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Phylogenetic Analysis of Hepatitis D Viruses Indicating a New Genotype I Subgroup among African Isolates

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Genetic analysis was performed on 13 hepatitis D virus (HDV) isolates from Ethiopia, Somalia, Jordan, Kuwait, Bulgaria, Moldavia, and Sweden. The complete nucleotide sequence and genomic organization are described for the first time for two African HDV isolates. Phylogenetic analysis showed all the African isolates to be intrarelated and to form a novel group within HDV genotype I; the suggested designation for this group is IC. The genetic distance to previously described type I isolates was about 0.15. The HDV genotype I isolates (total of 22 examined) phylogenetically formed three clusters, each of them corresponding to certain geographic regions; the "western" group consisted of six HDV isolates from western Europe and the United States plus one from Kuwait; the "eastern" group consisted of two isolates from Moldavia and one each from Bulgaria, Nauru, mainland China, and Taiwan; and the "African-Middle East" group consisted of six HDV isolates from Ethiopia and one each from Somalia, Jordan, and Lebanon.

Hepatitis D virus (HDV) is a 36-nm subviral agent, and the establishment of an HDV infection depends upon the presence of hepatitis B virus to provide the hepatitis B surface antigen as its envelope protein (29, 31).

Through analysis of HDV RNA from isolates from all continents but Africa, nucleotide sequence diversities that justify grouping the isolates into three major genotypes have been found (4, 9a, 17, 34, 35). It has been noticed that the severity of liver disease caused by HDV infection varies between different geographic areas from fulminant hepatitis to asymptomatic chronic liver disease (8, 10, 24, 28), and it has been discussed whether viral genetic factors might influence the pathogenesis of HDV infections. HDV genotype III, the type most distantly related to the other genotypes, is found in patients from northern South America, where outbreaks of severe hepatitis with particular high morbidity and mortality have been described (4, 21, 27). This has spurred efforts to characterize more HDV isolates from different parts of the world.

So far, there has been limited genetic information about HDV isolates from Africa, the Middle East, and east Europe, where a high prevalence of HDV infection among patients with chronic liver disease has been reported (30, 33). We have performed phylogenetic analysis of 13 HDV isolates from the above-mentioned regions, and for the first time the full sequence and genetic organization of two African isolates are described in detail.

MATERIALS AND METHODS

Patients. Serum samples from 13 HDV-infected patients, including 6 from Ethiopia, 2 from Moldavia, and 1 each from Somalia, Jordan, Kuwait, Bulgaria, and Sweden, were employed in this study. The clinical data are summarized in Table 1.

Strategy for amplification and sequencing of HDV genomes. Two isolates, one from Somalia and one from Ethiopia (E1), were fully sequenced. Five overlapping segments (here designated A to E) for amplification and sequencing were selected to cover the whole HDV genome. Conserved stretches where the primers could be placed were found by alignment analysis of 13 available full-length sequences, including all three major genotypes of HDV (2, 4, 6, 7, 9a, 12, 13, 15, 16, 20, 23, 32, 34, 35). Between segments B and C there was an 11-base overlap of the inner primers, prompting us to custom-make primer pairs flanking this junction (segment F), based on the sequence information available for the two African HDV isolates.

The sequence analysis of the rest of the samples was focused on the 777-base fragment between nucleotides (nt) 872 and 1648 (numbering according to reference 34), which covers the autocleavage site domain on the antigenomic strand and the whole hepatitis D antigen (HDAg)-encoding region through fragments E and A.

The primers were designed with the OLIGO program (version 5.0; National Biosciences, Inc., Plymouth, Minn.). All inner primers were also used as sequencing primers (Table 2).

RT-nested PCR for HDV RNA amplification. Reverse transcription (RT) and the first PCR were carried out in a single tube, but in separate steps. Briefly, 10 μl of extracted RNA was mixed with 2.5 μl of outer antisense primer (final concentration, 0.5 μM), denatured for 5 min at 98°C, and annealed for 5 min at 62°C before addition of 35 μl of the RT-PCR mixture (final concentrations: Tris buffer [pH 8.4], 20 mM; magnesium chloride, 2 mM; potassium chloride, 60 mM; each of the deoxyribonucleoside triphosphates, 200 μM) and 4 U of avian myeloblastosis virus reverse transcriptase (Promega, Madison, Wis.). RT was carried out at 42°C for 60 min, and then 2.5 μl of outer sense primer (final concentration, 0.5 μM) and 1 U of *Taq* DNA polymerase (Perkin-Elmer Cetus, Emeryville, Calif.) were added to the tube to complete the first PCR (35 cycles for 1.5 min at 95°C, 2 min at 62°C, and 3 min at 72°C). The second PCR was carried out by transferring 5 μl of the first-PCR product to a new tube containing 45 μl of the same PCR mixture with 1 U of *Taq* DNA polymerase and 0.5 μM concentrations of each of the inner primers, using the same temperature cycling conditions as for the first PCR in order to generate a sufficient amount of PCR amplimer for the sequence analysis.

