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Deletions of the Hypervariable Region (HVR) in Open Reading Frame 1 of Hepatitis E Virus Do Not Abolish Virus Infectivity: Evidence for Attenuation of HVR Deletion Mutants In Vivo

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Hepatitis E virus (HEV) is an important human pathogen, although little is known about its biology and replication. Comparative sequence analysis revealed a hypervariable region (HVR) with extensive sequence variations in open reading frame 1 of HEV. To elucidate the role of the HVR in HEV replication, we first constructed two HVR deletion mutants, hHVRd1 and hHVRd2, with in-frame deletion of amino acids (aa) 711 to 777 and 747 to 761 in the HVR of a genotype 1 human HEV replicon. Evidence of HEV replication was detected in Huh7 cells transfected with RNA transcripts from mutant hHVRd2, as evidenced by expression of enhanced green fluorescent protein. To confirm the in vitro results, we constructed three avian HEV mutants with various HVR deletions: mutants aHVRd1, with deletion of aa 557 to 585 (Δ557-585); aHVRd2 (Δ612-641); and aHVRd3 (Δ557-641). Chickens intrahepatically inoculated with capped RNA transcripts from mutants aHVRd1 and aHVRd2 developed active viral infection, as evidenced by seroconversion, viremia, and fecal virus shedding, although mutant aHVRd3, with complete HVR deletion, was apparently attenuated in chickens. To further verify the results, we constructed four additional HVR deletion mutants using the genotype 3 swine HEV as the backbone. Mutants sHVRd2 (Δ722-781), sHVRd3 (Δ735-765), and sHVRd4 (Δ712-765) were shown to tolerate deletions and were infectious in pigs intrahepatically inoculated with capped RNA transcripts from the mutants, whereas mutant sHVRd1 (Δ712-790), with a nearly complete HVR deletion, exhibited an attenuation phenotype in infected pigs. The data from these studies indicate that deletions in HVR do not abolish HEV infectivity in vivo, although evidence for attenuation was observed for HEV mutants with a larger or nearly complete HVR deletion.

Hepatitis E virus (HEV), the causative agent of human hepatitis E, is a nonenveloped, single-stranded, positive-sense RNA virus in the genus Hepeivirus of the family Hepeviridae (9). Hepatitis E is an important public health disease in many developing countries and is also endemic in some industrialized countries (1, 2, 4, 8, 19, 41). HEV transmission occurs primarily by the fecal-oral route through contaminated drinking water or water supplies in areas with poor sanitation (35). The disease mainly affects young adults, and the mortality rate is generally less than 1%, but it can reach up to 28% among infected pregnant women (17, 28). A relatively high prevalence of anti-HEV antibodies in healthy individuals has been reported in the United States and other industrialized countries where HEV infections are only sporadic (33, 34, 51). HEV antibodies have also been detected in several other animal species, including rodents, pigs, and chickens (12, 30, 35, 54). In 1997, the first animal strain of HEV, swine HEV, was discovered and characterized from pigs in the United States (58). More recently, another strain of HEV, avian HEV, from chickens with hepatitis-splenomegaly syndrome was discovered and characterized in the United States (18). The discovery of animal strains of HEV and the existence of a population of individuals in industrialized countries who are seropositive for HEV have led to a hypothesis that animal reservoirs exist for HEV (34). Increasing evidence indicates that hepatitis E is indeed a zoonotic disease (35, 39) and that pigs (and perhaps other species) are animal reservoirs for HEV (34).

There are at least four major genotypes of HEV: genotype 1 (primarily Burmese-like Asian strains) (3, 50, 52); genotype 2 (a single Mexican strain) (21); genotype 3 (strains from rare endemic cases in industrialized countries, including the United States, Europe, and Japan, and swine HEV strains from pigs worldwide) (11, 38, 45–48); and genotype 4 (variant strains from endemic cases in Asia and swine HEV strains from pigs in Asia) (49, 57). All swine HEV strains identified thus far worldwide belong to either genotype 3 or 4 (20, 22, 40, 47). The avian HEV from chickens likely represents a new genus in the family Hepeviridae (25).

The genome of HEV is approximately 7.2 kb in length and consists of three open reading frames (ORFs) (14, 31, 43, 50, 55) and short 5′ and 3′ noncoding regions (NCR), followed by a poly(A) tail (8, 15, 29, 50). The ORF1 encodes a nonstructural polyprotein, which contains putative functional motifs characteristic of methyltransferase, protease, helicase, and RNA-dependent RNA polymerase (31, 42). ORF2 encodes the capsid protein of about 660 amino acids (aa). The small ORF3 encodes a phosphoprotein of about 123 aa, the biological function of which has yet to be fully defined (14, 15, 31, 50, 55).
55, 58, 60). Though ORF1 is essential for HEV replication, it contains a highly heterogeneous and hypervariable region (HVR) among HEV strains (25, 32, 44, 53). The size differences among different HEV genomes are confined mainly to the HVR of ORF1 (25). The observed extensive inter- and intragenotypic sequence variations in the HVRs of HEV genomes suggest that the HVR may not be necessary for virus replication. However, sequences not required for virus infectivity or spread are normally rapidly lost in vivo, especially in small RNA viruses like HEV. Therefore, the fact that HEV does retain such a hypervariable sequence in its genome suggested a potential biological role for the HVR in HEV replication and/or pathogenesis, which warranted further investigation.

Reverse genetic systems for HEV have been recently established, permitting the manipulation of the HEV genome to explore the potential functions of viral genes (10, 24, 26). To elucidate the potential role of the HVR in HEV replication and/or pathogenesis, in this study we constructed various HVR deletion mutants using a genotype 1 human HEV replicon, an avian HEV infectious clone, and genotype 3 swine HEV infectious clones. The mutants were tested for infectivity in HuH7 liver cells, as well as in chickens and pigs. The results from this study indicate that deletions of HVR from the HEV genome do not affect virus viability in vitro or in vivo, although virus mutants with a larger or nearly complete HVR deletion were apparently attenuated in infected animals.

### MATERIALS AND METHODS

**Cells and infectious cDNA clones.** The genotype 1 human HEV (Sar55 strain) infectious clone (10) and a subclone of the HuH7 liver cell line (7, 15) were gifts from Suzanne Emerson and Robert Purcell at the Laboratory of Infectious Diseases, National Institute of Allergy and Infectious Diseases, NIH. The infectious cDNA clones of the genotype 3 swine HEV (26) and avian HEV (24) were reported previously.

**Sequence analysis of the HVR among known HEV strains.** In order to identify the length of the HVR and to determine the intragenotypic and intergenotypic sequence identities in the HVR among different HEV strains of the four major genotypes, the amino acid sequences flanking the HVR among known mammalian strains of HEV (Fig. 1) and the corresponding region of avian HEV were aligned and analyzed using the Clustal W method of the MegAlign program (DNASTAR, Inc.). The putative HVR for each genotype of mammalian HEV and the corresponding HVR in avian HEV were predicted on the basis of the putative HVR in avian HEV are derived from the corresponding HVR in mammalian strains of HEV (Fig. 1) and the corresponding region of avian HEV were predicted on the basis of the putative HVR in avian HEV.

**Construction of genotype 1 human HEV HVR deletion mutant replicons.** To further elucidate the role of the HVR in HEV replication, we utilized the genetically distinct avian HEV to produce the EGFP replicon from which the SphI-NsiI region had been deleted. The amplified EGFP replicon is intact, with part of the carboxy terminus of ORF2 fused to the EGFP gene. The amino terminus of the ORF2 gene (nt 5148 to 5816) downstream of the first methionine was removed, and the EGFP gene was inserted in frame with the ORF2 initiation codon. The EGFP replicon was shown to express the EGFP protein when transfected into HuH7 liver cells (Huang and Meng, unpublished).

Two HEV deletion mutants of the genotype 1 HEV replicon were constructed using fusion PCR (Fig. 2). Amino acid residues 711 to 777 and 747 to 761, corresponding to nucleotides (nt) 2131 to 2331 and 2233 to 2828, were deleted to construct the HVR deletion mutants hHVRd1 and hHVR2, respectively. The two fragments used for fusion PCR were first amplified with the primer sets Hu F/Hu r1 and Hu f1/Hu R for the mutant hHVRd1 and Hu F/Hu r2 and Hu f2/Hu R for the mutant hHVRd2 (Table 1). The PCR products amplified from each mutant were then used in the fusion PCR with primer set Hu F/Hu R (Table 1). To produce the two HVR deletion mutants, the fusion product was purified, digested with Sphl and NsiII, and ligated into the backbone of the genotype 1 HEV EGFP replicon from which the Sphl-NsiII region had been deleted.

**Construction of avian HEV HVR deletion mutants using the avian HEV infectious cDNA clone as the backbone.** To further elucidate the role of the HVR in HEV replication, we utilized the genetically distinct avian HEV to produce three avian HEV HVR deletion mutants. Avian HEV shares only approximately 50% nucleotide sequence identity with the mammalian HEV strains (23–25), although avian HEV and mammalian HEV have similar genomic organizations. Since the complete sequences of avian HEV are available for only two strains, the putative HVR in avian HEV is derived from the corresponding HVR in mammalian HEV.

To construct the three avian HEV HVR deletion mutants with various lengths, the avian infectious cDNA clone (24), p17a-HEV, was used as the backbone (Fig. 3). Mutant aHVRd1 was created by PCR to delete amino acid residues 557 to 585 (nt 1693 to 1779) using the primers Avf and Avr (Table 1). Similarly, to construct mutant aHVRd2, primers Avf and Avr were used to delete amino acid residues 612 to 641 (nt 1858 to 1947). All four primers contain a unique Hpal restriction site. The PCR products were purified with a Genclean II kit, digested with Hpal, and ligated into the backbone of the avian HEV infectious clone p17a-HEV from which the Hpal region had been deleted. For the construction of mutant aHVRd3, a 557 to 641 (nt 1693 to 1947) were deleted by

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**FIG. 1.** Schematic diagram showing the relative positions of the HVR of ORF1 from representative isolates in four major genotypes of mammalian HEV (genotypes 1 to 4, in parentheses), along with putative functional domains: MET, methyltransferase; P, papain-like cysteine protease; Y, Y domain; H, HVR; X, X domain; HEL, helicase; RDRP, RNA-dependent RNA polymerase. The amino acid sequences of different HEV strains are indicated by dots, and deletions are indicated by dashes. Representative strains from each genotype used in this study are underlined.
direct digestion of the avian HEV infectious cDNA clone with HpaI and religation of the ends after purification.

Construction of swine HEV HVR deletion mutants using the genotype 3 swine HEV infectious cDNA clone as the backbone. To more definitively verify the role of the HVR in HEV replication, we subsequently constructed four additional HVR deletion mutants with a different genotype, the genotype 3 swine HEV. Briefly, the infectious cDNA clone pSHEV-3 of the prototype genotype 3 swine HEV (26) was used as the backbone for the construction of four HVR deletion mutants using fusion PCR. Amino acid residues 712 to 790, 722 to 781, 735 to 765, and 712 to 765 (corresponding to nt 2160 to 2396, 2190 to 2369, 2229 to 2321, and 2160 to 2321, respectively) were deleted from the infectious cDNA clone pSHEV-3 to produce HVR deletion mutants sHVRd1, sHVRd2, sHVRd3, and sHVRd4, respectively (Fig. 4). The two fragments used for fusion PCR were first amplified with the primer sets Sw F/Sw r1 and Sw f1/Sw R for the mutant sHVRd1, Sw F/Sw r2 and Sw f2/Sw R for the mutant sHVRd2, Sw F/Sw r3 and Sw f3/Sw R for the mutant sHVRd3, and Sw F/Sw r1 and Sw f4/Sw R for the

FIG. 2. (A) Schematic diagram showing the HVR (aa 707 to 777) in ORF1 of the genotype 1 human HEV (strain Sar55) replicon expressing EGFP. MET, methyltransferase; P, papain-like cysteine protease; Y, Y domain; H, HVR; X, X domain; HEL, helicase; RDRP, RNA-dependent RNA polymerase. The amino acid sequence of each HVR deletion mutant is aligned with that of the wild-type Sar55 HEV replicon to show the relative positions of the in-frame amino acid deletions: mutants hHVRd1 (aa 711 to 777) and hHVRd2 (aa 747 to 761). (B) Fluorescence microscopy of Huh7 liver cells at 6 days posttransfection with similar amounts of capped RNA transcripts from the wild-type Sar55 replicon with the EGFP gene (A), HVR deletion mutants hHVRd1 (B) and hHVRd2 (C), and mock-transfected cells (D).
TABLE 1. Oligonucleotide primers used for construction of HVR deletion mutants, as well as for PCR and sequencing, in this study

<table>
<thead>
<tr>
<th>Primer ID</th>
<th>Primer sequence (5′ → 3′)</th>
</tr>
</thead>
<tbody>
<tr>
<td>For construction of avian HEV HVR-deletion mutants</td>
<td></td>
</tr>
<tr>
<td>AvF</td>
<td>CCCCCTGTTAAGGCGACACCGGGCC</td>
</tr>
<tr>
<td>Avf1</td>
<td>CCCCAGGTTAAGGCGACACCGGGCC</td>
</tr>
<tr>
<td>AvR</td>
<td>CCCCCTGTTAAGGCGACACCGGGCC</td>
</tr>
<tr>
<td>Avr</td>
<td>CCCCCTGTTAAGGCGACACCGGGCC</td>
</tr>
<tr>
<td>Av</td>
<td>CCCCCTGTTAAGGCGACACCGGGCC</td>
</tr>
<tr>
<td>For construction of swine HEV HVR-deletion mutants</td>
<td></td>
</tr>
<tr>
<td>SwF</td>
<td>GATCATCGGGCTCGACCTCCTCCC</td>
</tr>
<tr>
<td>Swf1</td>
<td>GATCATCGGGCTCGACCTCCTCCC</td>
</tr>
<tr>
<td>SwR</td>
<td>GATCATCGGGCTCGACCTCCTCCC</td>
</tr>
<tr>
<td>Swr1</td>
<td>GATCATCGGGCTCGACCTCCTCCC</td>
</tr>
<tr>
<td>Swr2</td>
<td>GATCATCGGGCTCGACCTCCTCCC</td>
</tr>
<tr>
<td>Sw3</td>
<td>GATCATCGGGCTCGACCTCCTCCC</td>
</tr>
</tbody>
</table>

For construction of genotype 1 human HEV HVR-deletion mutants

HuF | GGAGGAGTCTGTCGAGGCTCCG |
Hu1 | GGAGGAGTCTGTCGAGGCTCCG |
Hu2 | GGAGGAGTCTGTCGAGGCTCCG |
HuR | GGAGGAGTCTGTCGAGGCTCCG |
Hu1 | GGAGGAGTCTGTCGAGGCTCCG |
Hu2 | GGAGGAGTCTGTCGAGGCTCCG |

For sequencing of the HVR region from viruses recovered from pigs and chickens

Av | TTACCATGCTGTTGACTGCAAGTTAAGC |
Av3 | TTACCATGCTGTTGACTGCAAGTTAAGC |
Sw | CAGCTGATTCGATTTCTATAGG |
Sw2 | CAGCTGATTCGATTTCTATAGG |

For demonstration of the viability of the HVR deletion mutants in Huh7 liver cells, as previously described (7). Capped RNA transcripts were synthesized from each cDNA clone in a...
A

ORF1

ORF3

ORF2

Cap

MET

P

H

HEL

RDRP

(A)n

aa 557

aa 641

avian HEV

aHVRd1

aHVRd2

aHVRd3

B

Group A

Group B

Group C

Group D

Group E

Weeks post-inoculation

Weeks post-inoculation

Weeks post-inoculation

Weeks post-inoculation

Weeks post-inoculation

OD value

OD value

OD value

OD value

5337

5381

5368

5333

5372

5580

5353

5374

5362

5367

5354

5355

5354

5355

5352

5358

5309

5358
and sera were collected from all inoculated pigs at weekly intervals until they were necropsied at 10 weeks p.i. Fecal and serum samples were tested by reverse transcription (RT)-PCR (16, 36, 37, 59) for swine HEV RNA, and weekly serum samples were also tested by ELISA for immunoglobulin G (IgG) antibodies to swine HEV (16, 36).

Detection and sequencing of viruses recovered from experimentally infected chickens and pigs. For the chicken study, fecal materials collected from inoculated chickens at 3 weeks p.i. were tested by RT-PCR using the primers specific for the avian HEV HVR. A nested PCR with external primers Av N1 and Av N2 and internal primers Av N3 and Av N4 (Table 1) were used to amplify the region flanking the avian HEV HVR. Similarly, for the pig study, fecal materials collected from the inoculated pigs at 4 weeks p.i. were tested by RT-PCR using the primers specific for the genotype 3 swine HEV HVR. A One-step RT-PCR kit (Invitrogen) was used to amplify the region flanking the swine HEV HVR using the Sw N1 and Sw N2 primers (Table 1). The amplified PCR products from pigs and chickens were purified with a GeneClean II kit and sequenced at the Virginia Bioinformatic Institute. The sequences obtained from viruses recovered from the infected chickens and pigs were compared with the sequences of the original viruses used as the inocula.

RESULTS

The HVR is highly variable among HEV strains. Sequence analyses confirmed the existence of an HVR in ORF1 of HEV strains (Fig. 1). The intergenotypic amino acid sequence identity in the HVR among HEV isolates in different genotypes differed by as much as 71%, whereas the intragenotypic amino acid sequence identities among isolates within the same genotype differed by 31% among genotype 1 isolates, 41% among genotype 3 isolates, 46% among genotype 4 isolates, and 30% between the only two available avian HEV isolates (data not shown). The variability of the HVR in genotype 2 is unknown, since only one strain of genotype 2 HEV has been sequenced to date. The predicted HVR for the HEV strains used in the present study includes ORF1 aa 707 to 777 in genotype 1 human HEV (Sar55 strain) (Fig. 2), aa 707 to 790 in genotype 3 swine HEV (pSHV-3 infectious clone) (Fig. 4), and aa 557 to 641 in avian HEV (Fig. 3). It was previously predicted, based on sequence comparison of an apparently avirulent strain and the prototype pathogenic strain of avian HEV, that the region spanning aa 554 to 614 in ORF1 of avian HEV is hypervariable (5). However, further sequence comparisons with mammalian HEV strains revealed that the avian HEV genome downstream of the originally predicted HVR at aa 615 to 641 also displayed significant sequence variations. Therefore, we considered aa 557 to 641 the HVR of avian HEV for the purpose of constructing avian HEV HVR deletion mutants in this study (Fig. 3).

The HVR of genotype 1 human HEV is not required for virus replication in vitro. We constructed two genotype 1 human HEV HVR deletion mutants using a strain Sar55 HEV replicon expressing EGFP (Fig. 2). Huh7 cells were transfected with capped RNA transcripts from a wild-type replicon and the two mutants, hHVRd1 and hHVRd2. The transfected cells were examined by fluorescence microscopy on days 4, 5, and 6 posttransfection for evidence of EGFP expression. EGFP fluorescence signal was detected in Huh7 cells transfected with the wild-type Sar55 replicon, as well as in those transfected with the mutant hHVRd2 replicon (Fig. 2), but not in the cells transfected with the mutant hHVRd1 replicon. Fluorescence was first detected on day 4, and the EGFP signal intensity increased on days 5 and 6 posttransfection. Expression of EGFP by the HVR deletion mutant hHVRd2 indicated that the mutant is replication competent in Huh7 liver cells.

The HVR (aa 557 to 585 and aa 612 to 641) of avian HEV tolerated deletions. To validate the dispensability of the HVR for HEV replication observed in our in vitro study with the genotype 1 human HEV replicon, we selected the genetically distinct avian HEV for an in vivo study. Three avian HEV HVR deletion mutants were generated using the avian HEV infectious cDNA clone as the backbone (Fig. 3). The abilities of the three avian HEV HVR deletion mutants to infect chickens were tested by direct intrahepatic inoculation of SPF chickens with capped RNA transcripts from each mutant. Seroconversion to IgG anti-avian HEV was observed in all HVR deletion mutant groups (A, B, and C), as well as in the positive control group (D). In each group, however, only one or two chickens out of the three that were inoculated seroconverted (Fig. 3): only one chicken (no. 5361) in group A (aHVRd1) seroconverted at 6 weeks p.i., chickens 5388 and 5353 of group B (aHVRd2) seroconverted at 4 and 6 weeks p.i., and chicken 5354 of group C (aHVRd3) seroconverted at 5 weeks p.i. All the chickens in the positive control group D seroconverted at 4 weeks p.i. The three negative control chickens (5352, 5358, and 5369) remained seronegative through the experiment.

Avian HEV-specific RNA in feces was detected variably in inoculated chickens (Table 2). In group A chickens (aHVRd1), fecal virus shedding began at 3 weeks p.i. for chicken 5361. Fecal virus shedding was delayed until 6 weeks p.i. in chicken 5357 and was not detected in chicken 5368. In group B chickens (aHVRd2), fecal virus shedding began at 2 and 3 weeks p.i. for chickens 5388 and 5353 but was undetectable in chicken 5372. None of the chickens in group C (aHVRd3) had detectable avian HEV RNA in the feces. Viremia could not be detected in group A or C chickens (Table 1) and was transient in chicken 5353 but lasted for 4 weeks in chicken 5388 of group B. Transient viremia was detected in all chickens of positive control group D.

The HVR of genotype 3 swine HEV is not required for in vivo infectivity. To further verify the results from the avian HEV.
TABLE 2. Detection of avian HEV RNA

<table>
<thead>
<tr>
<th>Group</th>
<th>Chicken no.</th>
<th>Result (fecal/serum) at week p.i.:</th>
</tr>
</thead>
<tbody>
<tr>
<td>A (aHVRd1)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>5357</td>
<td>–/– –/– –/– –/– –/+ –/+ –/+ –/+</td>
</tr>
<tr>
<td></td>
<td>5361</td>
<td>–/– –/– –/– –/– –/+ –/+ –/+ –/+</td>
</tr>
<tr>
<td></td>
<td>5368</td>
<td>–/– –/– –/– –/– –/+ –/+ –/+ –/+</td>
</tr>
<tr>
<td>B (aHVRd2)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>5353</td>
<td>–/– –/– –/– –/– –/+ –/+ –/+ –/+</td>
</tr>
<tr>
<td></td>
<td>5372</td>
<td>–/– –/– –/– –/– –/+ –/+ –/+ –/+</td>
</tr>
<tr>
<td></td>
<td>5388</td>
<td>–/– –/– –/– –/– –/+ –/+ –/+ –/+</td>
</tr>
<tr>
<td>C (aHVRd3)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>5354</td>
<td>–/– –/– –/– –/– –/+ –/+ –/+ –/+</td>
</tr>
<tr>
<td></td>
<td>5355</td>
<td>–/– –/– –/– –/– –/+ –/+ –/+ –/+</td>
</tr>
<tr>
<td></td>
<td>5358</td>
<td>–/– –/– –/– –/– –/+ –/+ –/+ –/+</td>
</tr>
<tr>
<td>D (wild-type clone) (positive control group)</td>
<td>5362</td>
<td>–/– –/– –/+ –/+ –/+ –/+ –/+ –/+</td>
</tr>
<tr>
<td></td>
<td>5367</td>
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<tr>
<td></td>
<td>5374</td>
<td>–/– –/– –/+ –/+ –/+ –/+ –/+ –/+</td>
</tr>
<tr>
<td>E (negative control group)</td>
<td>5352</td>
<td>–/– –/– –/+ –/+ –/+ –/+ –/+ –/+</td>
</tr>
<tr>
<td></td>
<td>5358</td>
<td>–/– –/– –/+ –/+ –/+ –/+ –/+ –/+</td>
</tr>
<tr>
<td></td>
<td>5369</td>
<td>–/– –/– –/+ –/+ –/+ –/+ –/+ –/+</td>
</tr>
</tbody>
</table>

* Positive (+) or negative (–) samples at the indicated week p.i. in SPF chickens intrahepatically inoculated with capped RNA transcripts of avian HEV HVR deletion mutants, as well as the wild-type avian HEV infectious clone.

node study and the in vitro genotype 1 HEV replicon study, we subsequently constructed four swine HEV mutants using the genotype 3 swine HEV infectious cDNA clone as the backbone (Fig. 4). The abilities of the four genotype 3 swine HEV mutants to infect pigs were tested by direct intrahepatic inoculation of SPF pigs with capped RNA transcripts from each mutant. Since the intrahepatic inoculation of RNA transcripts of avian HEV HVR mutants in chickens was a blind percutaneous procedure (24), some chickens may not have received, or received much less, inocula in the livers. To ensure that all animals received equal amounts of RNA inocula, we subsequently constructed four swine HEV mutants using the genotype 3 swine HEV infectious cDNA clone as the backbone (Fig. 4). The abilities of the four genotype 3 swine HEV mutants to infect pigs were tested by direct intrahepatic inoculation of SPF pigs with capped RNA transcripts from each mutant. Since the intrahepatic inoculation of RNA transcripts of avian HEV HVR mutants in chickens was a blind percutaneous procedure (24), some chickens may not have received, or received much less, inocula in the livers. To ensure that all animals received equal amounts of RNA inocula, we used an ultrasound-guided technique for the intrahepatic injection to make sure that the RNA inocula were injected directly into the liver. All the pigs in groups A, B, C, and D, which were injected with capped RNA transcripts from respective HVR deletion mutants, seroconverted to IgG anti-HEV, indicating that active swine HEV infections had occurred in the inoculated pigs (Fig. 4). All the pigs in group B (shVRd2), group C (shVRd3), and group D (shVRd4) seroconverted at about 3 to 5 weeks p.i. The three pigs in group A (shVRd1) had a delayed seroconversion at 6 to 7 weeks p.i. The positive control pigs in group E (pSHEV-3) seroconverted at 3 to 4 weeks p.i. The three negative control pigs in group F remained seronegative throughout the course of study (Fig. 4).

Fecal virus shedding occurred variably in pigs of groups B, C, and D (Table 3). There was no fecal virus shedding in group A pigs. Delayed fecal virus shedding occurred in pigs 291 and 295 of group B at 9 to 10 weeks p.i., while there was no fecal virus shedding in pigs 613. Fecal virus shedding occurred as early as 1 week p.i. in group C pigs and at 2 weeks p.i. in group D pigs and lasted for 5 to 8 weeks. Viremia was not detected in group A or B pigs (Table 3). Viremia was detected only in pigs 604 of group C at 3 and 6 weeks p.i. and only in pigs 288 of group D at 5 weeks p.i. (Table 3).

Viruses recovered from infected chickens and pigs retained their respective deletions in the HVR. Viruses recovered from the feces of chicken no. 5361 from group A and chicken no. 5388 from group B at 3 weeks p.i. were sequenced to confirm the presence of deletions in the HVR. Sequence analyses revealed that the recovered virus from chicken no. 5361 retained its nt 1693 to 1779 deletion and the virus recovered from chicken no. 5388 also retained its nt 1858 to 1947 deletion.

Similarly, we also amplified and sequenced the HVRs of chickens infected with genotype 3 swine HEV.
the rescued viruses from selected pigs inoculated with the genotype 3 HVR deletion mutants. The introduced deletions nt 2190 to 2369 for group B pigs, nt 2229 to 2321 for group C pigs, and nt 2160 to 2321 for group D pigs were retained intact in the viruses recovered from the fecal samples collected at 4 weeks p.i.

**DISCUSSION**

The objective of this study was to assess the role of the HVR in ORF1 of HEV in virus replication and/or pathogenesis. Sequence analysis of known HEV strains revealed an HVR with a high degree of variability at both amino acid and nucleotide sequence levels. This region overlaps the proline-rich hinge region of ORF1 (25, 32, 44, 53). It is known that inherent structural constraints can influence the vulnerability of genomic segments to replication errors during virus infection, resulting in the accumulation of mutations for genetic diversity (13). The size differences in HEV genomes from different genotypes are confined mainly to the HVR of ORF1, which spanned 105 aa as originally proposed (31, 53). As the sequences of additional HEV isolates were published, it became clear that the first 35 aa in the originally described HVR among HEV strains (53) is not hypervariable. Thus, the true HVR is 70 to 72 aa for genotype 1 HEV, 68 aa for genotype 2 HEV, 80 to 86 aa for genotype 3 HEV, 84 aa for all genotype 4 HEVs, and 84 aa for avian HEV (based on the corresponding region in mammalian HEVs). HEV genomes exhibited increased divergence in the HVR encompassing aa 707 to 777 for genotype 1 human HEV, aa 707 to 790 for genotype 3 swine HEV, and aa 557 to 641 for avian HEV. Extensive sequence variations observed among isolates in the four major genotypes of mammalian HEV and avian HEV, as well as within each genotype, suggested that the HVR may not be necessary for virus replication. It has been shown that a 507-nt deletion in a variable nonstructural region of rubella virus, a virus distantly related to HEV, is not required for virus replication (56). Therefore, we hypothesize that the HVR of HEV is not required for virus infectivity.

