Identification of hepatitis A virus non-structural protein 2B and its release by the major virus protease 3C

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The RNA genome of hepatitis A virus (HAV) encodes a giant polyprotein that is putatively cleaved proteolytically into four structural and seven non-structural proteins. So far, most of the proposed non-structural proteins and their respective cleavage sites have not been identified. A vaccinia virus recombinant (vRGORF) containing the complete HAV ORF under the control of the bacteriophage T7 promoter was used to express HAV in recombinant animal cells (BT7-H) that constitutively expressed T7 DNA-dependent RNA polymerase. A HAV-specific 27.5 kDa expression product was identified as peptide 2B. The 27.5 kDa 2B antigen was also found in HAV-infected MRC-5 cells. The N-terminal amino acid residues of the new peptide 2B are Ala-Lys-Ile-Ser-Leu-Phe and polyprotein cleavage between 2A and 2B occurred at amino acids 836-837 (Gln-Ala). Furthermore, heterologous expression in the same system of regions P1-P2 and of the protease 3C (3Cpre) gene, showed that P1–P2 polyprotein is not cleaved autocatalytically but by 3Cpre. Hence, 3Cpre is effective in cleaving the polyprotein 2A–2B junction.

Introduction

Hepatitis A virus (HAV) was identified as an aetiological agent of acute hepatitis in humans (Feinstone et al., 1973) and was successfully adapted to growth in cultured cells by Provost & Hilleman (1979). Because of its distinct features, the virus has recently been classified taxonomically as the prototype virus of the genus Hepatovirus within the family Picornaviridae (Minor, 1991). Among the distinguishing features of this virus are its extreme physical stability (Siegl et al., 1984a), its protracted replication in cell culture (Siegl et al., 1981) and the few similarities of the HAV genome and protein sequences with those of other picornaviruses (Tiechurst et al., 1989).

The ssRNA genome is approximately 7500 nucleotides long (Cohen et al., 1987a) and has messenger polarity. Its 5' end is covalently linked to a small virus-encoded protein VPg (Weitz et al., 1986) and contains a large non-translated region (NTR) of 735 nucleotides (Tiechurst et al., 1989). The RNA codes for a giant polyprotein of 253 kDa (Cohen et al., 1987a) and is polyadenylated at its 3' end (for reviews see Tiechurst et al., 1989; Weitz & Siegl, 1993). The polyprotein may be divided into mature structural (VP1–VP4) and non-structural proteins (2A–2C, 3A–3D). It is co- and post-translationally processed primarily by the virus-encoded protease 3C (3Cpre; Gauss-Müller et al., 1991; Jia et al., 1991).

So far, five out of ten putative protease acceptor sites within the polyprotein have been confirmed by amino acid sequencing of the physically identified peptides VP2 (Gauss-Müller et al., 1986), VP1 (Linemeyer et al., 1985), 3A (Harmon et al., 1992), 3C (Gauss-Müller et al., 1991) and 3D (Tesar et al., 1994). Indirect experimental evidence has also been presented for the position of junctions VP2-VP3 (Wheeler et al., 1986) and 2B–2C (Malcolm et al., 1992). However, the structure of the polyprotein in the P2 region in particular has been scarcely investigated and little is known about the actual functions of proteins from this region.

Proteins 2C and 2BC of poliovirus are apparently functionally involved in proliferation of membranes in infected cells and protein 2C shares similarities with helicases (Gorbalenya & Koonin, 1989). The biochemical functions of 2B and 2C in HAV are not yet known. Nucleic acid sequence analyses of variant isolates imply that the 2B and 2C genes may code for products involved in adaptation of the virus to cell culture (Cohen et al., 1989; Emerson et al., 1993; Lemon et al., 1991). Experimental evidence of the physical existence of these products is, however, missing. In contrast, considerable knowledge has been accumulated on picornavirus protein 2A. Enterovirus and rhinovirus protein 2A functions primarily as a protease, releasing P1 polyprotein from nascent P2–P3 polyprotein (Toyoda et al., 1986), whereas
for cardioviruses and aphthoviruses, this primary cleavage occurs at the 2A–2B junction with the involvement of 2A amino acid residues (Ryan et al., 1991). A similar functional role of 2A of HAV is unlikely because it does not share significant amino acid sequence similarities with 2A of these other genera. In addition, the predicted size of peptide 2A is extraordinarily large compared to the respective proteins of other members in the virus family (Ticehurst et al., 1989). Furthermore, the actual existence of a HAV 2A equivalent has not been physically proven.

