Chapter 2
Replication Cycle of Astroviruses

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Abstract Astrovirus infections cause gastroenteritis in mammals and have been identified as causative agents of diverse pathologies in birds such as hepatitis in ducks and poult enteritis mortality syndrome (PEMS), which causes enteritis and thymic and bursal atrophy in turkeys. Human astroviruses are recognized as the second leading cause of childhood viral gastroenteritis worldwide. Eight traditional astrovirus serotypes have been identified in humans, but recently novel astrovirus strains isolated from humans have been associated with diseases other than gastroenteritis. Herein we summarize our current knowledge of the astrovirus life cycle. Though there are gaps in our understanding of astrovirus replication, similarities can be drawn from *Picornaviridae* and *Caliciviridae* virus families. There are, however, unique characteristics of the astrovirus life cycle, including intracellular proteolytic processing of viral particles by cellular caspases, which has been shown to be required for the maturation and exit of viral progeny.

Introduction

The family *Astroviridae* includes human and animal viruses that share a characteristic icosahedral morphology; they are nonenveloped and their genome is composed of positive-sense, single-stranded RNA, whose organization distinguishes them

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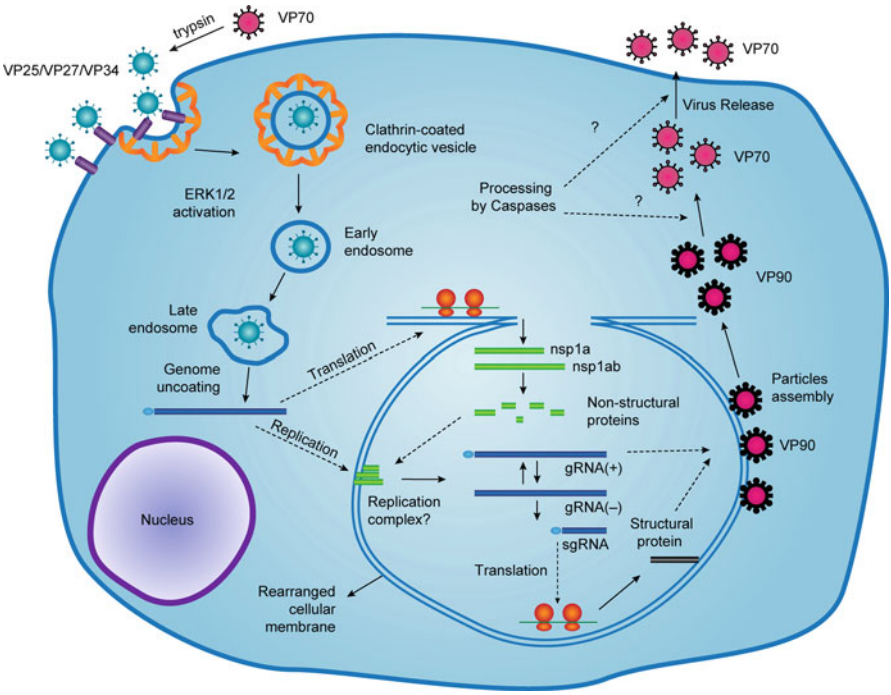


Fig. 2.1 Replication cycle of astroviruses. See details in the text

from other virus families with similar properties. Efficient propagation of astrovirus strains from both human and animal origin in cultured cells has permitted advances in the characterization of their life cycle (Fig. 2.1). After astrovirus cell entry, the genomic RNA is uncoated and is then translated into a polyprotein precursor that is subsequently cleaved into the proteins required for replication of the virus genome and assembly of progeny viruses. These processes most likely take place in association with cellular membranes, and completion of the virus life cycle requires the action of caspases for the virus particles to exit the cell.

Genome Structure and Organization

Astroviruses have a positive-sense, single-stranded RNA genome that varies in length from 6.17 kb for human astrovirus (HAstV) MLB-1 to 7.72 kb for duck astrovirus-2, excluding the poly(A) tail at the 3′-end [54]. The viral genome contains 5′ and 3′ untranslated regions (UTR) and three open reading frames (ORFs) (Fig. 2.2). The two ORFs located toward the 5′-end of the genome, designated ORF1a and ORF1b, encode nonstructural proteins that are presumed to be involved

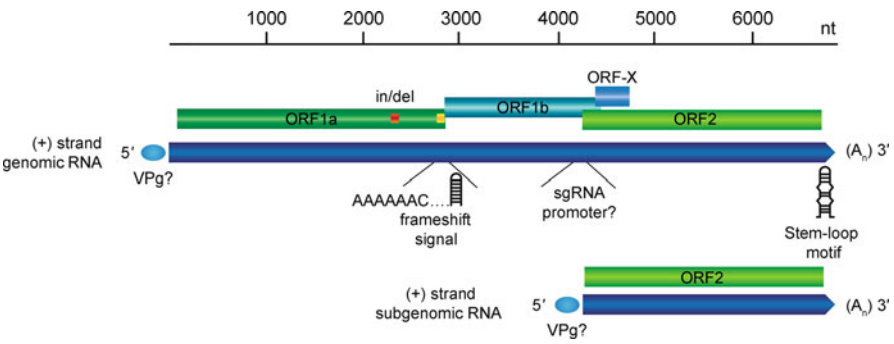


Fig. 2.2 Genome organization of human astrovirus. The genomic RNA, of approximately 6.8 kb, contains three recognized open reading frames (ORF1a, ORF1b, and ORF2). ORF-X has been proposed as functional, given its conservation among astrovirus The genome contains three elements conserved among all members of the family: the frameshift signal (*blue square*), the sequence upstream ORF2 that putatively acts as promoter for synthesis of the subgenomic (sg) RNA, and the stem-loop at the 3′-end. Also shown are the insertion/deletion (In/del) region (*red square*) and the presence of a poly(A) tail at the 3′-end. A putative VPg protein is depicted as bound to the 5′-end of the genomic and subgenomic RNAs

in RNA transcription and replication based on the sequence motifs they contain. The third ORF, found at the 3′-end of the genome, designated ORF2, is common to both the genomic and subgenomic RNAs and encodes the capsid proteins [50, 76]. An additional ORF (ORF-X) of 91–122 codons, contained within ORF2 in a +1 reading frame, has been described in all HAstV and some other mammalian viruses (Fig. 2.2) [18]. Its initiation codon is located 41–50 nucleotides downstream the ORF2 AUG and might be translated through a leaky scanning mechanism. It remains to be determined whether the putative protein encoded in ORF-X is synthesized in HAstV-infected cells and its significance for virus replication.

The length of all ORFs varies among different astrovirus strains, with the largest variation observed in ORF1a. The variation in this ORF is mainly due to insertions or deletions (in/del regions) present near the 3′-end [30] that have been associated with the cell line used to adapt the virus for growth in cell culture (Table 2.1) [73]. ORF1b is similar in length among all astroviruses. Comparative analysis of ORF2 among astrovirus has been more complete, because the sequences of a large number of strains of diverse origin have been determined. Its size is not as variable as that of ORF1a (Table 2.1), but it shows the highest sequence variability (Tables 2.2, 2.3, and 2.4).

A comparison of the complete genome sequences of astrovirus strains reported thus far has recognized common features. An overlap of 10–148 nt in the genome of mammalian viruses has been found between ORF1a and ORF1b. These two ORFs also overlap in avian viruses, but this overlapping is only 12–45 nt long [54]. The overlapping region contains signals that are essential for translation of the viral RNA polymerase encoded in ORF1b through a frameshift mechanism. During infection of susceptible cells, two positive-sense RNA species have been observed: the full-length genomic RNA (gRNA), and a subgenomic RNA (sgRNA, of ~2.4 kb) [38, 49].