Sequencing. The sequencing reaction was done with the Dyedeoxy terminator cycle sequencing kit (PRISM Ready reaction), which contains dye-labelled deoxyribonucleotides (Applied Biosystems Inc., Foster City, Calif.), according to the manufacturer's instructions but with a modification of the annealing temperature to 62°C instead of 50°C. The sequencing gels were run on an ABI 373A sequencer (Applied Biosystems). For each segment both strands were analyzed to obtain the consensus sequence. In order to compensate for the potential infidelity of the *Taq* polymerase, the full HDV cDNA sequences of the Somalian and the E1 Ethiopian samples were RT-PCR amplified and sequenced twice.

Sequence data analysis. Editing of raw sequence data and assembly of the segments into the continuous genome were accomplished by the programs Sequence Navigator (version 1.0.1; Applied Biosystems) and AutoAssembler (version 1.3.1b1; Applied Biosystems). Sequence alignments were done by use of the program MacMolly (Tetra, version 2.5; Soft Gene GmbH, Bocholt, Germany). RNA secondary structures were predicted by the program FoldRNA from the Genetics Computer Group program package (University of Wisconsin, Madison). Phylogenetic analysis of nucleotide sequences was conducted with the program DNA ML (maximum likelihood) (version 3.57c), with global rearrange-

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TABLE 1. Characteristics of HDV-infected patients investigated

Sample code	Origin	Age (yr)	Sex[a]	Diagnosis
E1	Ethiopia	25	M	Cirrhosis
E2	Ethiopia	18	M	Hepatoma
E3	Ethiopia	45	F	Cirrhosis
E4	Ethiopia	36	F	Cirrhosis
E5	Ethiopia	35	M	Cirrhosis
E6	Ethiopia	32	M	Cirrhosis
3239/94	Somalia	42	M	Chronic active hepatitis
J104	Jordan	NA[b]	NA	NA
K947	Kuwait	NA	NA	NA
15117/93	Bulgaria	32	M	Chronic hepatitis
M49	Moldavia	NA	NA	Chronic hepatitis
M64	Moldavia	NA	NA	Chronic hepatitis
236/95	Sweden	26	F	Acute hepatitis

[a] M, male; F, female.
[b] NA, not available.

ments. The programs ProtDist and Neighbor (version 3.57c) were used to analyze amino acid sequences. Unrooted trees were plotted by the program DrawTree and are presented with branch lengths drawn to scale. All programs used in the phylogenetic analysis are part of the PHYLIP program package, which was kindly provided by J. Felsenstein.

RESULTS

cDNA sequences and genomic organization of the Somalian and Ethiopian HDV isolates. Complete agreement between the results from the repeated RT-PCR sequence analyses of the two full-length sequences was found.

The complete HDV cDNA sequence of the Somalian and one Ethiopian (E1) isolates were shown to be 1,674 and 1,679 nt long, respectively. Although the two isolates did not contain the unique *Hin*dIII site which has been used as the start site in HDV sequence numbering (34), the similarity between the isolates in the sequence around this site allowed alignments with published sequences and corresponding numbering.

Overall, the Somalian and Ethiopian isolates have 83 to 85% and 84 to 86% sequence similarity with nine genotype I isolates (2, 6, 7, 15, 16, 20, 23, 32, 34, 35), 72 and 74% similarity with genotype II (9a), and 63 and 61% with genotype III (4), respectively. The sequence similarity between the two African isolates was 86%. A high degree of sequence conservation in some regions of the HDV genome, corresponding to the genomic and antigenomic cleavage domains as well as the HDV RNA binding domain of HDAg (14, 19, 25, 26), was observed in alignment analysis. Figure 1 shows full-length sequence alignment of the two African isolates with the HDV prototype strain (34, 35).