Investigating morphogenetic processes in HAV replication, Anderson & Ross (1990) reported the presence of a unique structural peptide precursor pX (apparent molecular mass 40 kDa), comprising VP1 and proposedly part of the 2A amino acid sequence. Peptides similar to pX have also been observed by others (Cho & Ehrenfeld, 1991; Winokur et al., 1991). However, the variation in apparent molecular mass (38 to 42 kDa) and the lack of precise characterization posed the questions whether pX contained a 2A fragment that was produced by cleavage within the predicted 2A or whether it contained authentic 2A, the size of which had been predicted on false positioning of the 2A–2B junction. To resolve these questions our investigation aimed to identify the authentic cleavage site and the proteolytic activity that separate 2A and 2B.

Methods

Enzymes and plasmids. Restriction enzymes, ligase and DNA polymerase I (Klenow fragment) used in cloning DNA were purchased from Boehringer Mannheim and New England Biolabs. Taq DNA polymerase for PCR and calf intestinal alkaline phosphatase were from Boehringer Mannheim. T4 polynucleotide kinase was purchased from Gibco BRL. Subeloning and PCR procedures were performed according to standard protocols (Sambrook et al., 1989; Ausubel et al., 1991).

For expression of 3C<sup>pp</sup>, a PCR fragment encoding the 3C<sup>pp</sup> region was amplified from plasmid pHAV/7 (Cohen et al., 1987b). To allow for high translation efficiency, the primer HAV3C2 (Table 1) comprised a Kozak consensus sequence, an artificial start codon and an upstream BamHI site for cloning. Via the reverse primer HAV3C4R (Table 1), two in-frame stop codons and a second BamHI site were introduced. The PCR product was cleaved with BamHI and subcloned into the unique BamHI site of the vaccinia virus transfer vector pTF7-5 (Fuerst et al., 1987) to yield plasmid pRG3C.

The complete HAV ORF was subcloned into pRG-1. This plasmid was derived by modification of pTF7-5. A synthetic linker 5' CCGGATCCCTATTACCTGACCATTTCAATTTT 3' comprising a Kozak consensus sequence and unique NcoI and SalI sites, respectively, had been introduced into the BamHI site of pTF7-5, resulting in plasmid pRG-1.

Generation of pRGORF was subsequently achieved in two steps. A DNA fragment coding for the P1–P2 region was cloned in plasmid pRG-1 by a PCR technique termed 'gene splicing by overlap extension' (Horton et al., 1990) using pHAV/7 as the template. A BspHI–SalI PCR fragment consisting of the N terminus of region P1 (sense primer 730at; antisense primer R1195at; Table 1) and the C terminus of region P2 (sense primer 4912at; antisense primer R4995at; Table 1) was introduced into pRG-1 linearized with NcoI and SalI to yield plasmid pRGAPI-ΔP2. This plasmid was linearized with BstEII and EcoRI and ligated to a pHAV/7 BstEII–EcoRI fragment (representing the remainder of regions P1, P2) resulting in plasmid pRGPI-P2.

An EcoRI–SalI fragment [representing region P3, the 3' NTR and the poly(A) tail of HAV] was asymmetrically ligated to pRGPI-P2 that had been cleaved with SalI, blunt-ended and had been linearized with EcoRI, to yield pRGORF.

Virus and cells. Wild-type vaccinia virus (strain WR; ATCC) was propagated in HeLa S3 suspension cultures and purified according to standard procedures (Ausubel et al., 1991). CV-1, BS-C-1 and human thymidine kinase-negative (TK) 143 cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% (v/v) fetal bovine serum (FBS). HeLa S3 cells were grown in suspension cultures in HAM F-12 medium containing 10% (v/v) FBS. MRC-5 cells were grown in MB medium containing 10% (v/v) FBS. BT7-H cells were grown in DMEM containing 10% (v/v) FBS and 500 μg/ml gentamicin (DMEM; H; Whetter et al., 1994).

