thesis. In addition to the primary receptor molecules, many if not all viruses need additional proteins, sometimes called coreceptors, for entry. CAV21 has been shown to require the presence of both ICAM-1 and the decay-accelerating factor (DAF) for cell entry (Shafren *et al.* 1997), and there is a post-attachment requirement for β2-microglobulin in the entry process of CAV9 (Triantafilou *et al.* 1999).

When rhinoviruses were found to have a narrow canyon around the fivefold axis, these canyons were suggested to be the receptor recognition sites (Rossmann *et al.* 1985). Similar canyons have been found in all entero- and rhinovirus structures determined to date, and recently, structural studies have unambiguously resolved the precise location of binding. Structures of HRV14 and HRV16 complexed with domains of ICAM-1, and PV1 in complex with PVR have verified that the receptor binding site actually is in the canyon (Olson *et al.* 1993, Bella *et al.* 1998, Kolatkar *et al.* 1999, Belnap *et al.* 2000a, He *et al.* 2000).

There are at least two alternative receptors for CAV9, the integrin $\alpha_v\beta_3$, which is the vitronectin receptor (Roivainen *et al.* 1991a, Roivainen *et al.* 1994), and an

unidentified cell surface receptor (Hughes *et al.* 1995, Roivainen *et al.* 1996). The integrin $\alpha_v\beta_3$ recognizes the tripeptide arginine-glysine-aspartate (RGD), which is a known recognition signal for integrins (Ruoslahti & Pierschbacher 1986) containing the α_v subunit (Hynes 1992). The RGD sequence is used in some other animal viruses as a receptor recognition sequence, as exemplified by the echovirus 9 Barty strain (Zimmermann *et al.* 1997), FMDV (Fox *et al.* 1989, Berinstein *et al.* 1995), and human parechovirus 1, that was formerly known as echovirus 22 (Stanway *et al.* 1994). CAV9 has this tripeptide close to the C terminus of VP1, in a sequence of approximately 15 amino acids that is not found in other enteroviruses (Chang *et al.* 1989).

Integrin $\alpha_v\beta_3$ was found to act as a receptor for CAV9 in GMK cells (Roivainen *et al.* 1991a, Roivainen *et al.* 1994), but proteolytic cleavage (Roivainen *et al.* 1991a) and site-directed mutagenesis studies (Hughes *et al.* 1995) thereafter revealed that CAV9 lacking the RGD motif can still propagate in GMK cells, although displaying a small-plaque phenotype. Furthermore, in RD cells, removal of RGD from VP1 apparently had no effect in infectivity (Hughes *et al.* 1995, Roivainen *et al.* 1996). Regardless of the capability of CAV9 to survive *in vitro* without the RGD motif, all natural isolates contain this motif (Chang *et al.* 1992, Santti *et al.* 2000). These results show that although CAV9 does use the integrin $\alpha_v\beta_3$ as a receptor, it can also use an alternative receptor. This is one of the several examples of viruses that are not dependent on a single receptor, underlining the assumption that the identity of the receptor may not be as important for virus survival as has been thought earlier, although it can still have a role as a determinant of pathogenesis.

Judging by the phenomena described above, it seems that viruses are somewhat opportunistic in their receptor usage, being capable of adapting to different receptors during virus-host coevolution. This view is supported by the finding that there is variation in receptor specificity within a given CBV serotype, depending on the passaging history and between various isolates (Bergelson *et al.* 1997b). It has

also been suggested that the ability to bind DAF may have originated more than once among enteroviruses (Powell *et al.* 1999).

Table 2. Current view of enterovirus receptors

Receptor	Virus	Reference
Immunoglobulin superfamily:		
PVR	PV1-3	Mendelsohn *et al.* 1989
intercellular adhesion molecule 1 (ICAM-1)	CAV13, 18, 21	Colonno *et al.* 1987
	CAV15, 20	Pulli *et al.* 1995
coxsackievirus-adenovirus receptor (CAR)	CBV1-6	Bergelson *et al.* 1997a
Integrins:		
integrin $\alpha_v\beta_3$	CAV9	Roivainen *et al.* 1994
	EV9 (Barty)	Nelsen-Salz *et al.* 1999
integrin $\alpha_2\beta_1$ (VLA-2)	EV1	Bergelson *et al.* 1992
Other:		
decay-accelerating factor (DAF, CD55)	CAV13, 18	Colonno *et al.* 1986, 1987
	CAV21	Shafren *et al.* 1997
	CBV3	Bergelson *et al.* 1995
	CBV1, 3, 5	Shafren *et al.* 1995
	EV7	Ward *et al.* 1994
	EV13, 21, 29, 33	Bergelson *et al.* 1994
	EV70	Karnauchow *et al.* 1996

Conformational transitions in the capsid

Upon attaching to the receptor, enteroviruses undergo a permanent reorganization of the capsid, characterized by a shift from a 160S sedimenting particle to a 135S particle. The 135S particle has lost the majority of the 60 copies of the capsid protein VP4, and the antigenicity and protease sensitivity are altered in comparison to the native virion (CBV3: Crowell and Philipson 1971; PV: Fenwick & Wall 1973, De Sena & Mandel 1977). One of the specific alterations is the exposure of the N terminus of VP1 (Fricks & Hogle 1990). This 135S altered particle (A particle) is thought to be an essential intermediate in initiating a new infectious cycle, while being itself noninfectious (PV: Joklik & Darnell 1961, Fenwick & Cooper 1962, Lonberg-Holm *et al.* 1975; CBV3: Crowell and Philipson 1971). Nevertheless, it has been shown that to a very small extent, it is capable of initiating infection in cells that are nonsusceptible to the intact 160S virion (Curry *et al.* 1996).

Poliovirus infection can proceed without accumulation of 135S particles, which could be interpreted as suggestive evidence of its lack of importance. In these studies, the infection was found to occur in the presence of a capsid-binding drug R78206 (Ofori-Anyinam *et al.* 1995), or the virus had been adapted to grow in HeLa cells at +25 °C (Dove & Racaniello 1997), at which temperature the conformational transitions do not occur (Yafal *et al.* 1993). These data are not conclusive, since the results could be interpreted as showing that one kinetic barrier has been overcome in the conditions used, resulting in lack of accumulation of the transformed particles, rather than lack of transformation. Another kind of evidence was provided by a kinetic experiment in which the amounts of intact poliovirions, 135S particles, and 80S particles were followed during incubation with purified PVR. In this study, the 80S particles appeared to be transformed from the 160S particles, but not from the 135S particles (Arita *et al.* 1998). This seems to suggest that the accumulating 135S particles are not intermediate forms on the productive infectious pathway. However, there are several lines of evidence suggesting that the

