Inhibition of cap-dependent gene expression induced by protein 2A of hepatitis A virus

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The viral protein 2A of hepatitis A virus (HAV) lacks the conserved 18 aa sequence found in other picornavirus proteases; hence, it is unclear whether the induction of CPE by culture-adapted HAV strains is due to 2A-mediated activity. Moreover, the cleavage sites and actual borders of HAV 2A are not known. Accordingly, a nested series of cDNA sequences encoding the segment of the HAV polyprotein (aa 760–1087) were linked to the 5'-UTR of poliovirus type 2 (Lansing strain) and inserted downstream of the gene encoding human growth hormone (GH). Following transfection of COS-1 cells, levels of GH (translation of which was entirely cap dependent) were determined in culture supernatants. Expression of HAV peptides extending from aa 764, 776 or 791 to 981 strongly inhibited cap-dependent translation of GH, whereas cap-independent expression of a reporter gene (CAT) directed by the poliovirus RNA 5'-UTR was unaffected. The inhibitory effect was absent in constructs expressing either the short peptide encompassing aa 760–836 or proteins initiated downstream of the putative cleavage site 836–837, suggesting that the boundaries of a functional HAV 2A may extend from the Gln/Ser junction 791–792 to residue 981, while peptides initiated at the Gln/Ala pair 836–837 may result from alternative cleavage. Point mutations that substituted members of the triad Ser916, His927 and Asp931 abolished the inhibitory effect on cap-dependent translation, suggesting that the HAV-induced CPE may be mediated by 2A protein.

Introduction

The aetiological agent of hepatitis A, hepatitis A virus (HAV), is a picornavirus of the hepatovirus group, but, in contrast to other representatives of this family, most isolates replicate very slowly in vitro, without any noticeable effect on host-cell macromolecular synthesis. This is a surprising observation, since picornaviruses are among the most cytopathic viruses. There is no explanation for such unusual behaviour.

Previous work from our laboratory led to the isolation of a strain of HAV endowed with the ability to replicate faster than any other isolate (7–10 days instead of weeks) and able to induce a strong cytopathic effect in cell lines to which it was adapted (Venuti et al., 1985; Divizia et al., 1986). Its entire genome has been sequenced (Beneduce et al., 1995; GenBank accession no. X83302). Following this report, several groups have independently identified cytopathic variants of HAV, isolated after several passages in cell culture.

It has been known for many years that infection of susceptible cells with a picornavirus leads to a rapid and irreversible inhibition of host-cell protein synthesis, although intact and functional cellular mRNAs can be extracted from infected cultures and translated correctly in vitro in cell-free systems. The molecular mechanism(s) responsible for such an exquisite discrimination between cellular and picornavirus mRNAs remained elusive for a long time.

The lack of a 5'-terminal cap and the ability to initiate translation internally (Pérez Bercoff, 1982; Pérez Bercoff & Kaempfer, 1982; Degener et al., 1983) by virtue of cis-acting sequences (Pelletier & Sonenberg, 1988; Jang et al., 1988) close to the initiation site are the most conspicuous differences between cellular and picornavirus RNAs. They also endow the RNA of picornaviruses with a distinct advantage in the event of failure (or inactivation) of the cap-recognition machinery and provide an easy way of inhibiting selectively the translation of cellular (but not viral) mRNAs. By preventing cap recognition, picornaviruses may be able to redirect the cellular translation machinery to the translation of their own mRNAs.
In poliovirus-infected cells, the eukaryotic translation initiation factors eIF-4G I and II (formerly known as p220) are the selected targets of the virus-encoded protease 2A. The protease cleaves the eIF-4G moiety of the cap-binding complex and, consequently, eliminates cap-binding activity (Krasslich et al., 1987; Wyckoff et al., 1990, 1992; Ehrenfeld, 1990). Cleavage of eIF-4G I and II correlates with the selective inhibition of cellular protein synthesis in poliovirus-infected cells (Gradi et al., 1998). Protein 2A, a 16-4 kDa polypeptide, is normally required to introduce the first proteolytic cleavage in the nascent poliovirus polyprotein (for a review see Ryan & Flint, 1997). The sensitivity of poliovirus 2A to heavy metals and alkylating reagents led to the suggestion that poliovirus 2A was a trypsin-like protease of the serine group. This view was further substantiated by the observation that poliovirus protease 3C and the peptide 2A share a similar protease consensus sequence related to the trypsin-like group of serine proteases (Lloyd et al., 1988). Moreover, mutational analysis confirmed that the predicted catalytic triad His²⁰, Asp³⁸ and Cys¹⁰⁹ is indeed the active site of poliovirus 2A (Yu & Lloyd, 1991).