The open reading frame presumed to code for HDAg is

TABLE 2. Sequences, polarities, and positions of HDV primers used for amplification and sequencing analysis[a]

Designation	Sequence	Polarity	Position (nt)
Segment A			
1216 OU	5′ TCG GTC AAC CTC CTG AGT TC 3′	Outer and sense	1195–1214
1694 OR	5′ GGG GAG TCC AGC AGT CTC 3′	Outer and antisense	1670–1653
Delta UP	5′ CTT GTT CTC GAG GGC CTT CC 3′	Inner and sense	1266–1285
1673 R	5′ CGA GTC CAG CAG TCT CCT CT 3′	Inner and antisense	1668–1649
Segment B			
1554 OU	5′ GGC CAC CCA CTG CTC GAG 3′	Outer and sense	1533–1550
337 OR	5′ TGT TCG CTG AAG GGG TCC T 3′	Outer and antisense	311–329
1603 IU	5′ CTC GAC TCG GAC CGG CTC AT 3′	Inner and sense	1579–1598
333 ID	5′ CGC TGA AGG GGT CCT CTG GA 3′	Inner and antisense	325–306
Segment C			
299 OU	5′ AGC AGA CAA ATC ACC TCC AGA GGA 3′	Outer and sense	291–314
746 OR	5′ CGG TCC CCT CGG AAT GTT G 3′	Outer and antisense	744–726
310 IU	5′ CAC CTC CAG AGG ACC CCT TC 3′	Inner and sense	302–321
DW	5′ AGC GAG GAG GCT GGG ACC AT 3′	Inner and antisense	693–712
Segment D			
454 OU	5′ CCG AGA GGG GAC GAG TGA GG 3′	Outer and sense	454–473
890 OD	5′ TTC CTC TTC GGG TCG GCA TG 3′	Outer and antisense	909–890
464 IU	5′ ACG AGT GAG GCT TAT CCC GG 3′	Inner and sense	464–483
880 ID	5′ GTC GGC ATG GCA TCT CCA C 3′	Inner and antisense	898–880
Segment E			
847 OU	5′ CTC CTT CGG ATG CCC AGG T 3′	Outer and sense	847–865
1302 OD	5′ GGA TTC ACC GAC AAG GAG AG 3′	Outer and antisense	1322–1303
853 IU	5′ CGG ATG CCC AGG TCG GAC C 3′	Inner and sense	853–871
1297 ID	5′ GGC AGG ATC ACC GAC GAA GG 3′	Inner and antisense	1303–1284
Segement F			
150 OU	5′ TGA AGC TCT AGG AAG CGG AA 3′	Outer and sense	150–168
487 OD	5′ TTC CCC GGG ATA AGC CTC A 3′	Outer and antisense	487–469
152 IU	5′ AGC TCT AGG AAG CGG AAG AA 3′	Inner and sense	152–171
471 ID	5′ TCA CTC TTC CCC TCT CGG 3′	Inner and antisense	471–454

[a] Numbering is based on that in references 34 and 35.