Vaccinia virus recombinants. Recombinant vaccinia viruses were prepared by transfection of wild-type virus-infected CV-1 cells with plasmid recombinants containing HAV genes (Ausubel et al., 1991). Virus recombinants were isolated and purified after selection through three consecutive rounds of plaqueing on TK<sup>−</sup> cells (Ausubel et al., 1991). Large stocks of virus recombinants were prepared in HeLa S3 cells. Identification of virus recombinants was achieved by PCR (Pasamontes et al., 1991; Ausubel et al., 1991).

Expression. Confluent BT7-H cell monolayers were infected with virus recombinants and wild-type virus at a m.o.i. of 10 p.f.u./cell and radiolabelled metabolically with 100 μCi/ml of <sup>35</sup>S]methionine (1000 Ci/mmol; NEN) in culture medium lacking [35S]methionine from 2 h post-infection (p.i.) until being harvested at the times indicated in the figure legends.

After lysis of cells (Fuerst et al., 1987), immunoprecipitation of proteins was performed with rabbit antisera raised against synthetic

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<th>Table 1. HAV-specific oligonucleotides used in PCR reactions</th>
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<tr>
<td>Oligonucleotide</td>
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<tr>
<td>-----------------</td>
</tr>
<tr>
<td>HAV3C2</td>
</tr>
<tr>
<td>HAV3C4R</td>
</tr>
<tr>
<td>730at</td>
</tr>
<tr>
<td>R1195at</td>
</tr>
<tr>
<td>4912at</td>
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<td>R4995at</td>
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* HAV-specific sequences are underlined.
† Nucleotides numbered according to HAV strain HM175/7 MK5 (Cohen et al., 1987b).
peptides (Table 2). Three μl of preimmune serum were added to 20–200 μl of lysate and the reaction volume was adjusted to 200 μl with Triple buffer [50 mM-Tris–HCl pH 7.5, 150 mM-NaCl, 1% (w/v) sodium deoxycholate, 1% (v/v) Triton X-100 and 0.1% (v/v) SDS]. Fifty μl of Protein A-Sepharose [50% (v/v); PAS; Pharmacia] were added and incubated at 4 °C for 1.5 h or overnight and non-specifically adhering components were then removed by centrifugation.

To preadsorbed antigen mixtures (supernatant), 3 μl of specific hyperimmune serum and 50 μl PAS were added and incubated at 4 °C for 1.5 h. Further processing was according to standard procedure (Ausubel et al., 1991). Immune complexes were analysed by SDS-PAGE (Laemmli, 1970). For detection by radiofluorography, gels were soaked in 1 M-sodium salicylate, dried and exposed to Fuji RX medical X-ray films.

**Amino acid sequence analyses.** Various preparations of metabolically radiolabelled peptide 2B (using different 3H-labelled amino acids) were purified by immunoprecipitation and SDS-PAGE. The peptides were subsequently electrophoretically transferred from the acrylamide gel to a PVDF membrane, excised and subjected to automated N-terminal amino acid sequence analyses directly from the solid support.

Automated Edman degradations were carried out on an Applied Biosystems 477A Protein Sequenator equipped with a reaction cartridge suited to PVDF membrane pieces. PTH amino acids released after each cycle were identified on-line on a model 120A Amino Acid Analyzer (Applied Biosystems). The injector of the PTH analyser was fitted with a 20 μl loop in order to collect approximately 80% of the converted amino acids into the fraction collector. All other sequencing parameters were as described (Jen6 et al., 1995). To determine radioactive cleavage products, fractions were collected and analysed in a Packard Tri Carb Liquid Scintillation Analyzer.

**Propagation of HAV strain CLF in MRC-5 cells.** Confluent MRC-5 cell monolayers were infected with HAV strain CLF (Siegl et al., 1984 b) at a m.o.i. of 1 TCID₅₀/cell and incubated at 32 °C for 3 h, then MB medium containing 2.5% (v/v) FBS was added and cells were incubated at 32 °C until day three. Metabolic labelling was the same as described above, but medium containing [³⁵S]methionine was renewed at 4 day intervals and cells were harvested and lysed at 11 days p.i. Preparation of cell lysates and immunoprecipitation was as above.

**Peptides and antibodies.** The amino acid sequence of synthetic peptides (Table 2) was predicted from the nucleotide sequence of cloned HAV cDNA. Antisera were raised against these synthetic peptides in rabbits as described earlier (Weitz et al., 1986).

### Results

**Construction of chimaeric vaccinia viruses**

The integrity of the regulatory elements and the correct nucleotide sequence of subcloned parts of the HAV genome in plasmids pRG-1, pRG3C, pRGPl-P2 and pRGORF were confirmed by nucleotide sequencing at all junctions of subcloning and showed intact reading frames of HAV sequences coding for 3Cᵣᵣᵣ, P1–P2 and P1–P2–P3 regions, respectively (Fig. 1). Owing to the introduction of a synthetic Kozak consensus sequence, initiation of translation was directed to an authentic AUG codon at position 730 of the HAV genome, or in cases of genes lacking the latter to an artificially AUG codon. Abbreviations: Tkᵣ, and Tkₗᵣ, parts of thymidine kinase gene; Tₗᵣ, and Tₗᵢ, bacteriophage T₇ promoter and terminator sequences; MCS, multiple cloning site; Kozak, Kozak consensus sequence; Stop, two in-frame stop codons. Only relevant regions of plasmid recombinants are shown.

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**Table 2. Synthetic peptides used to raise HAV-specific antisera in rabbits**

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Amino acids</th>
<th>Location on putative HAV polyprotein*</th>
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<tr>
<td>757 (2B)</td>
<td>CDSRMMELELTRQ</td>
<td>1078–1087</td>
</tr>
<tr>
<td>503 (2A)</td>
<td>INLADRMLGLSVGQEIK</td>
<td>961–980</td>
</tr>
<tr>
<td>426† (VP1)</td>
<td>VPI 4–139</td>
<td>495–630</td>
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* Amino acids numbered according to HM175 (Cohen et al., 1987a).
† Raised in rabbits against recombinant VP1-β-galactosidase fusion protein.
Cells. A very large peptide, supposedly representing the HAV complete polyprotein and only detected in minute amounts at 20 to 24 h p.i. (Fig. 2b). It was not isolated and SDS-PAGE. Lysates of vRGORF-infected BT7-H cells were then analysed by specific radioimmunoprecipitation with anti-2B antiserum and analysis of immune complexes by SDS-PAGE. Lane 1, mock-infected cells; lane 2, wild-type vaccinia virus-infected cells; lane 3, vRGORF-infected cells. The sizes of molecular mass markers are given in kDa.

Expression and identification of HAV peptide 2B

To identify expressed HAV-specific peptides, lysates were then analysed by specific radioimmunoprecipitation and SDS-PAGE. Lysates of vRGORF-infected BT7-H cells contained a 27.5 kDa peptide which could be recognized at 5 h p.i. by anti-2B antiserum (data not shown). This peptide was expressed at maximum concentrations of peptide 757 that had been used to produce the hyperimmune serum (Table 2). Formation of an immune complex with the 27.5 kDa peptide was inhibited in a concentration-dependent manner with more than 50 ng of the synthetic immunogen present. In contrast, background peptide patterns of the vaccinia virus- and mock-infected cells were not affected in the presence of 10 times higher concentrations of peptide 757 (data not shown).

Identification of peptide 2B in HAV-infected MRC-5 cells

To correlate the results of heterologous expression to authentic processes in HAV-infected cells, analyses were extended to lysates of HAV-infected MRC-5 cells. With anti-2B antiserum it was possible to specifically enrich a peptide corresponding to the complete HAV polyprotein and only detected in minute amounts after radioimmunoprecipitation with anti-2B antiserum, was not further investigated.