135S particles are in fact an intermediate in the infection process. First, the 135S particle is the dominant virus form found inside the infected cells in the early phase of infection (Lonberg-Holm *et al.* 1975, Everaert *et al.* 1989), but a portion of the attached virus particles are also eluted from the cells as 135S particles, and can be purified (PV: Fenwick & Cooper 1962, CBV3: Crowell & Philipson 1971). Second, the same reorganization of the capsid is seen when incubating polioviruses with solubilized poliovirus receptor (PVR) (Kaplan *et al.* 1990), or when incubating enteroviruses with membrane extracts (CBV3: Roesing *et al.* 1975; PV: De Sena & Mandel 1976, 1977; Guttman & Baltimore 1977). Third, several antiviral drugs, such as arildone (McSharry *et al.* 1979, Caliguiri *et al.* 1980) and the WIN compounds (McKinlay 1985), function by binding to enteroviruses, thereby hindering the conformational transitions. Fourth, 135S particles bind to lipid bilayers, forming ion channels (Fricks & Hogle 1990, Tosteson & Chow 1997). Fifth, the 135S particle is moderately infectious (Curry *et al.* 1996). Sixth, mutant polioviruses overcome defects, caused by mutated receptor, by lowering the thermal energy required for the structural change into 135S particles (Wien *et al.* 1997).

For the above reasons, the 135S form of the virus particle is generally seen as an intermediate in the process where the stable virion transforms due to receptor binding, in order to finally release the genome into the cytoplasm of the susceptible cell. Empty capsids ($VP123_{60}$) can be purified from infected cells soon after initiation of infection, and by releasing the RNA, the particles have shifted from 135S particles to 80S particles.

Enteroviruses can transform into 135S particles without the presence of receptors, when incubated at elevated temperatures and hypotonic conditions (CAV13: Cords *et al.* 1975; PV: Lonberg-Holm *et al.* 1976, Wetz & Kucinski 1991). The resulting 135S particles are indistinguishable from those appearing during infection. The receptor therefore functions like an enzyme, by reducing the thermal energy required in transforming the stable capsid into an entry-competent form (Chow *et*

al. 1997). Conversely, it has been shown that receptor defects can be overcome by viral mutations that lower the transformation energy (Wien *et al.* 1997).

Very recent data suggest that there is an intermediate structure in the transition pathway from 160S native virions to the 135S particles. These 147S particles contain VP4, they are infectious, and the N terminus of VP1 is apparently not exposed, as suggested by resistance to V8 protease digestion (Pelletier *et al.* 2000). In the conditions used, an alternative form of 135S particles was also detected, containing VP4.

In addition to the irreversible structural changes that occur in the capsid during uncoating, virus particles are known to undergo reversible transitions. In contrast to the static view of the virus particles that is given by the crystallographic studies, it is known that the capsids are actually flexible and dynamic entities. It has been known for a long time that polioviruses exist as two interconvertible forms that have different isoelectric points (pI 7 or 4; Mandel 1971). These two forms might reflect the apparent controversy observed between the crystal structures and various biochemical studies; namely, some of the regions that are inside the capsid in the crystal structures, are actually reachable by antibodies. The regions that have been shown to be involved in reversible exposure are the internal capsid protein VP4 (Li *et al.* 1994) and the N terminus of VP1 (Chow *et al.* 1985, Roivainen *et al.* 1993, Li *et al.* 1994).

The N termini of enterovirus VP1 proteins are very heterogeneous both in length and sequence, but there is a highly conserved region close to the N terminus of VP1 of all enteroviruses (Hovi & Roivainen 1993). This motif was first identified as a part of an immunodominant region of 20 amino acids (Roivainen *et al.* 1991b). As it was noticed that the immunogenicity was combined with a conserved sequence, it was shown that antibodies against this sequence can be used as a group reagent recognizing enteroviruses (Hovi & Roivainen 1993). Peptide antibodies against this motif (amino acids 42-52 in PV1/Mahoney) were shown to be capable of

precipitating purified poliovirus particles, showing that this region is exposed in solution (Roivainen *et al.* 1993), while being on the inner surface of the capsid in the crystal structure. It was also conclusively shown that the neighboring region, amino acids 24 to 40 of VP1 of PV1/Mahoney is transiently exposed (Li *et al.* 1994), by demonstrating that antibody recognition of this motif is dependent on coincubation of the virus and the antibodies at +37 °C. The transitions are thus reversible, occurring at physiological temperatures but not below that (Li *et al.* 1994). On the other hand, it has been shown that poliovirus can be immunoprecipitated at room temperature by overnight incubation with peptide antibodies against amino acids 42-52 (Roivainen, unpublished results). These apparently contrasting observations could be explained by a kinetic equilibrium of the two forms of the virion. The transiently exposed portions could bind to antibodies with kinetics determined by the incubation temperature, and then being locked in this conformation by the antibody.

Medium-resolution structures of the 135S and 80S particles (2.2 nm resolution) were recently revealed using cryo-electron microscopy and image reconstruction (Belnap *et al.* 2000b). The structures indicate that both 135S and 80S particles are approximately 4 % larger than the virion, therefore having thinner, as well as more angular capsids. They also indicate domain movements of up to 0.9 nm (3 % of the virion diameter), and gaps that are seen as plausible sites for the externalization of the VP4 and the N terminus of VP1. Other studies have suggested that VP4 and the N terminus of VP1 would exit through the fivefold axis (Hadfield *et al.* 1997, Hendry *et al.* 1999), or that VP4 would exit through a channel along the fivefold axis and the N terminus of VP1 through the pseudo threefold axis (Lentz *et al.* 1997). However, it was suggested that the exit site for both of these would be the base of the canyon, and the five extended N terminal arms of VP1 would follow the fivefold protrusion on the surface of the capsid, meeting each others at the fivefold axis, ideally positioned for membrane insertion (Belnap *et al.* 2000b). The fivefold vertex was not favored as the exit site for VP4 and the N termini of VP1, as there is a plug formed by the N termini of VP3 that is present in both 160S and 135S

particles, seeming to prevent the use of this location as the exit site. It was suggested that despite the VP3 plug, the RNA could be released through the fivefold axis, as the plug might act as a float-valve (Belnap *et al.* 2000b).

Interactions of enteroviruses with lipid bilayers

Poliovirus 135S particles are capable of attaching to liposomes, artificial lipid bilayers (Lonberg-Holm *et al.* 1976), and this attachment is dependent on the exposed N terminus of VP1 (Fricks & Hogle 1990). On the other hand, poliovirus infection is known to permeabilize the infected cell to various compounds (Fernández-Puentes & Carrasco 1980). These observations have led to the view that as the infection of the cell is initiated, the enteroviruses form a pore on one of the cellular membranes (Fricks & Hogle 1990). The pore is believed to be composed of at least the five N termini of VP1 that are present near the pentamer fivefold axis, and the myristate-linked VP4 is also very likely to be involved (Moscufo *et al.* 1993). It is possible that the receptor or some other cellular compound is also involved.