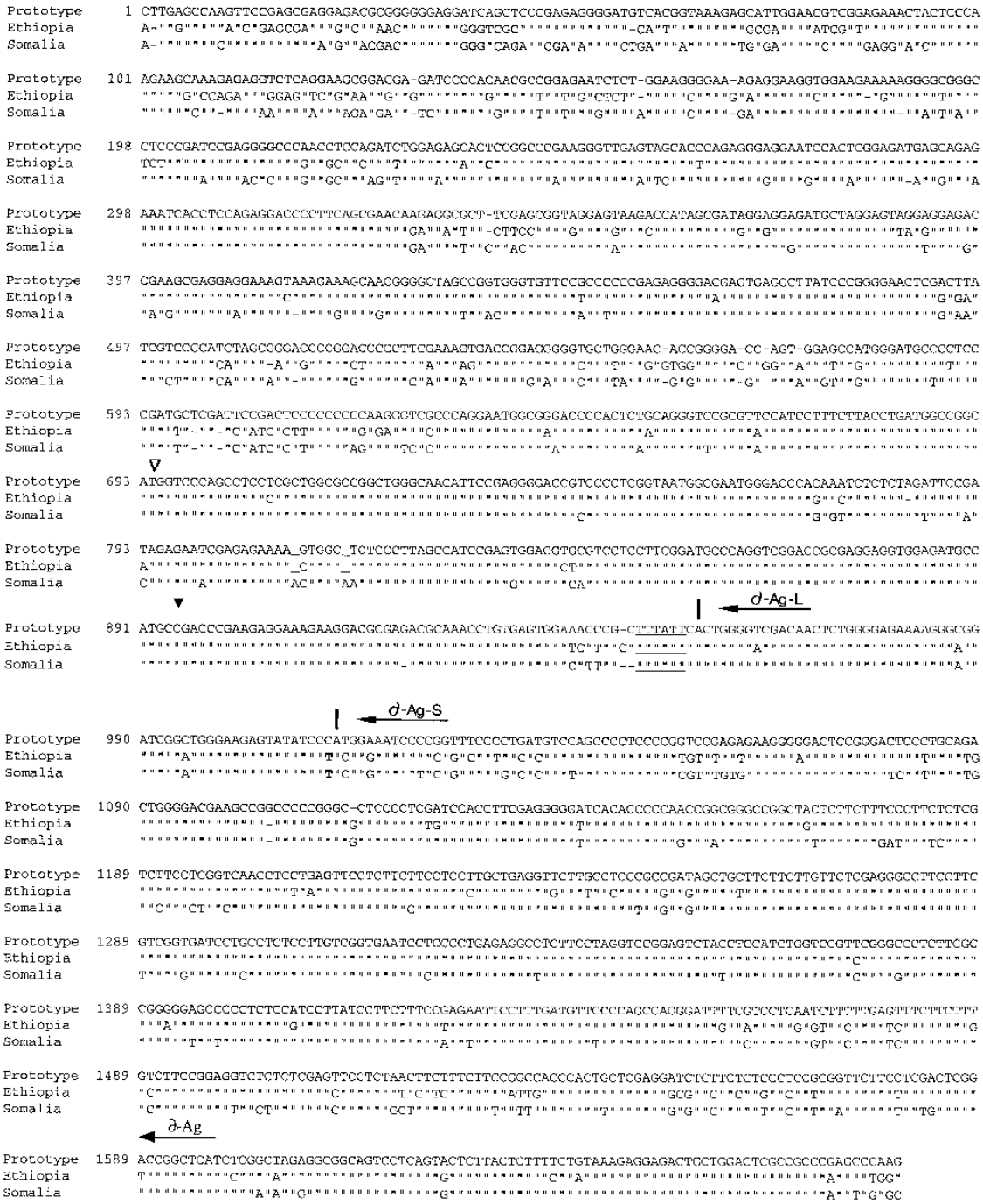

FIG. 1. Full-length-sequence alignment of the Somalian and Ethiopian isolates with the prototype strain. Open arrowhead, autocleavage site on genomic strand; filled arrowhead, autocleavage site on antigenomic strand. The poly(A) signal is underlined, and the RNA editing target is in boldface.

present in the antigenomic strand of the two isolates, with initiation and termination codon positions identical to those shown in Fig. 1, encoding the 195-amino-acid short form of HDAg (∂Ag-S). When RNA editing occurs (3, 5, 22, 37), a new frame is created; this frame has the same start codon but its stop codon is 57 bases further downstream, resulting in synthesis of the large form of HDAg (∂Ag-L), with 214 amino acids. Both the Somalian and the Ethiopian isolates contain presumed RNA editing sites (Fig. 1). The editing structure surrounding the RNA editing target required for maximum editing efficiency (3) as predicted by the program FoldRNA is similar to that described earlier, but with a 1-bp difference from AU to GC corresponding to positions 578 and 1014. The poly(A) signals and the autocleavage sites on the genomic and antigenomic strands are indicated in Fig. 1.

Nucleotide and amino acid sequence comparisons. Figure 2 shows an alignment of a 645-nt stretch encoding the HDAg from the six Ethiopian, the Somalian, the two Moldavian, and the Jordanian, Bulgarian, Kuwaiti, and Swedish isolates with the prototype strain. The sequence similarity between the pro-