Determination of anti-2B antiserum specificity

To assess the specificity of interaction of the anti-2B antiserum with the 27.5 kDa peptide, radioimmunoprecipitations were performed in the presence of increasing amounts of the synthetic peptide immunogen 757 that had been used to produce the hyperimmune serum (Table 2). Formation of an immune complex with the 27.5 kDa peptide was inhibited in a concentration-dependent manner with more than 50 ng of the synthetic immunogen present. In contrast, background peptide patterns of the vaccinia virus- and mock-infected cells were not affected in the presence of 10 times higher concentrations of peptide 757 (data not shown).
Specific formation of the 2B immune complex was again demonstrated by competition binding analyses with peptide 757 (data not shown).

The results showed that heterologous expression by vRGORF of the complete ORF of HAV and replication of HAV produced the same 27.5 kDa peptide 2B and that this peptide contained 2B sequences. However, the apparent molecular mass (27.5 kDa) of 2B differed significantly from that calculated (12 kDa) on the basis of published data for the HAV genome structure.

Characterization of HAV peptide 2B

To characterize the HAV-specific 27.5 kDa peptide 2B in more detail, lysates of cells infected with vRGORF were analysed with an additional antiserum. This antiserum had been raised in guinea pigs using a peptide that represented the 19 C-terminal amino acids of the originally predicted 2A (Table 2). This putative anti-2A antiserum revealed the presence of an abundant peptide with an apparent molecular mass of 27.5 kDa only in vRGORF-infected cells (Fig. 3b) but not in mock-infected or wild-type vaccinia virus-infected cells. This result indicated that 2B apparently also contained at least 19 amino acids of the originally predicted C-terminal region of 2A. A 68 kDa peptide was variably detected in vaccinia virus- or chimera-infected cells. It was also recognized by various preimmune serum preparations and was therefore disregarded as a common antigen already recognized by preimmunized animals (data not shown).

Analysis of N-terminal amino acid residues of peptide 2B

To determine precisely how much of the predicted 2A was actually part of 2B and to locate the exact position of the 2A–2B junction within the HAV polyprotein, the N terminus of purified peptide 2B was determined by Edman degradation. Individual preparations of peptide 2B (27.5 kDa) radiolabelled with tritiated amino acids Lys, Ile, Ser or Phe were expressed in BT7-H cells. Sequence analyses showed that amino acid residues were located within peptide 2B as follows: Lys at position 2, Ile at position 3, Ser at position 4 and Phe at position 6 (Fig. 4). The corresponding amino acid sequence was found at positions 838 to 843 of the predicted HAV polyprotein. Hence, the 2A–2B junction appeared to be a Gln–Ala dipeptide representing amino acids 836–837 of the HAV polyprotein. The data showed that the 2B region actually spanned two-thirds of the originally predicted 2A. Consequently, 2A would be expected to comprise only amino acids 792 (not confirmed) to 836 of the HAV polyprotein. The calculated molecular mass of 2A would then be 5.3 kDa; 2A would contain only 45 amino acids instead of the predicted 189.

Identification of VP1 precursor peptides

To correlate the modification of the polyprotein structure observed under conditions inherent to the system with observations of pX in HAV-infected cells, expression products were analysed for the presence of pX. Lysates of BT7-H and MRC-5 cells infected with vRGORF and with HAV were subjected to immunoprecipitation with an anti-VP1 antiserum. This antiserum had been raised in rabbits against a recombinant VP1–β-galactosidase fusion protein (Table 2). HAV-specific proteins with molecular masses between 39.5 and 27.0 kDa were observed (Fig. 5a). Two of these peptides had apparent molecular masses of 33.0 and 39.5 kDa, corresponding to VP1 and pX, and were present in both vRGORF- and HAV-infected cells. Additional anti-VP1-reactive peptides ΔVP1 (27.0 and 28.0 kDa) were observed but not investigated further.