Earlier it was thought that the conformational transition to the 135S particle is required before binding to the membrane can occur, but recently it was shown that also native poliovirus is capable of binding to a planar lipid membrane (Tosteson & Chow 1997). Furthermore, both 135S and 160S particles induce the formation of ion-permeable channels in such membranes (Tosteson & Chow 1997). This finding is in line with the observation that poliovirus infection changes the permeability of infected cells (Fernández-Puentes & Carrasco 1980), although the change in permeability allows entry of larger molecules than could be explained by these channels. The infected cells are permeabilized to compounds like luciferase, horseradish peroxidase (Otero & Carrasco 1987), and polysaccharides (Gonzáles & Carrasco 1987). The channels formed by the two forms of poliovirus were found to be different in nature, the ones formed by the 135S particle being larger and

independent of temperature or voltage across the membrane. It can therefore be speculated that the two forms of channel either have a different function in the infection process, or that one (or both) of them has no role in it. Uncoating of poliovirus appears to be necessary for the permeabilization of infected cells, since uncoating inhibitors also block membrane permeabilization (Almela *et al.* 1991). Based on the observed conductance of the channels, it was estimated that the channel formed by the 135S particle should have a diameter of approximately 2 nm, which could in theory allow the RNA genome to pass through (Tosteson & Chow 1997). The diameter of a fully extended RNA molecule is approximately 1 nm. This data is consistent with the model that enterovirus capsid proteins would form a pore in one of the cellular membranes, allowing the RNA genome to enter the cytoplasm either through a pore or by lysis of an endosome. While the channels formed by the 135S particles might be an essential step in enterovirus infection cycle, it is unclear whether the channels induced by the native virions can be associated with the normal infection process, or whether they merely reflect properties of the virion that are necessary for the uncoating to occur.

It can also be speculated that the channels as such would not have a function in the normal infection, but that they are merely an artificial effect demonstrating the physical properties of the viral particles. Permeabilization of the newly infected cell is a specific phenomenon and does not occur in nonsusceptible cells (Otero & Carrasco 1987, Carrasco 1995). This would suggest that the virus binding and channel formation phenomena that occur nonspecifically in liposomes and other artificial membranes, do not necessarily occur similarly in a normal infection. Specifically, when the 135S particles are studied, they have already lost the majority of the VP4 protein that is needed in the cell entry (Moscufo *et al.* 1993), and may be important in the functional channel formation during this process.

Entry route

The productive infectious pathway of enteroviruses is ill-understood between attachment to a cell surface receptor and initiation of replication in the cytoplasm. It is known that both the 135S particles and even native virions can be internalized into the cell under appropriate conditions (Fenwick & Wall 1973, Lonberg-Holm *et al.* 1975, Willingmann *et al.* 1989, Kronenberger *et al.* 1992, Kronenberger *et al.* 1998). A major problem in trying to elucidate the infectious pathway is the very large particle/infectious unit ratio of all enteroviruses. Only one virus particle out of 100 or more actually initiates an infectious cycle, while all direct chasing methods follow the bulk of the virus population. It is therefore clear that any straightforward observation concerning the fate of the virus or its subunits is unavoidably clouded and potentially misled by the bulk of the virus population engaged in apparently futile events.

In order for the infection to be initiated, the viral genome must pass at least two obstacles: the stable virus capsid and a cellular membrane. The suggested models of infection propose three locations as the site of crossing the protein-membrane barrier between the viral RNA and cellular cytoplasm. Direct penetration through the plasma membrane was suggested when electron microscopic images showed apparently intact poliovirus particles in the cytoplasm 3 min after infection (Dunnebacke *et al.* 1969). The endocytic pathway was also supported by electron microscopy, since poliovirus particles were found in endocytic vesicles (Dales 1973). Most recently, EV1 was found to co-localize with caveolin-1 but not with markers of the clathrin endocytic pathway, suggesting caveoli as the entry site for this virus (Marjomäki *et al.* 2000). It has also been proposed that polioviruses are not dependent on a single infectious pathway and that several alternative pathways could exist together (Arita *et al.* 1998, Arita *et al.* 1999).

Numerous experimental setups have been used in order to find out whether acidification, that occurs in the clathrin-coated pits pathway, is necessary for the

infection to occur. Some of the earlier results suggested that acidification of endosomes is needed for poliovirus infection (Madshus *et al.* 1984a, Madshus *et al.* 1984b), but increasing evidence argues the contrary (Gromeier & Wetz 1990, Pérez & Carrasco 1993, DeTulleo & Kirchhausen 1998).

Acidification appeared to be necessary when studying poliovirus infection with compounds that either dissipate proton gradients across membranes, or inhibit the acidification process of endosomes (Madshus *et al.* 1984a, Madshus *et al.* 1984b). These studies showed that both these types of compounds inhibited infection, and the inhibition was overcome by exposing the cells to low pH. Nevertheless, another study showed that use of these compounds resulted in a delay in infection, but not inhibition (Gromeier & Wetz 1990). The delay was due to side effects of these compounds (Cassell *et al.* 1984), and not the pH-elevating mechanism (Gromeier & Wetz 1990).

Bafilomycin A (BFLA) is an inhibitor of vacuolar proton-ATPases, enzymes that acidify endosomes as well as other vesicular organelles (Bowman *et al.* 1988). This drug has also been shown to have unwanted effects: it blocks endosomal transport in a cell line-dependent manner (Bayer *et al.* 1998). Therefore, inhibition of infection in the presence of BFLA does not necessarily imply that the virus is dependent on acidification, but a negative effect on inhibition apparently shows that the studied virus does not require acidification for infection. It has been shown that BFLA does not affect infection by poliovirus (Pérez & Carrasco 1993) or CAV9 (Roivainen, unpublished results), and these viruses therefore do not require an acidic environment for entry.

Further evidence against the necessity of using the clathrin endocytic pathway was obtained by using HeLa cell lines that expressed either dynamin^{K44A}, failing to load GTP, or dynamin^{ts}, failing to hydrolyze GTP at the non-permissive temperature (DeTulleo & Kirchhausen 1998). The phenotype of these cells appeared unaltered, except for the block in the formation of clathrin-coated pits and vesicles. Clathrin-

dependent endocytosis is blocked in these cells, because dynamin functions in a GTP-dependent manner in the budding of a clathrin-coated pit. The results showed that poliovirus 1/Mahoney was able to infect the cells in a clathrin-independent manner, while the three other viruses studied were not. Two of these viruses had envelopes (Semliki Forest virus and Sindbis virus), but even human rhinovirus 14, an unenveloped picornavirus, appeared to be dependent on the clathrin-coated vesicle formation.