Table 2.1 Length variation of astrovirus polyproteins

		nsp1a		nsp1b		ORF2	
Animal origin	Virus strain	Length (aa)	Acc. number	Length (aa)	Acc. number	Length (aa)	Acc. number
Human	HAstrovirus-1	920	AAC34715.1	519	AAC34716.1	786	NP_059444.1
	HAstrovirus-2					796	Q82446.1
	HAstrovirus-3	928	ADE09293.1	515	AEN74891.1	794	ADE09295.1
	HAstrovirus-4	920	AAY84777.1	515	AAW51878.1	771	BAA93448.1
	HAstrovirus-5	950	Q4TWH9.1	515	AAY46273.1	783	BAA90311.1
	HAstrovirus-6	921	ACX83562.2	515	ACV92106.1	778	ACX83561.1
	HAstrovirus-7					791	AAK31913.1
	HAstrovirus-8	921	Q9IFX3.1	518	AAF85963.1	842	AAF85964.1
	HAstrovirusMLB1	787	ACN44169.1	511	YP_002290967.1	755	ACN44171.1
	HAstrovirusMLB2	787	AER41412.1	511	ACX69837.1	745	AER41415.1
	HAstrovirus-SG	885	ADH93576.1	508	ADH93575.1	758	ADH93577.1
	HAstrovirus-VA1	886	YP_003090287.1	487	ACR23347.1	758	YP_003090288.1
	HAstrovirus-VA2	845	ACX83590.1	528	ACX69838.1	642	ACX83591.1
	HMOAstrovirus-A	874	YP_003275952.1	487	YP_003275951.1	731	YP_003275953.1
	HMOAstrovirus-B					755	ACX85474.1
	HMOAstrovirus-C					758	ACX85476.1
Porcine	PAstrovirus					783	CAB95000.1
	PAstrovirus-2	824	AER29998.1	498	AER29999.1	851	ADP21511.1
	PAstrovirus-4	850	AER30007.1	492	AER30008.1	825	AER30009.1
	PAstrovirus-5	872	AER30001.1	491	AER30002.1	735	AER30003.1
Bovine	BAstrovirus	815	AED89607.1	452	AED89583.1	772	AED89600.1
Deer	CcAstrovirus-1					755	AAV37187.1
	CcAstrovirus-2					749	ADO67581.1
Ovine	OAstrovirus	844	NP_059944.1	523	CAB95003.1	762	NP_059946.1
	OAstrovirus-2					737	AEP17838.1
Canine	CaAstrovirus					765	AEX00102.1
	SHDAstrovirus					824	ADL27736.1

Feline	FAstrovirus					816	AAC13556.1
Bat	BatAstrovirus-1					850	ACF75865.1
Mouse	MouseAstrovirus	849	YP_004782205.1			707	YP_004782206.1
Rat	RatAstrovirus			516	ADJ38390.1	810	ADJ38394.1
Mink	MAstrovirus	874	NP_795334.1	507	ADR65075.1	775	ADR65076.1
Sea lion	CslAstrovirus-1					780	ACR54272.1
	CslAstrovirus-2					859	ACR54274.1
	CslAstrovirus-4	912	AEM37614.1	530	AEM37615.1	828	AEM37616.1
	CslAstrovirus-5	929	AEM37617.1	515	AEM37618.1	766	AEM37619.1
	CslAstrovirus-6			530	AEM37621.1	840	AEM37622.1
	CslAstrovirus-7	888	AEM37623.1	535	AEM37624.1	839	AEM37625.1
	CslAstrovirus-8			530	AEM37627.1	825	AEM37628.1
	CslAstrovirus-9	888	AEM37629.1	535	AEM37630.1	838	AEM37631.1
	CslAstrovirus-10	888	AEM37632.1	536	AEM37633.1	825	AEM37634.1
	CslAstrovirus-11	934	AEM37638.1	515	AEM37639.1	752	AEM37640.1
Dolphin	BdAstrovirus-1					826	ACR54280.1
	BdAstrovirus-3					730	ADX97506.1
Whale	MWAstrovirus-2					779	ADX97522.1
Pigeon	FPAstrovirus					673	CBY02488.1
	WPAstrovirus					676	CBY02492.1
Duck	DAstrovirus	1,240	ADB79808.1	516	ADB79809.1	731	YP_002728003.1
Chicken	ANV-1	1,005	P0C6K7.1	507	Q9JGF2.1	680	ADG45753.1
	ANV-2					680	AEB15599.1
	Castrovirus	1,139	AEE88303.1	516	AEE88304.1	743	AEE88305.1
Turkey	Tastrovirus	1,126	ABX46576.1	517	ABX46574.1	723	ABX46584.1
	TAstrovirus-2	1,125	ABX46564.1	527	AAF60952.1	725	ABX46566.1
	TAstrovirus-3					724	AAV37187.1

Table 2.2 Sequence diversity of protein nsp1a of astroviruses[a]

	Virus	H-1	H-3	H-4	H-5	H-6	H-8	H-MLB1	H-MLB2	H-SG	H-VA1	H-VA2	HMO-A	P-2	P-4	P-5	B	O	Mouse	M	Csl-4	Csl-5	Csl-7	Csl-9	Csl-10	Csl-11	D	ANV-1	C	T	T-2
Human	HAstV-1	100																													
	HAstV-3	95	100																												
	HAstV-4	97	95	100																											
	HAstV-5	95	87	96	100																										
	HAstV-6	88	87	88	87	100																									
	HAstV-8	96	97	97	96	88	100																								
	HAstVMLB1	30	37	34	34	32	37	100																							
	HAstVMLB2	31	32	32	32	32	32	82	100																						
	HAstV-SG	26	26	26	26	26	27	28	29	100																					
	HAstV-VA1	29	28	27	27	28	28	30	29	97	100																				
	HAstV-VA2	26	27	26	27	26	27	30	31	66	66	100																			
	HMOAstV-A	27	28	27	26	27	27	29	30	65	65	98	100																		
Porcine	PAstV-2	32	33	33	33	33	33	30	30	31	28	30	30	100																	
	PAstV-4	32	32	32	32	33	32	34	31	30	30	28	31	36	100																
	PAstV-5	25	26	26	26	26	26	25	26	26	26	24	25	29	25	100															
Bovine	BAstV	29	29	29	29	29	29	30	29	29	29	32	32	71	34	30	100														
Ovine	OAstV	31	42	28	31	27	29	28	29	40	41	44	45	28	29	25	33	100													
Mouse	MouseAstV	26	27	27	26	26	26	28	27	25	26	26	26	27	26	25	27	28	100												
Mink	MAstV	29	29	28	29	29	28	29	29	41	41	42	43	29	27	28	30	40	26	100											
Sea lion	CslAstV-4	43	44	44	43	45	45	34	30	28	29	29	30	35	33	25	29	27	26	27	100										
	CslAstV-5	75	75	76	75	76	76	35	34	29	30	30	31	28	34	25	29	31	27	28	45	100									
	CslAstV-7	47	47	47	47	46	48	31	30	27	27	30	30	28	34	27	28	29	27	29	71	45	100								
	CslAstV-9	47	47	47	47	46	48	33	30	28	29	30	30	29	34	28	28	29	28	28	70	45	99	100							
	CslAstV-10	47	47	47	47	46	48	33	32	27	29	31	31	28	34	28	28	28	27	28	70	45	98	98	100						
	CslAstV-11	74	74	75	74	74	74	34	34	29	29	31	31	29	33	27	29	34	25	29	48	86	54	54	49	100					
Duck	DAstV	25	25	26	25	27	26	28	24	25	24	25	21	49	25	33	25	21	23	26	27	24	28	28	27	28	100				
Chicken	ANV-1	26	28	27	29	29	28	29	27	27	25	27	27	31	23	29	27	23	25	28	29	28	27	27	27	27	42	100			
	CAstV	25	26	26	25	23	25	25	24	25	25	23	24	27	26	25	25	24	24	25	24	26	24	24	23	25	59	28	100		
Turkey	TAstV	25	24	25	25	25	25	25	26	27	27	27	27	26	24	23	25	24	25	26	25	25	24	24	24	24	43	29	45	100	
	TAstV-2	25	24	25	24	27	24	25	26	28	28	28	28	27	23	26	26	24	24	27	25	24	24	24	22	23	50	29	45	98	100

[a]All available nsp1a sequences at the NCBI database were downloaded for each astrovirus strain. The sequences were pairwised compared, and their percent amino acid identities are shown in the figure.

Both RNAs are transcribed from the negative-sense full-length complement of the genomic RNA. Based on the transcription initiation site determined for the sgRNA in HAstV-1 and HAstV-2 [50, 76], ORF1b and ORF2 overlap by 8 nt; however, the length of this overlapping may vary, and it is not present in a duck astrovirus, in which it is 24 nt apart [19]. The synthesis of sgRNA requires an internal subgenomic promoter in the negative-sense RNA, and a highly conserved sequence in mammalian astroviruses around the ORF2 start codon that has been suggested to be part of the promoter for synthesis of the sgRNA [36]. This sequence is also conserved in avian astroviruses, but is slightly different in sequence and length [54]. This conserved sequence has a partial identity with the 5′-end of the gRNA [36], suggesting it has an important role for the synthesis of both gRNA and sgRNA.