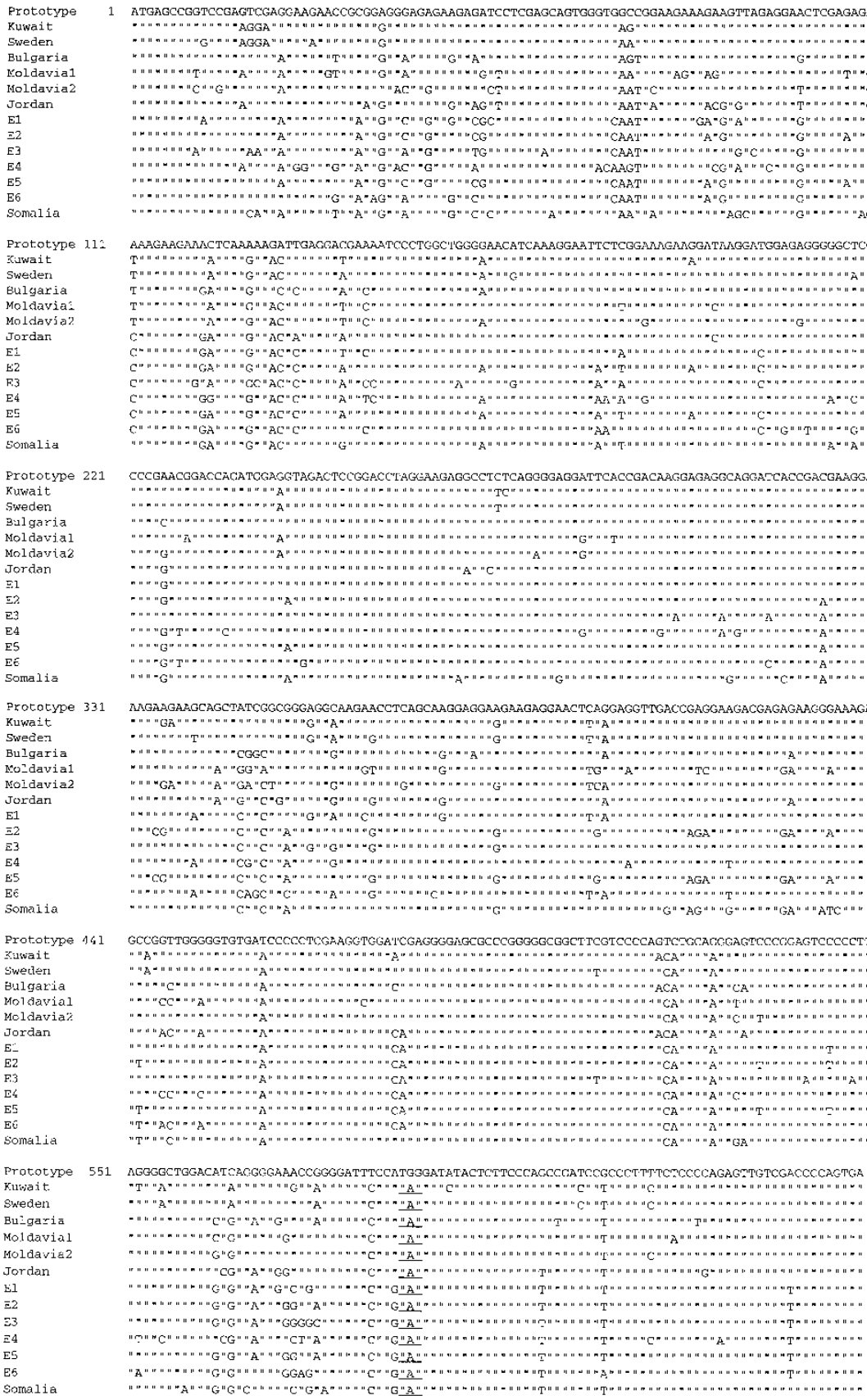

FIG. 2. Sequence alignment of the 645 bases of the HDAg-encoding region from the HDV isolates in this study and the prototype strain. The first stop codon of the open reading frame for HDAg is underlined.

