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CHAPTER

12

Genetics, Pathogenesis and Evolution of Picornaviruses

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INTRODUCTION

The discovery of viruses heralded an exciting new era for research in the medical and biological sciences. Many contemporary virologists do not know, however, that the first animal virus described was a picornavirus, the etiological agent of the dreaded foot-and-mouth disease in cloven-footed animals. The discovery of foot-and-mouth disease virus (FMDV) by F. Loeffler and P. Frosch in 1898 (Loeffler and Frosch, 1898) occurred at the same time as M.W. Beijerinck described the amazing "contagium vivum fluidum" in 1898. This liquid was a filtered leaf extract derived from tobacco plants suffering from tobacco mosaic disease. Free of bacteria, it was yet able to transmit the disease to uninfected plants. Already in 1892, I. Ivanovski had made a similar observation with tobacco mosaic virus but apparently he was unable to fully convince his peers of the significance of his discovery (Waterson and Wilkinsen, 1978).

Research on viruses, now formally in its hundred-and-first year, has yielded an immense harvest of biochemical and biological information. The studies were driven not only by an urgent need to understand, and possibly prevent, viral disease; they were also fueled by a strong curiosity about the minute biologicals called viruses, which we can view as chemicals, on the one hand and as "living" entities on the other. Poliovirus is an exquisite example of a chemical with a known empirical formula (Molla *et al.*, 1991) that can be crystallized (Schaffer and Schwerdt, 1955) yet causes a devastating disease in humans. Poliovirus was discovered 90 years ago by Landsteiner and Popper (1909) to be the causative agent of poliomyelitis. The current knowledge of its chemical and three-dimensional structure and of its life cycle and pathogenesis is second to none. Indeed, the intense research efforts on poliovirus over a period of nine decades will lead to its demise in the near future: global eradication of poliovirus is considered possible by the year 2000.

Following the identification of FMDV and poliovirus, a deluge of other viruses with similar properties were uncovered. These viruses have now been classified as Picornaviridae, a large family of small (Lat. *pico*) RNA (rna) viruses. Currently, Picornaviridae consists of six genera: *Enterovirus, Rhinovirus, Hepatovirus, Parechovirus, Cardiovirus* and *Aphthovirus* (Table 12.1). The first four genera include predominantly human pathogens, which cause a bewildering array of disease syndromes. Although a disease syndrome may be considered characteristic for a specific picornavirus group, the same syndrome can possibly be also produced by

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TABLE 12.1 Picornaviridae (nd, no data; italics denote a non-human pathogen)

Genus	Clusters	Major associated syndromes	Receptor	References
Enterovirus	A			
	Coxsackieviruses A 2,3,5,7,8,10,12,14,16	Herpangina, hand-foot-and-mouth disease, respiratory disease, meningitis, poliomyelitis (CAV7)		
	Enterovirus 71	Hand-foot-and-mouth disease, meningitis, paralysis	nd	
	B			
	Coxsackieviruses A9	(See CAV above), poliomyelitis,	$\alpha_v\beta_3$	(1)
	Coxsackieviruses B1–6	myocarditis, pleurodynia, meningitis, hand-foot-and-mouth disease, respiratory disease, neonatal, infections	HCAR*, DAF	(2)
	Echoviruses 1–9, 11–21, 24–27, 29–33	Meningitis, encephalitis, pleurodynia, exanthema	VLA-2(=$\alpha 2\beta 1$)	(3)
			DAF (=CD55)	(4)
	Enterovirus 69		nd	
	C			
	Poliovirus types 1–3	Poliomyelitis, meningitis	CD155	(5)
	Coxsackieviruses 1, 11, 13, 15, 17, 18–22, 24	Common cold, infantile diarrhea	ICAM-1†	(6)
	Coxsackievirus 24v‡	Acute hemorrhagic conjunctivitis		
	D			
	Enteroviruses 70 (68)	Acute hemorrhagic conjunctivitis	nd	
	E			
	Bovine enterovirus types 1 and 2	Diarrhea (cattle)	nd	
*Parechovirus***	Human echovirus types 22 and 23	Respiratory disease, encephalitis	nd	
Rhinovirus	Major receptor group rhinov. (>90 serotypes)	Common cold	ICAM-1	(7)
	Minor receptor group rhinov. (>10 serotypes)	Common cold	LDL receptor	(8)
Hepatovirus	Hepatitis A virus	Type A hepatitis	HAVcr-1	(9)
Aphthovirus	Foot-and-mouth-disease virus FMDV A12	Foot-and-mouth disease	$\alpha_v\beta_3$	(10)
	O1	(cloven-footed livestock)	Heparan sulfate	(11)
Cardiovirus	Encephalomyocarditis virus	Encephalitis, myocarditis	VCAM-1	(12)
	Mengovirus	(hoofed livestock)		
	Theiler's murine encephalomyocarditis virus	Encephalitis (murine)	nd	
	Vilyuisk virus	Encephalitis	nd	

The following viruses have been recognized as picornaviruses on the basis of their genome sequences and physico-chemical properties as well as the result of comparative sequence analyses (see the section on Evolution): equine rhinovirus types 1 and 2, Aichi virus, porcine enterovirus, avian encephalomyelitis virus, infectious flacherie virus of silkworm

Clusters of enteroviruses refer to groups of enteroviruses arranged predominantly according to genotypic kinship (Hyypia *et al.*, 1997). More clusters, including mainly animal enteroviruses, have been proposed.

List of human syndromes adapted from Melnick, 1996. Common syndromes in humans caused predominantly by one and/or other member(s) of the cluster but member viruses of other clusters may cause the same syndrome.

Receptors may be specific for specific serotypes. For details, see text.

References describing the identification of receptors: (1) Roivainen *et al.*, 1994, (2) Tomko and Philipson, 1997; Shafren *et al.*, 1997, (3) Bergelson *et al.*, 1992; (4) Bergelson *et al.*, 1994; Ward *et al.*, 1994; (5) Mendelsohn *et al.*, 1989; (6) Shafren *et al.*, 1997; (7) Staunton *et al.*, 1989; Greve *et al.*, 1989; (8) Hofer *et al.*, 1994; (9) Feigelstock *et al.*, 1998; (10) Neff *et al.*, 1998; Berinstein *et al.*, 1995; (11) Jackson *et al.*, 1996; (12) Huber, 1994.

* shared with adenovirus type 2.

† DAF (decay accelerating factor) may function as non-essential (infection-augmenting) coreceptor.

‡ Coxsackie virus A24v is a genetic variant of coxsackie virus A24.

** Pringle, 1996.

other picornaviruses. It has been realized that the cellular receptor guiding a virus to a target cell cannot be the sole determinant of a virus's pathogenic potential. Indeed, it is a major challenge of the day to decipher the molecular mechanism(s) that determine viral tissue tropism and disease.

What is the identity of picornaviruses? It relates to ancestral viruses whose identity we will never know. Comparative analyses of the structures of genomes and their products, however, have placed the picornaviruses into a large "picorna-like" virus family, in which they occupy a prominent place (discussed in the section on Evolution). These same analyses have led to an evolutionary tree of picornaviruses that reveals the extent of kinship (Figure 12.1A).

One result of these phylogenetic investigations was a radical reorganization of the taxonomy of *Enterovirus*, a genus of Picornaviridae comprising numerous members infecting the gastrointestinal tract. The enteroviruses have now been divided into clusters (Table 12.1; Figure 12.1B) grouping the viruses mainly corresponding to genotypes (Hyypia *et al.*, 1997). Earlier classifications were based (1) on specific properties of the virions, (2) on disease patterns, (3) on the apparent absence of pathogenesis (ECHO is an acronym for "enteric cytopathic human orphan" because no disease was originally correlated with these viruses), or (4) in reference to the site of discovery (e.g. the town of Coxsackie in New York State) and pathogenesis in suckling mice. As the number of known enteroviruses increased and the properties of these new isolates were elucidated, the need for a modified classification became apparent. However, even the latest dendrograms are likely to be modified again.

Principally, viruses that have been classified as belonging to a specific genus may be further divided into serotypes. A serotype is defined by the virus's ability to elicit a set of neutralizing antibodies ("antiserum") in a host animal; this set of neutralizing antibodies will generally *not* neutralize any other virus, regardless of the origin of the antiserum. Neutralizing antibodies, in turn, are elicited by structures specific for a virus's capsid, and they have been referred to as neutralization antigenic determinants (or sites). The poliovirion carries at its surface four distinct neutralization antigenic determinants (Nomoto and Wimmer, 1987; Minor, 1990). However, poliovirus expresses only three unique sets of these four determinants; hence poliovirus occurs in three serotypes. Hepatitis A virus, on the other hand, expresses only one set of neutralization antigenic determinants; hence, it occurs in only one serotype. In contrast, human rhinoviruses (HRV) can express more than 100 unique sets of four antigenic determinants. Rhinoviruses, therefore, occur in more than 100 serotypes. It should be noted that a poliovirus has been constructed that expresses neutralization antigenic determinants of all three serotypes. This virus, which is severely handicapped in proliferation, is trivalent as it can be neutralized by all three serotype-specific antibodies (Murdin *et al.*, 1992).

A genus consisting of viruses that cause the same disease syndrome can be subdivided further on the basis of receptor use. For example, all member viruses of the genus *Rhinovirus* cause the common cold, yet they use two different receptors (ICAM-1 and LDL receptor; Table 12.1). On the basis of genotypes, however, this division no longer holds up (Figure 12.1).

As mentioned, the enteroviruses have now been subdivided into clusters based on genotypes (Table 12.1, Figure 12.1B). For example, the C-cluster consists of the three serotypes of poliovirus and of serotypes 1, 11, 13, 15, 17, 18–22, 24 of coxsackie A virus (C-CAV). Originally the C-CAVs were not considered related to polioviruses because of the profound difference in pathogenesis (common cold and poliomyelitis, respectively) and the different use of receptor (ICAM-1 and CD155, respectively). However, their very close kinship was revealed by genome sequence. This proximity has led to the interesting question of whether the C-CAVs are genetic variants of poliovirus (Harber *et al.*, 1995) or *vice versa* (discussed in detail in Evolution

An interesting recent variant of CAV24 is CAV24v, an agent that emerged in the early 1970s and that causes acute hemorrhagic conjunctivitis. This syndrome is also associated with a new variant of enterovirus 70, a D-cluster enterovirus (Table 12.1; Yin-Murphy, 1973). The phenomenon of the sudden appearance of enterovirus strains causing human diseases not previously associated with picornaviruses is of

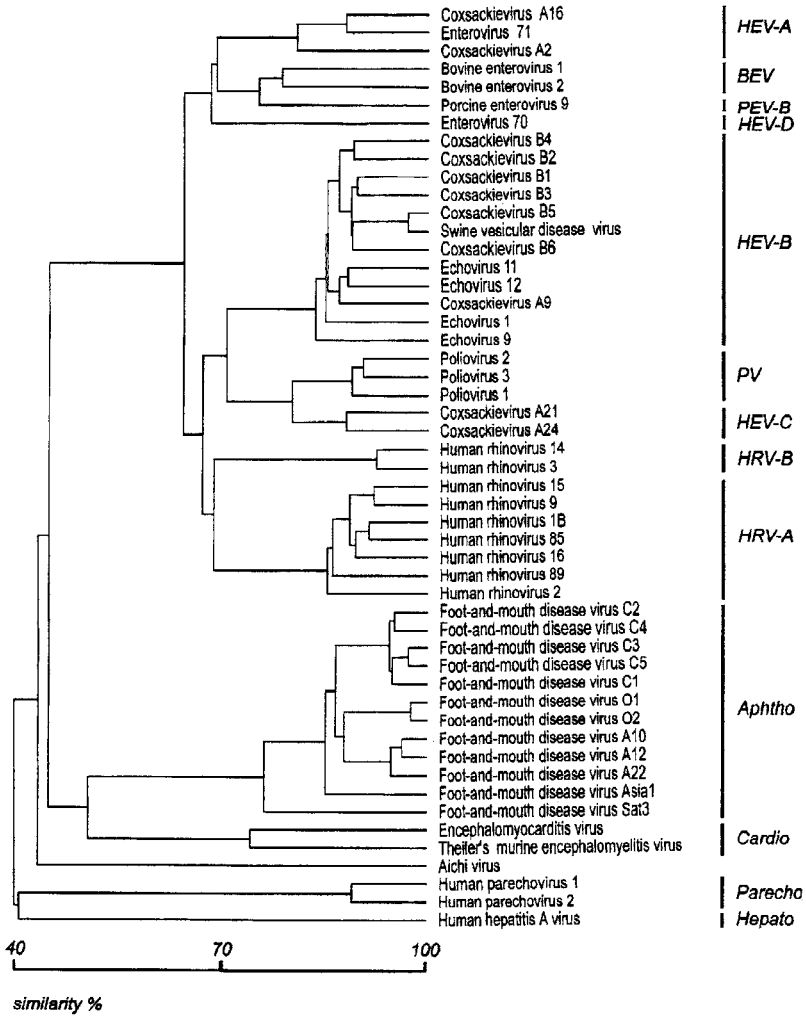


FIGURE 12.1 Evolutionary relationships of Picornaviridae. This part is a dendrogram of Picornaviridae showing amino-acid similarity in the capsid region. Sequences were aligned and the dendrogram was generated using the PILEUP program of the Genetics Computer Group software package. (*continued on facing page*)

greatest interest with respect to the dynamics of picornavirus diversification, particularly in view of the eradication of poliovirus.

What are the mechanisms by which the picornaviruses and other RNA virus families have diversified? Clearly, the genetic program inscribed into the viral genome is being changed as the viruses acquire new genetic traits. The predominant driving force of the changes in the genotype is largely an adaptation to new opportunities to proliferate. In the following, we will discuss some mechanisms and rules of genetic diversification and evolution of picornaviruses.

GENETICS OF PICORNAVIRUSES

The Picornavirus Genome

The picornavirus genome is single-stranded RNA of plus strand polarity, roughly 7500 nt long (for an overview, see Rueckert, 1996). The genome can be divided into three parts: the 5′ non-translated region (5′NTR), the open reading frame of the polyprotein and the 3′ non-translated region (3′NTR) (Figure 12.2).

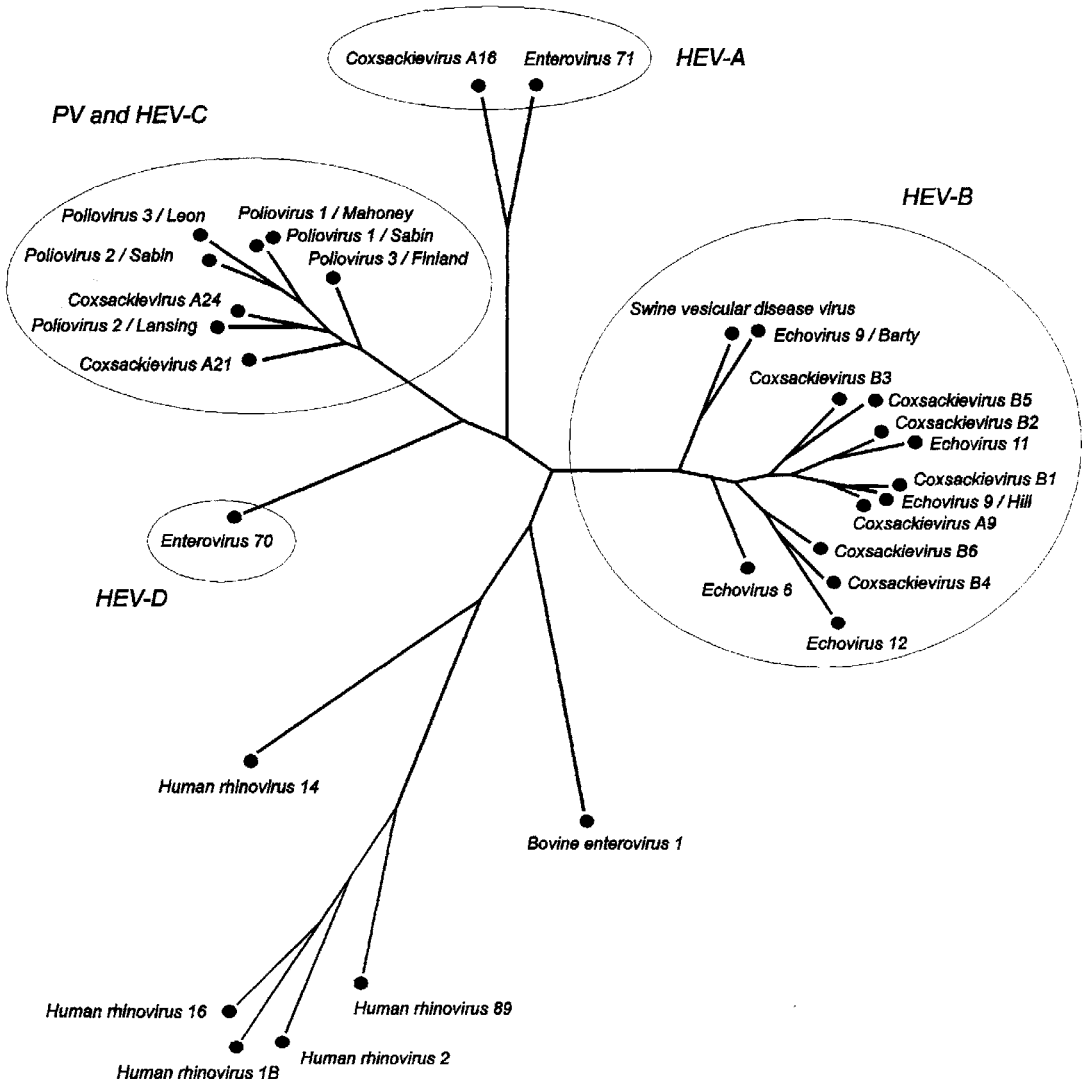


FIGURE 12.1 (*continued*) An unrooted tree based on the region for the RNA polymerase $3D^{pol}$. The tree was constructed using the maximum likelihood method (DNAML from the PHYLIP package). The branch lengths are proportional to genetic distance. In this tree the clusters are designated as Human Enterovirus-A, -B, -C, etc. (HEV-A; HEV-B; HEV-C, etc.). The groups boxed (PV and HEV-C, HEV-A, and HEV-D) are considered statistically significant by bootstrapping analysis. Figures kindly provided by Leena Kinnunen.

The 5′NTR

A signature of all picornaviruses is their unusually long 5′NTR (733–1199 nt; >10% of genome length), which seems to defy the notion that RNA viruses strive to keep their genome at minimum length (Wimmer *et al.*, 1993; see below). These 5′NTRs carry important structures: the terminal protein VPg, adjacent *cis*-acting sequences involved in RNA replication, and the internal ribosomal entry site (IRES), controlling translation.

The virus-encoded 5′-terminal protein, VPg (viral protein genome-linked) is covalently linked to the 5′-terminal uridylic acid via a O^4-(5′-uridylyl)tyrosine bond (Lee *et al.*, 1976, 1977; Flanegan *et al.*, 1977; Nomoto *et al.*, 1977b; Rothberg *et al.*, 1978). Picornavirus VPgs are

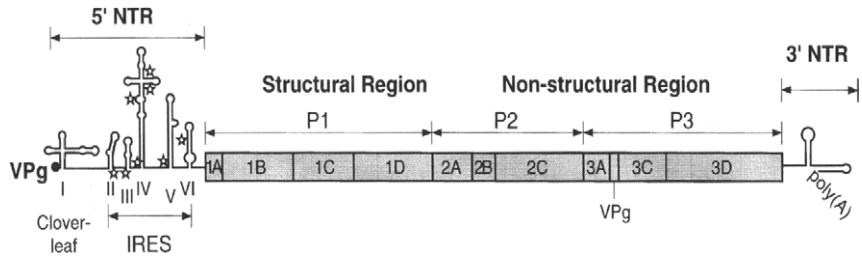


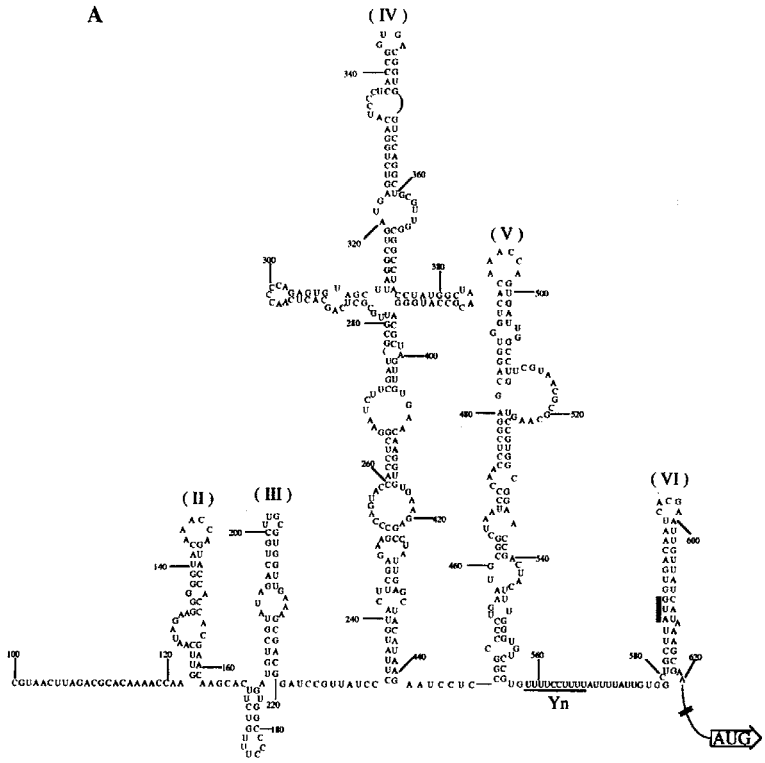
FIGURE 12.2 Structure of the poliovirus genome. The single-stranded RNA is shown with the genome-linked protein VPg (3B) at the 5′ end of the non-translated region (5′ NTR, single line) and the 3′ NTR (single line) connected to the poly(A) tail. The boxed region shows the polyprotein and vertical lines within the box indicate proteinase cleavage sites. The locations of the structural (P1) and non-structural (P2, P3) region are shown on top. RNA structural domains within the 5′ non-translated region are shown by Roman numerals, cloverleaf (I), IRES (II–VI). Stars indicate the positions of non-initiating AUG triplets.