The putative active site and an 18 amino acid sequence in the C-terminal third of poliovirus 2A are among the most conserved elements throughout the enteroviruses and rhinoviruses, but the protease consensus sequence is absent in the cognate peptides of aphthoviruses, cardio- and hepatoviruses. In aphthoviruses, the leader peptide (LPro) at the N terminus of the viral polyprotein is responsible for the proteolytic cleavage of eIF-4G (Devaney et al., 1988).

While it is tempting to speculate that all members of the picornavirus family may have evolved a similar mechanism to shut off host protein synthesis, several lines of evidence indicate that cardioviruses depart from this general scheme. Their ability to inhibit cellular protein synthesis has been shown to result from the complete dephosphorylation of eIF4E-BP, which in this form cannot complex with eIF-4E and consequently cannot participate in the recognition of the 5′-terminal cap of cellular mRNAs (Gingras et al., 1996).

As mentioned above, rapidly growing variants of HAV have been isolated. Comparison of the sequences of the genomes of several such HAV isolates revealed that they share common mutations, mainly in the 5′-terminal untranslated region (5′-UTR) and in the putative P2 and P3 regions of the genome (Jansen et al., 1988; Cohen et al., 1989; Anderson & Ross, 1990; Emerson et al., 1992; Robertson & Nainan, 1996). Moreover, studies conducted with chimeric infectious HAV cDNA clones indicated that the substitutions in the P2 region might act cooperatively with mutations in the 5′-UTR (Day et al., 1992; Emerson et al., 1992, 1993). While these findings implicated the gene product(s) encoded in the P2 region in the adaptation to tissue culture conditions, little was known about the functions of 2A, 2B and 2C in HAV and even the actual boundaries of these proteins were unclear (Anderson, 1996).

Computer-assisted analysis of the deduced amino acid sequence led to the suggestion that the cleavage between the capsid protein VP1 and 2A occurred at a Glu/Ser pair (amino acids 791/792 of the polyprotein). HAV 2A would then extend to the Gln/Gly junction 981/982 (Cohen et al., 1987). More recently, however, two additional Glu/Ser pairs in the proper environment have been identified, at positions 764/765 and 776/777 (Martin et al., 1995; Probst et al., 1997). Cleavage at any of these sites would generate a 2A protein considerably longer than that of other picornaviruses (189 amino acids compared with 140–150). However, the isolation from extracts of HAV-infected BSC-1 cells of a protein (pX) containing the entire VP1 plus a C-terminal extension (Anderson & Ross, 1990) and the identification of a scissile bond in the middle of what was expected to be 2A (namely between Gln²³⁶ and Ala⁸³⁷) (Jia et al., 1993) suggested that HAV 2A might instead be shorter than previously believed: a polypeptide of 45 (or 91) amino acids at most.

Whatever the length assigned to it, HAV 2A lacks the consensus sequence of the putative catalytic site of the trypsin-like proteases (Lloyd et al., 1988) and peptides generated by in vitro translation of RNA transcripts encoding 2A are apparently devoid of autocatalytic activity, although still susceptible to cleavage by added 3C (Schultheiss et al., 1994). Moreover, deletion of 45 nucleotides spanning positions 3155–3200 of HAV RNA (i.e. a 15 amino acid stretch encompassing amino acids 806–821) did not affect infectivity of cDNA clones (Harmon et al., 1995), with the obvious implications that, whatever the function(s) of HAV 2A, (i) the virus can dispense with it and (ii) the active site must map downstream of amino acid 821 of the polyprotein.

These observations raised the question as to whether the HAV 2A protein was involved in the virus-induced cytopathic effect, as in the case of entero- and rhinoviruses, and we report here that the expression of an HAV-encoded peptide encompassing amino acids 791 to 981 of the viral polyprotein inhibits cap-dependent gene expression, while internal initiation of translation is unaffected. Mutational analysis of the viral sequences indicated that the triad of Ser⁹¹⁶, His⁹²⁷ and Asp⁹³¹ is absolutely required for the inhibitory effect.