A short 5′ UTR between 11 and 85 nt long precedes ORF1a. Also, an 80–85-nt long 3′ UTR is found between ORF2 and the poly(A) tail of HAstV genomes, but this sequence can be longer (130–305 nt) in avian astroviruses [68]. Of note, in addition to the 3′ UTR, the terminal 19 nt of ORF2 are also highly conserved among HAstV serotypes [48], and similarities have been observed in the sequence and folding of the 3′ UTR of several animal astroviruses, as well as in avian infectious bronchitis virus (a coronavirus), a dog norovirus, and the equine rhinovirus serotype 2 [36]. These conserved sequences are thought to be important for interaction with the viral RNA replicase and with cellular proteins that are likely needed for replication of the viral genome.

General Features of Nonstructural Proteins

The nonstructural proteins synthesized by positive-sense, single-stranded RNA viruses usually include a protease, an RNA-dependent RNA polymerase (RdRp), a helicase, and, in some cases, a viral genome-linked protein (VPg). The helicase and NTPase unwind the RNA before replication, while the VPg is covalently attached to the 5′-end of the RNA genome and is required for its efficient translation and/or replication. With the exception of the helicase, there is evidence for the presence of the other activities in the nonstructural proteins of astroviruses.

The primary translation products of the astrovirus genome are polyproteins nsp1a and nsp1ab (Fig. 2.3). Nsp1a is encoded in ORF1a, while nsp1ab is translated from ORF1a and ORF1b through a ribosomal frameshift mechanism. The length of nsp1a varies in mammalian viruses from 787 to 950 aa long, and in avian viruses it can be as long as 1,240 aa (Table 2.1). The most evident feature of nsp1a is the presence of a protease motif. The sequence of the predicted protease is well conserved between HAstV strains, but less conserved between mammalian and avian astroviruses, with only 37% amino acid identity between HAstV-1, turkey astrovirus, and avian necrosis virus [34]. The protein displays the typical properties of trypsin-like enzymes. The crystal structure (2.0 Å resolution) of the predicted protease from a HAstV strain revealed a similarity with viral serine proteases from Sesbania mosaic virus and various picornaviruses (human rhinovirus 2, hepatitis A

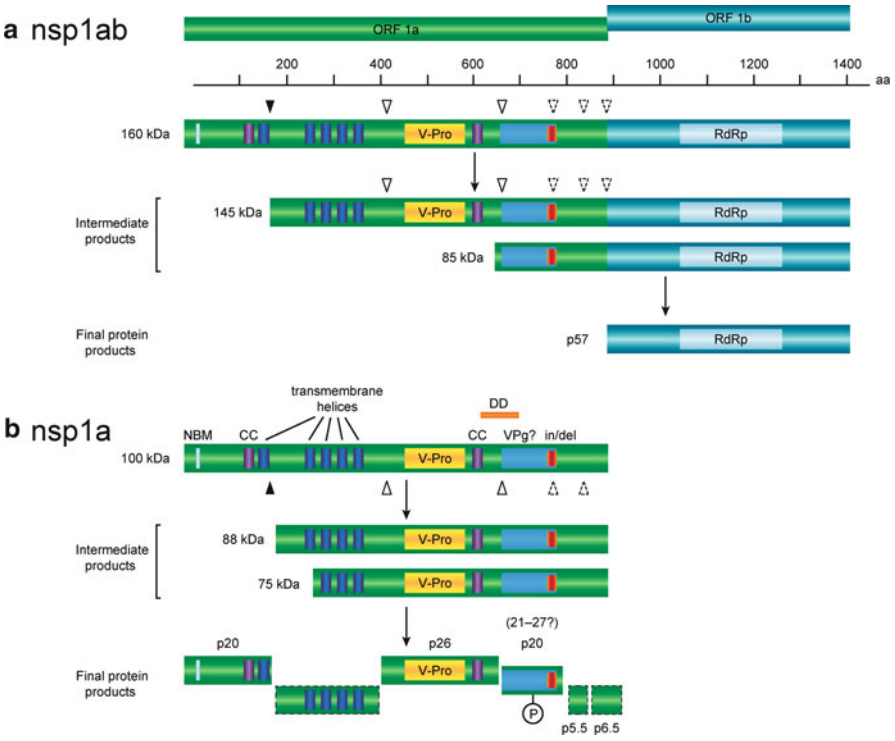


Fig. 2.3 Nonstructural proteins and protein structural motifs predicted from the nucleotide sequence of ORF1a and ORF1b. *NBM* nucleotide binding motif, *CC* coiled-coil, *TH* transmembrane helices, *v-Pro* viral protease, *VPg* viral protein attached to the genome, *in/del* insertion/deletion, *DD* putative death domain, *RdRp* RNA-dependent RNA polymerase. Nsp1ab (**a**) and nsp1a (**b**) are processed by cellular (*closed triangle*) and viral (*open triangles*) proteases. Although only the cleavages around amino acid residues 410 and 654 have been confirmed to be due to v-Pro, indirect evidences that would explain the protein processing products observed suggest that the downstream cleavages (*dashed triangles*) are also due to the viral protease. No products from the hydrophobic region (TH) have been identified, and the smaller products of 5.5 and 6.5 kDa have not been mapped into nsp1a (*dotted boxes*)

virus, and foot-and-mouth disease virus). The structural comparison confirmed that the catalytic triad is composed of His461, Asp489, and Ser551 [63], as was initially proposed using site-directed mutagenesis [37].

Sequence analysis of nsp1a showed the presence of five to six transmembrane helices upstream the protease motif, both in mammalian and avian viruses, although there is little conservation at the amino acid level between different astrovirus strains. Two regions with heptad repeat patterns were also identified in nsp1a as coiled-coil structures, one just upstream of the first helical motif and the second downstream of the protease motif [36]. The function of these structures or of the proteins that contain them remains unknown.

Table 2.3 Sequence diversity of protein nsp1b of astroviruses[a]

	Virus	H-1	H-3	H-4	H-5	H-6	H-8	H- MLB1	H-MLB2	H-SG	H-VA1	H-VA2	HMO-A	P-2	P-4	P-5	B	O-2	Rat	M	Csl-4	Csl-5	Csl-6	Csl-7	Csl-8	Csl-9	Csl-10	Csl-11	D	ANV-1	C	T	T-2
Human	HAstV-1	100																														
	HAstV-3	94	100																													
	HAstV-4	99	94	100																												
	HAstV-5	96	95	97	100																											
	HAstV-6	95	95	95	95	100																										
	HAstV-8	99	94	98	97	95	100																									
	HAstVMLB1	56	55	54	55	54	56	100																								
	HAstVMLB2	56	56	56	56	55	56	80	100																							
	HAstV-SG	52	53	52	53	53	52	48	50	100																						
	HAstV-VA1	52	53	52	52	52	51	48	49	98	100																					
	HAstV-VA2	51	51	51	51	50	51	48	49	74	75	100																				
	HMOAstV-A	52	52	52	52	51	52	48	48	74	75	97	100																			
Porcine	PAstV-2	57	58	58	56	58	57	53	54	52	54	51	52	100																		
	PAstV-4	57	58	58	56	58	57	53	54	51	54	51	52	60	100																	
	PAstV-5	49	49	49	49	48	49	47	47	47	47	44	45	49	49	100																
Bovine	BAstV	61	61	61	61	61	62	54	56	51	52	50	51	83	61	51	100															
Ovine	OAstV-2	51	51	51	51	50	51	48	47	63	64	64	66	50	50	46	51	100														
Rat	RatAstV	58	57	57	56	57	57	57	56	49	49	49	50	58	58	47	59	48	100													
Mink	MAstV	51	51	51	51	51	51	47	48	64	64	65	65	53	52	46	53	64	51	100												
Sea lion	CslAstV-4	72	72	72	72	72	72	55	56	52	53	52	53	57	57	46	59	50	57	50	100											
	CslAstV-5	90	92	90	90	90	91	54	57	54	55	51	53	58	58	50	62	52	56	52	72	100										
	CslAstV-6	72	72	72	72	72	72	55	55	52	54	52	53	57	57	46	59	51	58	51	98	72	100									
	CslAstV-7	72	72	73	73	73	72	55	56	52	54	52	54	57	58	46	60	50	59	51	88	72	88	100								
	CslAstV-8	72	72	72	72	72	72	55	56	52	53	52	53	57	58	45	59	50	57	51	98	71	97	87	100							
	CslAstV-9	72	73	73	73	73	72	55	56	52	54	52	54	57	59	46	59	50	58	51	88	72	88	99	87	100						
	CslAstV-10	68	68	69	69	68	68	51	53	48	49	49	50	55	54	44	56	47	56	48	83	68	83	94	82	93	100					
	CslAstV-11	90	92	90	90	90	90	54	57	54	55	51	53	58	58	49	62	52	56	52	72	99	72	72	71	72	68	100				
Duck	DAstV	42	42	42	42	43	42	40	40	40	41	39	41	42	41	43	42	41	39	37	42	42	42	43	42	43	40	42	100			
Turkey	ANV-1	44	44	43	44	45	44	43	43	39	39	39	39	42	42	41	42	39	41	39	42	43	42	43	41	43	40	42	54	100		
	CAstV	41	42	41	41	43	41	40	42	41	42	42	44	40	41	41	42	40	40	41	42	42	43	44	42	44	40	42	70	55	100	
	TAstV	41	42	41	42	41	41	42	43	41	42	42	43	41	41	41	42	42	37	41	42	44	42	42	42	42	38	44	70	54	70	100
	TAstV-2	42	41	41	42	41	42	40	42	41	43	41	43	41	40	40	42	42	38	41	42	43	42	41	41	41	38	43	70	54	70	95	100

