Hepatitis A Virus Genome Organization and Replication Strategy

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Hepatitis A virus (HAV) is a positive-strand RNA virus classified in the genus *Hepatovirus* of the family *Picornaviridae*. It is an ancient virus with a long evolutionary history and multiple features of its capsid structure, genome organization, and replication cycle that distinguish it from other mammalian picornaviruses. HAV proteins are produced by cap-independent translation of a single, long open reading frame under direction of an inefficient, upstream internal ribosome entry site (IRES). Genome replication occurs slowly and is noncytopathic, with transcription likely primed by a uridylated protein primer as in other picornaviruses. Newly produced quasi-enveloped virions (eHAV) are released from cells in a nonlytic fashion in a unique process mediated by interactions of capsid proteins with components of the host cell endosomal sorting complexes required for transport (ESCRT) system.

Following discovery of the hepatitis A virus (HAV) particle in human fecal material using immunoelectron microscopy in 1973 (Feinstone et al. 1973), there was considerable controversy concerning the nature of the HAV genome and classification of the virus. This was resolved by the molecular cloning of the genome in the 1980s (Ticehurst et al. 1983; Najarian et al. 1985), which confirmed that it was RNA and placed classification of the virus squarely within the picornavirus family (*Picornaviridae*). Early studies supported its inclusion within the *Enterovirus* genus (Gust et al. 1983), but as evidence distinguishing it from other picornaviruses continued to accumulate it was classified within a new genus, *Hepatovirus* (King et al. 2012). Recent studies have revealed that HAV is evolutionarily distinct from other picornaviruses, not only

in terms of the nucleotide sequence of its genome, but also in the structure of its capsid, which shares features in common with primitive insect viruses (Wang et al. 2015; Stuart et al. 2018). Although for many years only humans and other primate species were thought to be susceptible to infection by hepatoviruses, multiple distinct hepatovirus species closely related to human HAV have been recognized recently in various species of small mammals and are now classified within the same genus (Anthony et al. 2015; Drexler et al. 2015; Yu et al. 2016; Sander et al. 2018). These relatively recent insights have led to the realization that HAV is a truly ancient virus with a long association with a range of mammals.

In contrast to many other picornaviruses, including the well-studied poliovirus, HAV

Editors: Stanley M. Lemon and Christopher Walker

Additional Perspectives on Enteric Hepatitis Viruses available at www.perspectivesinmedicine.org

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Cite this article as *Cold Spring Harb Perspect Med* 2018;8:a033480

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does not shut down cellular protein synthesis in infected cells, and generally replicates without cytopathic effect (Provost et al. 1981; Binn et al. 1984; Gauss-Muller and Deinhardt 1984). One of the most interesting features of the virus is its recently recognized capacity for non-lytic release from infected cells as membrane-wrapped, quasi-enveloped infectious virions (eHAV) (Fig. 1) (Feng et al. 2013). eHAV particles have a specific infectivity similar to that of the naked virion, and represent a second form of infectious virus. Their mechanism of biogenesis appears to be unique among the *Picornaviridae*.

Given the public health threat posed by HAV infection, the main driver for HAV research following discovery of the virus was the need for a safe and efficacious vaccine. With proof that inactivated vaccines could be produced from infected cell cultures (Binn et al. 1988), and that commercially produced vaccines were highly protective against hepatitis A (Werzberger et al. 1992; Innis et al. 1994; Shouval 2018), interest in HAV research waned substantially. This interest has been rekindled in recent years by the discovery of quasi-enveloped virus and a deepening appreciation of the differences that exist between HAV and other mammalian picornaviruses (Feng et al. 2013; Drexler et al. 2015; Wang et al. 2015). However, much of what is known (or suspected) of the molecular mechanisms involved in HAV replication rests heavily on studies of other picornaviruses.

CELL CULTURE PROPAGATION OF HAV

HAV was first isolated in cell culture in 1979, and the infectivity of synthetic RNA transcripts derived from molecularly cloned HAV was first reported in the late 1980s (Provost and Hilleman 1979; Cohen et al. 1987a). Among all human hepatitis viruses, it is the most readily isolated in cultured cells. A variety of mammalian cell cultures, including African green monkey kidney cells and fetal rhesus kidney cells, are permissive for replication (Daemer et al. 1981; Binn et al. 1984; Feng et al. 2013). Huh-7.5 cells, derived from a human hepatoma and selected for the ability to support replication of a subgenomic hepatitis C virus (HCV) replicon (Blight et al. 2002), are especially permissive. In all cell types, HAV replication is slow and generally noncytopathic, typically resulting in persistent infection. Electron microscopic examination of HAV-infected cells usually fails to reveal morphologic changes other than relatively minor rearrangements of intracellular membranes (Teterina et al. 1997; Gosert et al. 2000b). Wild-type virus recovered directly from humans often requires several weeks incubation before viral antigen becomes detectable in cell culture (Daemer et al. 1981; Binn et al. 1984).

HAV can be adapted to more efficient replication in cell culture by continued passage. This is associated with mutations in an essential non-structural protein, 2B, and in the 5' untranslated region (5'UTR) of the genome (Emerson et al.

Figure 1. Quasi-enveloped and naked, nonenveloped hepatitis A virus (HAV) virions. Electron microscopic images of negatively stained quasi-enveloped virions (eHAV) (panels i–iv) and naked, 27 nm nonenveloped (panel v) virions recovered from supernatant fluids of infected Huh-7.5 cells. (From Feng et al. 2013; adapted, with permission.)