To test our hypothesis, we first constructed two genotype 1 human HEV HVR deletion mutants using the EGFP-expressing Sar55 HEV replicon as the backbone: mutants hHVRd1 (aa 711 to 777 deleted) and hHVRd2 (aa 747 to 761 deleted). The wild-type Sar55 EGFP replicon, which was constructed in our laboratory (Huang and Meng, unpublished), was shown to be replication competent and expressed EGFP when transfected into Huh7 liver cells. The two HVR deletion mutants (hHVRd1 and hHVRd2) were tested for viability and replication competency in Huh7 cells. EGFP fluorescence signal was detected in Huh7 cells transfected with the mutant hHVRd1, as well as with the wild-type Sar55 replicon, but not in the cells transfected with the HVR deletion mutant hHVRd1. The results from this experiment showed that the mutant hHVRd2 with partial HVR deletion is viable, and thus, the HVR is dispensable for virus replication in vitro. The absence of EGFP expression for mutant hHVRd1, which contains a deletion of the nearly complete HVR, suggested that this mutant is not replication competent. Therefore, it is likely that the end sequences of the HVR for genotype 1 human HEV may be important for virus viability. Nevertheless, partial deletion of HVR sequence in the middle region, as revealed by mutant hHVRd2 (Fig. 2), apparently does not affect the replication ability of the genotype 1 human HEV in vitro.

To further confirm our results from the in vitro study with genotype 1 human HEV replicon mutants, we utilized a genet-

**TABLE 3. Detection of HEV RNA**

<table>
<thead>
<tr>
<th>Group</th>
<th>Pig no.</th>
<th>Result (fecal/serum) at week p.i.:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>A (sHVRd1)</td>
<td>286</td>
<td>-/-</td>
</tr>
<tr>
<td></td>
<td>296</td>
<td>-/-</td>
</tr>
<tr>
<td></td>
<td>608</td>
<td>-/-</td>
</tr>
<tr>
<td>B (sHVRd2)</td>
<td>291</td>
<td>-/-</td>
</tr>
<tr>
<td></td>
<td>295</td>
<td>-/-</td>
</tr>
<tr>
<td></td>
<td>613</td>
<td>-/-</td>
</tr>
<tr>
<td>C (sHVRd3)</td>
<td>292</td>
<td>-/-</td>
</tr>
<tr>
<td></td>
<td>604</td>
<td>-/-</td>
</tr>
<tr>
<td></td>
<td>606</td>
<td>-/-</td>
</tr>
<tr>
<td>D (sHVRd4)</td>
<td>288</td>
<td>-/-</td>
</tr>
<tr>
<td></td>
<td>293</td>
<td>-/-</td>
</tr>
<tr>
<td></td>
<td>300</td>
<td>-/-</td>
</tr>
<tr>
<td>E (wild-type clone)</td>
<td>289</td>
<td>-/-</td>
</tr>
<tr>
<td>(Positive control group)</td>
<td>294</td>
<td>-/-</td>
</tr>
<tr>
<td></td>
<td>612</td>
<td>-/-</td>
</tr>
<tr>
<td>F (negative control group)</td>
<td>603</td>
<td>-/-</td>
</tr>
<tr>
<td></td>
<td>609</td>
<td>-/-</td>
</tr>
<tr>
<td></td>
<td>611</td>
<td>-/-</td>
</tr>
</tbody>
</table>