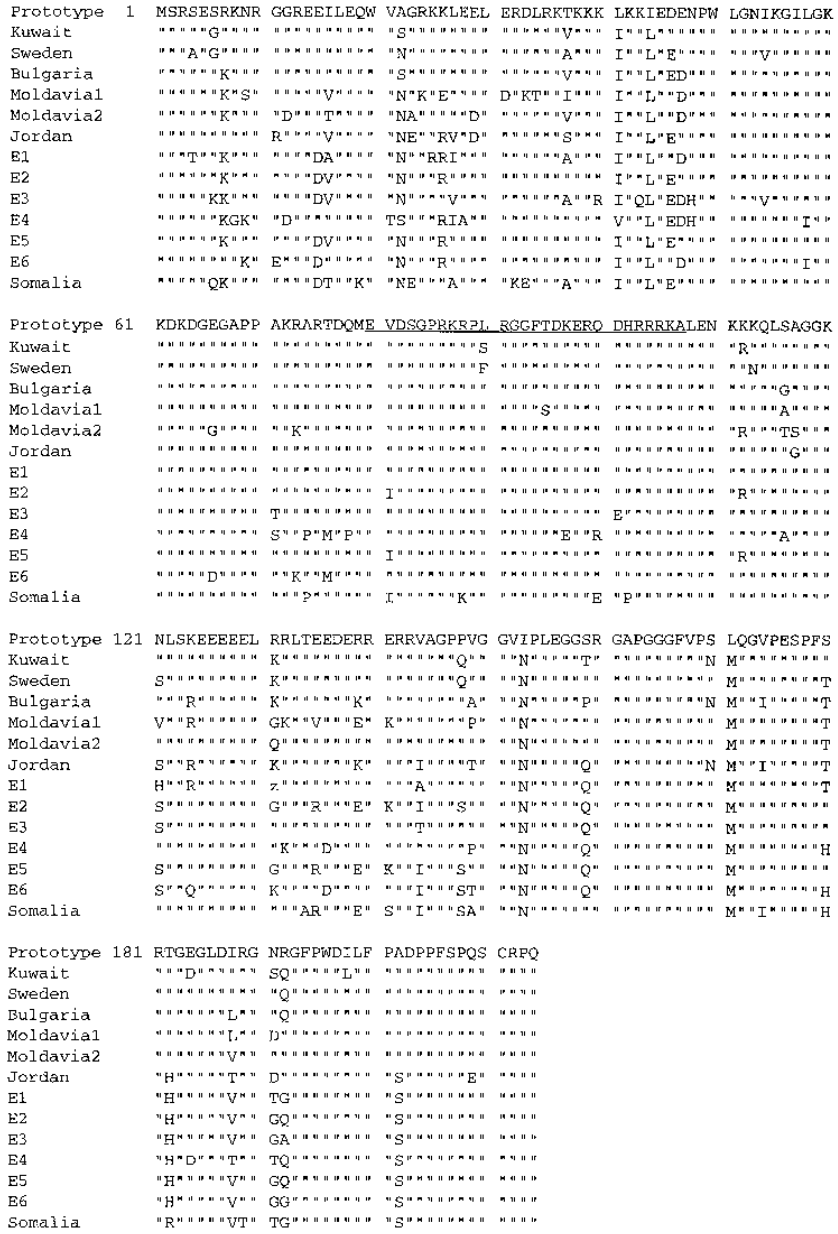

FIG. 3. Amino acid sequence alignment of the HDV isolates in this study and the prototype strain. The presumed HDV RNA binding domain is underlined.

totype strain and the African isolates was 85 to 89%, and that between the prototype strain and the other isolates was 90 to 93%.

About 56 and 37% of the nucleotide differences in the HDAg-encoding region of the two fully sequenced African isolates, compared with the HDV prototype strain, occurred in the first and/or second base of the codons, respectively. Therefore, the differences between the deduced 214-amino-acid sequences were greater than those between the corresponding nucleotide sequences. The Somalian and Ethiopian isolates had 81 to 85% and 78 to 90% amino acid sequence similarity with genotype I strains versus 86 to 88% and 85 to 90% nucleotide sequence similarity, respectively. Figure 3 shows an amino acid sequence alignment of the 13 HDV isolates investigated in this study with the prototype strain.

Phylogenetic analysis. (i) Evolutionary distances between new and previously described HDV isolates. The mean genetic distances between 24 HDV isolates at both the nucleotide and amino acid sequence levels were calculated from three data sets (the 358 bases between nt 908 and 1256 [4], the 645 bases of the whole HDAg-encoding region, and the 214 amino acids of the large form of HDAg) (Table 3).

The genetic distances varied depending upon whether the nucleotide or amino acid sequence data sets were used. Generally, the amino acid sequences indicated evolutionary distances between the HDV isolates greater than those obtained from the two nucleotide data sets. One of the exceptions was the distances between the Peru 1 and the other HDV isolates obtained with the 358-base stretch, where the high sequence divergence found in the 5′ part of the Peru 1 isolate resulted in

TABLE 3. Mean evolutionary distances between HDV isolates on both the nucleotide and amino acid levels

HDV (strain[a])	No. of isolates	Mean evolutionary distance calculated from 3 data sets[b]											
		I			Ethiopia			Somalia			II (Japan 1)		
		358 nt	645 nt	214 aa	358 nt	645 nt	214 aa	358 nt	645 nt	214 aa	358 nt	645 nt	214 aa
I	15	0.10	0.10	0.13									
Ethiopia	6	0.13	0.14	0.15	0.10	0.10	0.13						
Somalia	1	0.15	0.15	0.20	0.12	0.13	0.19						
II (Japan 1)	1	0.36	0.45	0.39	0.38	0.42	0.41	0.42	0.40	0.37			
III (Peru 1)	1	0.57	0.31	0.52	0.60	0.30	0.52	0.58	0.31	0.51	0.53	0.45	0.48

[a] I, II, and III represent genotypes I, II, and III, respectively.
[b] 358 nt, fragment between nt 908 and 1256; 645 nt, whole HDAg-coding region; 214 aa, large form of HDAg (aa, amino acids). Intragroup distances are underlined.

greater calculated evolutionary distances at the nucleotide than at the amino acid level.