AIMS OF THE STUDY

The intriguing properties of the conserved PALTAVETGHT motif (described above in “Conformational transitions in the capsid”) prompted us to study the nature of this amino acid sequence, and the functions that it might have in the coxsackievirus A9 infectious cycle. By using a site-saturation approach, we expected to be able to elucidate both the nature of conservation that is seen in the amino acid sequences, and to probe the function that the motif was hypothesized to have. It was hoped that the mutants would assist in understanding the reversible and irreversible conformational transitions that occur in the enterovirus structure. To reach this goal, we wanted to develop a new site-saturation strategy to be used in studying picornaviruses. The results obtained during the work prompted us to ask whether the liposome-binding properties of poliovirus are a common feature of enteroviruses.

MATERIALS AND METHODS

Numerous laboratory methods have been used in the studies described in this thesis. The majority of these methods (virus culturing and labeling, analysis of protein compositions and molar ratios, ultracentrifugations, polymerase chain reactions, DNA sequencing, etc.) are standard laboratory practices that are described in the original articles and manuscripts (I-IV), or can be found in laboratory manuals and textbooks. Only the techniques that are not normally used in virological laboratories, or contain a novel aspect, are described here.

Viruses and cell lines

Coxsackievirus A9 (Griggs) was obtained as an infectious cDNA clone from Dr. Glyn Stanway (University of Essex, UK) (Chang *et al.* 1989, Hughes *et al.* 1995). This cDNA was linearized and transcribed to RNA, that was then transfected to LLC Mk_2 cells. The cells were covered by 0.5 % carboxymethylcellulose (CMC), and well isolated virus plaques were used in growing the virus stocks.

In addition, we used the following viruses, originally obtained from the ATCC: poliovirus 1/Mahoney, poliovirus 3/Sabin, coxsackievirus A7 (USSR AB-IV), coxsackievirus A16 (G-10), coxsackievirus A21 (Kuykendall), coxsackievirus B5 (Faulkner), and enterovirus 68 (Fermon).

In propagating the viruses, we used green monkey kidney cell lines (LLC Mk_2, GMK, Vero) and human tumor-derived cell lines (HeLa-Ohio, RD, HEp-2c).

Vectors and primers

The M13mp18 vector and the *E. coli* TG1 strain were obtained in the Sculptor *In vitro* Mutagenesis Kit (Amersham, UK). Vector pBS was obtained together with the

Table 3. Oligonucleotides used in this thesis.

Oligo	Sequence (5'-3')	Use (ref.)	CAV9 position
I	CAATTCCGCGAGCGTAXYZGCACTCACTGCAGTTG	m (I)	2516-2550
II	GCACTCACTGCAGTTXYZACAGGGCACACCTCG	m (I)	2535-2567
III	GTACCTGCACTCACTXYZGTTGAGACAGGGCAC	m (I)	2529-2561
IV	GTTGAGACAGGGCACXYZTCGCAAGTTACTCCAAG	m (I)	2547-2581
V	CCGCGAGCGTACCTXYZCTCACTGCAGTTGAG	m (I)	2521-2552
VI	GCGAGCGTACCTGCAXYZACTGCAGTTGAGACAG	m (I)	2523-2556
VII	GCGTACCTGCACTCXYZGCAGTTGAGACAGGG	m (I)	2527-2558
VIII	CCTGCACTCACTGCAXYZGAGACAGGGCACAC	m (I)	2532-2563
A1	CTAGACGTACGGCGCGCT	a (I)	-
A2	CTAGAGCGCGCCGTACGT	a (I)	-
7625	GCGTGCAACGACTTCTCAGTAAGAATGTTGAG	p, s (II, III)	2379-2410
4934	CACAGTCGACTCAGATCTAGAATGATAGTTTTTCAC	p, s (II)	2639-2604
M13 –40	GTTTTCCCAGTCACGAC	s (II)	-
3869	TAATAAGCTTCAAGGGGAT	m (II)	2435-2453
6093	GACATGTGAAAAACTATCATTCTAGATCTGAGTCGACTGTGGAG	m (II)	2599-2642
6966	CTATCATTCTAGAAGTGAGTCGACTGTGG	m (II)	2612-2640
91856	CGGTGAGCAACAGAGCTATTGTGTATCTGTTTACTG	p, s (III)	641-676
91857	CCATCTCAGCCTCGGGCACACACAC	p, s (III)	1347-1323
92066	CAGAACATGCAATACCACTACCTGGGTCGTGCAG	p, s (III)	1230-1263
92067	CACGACCGAGTCCACTTCTGCTATTTCCATTAG	p, s (III)	1892-1860
91860	GGCCTTTGCCACAATTTGATGTGACGCCT	p, s (III)	1801-1829
91861	CAACTGCCCTCTCAATGGCTTCTTCCACAT	p, s (III)	2481-2451
9354	CACCCGTCATAAAAATTGCTGTATGCATTTCC	p, s (III)	3043-3012
91862	GATTCCAGCAAAAGTTGATGATTATGCCTGGCAG	p, s (III)	2903-2946
91863	CAATTTTGCCAATCCGTGTGTGTTG	p, s (III)	3436-3408

Primers are given with the names or numbers used in the original articles. Code: **a**, used in forming an adapter; **m**, used in mutagenesis; **p**, used in PCR; **s**, used in sequencing. If the oligonucleotide was used in mutagenesis, the mutagenic bases are underlined. Numbering of the nucleotide positions is according to Chang *et al.* (1989). In oligonucleotides I and II, the bases X, Y and Z each contained 25 % of A, C, G, and T. In oligonucleotides III-VIII, the proportions of bases were modified as described in article I.

CAV9 clone from from Dr. Glyn Stanway (University of Essex, UK).

Oligonucleotides were synthesized in the National Public Health Institute (Helsinki, Finland), or at the Institute of Biotechnology (University of Helsinki, Finland). Primers used in mutagenesis, polymerase chain reactions, sequencing, and in creating an adapter, are shown in Table 3.