1993; Schultz et al. 1996b; Yi and Lemon 2002). Continued passage of the virus in cell culture also leads to its attenuation in primates (Provost et al. 1983; Lemon et al. 1990; Funkhouser et al. 1994; Emerson et al. 2002). Although this raised early hopes for development of an oral, Sabin poliovirus-like attenuated hepatitis A vaccine, later studies showing that attenuation of HAV pathogenicity was associated with an unacceptable loss of immunogenicity led to this approach being abandoned (Midthun et al. 1991). Very highly passaged, rapidly replicating, cytopathic variants of HAV have been isolated that induce apoptosis in infected cells (Lemon et al. 1991; Brack et al. 1998). Their virulence has not been assessed in primates, but is likely to be low. The cytopathic effect is linked to enhanced replication capacity, and is associated with multiple mutations throughout the genome (Lemon et al. 1991; Zhang et al. 1995).

Although cytopathic, cell culture-adapted variants such as HM175/18f can be used in classical plaque assays (Zhang et al. 1995), methods for quantifying infectious HAV have generally relied on detection of intracellular viral antigen or RNA. A quantal radioimmuno-focus assay (RIFA) was developed in the early 1980s using radioiodinated anti-HAV antibody to demonstrate the presence of replication foci developing beneath an agarose overlay (Lemon et al. 1983). The assay was subsequently adapted for detection of neutralizing anti-HAV antibodies (Lemon and Binn 1983b; Lemon et al. 1997), and more recently modified to use infrared fluorescent anti-HAV probes (Counihan et al. 2006).

GENOME STRUCTURE AND ORGANIZATION

The HAV genome is a single-stranded, positive-sense RNA molecule approximately 7500 nucleotides in length that serves directly as messenger RNA for translation of HAV-encoded proteins (Fig. 2A). It has an exceptionally low G+C content compared to other picornaviruses (~38% overall) (Cohen et al. 1987b), and a very unusual pattern of codon usage (see Pintó et al. 2018). There is no cap at its 5' end, which terminates

instead in a covalent linkage to a small, virally encoded protein, VPg (or 3B), the putative protein primer for RNA synthesis (Weitz et al. 1986). Like cellular messenger RNAs (mRNAs), the 3' end terminates in a relatively lengthy poly (A) tail (Baroudy et al. 1985; Najarian et al. 1985).

Multiple strains of human HAV have been molecularly cloned and sequenced, but HM175, a strain recovered in Melbourne, Australia, has been the most intensively studied (Gust et al. 1985; Cohen et al. 1987b). The genome contains a single long open reading frame (ORF) flanked by a relatively lengthy 735 nt 5'UTR segment comprising ~10% of the genome, and a short 63 nt 3'UTR (Fig. 2A). Both UTRs contain significant RNA secondary structure. The 5'UTR fold was established by identifying covariant nucleotide substitutions in several different strains of HAV and mapping sites where single- and double-stranded RNA (dsRNA)-specific nucleases cleave synthetic RNA (Fig. 3A) (Brown et al. 1991a). A small 5' terminal hairpin is followed by two potential pseudoknots that may function as RNA replication signals, and a short polypyrimidine tract, pY1, extending from nts 99–138 (Brown et al. 1991a; Hardin et al. 1999). Small deletion mutations in this region of the 5'UTR confer a temperature-sensitive replication phenotype, but the pY1 tract is not essential for either efficient replication in cultured cells or pathogenicity in primates (Shaffer et al. 1994; Shaffer and Lemon 1995). Its function, if any, is unknown. Further downstream, comprising the 3' half of the 5'UTR, are a series of complex structural elements that act as an internal ribosome entry site (IRES) and direct the cap-independent translation of HAV proteins (Fig. 3A).

Secondary RNA structure within the 3'UTR (Fig. 3C) has not been as carefully studied, but may be important for binding specific host and/or viral proteins involved in the initiation of minus-strand RNA synthesis (Kusov et al. 1996, 1997). Elsewhere within the genome, located internally within the ORF, is a large complex stem-loop that functions as a *cis*-acting replication element (*cre*) that is essential for viral RNA synthesis (Fig. 3B) (Yang et al. 2008).

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Figure 2. Organization of the hepatitis A virus (HAV) RNA genome and processing of the polyprotein. (A) Genome organization, showing the 5' covalently-linked VPg (3B) protein, 5' untranslated region (5'UTR), the internal ribosome entry site (IRES), *cre*, 3'UTR, and 3' poly(A) tail. The open reading frame (ORF) encoding the polyprotein is shown as a box, with cleavage sites leading to production of mature structural (P1 segment, 1A–1D, shaded) and nonstructural (P2–P3 segment, 2B–3D, open) proteins. (B) Polyprotein processing. The primary cleavage between VP1pX (1D) and 2B is likely to occur cotranslationally. Processing of the nonstructural proteins results in multiple processing intermediates. The kinetics are not known in detail, but cleavages between 2B and 2C, and 3C and 3D appear to be favored (Martin and Lemon 2002). Most scission events are mediated by the 3C^{pro} cysteine proteinase (“*”). Exceptions are the VP4–VP2 cleavage, which is autocatalytic (“A”) and activated by packaging of the RNA genome, and excision of the pX domain from VP1pX after loss of the quasi-enveloped virion (eHAV) membrane, which is mediated by an unknown host protease (“H”).