[a]All available nsp1b sequences at the NCBI database were downloaded for each astrovirus strain. The sequences were pairwised compared, and their percent amino acid identities are shown in the figure.

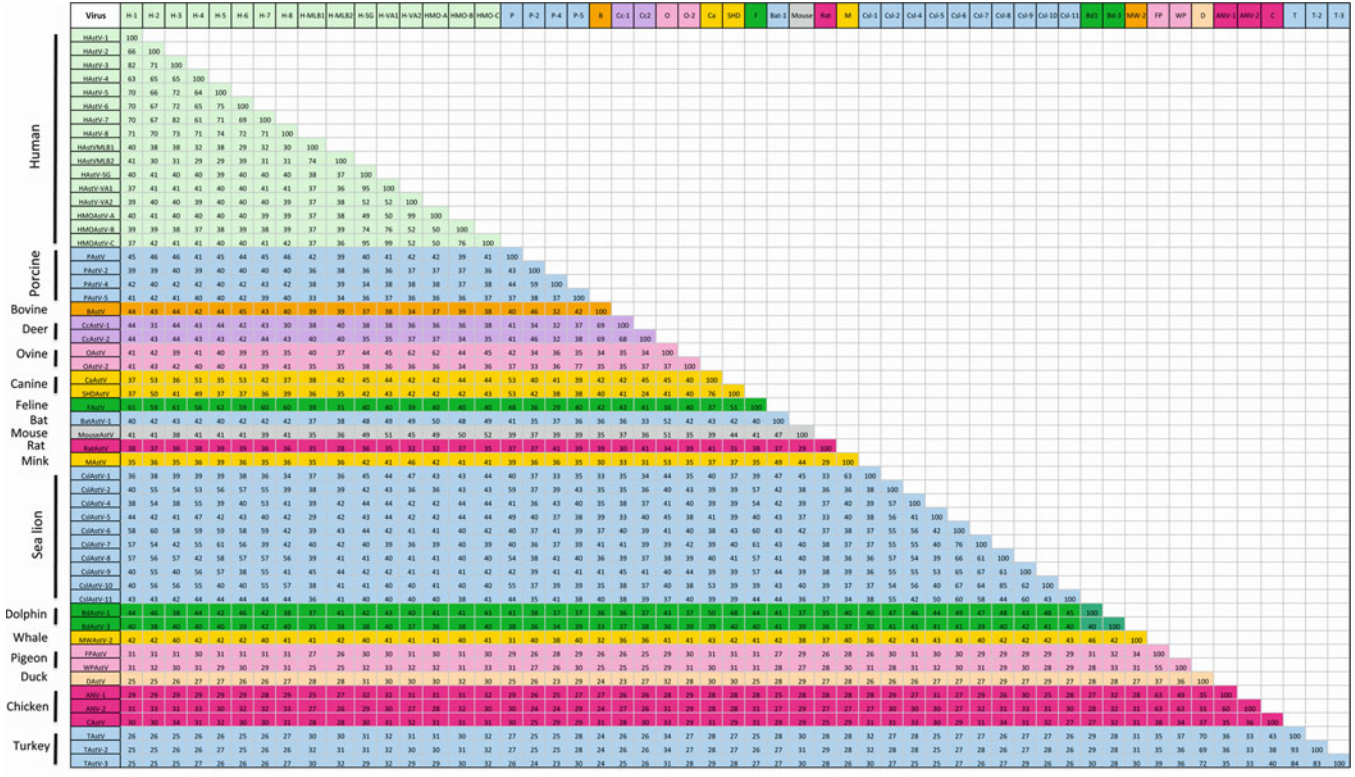
Phylogenetic and comparative analysis of the genome of astrovirus with other plus-stranded RNA viruses identified a putative VPg coding region between amino acids 664 and 758 of nsp1a, with high sequence similarity to the VPg coding region of caliciviruses [2]. Its synthesis in astrovirus-infected cells has not been investigated; however, similar to calicivirus, treatment of the viral genomic RNA with proteases markedly reduced the yield of infectious virus when the astrovirus genome was transfected into permissive cells, suggesting that the ability of the astrovirus genome to initiate a replication cycle depends on the presence of a genome-linked protein [31, 70]. A hypervariable region with deletions and insertions downstream the VPg motif has been related to adaptation of the virus to culture cells [73] and to efficient synthesis of viral RNA during infection [30]. However, the form in which this region might modulate these events is unknown. The role in viral replication of the C-terminal region of nsp1a, comprising from aa residue 568 to the C terminus of the protein has been analyzed. This region, named nsp1a/4 [29], includes the putative VPg protein and the hypervariable region, and was found to be phosphorylated and to form oligomers. Nsp1a/4 interacts with the RNA polymerase when translated *in vitro*, and this interaction depends on the phosphorylation status of the protein. The fact that the proposed phosphorylation site is in the putative VPg region supports the idea that nsp1a/4 could be implicated in the replication of the astrovirus genome [20]. In addition, nsp1a/4 has a perinuclear distribution near the endoplasmic reticulum and colocalizes with viral RNA [29]. Whether the region of nsp1a/4 that interacts with the replicase is equivalent to the predicted VPg protein remains to be determined.

A bipartite nuclear localization signal (NLS) was described downstream of the second coiled-coil motif in HAstV and avian necrosis virus. When a recombinant protein spanning the NLS of HAstV-1 was expressed using baculovirus, the protein localized to the nucleus of insect cells [74]; however, the NLS antigen has not been observed in astrovirus-infected cells [24]. Moreover, neither turkey nor ovine astrovirus genome has a conserved NLS motif [36] and the region where the NLS motif is located has been proposed to encode the astrovirus VPg [2]. Thus, the role of the NLS remains unknown.

Motifs for an RNA-dependent RNA polymerase (RdRp) have been identified in the polypeptide encoded in ORF1b. This ORF encodes a protein of 515–539 amino acids that contains a region similar to the RdRp of plant bymoviruses and potyviruses [34, 43]. The eight conserved motifs in this region are typical of the RdRp of positive strand RNA viruses of the Koonin's supergroup I [39], which includes picornaviruses and caliciviruses (Table 2.3).

A region potentially encoding an RNA helicase has not been found in the astrovirus genome. This is unusual in single-stranded RNA virus with a genome length of more than 6,000 nt [34]. A conserved sequence in the N-terminal region of nsp1a of mammalian astrovirus has similarity with motifs I and II (NTP binding motif) of the seven conserved motifs of the RNA helicase of pestivirus, but lacks other motifs, such as the substrate binding and NTP hydrolysis domains present in these enzymes [67]. However, other similarities were not found, and this region is not conserved in avian astroviruses [2].

Table 2.4 Sequence diversity of the capsid polyprotein of astroviruses[a]



[a]All available capsid amino acid sequences at the NCBI database were downloaded for each astrovirus strain. The sequences were pairwised compared, and their percent amino acid identities are shown in the figure.

General Features of Structural Proteins

The structural polyprotein shows the largest sequence variability among astrovirus proteins. It varies from 672 to 851 aa in length, and, in general, the ORF2 of avian viruses codes for shorter polyproteins (Table 2.4). The N-terminal half of the polyprotein is more conserved than the C-terminal domain, with the N-terminal region encompassing a region of basic amino acids that is conserved among astrovirus [35, 72] and is thought to interact with the genomic RNA in the virion [23]. The C-terminal half of the protein shows considerable sequence variability among astroviruses isolated from different animal species, or even among different isolates from the same species (Fig. 2.4) as it contains a delimited region with abundant

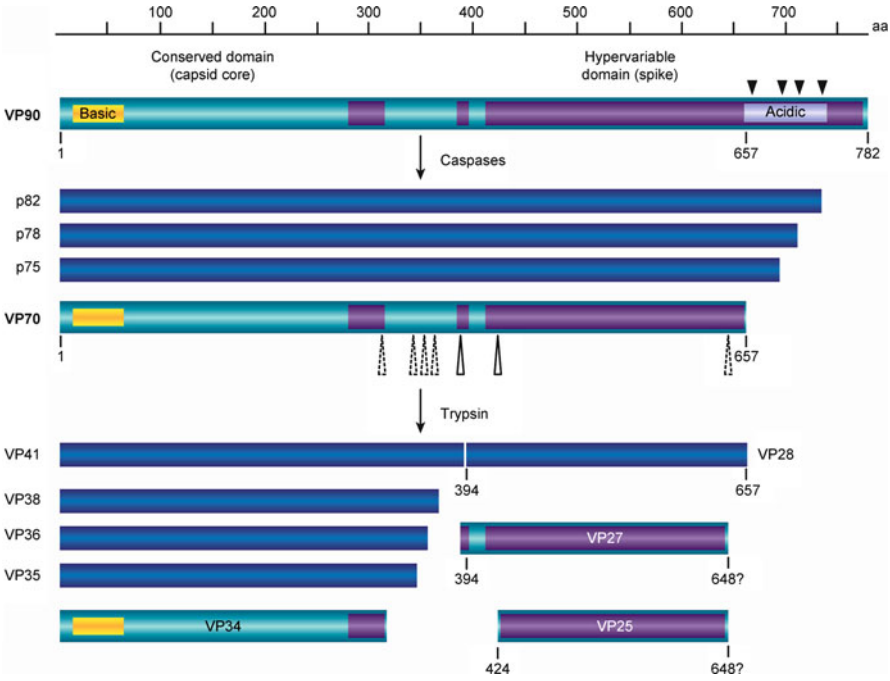


Fig. 2.4 Features of structural proteins. The primary ORF2 product (named VP90 in HAstV-8) contains two domains that can be distinguished by their degree of conservation: the N-terminal domain is highly conserved and forms the core of the capsid, while the hypervariable C-terminal domain forms the spikes of the virus particle. VP90 contains basic and acidic regions that are highly conserved among all astroviruses. VP90, the primary product of ORF2 is sequentially processed at its carboxy terminus by caspases to generate VP70 (intermediate cleavage products are shown in *gray*), the protein present in the extracellular virions. These particles are processed by trypsin to generate protein intermediates of variable size (in *gray*), and the final products VP34, VP27, and VP25. *Closed arrow heads* represent cleavages carried out by caspases, important for virus maturation and cell egress*; open long arrow heads*, cleavages by trypsin. The cleavage sites indicated by *dashed arrow heads* have not been precisely determined; therefore, the carboxy termini of the intermediate and final products are unknown

insertion or deletions [72]. The carboxy terminus of the polyprotein has an acidic character (residues 649–702 for HAstV-8) that is proposed to be highly conserved among all members of the *Astroviridae* family (Fig. 2.4) [54] and is the substrate of cellular caspases. The last five amino acid residues at the C-terminal end are also conserved among human and other mammalian virus strains. The conserved N-terminal domain of the structural protein forms the capsid core of the particle, whereas the hypervariable C-terminal domain forms the spikes of the virion. Thus, this last domain is predicted to participate in the early interactions of the virus with the host cell [16, 40].

Early Steps of Infection

The early interactions of astroviruses with their host cell have been poorly characterized. The cellular receptor is unknown; however, the observation that different HAstV serotypes show different tropism in cultured cells [7] suggests that more than one cellular attachment or receptor molecule may be used by these viruses. Caco-2 cells are susceptible to infection by all HAstV serotypes, but HTC-15, another colon carcinoma cell line, only weakly supports infection by HAstV-1 [7]. Likewise, baby hamster kidney (BHK-21) cells only permit the sustained passage of HAstV-2, but they are permissive for replication of other HAstV serotypes after transfection of *in vitro* transcribed genomic RNA [21], suggesting that the blockage is at the virus entry level. It has been found that HAstV-8 binds to the surface of Caco-2 cells with a half-time of about 9 min (Méndez et al., unpublished), and it is known that the infectivity of HAstV is greatly enhanced (3–5 logs) by, and probably is dependent on, trypsin cleavage of the capsid protein [4, 55, 61]. Although the trypsin cleavage products have been characterized (see below), the mechanism by which this treatment enhances virus infectivity is unknown. Trypsin cleavage of the precursor polyprotein produces in HAstV-8 three final products, VP34, VP25, and VP27 [55], with the concomitant induction of drastic structural changes in the particles [4]. VP34 represents the conserved domain and forms the capsid core, while both VP25 and VP27 form the spikes on the virion surface. The crystal structure of the HAstV-8 spike domain was recently solved at a 1.8 Å resolution [16], mapping conserved residues onto the astrovirus projection domain revealed a putative receptor binding site with amino acid compositions characteristic for polysaccharide recognition [16]. Antibodies that recognize the spike proteins VP25 and VP27 are serotype specific and neutralize virus infectivity [5, 61], probably by blocking virus binding.

Early studies with HAstV-1 in HEK293 Graham cells demonstrated the presence of virions in coated pits and coated vesicles, implicating clathrin-mediated endocytosis as the viral entry mechanism. This was supported by inhibition of viral infection by endocytosis-blocking drugs [15]. An acidic endosomal pH was also found to be required for HAstV-1 infection [15]. Recently, a clathrin-dependent

endocytosis was confirmed to be a functional pathway for the entry of HAstV-8 into Caco-2 cells. Drugs that affect clathrin assembly, endosome acidification, and actin filaments, as well as those that decrease cellular cholesterol and interfere with vesicular transport, reduced the infectivity of HAstV-8. The infection of this virus was also reduced by knocking down the expression of clathrin heavy chain by RNA interference and by expression of dominant-negative mutants of dynamin (Méndez et al., unpublished data).

The mechanism through which the astrovirus genome is released from the infecting virus particle into the cytoplasm for translation, the cell site where it occurs, and the cellular and viral factors involved in this event are unknown. However, it has been determined that in Caco-2 cells, HAstV-8 uncoats about 130 min after cell entry, and the infectious virus has to reach late endosomes for a productive infection, since virus infectivity is reduced by knocking down the expression of Rab7 (Méndez et al., unpublished data).

The interaction of HAstV with the host cell provokes activation of the extracellular signal-regulated kinase (ERK1/2) pathway within the first 15 min after virus attachment. Although the mechanism for this activation is unknown, it was independent of virus replication, suggesting that this pathway is triggered during virus binding or entry into the cell [51]. ERK1/2 seems to be required at early times to establish a productive infection, since inhibitors of this kinase blocked synthesis of viral proteins and RNA, and, consequently, reduced the virus yield. It has also been shown that astrovirus increases barrier permeability in polarized monolayers of Caco-2 cells following apical infection [52]. Permeability was increased when monolayers were treated not only with infectious virus, but also with inactivated virus or purified virus-like particles (VLPs), indicating that astrovirus-induced permeability occurs independently of viral replication and is modulated by the capsid protein. It has been suggested that HAstV could trigger tight junction instability to reach putative receptors present in the basolateral membrane; however, the fact that the increase in permeability is observed at least 16 h postinfection suggests that more than a requirement for virus entry, it is a consequence of the cellular signal transduction pathways induced by the virus.

Translation

After uncoating, the virus RNA genome is translated into nonstructural proteins. It has been suggested that the astrovirus genome contains a VPg 5′ moiety in place of a traditional 7-methyl cap, and, as observed for caliciviruses, it could recruit the cap binding protein eIF4E to initiate translation [2, 10, 70]. The VPg protein from calicivirus has been shown to directly interact with eIF4E and eIF3, a multi-subunit scaffolding initiation factor, to promote assembly of the preinitiation complex [13, 25]. This VPg-eIF4E interaction may be a preferential method of viral over host translation [60].