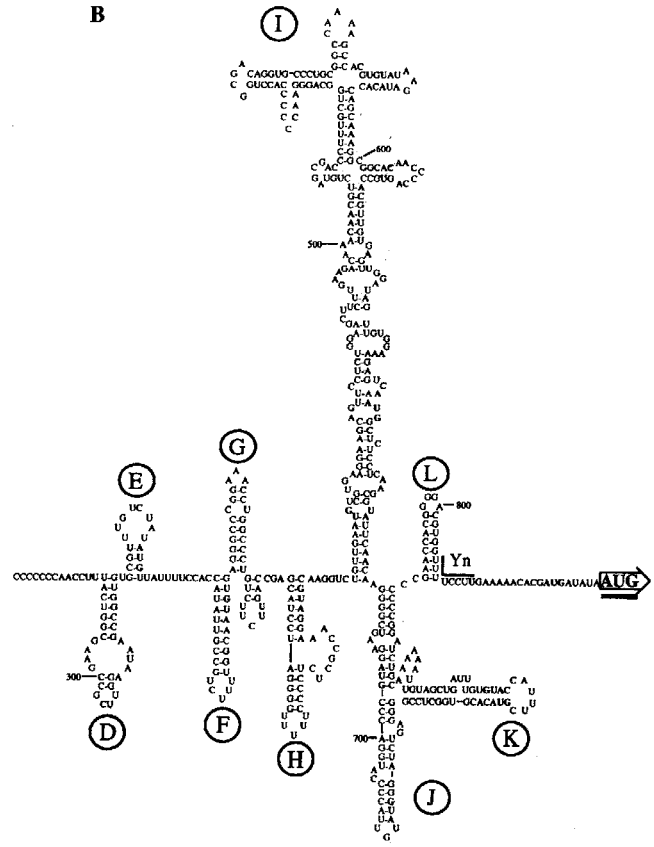
22–24 amino acids long; their third amino acid (from the N-terminus) is always a tyrosine, the residue linking VPg to the genome. Genome-linked proteins are quite common amongst viruses belonging to the picorna-like super family (see Figure 12.10)

Picornavirus VPgs are attached to 5′-terminal nucleotide sequences that form complex structures typical for entero- and rhinoviruses on the one hand, or cardio-, aphtho- and hepatoviruses on the other. These sequences are important signals in genome replication. Entero- and rhinoviruses share a cloverleaf structure (Rivera *et al.*, 1988; Andino *et al.*, 1990) that has been subject to intense studies (see below). Relatively little is known about the role of corresponding structural elements (which do not form cloverleaves) of cardio, aphtho- and hepatovirus genomes.

The cloverleaf is followed by the internal ribosomal entry site (IRES), arguably the most complex *cis*-acting element in any RNA virus genome known (Figures 12.2, 12.3; Wimmer *et al.*, 1993). Picornavirus IRES elements, which are approximately 400 nt long, regulate the initiation of polyprotein synthesis. In deviation to cap-dependent "scanning", IRESes promote internal ribosomal entry, i.e. they allow initiation of translation independently of a capping group and even a free 5′ end (Jang *et al.*, 1988, 1989; Pelletier and Sonenberg, 1988; Molla *et al.*, 1992; Chen and Sarnow, 1995). Remarkably, IRES elements are defined by their function, not by their sequences or apparent higher-order structure(s). This is illustrated in Figure 12.3, which depicts the sequence and folding pattern of the IRES elements of poliovirus and encephalomyocarditis virus (EMCV; Pilipenko *et al.*, 1989a,b). In spite of these differences, the poliovirus IRES has been exchanged with that of EMCV, leading to a novel chimeric virus with excellent growth properties (Alexander *et al.*, 1994). Similarly, the IRES of hepatitis C virus (HCV), a flavivirus, was found to functionally substitute for the poliovirus IRES, yielding a polio/HCV chimeric virus (Lu and Wimmer, 1996; Zhao *et al.*, 1999). Finally, a construct in which the IRES of human rhinovirus type 2 (HRV2) replaced that of poliovirus yielded a PV/HRV chimeric virus (PV1(RIPO)) that is

FIGURE 12.3 Sequences and secondary structures of IRES elements of poliovirus and encephalomyocarditis virus. **A.** Poliovirus IRES; individual domains have been labeled with Roman numerals. **B.** Encephalomyocarditis virus (EMCV) IRES; domains have been labeled with capital letters. Both IRESes contain a conserved YnXmAUG motif, of which the oligopyrimidine stretch (Yn) and the AUG triplet are indicated by solid bars. Note that in the EMCV IRES, the AUG triplet of the YnXmAUG motif is the initiating codon of the polyprotein. In the poliovirus IRES, this AUG triplet is silent and is separated from an AUG codon initiating the synthesis of the polyprotein by a "spacer sequence" of 154 nt (Jang *et al.*, 1990). Single attenuating mutations in the poliovirus vaccine strains map to domain V (Wimmer *et al.*, 1993).


A
(IV)
(V)
(II)
(III)
(VI)
Yn
AUG


B
I
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Yn
AUG

indistinguishable from wt poliovirus with respect to replication in HeLa cells yet is highly attenuated in poliovirus-receptor-transgenic mice and in monkeys (Gromeier *et al.*, 1996, 1999a; discussed in the section on Pathogenesis). The properties of this interesting novel virus will be discussed in a later section.

The mechanism by which IRES elements function is still obscure.

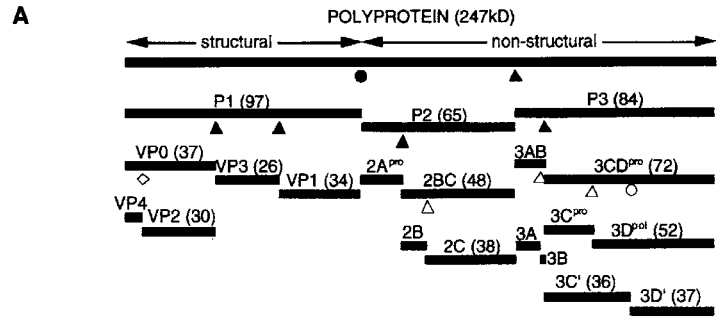
The Polyprotein

Translation of picornavirus mRNA is initiated downstream of the IRES to yield an unstable "polyprotein" that is rapidly cleaved by virus-encoded proteinases to proteins involved in viral proliferation (Figure 12.4; see also Evolution and Figure 12.10). It is important to note that the mRNA found in viral polyribosomes that encodes the polyprotein differs from virion RNA in one important aspect: it is terminated with pUp ... (Hewlett *et al.*, 1976; Nomoto *et al.*, 1976). Apparently, the terminal protein VPg has been cleaved from incoming or from newly synthesized RNA. It has been suggested that the enzyme cleaving the VPg–pUp phosphodiester bond is of cellular origin but the reason for the removal of the protein and the nature of the enzyme catalyzing it remain unknown. Moreover, it is not clear whether the incoming VPg-linked virion RNA will be processed immediately after entry or whether the removal of VPg will occur only after the first round(s) of viral protein synthesis.

Entero- and rhinoviruses encode the two proteinases $2A^{pro}$ and $3C/3CD^{pro}$, aphthoviruses the two proteinases L^{pro} and $3C^{pro}$, and cardioviruses only the proteinase $3C^{pro}$. Interestingly, both cardioviruses and aphthoviruses have evolved a peculiar cleavage mechanism between 2A and 2B that occurs only in *cis* and is an enzyme-independent reaction (reviewed by Ryan and Flint, 1997). A similar as yet unknown mechanism of proteolytic cleavage is that between VP4 and VP2 (Figure 12.4D), which occurs only during maturation of the virion (maturation cleavage) and appears also to be proteinase-independent (Harber *et al.*, 1991; see below). The origin of these fascinating enzymes and of specific cleavage events are discussed in the section on Evolution. Since most details of proteolytic processing have been accumulated for poliovirus, much of the following discussion will center on this viral system.

The two poliovirus proteinases $2A^{pro}$ and $3C/3CD^{pro}$ cleave at different sites, as determined by the sequences of the scissile bond (Figure 12.4B, C). Theoretically, the poliovirus polyprotein could give rise to 77 different cleavage products if proteolytic processing by these enzymes and the maturation cleavage were entirely random (Wimmer *et al.*, 1993). In fact, only roughly 29–30 cleavage products have been identified in poliovirus-infected cells (Nicklin *et al.*, 1986). It has thus been concluded that processing of the picornavirus polyproteins is not random but follows a pathway that is determined by protein folding (masking of cleavage sites) and by the amino-acid sequences surrounding the scissile bond (Figure 12.4B, C; Harris *et al.*, 1990). For example, the precursor $3CD^{pro}$ can be cleaved into $3C^{pro}$ and $3CD^{pol}$ by a (*cis*?) cleavage in which the $3C/3CD^{pro}$ proteinase is involved. Both $3C^{pro}$ and $3D^{pol}$ are quite stable end-products of processing. However, in the case of poliovirus type 1 (Mahoney) (PV1 (M)), $3CD^{pro}$ can also be efficiently processed in *trans* by $2A^{pro}$ to 3C′ and 3D′ (Figure 12.4C), two polypeptides with no apparent function in viral proliferation (Lee and Wimmer, 1988). Just like $3C^{pro}$ and $3D^{pol}$, 3C′ and 3D′ are quite stable end-products of processing, even though $3D^{pol}$ harbors a perfect cleavage site for $2A^{pro}$ and 3C′ harbors a cleavage site for $3C/3CD^{pro}$ (Figure 12.4). Indeed, in PV1(M)-infected cells, nearly equal amounts of the four cleavage products of precursor $3CD^{pro}$ are observed. It is assumed that structural constraints mask one or the other cleavage site from recognition and processing once the cleavage product has been formed (Lee and Wimmer, 1988).

The preferred cleavage sequence for $3C/3CD^{pro}$ in the poliovirus polyprotein is AxxQ*G; hence, cleavage sites with this sequence are usually rapidly processed. Numerous mutational studies have supported the identity of this $3C/3CD^{pro}$ cleavage motif (reviewed in Dougherty and Semler, 1993, and Wimmer *et al.*, 1993). An intriguing genetic analysis has made use of a viral construct that



	Site	P6	P5	P4	P3	P2	P1	P1'	P2'	P3'	P4'	P5'
B	VP0/VP3	T	L	P	R	L	Q	G	L	P	V	M
	VP3/VP1	Q	K	A	L	A	Q	G	L	G	Q	M
	2A/2B	E	E	A	M	E	Q	G	I	T	N	Y
	2B/2C	P	Y	V	I	K	Q	G	D	S	W	L
	2C/3A	M	E	A	L	F	Q	G	P	L	Q	Y
	3A/3B	L	F	A	G	H	Q	G	A	Y	T	G
	3B/3C	R	T	A	K	V	Q	G	P	G	F	D
	3C/3D	Y	F	T	Q	S	Q	G	E	I	Q	W
C	VP1/2A	K	D	L	T	T	Y	G	F	G	H	Q
	3C'/3D'	K	L	L	D	T	Y	G	I	N	L	P
D	VP4/VP2	T	A	P	M	L	N	S	P	N	I	E

FIGURE 12.4 Processing scheme and cleavage sites of the poliovirus polyprotein. **A.** Proteolytic cleavages of the polyprotein. Triangles indicate cleavage by $3C^{pro}$ and/or $3CD^{pro}$. Note that both enzymatic entities can efficiently cleave the non-structural proteins. In contrast, the P1 capsid precursor can be processed by $3CD^{pro}$ only. Solid triangles represent efficient cleavage sites, whereas open triangles represent slowly cleaved sites resulting in stable precursor proteins. The $2A^{pro}$-mediated cleavages are depicted with circles. Only the cleavage between P1 and P2–P3 (solid circle) is essential, whereas the cleavage of $3CD^{pro}$ to 3C′ and 3D′ is dispensable (open circle). The maturation cleavage is indicated by the open diamond. The mechanism by which this cleavage occurs is unknown. Numbers in brackets indicate the molecular weight in kDa. **B–D.** Amino-acid residues at sites cleaved by **(B)** $3C^{pro}$ and/or $3CD^{pro}$, **(C)** by $2A^{pro}$ and **(D)** during the maturation cleavage are shown in a single-letter code. The positions of the amino-acid residues are designated P1, P2, P3, . . . at the newly generated C-termini, or P1′, P2′, P3′, . . . at the newly generated N-termini. The fastest cleavages catalyzed by $3C^{pro}$/$3CD^{pro}$ occur at sites in which the P4 position is a small aliphatic amino acid (e.g. AxxQ*G). Cleavage at TQSQ*G between 3C and 3D is slow, giving rise to the $3CD^{pro}$ cleavage intermediate with a long half-life (Cao and Wimmer, 1996).

mutated this AxxQ*G cleavage motif at a specific site in order to avoid proteolytic processing. The amino acids placed by the mutants into the motif confirmed the proposed interaction between substrate and enzyme during cleavage (Cao and Wimmer, 1996, and references therein). As will be discussed later, poliovirus is a purist with respect to cleavage signals, since the scissile bond in all cleavages, catalysed by $3C/3CD^{pro}$, is Q*G (Kitamura *et al.*, 1981; Semler *et al.*, 1981a, 1981b). In other picornaviruses, or viruses of the large picorna-like superfamily, the cleavage site may differ from the canonical Q*G signal.

A most important observation in studies of picornavirus proliferation is that cleavage intermediates may have important functions that in some cases may even be distinct from that of

their end-products (e.g. $3CD^{pro}$ yielding $3C^{pro}$ and $3D^{pol}$).

The structure of picornavirus $3C^{pro}$ enzymes has been accurately predicted by Gorbalenya *et al.* (1986), leading to the genetic analyses alluded to above. The structures were proved to be correct by X-ray crystallographic studies of $3C^{pro}$ of human hepatitis A virus (Allaire *et al.*, 1994) and human rhinovirus 14 (Matthews *et al.*, 1994).

The 3′NTR

Following the ORF, there is a heteropolymeric region that may be different with respect to length (72–126 nt) and structure in different picornavirus genomes (Xiang *et al.*, 1997). However, all picornavirus genomes terminate with poly(A), as was shown first for poliovirus (Yogo and Wimmer, 1972). The role these sequences play in replication will be discussed below.

The Polarity of Picornavirus RNA and Reverse Genetics

The genomic RNA of picornaviruses can serve as mRNA and, consequently, it is of the same polarity as cellular mRNA. By convention, this polarity has been designated plus-strand polarity (Baltimore, 1971). Fortunately, the genomic RNA of picornaviruses is infectious; that is, upon transfection into suitable host cells, virion RNA will initiate a complete infectious cycle (Wimmer *et al.*, 1993). Interestingly, poliovirus and its purified genome will replicate even in enucleated cells (Morgan-Detjen *et al.*, 1978), an observation suggesting that the nucleus does not contribute factors essential for viral proliferation.

Using reverse transcriptase, Racaniello and Baltimore (1981) generated full-length "complementary" DNA (cDNA) that contained the entire genetic information of the viral genome (currently, cDNA refers to double-stranded DNA generated from the original complementary DNA strands). Transfections into HeLa cells of the cDNA that contained heterologous DNA sequences at either end of the virus-specific sequences generated, surprisingly, poliovirus. With this experiment, "reverse genetics" of RNA viruses was born as the RNA genome was now amenable to manipulations developed for DNA.

The efficiency with which the original cDNA clones induced an infectious cycle in HeLa cells was very low (about 10 PFU/μg DNA; Racaniello and Baltimore, 1981). Construction of plasmids that could replicate in transfected cells dramatically increased the specific infectivity to 10^3 PFU/μg DNA; Semler *et al.*, 1984). However, reverse genetics was made more practical when the cDNA was cloned downstream of the phage T7 RNA transcriptase promoter and, using purified T7 transcriptase, virtually unlimited amounts of highly infectious transcript RNA could be produced in a simple test-tube experiment (>10^5 PFU/μg of transcript RNA; van der Werf *et al.*, 1986). This was important because mutant genomes with highly debilitating replication phenotypes could not be recovered by the inefficient cDNA transfection method. It was known before that VPg is not required to be at the 5′ end for poliovirus RNA to be infectious (Nomoto *et al.*, 1977a). The 5′ end of the T7 transcripts is *ppp*GGUUAAAA . . . whereas that of virion RNA is VPg-pUUAAAA . . . The extra G residues do not prevent transfection but they reduce the specific infectivity of the transcript. In any event, picornavirus RNA is quite tolerant of modifications of the 5′ end of its genome and, in all cases, the virion RNAs isolated after transfections have the authentic terminus restored (Wimmer *et al.*, 1993).

Infectious cDNAs have now been generated from members of all picornaviruses. The method of choice to generate virus remains transfection of T7 transcripts. Recently developed methods of RT/PCR allow researchers to generate infectious cDNA clones in less than 1 month (Tellier *et al.*, 1996).

The Mechanism of Genome Replication

In general terms, genome replication proceeds in two steps: synthesis of a complementary RNA strand (–strand) that then serves as template for plus RNA strands (+strands; Figure

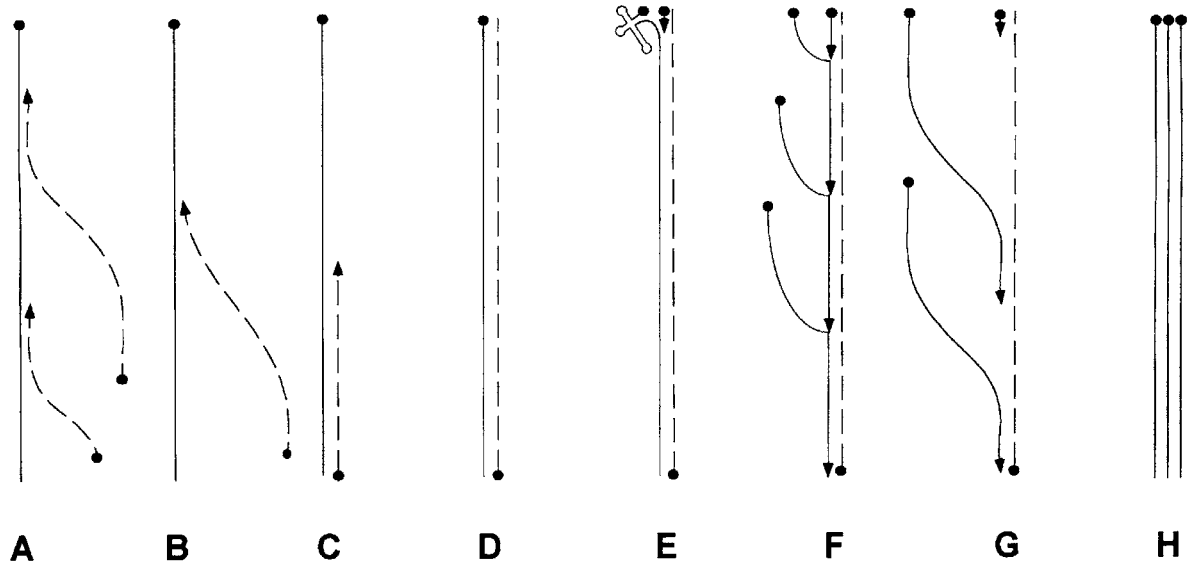


FIGURE 12.5 Steps in the replication of the poliovirus genome. Parental, positive-stranded virion RNA (solid line) is transcribed, yielding – RNA (broken line) after protein (VPg)-priming by the viral RNA-dependent RNA polymerase $3D^{pro}$ (enzyme or any other proteins involved are not shown). A replicative intermediate (RI) form consisting of a single +strand template and multiple nascent – RNA strands (**A**) has not been detected, so that, more probably, intermediates in – RNA synthesis are either mainly single-stranded (**B**) or double-stranded (**C**). Elongation of the nascent – RNA (**C**) yields the replicative form (RF) double-stranded RNA (**D**). Available evidence suggests that the RF is an intermediate in genome replication (discussed in Xiang *et al.*, 1997). Accordingly, a cloverleaf/RNP is formed at the end of the RF that promotes VPg-primed synthesis of +RNA (**E**). The structures formed after multiple initiation could either be "closed" (entirely base-paired; **F**) or "open" (**G**). Available evidence suggests that structure **F** is the correct intermediate (note that $3D^{pol}$ is an unwindase). For details, see Wimmer *et al.*, 1993, and Xiang *et al.*, 1997. Modified from Wimmer *et al.*, 1993.

12.5). The validity of this scheme has been known for almost three decades yet only very few details of the individual steps have been elucidated (Agol *et al.*, 1999). Because the vast majority of studies have been carried out with poliovirus, this review will concentrate predominantly on this viral system.