(ii) Phylogenetic groupings. Phylogenetic analysis was performed on the 358- and 645-nt and the 214-amino-acid sequences of the 13 HDV isolates of this study plus 11 previously known isolates, including 9 genotype I, 1 genotype II (Japan 1), and 1 genotype III (Peru 1) samples, and the results are presented as unrooted trees. The trees showed the African isolates emerging as a new phylogenetic group or branch from the previously characterized strains of genotypes I, II, and III (Fig. 4), although some of the isolates were given somewhat different positions in the different trees. In the one built from the 358-base data set, three distinctly separated clusters within genotype I were seen (Fig. 4a); the first cluster corresponded to HDV genotype IA and consisted of samples from Italy, France, Sweden, the United States, and Kuwait (the "western" branch); the second cluster corresponded to HDV genotype IB and consisted of isolates from mainland China, Taiwan, Nauru, Moldavia, and Bulgaria (the "eastern" branch); and the third cluster contained the six Ethiopian HDV isolates and the isolates from Jordan, Lebanon, and Somalia (the "African-Middle East" branch), now designated HDV genotype IC. The mean genetic distance between the African-Middle East and eastern branches was 0.14, and that between the African-Middle East and western branches was 0.15. The western and eastern branches were calculated to have a mean genetic distance of 0.10.

DISCUSSION

Identification of HDV variants. It has been proposed that differences in the pathogenesis of hepatitis D may depend on certain features of the HDV genome (4, 36). Because of the lack of a serotyping assay for HDV, classification is currently based upon genetic sequence comparisons. However, there is no consensus about the choice of subgenomic regions and methods for such comparisons. Either of two methods, measurement of pairwise sequence similarity or phylogenetic analysis of sequences, could be used to measure genetic distances. The two methods may yield similar results. However, pairwise sequence similarity determination does not allow for multiple substitutions and might underestimate the extent of divergence between sequences. Therefore, we favor the phylogenetic analysis as a tool for classification. An ideal region should be sufficiently variable to distinguish differences between isolates. The 358-base stretch between nt 908 and 1256 has previously been used in the designation of HDV genotypes (4). As noticed in this study as well as others (9, 17), the amino acid sequence divergences could be greater than those of the nucleotide sequences, and the evolutionary distances calculated from the amino acid sequence data set were also greater than those from comparison of the nucleotide sequences. Analysis of amino acid sequences could, therefore, provide information complementary to nucleotide sequence comparisons.

Classification of HDV isolates. In a recent sequence analysis of more than 100 HDV strains collected from different parts of the world, most of the HDV isolates were classified as genotype I, which was suggested to be divided into the subgroups IA and IB. IA included HDV isolates from western Europe and North America, and IB contained isolates from Asia (1a, 4). When our 13 HDV isolates were analyzed together with 11 previously described HDV strains, the Somalian and Ethiopian isolates could be phylogenetically classified as a new genotype subgroup, tentatively designated IC, while those previously classified as IA and IB isolates remained in the IA and IB branches, respectively. It appeared that the samples within each of the groups corresponded to certain geographic regions. In the present study the western (IA) group consisted of six HDV isolates from western Europe and the United States plus one sample from Kuwait. The eastern (IB) group contained two isolates from Moldavia and one each from Bulgaria, Nauru, mainland China, and Taiwan, and the new distinctly separated African-Middle East (IC) group consisted of the six HDV isolates from Ethiopia and those from Somalia, Jordan, and Lebanon. The HDV genome of the Somalian isolate was more distantly related to that of the genotype I prototype strain than the other African isolates; only genotypes II and III were more distant at the full-length-sequence level. The mean genetic distances to the Ethiopian isolates and to the other genotype I strains were 0.19 and 0.20, respectively, calculated at the amino acid level.

Our data indicate that at least one unique HDV phylogenetic group exists on the African continent. Severe cases of hepatitis D have been reported from the Central African Republic (18) with histological features similar to those seen in cases of fulminant hepatitis in South America. Investigation of more HDV isolates from different parts of the African continent will disclose whether further HDV genotypes or subtypes exist and to what extent clinical features could be linked to genetic variants.