Cloning and site-saturation mutagenesis (I, II)

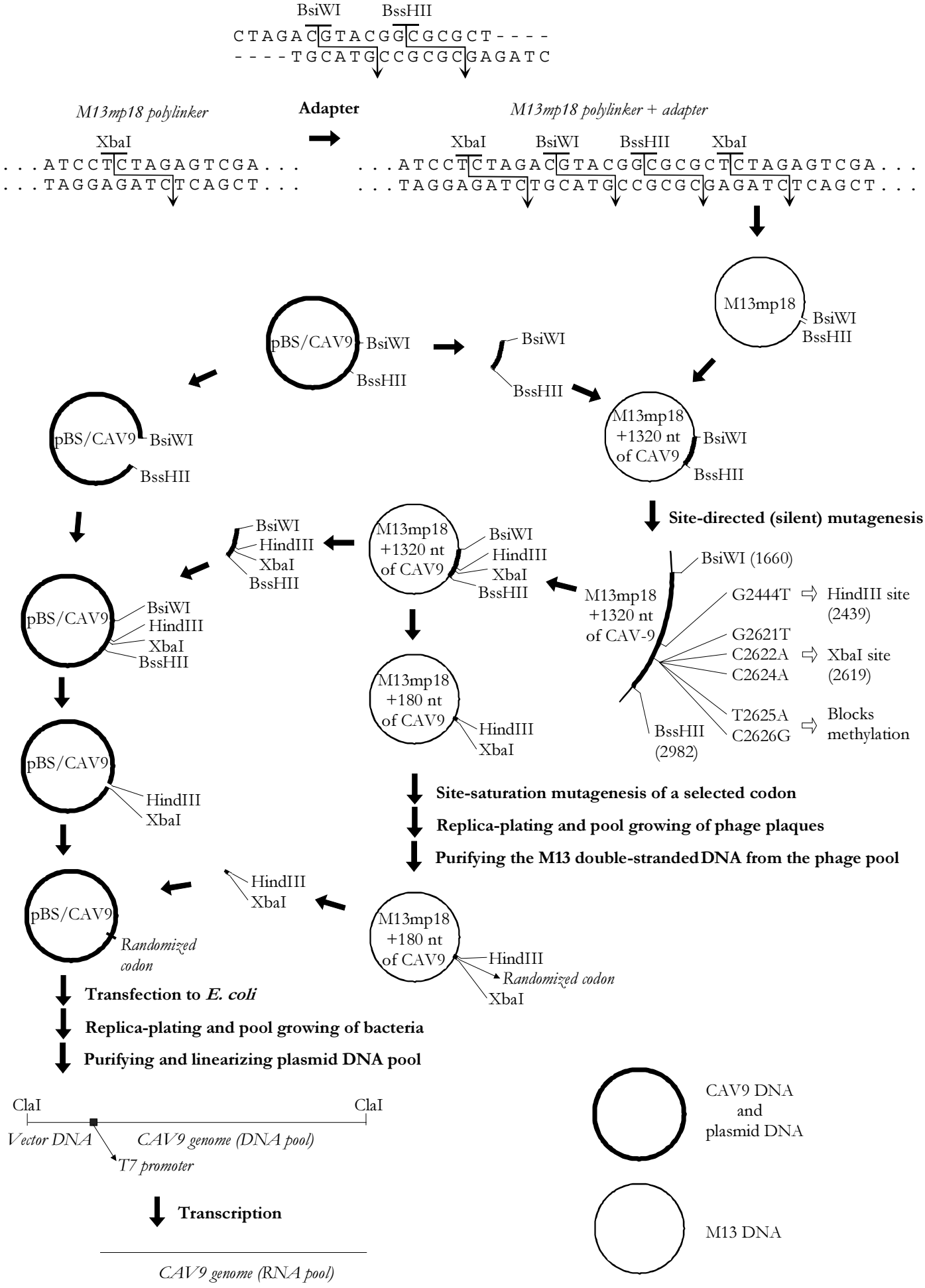
Nucleotides 1661 to 2982 (1322 bp) of the CAV9 genome in pBS vector were cleaved from the cDNA clone (Chang *et al.* 1989, Hughes *et al.* 1995) by restriction endonucleases BsiWI and BssHII, and ligated to an M13mp18 vector supplemented with additional cleavage sites for these enzymes, created by using an adapter. Site-directed (silent) mutagenesis was performed (see below) to introduce unique restriction sites for XbaI and HindIII, and the 1322 base pairs were cloned back into the genome. The silent mutations were G2444T, G2621T, C2622A, C2624A, T2625A, and C2626G. These mutations facilitated the cleavage of a fragment of 180 nt around the target area without changing the amino acids coded therein. This fragment was subcloned from the pBS/CAV9 plasmid into M13mp18 as above, and site-saturation mutagenesis was performed on four different codons.

Site-saturation mutagenesis was performed, using the single-stranded form of M13mp18 including the 180 bp CAV9 insert, by primer extension reaction with degenerate central trinucleotides in the oligonucleotides, using the Sculptor *In vitro* Mutagenesis Kit (Amersham, UK) with the *E. coli* TG1 strain included in the kit. The method is a modification of the original M13-based phosphorothioate technique (Sayers *et al.* 1992). Annealing of the mutagenic oligonucleotide to the single-stranded template DNA was performed by first incubating the annealing mix for 3 min at +80 °C, then moving the mix to a waterbath at +60 °C, and allowing the

waterbath to cool to +37 °C during 60 min. The primer extension reaction was performed using T7 DNA polymerase with a dNTP mix with three normal bases and the dCTP base analogue dCTPαS, in the presence of T4 DNA ligase. The phosphorothioate base has a sulphur atom substituted for oxygen at the alpha phosphate. Remaining single-stranded DNA was digested by T5 exonuclease. The non-mutant strand was nicked by the restriction endonuclease NciI (recognition sites: CCCGG or CCGGG), which does not cleave the phosphorothioate internucleotidic linkages, therefore leaving the mutated strand intact. The nicked non-mutant strand was partially digested using exonuclease III, and repolymerized by DNA polymerase I to include the mutated area. Ligation was again accomplished by T4 ligase. *E. coli* TG1 bacteria were transformed with the mutated DNA, and grown on petri plates containing a culture of *E. coli* TG1 to facilitate multiplication of the M13mp18 bacteriophage.

The M13mp18 phage plaques were transferred from culture plates by allowing the plaques to attach to a Magna Lift nylon membrane (Micron Separations) at +37 °C, that was then lifted to an Erlenmeyer flask with 2 x TY medium containing *E. coli* TG1. The flask was agitated vigorously at +37 °C for 4 h 40 min, after which double-stranded M13mp18 DNA was purified from the bacteria using the Qiagen Midi Plasmid kit. The 180 bp fragment was cut by XbaI and HindIII, purified from agarose gel, and ligated back to CAV9 cDNA. The cDNA pool was transfected to *E. coli*, and bacterial colonies were replica-plated as the M13 plaques above. The bacterial colonies on the nylon membrane were lifted to LB medium containing 100 µg/ml ampicillin, and allowed to grow overnight with vigorous agitation. The plasmid DNA was purified with the Qiagen Midi Plasmid kit.

Figure 4. Site-saturation mutagenesis and cloning scheme.