Trans-cleavage of polyprotein P1–P2 by 3Cpro

In order to identify the protease that cleaved within the P2 polyprotein region to release 2B from 2A, polyprotein
Fig. 5. Identification of HAV-specific peptides. Infected BT7-H cells were metabolically labelled with [35S]methionine from 2 h p.i. until 20 h p.i. Antigen was radioimmunoprecipitated with (a) anti-VP1 antiserum (kindly provided by Dr Y. Kusov, Institut für Medizinische Mikrobiologie, Lübeck, Germany) or with (b) anti-2B antiserum. Immune complexes were analysed by SDS-PAGE. (a) Identification of HAV peptide 2A precursors. Lane 1, mock-infected cells; lane 2, wild-type vaccinia virus-infected cells; lane 3, vRGORF-infected cells. (b) Trans-activity of protease 3C. Lane 1, mock-infected cells; lane 2, wild-type vaccinia virus-infected cells; lane 3, cells coinfected with wild-type vaccinia virus and vRGPI-P2; lane 4, cells coinfected with vRGPI-P2 and vRG3C. The sizes of molecular mass markers are given in kDa.

comprising only P1–P2 was separately expressed in BT7-H cells coinfected with vRGPI-P2 and wild-type vaccinia virus. A virus-specific peptide with an apparent molecular mass of approximately 160 kDa was precipitated with the anti-2B antiserum from lysates of these cells (Fig. 5b). This peptide was not present in mock-infected or in wild-type vaccinia virus-infected cells and it corresponded by apparent molecular mass to the predicted unprocessed polyprotein P1–P2 of HAV.

To test whether 3Cpro was capable of cleaving P1–P2 polyprotein, BT7-H cells were coinfected with vRGPI-P2 and vRG3C. When cellular lysates after coinfection were analysed with anti-2B antiserum, a prominent peptide 2B (27.5 kDa) was observed (Fig. 5b). Consequently, P1–P2 neither possessed inherent proteolytic activity for self-cleavage, nor was any relevant proteolytic cleavage performed by cellular or vaccinia virus-specific proteases. Furthermore, peptide 2B was not present in mock-infected or in wild-type vaccinia virus-infected cells. Hence, the P1–P2 precursor was specifically cleaved by 3Cpro at both junctions 2A–2B and 2B–2C.

Discussion

Heterologous expression of the complete ORF of HAV in BT7-H cells resulted in processing of the HAV polyprotein. Among the final products, a new protein 2B was identified and characterized by an apparent molecular mass of 27.5 kDa and an N-terminal amino acid sequence of Ala-Lys-Ile-Ser-Leu-Phe. Hence, the cleavage site between 2A and 2B was identified as a Gln–Ala dipeptide at position 836–837 within the predicted HAV polyprotein. These results imply that the originally predicted Gln–Gly dipeptide cleavage site at position 980–981 of the HAV polyprotein (Najarian et al., 1985; Cohen et al., 1987a) has to be relocated to position 836–837 and that 2B is significantly larger than previously assumed. Our results are independently corroborated by N-terminal amino acid sequencing of a 2B–poliovirus VP1 fusion peptide by A. Martin (Pasteur Institute, Paris, France; personal communication). The investigation also showed that 2B is specifically released by 3Cpro. This result is in agreement with the observations prior to identification of 2B that HAV protease 3Cpro might suffice to process the P2 region of the polyprotein (Harmon et al., 1992).

The lack of P1–P2 polyprotein processing observed in the absence of 3Cpro is consistent with observations in translation of synthetic RNAs in vitro. Analyses of translation products resulting from RNAs including intact 2A and 2B coding sequences suggested that cleavage occurred neither at the putative VP1–2A nor at the previously predicted 2A–2B junctions (Kusov et al., 1992; Jia et al., 1993).

The new 2A–2B (Gln–Ala) cleavage site adds variability to the specificity of 3Cpro. The observation is in accordance with earlier findings that 3Cpro of HAV shows considerable flexibility towards dipeptide composition of polyprotein substrate sites (Linemeyer et al.,...
cleaved more effectively by recombinant 3C\textsuperscript{pro} in peptide located at the relevant positions around the proposed positions 1 and 4. Intriguingly, Gln and Leu are also cleavage assays than molecules with other residues at new 2A2B cleavage site. 