**Methods**

- **Cells and bacteria.** COS-1 cells were routinely maintained in Dulbecco’s modified Eagle’s medium (DMEM) with 2% synthetic serum substitute Ultraser G (Seracor) and antibiotics. Cultures of *E. coli* (TG-1 strain) were transformed after treatment with calcium chloride and selected for ampicillin resistance according to standard procedures.

- **Plasmid construction.** The molecular cloning of the genome of the fast-growing strain of HAV has been described previously (Venuti et al., 1985; Divizia et al., 1986). Digestion of plasmid DNA containing poliovirus or HAV cDNA sequences, dephosphorylation by alkaline phosphatase treatment, purification of DNA fragments by agarose gel electrophoresis and QN–butanol (Langridge et al., 1980) or Sephagel extraction (Pharmacia), ligation of DNA fragments and propagation of the constructs in competent bacteria were performed by standard...
Determination of the boundaries of HAV 2A. cDNA clones of HAV sequences of different lengths were generated by PCR with pairs of synthetic oligonucleotides of opposing polarities that introduced HindIII restriction sites flanking AUG and stop codons that replaced the amino acids present at the positions of the HAV polyprotein indicated in Fig. 3. The cDNA fragments so generated were purified (Qiapquick spin column, Qagen), digested with HindIII ligated into a HindIII-digested, phosphatase-treated pSV-GH-Lansing vector and then propagated in competent bacteria (E. coli, TG-I strain). Amp^r colonies were screened for the presence of constructs carrying HAV sequences in the proper orientation by PCRs driven by pairs of oligonucleotides as described previously (Degener et al., 1995). The constructs were subsequently checked by direct DNA sequencing.

Results

Effect of HAV 2A on cap-dependent translation

Infection of susceptible cells with a cytopathic variant of HAV typically results in the inhibition of host-cell protein synthesis (Venuti et al., 1985; Divizia et al., 1986). We tried first to establish whether the putative HAV 2A (regardless of where its borders might be) was involved in this process. To this end, a series of bicistronic plasmids was constructed containing a tandem arrangement of the gene encoding human GH followed by the sequences encoding the putative protease 2A of either HAV or the highly cytolytic poliovirus type I (Mahoney), placed under the transcriptional control of the late promoter of SV40 (Fig. 1a). In-frame AUGs and termination codons were engineered to secure the expression of a free peptide even in the absence of self-cleavage. Since the actual boundaries of HAV 2A are still controversial, in a first series of experiments, we assumed that HAV 2A extended from amino acid 764 to 981 of the viral polyprotein. cDNA representations of the genome of the fast-growing strain of HAV (Venuti et al., 1985) were used in these studies. Comparison of its deduced

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Oligonucleotide-directed PCR-amplified site-directed mutagenesis. A cDNA representation spanning nucleotides 2993–3674 of the HAV genome was inserted into the EcoRI site of the phagemid BlueScript KS (+) (Stratagene) and used as the template to generate a set of overlapping fragments encoding 2A.

To introduce amino acid substitutions in the 2A coding region, pairs of complementary oligonucleotides containing the desired mutations were used to prime PCRs. Following gel purification and thermal denaturation, the cDNA fragments were allowed to hybridize in their overlapping sequences and then extended and subsequently amplified by PCR. The mutated fragments so generated were digested with EcoRI (which cuts HAV cDNA at bases 2998 and 3674), blunt-ended with T4 DNA polymerase and inserted into the blunt-ended HindIII site of the eukaryotic expression vector pSV-GH-Lansing downstream of the latter sequences as described previously (Degener et al., 1995). The constructs were propagated in competent bacteria. All mutations were checked by direct DNA sequencing.

Transfection and quantification of gene expression. COS-1 cell monolayers in 10 cm diameter plastic Petri dishes (Nunc) were transfected with 10 μg of each plasmid DNA by the DEAE-dextran/chloroquine procedure (Gorman, 1985). After incubation (37 °C, 72 h), the amount of human growth hormone (GH) present in the supernatants was determined by radioimmunoassay (RIA) in triplicate 100 μl aliquots (Silveira Carneiro et al., 1995). When indicated, inhibition of cap-dependent GH expression is presented as a percentage of the value of the control construct (e.g. GH-Lansing-CAT), as in Fig. 3. The level of chloramphenicol acetyltransferase (CAT) activity in cell lysates was measured as described previously (Silveira Carneiro et al., 1995). The acetylated forms of [14C]chloramphenicol were resolved from the unmodified form by ascending TLC on silica gel plates. After autoradiography, the relative activities were determined either by cutting out the corresponding areas of the TLC plates and counting the radioactivity by liquid scintillation, or by estimation in a photoimaging device (Silveira Carneiro et al., 1995).
a amino acid sequence with that of the standard wild-type isolate (Cohen et al., 1987; GenBank accession no. M14707) showed that they were identical throughout the region corresponding to the putative 2A and 2B genes.

Expression of the HAV (or poliovirus) 2A sequences by internal initiation of translation was achieved by inserting the entire 5'-UTR of poliovirus type 2 (Lansing strain) in the intergenic region (Fig. 1(a)).

In a series of control experiments, the 5'-UTR of the Lansing strain of poliovirus was replaced with its translationally inactive mutant S1, in which substitutions spanning nucleotides 138–143 destabilize the stem of domain III and consequently deprive the poliovirus IRES of its ability to direct internal initiation of translation (Nicholson et al., 1991). The inability of S1 to drive internal initiation of translation is illustrated in Fig. 1(b).

These constructs were transfected into COS-1 cells. The bicistronic mRNAs transcribed from these constructs carried a first cistron (GH), translation of which was entirely cap-dependent, and a second cistron (HAV or poliovirus 2A) expressed by cap-independent internal initiation of translation driven by the Lansing 5'-UTR. Expression of the latter was abolished in constructs containing the S1 instead of the Lansing 5'-UTR.

Upon transfection of COS-1 cells with plasmids containing HAV 2A preceded by an efficient 5'-UTR that secured its translation, strong inhibition of cap-mediated expression of the upstream GH cistron was observed. In the experiment depicted in Fig. 2, expression of HAV 2A reduced the amount of GH detected by RIA in 100 µl of the supernatant of the cultures from 8582 c.p.m. to a mere 1737 c.p.m. (an 80% reduction), while poliovirus 2A reduced the amount of GH by 95% to 482 c.p.m. While the level of inhibition varied from one experiment to another, the reduction of cap-dependent expression mediated by HAV 2A was typically 65% (vs 90% for poliovirus 2A). The inhibition of cap-dependent expression mediated by HAV 2A was always less intense than that induced by poliovirus. For the time being, the reasons for such a difference remain unclear.

Replacement of the Lansing 5'-UTR with the translationally inactive mutant S1 in the bicistronic constructs prevented the

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**Fig. 2.** Effect of HAV or poliovirus 2A on the cap-dependent expression of the GH gene. The amount of GH present in triplicate 100 µl samples of the supernatants of COS-1 cells transfected with constructs expressing (a) or not expressing (b) either HAV or poliovirus 2A was determined by RIA.

The amount of GH in 100 µl supernatant of cultures transfected with pSV-GH-Lansing-CAT was 8582 c.p.m.

**Fig. 3.** Effect of HAV or poliovirus 2A on the expression of cap-dependent (GH) and internally initiated (CAT) reporter genes. (a) COS-1 cells were transfected with the bicistronic plasmid GH-Lansing-CAT. The transient expression of GH was determined from triplicate 100 µl samples of the supernatants by RIA and this value (9012 c.p.m. in the experiment depicted) was used as 100% GH expression. CAT activity was assayed in the cell lysates. The acetylated and unacetylated forms of [14C]chloramphenicol were resolved by ascending TLC as described previously (Silveira Carneiro et al., 1995). Silica gel plates were autoradiographed and quantified by photoimaging. The ratio of acetylated to total [14C]chloramphenicol (16605 c.p.m. vs 22482 c.p.m. in the experiment presented here) was used as 100% transacetylation activity.

(b)–(e) COS-1 cells were co-transfected with the plasmid pSV-Lansing-CAT (shown at the top of each panel) and the various constructs indicated. The levels of GH and CAT present in the supernatants or lysates, respectively, were expressed as percentages of the values found in (a).
expression of HAV (or poliovirus) 2A and concomitantly abolished the inhibitory effect on cap-dependent expression of GH (Fig. 2b).

Differential inhibition of cap-dependent and internal initiation of translation

In order to ascertain whether the reduced expression of GH involved a cap-related mechanism, COS-1 cells were cotransfected with the expression vector pSV-Lansing-CAT (which expresses CAT in a cap-independent manner driven by the IRES of poliovirus type 2, Lansing strain) and the constructs described earlier containing the sequences encoding HAV or poliovirus 2A (Fig. 3b, d). The amount of GH expressed under these conditions was determined by RIA as above. Fig. 3 shows that the transient expression of HAV 2A resulted in a 65% reduction of cap-dependent expression of GH (2490 c.p.m. vs 9012 c.p.m. in the control shown in Fig. 3a), while poliovirus 2A reduced the amount of GH to 852 c.p.m., a 90% reduction.