BsiWI
BssHII
C T A G A C G T A C G G C G C G C T - - - -
- - - - T G C A T G C C G C G C G A G A T C
M13mp18 polylinker
Adapter
M13mp18 polylinker + adapter
XbaI
. . . A T C C T C T A G A G T C G A . . .
. . . T A G G A G A T C T C A G C T . . .
XbaI BsiWI BssHII XbaI
. . . A T C C T C T A G A C G T A C G G C G C G C T C T A G A G T C G A . . .
. . . T A G G A G A T C T G C A T G C C G C G C G A G A T C T C A G C T . . .
M13mp18
BsiWI
BssHII
pBS/CAV9
BsiWI
BssHII
M13mp18 +1320 nt of CAV9
Site-directed (silent) mutagenesis
BsiWI (1660)
M13mp18 +1320 nt of CAV-9
G2444T ⇨ HindIII site (2439)
G2621T
C2622A ⇨ XbaI site (2619)
C2624A
T2625A
C2626G ⇨ Blocks methylation
BssHII (2982)
HindIII
XbaI
M13mp18 +180 nt of CAV9
Site-saturation mutagenesis of a selected codon
Replica-plating and pool growing of phage plaques
Purifying the M13 double-stranded DNA from the phage pool
Randomized codon
Transfection to E. coli
Replica-plating and pool growing of bacteria
Purifying and linearizing plasmid DNA pool
ClaI
Vector DNA
CAV9 genome (DNA pool)
T7 promoter
Transcription
CAV9 genome (RNA pool)
CAV9 DNA and plasmid DNA
M13 DNA

This plasmid pool, now containing the infectious CAV9 clone with all possible codons at one selected amino acid-coding position, was linearized by cleaving with restriction endonuclease ClaI. Infectious viral RNA was synthesized by T7 RNA polymerase, and transfected into LLC Mk_2 cells by Lipofectin (Gibco-BRL). Lipofectin was replaced by medium containing 0.5 % CMC and 10 % fetal calf serum, and the plates were incubated at +37 °C for 1 to 3 days, picking the plaques in 2 µl of medium upon their appearance. These virus samples were plaque-purified once, and the resulting plaques were propagated on 6-well plates until full CPE was seen. Success of site-saturation was confirmed by sequencing. The complete cloning scheme is shown in Figure 4.

Liposome flotation (IV)

Lipid stocks were prepared from phosphatidylethanolamine, phosphatidylcholine, sphingomyelin, cholesterol, and phosphatidic acid in molar ratios of 1:1:1:1.5:0.3 (White & Helenius 1980, Fricks & Hogle 1990). In each experiment, 1 µmol of total lipid was used. Liposomes were prepared by dispersing dried lipids in buffer containing 10 mM sodium phosphate, 140 mM NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, and 0.1 % bovine serum albumin, warmed to +50 °C, vortexed for 1 min, and sonicated for 1 min at +50 °C.

Virus particles were mixed with liposomes at room temperature, and incubated for 5 min at various temperatures between room temperature and +50 °C. The mixture was brought back to room temperature and mixed with 67 % sucrose to obtain 50 % final sucrose. This sample layer was overlaid with two layers of 25 % and 10 % sucrose, respectively. These step gradients were centrifuged for 16 hours at 40 000 rpm at +18 °C, and the gradients were fractionated. Sample radioactivity was measured in a Wallac MicroBeta counter (Wallac, Finland), using 50 µl samples and 200 µl Optiphase Supermix SC/9235/21 scintillation liquid (Wallac, Finland).

RESULTS AND DISCUSSION

Site-saturation (I)

Site-saturation mutagenesis is a method that is used in replacing a selected codon by a set of codons that, upon translation, should yield all 20 amino acids in the mutant population. We decided to use this method in order to estimate what phenotypic features of the studied motif (see the next chapter) are associated with its evolutionary conservation, and to probe the function that the motif might have.

When performing the site-saturation mutagenesis in an M13 system, we noticed that the previously used methods did not yield a random set of codons. Instead, the individual positions of the resulting set of codons at the target position were found to have a strong bias, tending to conserve the original sequence. Percentages of original bases were between 35 % and 58 %, rather than the expected 25 %. We concluded that this is due to the differential melting temperatures (T_m) of the mutagenic degenerate oligonucleotides, and decided to study whether this bias could be corrected by modifying the nucleoside phosphoramidite compositions during the synthesis of the mutagenic oligonucleotides. Our aim was to find out whether there were some general rules that could be applied in correcting the compositions.

We synthesized six degenerate oligonucleotides, now with modified base compositions, and used them in site-saturation mutagenesis of six codons. In the degenerate positions of the mutagenic oligonucleotides, we reduced to varying extents the complementary bases, in favor of the mutagenic bases. We studied a total of 344 mutagenized codons, and made a statistical analysis of the results, in order to find out the underlying general rules that could be applied in further studies. We analyzed the effects of the various base compositions at the nucleotide and the codon level, the influence of the mismatching positions, and the possible significance of all non-Watson-Crick base pairs.

The oligonucleotides with modified compositions yielded an almost optimal randomization in the resulting codons. There was a drawback, however, that needed to be considered separately: the central position (p2) of the codon was too heavily balanced towards mutagenesis. This was presumably due to the lack of base pair stacking in the cases when the annealing mutagenic oligonucleotide has mismatches at both the first and the third positions (p1, p3) of the codon. In these cases, the central matching nucleotide contributes very little thermodynamic stability, and therefore, in the subset of mutagenic oligonucleotides having p1 and p3 mutated, the identity of p2 reflects the base composition of the mutagenic oligonucleotide at that position. In the analysis of all double mutants found in the study, p1 and p2 were mutated in 54 codons, p2 and p3 in 57 codons, but p1 and p3 in only 24 codons.

In addition to the differential stabilities of the normal Watson-Crick base pairing (C-G being stronger than A-T), the non-Watson-Crick base pairs are known to have differences in their stabilities (Werntges *et al.* 1986). We analyzed whether the effect of these differences could be seen in the set of mutagenized codons. Although there was some overrepresentation of the 2nd and 3rd strongest pairs G.G and A.G (Aboul-ela *et al.* 1985), and underrepresentation of the weakest base pair C.C, the results led us to conclude that non-Watson-Crick base pairing does not need to be considered in designing the oligonucleotides for site-saturation mutagenesis.

Based on the analysis of the data set, we proposed a general design for the degenerate positions in the mutagenic oligonucleotide. In this design, the differential stabilities of the two Watson-Crick base pairs, as well as the special nature of the central base pair have been considered (Table 3 in I).

The VP1 intracapsid hook (II, III)

In all enteroviruses, there is a highly conserved amino acid motif of 11 residues close to the N terminus of VP1 (II). The consensus sequence of this motif is PALTAVETGHT. In an alignment of 79 different enterovirus sequences of 68 different serotypes, five of the 11 amino acids were completely conserved (underlined residues above), and there were no insertions or gaps in this motif (II). The amino acids taking part in the β-barrel formation are known to be highly conserved among enteroviruses, while the loop regions between the β-strands and at the N and C termini of the proteins are more variable. Outside of the β-barrel-motivated conservation, the PALTAVETGHT region appears to be the most conserved region in the viral structural proteins.

This region has been shown to be transiently exposed in native poliovirus virions (Roivainen *et al.* 1993, Li *et al.* 1994), although being inside the capsid in the crystal structure. These properties of the conserved motif lead us to use site-saturation mutagenesis in order to probe the nature of the conservation, and to make an attempt to elucidate the functions that this motif might have in the enterovirus life cycle. To facilitate the site-saturation mutagenesis, we made several improvements to the previously used methods, allowing the transfection of a site-saturated viral genome pool (II). This “single-tube” site-saturation method should be useful in studying any virus, provided that the viral genome is cloned and the cDNA, or its derivatives, are infectious in cell culture.

When studying the structures of the six enteroviruses that have been published, we found that this region is always in exactly the same position (III). Furthermore, exactly the same organization is seen in rhinoviruses (III). The β-barrel of the VP1 protein is separate from the N terminal 60 to 80 residues, the β-barrels of five copies of VP1 forming the protrusion at the fivefold axis, and the 'north' rim of the canyon. On the N terminal side of the β-barrel, the chain first dives to the inner surface of the capsid, and there forms a long loop of approximately 45 to 50 amino

acids. The very N terminus then forms an extended arm towards the fivefold axis (Figure 7 in III). The loop region makes a tour on the inner surface of the capsid, with approximately 20 amino acids forming a hook-like structure at the end of the loop. In this hook, the PALTAVETGHT motif is covered in a cavity formed by the other three capsid proteins.

When viewed from within the capsid, the bottom of the hook cavity is formed by the BIDG β-sheet of VP3, the tip is formed by VP2, and the hook is loosely covered by the approximately 16 C terminal residues of VP4. When studying the contacts that the hook amino acid residues have with the neighboring capsid proteins, we found that there are completely conserved contacts with all the other capsid proteins (III). In particular, the tripeptide Gln-Ser-Ser (QSS) in VP3 was found to be identical in all enterovirus structures, and always in contact with leucine (L) and glutamate (E) of the PALTAVETGHT motif (III). The same organization is seen in rhinoviruses, except that the latter serine of the QSS motif is replaced by threonine.

We performed oligonucleotide-mediated site-saturation mutagenesis of four codons in the hook. When trying to mutate the CAV9 residues A30, L31, T32 and V34 (PALTAVETGHT), we were unable to recover a viable virus carrying a mutation at the leucine residue L31 (II). This was not due to a problem in the site-saturation method, since we could recover mutant viruses with three different leucine codons at the target location (II). It was not surprising that this amino acid does not seem to allow variation, as it was the only completely conserved residue of the ones that we tried to mutate. It is also the residue that is always found in contact with the QSS motif of VP3.

At the other mutated positions, we found a number of various substitutive amino acids among the viruses recovered (II). Alanine 30 allows clearly more variation than the other amino acids, and we recovered viruses carrying both polar (glutamine) and nonpolar substitutions (cysteine, leucine), in addition to intermediate ones (asparagine, glycine, serine, and the wild-type alanine). At

position 32, we found two substitutions of intermediate polarity (histidine and serine), in addition to the wild-type threonine, which is polar. At position 34, we found two intermediate-polarity substitutions (alanine and serine), and three nonpolar residues (cysteine, isoleucine, and the wild-type valine). In other words, the intermediate-polarity alanine could be mutated to both polar and nonpolar amino acids, the polar threonine could be changed to intermediate polarity (but not nonpolar), and the nonpolar valine could be changed to intermediate polarity (but not polar). The variation in the viable mutant viruses faithfully follows the variation that is observed among enteroviruses (Table 2 in II), strongly suggesting that it is the polarity in the region around the five completely conserved amino acids, that needs to be conserved (II).

The hook region of the VP1 of CAV9, at positions 28 to 51, has a clearly periodic hydrophobicity profile, characteristic of amphipathic structures (II). The conservation of this profile and the finding that site-saturation did not yield any mutant viruses in which a polar amino acid would be replaced by a nonpolar one, or vice versa, suggests that the studied motif may have a function where amphipathic nature is needed. No apparent explanation for the conservation of periodic hydrophobicity could be found by studying the virus structures. The polar and nonpolar amino acids group together as always, but there is no apparent reason for periodicity, or even conservation. The recently published model of the 135S particle (Belnap *et al.* 2000b) offers an attractive explanation. Namely, it is suggested that the N termini of the VP1 proteins are externalized through the base of the canyon, necessitating the release of the pocket factor from the bottom of the canyon. The N termini of VP1 molecules would then form extended arms towards the fivefold axis, where the N termini meet and probably form a five-stranded structure that is inserted into the cell membrane (Belnap *et al.* 2000b). The arm should therefore be on the outside of the virus capsid, snaking up the fivefold protrusion, and probably making a contact with the membrane. This would explain why the potential for amphipathicity of this region needs to be conserved.

From the site-saturated virus pools, we purified 12 different mutants, all of which had defects in producing infectious progeny virus (II). Based on preliminary results of all viable mutant phenotypes, five mutants were selected for detailed characterization. The selection was based on three criteria: (i) apparently homogeneous stocks of the selected viruses could be grown, (ii) all three successfully mutated positions were represented, and (iii) judging by the initial characterization, the selected viruses represented a maximal range of phenotypes.

We found two general properties that were altered in the mutant viruses: the capability to produce infectious progeny virus was decreased, and the particle/infectious unit ratio was increased (III). In addition, three variables in the virus life cycle were altered. First, the stability of the virions was changed, two of the mutants being hyperlabile (A30G and V34A), two being moderately more labile than the wild-type (A30S and V34I), and one mutant being more stable than the wild-type (T32S). Second, attachment efficiency to LLC Mk_2 cells was significantly lower for at least two mutants (A30G and V34A). Third, formation of subviral particles upon attaching to the host cell was altered, and correlated with the stability of each mutant. We were unable to find any differences in the time scale of infection, efficiency of protein synthesis, accumulation of assembly intermediates, release from cells, or sedimentation coefficients of the virions.

All the differences found between the mutant viruses and the wild-type virus can be explained by variables related to uncoating (III). The hyperlabile mutants were apparently too easily triggered to transform into A particles, rendering a large proportion of the viruses unable to initiate infection. This would be the direct reason for increased particle/infectious unit ratio as well. It can also offer an explanation for the defects in cell attachment, as the A particles do not attach to cells. It should be noted that the protein production was not hindered when using similar titers of input virus, suggesting that production of progeny virus was perhaps not altered, but the progeny was inactivated too easily. Properties of the moderately labilized mutants can be explained with same arguments. The stabilized mutant, on the other

hand, is apparently less sensitive to signals that trigger uncoating, therefore being ineffective in initiating infection.

The mere location of the PALTAVETGHT motif in the sequence, being close to the N terminus of VP1, suggests a function involved in uncoating or cell entry, due to the membrane-binding properties of the N terminus, and its suggested role in channel formation (Fricks & Hogle 1990). The periodicity in the hydrophobic profile suggests a function related to membrane interactions (see above), and properties of the viable mutants suggest that the high degree of conservation may be needed due to the conserved contacts between residues of this motif and all other capsid proteins (III). These contacts may, in part, determine the kinetic equilibrium between the two interconvertible forms of the virus.

Liposome interactions (IV)

Liposome binding of PV1 has been demonstrated earlier (Fricks & Hogle 1990, Tosteson & Chow 1997), and based on sequence analysis, it has been suggested to be common to enteroviruses (Fricks & Hogle 1990). Since the binding is dependent on the N terminus of VP1, and the motif that we have studied is close to this region, we decided to study whether the liposome binding properties of the CAV9 mutants would be altered. To our surprise, we were unable to find liposome-associated virus in these experiments. This prompted us to ask whether liposome binding is a property that is common to enteroviruses.

We used the liposome flotation method in assaying the binding (White & Helenius 1980, Fricks & Hogle 1990). The studied virus particles were incubated with liposomes under various conditions, and mixed with sucrose to obtain 50 % final sucrose concentration (W/V). On top of this sample layer, we carefully added two layers of more diluted sucrose (25 % and 10 %), and this step gradient was

ultracentrifugated. During ultracentrifugation, virus particles that are bound to liposomes, are expected to elevate towards the top of the step gradient.

We characterized the liposome binding of eight enteroviruses, representing all five enterovirus species (PV and HEV-A to HEV-D). The differences in binding were found to be remarkably clear. Representatives of the genetic cluster formed by polioviruses and HEV-C readily bound to liposomes, whereas members of the HEV-A, HEV-B, and HEV-D species showed only marginal binding under all conditions used. The binding capacity was not altered by different pH conditions, or by introducing a pH gradient across the liposome membrane, and the percentage of attached virus did not increase after five minutes.

We studied the binding of both 135S particles and native virions, and found that the 135S particles bound at +22 °C, while the native virions bound at +37 °C and temperatures above that. Temperatures between +22 °C and +37 °C were not studied. At elevated temperatures (+50 °C and above), a minority of the non-binding viruses were found to form a peak in the flotation pattern, similar to the ones formed by polioviruses and CAV21. A surprising difference between the liposome-binding viruses was that while PV1 and CAV21 bound as both 135S particles and native virions, PV3 bound only as a native virion. Furthermore, the flotation pattern of PV3 was remarkably different from that of PV1 and CAV21, the majority of PV3 particles being only slightly elevated. A possible interpretation of these observations would be that PV1 and CAV21 formed networks through virus particles that are bound to two or more liposomes, whereas the majority of PV3 would be bound to a single liposome. Another explanation would be that a small amount of lipids becomes associated with degraded PV3 particles or viral protein aggregates, forming the slowly elevating material. The latter option seems unlikely, since this peak was formed by native virions and liposomes, but not by 135S particles and liposomes.

Under the conditions used, it seems that out of the eight enteroviruses studied, only polioviruses and CAV21 are capable of binding to liposomes. There are, however, two observations that oppose this interpretation. First, at elevated temperatures, the non-binding viruses did show signs of binding to liposomes. Second, sequence alignments of the N terminal regions of the viruses show that they all have a theoretical capacity to be arranged into an amphipathic structure, albeit they share little or no homology at the level of amino acid identity. The variation in liposome binding is likely to reflect functional differences in uncoating and cell entry between the different enterovirus species.

CONCLUSIONS

The biological properties of two elements in the VP1 protein of enteroviruses have been studied. One of the elements was a highly conserved amino acid motif of 11 residues (PALTAVETGHT) that lies inside the capsid, but is known to be transiently exposed. The other element was the N terminus of the same protein, known to be capable of interactions with lipid membranes.

Four codons corresponding to residues Ala30, Leu31, Thr32, and Val34 of coxsackievirus A9 capsid protein VP1 were randomized and cloned back to the infectious cDNA, yielding a pool of genomes that were then transcribed to RNA and transfected to green monkey kidney cells (LLC Mk_2). The spectrum of viable mutant viruses suggests that the periodic hydrophobicity pattern is required for viability of the virus. It appears that changes of Thr32 or Val34 to amino acids of opposite polarity abolished the viability of the virus, but even mutations that retained the polarity were defective in producing infectious progeny virus. All mutations affected formation of the A particles, a structurally altered form of virus that is thought to be an essential intermediate in cell entry. Two mutations strongly enhanced A particle formation, two others enhanced it to a moderate degree, and one mutant appeared more stable than the wild-type virus. By structural comparisons of all entero- and rhinovirus structures available, completely conserved Van der Waals contacts of the studied motif with all other capsid proteins were identified.

It is concluded that the PALTAVETGHT motif is one of the key factors that affect the uncoating process. The motif is suggested to have a kinetic equilibrium between two states, one being exposed on the surface of the capsid, and the other lying at the highly conserved pocket formed by the three other capsid proteins. Receptor binding, or the presence of a membrane, may shift the equilibrium, triggering the irreversible changes that initiate uncoating through externalization of the N terminal region of VP1 and the internal capsid protein VP4. Five copies of the very N

terminus of VP1 are thought to form a channel through the membrane at the fivefold axis of the virus, while the PALTAVETGHT is suggested to form an amphipathic arm between the membrane-spanning N terminus and its exit site at the base of the canyon surrounding the fivefold axis.

The liposome binding properties of eight enterovirus serotypes were characterized, using representatives of all five human enterovirus species. The readily binding viruses were found in the genetic cluster that includes polioviruses and the human enterovirus C species. Human enteroviruses A, B, and D were found inefficient in binding to liposomes, and very limited binding was only seen at elevated temperatures. The differences suggest that there is a functional difference in the uncoating between the genetic cluster formed by polioviruses and human enterovirus C species, and the other enteroviruses. The difference may lie in the uncoating mechanism, or in the channel formation properties of the virus particles, that may be assisted by cellular compounds.

Two methodological improvements were developed. First, randomization of site-saturated codons was markedly enhanced by modifying base compositions at the degenerate positions of the mutagenic primer. Second, cloning strategies were developed to enable direct transfection of viral genome pools that are otherwise identical, but have a selected codon randomized. This strategy enables direct phenotypic selection of the resulting mutant virus populations, and by allowing simultaneous processing of all mutant genomes, it drastically reduces the laboratory work needed.

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