Viral and Cellular Polypeptides Involved in Genome Replication

With the exception of the capsid proteins, all viral non-structural proteins and even processing intermediates have been implicated in genome replication (Xiang *et al.*, 1997). The evidence for the involvement of these proteins ($2A^{pro}$, 2B, 2BC, 2C, 3A, 3AB, VPg, $3C/3CD^{pro}$, $3D^{pol}$) is based largely on genetic data or on biochemical experiments assumed to be indicative of genome replication (Wimmer *et al.*, 1993; Xiang *et al.*, 1997). For example, genetic and biochemical analyses of 3AB strongly suggest that this protein, a non-specific RNA binding protein, and the proteinase $3CD^{pro}$ participate in the formation of an initiation complex for +strands (Xiang *et al.*, 1997). Another example is the involvement of 2C in RNA replication. Briefly, poliovirus RNA synthesis is highly sensitive to the presence of 2 mM guanidine hydrochloride (Gua HCl); poliovirus mutants resistant to 2 mM Gua HCl harbor a single amino-acid exchange (N179A/G) in polypeptide 2C. It has recently been established that 2C is an ATPase (and not a GTPase) and we now refer to it as $2C^{ATPase}$ (Pfister and Wimmer, 1999). The ATPase activity of purified $2C^{ATPase}$ is inhibited by 2 mM Gua HCl, whereas that of purified $2C^{ATPase}$ with a N179/G mutation is resistant to this concentration of the drug (Pfister and Wimmer, 1999). On the basis of these considerations, it can be assumed that the ATPase activity of $2C^{ATPase}$ is essential for genome replication. Just as with

3AB or $3CD^{pro}$, however, the step(s) by which $2C^{ATPase}$ is exerting its essential function are still unknown.

The only proteins whose role in genome replication has been firmly established are VPg and $3D^{pol}$. The crystal structure of $3D^{pol}$ has recently been solved (Hansen and Schultz, 1997), a result that will greatly advance our (limited) understanding of this important enzyme. Importantly, $3D^{pol}$ was established already in 1977 as being a primer-dependent and RNA-dependent RNA polymerase (Flanegan and Baltimore, 1977). Although a deluge of circumstantial evidence suggested that a uridylylated form of VPg might serve as primer for $3D^{pol}$ (Nomoto *et al.*, 1977b; Wimmer, 1982; Takeda *et al.*, 1986; Toyoda *et al.*, 1987), direct evidence for this mechanism has been obtained only very recently (Paul *et al.*, 1998b). Briefly, VPg is being uridylylated to VPg-pU(pU) by the viral RNA polymerase $3D^{pol}$ in the presence of template (poly(A)). VPg-pU(pU) then primes the transcription of poly(A), leading to the synthesis of poly(U), which is the 5′ terminus of –strands (Paul *et al.*, 1998b).

In spite of these seemingly simple experiments (Paul *et al.*, 1998b), the mechanism of initiation of RNA synthesis was a matter of controversy for almost two decades. Baltimore's and Flanegan's groups presented evidence favoring "hairpin priming", whereas Wimmer's group accumulated data suggesting "protein priming" (reviewed by Richards and Ehrenfeld, 1990). The controversy has finally been settled in favor of protein priming.

At low concentration of enzyme, poliovirus polypeptide 3AB stimulates the transcriptional activity of $3D^{pol}$ up to 100-fold (Lama *et al.*, 1994; Plotch *et al.*, 1989; Paul *et al.*, 1994). Indeed, biochemical and genetic evidence suggests that $3D^{pol}$ and 3AB form a complex in solution (Molla *et al.*, 1994). The significance of these observations is not yet known.

An important additional property of $3D^{pol}$ is its ability to unwind double-stranded RNA. That is, the enzyme, while transcribing a template, can replace a dormant RNA strand that is hybridized to the template with the new strand that is just being synthesized (Cho *et al.*, 1993). It should be noted, however, that $3D^{pol}$ is not a helicase as it will not separate two strands without transcribing one of them (Cho *et al.*, 1993).

The participation in picornavirus replication of cellular proteins, referred to by investigators as "host factors", has also had a history of controversies. Several polypeptides were proposed to be involved in replication (e.g. a kinase or a uridylic acid transferase) but these proteins have disappeared after further analysis (Richards and Ehrenfeld, 1990). Ehrenfeld's and Semler's groups have recently identified a cellular 38 kDa RNA binding protein, poly(rC) binding protein 2 (PCBP2), that is not only required for the function of the poliovirus IRES but it has also the propensity to bind, together with $3CD^{pro}$, to the poliovirus 5′-terminal cloverleaf (Blyn *et al.*, 1996). PCBP2 (or PCBP1, a protein related to PCBP2; Gamarnik and Andino, 1997) is undoubtedly the "host factor p36" that was originally proposed by Baltimore's group to effect the binding of $3CD^{pro}$ to the poliovirus cloverleaf (Andino *et al.*, 1990, 1993).

Andino *et al.* (1993) provided first evidence suggesting that the formation of a specific protein/cloverleaf RNP complex consisting of viral protein 3CD, a cellular protein ("p36") and the viral RNA is required for the initiation of +strand synthesis (Andino *et al.*, 1993). This hypothesis has been further supported by the discovery of PCBP2 (Gamarnik and Andino, 1997; Parsley *et al.*, 1997). PCBP2 is therefore a sensible candidate for a "host factor" involved in poliovirus RNA replication. However, poliovirus protein 3AB can replace PCBP2 in all biochemical reactions characteristic of the formation of a 5′ terminal RNP. Moreover, 3AB and $3CD^{pro}$, both cleavage products of the P3 precursor (Figure 12.4A), are associated in solution (Molla *et al.*, 1994). Finally, the phenotypes of mutants of 3AB *in vivo* and *in vitro* support the conjecture that 3AB is involved in the formation of a cloverleaf/$3CD^{pro}$/3AB complex (Harris *et al.*, 1994; Xiang *et al.*, 1995a,b). Currently, there is no compelling evidence in favor of the cloverleaf/$3CD^{pro}$/PCBP2 complex over that of cloverleaf/$3CD^{pro}$/3AB with respect to poliovirus genome replication (see a discussion in Xiang *et al.*, 1997).

Cis-*acting RNA Signals*

Recognition of RNA signals located somewhere in the RNA genome is a prerequisite for specificity in genome replication. This review will concentrate only on *cis*-acting elements of entero- and rhinoviruses because, as mentioned earlier, the overwhelming number of experiments deal with these viruses.

Currently, only the 5'-terminal cloverleaf has been firmly established as a *cis*-acting signal in enterovirus genome replication (see previous section), although the mechanism by which it functions is still obscure. Clearly, the formation of a specific RNP plays a role the significance of which will be discussed below.

More complicated is the recognition of the +strand template for the initiation of –strands. Since replication of picornavirus RNAs commences at the 3'-terminal poly(A), a homopolymeric sequence found also in most cellular mRNAs, poly(A) alone cannot be a determinant for virus-specific –strand synthesis. VPg-pU(pU) can be synthesized in the presence of poly(A), and VPg-poly(U), the 5' end of –strands, will follow the synthesis of the primer (Paul *et al.*, 1998). This reaction, however, does not reveal the mechanism of specificity.

Mutational analysis of the heteropolymeric sequence of the 3'NTR of enteroviruses indicated that this region was critically important for replication (Pilipenko *et al.*, 1996; Melchers *et al.*, 1997). However, the poliovirus 3'-terminal heteropolymeric sequence can be replaced with that of HRV14, a hairpin with no apparent homology with the poliovirus structure, and the resultant poliovirus/HRV14 hybrid genome replicated with wt kinetics (Rohll *et al.*, 1995). Even more startling was a report from Semler's group that presented evidence that the heteropolymeric region could be deleted altogether without loss of viability (Todd *et al.*, 1997). Currently, the paradox intrinsic to these findings remains unsolved (Agol *et al.*, 1999).

It is possible that the 3' heteropolymeric region plays an important role in the efficient formation of an initiation complex for replication but to a much lesser extent in +strand template recognition. The authentic recognition signal may reside in RNA-internal sequences, as proposed by McKnight and Lemon (1996). These authors reported that, surprisingly, a stem loop structure mapping to the coding region of the HRV14 capsid proteins was absolutely necessary for genome replication. Fittingly, a stem-loop RNA structure that has been uncovered in poliovirus RNA also appears to play a role in genome replication; it maps to the coding region of $2C^{AlPase}$ (Goodfellow *et al.*, 1998). The mechanism by which these new elements influence replication has yet to be resolved.

Finally, evidence has been presented suggesting that sequences within the IRES play a role in genome replication (Borman *et al.*, 1994; Shiroki *et al.*, 1995). This is difficult to comprehend if one considers chimeric IRES viruses. As mentioned above, the cognate IRES of poliovirus can be replaced with IRES elements from different viruses whose IRES are merely related (HRV2, HRV14, CBV4, CAV9, CAV24, EV71; Gromeier *et al.*, 1996, 1999a and unpublished results) or entirely different (EMCV; Alexander *et al.*, 1994; HCV, Lu and Wimmer, 1996; Zhao *et al.*, 1999) without loss of genome replication.

Cis-*dominance of Translation in Genome Replication*

Defective interfering particles (DI particles; see below) of poliovirus are naturally occurring variants with deletions (of varying sizes) in the P1 region, encoding the capsid proteins. DI particles can replicate their RNA without helper function but they need wt virus for encapsidation. Sequence analyses of genomic RNAs of DI particles led Nomoto and his colleagues to the surprising observation that in all cases the deletions were in-frame of the polyprotein coding sequence. On the other hand, artificial genomes engineered with out-of-frame deletions were unable to replicate their RNA, even in the presence of wt helper virus (Kuge *et al.*, 1986; Hagino-Yamagushi and Nomoto, 1989). It was concluded that translation was necessary for the cognate genome to replicate. That is, translation had a *cis* effect on replication that could not be complemented in *trans* by a helper genome. These observations were later confirmed and extended (Wimmer *et al.*, 1993; Novak and Kirkegaard, 1994; Agol *et al.*, 1999).

There are several hypotheses that are used to explain the phenomenon. The least likely is that certain replication proteins can only function in *cis*. If so, only viral mRNA could serve as template in RNA synthesis. Since viral mRNAs lack VPg (Hewlett *et al.*, 1976; Nomoto *et al.*, 1976; see above), every +strand RNA that functions as template in RNA synthesis should also lack VPg. Available evidence suggests that all RNA templates involved in replication are terminated with VPg (Nomoto *et al.*, 1977b; Petterson *et al.*, 1977; Wu *et al.*, 1978; Larsen *et al.*, 1980). Furthermore, RNA replication occurs in a tight membranous environment (Bienz *et al.*, 1992). Thus, it is unlikely that these genome replicating membranous complexes also harbor viral polysomes (Wimmer *et al.*, 1993). Indeed, crude, membranous replication complexes can be isolated from infected cells that can replicate poliovirus RNA yet they are free of ribosomes (Takegami *et al.*, 1983; Takeda *et al.*, 1986; Toyoda *et al.*, 1987).

An alternative explanation is that the observed *cis* effect is operating only during the very first round of translation at the onset of infection. Clearly, translation of an infecting genome will have to be somehow arrested to allow the template to switch from translation to transcription. It is possible that, once the switch has been made, replication can proceed independently of translation. This does not exclude the possibility that viral proteins, perhaps intermediates with a short half-life or short-lived protein complexes, must be continuously supplied to the RNA synthesizing machinery.

The question of the switch from translation to RNA synthesis of infecting + stranded genomic RNA has been subject of much speculation. The classical study of Kolakofsky and Weissmann (1971) on phage Qβ replication solved the dilemma by showing that the phage replicase (a complex of four proteins) can repress translation of viral mRNA. A similar model has been proposed for poliovirus by Gamarnik and Andino (1998): the formation of an RNP consisting of cloverleaf/$3CD^{pro}$/PCBP2 at the 5′ end of the viral mRNA inhibits further translation, thereby switching the template to replication. One problem with this model is that at the peak of poliovirus replication, translation and RNA synthesis occur concomitantly in the presence of an excess of $3CD^{pro}$ molecules (note that for each virus particle, 60 molecules of $3CD^{pro}$ are synthesized; the ratio of viral +strand RNA to unprocessed $3CD^{pro}$ may be 1 : 100 through most of the replicative cycle). Moreover, if the genome has to be translated for replication to occur, how can inhibition of translation promote RNA synthesis?

Genome Replication: An Overview

A very schematic representation of steps in genome replication is shown in Figure 12.5 (Wimmer *et al.*, 1993). The possible RNA structures involved in replication have been divided into three categories: (1) single-stranded +RNA (continuous line; H), –strand RNA (dotted lines); (2) closed replicative form (RF: D) and open replicative form (B); and (3) closed (C, F) and open (G) replicative intermediate RNA (RI). The structures differ in an important aspect: in one case the nascent RNA strands are always fully base-paired to the template ("closed form"; C, F), in the other case, the nascent strands are only base-paired at the replication fork ("open form"; A, B, G). All strands are VPg-linked (indicated by closed circles; Nomoto *et al.*, 1977b).

We have argued before that the cumulative evidence favors the "closed forms" for RF and RI but this view may not be shared by others (Wimmer *et al.*, 1993; Xiang *et al.*, 1997). Since $3D^{pol}$ is an "unwindase" (Cho *et al.*, 1993; see above), the scheme does not necessarily require a helicase. Indeed, so far no picornaviral helicase has been identified, and purified $2C^{ATPase}$ has stubbornly refused to exhibit such activity (Pfister and Wimmer, 1999).

Briefly, VPg will be uridylylated at the 3′-terminal poly(A). VPg-pU(pU), in turn, will then prime synthesis of –strands (Figure 12.5C). It is unlikely that multiple initiation of –strands on the same template (prior to completion of the first –strand) occurs, since an RI with multiple –strands (such as in Figure 12.5A) has not been found in infected cells (Bishop and Koch, 1969). It is even possible that initiation at the poly(A) tail of poliovirus RNA occurs only once. Completion of the –strand will thus yield RF

(Figure 12.5D), which we consider an intermediate in replication and not a byproduct (Wimmer *et al.*, 1993). One compelling argument in favor of this assumption is that in the RF the 5′ end of +strands is in the close vicinity of the 3′ end of –strands, a prerequisite first proposed by Baltimore and his colleagues (Andino *et al.*, 1993; Harris *et al.*, 1994). Destabilization of this end of the RNA will lead to the formation of an RNP consisting either of cloverleaf/3CD^{pro}/3AB or cloverleaf/3CD^{pro}/PCBP2, which, in turn, will free the 3′ end of the –strand for VPg-primed +strand synthesis to occur (Figure 12.5E). Multiple initiation at this end will lead to the multistranded RI (Figure 12.5F), the nascent or full-length +strands being replaced during transcription by the 3D^{pol} unwindase.

Initiation of +strands may be more efficient than initiation of –strands; hence the large excess of +strands over –strands in infected cells. Note that a reconstituted replication system of purified viral and cellular components capable of synthesizing +strands from input +strands has not been achieved; thus many of the hypotheses put forward in this scheme have not yet been tested.

Replication of Poliovirus in Oocytes

Gamarnik and Andino (1996) have described a novel system to study poliovirus replication in *Xenopus* oocytes by injecting poliovirus RNA into these cells. However, virus will replicate only if a HeLa cell S10 extract was co-injected with the RNA. Interestingly, the authors have been able to separate the HeLa supporting activities (S10) into two factors, one necessary for poliovirus IRES-driven translation, the other for poliovirus RNA synthesis. This system offers an excellent opportunity to separate and characterize viral and cellular factors involved in virus replication.

Cell-free, De Novo *Synthesis of Poliovirus*

Viruses, lacking the genetic information as well as the tools to provide most of the essential components to replicate, are obligatory intracellular parasites. The complexity of viral proliferation – macromolecular synthesis of polypeptides and genomic nucleic acid, and encapsidation – has led to the text book wisdom that viruses are obligatory intracellular parasites unable to proliferate outside living cells.

However, poliovirus RNA (obtained either from virions or by transcription with phage T7 RNA polymerase from plasmid DNA), when incubated in an extract of uninfected HeLa cells void of nuclei, mitochondria and cellular mRNA, will direct translation, genome replication and genome encapsidation such that infectious particles are formed. These newly synthesized virions are indistinguishable from poliovirus isolated from tissue cultures. Thus, a picornavirus (poliovirus) is the first virus that has been synthesized *de novo* in a cell-free extract of mammalian cells (Molla *et al.*, 1991).

This experiment has nullified the notion that viruses can proliferate exclusively in living cells. Moreover, the novel approach can be used to study individual steps of viral replication in the absence of cell-membrane barriers. Several interesting observations regarding protein–protein interactions, the role of membranes, of cellular membranous components or soluble cellular factors, or of inhibitors of viral RNA synthesis, have been published (Barton and Flanegan, 1993; Molla *et al.*, 1993c, 1994; Barton *et al.*, 1995; Parsley *et al.*, 1997; Cuconati *et al.*, 1998; Towner *et al.*, 1998). The use of the cell-free cellular extract for studies of poliovirus RNA replication, however, is still in the early stages of exploitation. Nevertheless, it has been possible to even achieve genetic recombination of poliovirus in cell-free HeLa extracts (Duggal *et al.*, 1997; Tang *et al.*, 1997; Duggal and Wimmer, 1999; see below).

Error Rate in Genome Replication

General Observations

In the course of transcription, all template-dependent nucleic acid polymerases make errors in incorporating nucleotides with roughly the same frequency (10^{-3}–10^{-4}). As is discussed in Chapter 7, this phenomenon has profound biological consequences for RNA viruses. Because RNA viruses have chosen not to

develop mechanisms by which misincorporations of nucleotides can be recognized and corrected, the average number of "spontaneous" mutations per replication of the genome, referred to as *error rate*, is around 10^{-4}.

The high error rate in the absence of mechanisms of proofreading and editing has several consequences.

First, the average genome length of animal RNA viruses is small (12 000 nt). Notwithstanding the genome of the exceptional coronavirus (30 000 nt), RNA viruses with genomes exceeding 150 kb (e.g. the DNA viruses, herpes viruses, poxviruses, iridoviruses) are inconceivable because of the high probability that each genome would carry multiple mutations after each round of synthesis. It should be noted that these considerations by no means imply that DNA viruses with very small genomes do not exist. In fact, the animal virus with the smallest known genome is hepatitis B virus (3.2 kb). As to picornaviruses, their average genome length is 8000 nt (see also Wimmer *et al.*, 1993).

Second, RNA viruses replicate near the threshold of error catastrophe (Holland *et al.*, 1990). That is, the artificial increase of misincorporation of nucleotides (e.g. by chemical mutagens) may lead to a rapid decline of the viability of the entire virus population.

Third, plaque-purified clones of RNA viruses are not homogeneous but populations of many different, albeit very closely related genotypes; hence the term "quasispecies" (Eigen, 1993).

Fourth, the genetic heterogeneity allows an RNA to rapidly adapt to a changing environment.

Mutations and "Quasi-infectious" Genomes

A simple example should demonstrate the ease with which a drug-resistant mutant of poliovirus can be isolated. As mentioned, poliovirus RNA replication is highly sensitive to the presence of 2 mM guanidine hydrochloride (Gua HCl). After plating a stock of plaque-purified poliovirus on a monolayer of HeLa cells in the presence of 2 mM Gua HCl, a few plaques will arise corresponding to resistant variants (*gr*) with mutations mapping to $2C^{ATPase}$ (Pincus *et al.*, 1986; Tolskaya *et al.*, 1994).

In the case of the selection of *gr* poliovirus mutants, it should be noted that the resistant variants already existed in the population of the inoculating virus. If the virus inoculum had been entirely free of *gr* variants, no selection of *gr* mutants could have occurred since the drug inhibits RNA synthesis; hence, there would have been no misincorporation of nucleotides to generate the *gr* mutations in $2C^{ATPase}$. Although it may be a trivial thing to repeat, it is important to remember that genetic variation by misincorporation of nucleotides (just as recombination) requires replication. No replication, no mutants.

The genetic plasticity of genotypes and the dynamics of genetic variation can be studied conveniently when transcript RNA, produced by transcription of cDNA with T7 RNA polymerase, is transfected on to HeLa cell monolayers and the corresponding plaque phenotype of progeny virus is analysed. In the case of wt virus, the plaques are, by convention, "large". If mutant RNAs are analysed in plaque assays, one may observe only "small plaques" with a rare "large" plaque emerging on the plate. This rare large plaque may signal a reversion (either directly or through suppresser mutations) to a fast-replicating genotype. Passage of the population of small and large plaque phenotype viruses (at multiplicities of infection of more than 5) will rapidly yield populations of only the faster-growing virus because the impaired genomes are eliminated by competition. An example of this phenomenon has been described by Lu *et al.*, (1995b), who analysed a hybrid poliovirus in which the cognate $2A^{pro}$ coding sequence was exchanged to that of coxsackie B4 virus.

A special case of a genetic phenomenon is that of a "quasi-infectious" genome. This term was originally introduced by Agol and his colleagues (Gmyl *et al.*, 1993) to describe the following phenomenon. Genetically engineered poliovirus variant RNA was transfected into HeLa cells. Progeny virus was harvested, sometimes only after prolonged incubations of the transfected tissue cell cultures. Analysis of the genotypes of progeny virus genomes (by RT/PCR) revealed only revertant or pseudorevertant RNAs. None of the original mutant genotypes were detectable. This phenomenon

can be explained if the original mutant genotype was able to replicate its RNA, albeit only at levels too low for virus production or even for the development of CPE. Nevertheless, the slowly replicating mutant genome allowed for mutation (either misincorporation or deletions, insertions), eventually leading to fast-growing genotypes. By definition, the progeny of quasi-infectious genomes will not yield virus with the parental genotype.

If a mutation (point mutation, linker insertion, etc.) engineered into the genome RNA is lethal, the lesion may effect complete abrogation of genome replication. Hence, reversion to viability cannot be expected. An interesting example of quasi-infectious versus lethal mutations in the poliovirus genome was described when mutations in VPg were studied (Kuhn *et al.*, 1988; Reuer *et al.*, 1990; Cao and Wimmer, 1995). As mentioned, VPg is linked to the genome via a O^4-(5′-uridylyl) tyrosine (the tyrosine in position three of VPg). A mutation of tyrosine to phenylalanine (Y3F) was originally described as being lethal (Reuer *et al.*, 1990). This conclusion made sense, since phenylalanine lacks a O^4 hydroxyl group for phosphodiester formation. However, Cao and Wimmer (1995) later observed that cells transfected with VPg(Y3F) variant RNA produced viable virus, albeit only at very low frequency and only after prolonged incubation of the cultures. All of the progeny genomes carried a F3Y reversion. The possibility of contamination of the cultures with wt virus was excluded. The only explanation for this surprising result was that the VPg(Y3F) variant was quasi-infectious, presumably in that the threonine residue in position four of VPg may have served as a (poor) surrogate acceptor for uridylylation and protein priming of RNA synthesis (it should be noted that genome-linked terminal proteins are often attached to serine residues; Salas, 1991). Further analyses supported this hypothesis. A VPg(T4A) variant was found to be viable, expressing good growth kinetics. In contrast, VPg(F3Y, T4A) variant RNA never yielded progeny virus and was, therefore, considered unable to replicate its RNA. This mutation then can be considered to be lethal.

Genetic analysis of mutant genomes and their revertants has been an invaluable tool to study the structure and function of picornavirus genetic elements and picornavirus proteins (Wimmer *et al.*, 1993).

Genetic Recombination

Genetic recombination of picornaviruses is the exchange of genetic elements between two viruses that may occur during replication in the same cell. Discovered by Hirst (1962), and used first by Cooper and his colleagues (Cooper, 1977) to map poliovirus genetic units, lingering skepticism about the phenomenon was dispelled through biochemical analyses of poliovirus recombinant proteins (Romanova *et al.*, 1980; Tolskaya *et al.*, 1983) or FMDV recombinant genomes (King *et al.*, 1982). (For a detailed review of recombination, the reader is referred to Wimmer *et al.*, 1993.)

Detection of Recombinants

Picornavirus recombinants have been detected because (1) they acquired genetic traits from the parental strains allowing them to proliferate under conditions restricting the growth of either parent and (2) they arose in excess over (replication competent) revertants of the parental strains (Cooper, 1977). Restricting conditions for the selection of recombinants can include specific drugs, such as 2 mM Gua HCl, monoclonal neutralizing antibodies (Emini *et al.*, 1984) or host-cell specificity (Duggal *et al.*, 1997). An elegant method to study poliovirus recombination under normal growth conditions (without selection) has been developed by Jarvis and Kirkegaard (1992).

Mechanism of Recombination

A wealth of experimental data has shed light on the most important steps in recombination. Details of individual steps in recombination, however, remain to be elucidated. The current knowledge can be summarized as follows.

1. Recombination is homologous and it occurs by copy choice; i.e. an incomplete (nascent) RNA strand may switch template strands

during genome replication. The probability of crossover depends strongly upon the degree of homology between the two recombining viral genomes.

2. Genetic analyses have indicated that template switching occurs (predominantly?) during –strand synthesis (Kirkegaard and Baltimore, 1986).
3. Recombination is precise: no deletions or insertions at sites of homologous recombination have been observed. This is true even if crossover occurred in the 154 nt long noncoding region (spacer region) between IRES and initiating AUG of poliovirus (Jarvis and Kirkegaard, 1992) even though the sequence of this "spacer" is not conserved amongst the three poliovirus serotypes (Toyoda *et al.*, 1984). Indeed, deletions in the "spacer" could conceivably be tolerated in view of the observations by Kuge and Nomoto (1987) and others that the spacer can be partially or completely deleted without loss of viability.
4. Template switching requires that the replication complex pauses, allowing a heterologous (invading) +strand to offer its service as template. An unsolved question is whether pausing and crossover is random (Jarvis and Kirkegaard, 1991) or non-random (Romanova *et al.*, 1986). The latter is in all probability true.

Agol and his colleagues have proposed that higher order structures formed on template RNAs may favor pausing of RNA synthesis and crossover (Romanova *et al.*, 1986) In addition, King (1988) suggested that there are preferred sites of recombination in poliovirus RNA, and that crossover may be favored immediately after synthesis of two uridylate residues (UU) in the nascent strand.

Duggal and Wimmer (1999) observed that crossover patterns changed significantly when recombination occurred at different temperatures. Specifically, crossover between two genetically marked RNA strands at 34°C occurred over a wide range of the genome with preference for sequences coding for structural proteins in the 5′-terminal half of the genome. In contrast, recombination *in vivo* at 37°C and 40°C yielded crossover patterns that had shifted dramatically to a region encoding nonstructural proteins (Duggal and Wimmer, 1999). Preferential selection of recombinants at 37°C and 40°C was ruled out by analyses of the growth kinetics of the recombinants. The reason for the temperature effect is unknown. Temperature-dependent stability of higher order RNA structures seems possible.

Recombination Frequencies

Recombination frequencies are calculated by dividing the yield of the recombinant virus by the sum of the yield of the parental virus. For picornaviruses with linear genomes, the distance between genetic markers used to determine recombination is proportional to the recombination frequency. As mentioned above, the degree of homology between the parental genomes strongly influences the probability for crossover.

The frequency of recombination between homologous genomes is remarkably high (2×10^{-3} between markers only 600 nt apart; Jarvis and Kirkegaard, 1992). It has been estimated that 10–20% of the homologous viral genomes may undergo genetic recombination within a single growth cycle (King, 1988). This would mean an unprecedented genetic shuffling between genotypes of which the fittest retain the "wt" phenotype.

Experimental results that support a high frequency of recombination between sibling strands were obtained with engineered, quasi-infectious poliovirus genomes carrying two adjacent VPg sequences (Cao and Wimmer, 1996). After transfection, all progeny viruses had lost the downstream VPg, most probably by homologous recombination during –strand synthesis. On studying recombination using genetically marked genomes, 90% of the recombination events occurred between sibling strands.

Finally, Jarvis and Kirkegaard (1992) have demonstrated that the frequency of recombination increases with the progression of the infectious cycle; i.e. the larger the concentration of intracellular viral RNA the higher the probability of recombination.

Cell-free Recombination of Poliovirus

Recombination in a cell-free extract of uninfected HeLa cells (Molla *et al.*, 1991) has recently been reported by two groups. Recombination of parental viruses in the cell-free medium was detected either by RT/PCR in the absence of selection (Tang *et al.*, 1997), or by plating the progeny virus under conditions that were restricted for either parent (Duggal *et al.*, 1997). The recombination frequencies were found to be roughly the same as that *in vivo* (in tissue culture cells).

The crossover pattern of recombination *in vitro* and *in vivo* at 34°C was the same, lending credibility to the cell-free system as reflecting an *in-vivo* environment (Duggal and Wimmer, 1999). The *in-vitro* approach has the potential to decipher some basic steps in recombination, as, for example, the invasion of heterologous template strands into the replication complex. As mentioned before, the pattern of recombination changed significantly when recombination was carried out *in vivo* at 37°C or 40°C. Unfortunately, this effect at higher temperature cannot be analysed *in vitro* because cell-free synthesis of poliovirus is highly inefficient or completely absent at temperatures above 36°C (Molla *et al.*, 1993c).

Poliovirus Vectors, Defective Interfering Particles and Illegitimate Recombination

Picornavirus genomes are extremely "plastic" in that any change in their genotype can lead to unexpected nucleotide rearrangements. This has been observed, for example, in analyses of IRES elements, where deletions or insertions lead to unexpected new genotypes with excellent growth properties (see, for example, Dildine and Semler, 1989; Gmyl *et al.*, 1993; Pilipenko *et al.*, 1992; Alexander *et al.*, 1994; Charini *et al.*, 1994; Cao and Wimmer, 1995). Genetic plasticity is particularly apparent when poliovirus genomes are constructed harboring foreign sequences. The analysis of genetic rearrangements and deletions is especially important when picornavirus genomes are to be used as vectors for the delivery of foreign genes.

Picornavirus vectors and illegitimate recombination Generally, polioviruses respond to the insertion of foreign sequences by rapidly deleting these sequences either partially or completely. The driving force behind the selection of deletion variants may be: (1) excessive length of the genome, restricting encapsidation; (2) interference with efficient processing of the polyprotein; (3) interference with initiation of translation; (4) alteration of RNA structures necessary for replication; or others. There are examples, however, where poliovirus, or deletion mutants thereof, appear to tolerate a foreign sequence inserted into the genome.

An interesting approach to studying IRES elements was the insertion of the EMCV IRES into the ORF of poliovirus, thereby making unnecessary the primary cleavage between P1*P2 catalyzed normally by $2A^{pro}$ (Figure 12.6B; Molla *et al.*, 1992). The resulting RNA transcripts proved highly infectious (Molla *et al.*, 1992). Although this dicistronic virus expressed a small plaque phenotype, neither the plaque size nor the genotype surrounding the insertion changed over six passages. These observations suggested that the insertion was stable, at least under the conditions studied. The EMCV IRES has no apparent sequence homology with any sequence of the poliovirus genome, the poliovirus IRES included. This eliminated the possibility that the EMCV IRES was rapidly removed by homologous recombination. Perhaps, illegitimate recombination (see below) to delete the IRES without debilitating the virus was a very rare event and not apparent in progeny virus. It should be noted in parenthesis that the viability of the dicistronic virus shown in Figure 12.6B proved for the first time the function of the EMCV IRES as a true internal ribosomal entry site (Molla *et al.*, 1992).

On the basis of these experiments, a specific version of novel expression vectors was constructed (Figure 12.6C,D) that included, in addition to the foreign IRES, a foreign ORF (Alexander *et al.*, 1994; Lu *et al.*, 1995a). None of these vectors was genetically stable over extended numbers of passages, and in some cases the deletion occurred during first passage (Lu *et al.*, 1995a). Nevertheless, the insertion of a foreign ORF between two IRESes in the 5'NTR,

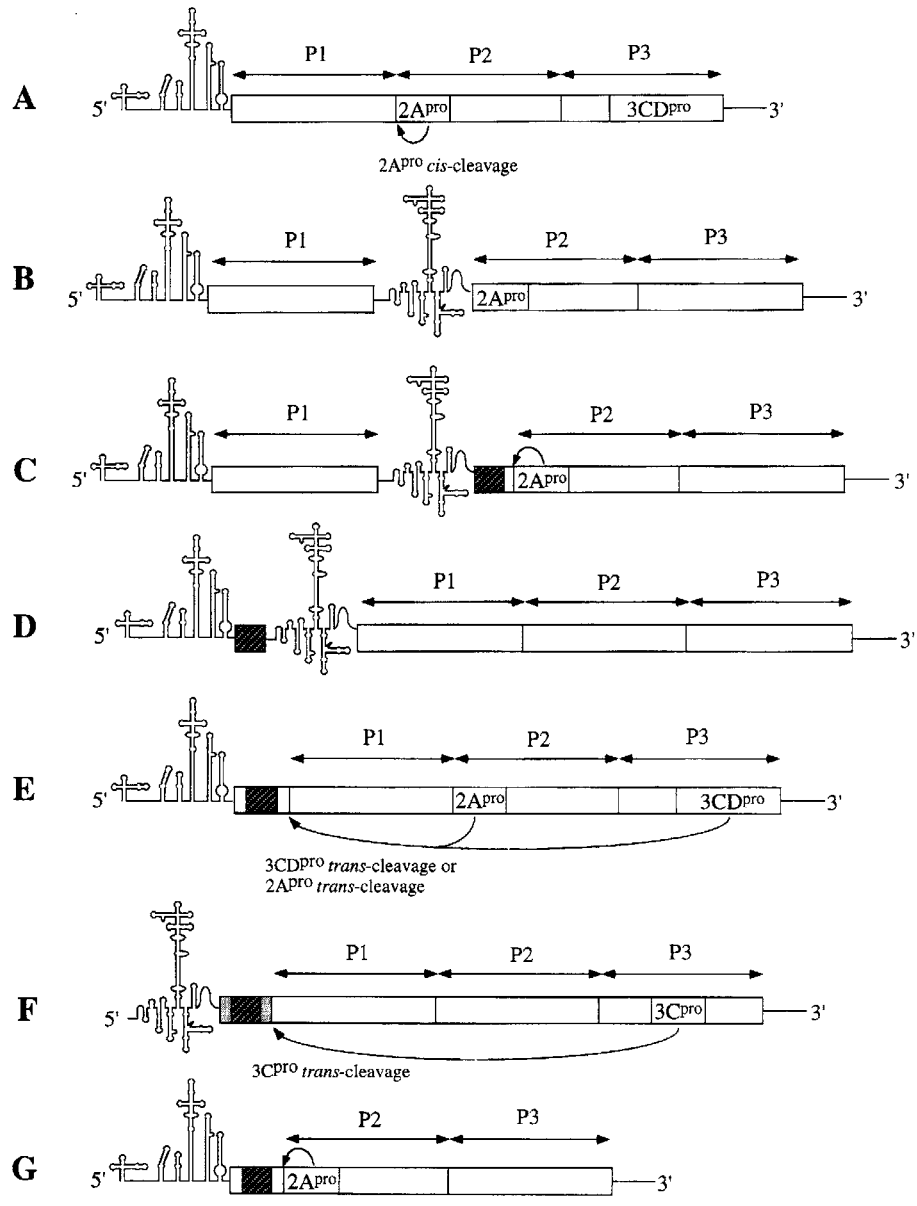


FIGURE 12.6 Picornavirus genomes and expression vectors. **A.** The poliovirus genome. **B.** Dicistronic poliovirus RNA in which the EMCV IRES (Figure 12.3) has been inserted into the genome at the site specifying primary $2A^{pro}$ cleavage. In this case, the function of the $2A^{pro}$ has been rendered superfluous. **C.** Expression vector based on the dicistronic genome depicted in **B**. The foreign gene (dark stippled box) was inserted upstream of $2A^{pro}$, which now delivers the foreign protein by a *cis* cleavage. **D.** Dicistronic poliovirus generated by inserting a foreign gene and the EMCV IRES into the 5'NTR. In this case the foreign gene is synthesized independently from the polyprotein. **E.** Generation of an expression vector by fusing the coding sequence of a foreign gene to the N-terminus of the poliovirus polyprotein. The foreign gene product is liberated through *trans* cleavage, by either $3D^{pro}/3CD^{pro}$ or $2A^{pro}$. **F.** Expression vector based on mengovirus, a cardiovirus that carries a small leader sequence preceding the P1 region of the polyprotein (see Figure 12.9). In this case, the foreign gene is inserted into the L coding sequence. Note that the organization of the genomes in **E** and **F** is identical. **G.** Encapsidation-incompetent poliovirus expression vector in which a portion of the P1 coding sequence has been replaced by a foreign gene. This genome can be encapsidated in *trans* but, by itself, it can only go through one cellular cycle of replication.

as shown in Figure 12.6D, yielded a replicating poliovirus vector that efficiently expressed the CAT gene over several passages (Alexander *et al.*, 1994). No deletion was apparent after the first passage. Remarkably, the genome of this construct is 17% larger than that of the wt genome, an observation indicating that the capsid of naturally occurring polioviruses is not "full". However, attempts failed to encapsidate and express the larger luciferase gene instead of the CAT gene in the context of the dicistronic virus. The luciferase activity was clearly detectable in cells transfected with the appropriate dicistronic transcript, but the genome harboring luciferase was not encapsidated (Alexander *et al.*, 1994). Apparently, an increase of genome length to 31% (luciferase gene plus EMCV IRES) was not tolerable for encapsidation (Alexander *et al.*, 1994).

A different strategy to convert poliovirus to an expression vector was the fusion of a foreign ORF directly to the poliovirus polyprotein (Figure 12.6E; Andino *et al.*, 1994). In these experiments, the strategy of Altmeyer *et al.* (1994) was mirrored, which made use of the genetic make-up of cardioviruses (mengovirus or EMCV). Specifically, the cardiovirus polyprotein is preceded by a small leader protein (67 aa) that is cleaved from the capsid region P1 by the viral $3C^{pro}$ proteinase, thereby allowing maturation and encapsidation of the virion. Altmeyer *et al.* (1994) inserted into the leader sequence of the mengovirus genome a foreign gene (Figure 12.6F) and expressed the product of this fusion protein over several passages in tissue culture (Altmeyer *et al.*, 1994, 1995). Andino *et al.*, (1994) generated a similar "leader" protein in front of the poliovirus polyprotein. In this case, however, it was necessary to engineer a novel $3C/3CD^{pro}$ cleavage site between the foreign ORF (the new "leader") and the viral polyprotein such that the foreign polypeptide can be cleaved from the poliovirus capsid precursor (Figure 12.6E). Although these poliovirus constructs were originally claimed to express excellent growth properties and, more importantly, were reported to be genetically highly stable (Andino *et al.*, 1994), the poliovirus-based vectors proved, in fact, impaired in replication and prone to rapid deletions, at least if the insert was more than 500 nt in size (Mueller and Wimmer, 1998, and references therein; see below). It should be noted that the cardiovirus-based vectors also suffered from loss of the inserts of a foreign gene upon repeated passage, an observation suggesting that even cardioviruses do not tolerate an extended leader protein for the purpose of gene therapy (Altmeyer *et al.*, 1994, 1995).

In a third strategy of the construction of picornavirus vectors, the P1 capsid region of the picornavirus genome is partially replaced with a foreign ORF (Figure 12.6G), yielding proliferation-incompetent replicons that appear to be genetically quite stable (see, for example, Porter *et al.*, 1993). For a possible application as vectors in gene therapy, the replicons are *trans*-encapsidated via a vaccinia virus-based P1 expression vector, with relatively low yields of proliferation-incompetent virions. The apparent genetic stability of these replicons may be due to the fact that the RNAs are similar in size when compared to the wt genome, and that the naturally occurring *cis* cleavage (between P1*P2) catalyzed by $2A^{pro}$ is highly efficient, placing no restriction on this step of polyprotein processing. However, the rapid selection of faster growing variants that lost the foreign gene is unlikely, since the *trans*-encapsidated replicons can only proceed to a one-step infectious cycle. This is very different from the selection pressure in proliferation-competent vectors, which engage in second-round infections.

What is the mechanism by which poliovirus may eliminate foreign sequences? Homologous recombination cannot function because there is not enough sequence homology to engage in crossover. Pilipenko *et al.* (1995) have nevertheless proposed that short sequences may serve as parting and anchoring sites for template switching in illegitimate (non-homologous) recombination. An alternative mechanism is "loop-out" deletion, in which the nascent strand skips endogenous sequences, jumping to an upstream sequence that serves as anchoring sequence. Given the high frequency by which recombination occurs among sibling strands, a crossover mechanism may be favored, but decisive experiments to decide between these two mechanisms are lacking. In any case, a detailed study

of genetic variations of polyprotein fusion vectors (Figure 12.6D) strongly supports the model of parting and anchoring sites for template switching or loop-out deletion (Mueller and Wimmer, 1997; see below).

Briefly, when expression vectors (Figure 12.6E) consisting of a *gag* gene (encoding p17-p24; 1161 nt) of human immunodeficiency virus that was fused to the N-terminus of the poliovirus polyprotein (Andino *et al.*, 1994; Mueller and Wimmer, 1998) were analysed after transfection into HeLa cells, the genomes were not only found to be severely impaired in viral replication but they were also genetically unstable (Mueller and Wimmer, 1997). Upon replication, the inserted sequences were rapidly deleted as early as the first growth cycle in HeLa cells. Interestingly, the vector viruses did not readily revert to wt sequences but rather retained some of the insert plus the artificial 3C/3CD^{pro} cleavage site (to allow processing at the N-terminus of the polyprotein). Thus, variants of different genotypes that replicated nearly as well as wt poliovirus had followed an evolutionary pathway towards the genetic organization of cardioviruses (Mueller and Wimmer, 1998). That is, the poliovirus polyprotein of these variants was preceded by *gag*-derived "leader" proteins of different but distinct sizes (predominantly between 20 and 50 aa long), the most prominent leader size reflecting the length of that in cardioviruses (67 aa). In the immediate vicinity of the deletion borders of several isolates, short direct sequence repeats were observed that are likely to allow alignment of RNA strands for non-homologous (illegitimate) recombination during –strand synthesis (Figure 12.7; Mueller and Wimmer, 1998). Interestingly, the selection of the leader size occurred during the very first rounds of replication of the transfected RNA; in most cases, as sequential shortening of the leader sequence was not observed.

Defective interfering particles An interesting phenomenon of naturally occurring deletion mutants of picornaviruses are defective interfering particles (DI particles) that can be (rarely) discovered in laboratory stocks of virus or generated (with difficulty) by passage of virus at high multiplicities (reviewed in Wimmer *et al.*, 1993). All naturally occurring DI particles carry deletions in the P1 capsid precursor region (Cole et al., 1971; Cole and Baltimore, 1973; Lundquist *et al.*, 1979; Nomoto *et al.*, 1979; Kajigaya *et al.*, 1985; Kuge *et al.*, 1986). As mentioned before, Nomoto and his colleagues have found that the deletions in all DI particles are in-frame (Kuge *et al.*, 1986). Since genetically engineered DI genomes (replicons) with an out-of-frame deletion in the P1 region are unable to replicate, Nomoto and his colleagues (Kuge *et al.*, 1986; Hagino-Yamagushi and Nomoto, 1989) correctly concluded that poliovirus RNA replication requires, at some stage of the replicative cycle, translation of the replicating RNA (*cis* requirement of translation), and that the DI particles with out-of-frame deletion cannot be complemented in *trans*. Novak and Kirkegaard (1994) confirmed this hypothesis in that replicons with translation termination codons downstream of the P1 coding region could not be rescued in *trans*. For hypotheses to explain this phenomenon, see above.

Genetic Recombination by Non-replicative Mechanisms

Chetverin *et al.* (1997) have recently made the startling observation that certain RNA fragments can join to one another via a molecular pathway determined by the intrinsic chemical properties of the RNA molecules (Chetverin *et al.*, 1997). The fragments that formed chemically stable duplexes were selected by the replicase of phage Qβ: only those dimers that had acquired signals from two different RNA molecules were able to replicate.

Gmyl *et al.* (1999) have now reported that viable recombinants could also be generated from non-replicating and non-translatable segments of the poliovirus genome. These fragments by themselves were unable to generate the viral RNA-dependent RNA polymerase necessary for a replication-dependent recombination event. The "crossovers" were targeted to the highly variable segment of 154 nucleotides located within the 5'NTR upstream of the initiating AUG codon for the polyprotein. A great number of recombinants have been obtained by transfection of mixtures of RNA fragments. Analyses of viruses that evolved after the mix-

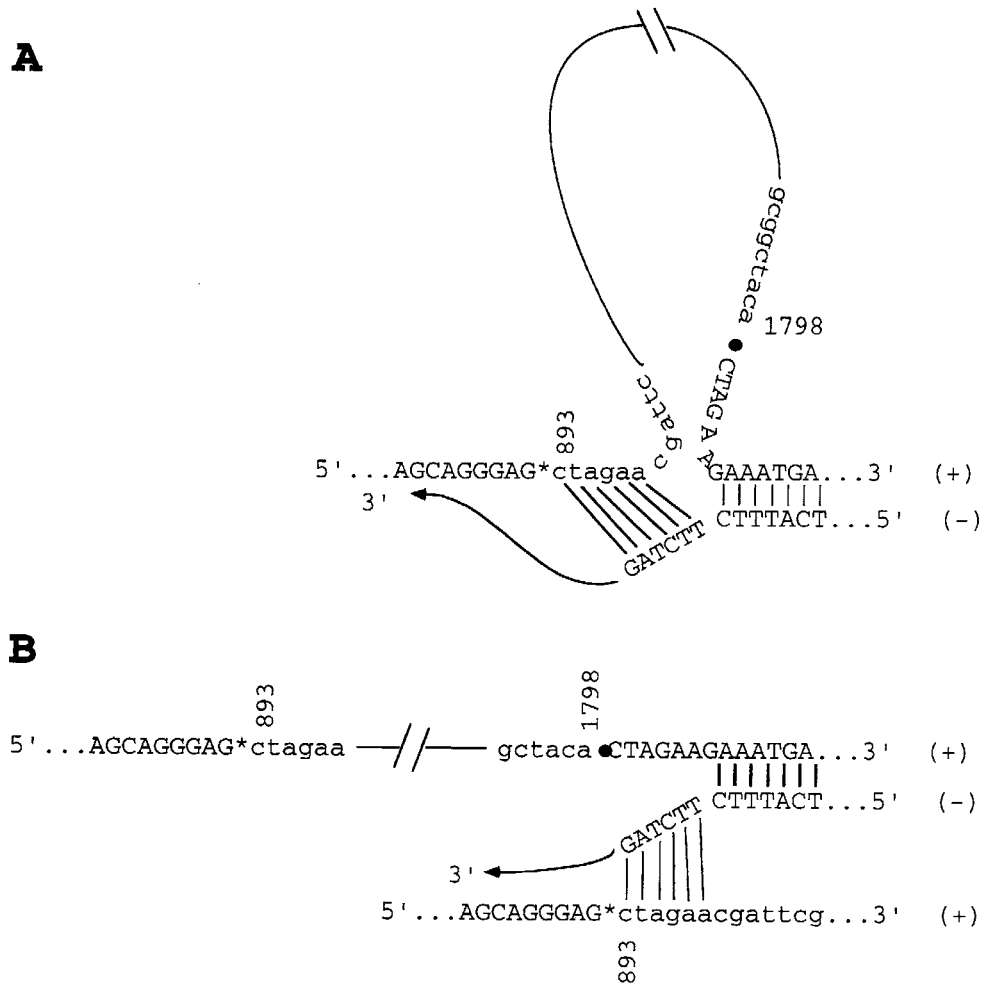


FIGURE 12.7 Two models of illegitimate recombination during – RNA synthesis as was observed with an expression vector shown in Figure 12.6E (Mueller and Wimmer, 1998). Both models require a partial dissociation of the nascent – RNA from the template +RNA, caused presumably by pausing of the RNA polymerase. The free 3′ end of the nascent – RNA can re-anneal to a short complementary sequence further upstream on the same template strand, thereby looping out the intervening sequence (**A**), or it can re-anneal to the same complementary sequence but on a sibling +strand, and complete synthesis on this second template (**B**; strand switching). In both cases, the resulting strands would have excised the same sequence and could now, in turn, give rise to truncated +RNA genomes. Note that this deletion event can occur even during the first round of replication of the expression vector leading to partial or complete deletion of the foreign coding sequences. Reproduced, with permission, from Mueller and Wimmer, 1998.

ture of the RNA species strongly suggested that the connection between two fragments was the result of chemical reactions between the fragments rather than of template-switching (Gmyl *et al.*, 1999).

The mechanism by which the chemical linkage between two fragments is formed is obscure but it could involve structures reminiscent of ribozyme-like activities in the viral RNAs. Nevertheless, this observation could have profound implications for the generation of novel genotypes in nature (Gmyl *et al.*, 1999).

Genetic Complementation

Is the Picornavirus Genome Monocistronic?

Genetic complementation is the compensatory action of gene products of two homologous

genetic systems to alleviate defects of mutant genes. Genetic complementation has been firmly established in picornavirus replication. However, because of the complexity of diverse function(s) of precursor proteins and their cleavage products, it has not been possible to define complementation groups (Wimmer *et al.*, 1993). Complementation groups are indicative of genetic elements that can function independently, and they have been the basis of the definition of cistrons (Benzer, 1957). A cistron, therefore, may be equated with a gene, i.e. a functional unit of genetic material specifying a single protein. On the basis of these definitions, the picornavirus genome, encoding only the polyprotein whose products function in many cases in overlapping or even opposing fashions, cannot be called multicistronic.

In general genetics, mutations affecting the same polypeptide can occasionally complement each other, a phenomenon referred to as intracistronic complementation (Schlesinger and Levinthal, 1963). Based on these considerations, we have suggested that the picornavirus genome be considered "monocistronic" (Wimmer *et al.*, 1993). It follows that the genome encodes only one gene product, the polyprotein. The polyprotein, in turn, contains multiple genetic units whose products may or may not be capable of intracistronic complementation. If this definition is accepted, one should avoid referring to individual coding regions of the picornavirus genome as "genes". Thus, there would be no "$3D^{pol}$ gene". This convention makes good sense if one considers that a "gene for $3D^{pol}$" is for the most part also the gene for $3CD^{pro}$, a proteinase with properties unrelated to the polymerase $3D^{pol}$. It should be noted that Theiler's virus is an exception to the monocistronic nature of picornaviruses in that it encodes a small protein in a separate reading frame, mapping towards the N-terminus of the polyprotein (Chen *et al.*, 1995).

It is interesting to consider that there is no absolute requirement that picornaviruses must exist as monocistronic (single-polyprotein-producing) entities. For example, the insertion of a second IRES into the genome would represent a viable dicistronic virus (Figure 12.6B), an entity artificially generated by Molla *et al.* (1992, 1993b). Apparently, during the evolution of picornaviruses, the elimination of genetic elements regulating the expression of different picornavirus proteins was favored over retaining them. Similarly, there was no pressure to generate such regulatory sequences and insert them into the genome. In other words, proteolytic processing of a single polyprotein evolved not only to be highly efficient but also as a means to regulate the temporal appearance of viral proteins (e.g. precursor proteins versus end-products of proteolytic cleavage). In contrast, in prokaryotic RNA phages or in –strand RNA viruses, the expression of proteins is regulated by sequence elements located between different cistrons.

Interestingly, the dicistronic poliovirus depicted in Figure 12.6B resembles the genetic composition of cow pea mosaic virus (CPMV), a plant virus (Hellen *et al.*, 1989; Molla *et al.*, 1992). Indeed, CPMV and the dicistronic poliovirus shown in Figure 12.6B have similar gene order and amino acid sequences, the main difference being that the genome of CPMV is bipartite. That is, rather than inserting a sequence such as an IRES into the genome, CPMV preferred to divide the genome into two portions, one coding for the capsid proteins the other for the replication proteins. Such a genetic arrangement, which requires two particles to initiate a complete infectious cycle, may be suitable for a plant virus (where the yield of virus per host can be extremely high and, equally importantly, the local concentration of host organisms can be high) but it would be highly disadvantageous for an animal virus.

The fascinating topic of the structure and evolution of polyproteins within the RNA-like virus superfamily is discussed later.

General Observations of Picornavirus Complementation

Evidence for genetic complementation *in vivo* has existed for decades, the best-known involving guanidine-generated mutants. Complementation of guanidine mutants seemed unidirecional (Wimmer *et al.*, 1993). Bernstein *et al.* (1986) provided the first conclusive evidence for symmetric complementation, using mutants

that were generated in $2A^{pro}$ and 3A. These authors, however, also made the unexpected observation that mutants mapping to 2B or $3D^{pol}$ (which they had also generated by genetic engineering) could not be complemented (Bernstein *et al.*, 1986). On the other hand, Charini *et al.* (1991) clearly showed that mutations in $3D^{pol}$ mapping to a different region of the coding sequence could be rescued in *trans*. This example and many others (Wimmer *et al.*, 1993) support the notion that the polyprotein is a single genetic unit that does not consist of non-overlapping genes whose functions can be separated by complementation grouping.

A special case of complementation was tested using dicistronic polioviruses. Briefly, Cao and Wimmer (1995) constructed a virus with a genotype shown in Figure 12.6B, the extra cistron being the coding region for poliovirus 3AB. The dicistronic construct yielded a virus expressing a small plaque phenotype but it was genetically unstable, losing its inserts after several passages. Nevertheless, if the lethal mutation VPg(Y3F, T4A) was engineered into the second cistron of the dicistronic virus (see above), the first cistron (3AB) could rescue the genome, albeit inefficiently.

A much more efficient rescue of lesions in the 3AB coding sequence was reported by Towner *et al.* (1998), who used the cell-free system of poliovirus replication developed by Molla *et al.* (1991). Apparently, the supply in *trans* of P3 polypeptides *in vitro* is more efficient that *in vivo*, a phenomenon that remains as yet unexplained. Interestingly, it appeared as if a mutation in 3AB could be rescued only if the complementing polypeptide was a precursor of 3AB, preferably P3 (see Figure 12.4). Perhaps, efficient complex formation between $3AB/3CD^{pro}$ (see above) in this system depends on the cleavage of the P3 precursor *in situ*.

PATHOGENESIS OF PICORNAVIRUSES

Picornaviridae combines species that infect animals with exceedingly varied pathogenic features affecting almost every organ system (Table 12.1). In the following, we will concentrate only on human picornavirus infections, which range in severity from protean symptoms associated with the common cold (e.g. rhinoviruses), mild gastroenteritis (e.g. echoviruses), hepatitis (e.g. hepatitis A virus), to fatal CNS manifestations (e.g. PV, enterovirus 71) or lethal myocarditis (coxsackievirus B group). Despite the enormous variety in organ tropism observed with different species of the picornavirus group, pathogenic features of every single species are recognized in the form of highly distinct disease syndromes (exceptionally, coxsackieviruses can cause fatal disseminated infections in neonates with widespread viral propagation in multiple organs).

We will define pathogenic properties of picornaviruses as a combination of different viral traits: (1) those that affect tropism (determining the target cell type of, and influencing spread in, the host); (2) those that affect virulence (determining kinetics of particle propagation); and (3) those determining the progression of a disease syndrome ("pathogenicity proper", the propensity to cause clinical symptoms). There is a fourth parameter, which is strictly related to a condition of the host. An example is injury-provoked ("provocation") poliomyelitis, which will also be discussed below.

Surprisingly, the disparity in pathogenic properties may be contrasted with a high degree of sequence conservation among certain groups of picornaviruses. This is most evident with the cluster C enteroviruses (Figure 12.1B). For example, on the basis of sequences of the $3D^{pol}$, poliovirus serotype 2 (Lansing) (PV2(L)) shares more than 90% sequence homology with its close relative coxsackievirus A24 (CAV24). Indeed, their sequence similarity exceeds that between PV2(L) and PV1 (Mahoney) or PV3 (Leon) (Figure 12.1B). Yet, whereas all PV serotypes are associated with poliomyelitis, a severe and frequently fatal infection of the CNS, CAV24 causes mild upper respiratory tract infections only. Since minor sequence variations of picornaviruses can account for drastically different disease syndromes it may be assumed that the pathogenic phenotype of picornaviruses is encrypted within a few crucial genetic determinants.

These basic determinants of pathogenic

features appear to be dynamic, leading occasionally to the emergence of novel virus variants causing clinical syndromes not previously observed with their ancestors. This was the case when widespread epidemics of acute hemorrhagic conjunctivitis ravaged Africa and the pacific rim (Yin-Murphy, 1973) and quickly expanded worldwide. Two picornaviruses were associated with this previously unknown clinical syndrome, coxsackievirus A24 variant (CAV24v) and enterovirus 70 (EV70; Table 12.1). The former evolved from its ancestral CAV24, causing mild upper respiratory tract infections, whereas EV70 was primarily recognized for its association with a poliomyelitis-like neurological disorder (Melnick *et al.*, 1974). The deviation of tropism toward ocular tissues resulting in acute hemorrhagic conjunctivitis suggests a switch or an expansion in receptor specificity. This hypothesis, however, awaits confirmation, since the cellular receptor(s) of EV70 is unknown (Table 12.1). Available evidence suggests that CAV24v can still use ICAM-1 as receptor but may have extended its cellular binding activity to a second unknown protein. CAV24v and EV70 do not share receptors (N. Takeda, personal communication; see later).

The circumstances and conditions that may favor a switch in host cell tropism with resulting changes in the pathogenic phenotype are unknown. A detailed discussion of our current view of the evolution of enteroviruses, however, is presented in the following section on Evolution. This is particularly relevant in the context of the imminent global eradication of poliovirus. It is known that coxsackieviruses A7 and A9 (CAV7, CAV9) as well as enteroviruses 70 and 71 (EV71) occasionally cause a clinical syndrome with striking resemblance to poliomyelitis (Melnick *et al.*, 1974). Occurrence of poliomyelitis caused by these virus species has only rarely been reported in epidemic proportions (Voroshilova and Chumakov, 1959; Melnick *et al.*, 1980); generally they occur as isolated incidents. Fortunately, preliminary evidence (da Silva *et al.*, 1996) suggests that to date no surge in non-PV-caused poliomyelitis has occurred in response to the eradication of poliovirus in Latin America. However, the time elapsed since the eradication of wt polioviruses in the western hemisphere is too short in terms of evolution to conclude that the incidence of non-PV-caused poliomyelitis and poliovirus eradication are unrelated (see section on Evolution).

The observation of diverse specific clinical syndromes caused by closely related picornaviruses (particularly enteroviruses) has sparked interest amongst virologists in identifying those factors that may determine the clinical outcome of picornaviral infections. Generally, signals for pathogenic phenotypes can be found in all parts of the viral genome. However, factors that determine cell and tissue tropism are not necessarily the same as factors determining virulence or attenuation. For example, the capsid mutations of the live attenuated strains of PV (the Sabin strains) have an attenuating effect without altering the tropism of the Sabin strains for the prime target of PV: spinal anterior horn motor neurons.

A different effect of capsid proteins on an extended host tropism has been reported for several poliovirus type 2 strains, e.g. PV2(L). A small segment in capsid protein VP1 of PV2(L) (the B-C loop) has been identified as carrying determinants of host range extension from primates to rodents (Murray *et al.*, 1988). However, whereas PV2(L) infection causes poliomyelitis in primates and in *CD155* tg mice, normal mice developed histopathology indicative of panencephalomyelitis, which was radically distinct from poliomyelitis observed in primates and *CD155* tg mice (Gromeier *et al.*, 1995). This observation suggests PV2(L) tropism toward a cell type in mice that is not targeted in primates. It is likely that the mouse-adapted PV2(L) acquired additional receptor specificity but the nature of the receptor for PV2(L) in normal mice is unknown. It should be noted that although PV2(L) causes disease in mice after intracerebral injection, cultivated mouse L cells cannot be infected with this strain (Gromeier *et al.*, 1995).

The fact that single determinants of pathogenicity (e.g. the PV capsid) can carry signals that influence either tropism (e.g. PV2(L)) and/or virulence (Sabin strains of poliovirus) indicates different dimensions of picornaviral pathogenesis. Taking multiple determinants of tissue tropism and virulence (shared between the capsid, non-

structural viral proteins and non-coding sequences) into account, the enormous complexity of the molecular basis of picornaviral pathogenesis comes into perspective.

Capsid protein structure determines the interaction of a virus with its cellular receptor. As pointed out for the poliovirus Sabin strains and PV2(L), small differences in the structure of viral capsids are critical for cell and organ tropism as they ultimately determine the pathognomic features of the resulting infection. Similarly, the capsid was determined to harbor sequences critical for cardiotropism (Tracy *et al.*, 1995; Cameron-Wilson *et al.*, 1998) as well as diabetogenicity (Kang *et al.*, 1994) of coxsackie B viruses (CBV). Moreover, diabetogenicity of EMCV in mice mapped to the capsid (Jun *et al.*, 1998).

As pointed out, the mechanism by which the changes in the capsid may affect pathogenesis may be related to differences in the interaction between virus and receptor. Apart from direct receptor switching (or extending receptor specificity to more than one cell surface molecule), virus capsid alterations may affect the kinetics of virus/receptor binding or particle stability. This would influence the virulence of that virus without a concurrent change in host cell tropism. Reduced particle integrity has been proposed to participate in the attenuation phenotype of the Sabin strains of poliovirus (Filman *et al.*, 1989). However, theories linking capsid mutations within the Sabin strains with structural elements important for protomer cohesion and capsid integrity remain inconclusive.

In contrast to a likely relationship between capsid structure and pathogenic phenotype, the role of non-structural viral gene products in the determination of disease has been less obvious. Non-structural viral proteins are cell-internal and, hence, do not influence tropism in a strict sense. Cell-type-specific restrictions in viral replication regulated by non-coding regions of the viral proteins or by non-structural proteins will not change the spectrum of target cells infected but may critically influence virulence. Viruses normally evolve to adapt to host cells offering adequate portal of entries (receptors), thereby exposing the viral particles to an intracellular milieu supportive of particle propagation. Thus, favorable cell-internal conditions for viral replication would ideally be matched by a suitable viral receptor to avoid virus entry into cells that do not permit replication. For most viruses invading a host organism, the match is not perfect and, hence, the viruses are restricted to replication in fewer cells or organs than the distribution of receptor molecules would suggest.

Cell-internal determinants mapping to the viral genome of various picornaviruses have been suggested to influence virulence. These can be divided into loci mapping to viral proteins or to non-coding regions (e.g. the 5'NTR). For example, mutations within the coding region for the RNA-dependent RNA polymerase $3D^{pol}$ of the PV1 Sabin vaccine have been implicated in the attenuation phenotype (Toyoda *et al.*, 1987; Tardy-Panit *et al.*, 1993). It was found that these mutations contributed to the *ts* phenotype of this Sabin vaccine strain (Toyoda *et al.*, 1987). Mutations in the P2 region of hepatitis A virus have been correlated with an attenuated phenotype of HAV (Raychaudhuri *et al.*, 1998). In many instances the genetic loci of pathogenesis mapping to viral non-structural proteins have been identified through sequence comparison. This approach, of course, did not reveal mechanisms to account for reduced virulence.

The non-coding genetic elements of picornaviruses have also been shown to carry signals determining virulence (Evans *et al.*, 1985; Duke *et al.*, 1990; Gromeier *et al.*, 1996; Tracy *et al.*, 1996). As has been discussed in the section on Genetics, the 5'NTR harbors the internal ribosomal entry site (IRES) that, on the basis of the early observation by Evans *et al.* (1985) with the Sabin type 3 vaccine strain, has been identified as a major determinant of virulence for a number of picornaviruses. Sequence comparison of the Sabin strains of poliovirus with their *wt* progenitors revealed point mutations within a confined region of the 5'NTR in all three serotypes, known as domain V (Figure 12.3; reviewed in Wimmer *et al.*, 1993). In analyses of viral strains recovered from patients who acquired paralytic polio after vaccination, a point mutation at position 472 (direct reversion in domain V) of the IRES of PV3(Sabin) was proposed to contribute to the neurovirulence of the

isolate (Evans *et al.*, 1985). Recent analyses led to a different hypothesis stressing a co-operative attenuating effect of capsid mutations with mutations in the IRES and other locations in the Sabin vaccine genomes (McGoldrick *et al.*, 1995). The mechanism responsible for IRES-mediated attenuation of neurovirulence remains obscure. Analyses of cell-type-specific growth restrictions in cell lines of neuronal origin and biochemical studies of cell-type-specific IRES function suggested impairment of initiation of translation in a cell-type-specific manner (Haller *et al.*, 1996; Agol *et al.*, 1989; La Monica and Racaniello, 1989). Following the example of poliovirus, IRES elements or surrounding sequences of a large number of picornaviruses were found to contain genetic markers with a role in the determination of a pathogenic phenotype. This was most impressively demonstrated by the drastic attenuating effect of a deletion within the poly(C) tract of the 5'NTR of EMCV (Duke *et al.*, 1990).

How do the multiple mechanisms alluded to interlace to produce a specific picornavirus disease syndrome? The intricacies of dual cell external and internal determinants of viral pathogenic features are best illustrated using the example of poliovirus. This most thoroughly studied prototype picornavirus is characterized by pathogenic properties of specificity untypical of viral pathogens of the CNS. Poliovirus host range is limited to primates only. Within the primate organism poliovirus replicates within an unknown site in the gastrointestinal tract and within associated lymphatic structures, leading to viremia (Bodian, 1972). At this stage, the virus causes hardly any disease symptoms. However, viremia may, in only about 1% of infections, lead to CNS invasion, presumably through passive passage of the blood–brain barrier (Yang *et al.*, 1997). On muscle injury, the virus may also reach the CNS via retrograde axonal transport (Gromeier and Wimmer, 1998, 1999). Within the CNS, poliovirus uniquely targets spinal cord anterior horn motor neurons. Lytic destruction of anterior horn motor neurons results in flaccid paralysis, the hallmark clinical sign of paralytic poliomyelitis (Bodian, 1972).

Whereas efficient poliovirus proliferation occurring in the human gastrointestinal tract produces few or no symptoms, this virus's pathological potential is expressed in a small and relatively inaccessible subpopulation of neurons in the CNS. This peculiar restriction has been the subject of research interest for many years. Available evidence clearly suggests that the restriction of the host range of poliovirus to primates is determined predominantly by the receptor. In humans, this receptor is the immunoglobulin superfamily molecule CD155 (Mendelsohn *et al.*, 1989) and two proteins closely related to CD155 function as poliovirus receptor in simians (Koike *et al.*, 1990). Clearly, the observed organ and cell tropism are co-determined by the virus's dependence on the cellular receptor. We believe that, at least in part, the expression of CD155 must also determine the highly restrictive cell tropism of poliovirus within the CNS, because mice transgenic for the *CD155* gene develop a neurological condition with pathologic and clinical features identical to those observed in primates (Ren *et al.*, 1990; Koike *et al.*, 1991; Gromeier *et al.*, 1996). Furthermore, support for this hypothesis comes from studies of transcriptional control (Solecki *et al.*, 1999) and developmental expression of the *CD155* gene (Gromeier *et al.*, 1999b). It has been reported that *CD155* expression is restricted to structures in close anatomical and functional relationship with spinal cord anterior horn neurons during embryonic development of the CNS (Gromeier *et al.*, 1999b). It is thus likely that the restrictive expression pattern of *CD155* may indeed direct poliovirus tropism toward a specific cellular compartment of the CNS.

Analyses of pathogenesis related to genetic determinants mapping to the capsid, proteins, IRES, or $3D^{pol}$ have recently been extended using poliovirus hybrid viruses. Specifically, polioviruses have been constructed in which the cognate IRES was replaced by that of other picornaviruses (Gromeier *et al.*, 1996). By exchanging the cognate IRES element of PV by that of other picornavirus species, it could be shown that neuropathogenicity of PV can be eliminated without affecting growth properties in non-neuronal cell types normally susceptible to poliovirus (Gromeier *et al.*, 1996). Thus, it was determined that the IRES of rhinovirus type 2, a virus species never associated with neurological

disease, confers the attenuation phenotype to poliovirus. Significantly, this chimeric virus, called PV1(RIPO), did not carry any attenuating mutations in the poliovirus-specific sequence of its genome.

The neuropathogenic potential of a picornavirus IRES cannot be predicted but it is innate, of course, in its sequence. Certainly, IRES elements of enteroviruses known to cause poliomyelitis (CAV7, CAV9, EV70, and EV71) are candidates for "neurovirulent IRESes" and, indeed, a corresponding PV/CAV chimera has proved this hypothesis (Gromeier *et al.*, unpublished results). C-cluster enteroviruses, on the other hand, never cause poliomyelitis. However, because of their close genetic kinship, the IRESes of C-cluster coxsackieviruses confer a highly neurovirulent phenotype to the PV/CAV chimeras (Gromeier *et al.*, unpublished). Finally, IRES elements of the genus *Rhinovirus* ablate neuropathogenesis in poliovirus chimeric viruses (Gromeier *et al.*, 1996).

These observations indicate that, indeed, cell-external restriction in cell tropism as well as cell-internal factors exert powerful limitations toward enterovirus pathogenesis. Many relatives of poliovirus of the *Enterovirus* genus (particularly the C cluster; Table 12.1) presumably would equal the neuropathogenic properties of PV if their capsid structure allowed interaction with neuronal cells. Thus, non-neurovirulent C-cluster enteroviruses with high sequence homology to PV and "neurovirulent" IRES elements may gain tropism for neurons in the future (see under Evolution).

In addition to virus-encoded factors of PV neuropathogenicity, the circumstances within the host organism at the time of infection or shortly thereafter may influence the outcome of poliovirus infection. Trivial muscle injury has been shown to increase the probability of neurological complications of concurrent poliovirus infection (McCloskey, 1950). A proposed pathogenic mechanism for provocation polio identified a deviation of the route of CNS invasion toward retrograde axonal transport to account for the increased risk of polio among individuals who received intramuscular injections (Gromeier and Wimmer, 1998, 1999).

Picornaviruses have adapted to a wide variety of cellular components of their hosts. The diverse spectrum of disease syndromes associated with human picornaviruses provides an excellent field of study to examine the factors that determine the clinical outcome of a viral infection. The enormous amount of sequence information, combined with a broad knowledge of the molecular biology of many of these agents, have sparked hopes of a rapid elucidation of the molecular basis for their pathogenic properties. Initial optimism that sequence comparison of virulent strains with their attenuated variants alone would rapidly identify those elements responsible for a pathogenic phenotype and unravel mechanisms of pathogenesis is, however, unjustified. This is particularly true for poliovirus, the most thoroughly studied picornavirus. Progress in the analysis of poliovirus neuropathogenicity has revealed that the interactions of poliovirus with the host are characterized by a degree of complexity not previously appreciated. Mechanistic concepts of viral pathogenesis, combined with one-dimensional views of virus replication and its relation to the host organism, have helped little in increasing our understanding of the selective susceptibility to poliovirus of motor neurons. Viral infections result in complex clinical syndromes that are difficult to explain in terms of single viral genetic elements. Using poliovirus as an example, picornavirus-induced disease is the result of an intricate interplay of numerous factors, of both viral and host origin, that coordinately affect the ability of the virus to propagate in any particular cell type or organ.

EVOLUTION

General Remarks

Numerous investigations, particularly those of J.J. Holland, E. Domingo and their colleagues (see Chapter 7), have led to the realization that the rapid evolution of RNA viruses results from high mutation rates combined with exceedingly large heterogenic populations. This is true for Picornaviridae that encode variants of conserved protein folds as well as catalytic systems

not found in the cellular world and, hence, have explored an enormous evolutionary space. It is less appreciated, although equally true, that it is the host environment that has provided new opportunities for viruses to proliferate and select new variants out of a huge number of mutants. Without this "co-operation" between host and parasites, picornavirus evolution would not have resulted in the tremendous diversity of genotypes whose number, now counting in the hundreds, is currently biased towards those infecting mammals.

The key role of virus–host interaction is evident in the evolution of RNA viruses. Indeed, the relatively slow pace by which the cellular environment is changing imposes severe restrictions on the mode of RNA virus evolution. A model has been proposed suggesting that, in each moment of protein evolution, mutations could only be accepted in a very limited number of positions of a polypeptide chain; otherwise, protein structure and function would have been severely compromised. These limited places of acceptable variation have been called covarions (Fitch, 1971). It has been argued that the fast evolution of RNA viruses in a constrained environment has to proceed through the exploitation of constantly emerging but vastly overlapping covarions (Koonin and Gorbalenya, 1989). This model of virus evolution provides a sensible hypothesis as to how picornaviral proteins have managed to accept a heavy load of mutations that are quite frequently unique and rarely seen at sites in cellular proteins, while still not entirely losing some discernible similarity with other viral and cellular homologs. This has important practical consequences, since structural similarities can be used to reconstruct the evolution of RNA viruses, an undertaking impossible just 20 years ago.

Apart from the biological properties generally associated with all RNA viruses, the current end-product of evolution makes each virus species unique. This is engraved in the virus' genetic plan: the organization of the genome and the mode of gene expression. During the past years we have learned that +strand RNA viruses employ variations of surprisingly few basic genetic plans. The genetic organization and genetic expression of picornaviruses has been outlined in detail above. In the following, the genetic organization of different picornaviruses will be put into an evolutionary perspective. In addition, we will discuss in a rather speculative manner some hypothetical implications of evolutionary consequences of the eradication of poliovirus.

Classification and Phylogenetic Relationships of Picornaviridae

Numerous RNA viruses have been combined into a picorna-like supergroup of which the Picornaviridae comprise a rather compact domain (Figure 12.8). The viruses of the Picorna-like supergroup, a taxon not yet recognized as higher-than-family rank, share a conserved array of replicative proteins (see below). They infect plants, insects, birds and mammals. Most of the established members of Picornaviridae are mammalian viruses, and they fall principally into six genera (Table 12.1). The newly characterized avian encephalomyelitis virus (AEV), which is a member of Picornaviridae, is most closely related to hepatitis A virus (Marvil *et al.*, 1999).

The latest revision of the classification of picornaviruses, although closely related to the original version, has a clear evolutionary flavor since it tends to combine viruses in accord with phylogenetic kinship rather than relying on phenotypic properties (see the Introduction). The largest number of picornaviruses fall into the most closely related genera, *Enterovirus* and *Rhinovirus* (Table 12.1). *Cardiovirus* and *Aphthovirus* comprise two other genera that have most probably emerged from a common ancestor. It appears that the two pairs of picornavirus genera diverged after hepato- and parechoviruses split from the main trunk of the picornavirus tree (Figures 12.1, 12.8). The exact phylogenetic interrelationship between hepato- and parechoviruses remains somewhat uncertain and may be different from that shown in Figure 12.1 (see also below). In addition, three other picornaviruses, equine rhinovirus types 1 and 2 (ERV1 and 2 respectively) and Aichi virus (AiV), remain to be classified. ERV1 was shown

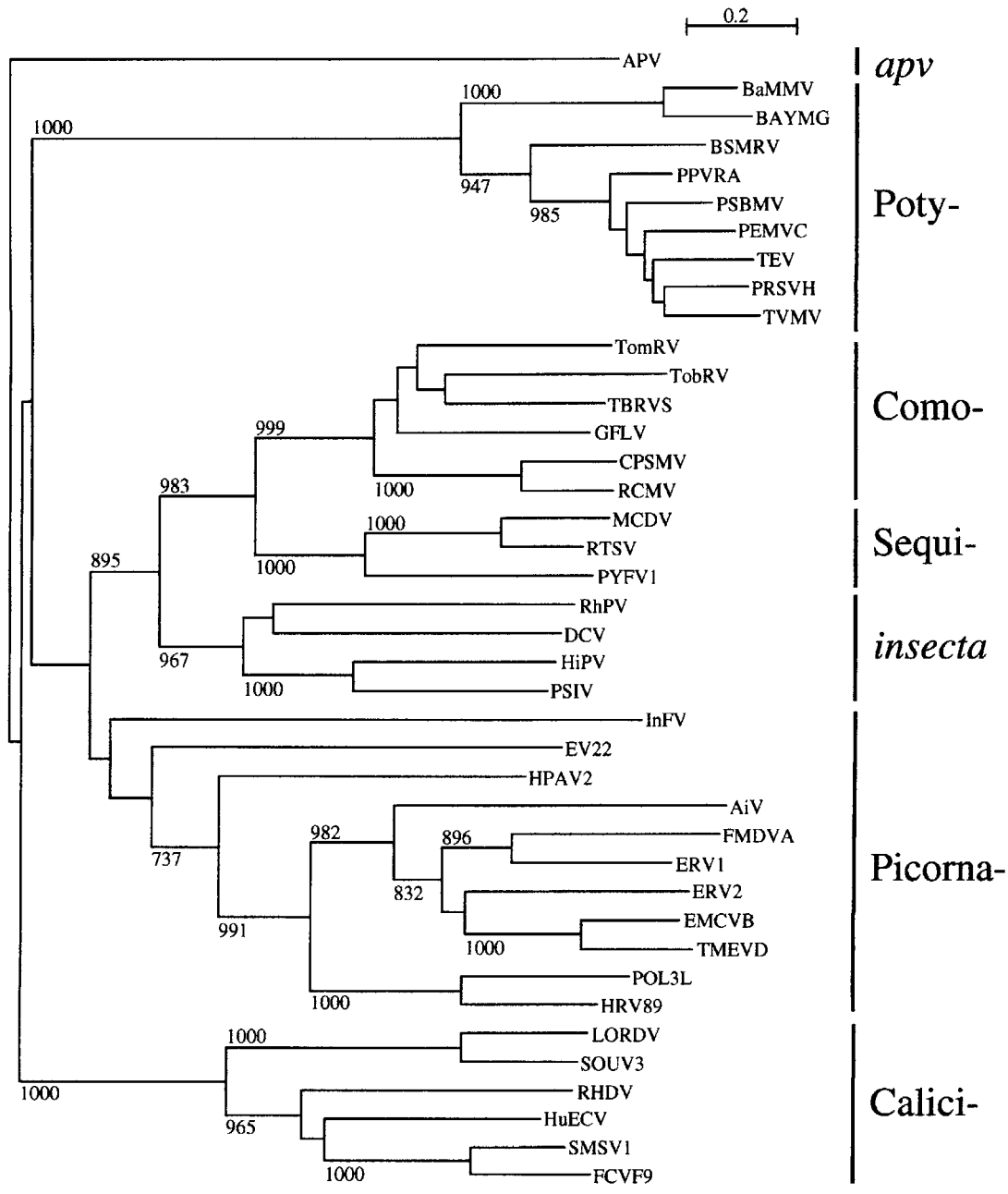


FIGURE 12.8 Tree of $3D^{Pol}$ and its homologs encoded by viruses of the picorna-like supergroup. The tree was generated for 3D (-like) sequences of a representative set of picorna-like viruses, including the five established families Potyviridae, Comoviridae, Sequiviridae, Caliciviridae and Picornaviridae, and two provisional groups of insect viruses, one dubbed *insecta*, the other *apv* (A.E. Gorbalenya, unpublished results). The neighbor-joining method with the Kimura correction for multiple substitutions, as implemented in the ClustalX program, was used to calculate the tree. Those radiations that have persisted in more than 700 out of 1000 bootstrap replications are marked at the bifurcations.

to be a distant relative of FMDV (Wutz *et al.*, 1996), while ERV2 (Li *et al.*, 1996; Wutz *et al.*, 1996) and AiV (Yamashita *et al.*, 1998) appear to be related to the cardio- and cardio/aphthovirus branches respectively. They are not recognized, however, as cardio- or aphthoviruses.

In addition to mammalian viruses, a number of insect viruses have been previously included into the Picornaviridae on the basis of phenotypic criteria. During the last 2 years, however, the complete genome structure of six picorna-like insect viruses has been reported (van der Wilk *et al.*, 1997; Isawa *et al.*, 1998; Johnson and Christian, 1998; Moon *et al.*, 1998; Nakashima *et al.*, 1998; Sasaki *et al.*, 1998). Some of these viruses feature different genome organizations, and only infectious flacherie virus of silkworm (InFV; Isawa *et al.*, 1998) was shown to possess a genotype and gene organization that may justify placing it in the Picornaviridae (A.E. Gorbalenya, unpublished results). It is likely that an ancestor of InFV separated from the main branch of Picornaviridae before the radiation of mammalian viruses (Figure 12.8).

The rapid accumulation of new virus genotypes has not been matched by an understanding of its evolutionary meaning. Therefore, the basis of picornavirus classification may need to be revisited. Moreover, the relationship between Picornaviridae and other genetic systems may have to be defined within a new classification. Although confusing for virologists on first sight, any reclassification into hierarchically organized taxa will ultimately aid our understanding of evolution, host range of viruses and pathogenesis. It should be kept in mind, however, that any classification is, at best, an approximation of true phylogenetic relationships, and the current classification of Picornaviridae should be treated as such.

The Conserved Backbone and Hot Spots of Genome Organization

Picornaviridae have evolved by speciation from a common ancestor. This plausible statement has been supported by computer analyses of nucleotide and protein sequences as well as by studies of the tertiary structure of capsid proteins and $3C^{pro}$ proteinases. There is every reason to believe that the putative ancestral viral entity had a genetic organization that has been conserved largely in contemporary picornaviruses. Its signature is a long 5'NTR-long open reading frame-3'NTR-poly(A) (Figure 12.2). With the sole exception of a strain of Theiler's virus (TMEV; see below), all picornavirus proteins are generated by autocatalytic processing of the gigantic polyprotein (Figure 12.3).

Proteins

The backbone of the polyprotein is formed by a set of polypeptides conserved in all known picornaviruses. In addition, the backbone may be decorated with a few optional proteins unique to a particular virus or virus group. Among the proteins, the conservation increases in the order (Figures 12.4, 12.9):

$L < 2A^{(pro)} < VP4 < 3A, 2B < 3B^{VPg} < VP1,VP2 < VP3 < 2C^{ATPase} < 3C^{pro} < 3D^{pol}$.

This order can be deduced from analyses of virus groups belonging to different phylogenetic ranks – clusters of closely related viruses, of distinct genera, of an entire family, or of the entire picorna-like supergroup (Figure 12.10; A.E. Gorbalenya, unpublished results). Slight deviations can only be seen upon analysis of some small taxonomic groups. As already noted, TMEV encodes a small, unique L* polypeptide outside the main reading frame at a locus overlapping the L reading frame. Additionally, the insect picornavirus InFV was predicted to encode unique domains as part of the polyprotein (Isawa *et al.*, 1998; A.E. Gorbalenya, unpublished results). A closer look at these proteins reveals the following.

The L polypeptide preceding the capsid coding region (Figure 12.9) is encoded by all picornaviruses except the entero- and rhinoviruses. The L protein is the most variable of all picornavirus proteins, and it exists in five different versions. L proteins with proteinase activity are encoded only by FMDV, ERV1 and ERV2 (Skern, 1998). Cardioviruses and AiV encode three different versions of L polypeptides containing a putative Zn finger (Chen *et al.*, 1995; A.E.

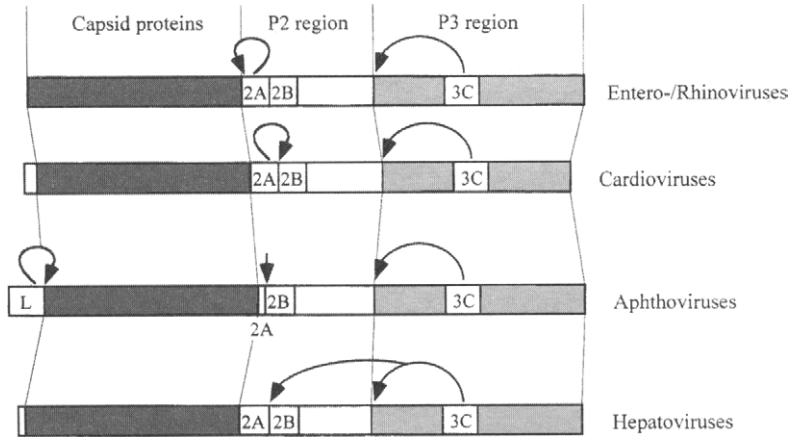


FIGURE 12.9 Primary cleavage in picornavirus polyproteins. Open boxes at the left end depict L proteins, of which only that of aphthoviruses is a proteinase. Of the 2A coding sequences, only $2A^{pro}$ of entero- and rhinoviruses is a proteinase. In cardio-and aphthoviruses, processing at the C-terminus of 2A is strictly a *cis* cleavage event. In hepatoviruses, even this cleavage is catalyzed by $3C^{pro}$. Modified from Ryan and Flint, 1997.

Gorbalenya, unpublished observations), while hepato-, parechoviruses and InFV appear to encode unique L proteins (Najarian *et al.*, 1985; Hyypia *et al.*, 1992; Isawa *et al.*, 1998).

Some evolutionary characteristics of the 2A polypeptides parallel those of the L proteins. Entero- and rhinoviruses encode 2A of the same protein family, known as 2A cysteine chymotrypsin-like proteinases (Bazan and Fletterick, 1988), whereas each of the three groups of hepato- and parechoviruses and InFV encodes a unique 2A with unknown function(s). The other picornaviruses, cardio- and aphthoviruses and AiV, encode a 2A protein having a characteristic C-terminal motif (or a derivative thereof) that has been implicated in the spontaneous separation of 2A and 2B proteins during polyprotein synthesis (reviewed in Ryan and Flint, 1997).

Besides L and 2A, the only other protein of the family not conserved at the primary structure is VP4 (Palmenberg, 1989; A.E. Gorbalenya, unpublished observation). It may therefore not come as a surprise that VP4 has been poorly resolved in X-ray analyses of picornavirions whose structure has been solved (Lentz *et al.*, 1997). It is interesting to note that this small protein, which occupies a position upstream of VP2, has moved its position to between VP2 and VP3 in insect InFV (Sasaki *et al.*, 1998).

Other picornavirus proteins are conserved, albeit to varying degrees. These polypeptides therefore may play similar role(s) in the life cycle of different picornaviruses. For example, 2B and 3A are of variable sizes but they contain hydrophobic regions thought to be involved in the anchoring of these proteins to membranes in RNA replication complexes. Only the hydrophobic patches of 2B and 3A polypeptides, however, have been conserved (A.E. Gorbalenya, unpublished results). In capsid proteins, the most pronounced conservation is evident in residues critically important for fold maintenance. Finally, in the key replicative enzymes $2C^{ATPase}$, $3C^{pro}$ and $3D^{pol}$, as well as in $3B^{VPg}$, the active site residues are amongst the most highly conserved (Gorbalenya and Koonin, 1993a).

The majority of proteins of picornaviruses, regardless of how well they have been conserved within their own family, have homologs among cellular and other viral proteins. First, the three capsid proteins, VP1, VP2 and VP3, have adopted different versions of an eight-

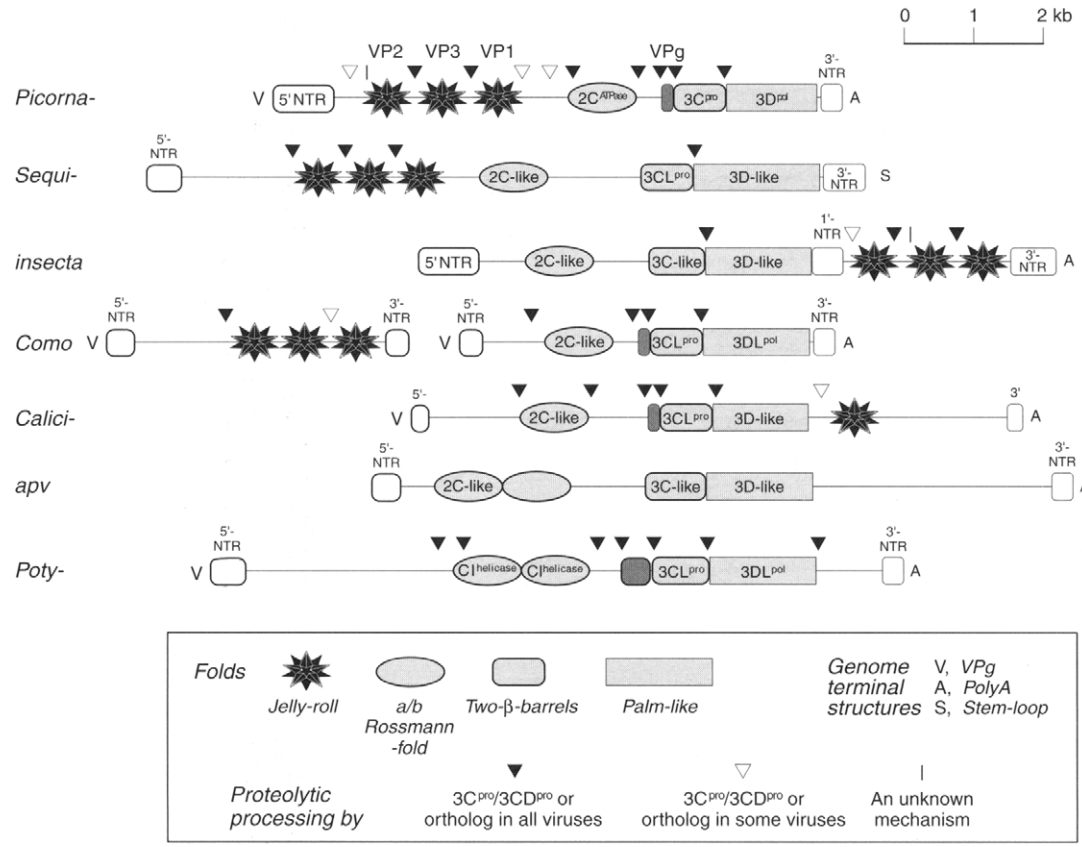


FIGURE 12.10 Comparison of the genome organizations of the main groups of the picorna-like supergroup. For each picorna-like family group, excluding APV, the conserved organization of an "averaged" genome typical for this group is shown and compared with that of picornaviruses. The genomes are aligned with respect to the position of the 3D (-like) locus (the RNA polymerase). "Averaging" was carried out with respect to genome size so that most conserved genome features could be shown. Note that bymoviruses, comprising a genus of Potyviridae, have a bipartite genome. It is believed that all picorna-like viruses contain VPg at the 5'-end, although a VPg has not yet been demonstrated for every group of viruses. In picorna-like viruses, proteins were designated so as to reflect their similarity to the prototype picornavirus enzymes, although other nomenclatures may be in use by other investigators. Apart from studies with picornaviruses, enzymatic activities have been ascribed to some proteins of como-, poty-, calici- and sequiviruses, but the complete processing map of polyproteins has been established only for como- and potyviruses. The conserved α/β Rossmann-fold and palm-like fold comprise only one of the domains of 2C and 3D, respectively, or their homologs. For further details, see legends to Figures 12.2 and 12.4, and the text.

stranded antiparallel beta-barrel fold, dubbed "jelly-roll" (Rossmann and Johnson, 1989). Amongst RNA and DNA viruses of different families, this fold is the most common to build icosahedral capsids (Rux and Burnett, 1998). It is also conserved in a number of cellular proteins (Rossmann, 1987; Orengo *et al.*, 1997). Second, the core domain of $3D^{pol}$, containing several highly conserved sequence motifs, is related to a number of polynucleotide polymerases including RNA-dependent RNA polymerases of RNA viruses, reverse transcriptases of viral and cellular origins, and DNA-dependent DNA polymerases (Hansen *et al.*, 1997). An analysis of the crystal structure of PV $3D^{pol}$ has also identified a (palm) subdomain adopting a RRM-like fold conserved among a number of functionally different proteins, including ribosomal proteins L7/L12 and S6 as well as the U1A splicing factor (Hansen *et al.*, 1997). Third, two picornavirus

proteinases, the ubiquitous $3C^{pro}$ and entero/rhinovirus-specific $2A^{pro}$, have adopted 12-stranded antiparallel two beta-stranded barrel folds, conserved in cellular serine proteases with chymotrypsin as the prototype (reviewed in Skern, 1998). These picornavirus proteinases have also relatives that are encoded by (+)RNA viruses belonging to dozens of different species (Gorbalenya and Snijder, 1996; Ryan and Flint, 1997). Unlike cellular proteases, the picornaviruses $3C^{pro}$ and $2A^{pro}$ employ cysteine as the principal catalytic nucleophile and, in some lineages, have another unique replacement – instead of the catalytic Asp they use a Glu (Gorbalenya *et al.*, 1989). The other small family of picornavirus proteinases, L^{pro} of aphthoviruses, ERV1 and ERV2, is related to cellular-papain-like proteases (Gorbalenya *et al.*, 1991; Skern, 1998) whose homologs have been identified in many animal and plant RNA viruses as well (Gorbalenya and Snijder, 1996). Finally, $2C^{ATPase}$, whose structure is yet to be solved, belongs to the so-called helicase superfamily III. This protein group includes polynucleotide-stimulated ATPases, some with helicase activity, which are encoded by (+)RNA and small DNA viruses as well as proteins of cellular origin (Gorbalenya and Koonin, 1990, 1993b). The $2C^{ATPase}$ has been predicted to be a three-domain protein. Two α/α domains flank an ATP-binding domain adopting a variation of the α/β "Rossmann" fold, which is widespread in the protein world (Teterina *et al.*, 1997).

With respect to details, our current understanding of the function of picornavirus proteins is rather fragmentary. Nevertheless, a preliminary functional profile of picornavirus proteins fits patterns of conservation evident at the structural level. The most conserved non-structural proteins provide the basic enzymatic activities needed for the synthesis and expression of viral RNAs inside the cell. The three conserved capsid proteins form the scaffold of virions shielding virus RNA from the detrimental environment outside the cell. All these activities appear to be virus-specific, although they may be modulated by cell-encoded components. In contrast, non-conserved viral proteins seem to sense and modify the host environment in addition to serving basic biosynthetic processes programmed by the viral genomes (for instance, see Piccone *et al.*, 1995; Zoll *et al.*, 1996; Svitkin *et al.*, 1998; Ventoso *et al.*, 1998). Virions also have host-dependent functions, such as the recognition of the cellular receptor, entry and, possibly, virion maturation. Different lines of evidence have shown that the least conserved regions of three capsid proteins, as well as VP4, may mediate these early activities of host cell entry (for recent work, see Hadfield *et al.*, 1997, and Lentz *et al.*, 1997).

NTRs

Much of what has been said about proteins applies also to the terminal NTRs of the picornavirus genomes. These regions are conserved within related genera but they may diverge when groups are compared (e.g. *Enterovirus/Rhinovirus* versus *Cardiovirus/Aphthovirus*) even although they play identical roles in viral proliferation (discussed in the section on Genetics). Variants of two very different conserved secondary structure organizations of IRES elements are shown in Figure 12.3, prototyped by those of PV and EMCV. It is unclear what type of 5'NTR was encoded by an ancestor of picornaviruses – that resembling one of the contemporary prototypes or rather a "consensus" one (Le and Maizel, 1998). In contrast, the 3'NTR region has diverged profoundly amongst picornaviruses and it is not conserved even within the otherwise closely related entero- and rhinoviruses (Poyry *et al.*, 1996).

Conservation of the Organization of Proteins Within the Polyprotein

The polyprotein of numerous +strand RNA viruses has evolved such that its organization reveals an additional level of conservation – the order of mature proteins in this large precursor (Figure 12.10; see also the order of protein domains in the prototype PV in Figure 12.4). This order is inflexible and none of the picornaviruses violates it, although entero- and rhinoviruses do not encode L proteins (see above). Despite a near absolute conservation of the order of protein domains, there is some plasticity (Figure 12.10). Upon computer

sequence analyses of the picorna-like viruses, it has become evident that the polyprotein can be divided into two parts, one comprising capsid proteins and the other the non-structural proteins. These parts are expressed rather independently. In a group of picorna-like insect viruses, *Rhopalosiphum padi* virus (RhPV), *Drosophila* C virus (DCV), *Plautia stali* intestine virus (PSIV) and cricket paralysis virus (CrPV), non-structural and capsid proteins are encoded by two ORFs, separated by a NTR (Koonin and Gorbalenya, 1992; Johnson and Christian, 1998; Moon *et al.*, 1998; Nakashima *et al.*, 1998; Sasaki *et al.*, 1998). In Comoviridae, a family of plant viruses, the capsid and non-structural proteins are encoded by two distinct RNAs, RNA2 and RNA1 (Goldbach, 1986). Remarkably, in the dendrogram shown in Figure 12.8, Comoviridae, Sequiviridae, a plant virus family having the same polyprotein organization as Picornaviridae (Turnbull-Ross *et al.*, 1993) and picorna-like insect viruses form a division immediately adjacent to the Picornaviridae.

It is important to stress that comparison of the sequences of many viruses of the picorna-like supergroup has revealed a profile of sequence conservation that parallels that observed for Picornaviridae. Thus, two groups of highly conserved clusters can be distinguished in polyproteins. The first group comprises the capsid proteins VP2–VP3–VP1, the second the non-structural proteins $2C^{ATPase}$–(VPg)–$3C^{pro}$–$3D^{pol}$ (or equivalents). The functions assigned to the individual members of the group of non-structural proteins remain provisional for the majority of known viruses. They have been inferred largely on the basis of sequence similarities with proteins of well-characterized viruses like poliovirus. Among the positionally highly conserved non-structural proteins, the genome-linked protein VPg has a special standing (highlighted by bracketing) since it is conserved functionally rather than structurally in the picorna-like viruses (Figure 12.10; Gorbalenya and Koonin, 1993a).

The combination of conserved non-structural proteins of picorna-like viruses has been termed "replicative module" (Goldbach, 1986). Such module of related proteins has been recognized also as "capsid modules" built of three "jelly-roll" proteins. Animal Caliciviridae, plant Potyviridae and insect *Acyrthosiphon pisum* virus, all of which are distantly related to Picornaviridae, encode a distinct variety of the replicative module that is associated with one of three unique sets of capsid protein(s) encoded in the 3′-region of viral genomes (Domier *et al.*, 1986; Meyers *et al.*, 1991; van der Wilk *et al.*, 1997; Figure 12.10).

Conservation of Proteins is Paralleled by the Mechanism of Processing

The conservation of protein order in the picornavirus polyprotein and the patterns of expression (proteolytic processing) have been conserved. Pairs of neighboring proteins are separated at scissile bonds cleaved by a virus proteinase or, in case of the VP4/VP2 junction, by an unknown mechanism. It could be hypothesized that the position of protein domains could be changed as long as the corresponding proteins were released more or less independently from the precursor. This, however, is not the case, as the pathway of proteolytic processing in picornavirus polyproteins is not random. Furthermore, at least some intermediate precursors, e.g. 2BC, 3AB and $3CD^{pro}$, have essential functions that differ from those of the end-product of processing (see the section on Genetics). These considerations provide a biological reasoning for the observed conservation of the protein order in polyproteins.

We have already pointed out that the order of two conserved units within the polyprotein, the capsid precursor and replicative modules, is flexible. The two least conserved proteins, L and 2A, flank the capsid precursor at the N- and C-termini, respectively, and bring additional plasticity to the organization of the polyprotein. This is reflected also in terms of expression, i.e. the mechanism of proteolytic processing. Processing of the capsid precursors as well as of the replicative module at junctions separating conserved proteins involves exclusively the conserved $3C/3CD^{pro}$ proteinases, a mechanism functioning not only in picornaviruses but also in other picorna-like viruses (Figure 12.10; Ryan and Flint, 1997). In contrast, the three cleavages separating the poorly conserved L and 2A pro-

teins from the neighboring polypeptide chains (L/VP4, VP1/2A and 2A/2B) are processed by a range of mechanisms in a genus-specific manner. Furthermore, whereas picornaviruses use two general pathways of cleavages – $3C^{pro}/3CD^{pro}$ versus distinct mechanisms involving L and 2A – this genetic repertoire may be further diversified in some picorna-like viruses. For instance, in Comoviridae and insect viruses, capsid precursor and non-structural proteins are encoded by distinct ORFs (Figure 12.10), which eliminates the need for cleavages separating these polypeptide chains.

$3C^{pro}/3CD^{pro}$ have emerged as the major enzymatic factors in the regulation of protein expression in all picorna- and related viruses. Interestingly, the primary structure of sites recognized by these proteases is virus-specific rather than position-specific. Among picornaviruses, entero- and rhinoviruses employ sets of structurally uniform sites while viruses of the other genera use more diversified sets. Poliovirus and HAV exemplify the most extreme diversity. In poliovirus, all eight cleavage sites have the same ("canonical") Q/G structure (Figure 12.4), whereas in HAV, six variations of this structure were described in different sites (Palmenberg, 1990).

Poliovirus proteins produced from its replicative module seem to have been exceptionally strongly constrained not only with respect to the type of the terminal amino acids but also with respect to size. Mature poliovirus proteins (except $3C^{pro}$), as well as processing intermediates, have sizes that can be divided by 11 without remainder or with only a small remainder (Gorbalenya *et al.*, 1986). This feature separates poliovirus proteins from the overwhelming majority of cellular and viral proteins. The latter are heterogeneous both in size and sequence, particularly at their termini, because of a relative abundance of mutations, including insertions and deletions. Structural regularities documented for poliovirus can be visualized in a form of weak primary structure periodicities with the common denominator of 11 comprising the major portion of the replicative module. On the basis of these observations, it has been proposed that the replicative module of picornaviruses has originated from a primitive self-replicating RNA molecule through consecutive multistep duplications (Gorbalenya, 1995; Gorbalenya *et al.*, 1986).

Does the History of a Polyprotein Determine How it is Likely to Evolve in the Future?

We have briefly described different levels of evolutionary conservation in picornaviruses by using results of comparative sequence analyses. The conservation of different properties is the result of a long evolutionary process, accompanied by numerous radiations. Does the history of the polyprotein determine how picornaviruses may evolve in the future? We are unaware that this question has ever been directly addressed in experimental studies, although many results obtained by using genetic engineering seem to be quite relevant. These data can technically be separated into two sets – those obtained in studies using site-directed mutagenesis and those aimed at constructing chimeras.

In numerous studies of the first type, it has been observed that different regions of the picornavirus genome express a differential tolerance to replacements (Wimmer *et al.*, 1993). It can be predicted that a profile of the "accepted" mutability, drawn over the entire genome, would fit the conservation profiles described above. Such a result would support the hypothesis that the past of picornaviruses influences their future in terms of evolution. However, mutagenesis saturating the genome has never been systematically carried out. Therefore, the available "mutagenesis profile" can only be used as a rough approximation of the yet-to-be-defined "accepted" mutability profile in relation to the conservation of modules. The "resolution" of the mutagenesis studies that remained unresolved is potentially relevant to an understanding of the evolution of contemporary picornaviruses, regardless of whether this relates to recent evolutionary events or to the complete historical past.

The second group of data involving genome engineering complements the mutagenesis studies and helps to address the question posed above. In wt genomes of entero- and rhinoviruses, the ORF of the capsid precursor is preceded

by the cognate 5'NTR (Figure 12.2). As was observed in studies of poliovirus expression vectors (Figure 12.5), genetically stable variants of poliovirus have been selected (Mueller and Wimmer, 1997) in which an additional leader peptide is encoded that is fused to the N-terminus of the polyprotein, just downstream from the 5'NTR. This organization may look unique on first sight, but in fact it resembles that of all other picornaviruses distantly related to entero- and rhinoviruses. These terminal appendices in the poliovirus variants resemble the L proteins and, hence, these poliovirus chimeras have a "cardio-like" organization (Figure 12.6E). We can speculate that PV has "accepted" an artificial L peptide because a similar event has already happened in the past history of its ancestors.

In a different set of experiments, several poliovirus chimeras have been generated in which the heterologous EMCV IRES was placed into the sequences specifying scissile bonds of the polyprotein, thereby dividing the polyprotein into two parts (Figure 12.6B). This insertion radically modified the conserved protein expression mechanism of picornaviruses, since it functionally replaced a proteolytic cleavage event by an event of internal initiation of translation directed by the alien IRES. In all, poliovirus genomes were constructed in which the EMCV IRES was placed between the Y*G cleavage site of $2A^{pro}$ (Figure 12.6B) or all possible Q*G cleavage sites involving the $3C^{pro}/3CD^{pro}$ proteinase (Molla *et al.*, 1992, 1993b; Paul *et al.*, 1998a). Only two poliovirus-EMCV dicistronic chimeras, specifically those carrying EMCV IRES between VP1 and 2A and between 2A and 2B, have given rise to viable and stable virus progeny (Molla *et al.*, 1992, 1993b; Paul *et al.*, 1998a). Although the genome organizations of these chimeras do not match anything found in nature, immediate parallels come to mind with genomes of picorna-like viruses in which capsid and replicative modules are encoded by different ORFs (for example Comoviridae, see above and the section on Genetics).

These considerations imply that the conserved and non-conserved features in organization and structure of genomes of picornaviruses and even picorna-like viruses are indicative of an evolutionary plasticity and of possible future changes of a picornavirus. Perhaps an "evolutionary space" of a picornavirus can be approximated from the past. Mechanistically, this can be seen as if the past evolution of the entire family has been "imprinted" in the organization of the genome of each of the contemporary picornaviruses.

Mechanism of Evolution

Phylogenetic trees that have been built for different picornaviral proteins (most often VP3, $2C^{ATPase}$ and $3D^{pol}$) by employing parsimonious and maximum-likelihood methods proved roughly topologically equivalent even though different regions of the polyproteins have definitely evolved at different rates (Stanway, 1990; Rodrigo and Dopazo, 1995; Hyypia *et al.*, 1997). These observations strongly favor a concerted evolution of (the majority of) the picornavirus proteins. This conclusion is not compromised by some incongruity in the tree topology of closely related viruses, e.g. the C cluster of the enteroviruses (Poyry *et al.*, 1996), or very distantly related groups, e.g. hepato- and parechoviruses. It is likely that some trees generated for different regions look different, as a result of technical limitations related to phylogenetic and biopolymer sequence analyses as well as a biased representation of some groups. Also, possible recombination events between closely related viruses may have complicated phylogenetic analyses. We shall analyse sequence alignments of picornavirus proteins and polynucleotides aimed at deducing the mechanisms functioning in picornavirus evolution.

Uniform sizes of each of the VP3, $2C^{ATPase}$, $3C^{pro}$, or $3D^{pol}$ polypeptides have been maintained in all picornaviruses. The diversity of the proteins is therefore most probably the result of numerous in-frame mutations. For the other proteins, some additional mechanism of diversification may have been functioning in the course of evolution. Among the viruses encoding 2A proteins sharing the NPGP motif, the two viruses FMDV and ERV1 encode a 2A consisting of only 16 amino acids, whereas the cardioviruses ERV2

and AiV encode a 2A ranging between 67 and 150 residues. It can be speculated that deletion events in the 2A coding region of FMDV and ERV1 are the result of "jumping" of $3D^{pol}$, perhaps by loop-out deletion or by illegitimate recombination (Figure 12.7). On the other hand, the three adjacent coding regions for VPg uniquely found in all strains of FMDV suggest duplication events. In other viruses, e.g. ERV2 or TMEV, genetic events such as local duplication and deletions may have occurred, leading to considerable size heterogeneity of the corresponding VPgs and adjacent sequences (Wutz *et al.*, 1996; A.E. Gorbalenya, unpublished results). Duplications have also been discovered in the 5'NTR of enteroviruses (Pilipenko *et al.*, 1989a). Picornavirus genomic redundancy, known as duplications, may have been generated by intragenomic recombination. After duplications, however, the sequences must have undergone some variation so as to avoid elimination by homologous recombination. Indeed, the nucleotide sequences (and to a small extent also the amino acid sequences) of the three VPgs of FMDV differ such that homologous recombination at this locus is unlikely (Cao and Wimmer, 1996).

In spite of lack of evidence, duplications by intragenomic recombination might have been involved in the production of large differences in size found in capsid proteins VP1 and VP2, or in non-structural 3A and 2B proteins of some picornaviruses. The capsid proteins contain long extra loops while the 2B protein of ERV1 has an enormous size relative to the 2B proteins of all other picornaviruses (283 versus 100–150 amino acids; Wutz *et al.*, 1996). On the other hand, Charini *et al.* (1994) have reported that, surprisingly, a viable poliovirus isolate they selected from a swarm of revertants had captured a short segment of cellular ribosomal RNA. Thus, capture of entirely foreign RNA sequences, although very rare, cannot be excluded from the mechanisms of diversification.

At least two different mechanisms could have given rise to the contemporary diversity of 2A and L protein families. The diversity includes, amongst others, chymotrypsin-like proteinase and NPGP motif-containing polypeptides for 2A and papain-like proteinase and Zn-finger proteins for L. Phylogenetic analyses suggest that "new" unrelated 2A and L proteins have emerged in the course of evolution of picornaviruses on several occasions, following the split of the major groups of the picornavirus tree. It is logical to assume that, following each split, one of the two descendants has arisen from an ancestral viral source, the other from an "independent" source. As to the latter, the coding sequence of either 2A or L could have recombined with a gene of either another virus or of the cell, leading to the replacement of the ancestral coding sequence. For example, this replacement mechanism could have resulted in the capture of cellular chymotrypsin-like ($2A^{pro}$) or papain-like (L^{pro}) activities. This hypothesis is, of course, purely speculative since no potential partners in recombination have been identified as yet.

Alternatively, the diversity of the 2A and L families may be the result of frame-shifting events. For example, enteroviruses have a "spacer sequence" between the IRES and the ORF of the polyprotein. This spacer commences with an unused ("silent") AUG at the 3' border of the IRES. In poliovirus, it is 154 nt long and represents a small out-of-frame ORF terminating inside the polyprotein ORF. If the silent AUG at the 5' end of the spacer were to trigger initiation of translation and, in addition, a frame-shift mutation connected the small ORF with the main ORF, a small "leader" peptide would be created fused to the polyprotein. All that is then necessary is a $3C^{pro}$ cleavage site to sever the "leader" from VP4 – and a genetic arrangement would have been created resembling that of cardio- and aphthoviruses (Jang *et al.*, 1990). Indeed, the silent AUG of poliovirus can be turned on by changing its Kozak context (Pestova *et al.*, 1994), and stable poliovirus variants can be isolated that carry short foreign leaders (see above; Mueller and Wimmer, 1998). Thus, the conversion of an enterovirus to a cardiovirus genotype with respect to an L protein can be envisioned by relatively simple genetic changes. Similarly, it should be possible to convert a cardiovirus genotype in this region into an enterovirus genotype by silencing its L ORF.

It is relevant that, as already mentioned, a

strain of Theiler's virus has been identified that, just like the normal cardioviruses, synthesizes a polyprotein-fused L protein and, in addition, a polypeptide L* in a separate ORF. L* synthesis is initiated at its own AUG initiation codon (Takata *et al.*, 1998). Apparently, the synthesis of L* may present the virus with an advantage in the natural host, a fact that may have contributed to its selection.

By comparison with the L protein region in TMEV, two 2A proteins may have existed in the ancestral picornavirus genome, one active in the polyprotein, the other "silent". In the course of subsequent speciation, each of these 2A variants may have been used in separate picornavirus lineages. The activation of the "silent" 2A may have led to a concomitant inactivation of the other 2A.

It should be mentioned that the presence of multiple alternative ORFs in ancestral picornavirus genomes may have been the rule rather than the exception, particularly if the polyprotein evolved by amplification of 11-mers (Gorbalenya, 1995; see also above). Ohno (1984) has demonstrated that periodicity-organized polynucleotides with a period that cannot be divided by 3 (11-long periodicity included) have an identical coding capacity in each frame. In other words, if one ORF is open the two other frames are open also. In the course of evolution, two out of three reading frames may have deteriorated or may have given rise to genetic variation as speculated for the generation of the diversity in 2A and L proteins.

Driving Force of Evolution

Numerous studies attest to a remarkable stability of the picornavirus genotype if grown under identical conditions (Wimmer *et al.*, 1993). On the other hand, if exposed to altered conditions in the environment, a shift to new variants can be readily observed. Just like other biological systems, it can be assumed that picornavirus speciation has been driven by a changing environment.

Circumstances upon which a picornavirus may encounter a "new" environment include: (1) horizontal or vertical transfer to a new (different) host; (2) entering a natural host through a non-natural gate; (3) infecting immunized (natural) hosts previously exposed to the same virus. Although there is no proof, it is intuitively highly likely that all three scenarios have played a role in picornavirus speciation. In the following, a speculative reconstruction of forces will be presented that may have contributed to the evolution of picornaviruses.

Picornaviruses belonging to a genus or a cluster may have almost identical phenotypes with respect to growth properties and even in regard to pathogenic potential. A most important characteristic, however, does further divide a group of very closely related picornaviruses (e.g. polioviruses): the susceptibility to activation by different neutralizing antibodies and, hence, the separation into serotypes (see the Introduction). It is logical to assume that the (negative) pressure of the immune system may be largely accountable for serotype diversification of picornaviruses. That is, the immune response can lead to the selection of viral variants resistant to the neutralizing immune response produced by the surviving host. Such variants would form a pool from which a new serotype could be further selected. In fact, such mechanism of virus evolution seems to dominate in the case of influenza A virus or immunodeficiency virus (HIV). However, the sheer unlimited degree of serotype diversification observed in influenza viruses or HIV is an exception rather than the rule amongst viruses. Indeed, not all picornaviruses seem to be able to easily produce new serotypes. For example, the genus *Hepatovirus* encompasses only one serotype while others are restricted to a few serotypes (e.g. poliovirus).

New viral variants that have escaped the immune surveillance must, of course, interact with multiple host components at virtually every stage of their reproduction in order to survive. This includes virus entry into the host cell, translation and processing, genome replication, encapsidation and maturation, spread in the host. Each of these steps are checkpoints and every new viral variant must be fit to pass these barriers.

The earliest events in the infectious cycle –

receptor interaction, uptake, uncoating – and the mechanisms of neutralization are amongst the least understood in the molecular biology of picornaviruses. The crystal structures of some member viruses of four picornavirus genera have been solved; examples are: *Enterovirus*, poliovirus 1 and 3 (Hogle *et al.*, 1985); *Rhinovirus*, human rhinoviruses 2, 3, 14, 16 (Rossman *et al.*, 1985); *Cardiovirus*, mengovirus (Luo *et al.*, 1987), Theiler's virus (Luo *et al.*, 1996); *Aphthovirus*, FMDV (Acharya *et al.*, 1989). (For a complete list, see Lentz *et al.*, 1999). However, the precise localization and structures of different neutralization antigenic sites (the structures interacting with neutralizing antibodies) is known only for polioviruses, rhinoviruses and aphthoviruses. For aphthoviruses and for polioviruses, the available evidence suggests that the same structures that determine in part the serotype identity are also involved in receptor recognition (Domingo *et al.*, 1993; Mason *et al.*, 1994; Harber *et al.*, 1995). Thus, immune-escaping viral mutants are likely to be enriched in those variants that have maintained the ability to efficiently interact with the cognate receptor and follow the pathway of uptake and uncoating. This is, of course, only speculative but, if correct, it would explain in part serotype restriction (Harber *et al.*, 1995).

In this respect it may be informative to compare receptor specificities with serotype diversities of human enteroviruses, on the one hand and rhinoviruses on the other. These two genera encompass viruses that have diverged from an immediate common ancestor and radiated during the same time period (Figure 12.1). In the course of evolution, different serotypes in roughly the same numbers have been generated in these two picornavirus branches: there are about 66 enterovirus and over 100 rhinovirus serotypes. This implies that viruses of the two genera are similarly prone to accumulation of changes in those capsid structures giving rise to new serotypes. But what about receptor specificity of these viruses?

At the time of writing, two receptors have been assigned for human rhinoviruses (which is probably all that will be found) and six receptors for human enteroviruses (at least four more are awaiting identification; Table 12.1). Thus, in contrast to the quite similar extent of serotype diversification in both genera, adaptation to new receptors is significantly more restricted in rhinoviruses than in the closely related enteroviruses. Importantly, there is an overlap between the two receptor patterns and, taken together, the ICAM-1 receptor specificity appears to be dominant among entero- and rhinoviruses. This can be interpreted to mean that the immediate common ancestor of both entero- and rhinoviruses may have used a receptor related to ICAM-1. Regardless of whether this is true or not, the subsequent evolution of ICAM-1-recognizing picornaviruses has proceeded differently, as seen in the disparity of the current use of this cellular receptor (>90 for rhinoviruses versus 11 for C-cluster coxsackieviruses). Given that the serotype diversification has proceeded at a similar pace in entero- and rhinoviruses, enteroviruses may have had greater opportunities – or a greater need – to adapt to new receptors in order to initiate an infection. This may be related to the function(s) of receptors in viral docking and uncoating: whereas rhinoviruses may need the receptor only for docking and uptake (because of their inherent sensitivity to the acidic pH inside late endosomes), the exceedingly stable enteroviruses do need the receptor (and possibly a co-receptor) for docking, uptake *and* uncoating. With poliovirus, a particle stable to detergents, proteases and low pH (pH 2), this is exemplified in the formation of A-particles, a labile product of receptor/virion interaction and an intermediate in uncoating (Wimmer *et al.*, 1994). A-particle formation appears to involve also sequences of neutralization antigenic sites (Harber *et al.*, 1995). Thus, the intercourse between receptor and enterovirion may be much more complex than that between receptor and rhinovirion. Consequently, a change in the serotype may have forced enteroviruses to search for new receptors to retain the uncoating capacity of the cellular receptor.

The unusually large serotype diversity of the major receptor group human rhinoviruses may then be explained as follows. It seems possible that the initiation of an infectious cycle of HRV *does not critically require* an interaction between structures of the neutralization antigenic sites of

the virion and ICAM-1. That is, the N-terminal domain of ICAM-1, by inserting itself into the virion's canyon, can effect docking, uptake and uncoating of the particle. Progression through any of these events is *not critically dependent* on sequences of the neutralization antigenic sites. If correct, it follows that variation of the antigenic sites does not restrict viral proliferation and serotype evolution. Consistently, in other picornaviruses the neutralization antigenic sites and the determinants recognizing the receptor would be much more overlapping and mutually dependent.

It is likely that an initial immune-driven selection might also finally result in a virus variant with changed or extended tissue tropism. This might have happened with CAV24v, a C-cluster human enterovirus. Immune pressure might have initiated the selection of the CAV24v mutant derived from a CAV24 swarm. As mentioned before, CAV24v is a very recent variant of CAV24 and, unlike its parent and the other members of the C-cluster, it can cause acute hemorrhagic conjunctivitis. Apart from the possibility that CAV24v emerged through immune selection, it could also have been selected from a swarm when the parental CAV24 was accidentally inoculated into the eye.

Another type of selection might have been responsible for the emergence of swine vesicular disease virus (SVDV). Phylogenetic analysis of genomes of human enteroviruses identified SVDV as being interleaved with human viruses comprising the CBV-like cluster (Poyry *et al.*, 1996). This observation is strongly indicative of selection of SVDV from a mutant of a human coxsackie-B virus entering the new host through frequent contacts of these domestic animals with (infected) humans.

Picornaviridae are a Prosperous Virus Family

We have discussed different aspects relevant to picornavirus evolution, but we did not address one crucial question: Are picornaviruses a successful family? We believe that the answer is: yes. In discussing this issue, we will also formulate considerations regarding the worldwide eradication of poliovirus.

One of the strongest criteria of biological prosperity is the diversity of a taxonomic group. Despite some bias inherent in current analyses, phylogenetic studies of picornaviral genomes suggest that Picornaviridae have radiated densely over the course of evolution, at both early and late stages (Figure 12.1). Furthermore, picornaviruses are members of a superfamily with numerous distant relatives (Figure 12.8) that infect a wide range of organisms, including both plants and animals. Some of these viruses, like Sequiviridae, employ a genetic plan that is basically a variation of the genetic plan used by picornaviruses (Figure 12.10).

Prosperity of the host is another prerequisite for a virus to be successful. By this criterion also, picornaviruses are successful, since the majority of them, representing different branches of the picornavirus tree, infect humans. Humans are arguably one of the most successful species in the biological world. In truth, picornaviruses are relatively harmless even though few humans, if any, can escape picornavirus infections. This too can be viewed as evidence that these viruses have adapted well to their host, as they have not significantly undermined human affairs. This is true even for poliovirus, an agent that is commonly regarded as a deadly virus following epidemics of poliomyelitis. However, prior to this century, poliovirus did not cause epidemics, even though it infected humans at rates approaching 100%. Epidemics emerged because human behavior changed through the invention of modern hygiene. Hygiene broke the chain of natural immunization through infant infection combined with infant protection by maternal antibodies. Even in this century's devastating epidemics, however only 1–2% of infected individuals developed poliomyelitis.

A Special Case of Picornavirus Evolution – the Human Battle with Poliovirus

The poliovirus–human relationship alluded to above deserves to be discussed in more detail. Humans, who occupy a unique niche in the bio-

logical world (because they care about *each* human life), did not accept their potential defeat as poliomyelitis became an epidemic. Unprecedented efforts combining medical research with modern technologies led to the development of two highly effective poliovirus vaccines, the inactivated poliovirus vaccine by Jonas Salk and the live attenuated vaccine by Albert Sabin (Wimmer *et al.*, 1993). Through education of the populace and advanced health-care measures, mass vaccinations have gradually eliminated wild-type poliovirus, first in the developed countries and later in most of the world. Incredibly, the few cases of poliomyelitis in the western hemisphere now result from vaccination with the live Sabin strains. Overall, polio vaccination is a success story of greatest consequence. Indeed, through worldwide efforts led by the World Health Organization, it is likely that wild-type polioviruses will be eradicated globally by the turn of the century (WHO, 1985).

Do these considerations allow us to safely conclude that, after its global eradication, poliovirus will have no chance to re-emerge through enterovirus evolution? For discussion of this issue, we will first summarize hypotheses about the possible origin of polioviruses and their closest relatives, the C-cluster coxsackieviruses.

The three serotypes of poliovirus belong to the C-cluster of enteroviruses (Table 12.1; Figure 12.1B). The most comprehensive analysis of the C-cluster has been performed with sequences of the VP4-VP2 capsids and with sequences of the $3D^{pol}$ RNA polymerase (Pulli *et al.*, 1995). Results of these analyses are consistent with data obtained in a study of the other regions of the viral genome using a less representative set of sequences (Poyry *et al.*, 1996). Therefore, these relationships shown in Figure 12.1B can be assumed to be quite reliable. A phylogenetic analysis of the capsid VP4-VP2 region of C-cluster viruses indicated that the tree has split at least twice, perhaps before the emergence of an immediate ancestor of polioviruses. The first split led to the separation of a branch encompassing CAV1, CAV21 and CAV24 from the main C-cluster trunk, and the second, more recent one resulted in the separation of the ancestor for PV and the ancestor for CAV11, CAV13, CAV17, CAV18, CAV20 and CAV20b. The results obtained with sequences of $3D^{pol}$ favor an even more complex evolutionary history of poliovirus, including more than five intermediate steps (Pulli *et al.*, 1995). Consistent with the results of the analysis of the capsid region, CAV21 and CAV24 were among those viruses that diverged from the main trunk relatively early in evolution while the three poliovirus serotypes clustered together with CAV11, CAV13, CAV17 and CAV18. Remarkably, in the tree based on $3D^{pol}$ sequences the latter four coxsackieviruses (as well as several other coxsackieviruses) are interleaved with, rather than separated from, the three poliovirus serotypes (Figure 12.1). This stands in contrast to the tree of the capsid region. Assuming the most parsimonious scenario of evolution, the combination of these results strongly implies that coxsackieviruses that recognize the ICAM-1 receptor formed a pool from which polioviruses, interacting with the CD155 receptor, have evolved. This conclusion is compatible with a hypothesis of the immune-driven evolution of entero- and rhinoviruses presented above. Furthermore, the analyses do not indicate that three polioviruses comprise a monophyletic subgroup within the C-cluster enteroviruses and, hence, have emerged from an ancestral virus by speciation, as one could expect from a distinct phenotypic profile of these viruses.

We have previously hypothesized that the coxsackiviruses may have derived from polioviruses by switching receptors from CD155 to ICAM-1 (Harber *et al.*, 1995). This possibility may be supported from the fact that the IRESes of C-cluster coxsackieviruses are highly 'neuropathogenic". On the other hand, the assessment presented above favors an evolutionary relationship in the opposite direction. Regardless of the direction in which these viruses emerged, the receptor switch has profound consequences for their pathogenic properties: whereas the C-cluster coxsackieviruses cause respiratory disease, poliovirus can cause deadly neurological disease. These considerations may also have important practical implications. For the sake of the argument, we will assume that the poliovirus eradication campaign has been

successfully completed and no more poliovirus particles, including those of the vaccine strains, are circulating worldwide. Furthermore, we will assume that all vaccination against poliovirus (including vaccination by inactivated vaccines) has been terminated, a scenario that has been envisioned to be a reality by the end of the next decade. These measures would mark the beginning of a new era in the history of mankind: there will be no human exposure to polioviruses and their antigens.

Generations of humans will be born that have not been infected with wild-type or vaccine polioviruses and, gradually, they will replace the older generations who carry anti-poliovirus antibodies. At that point, the world will not only be free of poliovirus, but its human population will also no longer carry anti-poliovirus antibodies. Thus, a new environment will emerge for human viruses, in particular for C-cluster coxsackieviruses, which are the closest genetic relatives of poliovirus. These C-CAVs are expected to circulate widely in the human population, exploring a new evolutionary space.

Within the human space populated by the C-CAVs, there will then exist also a free space that was previously occupied by the three (extinct) poliovirus serotypes. It is possible that mutations in antigenic sites of the C-CAVs may (re)generate affinity to CD155. Prior to eradication, C-CAVs carrying such mutations could conceivably be eliminated by anti-poliovirus antibodies (Harber *et al.*, 1995) but in the poliovirus-free world they may remain unchecked. This means that, once emerged, these new viruses carrying poliovirus-like neutralization antigenic sites with CD155 receptor affinity are less likely to be eliminated from the human population after eradication than before. Since all enteroviruses, the variants included, lead to enteric infections, these variants may find a passage to the CNS and, mediated by their affinity to CD155, may cause neurological disease. It is relevant to point out (Gromeier and Wimmer, unpublished results) that poliovirus chimeric viruses in which the poliovirus IRES has been replaced with that of C-cluster IRES elements have been found to be highly neurovirulent in *CD155* tg mice (see the section on Pathogenesis). Thus, there is reason to fear that in a poliovirus-free world new coxsackievirus-related, poliovirus-like pathogens that can cause poliomyelitis may emerge in the course of natural viral evolution. The time frame, however, cannot be predicted. It could be one generation or 1000 years.

The human condition favors an increasing rate of diversity of human viruses simply because of the increasing size of the human population (estimated to stabilize at 8–12 billion during the next century). This population explosion will lead to a dramatic increase of human contacts, either in cities, particularly megacities (harboring more than 50% of the world's population), through travel or otherwise. Clearly, this presents a fertile ground for proliferation and diversification of the highly infectious human picornaviruses. Thus, the possibilities of genetic variation of picornaviruses leading to new or renewed human pathogens, such as CAV24v, must always be kept in mind.

At this point, however, our considerations of the possible re-emergence of poliovirus-like pathogens in the post-eradication era pale in the face of mankind's heroic attempt to eradicate an RNA virus for the first time. After all, poliovirus has caused, and is still causing, terrible human suffering.

CONCLUDING REMARKS

Picornaviruses have been discovered because they cause diseases in animals and humans. Fortunately, most human picornavirus infections are self-limiting. Yet the enormously high rate of picornavirus infections in the human population can lead to a significant incidence of disease complications that may be permanently debilitating or even fatal. The case of poliovirus has taught us that a change of human behavior, which, paradoxically, was the invention of modern hygiene, has greatly aggravated the impact of infection by this specific agent. Clearly, this scenario could repeat itself with other human picornavirus species. The terror of this century's poliomyelitis epidemics has driven picornavirus research forward more than any other factor. This work has led to a wealth of discov-

eries in biology in general, and to an abundance of data describing the unique biology of picornaviruses and their evolution in particular.

Picornaviruses employ one of the simplest imaginable genetic systems: they consist of single-stranded RNA that encodes only a single multidomain polypeptide, the polyprotein. The RNA is packaged into a small, rigid, naked, icosahedral virion whose proteins are unmodified except for a myristate at the N-termini of VP4. The RNA itself does not contain modified bases. Thus, picornaviruses travel with light baggage. On first sight, the replication of picornaviruses is exceedingly simple. After having chosen a receptor from a large menu of cell-surface proteins, the virion enters the cytoplasm and immediately translates its genome, controlled by its IRES element. Thereafter, the polyprotein is processed by its own proteinases. RNA replication occurs by a unique, protein-primed mechanism catalyzed by the RNA-dependent RNA polymerase. Assembly appears to be linked to RNA synthesis, and release of the progeny virions follows a passive mechanism. There is no need for a cellular nucleus. Indeed, the entire replication cycle can occur in a cell-free system free of nuclei, mitochondria and perhaps of all other cellular organelles.

Yet as of now we understand only a small fraction of these viruses' life cycle, and we are awed by the sophistication with which the viruses express their genetic information. The IRES, arguably one of the most complex *cis*-acting signals known in RNA systems, has freed picornaviruses from the cellular constraint of cap-dependent translation. This, in turn, allows the primer-dependent RNA polymerase, an enzyme with properties generally ascribed only to DNA polymerases or reverse transcriptase, to prime with VPg and leave the RNA uncapped. Polyprotein processing proceeds in a controlled manner yielding cleavage intermediates and end-products that can be used for different functions. Thus, the menu of gene products is expanded through the temporal regulation of proteolytic processing. Details of all of these steps in replication are still obscure (Agol *et al.*, 1999).

The key to ultimately understanding picornaviruses may be to rationalize the huge amount of information about these viruses from the perspective of evolution. It is possible that the replicative apparatus of picornaviruses originated in the precellular world and was subsequently refined in the course of thousands of generations in a slowly evolving environment. Picornaviruses cultivated the art of adaptation, which has allowed them to "jump" into new niches offered in the biological world. Also, by having chosen humans as an additional host, they were offered an abundance of opportunities to proliferate in different tissues, which has contributed to their diversification. These opportunities have further increased through the human population explosion and through changes in human behavior.

We suggest that, in addition to drastic and expansive measures such as global eradication, strategies should be developed that aim at predicting the possible evolution of new picornavirus pathogens and preparing for their control. The results reviewed in this article may contribute to achieving this tantalizing and desirable goal.

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