Nonstructural Polyprotein Synthesis and Processing

The nonstructural astrovirus proteins are translated as two polyproteins, nsp1a (~101 kDa) and nsp1ab (~160 kDa). The translation of ORF1a to produce nsp1a stops at an amber codon, while a translational fusion of ORF1a with ORF1b that results from a −1 frameshift at the junction of these two ORFs and leads to the synthesis of nsp1ab. Nsp1ab is translated between 7% (*in vitro*) and 25% (*in vivo*) of the time [34, 44] and requires a highly conserved heptameric "slippery" sequence (AAAAAAC) and a downstream hairpin structure for induction of the frameshift (Fig. 2.2) [43, 75]. Of note, mechanisms for attenuated translation of the RdRp are common in positive-sense RNA viruses [46].

The nsp1a and nsp1ab polyproteins are cleaved by the viral serine protease to produce the individual nonstructural proteins, although a cellular protease seems to be involved in at least one of the cleavage events [23, 24, 37, 56]. The number and size of the final nonstructural proteins is not fully elucidated, and studies using different cell lines, human virus serotypes, and expression systems have described different intermediate and final processing products [54]. However, conclusions drawn from these reports suggest that at least five nonstructural proteins exist (Fig. 2.3) (1) an N-terminal 20-kDa protein, likely cleaved co-translationally by a cellular signalase that contains one predicted transmembrane domain and a coiled-coil domain [56]; (2) a protein predicted to contain four transmembrane domains, although a protein from this hydrophobic region of nsp1a has not been identified; (3) a 27-kDa serine protease [22, 37]; (4) a phosphoprotein of 21–27 kDa depending on its level of phosphorylation or differential processing that contains the hypervariable nsp1a region and possibly is the VPg protein [2, 20, 29]; and (5) a 57-kDa RNA-dependent RNA polymerase [22, 43]. Finally, two reported small products of 5.5 and 6.5 kDa have not been mapped in nsp1a [74].

Structural Polyprotein Synthesis and Processing

The primary translation product of astrovirus ORF2 is an 87–90 kDa polyprotein synthesized from the viral sgRNA. The mechanism of translation of the sgRNA remains to be determined; however, as proposed for the astrovirus gRNA, it could be translated through a mechanism involving the presence of a 5′-end VPg, as it has been shown for the gRNA and sgRNA of caliciviruses [31, 70].

In HAstV-8-infected Caco-2 cells, a 90-kDa protein (VP90) is synthesized from ORF2. This protein assembles into viral particles and is then cleaved at the carboxy terminus by cellular caspases to produce a 70-kDa protein (VP70) through several intermediate cleavage events (Fig. 2.4) [55]. The VP90 to VP70 cleavage is associated with the exit of the virus from the cell. A 90 kDa protein in HAstV-1 infected cells has also been detected [49, 70]. The intracellular cleavage of the primary translation product of ORF2 still requires further investigation; however, it seems clear

that extracellular virions are only constituted by VP70 [55]. These virions require trypsin treatment to fully activate their infectivity. The proteolytic treatment cleaves VP70 into three smaller products and enhances virus infectivity up to 105-fold [4, 42]. The trypsin cleavage pathway of VP70 has been best characterized for HAstV-8 (Fig. 2.4) and yields cleavage products of 25, 27, and 34 kDa. The cleavage products are similar in other astrovirus strains studied, although their sizes range from 24 to 26, 27 to 29, and 32 to 34 kDa [4, 6, 12, 23, 55]. Of interest, if intracellular viral particles formed by VP90 are isolated and treated with trypsin *in vitro* they also become infectious [53].

Transcription/Replication

Genome replication of positive-sense, single-stranded RNA viruses belonging to several different families has been well documented. Few details are known about astrovirus, but the available information is consistent with previous studies in other positive-sense RNA viruses, making it reasonable to assume that replication of the astrovirus genome occurs in a similar manner. Based on this premise, it is thought that after translation of the gRNA and proteolytic processing of the nonstructural proteins a viral replicase complex is formed, which uses the gRNA to produce a full-length negative-sense RNA. This negative-sense RNA is detected 6–12 h postinfection, and it accumulates to 0.7–4% of gRNA [33]. The replicase complex of positive-sense RNA viruses usually contains the viral RdRp and other nonstructural proteins, as well as host proteins necessary for replication of the viral genome. In the case of astrovirus, the nonstructural protein nsp1a/4 protein (corresponding to the C-terminal end of nsp1a) has been shown to be required for virus replication in addition to the viral polymerase [20, 30]. Also, a predicted VPg has been suggested as a possible product of nsp1a [2], opening the possibility that astrovirus RNA synthesis could be primer dependent. However, further studies are required to confirm this hypothesis. Whether the nonstructural proteins other than nsp1a/4 are required for astrovirus genome replication is unknown. Transcription of gRNA and sgRNA from an internal promoter of the full-length negative-sense RNA is detected starting at 8 h postinfection [54]. Requirements for the replicase complex to switch from minus-strand synthesis to positive-sense and sgRNA synthesis are unknown, but could pattern alphaviruses, which use nonstructural polyprotein processing to direct template activities [65].

The sgRNA is transcribed at an estimated tenfold greater amount over viral genome at 12 h postinfection [49]. Delayed structural protein synthesis and transcription of large amounts of sgRNA is a common theme in positive-sense RNA viruses and has been suggested to be a mechanism to overcome host translational repression, and possibly to promote packaging into virions of the newly synthesized viral genomes through inhibiting their translation [66]. The genome of HAstV contains highly conserved 5′ and 3′ UTRs [48]. UTRs from other viruses have been shown to facilitate 5′ and 3′ interactions during gRNA replication, recruit host factors for replication, are

sites of recognition and initiation by the viral polymerase, and enhance the stability of the RNA genome. It is likely that astrovirus UTRs play a similar role in the virus gRNA replication, although this remains to be determined [45, 59, 64, 69, 71]. Very little information is available regarding specific host proteins involved in replication of the astrovirus genome, although this process could require several RNA binding proteins, as reported for other plus-strand RNA viruses [1, 8, 11].

Infectious cDNA Clone

As a positive-sense RNA virus, the astrovirus genome is able to initiate a productive infection [21]. *In vitro* transcribed RNA from a full-length cDNA clone of the HAstV-1 genome has also proved to be infectious [21]. Using this clone, a reverse genetics system for HAstV-1 was established and used to analyze specific mutations in the structural [23] and nonstructural proteins [30]. This system consists of the *in vitro* transcribed RNA, which is transfected into the highly transfectable, but nonsusceptible and poorly permissive BHK-21 cells, harvesting the virus produced, and propagating this virus in the astrovirus-susceptible Caco-2 cell line. This protocol, although efficient in terms of virus yield after amplification in Caco-2 cells, has the drawback that the mutant virus is finally obtained after several rounds of replication in the latter cells, which might induce accumulation of additional mutations in the viral progeny. Thus, analysis of the phenotype associated with specific mutations introduced in the cDNA clone may be disguised. In fact, viruses with changes not related to the mutations originally introduced in the nonstructural region of HAstV-1 were found after virus recovery by this method [30]. More recently, a protocol that improves the reverse genetics system of astrovirus has been described [70]. It uses the highly transfectable and HAstV-susceptible and highly permissive hepatocyte cell line Huh7.5.1 making it possible to obtain higher titer virus yield in a single replication cycle, decreasing the frequency of potentially undesired mutations [70].

Cellular Sites of Viral Genome Replication

The replication of all positive-sense RNA viruses characterized takes place in association with membranes [14, 47, 58]. Infection with these viruses promotes intracellular membrane rearrangement yielding networked structures of vesicles, single- and double-membrane vesicles, and often invaginations or spherules that are associated with the synthesis of viral RNA and proteins, and with the assembly of virus particles [14]. The induced organelle-like structures can be derived from the ER, Golgi apparatus, and lysosomal or endosomal membranes, and the viral RNA and proteins, as well as the cellular proteins involved in these processes, have been found to form replication complex at these sites [14].

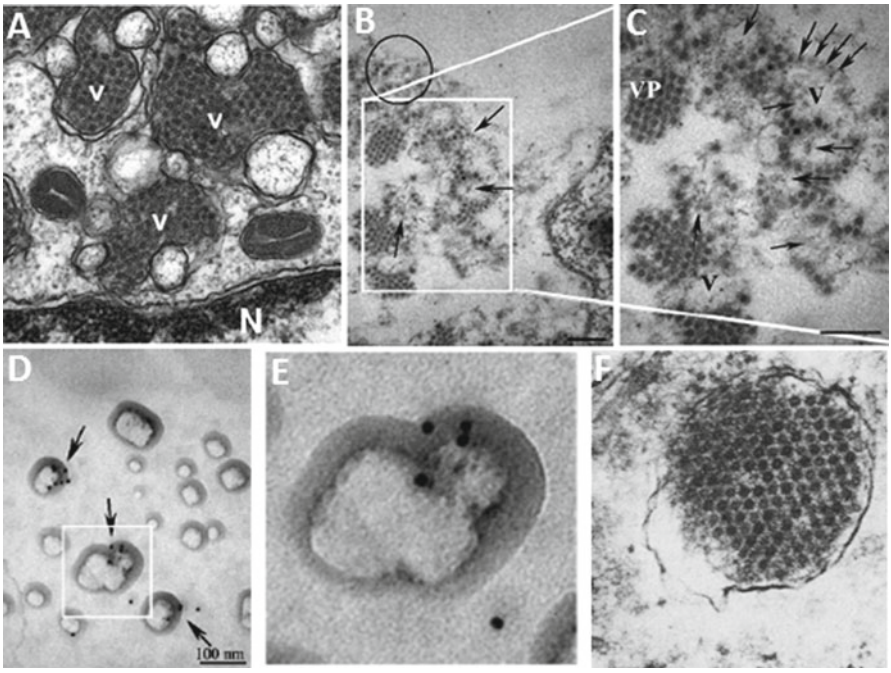


Fig. 2.5 Astrovirus proteins and particles are associated with membranes in infected cells. Ultrastructural analysis of Caco-2 cells infected with (**a**) HAstV-4 (48 hpi; *N* nucleus, *V* viral aggregates); (**b** and **c**) HAstV-8 (24 hpi; *V* vesicles, *VP* viral particles); (**d**) immunoelectron microscopy of membrane-enriched density gradient fractions of Caco-2 cells infected with HAstV-8. *Arrows* indicate positive immunogold signals; (**e**) enlarged image of (**d**); (**f**) electron microscopy of lamb mid-gut cells at 23 h postinfection with lamb astrovirus. Crystalline array of hollow-cored particles enclosed by a membrane. Photographs were reproduced from Guix et al. [29] (**a**), Mendez et al. [53] (**b**–**d**, **e**), and Gray et al. [27] (**f**), with permission

The membrane rearrangements induced by astrovirus infection have been poorly characterized. Viral aggregates in HAstV-4-infected cells were observed at 48 h postinfection near the periphery of the nucleus, surrounded by a large number of double-membrane vacuoles that were not observed in noninfected cells (Fig. 2.5a) [29]. Similar structures were found in HAstV-8-infected Caco-2 cells, where large groups of viral particles surrounding "O-ring" structures that probably correspond to double-membrane vesicles were described (Fig. 2.5b, c) [53]. Virus-like particles were observed inside and at the borders of the "O-ring" and could represent subviral particles at different assembling stages [53]. Moreover, the VP90 structural precursor protein was detected in membrane fractions resolved by density gradient centrifugation and analyzed by immunoelectron microscopy (Fig. 2.5d, e) [53]. Of interest, ultrastructural analysis of the jejunum and ileum of astrovirus-infected lambs showed viral particles aggregates within lysosomes and autophagic vacuoles of epithelial cells, as well as along microvilli (Fig. 2.5f) [27], suggesting

that astrovirus replication associated with membranes also occurs in naturally targeted intestinal epithelial cells.

Structural and nonstructural astrovirus proteins associate with cellular membranes. The structural polyprotein of HAstV-8 interacts with membranous structures through its C-terminal region, since this region is protected from trypsin cleavage when VP90 is associated to membranes, but not when the protein is membrane free [53] and this interaction can occur in the absence of other viral proteins, as judged from *in vitro* translation assays [53].

The nsp1a/4 nonstructural protein of HAstV-4, which interacts with the RdRp and is involved in viral gRNA replication, has been reported to associate to the ER membrane in close vicinity to viral RNA and structural proteins [20, 29]. The protease and RdRp, as well as genomic and antigenomic (negative-sense) RNAs, have also been reported to associate with membranes in HAstV-8-infected cells [53]. The presence of structural (VP90) and nonstructural (nsp1a/4, protease, RdRp) viral proteins, as well as viral RNAs, in membranous fractions suggests that RNA replication and the first steps of virus morphogenesis are carried out in association with the observed membranous structures. It is still unknown, however, if RNA replication and virus assembly occur at the same subcellular membranous structure. The putative replicase complex of astrovirus seems to associate with membranes that probably derive from the ER, since viral phosphoproteins of 21–27 kDa, which interact with the RdRp [20], and are likely involved in RNA replication, localize to this organelle [29].

Virus Morphogenesis and Release

The assembly of viral particles is a highly complex process. This process requires the coordinated expression of genes encoding the structural proteins, the proper interaction of the proteins among themselves and with the virus genetic material, and in many instances coupling of the capsid assembly with the replication of the viral genome. In addition, the assembly process frequently occurs in association with cell membranes and with the help of cellular proteins. The knowledge on the assembly process of infectious astrovirus particles is limited, but it is known that the capsid assembles in the absence of viral RNA, carried out in membranous structures, and requires the activity of cellular caspases during the final assembly step.

Assembly of Viral Particles

Virus-like particles (VLPs) spontaneously assemble both in mammalian and insect cells when astrovirus ORF2 is expressed using heterologous systems. The expression of HAstV-2 ORF2 in LLCMK2 cells using a recombinant vaccinia virus led to the assembly of VLPs similar in size and morphology to native

HAstV-2 virus particles when analyzed by electron microscopy [12]. Moreover, when these VLPs were purified and treated with trypsin, proteins VP32, VP29, and VP26 were generated, in coincidence with the structural proteins found in native viral particles [12], indicating that the HAstV-2 capsids can assemble correctly in the absence of viral RNA.

VLPs were also obtained when the complete ORF2 of HAstV-1 was expressed using the baculovirus–insect cells system [9], although the particles presented an atypical morphology when purified [54]. VLPs were assembled in this system when a truncated form of the structural polyprotein lacking the N-terminal 70 amino acids was expressed, or when a protein whose 70 N-terminal amino acids had been replaced by the green fluorescent protein was synthesized [9], indicating that the 70 N-terminal amino acids of the structural polyprotein are not essential for virus particle assembly; although it is believed that this region, of basic nature, is important for interaction with the viral genome during morphogenesis of the viral particle [54]. When the VLPs assembled in insect cells were further characterized by ultracentrifugation in sucrose gradients, the particles banded at a density of 1.08 g/ml, and electron microscopy analysis showed the presence in this fraction of VLPs around 38 nm in diameter, similar to HAstV-1 purified particles, as well as ring-like structures of 16 nm in diameter [9]. Of interest, these ring-like structures could be converted into VLPs in the presence of Mg^{2+}, while sequestration of divalent cations promoted the disassembly of VLPs into ring-like structures [9]. These observations showed the importance of divalent ions to maintain the capsid structure and suggest that the ring-like structures could be directly involved in VLP morphogenesis. The ring-like structures were also observed when native HAstV-1 viruses were purified by CsCl centrifugation in the presence of EDTA.

The assembly of gRNA into viral particles requires the specific interaction of structural protein(s) with encapsidation signals on the viral RNA and, in the case of positive-sense RNA viruses, the process of particle assembly is thought to occur through condensation of the capsid protein(s) around the nucleic acid. In this context, it is interesting that VP90 has been observed to form oligomers (dimers and trimers) in virus-infected Caco-2 cells, suggesting that this protein can assemble into intermediary structures during virion assembly [53]. Moreover, the N-terminal region of VP90 has a basic character that is conserved among astrovirus and is believed to interact with gRNA during packaging and assembly [54]; however, there is no information yet about encapsidation signals in the astrovirus gRNA.