Genetic features of the two African full-length HDV sequences. It has been considered that the delta antigen is the only HDV-encoded protein expressed, although a number of other open reading frames can be identified on both the genomic and antigenomic strands. However, the number of reading frames as well as their lengths and the positions of start and stop codons vary among isolates, strongly indicating that they do not code for any viral proteins. It was therefore noteworthy when, in 1993, Khudyakov et al. (11) reported antibodies against a synthetic peptide corresponding to a region designated ORF-K positioned in a −1 reading frame relative to that of ∂Ag-L and ∂Ag-S. This reading frame was predicted to

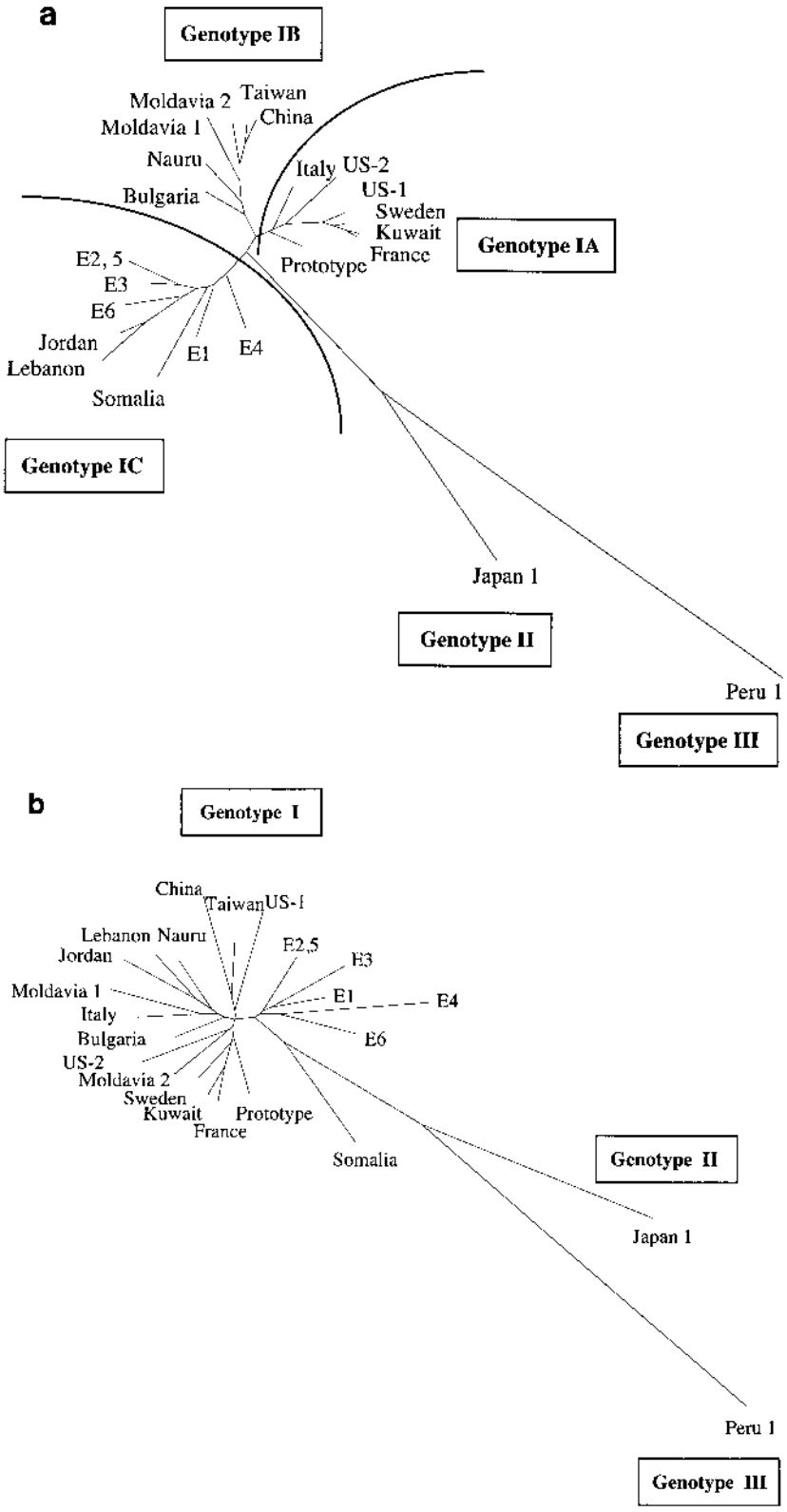

FIG. 4. