Characterization of Replication-Competent Hepatitis A Virus Constructs Containing Insertions at the N Terminus of the Polyprotein

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To determine whether hepatitis A virus (HAV) could tolerate the insertion of exogenous sequences, we constructed full-length HAV cDNAs containing in-frame insertions at the N terminus of the polyprotein and transfected the derived T7 RNA polymerase in vitro transcripts into FRhK-4 cells. Replication of HAVvec1, a construct containing an insertion of 60 nucleotides coding for a polylinker, a 2B/2C cleavage site for HAV protease 3Cpro, and two initiation codons that restored the sequence of the N terminus of the polyprotein, was detected 2 weeks after transfection by indirect immunofluorescence analysis using anti-HAV monoclonal antibodies. Western blot analysis of HAVvec1-infected cells using anti-VP2 and anti-VP4 antibodies failed to detect the expression of the inserted sequences. Insertion of a 24-mer oligonucleotide coding for a FLAG epitope into HAVvec1 resulted in its HAV-mediated expression which was retained upon deletion of a Glu residue from the inserted 2B/2C cleavage site. Western blot analysis using anti-FLAG and anti-VP2 antibodies showed that the FLAG epitope accumulated in infected cells fused to VP0. Replacement of the FLAG epitope with an epitope of the circumsporozoite protein (CSP) of Plasmodium falciparum resulted in its stable HAV-mediated expression for at least six serial passages in FRhK-4 cells. Sedimentation analysis in sucrose density gradients showed that the CSP epitope accumulated in infected cells fused to VP0, forming 80S empty capsids which also contained native VP0. Our data suggest that the HAV internal ribosome entry site can efficiently direct dual initiation of translation of the polyprotein from AUG codons separated by 66 to 78 nucleotides and show that HAV can tolerate insertions at the N terminal of the polyprotein.

Hepatitis A virus (HAV), the causative agent of acute hepatitis in humans (23), is the single member of the Hepatovirus genus of the Picornaviridae (21). This family of small, nonenveloped, positive-strand RNA viruses include human pathogens such as poliovirus (PV) and rhinovirus and animal pathogens such as foot-and-mouth disease virus and encephalomyocarditis virus, which are grouped within the Enterovirus, Rhinovirus, Aphthovirus, and Cardioivirus genera, respectively. HAV is transmitted via the oral-fecal route and can be prevented with formalin-inactivated vaccines containing HAV propagated in cell culture (24, 47).

The RNA genome of HAV is about 7,500 bases long and has a small virus-encoded VPg protein covalently linked to its 5′ end. After binding to a cellular receptor identified in African green monkey kidney cells as HAVcr-1 (28), the HAV genome is delivered to the cytoplasm by an unknown mechanism. The 5′ nontranslated region of the HAV genome, which is a long and complex structure containing an internal ribosome entry site (IRES), directs the cap-independent translation of the viral message (reference 26 and references therein). A single long open reading frame codes for a polyprotein from which structural proteins VP0, VP3, and VP1 and nonstructural proteins are cleaved by 3Cpro, the only HAV-encoded protease (41). Sixty copies of each of the structural proteins assemble into viral capsids, which in association with the HAV genome form viral particles that undergo a slow RNA-dependent maturation cleavage of VP0 into VP4 and VP2 (13). Downstream from the IRES there are two in-frame AUG codons at nucleotides (nt) 735 to 737 and 741 to 743; although both are used, translation of the polyprotein preferentially starts from the second AUG, which has a less favorable initiation context (43). Cardioviruses and aphthoviruses also have two in-frame AUG initiation codons separated by 9 and 84 nt, respectively, and both are used to initiate translation of the polyprotein producing two forms of the leader (L) protein, a nonstructural protein which precedes the structural proteins. HAV, on the other hand, does not code for an L protein, and its first translated protein is an unusually small VP4 protein of 21 to 23 amino acids that has not yet been found in the viral capsid (22, 29, 44). Although the N-terminal amino acid of VP4 of other picornaviruses is a myristylated Gly residue essential for virus viability (9, 16), there is no evidence that internal residues of VP4 of HAV are exposed and myristylated (44). Furthermore, it has recently been shown that addition of a Pro residue preceding the N-terminal Gly residue, which blocks its myristylation, is lethal for PV (35).

To further study the mechanism of initiation of translation in HAV and to determine whether this virus could tolerate the addition of sequences immediately downstream of the polyprotein initiation codons, we made in-frame insertions at the N terminus of the polyprotein. Infection of FRhK-4 cells with HAV constructs coding for a FLAG peptide or an NANP immunodominant epitope of the circumsporozoite protein (CSP) of Plasmodium falciparum inserted at the N terminus of the polyprotein resulted in the HAV-mediated expression of the tag epitopes fused to VP0 and termed ExtVP0. The simultaneous expression of ExtVP0 and a protein that comigrated with native VP0 in cells infected with our constructs pointed to
FIG. 1. Nucleotide and amino acid sequences of constructs containing insertions in the unique XbaI site at nt 744 of the HAV cDNA. Synthetic oligonucleotides coding for a putative polypeptide containing two adjacent initiation codons, a 2B/2C cleavage site for the HAV 3Cpro, and peptide MNVRK containing two initiation codons which restore the native sequence of VP0 were cloned into the unique XbaI site of pT7HAVxbaI and termed pT7HAVvecl. Synthetic oligonucleotides coding for the DYKDDDDK FLAG peptide were inserted into the SalI and KpnI sites of pT7HAVvecl, and the resulting construct was termed pT7HAVvec1Flag12. Deletion of the Gin residue required for the 3Cpro-mediated cleavage of the inserted 2B/2C cleavage site was introduced into pT7HAVvec1Flag12 by replacing the KpnI-XbaI DNA fragment with synthetic oligonucleotides, and the construct was termed pT7HAVvec1Flag34. The SalI-KpnI DNA fragment coding for the FLAG peptide in pT7HAVvec1Flag34 was replaced with synthetic oligonucleotides coding for an immunodominant NAP epitope of the CSP of P. falciparum (15) is indicated with a vertical arrow. The first (or native) set of initiation codons is boxed in white, and the second (or inserted) set of initiation codons is boxed in grey.

MATERIALS AND METHODS

Cells and viruses. Fetal thymus monkey kidney (FRhK-4) cells were a gift of S. Emerson, National Institutes of Health. Cells were grown in monolayer cultures in Eagle minimal essential medium supplemented with 10% horse serum.

The stocks of tissue culture adapted HM175 strain of HAV (HAV HM175) were derived from infectious cDNA (17) and grown in FRhK-4 cell monolayers. Nucleotide positions of the HAV genome are according to the published HAV HM175 cDNA sequence (17).

PV type 1 Mahoney strain (PV1/M) was obtained from the American Type Culture Collection and grown in HeLa S3 cell monolayers (27).

Antisera. Anti-VP4 antiserum was obtained from rabbits immunized with a polypeptide containing two AUG codons at the N terminus of the polyprotein of the following plasmids constructed for this work.

(i) pT7HAV. The Nhel-BglII DNA fragment of pHAV/7 (17), which contains the bacteriophage SP6 RNA polymerase promoter and nt 1 to 23 of the HAV cDNA, was replaced with annealed synthetic oligonucleotides 5'-CTAGCTAA TACGACTCTATAATGTCAGAGGGGAGTCTCCGGAATT-3' and 5'-CC CGGAATATTCGGGACCCCTCCTCGGGAATT-3' followed by the bacteriophage T7 RNA polymerase promoter followed by a G residue and the first 23 nt of the HAV cDNA. The synthetic oligonucleotides were used as primers for in vitro transcription of the resultant plasmid, pT7HAV, linearized with HaeIII were infectious upon transfection into FRhK-4 cells.

(ii) pT7HAVxbaI. The XbaI site immediately downstream of the poly(A) tail in pT7HAV was eliminated by filling in with DNA polymerase I Klenow enzyme. The resulting plasmid, pT7HAVxbaI, contains a unique XbaI site at position 744 of the HAV cDNA. T7 RNA polymerase was used to transcribe the plasmid, pT7HAVxbaI, linearized with Sahl was infectious upon transfection into FRhK-4 cells.

(iii) pT7HAVvec1. Annealed synthetic oligonucleotides 5'-CTAGCTAAC TACGACTCTATAATGTCAGAGGGGAGTCTCCGGAATT-3' and 5'-CTAGCTAAC TACGACTCTATAATGTCAGAGGGGAGTCTCCGGAATT-3' were used as primers for in vitro transcription of the resultant plasmid, pT7HAVvec1, which contains an in-frame insertion of 20 codons at the N terminus of the HAV cDNA. T7 RNA polymerase was used to transcribe the plasmid, pT7HAVvec1, linearized with HaeIII were infectious upon transfection into FRhK-4 cells.
the HAV polyprotein. The synthetic oligonucleotides restored only the XbOl site within the VP0 coding sequence.

(iv) \textit{pT7HAVvecFlag12}. Annealed synthetic oligonucleotides 5'-TCGACTA CAGGGACGACATGACAGCCACG-3’ and 5'-CTCCTTTGCATCTGCGTCG CTTGGA-3’, coding for the FLAG octapeptide DTKDDDDK, were cloned into the SalI and KpnI sites in the polylinker of \textit{pT7HAVvec}. The resulting plasmid, \textit{pT7HAVvecFlag12}, contains an in-frame insertion of 24 codons at the N terminus of the HAV polyprotein.

\textit{pT7HAVvecFlag13}. The KpnI-XhoI fragment of \textit{pT7HAVvecFlag12} was exchanged with annealed synthetic oligonucleotides 5'-GACATCGACTGACATGACAGCCACG-3’ and 5'-GATCCGTCGTCCTGGA-3’, resulting in a SalI and KpnI site in the polylinker of \textit{pT7HAVvec}. The resulting plasmid, \textit{pT7HAVvecFlag13}, coding for the FLAG octapeptide DTKDDDDK, was cloned and has an in-frame insertion of 24 codons at the N terminus of the HAV polyprotein.