Role of Caspases

Although there are no reports exploring the role in virus assembly of the C-terminal region of the structural polyprotein, it is known that it has to be cleaved by caspases to yield the mature virion and to promote egress of the virus. Analysis of HAstV-8-infected cells by density ultracentrifugation showed that intracellular viral particles

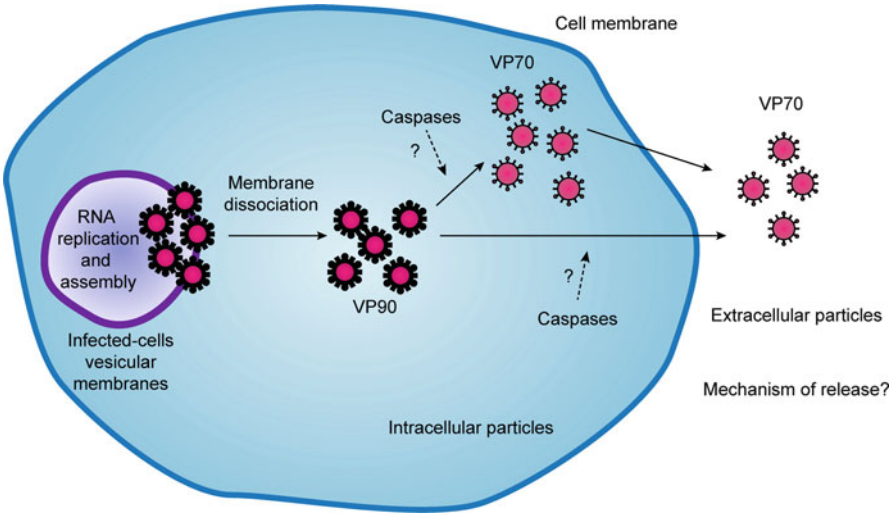


Fig. 2.6 Model of astrovirus particle maturation and cell egress. See details in the text. Modified from Méndez and Arias [53]

consisting of VP90 were present in association with membranes as well as in membrane-free fractions [53]. Based on these and related findings [3], a model was proposed in which the viral progeny assemble in membranous structures, and then dissociate from the membranes by an unknown mechanism; once in a membrane-free environment the assembled VP90 is then proteolytically cleaved at the C-terminal end to yield viral particles containing VP70 (Fig. 2.6). This cleavage is blocked by caspase inhibitors and induced by TRAIL (TNF-related apoptosis ligand), suggesting an active role of caspases in the process [57], although it is not clear if VP90 processing occurs intracellularly or concomitantly with cell egress of the virus. The involvement of caspases in this process is supported by the presence of an acidic region in the C-terminal region of VP90, conserved in astroviruses, that contains several putative caspases recognition motifs [57].

It has been reported that caspases-3 and -6 were activated during HAstV-8 infection of Caco-2 cells, as judged by cleavage of their respective substrates lamin A and PARP (poly ADP-ribose polymerase). The cleavage of PARP occurred at 12 h postinfection, in coincidence with the cleavage of VP90 to VP70 [3, 57]. Furthermore, executioner caspases-3/7, as well as initiator caspases-4, -8, and -9, were all activated at 12 h postinfection [3] as determined by the cleavage of preferential substrates of these caspases [3]. In addition, the precursor forms of all evaluated caspases were processed in these cells, confirming their active status, and suggesting that more than one caspase is active during astrovirus infection [3]. In contrast with these results, neither the apoptosis mediated by the mitochondrial pathway nor caspase-9 was activated in HAstV-4-infected Caco-2 cells, although the processed product of pro-caspase-8 was detected, suggesting its activation [28].

Although several caspases become active in astrovirus-infected cells, it is not clear which of these proteases mediate the VP90–VP70 cleavage. Knocking down the activity of caspases by RNA interference showed that caspases-3, -4, and -9, but not caspase-8, are involved in this process in infected cells [3]. *In vitro* translation of truncated forms of HAstV-8 VP90 suggests that the caspase motif $TYVD_{657}$, in the C-terminal acid region of VP90, is the most likely to be cleaved to yield VP70, although cleavages at motifs $DEVD_{666}$ and $EETD_{672}$ could generate the 75 to 82 kDa processing intermediate products of VP90 that have been observed in infected cells (Fig. 2.4) [3, 54]. In agreement with this observation, the recently reported crystallographic structure of the HAstV-8 capsid spike suggested that the aspartic acid residue at position 657 should be accessible to caspases in intact particles [16].

Caspase activation requires the translation and/or replication of the astrovirus genome, since psoralen-inactivated viruses failed to induce caspase activity [3]. The viral component responsible for this induction is still unknown; however, a putative death domain, similar to those present in proteins known to actively induce caspase activation and apoptosis, was found in nsp1a by bioinformatic analysis (Fig. 2.3) [28] . The cell expression of ORF1a has also been reported to induce apoptosis [29]; whether this effect is connected with the presence of the proposed death domain needs further investigation. Processing of viral proteins by caspases that result in the pro- or antiapoptotic activities of the processed viral polypeptides has been reported for other viruses such as influenza A and B [78], hepatitis C [62], and coronavirus [17]. However, astrovirus is one of the few viruses described in which caspase cleavage of viral proteins is required for the life cycle of the virus.

Virus Egress

The assembly of viral particles whose infectivity can be activated by *in vitro* treatment with trypsin starts after 8 h postinfection, reaching the highest level at 12 h postinfection (Fig. 2.7). The number of viral particles increased both intracellularly and in the cell medium. At 12 h postinfection only about 10% of the total particles were found extracellularly [3], and at 24 h postinfection the released virus accounted for about 30–50% of total viruses. The VP90–VP70 processing is increased by treatment of cells with the apoptosis inducer TRAIL, and concomitantly with this enhanced cleavage, a four to fivefold increase in the virus released to the cell medium was observed [57].

Although the cellular process triggered by caspase cleavage of VP90 to allow virus exit is unknown, it seems that cell death is neither induced by, nor required for this process, and rather a nonlytic mechanism seems to be involved [3]. Caspase activation does not always result in cell death or secondary necrosis and the activation level of these proteases and their interaction with cellular proteins modulate cell death progression [41]. The requirement of VP90 processing by caspases for virus exit has been confirmed through the use of caspase inhibitors. A potent inhibitor for executioner caspases-3/7, Ac-DEVD-CHO, blocked virus release at levels similar to

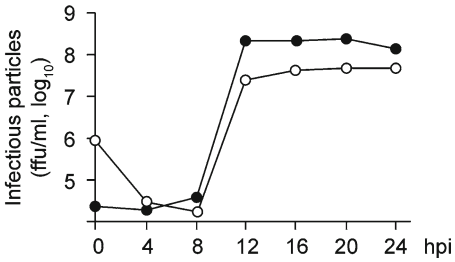


Fig. 2.7 Kinetics of infectious virus production. Caco-2 cells were infected with HAstV-8, and at different time points (from 0 to 24 hpi) the extracellular and cell-associated fractions were analyzed for infectious viral particles. Virus in the extracellular (*empty circles*) and cell-associated (*filled circles*) fractions was determined after trypsin treatment [3]

those induced by the pan-caspase inhibitor Z-VAD-fmk. On the other hand, inhibitors of initiator caspases-8 and -9 had no effect on virus egress [3]. Thus, although VP90 can be cleaved in astrovirus-infected cells by caspases-3 and -9, as described earlier, virus exit seems to depend on the activity of caspases 3/7 but not of caspase-9, and most probably on the activity of caspase-3, since RNA interference of the expression of this caspase has a strong negative effect on virus release [3].

Previous reports have associated the activity of executioner caspases with the intracellular transport of viral proteins, as in the case of influenza virus, where the activity of caspase-3 was required for the intracellular transport of NP protein [77]. The release of nonenveloped viruses, such as astrovirus, is generally believed to occur by cell membrane destabilization. However, rotavirus and poliovirus exit the cells through a nonlytic mechanism, and in the later case, probably through autophagic vesicles [32]. Thus, intracellular transport of astrovirus particles could be important for cell exit. It has not been determined whether autophagy is induced during astrovirus infection, although double membrane vesicles have been observed in astrovirus-infected cells [26, 29]. Some astrovirus strains have been shown to cause cytopathic effect on cultured cells and necrosis of hepatic cells in natural infections [26]. It is unclear which astrovirus-cell interaction leads to virus egress via a lytic or nonlytic process.

Perspectives

The perception of the relevance of astroviruses in public health is changing with the recent discovery of novel viruses isolated from humans that show genetic similarities with animal strains, some of which have been associated with diseases other than gastroenteritis. The continued application of high-resolution structural cryo-electron microscopy and X-ray crystallography of the virus and viral proteins will allow us to gain insight into the different stages of the life cycle of these viruses. Identification of the cell receptors and the entry pathway used by astroviruses to infect susceptible cells in culture, but most importantly to infect the target cells in a natural infection, will be of utmost importance to understand the biology of these

viruses and to control infection. Significant gaps exist in our understanding of the translation and replication of the viral genome; identification of viral and cellular proteins that participate in these processes will be fundamental to elucidate the mechanisms involved. The cell response to astrovirus infection has proved already to be beneficial for the early interactions of the virus with the cell, as well as for the last step of virus morphogenesis; however, genomic and proteomic approaches will be required to significantly advance our understanding of the virus–host cell interactions that lead to productive and efficient infection. The recently improved reverse genetics system developed for astroviruses will also be key to identify the molecular determinants involved in the virus replication cycle.

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