*In samples that were positive (+) or negative (−) at the indicated week p.i. in SPF chickens intrahepatically inoculated with capped RNA transcripts of swine HEV HVR deletion mutants, as well as the wild-type infectious clone.
phically distinct chicken strain of HEV (avian HEV); we constructed three avian HEV mutants with various deletions in the HVR and tested the mutants for the ability to infect chickens. Based on the amino acid sequence alignment of avian HEV with other mammalian HEV strains, we found that the region spanning aa 557 to 641 in avian HEV is highly divergent and thus termed it the HVR for avian HEV. A total of three avian HEV HVR deletion mutants with various lengths were constructed: aHVRd1, with a partial deletion in the 5’ end of the HVR; aHVRd2, with a partial deletion in the 3’ end of the HVR; and aHVRd3, with the deletion of the nearly complete HVR (Fig. 3). The infectivities of the three avian HEV mutants were tested in chickens by intrahepatically inoculating the RNA transcripts from each mutant into the livers of live chickens. The kinetics of virus replication appears to be different in chickens infected with different mutants and wild-type avian HEV (Fig. 3). Although seroconversion was observed in chickens inoculated with all three mutants, only one or two out of the three inoculated chickens had seroconverted (Fig. 3). Since the percutaneous intrahepatic-injection procedure used in this study to inoculate RNA transcripts into chicken livers is a blind procedure (24), it was quite possible that the RNA transcripts were not injected into the livers of some chickens or that only a small amount was injected (24). This may explain why not all inoculated chickens seroconverted to avian HEV antibodies. Fecal virus shedding and viremia were detected only in mutants aHVRd1 and aHVRd2. Deletions in the HVR may influence the replicative competence of the virus and thus may attenuate avian HEV. Therefore, attenuation of HVR deletion mutants to replicate at lower levels could explain why viral RNA was not detected in sera from group A chickens (aHVRd1) or in feces and sera of group C chickens (aHVRd3). Clearly, future studies are warranted to explore any potential role of the HVR in virus attenuation, which is beyond the scope of this study. The results from this avian HEV and chicken study indicated that the HVR of avian HEV is not essential for virus infectivity in vivo, although the avian HEV mutant with complete HVR deletion displayed an apparent attenuation phenotype.

In order to definitively verify our results from the avian HEV and chicken study, as well as from the in vitro genotype 1 HEV replicon mutant study, we subsequently constructed four genotype 3 swine HEV mutants with various HVR deletions: sHVRd1, with the deletion of the nearly complete HVR sequence; sHVRd2 and sHVRd3, with partial deletions of HVR sequences in the middle region; and sHVRd4, with a deletion of partial HVR sequence at the 5’ end (Fig. 4). The infectivities of these four mutants were tested in pigs by intrahepatic inoculation of capped RNA transcripts from each mutant via an ultrasound-guided inoculation procedure. Similar to our observations in the chicken study, we found that mutants sHVRd2, sHVRd3, and sHVRd4, with partial deletions of the HVR sequences at the 5’ end and in the middle region, are viable and infectious in pigs. Seroconversion was observed for all HVR deletion mutants; however, there was a delayed seroconversion with no detectable viral RNA in feces or sera for pigs inoculated with mutant sHVRd1, which contains the nearly complete HVR deletion, an indication of attenuation for the sHVRd1 virus. Viral RNA was detected much later during infection, at 9 weeks p.i., in pigs (no. 291 and no. 295) infected with mutant sHVRd2, which contains a larger sequence deletion of the HVR than mutants sHVRd3 and sHVRd4. Fecal virus shedding was detected at 1 and 2 weeks p.i. in pigs inoculated with mutants sHVRd3 and sHVRd4 and lasted for 5 to 8 weeks. These results suggest that mutants sHVRd1 and sHVRd2, with larger sequence deletions of the HVR, may be attenuated to replicate at lower levels, and it appears that the lengths of HVR deletions may affect the level of virus replication and attenuation. Again, additional studies to explore the role of the HVR in HEV attenuation, which is not within the scope of this study, will provide more insights into the role of the HVR in the biology and pathogenesis of HEV.

The patterns of viremia and fecal virus shedding in experimentally infected pigs and chickens (Tables 2 and 3) are somewhat different from that observed in HEV-infected humans. In humans, viremia usually precedes fecal virus shedding, whereas in the pig and chicken studies fecal virus shedding was detected prior to viremia, which is consistent with our previous animal studies (5, 16). It is believed that tissues in the gastrointestinal tract are the initial sites of swine HEV and avian HEV replication, and thus, the virus is excreted to the feces before entering the bloodstream. In fact, it has recently been demonstrated that after oral injection or intravenous injection, swine HEV and avian HEV first replicate in various gastrointestinal tissues in chickens and pigs before reaching the target organ, the liver, via the bloodstream (6, 59), and this may explain why fecal virus shedding precedes viremia in HEV-infected pigs and chickens.

Since ORF1 contains domains essential for HEV replication, proper folding of the encoded polyprotein is essential for its role in virus replication either individually or by interacting with host alleles. The results from our in vitro, as well as in vivo, animal studies showed that the deletions in the HVR did not influence the viability of the virus, and thus, the polyprotein encoded by ORF1 appears to be properly folded in viable mutants. The HVR, aa 747 to 761 of genotype 1 human HEV, aa 557 to 585 and 612 to 641 of avian HEV, and aa 712 to 765 of genotype 3 swine HEV, apparently has no major effect on the host-mediated processing of the polyprotein, as the mutant viruses are viable and infectious in animals. Since unneeded sequences in virus genomes normally are lost rapidly during in vivo replication, it is possible that the HVR, although not essential for virus infectivity, may play a biological role in HEV pathogenesis. In fact, the results from the animal studies with limited numbers of pigs and chickens suggested that deletions of a larger or nearly complete HVR from the HEV genome apparently attenuated the virus. Therefore, additional studies with larger numbers of animals are warranted to fully evaluate the biological role of HVR in HEV replication and pathogenesis.

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