\textit{pT7HAVvecINANP2}. The SalI-BamHI fragment of \textit{pT7HAVvecFlag3} coding for the FLAG peptide was replaced with annealed synthetic oligonucleotides 5'-GACAATCCGAACGCCAATCCAAACG-3’. The resulting plasmid, \textit{pT7HAVvecFlag3}, coding for the FLAG octapeptide DTKDDDDK, was cloned and has an in-frame insertion of 24 codons at the N terminus of the HAV polyprotein.

\textit{pT7HAVvecINAP2}. The SalI-BamHI fragment of \textit{pT7HAVvecFlag3} coding for the FLAG peptide was replaced with annealed synthetic oligonucleotides 5'-GACAATCCGAACGCCAATCCAAACG-3’ and 5'-GATCCGTCGTCCTGGA-3’. The resulting plasmid, \textit{pT7HAVvecFlag3}, coding for the FLAG octapeptide DTKDDDDK, was cloned and has an in-frame insertion of 24 codons at the N terminus of the HAV polyprotein.

\textit{pT7HAVvecINANP2}. The SalI-BamHI fragment of \textit{pT7HAVvecFlag3} coding for the FLAG peptide was replaced with annealed synthetic oligonucleotides 5'-GACAATCCGAACGCCAATCCAAACG-3’ and 5'-GATCCGTCGTCCTGGA-3’. The resulting plasmid, \textit{pT7HAVvecFlag3}, coding for the FLAG octapeptide DTKDDDDK, was cloned and has an in-frame insertion of 24 codons at the N terminus of the HAV polyprotein.

\textbf{Results}

\textbf{Insertion of a multiple cloning site immediately downstream of the polyprotein initiation codons.} To test whether HAV can tolerate the insertion of exogenous sequences at the N terminus of the polyprotein, we cloned synthetic oligonucleotides immediately downstream of the AUG initiation triplets at the unique XbOl site (nt 744) of \textit{pT7HAVXba1}. Preliminary results indicated that cloning of oligonucleotides coding for a poly-linker and a tag epitope at this XbOl site resulted in highly unstable HAV constructs which deleted the inserted sequences upon few passages in FRhK-4 cells. To circumvent this stability problem, we cloned into the unique XbOl site of \textit{pT7HAVXba1} complementary 60-mer synthetic oligonucleotides coding for a polylinker containing SalI, SnaBI, and KpnI restriction sites, followed by the 2B/2C cleavage site ELRTQ/SFSN for HAV protease C3Pro (33) and peptide MNNS, which restored the native VP4 sequence (Fig. 1). The resulting construct, \textit{pT7HAVvec1}, contains two in-frame sets of adjacent AUG initiation codons immediately downstream of the HAV IRES. Confirmation could be obtained by expression of the polyprotein at (i) the first set of AUG codons, giving rise to a protein larger-than-VP0 that we named ExtVP0, (ii) the second set of AUG codons, giving rise to native VP0, or (iii) both sets of AUG codons, giving rise simultaneously to ExtVP0 and VP0 (Fig. 1). It is also possible that C3Pro could recognize the
mock-infected FRhK-4 cells stained with anti-HAV Mabs (Fig. 2I) did not fluoresce, confirming that the staining was HAV specific. RT-PCR and nucleotide sequence analysis of the amplified cDNA revealed that HAVvec1 coded for the expected 60 nt inserted at the N terminus of the polyprotein (Fig. 1) and that these sequences were stably maintained for at least six serial passages in FRhK-4 cells.

**HAV-mediated expression of a FLAG epitope.** Since our data indicated that HAV could tolerate insertions at the N terminus of the polyprotein, it was of interest to determine whether the inserted sequences were expressed in infected cells. To do so, we cloned complementary synthetic oligonucleotides coding for the FLAG octapeptide DTKDDDDK (IBI) into the SalI and KpnI sites of the polylinker of pHAVvec1. The resulting plasmid, pT7HAVvec1Flag12 (Fig. 1), was transcribed in vitro and transfected into FRhK-4 cells. Replication of HAVvec1Flag12 was detected 25 days posttransfection by indirect IF analysis using anti-HAV Mabs. A stock of HAVvec1Flag12 was prepared and passaged twice in FRhK-4 cells, and infected cells were analyzed by indirect IF using anti-Flag M2 and anti-HAV Mabs (Fig. 2), which showed the presence of FLAG (Fig. 2F) and HAV epitopes (Fig. 2G), respectively. Mock-infected (Fig. 2J), HAV HM175-infected (Fig. 2B), and HAVvec1-infected (Fig. 2D) FRhK-4 cells stained with anti-FLAG Mab M2 did not fluoresce, indicating that the staining was FLAG specific. These data confirmed that HAV can mediate the expression of an epitope tag inserted at the N terminus of the polyprotein.

To analyze whether the inserted 2B/2C cleavage site was necessary for the HAV-mediated expression of the FLAG epitope, we deleted the Gln residue required for the 3Cpro-mediated cleavage of this site (2). The resulting plasmid, pT7HAVvec1Flag34, was transcribed in vitro and transfected into FRhK-4 cells. After 20 days posttransfection, indirect IF analysis using anti-HAV Mabs revealed the characteristic granular fluorescence of HAV-infected cells. A stock of HAVvec1Flag34 virus was prepared and passaged twice in FRhK-4 cells, and infected cells were analyzed by indirect IF using anti-Flag M2 and anti-HAV Mabs (Fig. 2), which showed the presence of FLAG (Fig. 2H) and HAV (Fig. 2G) epitopes, indicating that the 3Cpro-mediated cleavage of the inserted 2B/2C site was not required for the HAV-mediated expression of the tag epitope. It is possible that the lower level of FLAG-specific fluorescence (Fig. 2F and H) compared to the HAV-specific fluorescence (Fig. 2E and G) was due to the intrinsic characteristics of the Mabs used in the staining. However, the preferential initiation of translation from the second set of initiation codons, the possible deletion of the inserted sequences, and/or the proteolytic degradation of ExtVP0 could also play a role in the lower level of FLAG-specific fluorescence.

**The FLAG peptide accumulates as ExtVP0 in infected cells.** To further analyze the HAV-mediated expression of the tag epitope, we studied cytoplasmic extracts of infected and mock-infected FRhK-4 cells by Western blot analysis probing with anti-FLAG Mab M2, rabbit anti-VP4, and human anti-HAV antibodies (Fig. 3). Anti-FLAG Mab M2 recognized a 31-kDa band in HAVvec1Flag12-infected cells (closed arrowhead, lane 1) and a 32-kDa band in HAVvec1Flag34-infected cells (open arrowhead, lane 2), but it did not react with HAVvec1-, HAV-, and mock-infected cells (lanes 3 to 5). The anti-VP4 antibody also recognized a 31-kDa band in HAVvec1Flag12-infected cells (closed arrowhead, lane 6) and a 32-kDa band in HAVvec1Flag34-infected cells (open arrowhead, lane 7) which comigrated with similar bands detected with Mab M2 (compare lanes 1 and 2 with lanes 6 and 7). As expected, the
ExtVP0 band in HAVvec1Flag34-infected cells. The positions and sizes of band in HAVvec1Flag12-infected cells; the open arrowheads point to a 32-kDa anti-HAV Ab (lanes 11 to 15). The closed arrowheads point to a 31-kDa ExtVP0 anti-FLAG MAb M2 (lanes 1 to 5), rabbit anti-VP4 Ab (lanes 6 to 10), or human SDS-PAGE (12.5% gel), transferred to nylon membranes, and probed with HM175 HAV-infected (lanes 4, 9, and 14), and mock-infected (lanes 5, 10, and 15) FRhK-4 cells were prepared in RSB–1% NP-40. Proteins were separated by SDS-PAGE (12.5% gel), transferred to nylon membranes, and probed with anti-FLAG MAb M2 (lanes 1 to 5), rabbit anti-VP4 Ab (lanes 6 to 10), or human anti-HAV Ab (lanes 11 to 15). The closed arrowheads point to a 31-kDa ExtVP0 band in HAVvec1Flag12-infected cells; the open arrowheads point to a 32-kDa ExtVP0 band in HAVvec1Flag34-infected cells. The positions and sizes of prestained molecular weight markers are shown on the left. The migration of VP0 and VP1 is indicated with arrows.

anti-VP4 Ab reacted specifically against a 29-kDa VP0 band present in HAV-infected (lanes 6 to 9) but not in mock-infected cells (lane 10) (23); this Ab also detected a faint cellular band of approximately 20 kDa in HAV-infected (lanes 6 to 9) and mock-infected (lane 10) cells. Surprisingly, the anti-VP4 Ab did not detect larger-than-VP0 proteins in HAVvec1-infected cells (lane 8), indicating that the sequences inserted at the N terminus of the polyprotein were poorly translated, not translated at all, or degraded. The expression of larger-than-VP0 proteins in HAVvec1Flag12- and HAVvec1Flag34-infected cells that reacted with anti-VP4 Ab and anti-FLAG MAB M2 indicated that the FLAG epitope accumulated fused to VP0, forming a novel set of proteins that we defined as ExtVP0 proteins. The putative 3Cpro-mediated cleavage of ExtVP0 of HAVvec1Flag12 should have yielded a FLAG-containing peptide of 19 to 21 residues and VP0 containing 4 residues more than native VP0; however, we did not detect such protein species by Western blot analysis. On the other hand, the presence of native VP0 and ExtVP0 in HAVvec1Flag12- and HAVvec1Flag34-infected cells suggested the possibility that there was a dual initiation of translation of the polyprotein starting from two sets of AUG initiation codons: the first set located immediately downstream of the 3′ end of the IRES that initiates translation of ExtVP0, and the second set located 24 to 25 codons downstream that initiates translation of native VP0. Our results did not rule out the unlikely possibility that ExtVP0 was digested by cellular proteases, giving rise to a single VP0 molecule that comigrated with native VP0. It is also interesting that we did not detect larger-than-VP4 proteins corresponding to VP4 fused to the FLAG epitope. Such proteins would result from the putative maturation cleavage of ExtVP0 to form VP2 and a VP4-FLAG fusion peptide ranging in size from the 21- to 23-residue VP4 to the 46- to 48-residue VP4-FLAG fusion peptide in HAVvec1Flag12. The human anti-HAV polyclonal antisera reacted strongly against a 33-kDa protein present in HAV-infected cells (lane 8), indicating that the sequences inserted at the N terminus of the polyprotein were poorly translated, not translated at all, or degraded. The expression of larger-than-VP0 proteins was not affected by the insertions at the N terminus of the polyprotein. It should be pointed out that cytoplasmic extracts from similar amounts of cells were loaded in each well; therefore, the weak reaction of the anti-VP4 Ab with the HAVvec1Flag34-infected cell extract (lane 7) reflects a lower amount of viral proteins present in that particular extract.

To improve the specificity and sensitivity of the detection of HAV proteins in infected cells, cytoplasmic extracts were immunoprecipitated with human anti-HAV antiserum prior to Western blot analysis, using anti-VP2 Ab and anti-FLAG MAb M2 (Fig. 4). The anti-VP2 Ab detected a 27-kDa VP2 band in HAV-infected (lanes 1 to 4) but not mock-infected (lane 5) cells. A faint band migrating faster than VP2 was also detected by the anti-VP2 Ab in infected (lanes 1 to 4) but not mock-infected (lane 5) cells, indicating that it was of viral origin and typically present in HAV-infected cells. The anti-VP2 Ab also reacted with a 29-kDa VP0 band in HAV HM175-, HAVvec1-, HAVvec1Flag12-, and HAVvec1Flag34-infected cells (lanes 1 to 4). ExtVP0 bands of 31 and 32 kDa, which were previously recognized by the anti-VP4 Ab (Fig. 3), also reacted with the anti-VP2 Ab in HAVvec1Flag12-infected (lane 1) and HAVvec1Flag34-infected (lane 2) cells, respectively. The anti-FLAG MAB M2 also recognized ExtVP0 bands of 31 and 32 kDa in HAVvec1Flag12-infected (lane 6) and HAVvec1Flag34-infected (lane 7) cells, but it did not react with HAVvec1-, HAV HM175-, and mock-infected cells (lanes 8 to 10), indicating that the reaction was FLAG specific. Although the immunoprecipitation step prior to the Western blot analysis resulted in a stronger signal of the viral proteins and a lower background of the assay, we were unable to detect bands corresponding to (i) VP4 and VP4-FLAG fusion proteins coming from the maturation cleavage of VP0 and ExtVP0, respectively, and (ii) FLAG peptides and VP0 containing four extra residues coming from the 3Cpro-mediated cleavage of the inserted 2B/C site.

ExtVP0 in HAVvec1Flag12-infected cells should have contained one Gln residue more than in pHAVvec1Flag34-in-
fected cells (Fig. 1); however, Western blot analysis showed that it migrated faster than ExtVP0 of HAVvec1Flag34 (Fig. 3 and 4). This discrepancy in the migration of the ExtVP0 proteins suggested that deletions occurred during viral replication of the FLAG-containing constructs. Since HAVvec1Flag12- and HAVvec1Flag34-infected cells contained similar amounts of ExtVP0 (Fig. 3, lanes 6 and 7), it is likely that the higher level of VP0 in the HAVvec1Flag12-infected cells was due to a greater proportion of revertant viruses that deleted the inserted sequences.