POLYPROTEIN PROCESSING

The large ORF encodes a single ~2230 amino acid polyprotein that is processed into 10 mature proteins primarily by a single virally encoded proteinase, 3C^{pro} (Fig. 2B) (Schultheiss et al. 1994; Gosert et al. 1996; Martin and Lemon 2002). Three large structural proteins are produced from the amino terminal P1 segment of the polyprotein: VP0, VP3, and VP1pX. These are alternatively referred to as 1AB, 1C, and 1D, according to a well-established, systematic nomenclature for picornaviruses (Rueckert and Wimmer 1984). Sixty copies of each of these assemble to form the viral capsid, with VP0 (1AB) undergoing a maturation cleavage resulting in VP4 (1A) and VP2 (1B) (Fig. 2B) following packaging of the RNA genome. VP1pX is the largest of the structural proteins, 38.5 kDa in

mass, and is found in early morphogenesis intermediates and in eHAV released from cells (Anderson and Ross 1990; Feng et al. 2013). An 8.3 kDa fragment (“pX”) is cleaved from the carboxy terminus of VP1pX by an unknown cellular protease (Fig. 2B) following disruption of the eHAV membrane (Graff et al. 1999; Martin et al. 1999; Feng et al. 2013), resulting in the mature 31.2 kDa VP1 protein found in naked, nonenveloped virions excreted from infected animals and humans. The VP1–pX cleavage is efficient, but leaves VP1 with a heterogeneous carboxyl terminus (Graff et al. 1999).

The primary cleavage event in polyprotein processing occurs between VP1pX (1D) and 2B (Fig. 2B) and has been a source of confusion in the field as early, inaccurate annotations of genome sequences have persisted in on-line databases. In contrast to most other picornaviruses,

Figure 3. Secondary RNA structure within the positive-strand hepatitis A virus (HAV) RNA. (A) Secondary structure within the 5' untranslated region (5'UTR) has been established by analysis of covariant substitutions in sequences of different hepatoviruses and nuclease mapping coupled with Mfold predictions (Brown et al. 1991a). Functional assays show that internal ribosome entry site (IRES) activity is dependent upon stem-loops III, IV, and V (Brown et al. 1991a). Nuclease mapping suggests the polypurimidine pY1 track is ordered, and biophysical studies suggest it may form several short stem-loops stabilized by noncanonical U-U and C-C base pairs (Hardin et al. 1999). The two initiator AUG codons are highlighted. (B) Secondary structure of the HAV *cre*, which is located in the 3D-coding region. It is essential for RNA replication and functions in a position-independent fashion (Yang et al. 2008). Adenines in the apical loop (circled bases) likely template slide-back uridylation of VPg. (C) Mfold prediction of secondary RNA structure in the 3'UTR ($\Delta G = -37.6$). The UGA terminator codon is highlighted. Additional stem-loop structures are predicted to exist within the 3D^{pol}-coding sequence.

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HAV lacks a nonstructural 2A protein, which is a proteinase in enteroviruses that cleaves structural protein precursors (the “P1” segment) from the remainder of the polyprotein, and an autocatalytic polypeptide in aphthoviruses and cardioviruses that serves a similar function (Martin and Lemon 2002). The carboxy-terminal 8 kDa pX fragment of VP1pX has often been referred to as “2A,” but it is a structural protein with major roles in capsid assembly (Harmon et al. 1995; Martin et al. 1995) and (in unpublished work from our laboratory) quasi-envelope of the capsid. Mutations in pX have been shown to influence virulence in tamarins, which additionally points to its critical involvement in pathogenesis (Harmon et al. 1995; Emerson et al. 2002).

Six nonstructural proteins, each involved in replication of the HAV RNA, are derived from the remaining P2 and P3 segments of the polyprotein: 2B, 2C, 3A, 3B, 3C^{pro}, and 3D^{pol} (Fig. 2B). These proteins are all intimately associated with membranes (Jecht et al. 1998). Like other picornaviruses, the 2C protein contains a helicase motif and, when overexpressed (with or without 2B as 2BC), induces substantial membrane rearrangements in cells (Teterina et al. 1997). The 3A protein provides a membrane anchor for 3B (Pisani et al. 1995), the genome-linked protein (VPg) that likely serves as a protein primer for RNA synthesis. Interestingly, 3A is targeted to mitochondrial membranes (Yang et al. 2007). 3C^{pro} is a cysteine protease, albeit with a fold similar to the serine protease chymotrypsin, and was one of the first viral proteases to be characterized structurally (Chernaia et al. 1993; Allaire et al. 1994). The 3ABC processing intermediate is unique among picornaviruses in that it is relatively stable and has distinct activities in particle assembly (Probst et al. 1998) as well as disruption of innate immunity (Yang et al. 2007; Feng and Lemon 2018). 3D^{pol} is the viral RNA-dependent RNA polymerase and the catalytic core of the replicase complex, but unlike the poliovirus polymerase, has never been expressed in active form (Tesar et al. 1994). Processing at the 3CD site is more efficient than at 3AB and 3BC, but like 3ABC, 3CD is proteolytically active and contributes to disruption of in-

nate immunity (Probst et al. 1998; Qu et al. 2011).

VIRION STRUCTURE

Nonenveloped, “Naked” HAV Virions

Although naked HAV virions have a composition similar to other picornaviral capsids, with 60 copies of each of the four mature capsid proteins (VP4, VP2, VP3, and VP1) arranged in a pseudo $T=3$ symmetry, the HAV capsid has numerous distinguishing features (Wang et al. 2015; Stuart et al. 2018). Morphogenesis begins with the formation of protomers composed of VP0, VP3, and VP1 that assemble into 14S pentamer subunits that share partial antigenicity with the mature icosahedral capsid (Stapleton et al. 1993). In other picornaviruses, VP4 is amino-terminally myristoylated and serves to nucleate the assembly of pentamers. This is not the case in HAV, in which VP4 has a much smaller molecular mass and is not myristoylated (Tesar et al. 1993). Pentamer assembly is driven instead by intrinsic signals in the amino terminal half of the pX domain in VP1pX, whereas VP4 appears essential for further assembly of pentamers into 70S empty capsids (Probst et al. 1999; Cohen et al. 2002). Empty capsids are abundant in virus preparations, but whether they are intermediates in the assembly of infectious virions is unknown.

As mentioned above, VP0 is cleaved to VP4 and VP2 following packaging of the RNA genome. The packaging mechanism is poorly understood, but studies with enteroviruses suggest a key role for the 2C helicase, and RNA-activated autocatalytic processing of VP2 (Jiang et al. 2014a). As in other picornaviruses, VP0 cleavage is associated with an increase in specific infectivity and likely conformational changes in the HAV capsid (Bishop and Anderson 1993). However, it is inefficient and proceeds with slow kinetics in HAV capsids isolated from infected cell cultures.

Mature nonenveloped virus has a sedimentation coefficient of 144S and bands in CsCl at a density of 1.325 gm/cm³, indicating a tight capsid structure (Lemon et al. 1985; Wang et al.

2015). X-ray crystallography has recently revealed details of the capsid structure at high resolution, including the existence of a unique VP2 domain swap found in no other mammalian picornavirus (Wang et al. 2015; Stuart et al. 2018). The surface of the capsid is relatively featureless: there is no “canyon” surrounding the fivefold axis of symmetry like that into which the enteroviral receptor fits. The HAV capsid is substantially more stable at low pH and high temperatures than other picornaviruses (Siegl et al. 1984; Scholz et al. 1989).

There is a high degree of antigenic conservation among different strains of human HAV (Lemon and Binn 1983a; Stapleton and Lemon 1987; Ping and Lemon 1992). Important antigenic determinants are conformational in nature, and not recapitulated by independently expressed capsid proteins. Remarkably, the binding of postconvalescent, polyclonal human antibodies to the virus can be blocked almost completely by a combination of only two monoclonal antibodies (Ping and Lemon 1992). Neutralization epitopes map to one large cluster, with substitutions at residue 70 of VP3 dominating selection of monoclonal antibody escape mutants (Ping and Lemon 1992). However, more recent studies have expanded the range of capsid residues contributing to antigenic sites (Wang et al. 2015). The antigenic conservation of the HAV capsid extends to distantly related hepatoviruses identified recently in bats, which bind monoclonal antibodies raised to human HAV (Drexler et al. 2015).

Quasi-Enveloped eHAV Virions

Although naked, nonenveloped virions are shed in the feces of infected humans and nonhuman primates, only quasi-enveloped eHAV virus is detected in serum (Feng et al. 2013). The available evidence suggests that eHAV is secreted across the apical plasma membrane of hepatocytes into the proximal biliary canaliculi, where the membrane is stripped from the capsid by the detergent action of bile salts (Hirai-Yuki et al. 2016b). Most virions present in supernatant fluids of infected cell cultures are also quasi-envel-

oped (Feng et al. 2013; Hirai-Yuki et al. 2016b; Das et al. 2017).

Quasi-enveloped eHAV virions are between 50 and 110 nm in diameter and have a buoyant density of ~ 1.10 gm/cm³ in iodixonal, in contrast to naked virions that are ~ 27 nm in diameter and band in iodixanol at a buoyant density of ~ 1.25 gm/cm³ (Feng et al. 2013). Electron microscopy of negatively stained eHAV preparations show membranous vesicles containing one or two, occasionally three, RNA-containing capsids (Fig. 1). As mentioned above, these capsids contain predominantly unprocessed VP1pX, rather than the mature VP1 found in mature naked virions (Fig. 2B). The enveloping membranes completely occlude the capsid, preventing detection of capsid antigen in the absence of detergents and protecting it from antibody-mediated neutralization (Feng et al. 2013). Quasi-enveloped virions resemble exosomes and present no viral glycoproteins (peplomers) on the membrane surface, a feature that distinguishes them from conventional enveloped viruses (Feng et al. 2014). Yet, their specific infectivity is similar in cultured cells to that of naked virions (Feng et al. 2013). Consistent with the lipid membranes that cloak these virions, eHAV infectivity is lost upon extraction with organic solvents such as chloroform.

A recent quantitative proteomics analysis using stable isotope labeling with [¹³C] and [¹²C] (SILAC) followed by tandem mass spectrometry (MS/MS) identified 105 host proteins that were enriched in gradient-purified eHAV compared to unrelated extracellular vesicles banding at a similar density in iodixanol gradients (McKnight et al. 2017). The eHAV-associated proteins were highly enriched for endolysosomal components ($>60\%$, $p < 0.001$) and included many common exosome-associated proteins, such as the tetraspanin CD9 and dipeptidyl peptidase 4 (DPP4) as well as multiple proteins associated with the endosomal sorting complexes required for transport complex III (ESCRT-III), including charged multivesicular body protein (CHMP)1A/1B, CHMP4B, IST, and apoptosis-linked gene 2-interacting protein X (ALIX). eHAV biogenesis is discussed below. Importantly, antibodies to ALIX and DPP4 pre-

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cipitate eHAV present in serum from infected humans (McKnight et al. 2017).

THE HAV REPLICATION CYCLE

Cell Entry

Quasi-envelopment has important implications for cellular entry of the virus. Although infection typically results from ingestion of naked, non-enveloped virions, it is not clear which cell types are first infected, or even whether there is a primary site of replication within the gut (Mathiesen et al. 1978; Asher et al. 1995). On the other hand, once infected, spread of the virus within the host appears to involve quasi-enveloped virus, as only eHAV is detected in serum or plasma during acute infection (Feng et al. 2013). The mechanism by which eHAV enters cells must a priori differ from that of the naked virion, although it seems likely that they might share later steps in the entry process, such as interactions of the capsid with a specific cellular receptor. Such receptor interactions are central to the entry and uncoating of other picornaviruses, as well as delivery of their RNA genomes across endosomal membranes (Strauss et al. 2015).

T-cell immunoglobulin and mucin domain-containing protein 1 (TIM1) is a phosphatidylserine (PtdSer) receptor involved in scavenging of apoptotic cells, and it facilitates the attachment and entry of many enveloped viruses that display PtdSer on their surface (Moller-Tank et al. 2013). It was reported to be an essential receptor for HAV in 1996, and has otherwise become known as “HAV cellular receptor 1 protein” (HAVCR1) (Kaplan et al. 1996; Feigelstock et al. 1998). However, recent studies show TIM1 is not an essential entry factor for HAV. Clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9-mediated TIM1 knockout does not impair the ability of quasi-enveloped eHAV or HAV to infect Huh-7.5 human hepatoma cells (Das et al. 2017). TIM1 does seem to play a minor role in facilitating eHAV entry in Vero cells, most likely by binding PtdSer residues present on the surface of the eHAV membrane (Feng et al. 2015; Das et al. 2017). However, *Tim1*^{-/-}*Ifnar1*^{-/-} double-knockout

mice are susceptible to infection on intravenous virus challenge, with only modest reductions in fecal HAV shedding and serum alanine aminotransferase (ALT) elevations compared to *Ifnar1*^{-/-} mice (Das et al. 2017). These recent results point to a large gap in our understanding of HAV biology. Most studies investigating cellular entry of the virus were done prior to the recognition of quasi-enveloped eHAV, and in retrospect the nature of the virus inoculum used in these studies is often uncertain.

The initial uptake of eHAV into cells is likely to occur by mechanisms similar to those by which cells internalize exosomes. Unfortunately, these remain poorly understood. Clathrin-mediated endocytosis, macropinocytosis, and phagocytosis have all been implicated (Feng et al. 2010; Tian et al. 2014; van Dongen et al. 2016). PtdSer residues and adhesion molecules (integrins and tetraspanins) on exosome membranes contribute to the uptake of exosomes (Clayton et al. 2004; Hoshino et al. 2015; van Dongen et al. 2016), and PtdSer exists on the eHAV surface and promotes its efficient uptake by plasmacytoid dendritic cells (Feng et al. 2015). Integrin-β3 and the tetraspanins CD9 and CD63 are also associated with the eHAV membrane (McKnight et al. 2017).

eHAV has been shown to enter cells via an endocytic pathway with slow removal of its enveloping membrane rendering the capsid subject to neutralization by antibody in an endosomal compartment, even when antibody is added to cell cultures 6–8 h after removal of an eHAV inoculum (Feng et al. 2013; Das et al. 2017). The naked virion enters more rapidly, and is not subject to postendocytic neutralization. The lysosomal poison, chloroquine, strongly inhibits eHAV entry, and has much less effect on entry of naked HAV virions (Feng et al. 2013). It seems likely that endocytosed eHAV undergoes transport to a late endosome/lysosomal compartment where its membranes are degraded by lysosomal enzymes, providing the capsid access to a cellular receptor. A similar entry scheme has recently been suggested for quasi-enveloped hepatitis E virus (eHEV) (Yin et al. 2016). Naked HAV may interact with the same receptor, but in an early

endosome. Specific mechanisms of endocytosis of the two types of virions remain to be established.

Uncoating of the Genome

The final step in picornaviral entry is delivery of the RNA genome across the endosomal membrane to the cytosol. Early studies demonstrated that uncoating of HAV proceeds in a slow and asynchronous fashion (Bishop and Anderson 2000). Enteroviral capsids deliver their genomes to the cytosol via an “umbilicus” leading to a membrane pore formed by the myristoylated VP4 protein and the amino terminus of VP1 that extends from the capsid upon receptor binding (Strauss et al. 2013; Panjwani et al. 2014). VP4 may contribute to a similar process in HAV entry, even though it is not myristoylated (Tesar et al. 1993), as it appears to have direct membrane pore-forming activity (Shukla et al. 2014). However, the absence of any apparent receptor interaction site in the recently solved X-ray structure of the HAV capsid has fueled speculation that the virus may enter and uncoat by a mechanism completely different from other picornaviruses, with the capsid possibly entering the cytosol intact and uncoating intracellularly (Wang et al. 2015).

Viral Protein Translation

Once HAV RNA has been delivered to the cytosol, the replication cycle commences with protein translation to produce both structural proteins as well as the nonstructural proteins necessary for establishing an RNA replication complex. VPg is likely removed from the genomic RNA soon after its arrival in the cytosol, and translation of the polyprotein is initiated in a 5' cap-independent fashion under control of the IRES, which is comprised of stem-loops III, IV, and V (Fig. 3A) of the 5'UTR (Brown et al. 1994). The highly ordered RNA structure of the IRES functions in association with canonical host-cell translation initiation factors and various accessory proteins (Brown et al. 1991a,b; Glass et al. 1993). 40S ribosome subunits appear likely to land on the viral genome close to or at

the initiation codon, with little if any 5' to 3' scanning on the RNA. Translation initiates at one of two closely spaced AUG initiator codons, resulting in a heterogeneous amino-terminal VP4 sequence (Tesar et al. 1992).

The HAV IRES is a type III IRES (Brown et al. 1994; Beales et al. 2003), differing significantly in its structure (Fig. 3A) from other picornaviral IRES elements, and it is relatively inefficient in directing translation in cultured cells (Whetter et al. 1994). It requires intact cellular eIF4G to initiate translation (Borman and Kean 1997; Ali et al. 2001), which distinguishes it from the IRES elements of enteroviruses or aphthoviruses that encode proteases that shut down cellular mRNA translation by cleaving this host translation initiation factor. HAV does not shut down host mRNA translation, which proceeds in parallel with viral translation (Gauss-Muller and Deinhardt 1984).

Cell culture-adaptive mutations within the HAV IRES contribute to enhanced replication and cell-type-specific translation efficiency (Day et al. 1992; Schultz et al. 1996a). Poly(rC)-binding protein 2 (PCBP2), polypyrimidine-tract-binding protein (PTB), and La protein, as well as GAPDH, which binds the RNA specifically, have all been found to positively or negatively regulate IRES activity (Chang et al. 1993; Graff et al. 1998; Gosert et al. 2000a; Yi et al. 2000; Jiang et al. 2014b). The relatively weak activity of the HAV IRES coupled with suboptimal codon bias throughout the ORF contribute to slow and inefficient translation (Whetter et al. 1994; Pintó et al. 2007, 2018), an HAV attribute that may have evolved to reduce cytopathology in the liver and/or facilitate folding of the very stable capsid. 3C^{pro}-mediated processing of the polyprotein, as outlined above (Fig. 2B), probably commences simultaneously with translation as soon as the proteinase is expressed.

Viral RNA Synthesis

Picornaviruses usurp cellular proteins and membranes to establish membranous replication factories that are essentially mini-organelles. The membranes likely protect viral replication intermediates from being sensed by

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cytoplasmic pathogen recognition receptors such as the RIG-I-like helicases, and also provide a platform for assembly of a multiprotein replication complex. Ectopic expression of the HAV 2BC and 2C proteins induces cytoplasmic membrane rearrangements as well as increases in membrane permeability (Teterina et al. 1997; Jecht et al. 1998; Kusov et al. 1998; Gosert et al. 2000b). Thus, as with other picornaviruses, 2B and 2C appear to cooperatively orchestrate the redirection of intracellular membranes to the formation of replication complexes. These membranes are likely derived from the endoplasmic reticulum, although it is interesting that 3A, which anchors 3ABC to membranes, has a transmembrane domain that targets its delivery to mitochondrial membranes (Pisani et al. 1995; Ciervo et al. 1998; Yang et al. 2007). Replication complexes, identified by staining with antibodies specific for dsRNA, lie in close proximity to both mitochondria and ER. Host factors are clearly involved in establishing and maintaining these HAV replication factories, but have not been extensively studied. Recent studies demonstrate the dependence of RNA replication on adenosine-triphosphate-binding cassette transporters and FK506-binding proteins (Esser-Nobis et al. 2015). Unlike the membranous replication complexes of HCV, also a positive-strand RNA virus, HAV replication complexes are not dependent upon host phosphatidylinositol 4-kinase III α / β (PI4K III α / β). HAV RNA synthesis can be inhibited by guanidine as well as cyclosporine A (Cho and Ehrenfeld 1991; Esser-Nobis et al. 2015).

HAV probably uses the same strategy as other picornaviruses to synthesize new genomes (Fig. 4). A minus-strand copy of the incoming positive-strand genome is first made, and the resulting dsRNA intermediate is then used as a template to produce new positive-strand genomes. Minus-strand RNA is of low abundance and difficult to detect in infected cells (Schmitz and Dotzauer 1998). The 3D^{pol} polymerase is the catalytic core of the replication complex, with minus-strand RNA synthesis likely primed by a uridylated form of VPg (3B, VPg-pUpU) binding to the genomic 3' poly(A) sequence (Fig. 4 inset). Poliovirus VPg is uridylated via a

slide-back mechanism by a complex of 3D^{pol} and 3CD interacting with the adenine residues in the poliovirus *cre* (Paul et al. 2003). The HAV *cre* (Fig. 3B), located within the 3D-coding region of the ORF (Fig. 2A), has a similar arrangement of adenines within its terminal loop and functions in a position-independent fashion (Yang et al. 2008), which suggests a similar mechanism for uridylation. The 3'UTR likely adopts secondary structure (Fig. 3C) that scaffolds the binding and engagement of viral proteins necessary for initiating RNA replication (Fig. 3) (Nuesch et al. 1993; Kusov et al. 1996). However, this has not been studied in detail.

Positive-strand RNA synthesis is similarly primed by VPg-pUpU, explaining the presence of VPg at the 5' end of positive-strand viral RNA (Weitz et al. 1986). Secondary RNA structure within stem-loops I-II of the 5'UTR, upstream of the viral IRES (Fig. 3A), may act in coordination with viral proteins to melt the end of the newly formed duplex dsRNA such that VPg-pUpU can anneal to the 3' end of the minus-strand template to prime positive-strand synthesis, but these potential RNA replication signals have not been studied in detail. Poliovirus minus- and positive-strand RNA synthesis, and the inherent conflict between the 5' to 3' movement of ribosomes and 3' to 5' translocation of the replicase on the positive-strand genome, are tightly regulated by key interactions between viral and host cell RNA-binding proteins (Ogram and Flanagan 2011). HAV replication is no doubt similarly regulated, but this remains largely a black box. Multiple viral and cellular proteins bind the HAV 5'UTR with specificity (Chang et al. 1993; Schultz et al. 1996a; Graff et al. 1998; Beneduce et al. 1999), but their involvement in replication of the RNA has yet to be determined.

Virion Assembly

Little is known about how HAV capsid assembly (see above) and packaging of the viral RNA proceeds within infected cells. A role for the helicase activity of poliovirus 2C has been established recently in packaging of the poliovirus genome (Jiang et al. 2014a). The HAV 2C sequence suggests it also possesses helicase activity, and it is likely

Figure 4. RNA replication. Picornaviral replication is nonconservative, with more positive-strand RNA (+, solid line) than minus-strand RNA (–, dashed line) synthesized. The process begins after delivery of the (+)-strand genome to the cytosol and internal ribosome entry site (IRES)-directed translation has produced the nonstructural proteins required for replicase assembly. The incoming (+)-strand serves as template for initial synthesis of a (–)-strand, resulting in a full-length duplex double-stranded RNA (dsRNA). 3D^{pol} catalyzes RNA synthesis, but other viral and host proteins (HPs) are likely to be required for transcription. In a second step, the 5' end of the (+)-strand dissociates from the (–)-strand in the duplex, allowing priming for synthesis of a new (+)-strand, a process that then repeats. Little if any single-stranded (–)-strand RNA is produced. Uridylated VPg-pUpU serves as the protein primer for both (–)-strand and (+)-strand synthesis in tightly coupled reactions, annealing with terminal adenines at the 3' ends of both (+)-strand (see *inset*) and (–)-strand RNA. New (+)-strand RNA molecules are either packaged into nascent capsids, or recycled as a template for more (–)-strand synthesis.

to play a similar role in packaging. Confocal microscopy shows that mature HAV capsid and dsRNA colocalize at foci within infected cells, suggesting that virion maturation occurs close to sites of RNA replication (Fig. 5). However, extensive spatial segregation of capsid and RNA replication intermediates is also evident. In part, this may reflect the rapid recruitment of fully formed capsids to endosomes for export from the cell, as described in the following section.

Quasi-Envelopment and Viral Egress

Although minor amounts of naked HAV can be found in the supernatant fluids of infected cell cultures, this likely represents nonspecific release of virus through cell death or possibly loss of the membrane from secreted eHAV virions. Egress of newly replicated infectious virus is

predominantly nonlytic (Feng et al. 2013). Although not visualized directly, HAV capsids appear to be recruited to endosomes via interactions with components of the ESCRT system, budding into endosomes to form multivesicular bodies (MVBs) destined for transport to the plasma membrane where membrane fusion leads to the release of capsids wrapped within a single membrane (Fig. 6).

MVBs are formed by three distinct ESCRT protein complexes, acting sequentially to sort and load cellular cargo destined for degradation in lysosomes or export from the cell (Hurley 2015). ESCRT-I and ESCRT-II are core ESCRT machinery that sequester cargo at the endosomal membrane, often recognizing cargo as a result of its ubiquitylation, thereby creating an inward bud (Hurley 2015). ESCRT-III oligomers are recruited and pinch the neck of the

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Figure 5. Confocal fluorescence microscopy showing viral RNA and capsid proteins in hepatitis A virus (HAV)-infected cells. Huh-7.5 cells were fixed on chamber slides at 14-days posttransfection of infectious HM175/p16 synthetic RNA. Double-stranded RNA (dsRNA) (red) was visualized by staining with a specific monoclonal antibody J2 (Scicons), and conformational HAV capsid antigen (green) by staining with JC serum (Feng et al. 2013). Most cells show foci where the two signals colocalize, but there is extensive segregation of dsRNA (RNA synthesis) and capsid antigen (assembled capsids). Nuclei have been counterstained with DAPI (blue).

membrane invagination, releasing the buds into the endosomal lumen as intraluminal vesicles (ILVs) to form MVBs (Fig. 6). In later steps, VPS4 acts to dissociate the ESCRT-III oligomers. MVBs may then fuse with lysosomes for degradation of their cargo, or be directed to the plasma membrane where fusion releases ILVs as extracellular exosomes (Mathivanan et al. 2010; Bobrie et al. 2011).

ALIX, also known as programmed cell death 6 interacting protein (PCD6IP), is an accessory ESCRT protein that feeds into ESCRT-III complexes in parallel with ESCRT-I/II, and interactions with it represent a second pathway for cargo selection (Hurley 2015). HAV has hijacked this pathway to exit the cell, as RNAi-mediated knockdown of ALIX, CHMP2A (ESCRT-III), or VPS4B strongly inhibit eHAV release (Feng et al. 2013; McKnight et al. 2017). ALIX, VPS4, and several CHMP proteins are physically associated with extracellular eHAV, as noted above.

Two distinct, possibly redundant sorting signals appear to be involved in the recruitment of HAV capsids by the ESCRT system. Structural proteins of conventional enveloped viruses con-

tain “late domains” (PPXY, P(S/T)AP, GPPX₃Y, or YPX_{1or3}L) that mediate interactions with ESCRT machinery (Ren and Hurley 2011). Two tandem YPX₃L motifs, separated by 28 residues in the HAV VP2 capsid protein, are the only such motifs in the HAV polyprotein, and are conserved in all 94 human HAV sequences in GenBank as well as 10 novel hepatovirus species recently identified in bats and other small mammals (Feng et al. 2013; Drexler et al. 2015). YPX₃L motifs mediate interactions with ALIX, which in turn binds CHMP4 (ESCRT-III) in a step critical for membrane scission (Hurley 2010). Both CHMP4 and ALIX are present in eHAV, and anti-ALIX antibodies precipitate the HAV capsid in a YPX₃L motif-dependent manner (Feng et al. 2013; McKnight et al. 2017). Paradoxically, however, recent crystallographic studies of the naked HAV capsid show that both YPX₃L motifs are partially buried beneath the surface (Wang et al. 2015; Stuart et al. 2018), where the motifs are unlikely to be accessible for binding by ALIX. These contradictory findings have yet to be reconciled, but it may be that the conformation of the capsid is different within eHAV (where unprocessed VP1pX

Figure 6. Biogenesis of extracellular quasi-enveloped (eHAV) and nonenveloped virions. Following assembly and packaging of the viral RNA, capsids are recruited to the surface of endosomes through VP2 and VP1pX interactions with components of the endosomal sorting complexes required for transport (ESCRT) system (apoptosis-linked gene 2-interacting protein X [ALIX] and others). Inward budding of the capsids into the endosome results in intraluminal vesicles (ILVs) and formation of multivesicular bodies (MVBs). The MVBs undergo transport to the plasma membrane, where membrane fusion leads to the release of membrane-wrapped eHAV virions to the extracellular space. eHAV virions that are shed from the basolateral hepatocyte membrane into hepatic sinusoids circulate in the blood, whereas eHAV secreted across the apical membrane undergoes conversion to naked, nonenveloped virions mediated by bile salts in the proximal biliary system.

predominates) than in the mature, naked virion, and allows for physical interaction of the motifs with ALIX.

A second signal for quasi-envelope appears to reside within the pX domain itself, which shares no sequence identity with any other protein in the database. Unpublished studies from our laboratory show that specific mutations within this domain prevent the release of assembled, infectious capsids from infected cells. The nature of this signal remains under investigation.

Most of the above has been gleaned from studies of infected cell cultures. Confirming details of HAV replication or quasi-envelope

in vivo is not a trivial exercise. However, quasi-enveloped eHAV is the only form of the virus that can be detected in serum or plasma from acutely infected primates, and gradient-purified membrane-associated virions with the buoyant density of eHAV are infectious for *Ifnar1*^{-/-} mice (Feng et al. 2013; Das et al. 2017).

Hepatocytes are polarized epithelial cells, with apical membranes that abut the proximal biliary canaliculus into which bile salts are secreted, and basolateral membranes facing onto hepatic sinusoids that are bathed continuously by blood. Studies of polarized Caco-2 human colonic carcinoma cells or HepG2-N6 hepatocytes show differences in vectorial release of HAV, with release from Caco-2 cells being almost entirely apical in direction, whereas it is largely basolateral in HepG2-N6 cells (Blank et al. 2000; Snooks et al. 2008). However, most HAV released from either the basolateral or apical surfaces of these cells is quasi-enveloped, and membrane-associated virus has been identified within bile from infected mice (Hirai-Yuki et al. 2016b). eHAV released across the apical surface appears to be stripped of its membranes by the detergent action of bile salts, and this is the primary source of naked virions excreted in feces (Hirai-Yuki et al. 2016a,b). The virus is thus ideally positioned for both spread within the environment (as a highly stable, naked virion), and within the individual infected host (as membrane-cloaked virions that are hidden from the immune system and impervious to antibody-mediated neutralization) (Feng et al. 2013).

CONCLUDING THOUGHTS

The successful deployment of inactivated vaccines for HAV in the mid-1990s resulted in a demonstrable drop in research on this ancient but remarkable pathogen. Research funding and interest in the field shifted to other hepatotropic pathogens, such as hepatitis B virus (HBV) and HCV, which cause chronic liver disease. As a result, numerous important questions remain unanswered today concerning the virus and the disease it causes. However, there has been a reawakening of interest in recent years, fueled largely by discovery of eHAV quasi-envelope

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opment. How HAV manages to evade innate and adaptive immune responses for weeks after initial infection has been answered in part by the discovery of eHAV (Feng et al. 2013), and the role of HAV proteinase precursors in disrupting innate immune responses (Yang et al. 2007; Qu et al. 2011). The discovery of eHAV has also increased appreciation of the potential for nonlytic release of other classically “nonenveloped” viruses, including other picornaviruses (Altan-Bonnet 2016). A mouse model for hepatitis A now exists with which questions related to pathogenesis can be addressed (Hirai-Yuki et al. 2016a). Numerous distantly related hepatoviruses have been recently discovered in a large number of species of small mammals, providing novel insight into evolution of HAV (Drexler et al. 2015). And, finally, the structure of the HAV capsid has been solved using X-ray crystallography (Wang et al. 2015), information that ultimately can be used to answer questions about antigenicity, neutralization, and possibly why the HAV vaccine is so successful. With only a small research effort, big gains have been made and continued interest in HAV research seems assured.

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Cold Spring Harb Perspect Med 2018; doi: 10.1101/cshperspect.a033480 originally published online April 2, 2018

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