Evidence for a crucial role of these two residues was reported for 3C\textsuperscript{pro} of HAV expressed in prokaryotes (Schultheiss et al., 1995). Such assays are useful to determine the specificity of proteolytic enzymes and the findings corroborate results presented here. However, for the 2B–2C cleavage site, it was shown that efficiency of cleavage in such assays does not necessarily accurately reflect processes observed with authentic substrate (polyprotein) and virus-driven expression of 3C\textsuperscript{pro} (Jewell et al., 1992).

The results presented here not only redefined peptide 2B but also showed that 2A of HAV is much smaller than previously assumed (Najarian et al., 1985; Cohen et al., 1987 a). With the new 2A–2B junction, 2A would be expected to have an approximate molecular mass of 5.3 kDa when calculated on the basis of its amino acid sequence. But its exact N terminus and, conversely, the C terminus of VP1 remains to be determined to characterize 2A precisely.

The newly predicted region for 2A (45 amino acids) shows very little similarity to the corresponding region in other picornaviruses. In particular, it neither contains the amino acid residues (catalytic triad) of the putative active site of 2A proteases of rhinoviruses (Palmenberg, 1990), nor does it share any significant similarity in its C terminus with cardio- and aphthoviruses (Ryan et al., 1991). In addition, lack of p220 inactivation by HAV and thus failure of host cell shut-off (De Chastonay & Siegl, 1987) suggest that 2A of HAV has features distinct from that of poliovirus 2A (Toyoda et al., 1986; Macadam et al., 1994). This is consistent with the idea that the diverse functions of a related gene product encoded by different picornaviruses may result from the varied requirements of the virus for growth in different host cells (Kong et al., 1994). Hence, it is unlikely that 2A of HAV functions in the same manner as the 2A proteins of other picornaviruses, and its function remains unknown.

Moreover, it has recently been shown by deletion studies that up to 15% of the central 2A sequence is not essential for virus viability in cell culture or in susceptible primates (Harmon et al., 1995).

The results of our investigation with the vaccinia virus chimera, vRGORF, were confirmed by identification of a peptide 2B in lysates of HAV-infected MRC-5 cells. Hence, not only was heterologous expression effective, but it was also accompanied by authentic processing of the polyprotein. The results presented imply that the VP1 precursor pX contains not only part but all of 2A. Because vRGORF comprised the complete HAV genome except for the 5'NTR, infectivity should be abolished (Ura kawa et al., 1989). In contrast, the presence of only trace amounts of high molecular mass precursors suggested that processing of the polyprotein was nearly complete and thus supported the conclusion that authentic processing had occurred. However, much higher amounts of pX than VP1 were present in vRGORF-infected cell lysates. This is consistent with morphogenesis studies on HAV that revealed subviral particles preferentially containing pX rather than VP1. In contrast, mature HAV virions contain predominantly VP1 (Anderson & Ross, 1990). These results substantiate the assumption that pX is a precursor to VP1 and is most likely VP1–2A. Furthermore, these results imply that the precursor pX is cleaved by an unknown mechanism concomitantly with packaging of the viral RNA and thus with morphogenesis of virions. The cleavage activity responsible for this scission has yet to be identified. The primary processing of the HAV polyprotein was found to resemble neither that of aphtho- and cardioviruses (Vakharia et al., 1987; Jackson, 1986), nor that of entero- and rhinoviruses (Toyoda et al., 1986; Sommergruber et al., 1989). Therefore, it may be a novel type of polyprotein cleavage mediated not by an activity specific to the 2A region, but by 3C\textsuperscript{pro}.

Relocation of the 2A–2B junction within the polyprotein has considerable impact on functional models of proteins 2A and 2B. Cytopathic variants of HAV have mutations in both the 5'- and 3'NTR and in non-structural protein regions P2–P3. In particular, mutations within region P2, mainly in 2B and 2C but also in 2A were linked to growth characteristics of HAV isolates (Lemon et al., 1991; Cohen et al., 1987b, 1989; Emerson
et al., 1993). Our data indicate that the observed mutations now are exclusively located in genes 2B and 2C.

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