**Stability of the FLAG-containing HAV constructs.** The stability of the HAV constructs was tested by reverse transcription of viral RNA, PCR amplification using HAV primers flanking the inserted sequences, and nucleotide sequence analysis of the amplified cDNA. While HAVvec1 had the expected sequence (Fig. 1) and remained stable for at least six serial passages, the RT-PCR analysis of stocks of HAVvec1Flag12 showed several bands that migrated at and below the expected size. Nucleotide sequence analysis of the amplified HAVvec1Flag12 cDNAs was ambiguous due to the superimposition of deleted sequences accumulated during viral replication. These data agree well with the data obtained by Western blot analysis and indicate that the FLAG-containing constructs are unstable. To analyze individual sequences, viral stocks were cloned by limiting dilution in 96-well plates containing confluent FRhK-4 cell monolayers. Expression of the FLAG epitope was determined in 16 randomly selected clones of each construct by Western blot analysis using MAb M2. Western blot analysis of the eight HAVvec1Flag12 clones that expressed the FLAG epitope showed that the anti-FLAG MAb M2 reacted very strongly with the 31-kDa ExtVP0 band and the anti-VP2 Ab reacted with the 31-kDa ExtVP0, 29-kDa VP0, and 27-kDa VP2 bands, which further suggested that the FLAG epitope was expressed by replication-competent HAV recombinants. Two clones that did not react (clones 3 and 7) and one that did react (clone 10) strongly with anti-FLAG MAb M2 were subjected to nucleotide sequence analysis (Fig. 5A). Clones 3 and 7 deleted 43 and 71 nt of the inserted sequences, respectively, resulting in smaller out-of-frame insertions, whereas clone 10 deleted only 38 nt of the inserted sequences and still coded for the DYKXXD core epitope recognized by the anti-FLAG MAb M2 (38) inserted in frame with the polyprotein. The nucleotide sequence of an additional HAVvec1Flag12 clone that reacted with the anti-FLAG MAb M2 (clone 6) was identical to that of clone 10, which further confirmed that the M2 core epitope in the context of the HAV recombinants was recognized by the anti-FLAG MAb. It should be pointed out that in clone 10, the A residue of the first AUG triplet from the second set of initiation codons was also deleted. A similar analysis showed that only 3 of 16 randomly selected HAVvec1Flag34 clones strongly expressed the FLAG epitope and produced mature VP0 and ExtVP0, as assessed by Western blot analysis probing with anti-VP2 Ab. Nucleotide sequence analysis (Fig. 5B) revealed that while clones 8 and 9, which did not express the FLAG epitope, deleted all inserted sequences, clone 10, which expressed the FLAG epitope, retained all 72 inserted nucleotides. In addition, an A residue preceding the AUG initiation codons of the polyprotein was deleted in clone 8. These results showed that stocks of HAVvec1Flag12 and HAVvec1Flag34 are heterogeneous and contain viruses with different deletions at the N terminus of the polyprotein. Moreover, these experiments allowed us to estimate the proportion of revertants that deleted the inserted sequences in the stocks of HAV recombinants. Since 50% of the HAVvec1Flag12 clones expressed ExtVP0 with a molecular weight smaller than expected (due to the deletion of part of the inserted sequences [Fig. 5A]) and none of the isolated clones contained the full-length inserted sequence, we concluded that 100% of the viruses of the HAVvec1Flag12 stock...
or anti-CSP MAb 2A10. Arrows point to ExtVP0, VP0, and VP2 as revealed by (12% gel), transferred to nylon membranes, and probed with rabbit anti-VP2 Ab or anti-CSP MAb 2A10. Arrows point to ExtVP0, VP0, and VP2 as revealed by Western blot analysis, which indicated that the stock of this virus is composed of a homogeneous population and not a mixture of any viral protein in HAV HM175-infected (lane 5) and mock-infected (lane 3). Anti-CSP MAb 2A10 recognized only a 33-kDa ExtVP0 protein in HAV HM175-infected cells (lane 2), whereas no virus-specific bands comigrated with the VP0 and VP2 bands detected in HAV vec1NANP2-infected cells (lane 1). The latter two bands contained deletions in the inserted sequences. On the other hand, 3 of 16 HAV vec1Flag34 clones expressed full-length ExtVP0 (Fig. 5B, clone 10), which indicated that approximately 80% of the viruses of the HAV vec1Flag34 stock contained deletions in the inserted sequences.

**Stable HAV-mediated expression of an NANP epitope.** To determine whether the instability of the recombinant viruses was due to the composition of the FLAG epitope, we exchanged it with an immunodominant NANP epitope of the CSP of *P. falciparum*. To do so, synthetic oligonucleotides coding for the NP-NANP1 peptide were cloned into the SalI and KpnI sites of pT7HAVvec1NANP2 and transfected into FRhK-4 cells, and replication of HAV vec1NANP2 was detected 3 weeks posttransfection by indirect IF using anti-HAV Mabs. A stock of HAV vec1NANP2 was prepared, passaged twice in FRhK-4 cells, and studied by Western blot analysis (Fig. 6). Anti-VP2 Ab recognized a 33-kDa ExtVP0, a 29-kDa VP0, and a 27-kDa VP2 band in HAV vec1NANP2-infected cells (lane 1). The latter two bands comigrated with the VP0 and VP2 bands detected in HAV HM175-infected cells (lane 2), whereas no virus-specific bands were recognized in mock-infected cells (lane 3). Anti-CSP MAb 2A10 recognized only a 33-kDa ExtVP0 protein in HAV vec1NANP2-infected cells (lane 4) and did not react with any viral protein in HAV HM175-infected (lane 5) and mock-infected (lane 6) cells. These data indicated that the HAV vec1NANP2 ExtVP0 protein had the expected molecular weight and contained CSP and VP2 epitopes. Western blot analysis using anti-VP2 Ab and anti-CSP MAb 2A10 indicated that HAV vec1NANP2 was stable for at least six serial passages in FRhK-4 cells, which was further confirmed by RT-PCR and nucleotide sequence analysis. Moreover, HAV vec1NANP2 grew to similar titers in FRhK-4 cells as HAV HM175 and HAV vec1 and produced similar amounts of viral proteins and RNA. The stability of HAV vec1NANP2 suggested that the instability of HAV vec1Flag34, which only differ in the sequence of the epitope tag, was mainly due to the FLAG sequence itself. We did not detect deletions in the inserted sequences of HAV vec1NANP2 during six serial passages, as assessed by RT-PCR, nucleotide sequence analysis, and Western blot analysis, which indicated that the stock of this virus is composed of a homogeneous population and not a mixture of HAV mutants containing deletions in the inserted sequences as found in the FLAG-containing HAV recombinant stocks (Fig. 5). The lack of deletions in the inserted sequences and the similar levels of ExtVP0 and VP0 proteins in HAV vec1NANP2-infected cells (Fig. 6, lane 1) further supported the model of a dual initiation of translation of its polyprotein and confirmed that peptides fused to the N terminus of the polyprotein can be expressed by replication-competent HAV recombinants.

The **NANP epitope accumulates in HAV vec1NANP2-infected cells as 80S empty particles.** To determine whether the NANP epitope was present in HAV particles, cytoplasmic extracts of HAV HM175-infected and HAV vec1NANP2-infected cells were sedimented over 15 to 30% sucrose density gradients. Fractions of each gradient were analyzed by Western blotting using anti-VP2 Ab and anti-CSP MAb 2A10 (Fig. 7). Cytoplasmic extracts of FRhK-4 cells infected with PV1/M and labeled with [35S]Met were sedimented in similar gradients, and the positions of the 160S and 80S particles were determined by scintillation counting and used as sedimentation markers for HAV virions/provirions and empty capsids (5), respectively. In HAV HM175-infected cells (Fig. 7A), the anti-VP2 Ab detected a peak of virions and provirions (fractions 1 to 4) containing VP2 and traces of VP0 which comigrated with PV1/M 160S particles. The same Ab detected a peak of empty capsids (fractions 8 to 13) containing mainly VP0 which comigrated with PV1/M 80S particles. In HAV vec1NANP2-infected cells, the anti-VP2 Ab (Fig. 7B) detected a peak of virions (fractions 1 to 4) containing VP2 and a peak of empty capsids (fractions 8 to 13) containing VP0 and ExtVP0 which also comigrated with PV1/M 160S and 80S markers, respectively. MAb 2A10, on the other hand, detected only ExtVP0 in HAV vec1NANP2-infected cells forming a peak of empty capsids (Fig. 7C, fractions 8 to 13). We do not know whether ExtVP0 could form provirions and undergo maturation cleavage into a NANP-VP4 fusion protein and VP2, and we are currently performing experiments trying to address this important question. We were unable to detect NANP-VP4 fusion protein in HAV vec1NANP2-infected cells by Western blot analysis, suggesting that this peptide is never formed, is produced after the maturation cleavage of ExtVP0 but is unstable, or is technically difficult to detect. However, our results clearly indicate that in vivo HAV vec1NANP2 is expressed as 80S empty particles.
showed that the NANNP epitope contained in ExtVP0 accumulated in infected cells as empty capsids. Similar results were obtained with HAVvec1Flag12 and HAVvec1Flag34 where, despite the instability of the constructs, the FLAG peptide also accumulated in infected cells as empty capsids.

**DISCUSSION**

Although IRES elements of the different picornaviruses show little sequence homology, computer-assisted RNA folding predictions showed three distinct groups (for a review, see reference 25): (i) enteroviruses and rhinoviruses, (ii) cardioviruses and aphthoviruses, and (iii) hepatoviruses. The 3' end of the IRES element in cardioviruses, aphthoviruses, and hepatoviruses is adjacent to the authentic initiation site of the polyprotein, whereas in polioviruses and rhinoviruses, it is far upstream and initiation probably occurs following internal entry and scanning of ribosomes (for a review, see reference 12). In the present study, the sequences inserted at the N terminus of the polyprotein were placed in a location analogous to that of the nonstructural L protein of cardioviruses and aphthoviruses. In some cardioviruses, the dual initiation of translation of the polyprotein proceeds from two AUG triplets separated by only 9 nt. In aphthoviruses, two AUG triplets located 84 bases apart initiate translation of two forms of the polyprotein, producing the Lab and Lb forms of the L protein (40) through a leaky scanning mechanism (11). Some of the viruses constructed in this work, such as HAVvec1Flag12, HAVvec1Flag34, and HAVvec1NANP2, mimic the aphthovirus dual initiation configuration. Infection with HAVvec1NANP2, which is the most stable of our HAV constructs expressing a tag epitope, resulted in the expression of similar levels of ExtVP0 and VP0 (Fig. 6), indicating that the HAV IRES can most likely direct a dual initiation of translation. Since it has been suggested that the cardiovirus, aphthovirus, and hepatovirus IRES elements are related (14, 31), it is also interesting to speculate that their capability to direct the dual initiation of translation of the polyprotein is also a characteristic of this type of IRES.

HAVvec1 contains two sets of AUG triplets separated by 17 codons, but initiation of translation of the polyprotein was detected only from the second set, resulting in the production native VP0 (Fig. 3 and 4). If translation of the polyprotein had started from the first set of initiation codons in HAVvec1, Western blot analysis using anti-VP2 and anti-VP4 Abs most likely would have detected a shift in the migration of VP0 due to the additional sequences inserted at the N terminus of the polyprotein. Although initiation of translation from the second set of AUG triplets in HAVvec1 seems the most likely scenario, we cannot completely rule out the possibility that proteolytic degradation of the inserted sequences resulted in a protein that comigrated with native VP0. Unfortunately, deletion of the second set of AUG triplets in the FLAG-containing HAV recombinants resulted in highly unstable viruses which rapidly deleted the inserted sequences (data not shown); therefore, we could not use such constructs to determine whether proteases can mediate the unlikely cleavage of ExtVP0 into mature VP0. Dual initiation of translation of the polyprotein from both sets of initiation codons was detected only after a tag epitope was inserted into the polylinker of HAVvec1. Since sequences at and upstream of the first set of initiation codons were not modified, our data suggested that determinants downstream from the native initiation codons could play a role in regulating initiation of translation of HAV. Therefore, the insertion of foreign epitopes in HAV could be a useful tool to further understand the mechanism initiation of translation in HAV.

The rationale for introducing a 3C<sup>pro</sup> cleavage site at the N terminus of the polyprotein was to determine whether the viral protease could recognize it cutting some of the inserted sequences, thus increasing the possibility of obtaining an infectious HAV cDNA construct. A similar approach was successfully used with PV expression vectors by engineering a PV 3C<sup>pro</sup> cleavage site at the N terminus of the polyprotein, which, after being cleaved, restored the native sequence of VP4 (7). An identical approach of restoring the native VP4 sequence of HAV could not have been undertaken because, unlike the case for PV, the initiation AUGs of the HAV polyprotein are not posttranslationally cleaved, exposing a Gly residue (44). Since it was shown that purified 3C<sup>pro</sup> efficiently cleaved synthetic peptide ELRTQ/SFSN coding for the 2B/2C junction (33), we inserted such sequences in HAVvec1 (Fig. 1). Unfortunately, due to the instability of the HAVvec1Flag12 construct, we were unable to determine whether 3C<sup>pro</sup> recognized the inserted 2B/2C cleavage site in the context of the expression vector. Moreover, it is possible that deletion of a Gln residue from the inserted 2B/2C cleavage site in HAVvec1Flag12 to generate HAVvec1Flag34 increased the stability of this construct and allowed the isolation of clones containing all the inserted sequences (Fig. 2, 3, and 5). It has recently been shown that the 2A/2B and 2C/3A junctions are the preferred HAV 3C<sup>pro</sup> cleavage sites and that longer periods are required for cleavage of the 2B/2C junction (41). Therefore, it is possible that efficient cleavage of the inserted sequences can be achieved by exchanging the 2B/2C for the 2A/2B or 2C/3A site. Modification of the HAV vec1NANP2 construct to include other protease cleavage sites will help us to understand and possibly improve the proteolytic processing in the context of the recombinant HAV vectors.

Since the HAV constructs containing the FLAG epitope tended to be unstable, we constructed HAVvec1NANP2, in which the FLAG epitope was replaced by the CSP NPNANPN peptide. HAVvec1NANP2 was stable for more than six passages as assessed by RT-PCR and nucleotide sequence analysis, suggesting that the amino acid composition of the FLAG peptide contributed to the instability of the construct. The stability of HAVvec1NANP2 allowed us to determine that the tag epitope accumulated in infected cells as empty capsids (Fig. 7). Although we did not detect ExtVP0 and VP0 forming HAVvec1NANP2 provirions and virions (Fig. 7B and C), we cannot rule out the possibility that ExtVP0 undergoes maturation cleavage into an NANNP-VP4 fusion protein and VP2. More detailed experiments will be required to analyze the possible formation of HAVvec1NANP2 provirions and their maturation cleavage into virions (13).

Picornaviruses have been used as expression vectors to produce live recombinant vaccines (1, 3, 4, 6–8, 10, 18, 20, 35–37, 39, 48). Although construction of HAV expression vectors has been difficult due to the poor growth characteristics of the virus, it is possible that such vectors can also be used to develop recombinant vaccines. In this study, we clearly showed that exogenous sequences can be cloned into the N terminus of the HAV polyprotein, resulting in replication-competent viruses. We also showed that the inserted sequences are expressed as ExtVP0 proteins and accumulate in infected cells as 80S empty particles. Our data, therefore, support the development of HAV expression vectors as a tool to study this poorly understood picornavirus and to produce recombinant vaccines.

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