Cells were then detached from the plate and lysed and the level of CAT activity was quantified as described previously (Silveira Carneiro et al., 1995). Aliquots of 35 µl of lysates of cultures transfected with the construct pSV-GH-Lansing-CAT (Fig. 3a) containing 20 µg total protein were able to transacylate 75% of the [14C]chloramphenicol present in the reaction (43900 c.p.m. of a total of 58500 c.p.m.). Similarly, lysates of cells expressing poliovirus and HAV 2A (Fig. 3b, d) were able to drive the transacylation of 95 and 97% of the control (Fig. 3b, d; right column). The transient expression of HAV 2A was therefore accompanied by a drastic reduction (65%) of the cap-dependent expression of GH, while the cap-independent expression of the CAT cistron was unaffected.

As expected, co-transfection with constructs containing the 5'-UTR of the S1 mutant preceding poliovirus 2A or the cognate protein of HAV did not modify the expression of GH or interfere with translation of the CAT gene (Fig. 3c, e).

The functional borders of HAV 2A

As already mentioned, the actual borders of 2A are still the subject of controversy, and four putative cleavage sites spanning amino acids 760–836 had been identified as potential N-terminal boundaries of 2A at the Gln/Ser junctions 764/765, 776/777 and 791/792 and the Gln/Ala pair 836/837. Similarly, the C terminus of HAV 2A has been tentatively located at the Gln/Gly junction 981/982 or at the Gln/Ala pair 836/837.

In order to discriminate between these possibilities, a nested series of cDNA representations of the sequences encoding HAV peptides of various lengths encompassing amino acids 764–1087 (i.e. the different sizes proposed for the
putative HAV 2A and 2B proteins) were linked to the 5'-UTR of either the Lansing strain of poliovirus or its inactive mutant S1. These are illustrated schematically in Fig. 4. AUG codons were engineered in-frame at the putative N-terminal cleavage sites (i.e. replacing amino acids 765, 777, 792, 837 and/or 982) and stop codons were introduced at positions 837, 982 or 1088 of the polyprotein to generate the proteins depicted in Fig. 4. After transfection of COS-1 cells, the levels of GH present in the supernatants of the cultures were determined. Expression of HAV peptides extending upstream of amino acid 791 caused the same observed inhibition of the cap-dependent expression of GH (Table 1). This inhibitory effect was absent in constructs expressing the peptides 791–836 (45 aa) and 837–1087 (105 aa) and all those that initiated at the putative N-terminal cleavage sites (i.e. replacing amino acids 765, 777, 792, 837 and 982) or stop codons were introduced at positions 837, 982 or 1088 of the polyprotein to generate the proteins depicted in Fig. 4. After transfection of COS-1 cells, the levels of GH present in the supernatants of the cultures were determined. Expression of HAV peptides extending upstream of amino acid 791 caused the same observed inhibition of the cap-dependent expression of GH (Table 1). This inhibitory effect was absent in constructs expressing the peptides 791–836 (45 aa) and 837–1087 (105 aa) and all those that initiated at the putative cleavage site 836/837 (Fig. 4a, b), suggesting that the N-terminal border of the HAV peptide that exerts the inhibitory effects may be located at amino acid 791.

**Mutational analysis of HAV 2A**

Typically, proteins belonging to the same functional family show some degree of structural relatedness, but this rule is far from absolute; the bacterial serine protease subtilisin constitutes a well-known example of total deviance, and its amino acid sequence and tertiary structure are totally unrelated to the serine proteases of mammalian cells. Nonetheless, the bacterial subtilisin is indeed a protease.

HAV 2A bears no sequence similarity to the cognate peptide of entero- and rhinoviruses and lacks a most conserved stretch, 18 amino acids in length, present in trypsin-like proteases. Consequently, the mechanism responsible for the inhibition of cap-dependent protein synthesis observed upon expression of HAV 2A was unclear. To clarify this point, we conducted a mutational analysis of HAV 2A in search of a putative HAV 2A peptide that exerts the inhibitory effects of HAV 2A to inhibit cap-dependent translation. All Asp and Ser residues were tested in a similar way, and only Asp931 and Ser916 were found to eliminate the inhibitory effect on translation (Table 2).

**Discussion**

The transient expression of a peptide encompassing amino acids 791–981 of the HAV polyprotein results in strong inhibition of the cap-dependent translation of a reporter gene in a bicistronic construct. The borders of this peptide correspond exactly to those originally predicted by Cohen et al. (1987) as the cleavage sites of HAV 2A, and therefore we will indicate this peptide as such.

The shut-off of expression of the upstream GH gene is not due to a poisoning effect induced by the mere presence in the construct of the sequences encoding either poliovirus or HAV 2A, but requires their efficient translation. In fact, replacement of the Lansing IRES (which led to the expression of 2A) with its translationally inactive mutant S1 in the construct eliminates both the expression of 2A and, concomitantly, the shut-off of GH expression. Nor can this inhibition be explained as the mere result of competition for initiation of translation between the 5'-terminal cap and the internal IRES of the bicistronic mRNAs. The different degrees of inhibition induced by poliovirus and HAV 2A (both preceded by the same IRES sequence) exclude this alternative. HAV and poliovirus 2A appear to block only the cap-dependent expression of the reporter gene and not to interfere with that mediated by internal initiation of translation, suggesting that entero- and hepatoviruses may have evolved a similar (if not identical) mechanism responsible for the shut-off of host protein synthesis. This view is substantiated further (though not proven) indirectly by the observation that mutations introduced in the triad Ser916, His927 and/or Asp931 eliminate the inhibitory effect of HAV 2A. HAV and poliovirus 2A were both expressed by internal initiation of translation driven by the 5'-UTR of poliovirus 2 (Lansing strain). Two main reasons justified this choice. Firstly, the higher efficiency of the Lansing IRES compared with that of HAV is well documented (Silveira

### Table 1. Inhibition of cap-dependent GH expression mediated by HAV peptides

<table>
<thead>
<tr>
<th>2A Sequence</th>
<th>GH Expression (c.p.m.)</th>
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<tbody>
<tr>
<td>pSV-GH-Lansing-CAT</td>
<td>9027</td>
</tr>
<tr>
<td>Poliovirus 2A</td>
<td>751</td>
</tr>
<tr>
<td>HAV 764–981*</td>
<td>3400</td>
</tr>
<tr>
<td>HAV 776–981*</td>
<td>3821</td>
</tr>
<tr>
<td>HAV 791–981*</td>
<td>3780</td>
</tr>
</tbody>
</table>

* The numbers represent the residues of the HAV polyprotein expressed by the various constructs.

### Table 2. Effect of amino acid substitutions on HAV 2A inhibitory activity

<table>
<thead>
<tr>
<th>2A Sequence</th>
<th>GH Expression (c.p.m.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>pSV-GH-Lansing-CAT</td>
<td>2790</td>
</tr>
<tr>
<td>Poliovirus 2A</td>
<td>228</td>
</tr>
<tr>
<td>HAV 791–981 wt</td>
<td>1085</td>
</tr>
<tr>
<td>His*916 mutant</td>
<td>2415</td>
</tr>
<tr>
<td>Ser*916 mutant</td>
<td>1958</td>
</tr>
<tr>
<td>Asp*931 mutant</td>
<td>2654</td>
</tr>
</tbody>
</table>
Carneiro et al., 1995). Secondly, in the case of the Lansing IRES, a translationally inactive mutant was available (S1; Nicholson et al., 1991) and could be used in these studies as an indispensable control.

The nature of the cellular target of HAV 2A remains unclear. Interestingly, Borman & Kean (1997) have reported that the internal initiation of translation driven by the HAV IRES in vitro requires intact eIF-4G. Presumably, however, the non-cytolytic variants of HAV may also differ from the cytolytic forms in their specific requirements for the cap-recognition machinery. Moreover, the conditions of in vitro translation under which these conclusions were drawn may not necessarily mirror the situation of in vivo expression of the constructs reported here.

The amino acid sequence of the protein 2A encoded by the fast-growing strain of HAV used in these studies is identical to that of slow-replicating isolates, apparently unable to induce a noticeable cytopathic effect. However, given the low m.o.i. attainable with any HAV isolate before adaptation to tissue culture conditions, the absence of detectable cytopathic effect is the logical, predictable consequence of the faster rate of cell division compared with the protracted replication cycle of HAV. Under these conditions, the still-dividing, non-infected cells would easily replace the few infected ones, masking the effect of infection. As the virus adapts to cell culture conditions, the replication cycle shortens and the effects on host-cell protein synthesis become apparent. Thus, the ability of cell-adapted HAV strains to induce a visible cytopathic effect may not be due to a mutated 2A, but may be a consequence of its ability to replicate and spread faster.

As for other picornaviruses, the genomic RNA of HAV is translated into a single polyprotein that is subsequently cleaved into the functional peptides. The peculiar replication cycle of HAV renders the kinetic studies required to establish the precursor–product relationship among different peptides very difficult to perform. However, such relationships are clearly identifiable in cells infected by polio- or rhinoviruses. In the case of HAV, the cleavage sites were predicted by comparison with those of other members of the family Picornaviridae. This led to conflicting reports according to the model group considered (cardio- or enteroviruses). The putative cleavage sites and the nature of the enzymes involved in the proteolytic processing were subsequently studied using a variety of expression systems (bacterial or eukaryotic) in which truncated HAV-encoded proteins were used as the substrate of the presumed viral proteases. Since peptides containing HAV 2A were unable to undergo self-cleavage but were still susceptible to proteolytic processing by added 3C, it was concluded that HAV 2A was devoid of proteolytic activity and the sequences C-distal of VP1 (regardless of where its borders might be) were supposed to be just a spacer (Anderson, 1996). The finding by Harmon et al. (1995) that HAV cDNA clones carrying a 15 aa deletion between positions 807 and 821 of the polyprotein were still infectious led to the suggestion that HAV 2A had no function at all. However, in aphthovirus-infected cells, L<sup>Pro</sup> is known to mediate the cleavage of cellular eIF-4G and consequently to play a major role in the shut-off of host-cell protein synthesis. Nevertheless, foot-and-mouth disease virus cDNA clones from which the sequence encoding the entire L<sup>Pro</sup> has been deleted are infectious (Piccone et al., 1995), consistent with the notion that the inhibition of cellular macromolecular synthesis is not necessarily a prerequisite for virus replication to occur.

The HAV-encoded protein described in these studies extends from amino acid 792 to the junction at positions 981/982. These boundaries correspond to the borders proposed originally by Cohen et al. (1987) as the limits of HAV 2A. The N terminus of this protein corresponds to the cleavage site reported recently to produce the shortest VP1 precursor compatible with pentamer and empty capsid formation (Martin et al., 1998).

The predicted cleavage at the Gln/Gly pair 981/982 that forms the C terminus, on the other hand, has been questioned recently, since immune precipitation of the processing products present in a vaccinia virus recombinant expression system failed to detect the expected 189 and 106 aa proteins when antibodies raised against two synthetic peptides were used (Gosert et al., 1996). The absence of evidence under these experimental conditions, however, does not necessarily preclude the possibility that HAV may use this cleavage site during the normal replication cycle.

These findings apparently contradict the well-documented observation that HAV protease 3C preferentially cleaves at the Gln/Ala pair 836/837 in either truncated HAV proteins or in short synthetic peptides that reproduced the correct amino acid sequence (Martin et al., 1995; Schultheiss et al., 1995). However, the substrate specificity of picornavirus 3C<sup>Pro</sup> is strongly modified by the flanking peptides, as in 3CD, and the processing of the P1–2A border is known to be sensitive to conformational disturbances in 2A (Hahn & Palmenberg, 1996). Hence, products with N termini mapping to either position 791 or 836 may be generated in vivo by alternative cleavage. The former may be a functional 2A able to induce cytopathic effect, whereas the shorter peptide would be devoid of inhibitory activity.

Since cleavage of the Glu/Ser junction 791/792 (but not at positions 836/837) seems to be required for proper capsid formation (Martin et al., 1998), the possibility should be considered that the various peptides reported so far may be the intermediate products of a cascade proteolytic process that uses mutually exclusive alternative cleavage pathways. Cleavage at the Glu/Ala pair 836/837 may prevent the subsequent cleavage of the Glu/Gly junction at residues 981/982 by some kind of allosteric hindrance, while cleavage at positions 791/792 would generate a proper VP1 precursor and would allow cleavage at positions 981/982.

The question remains open, therefore, as to whether a single protease with modified substrate specificity (due perhaps...
to alternative flanking regions) would suffice to secure the proposed alternative cleavage pathways.

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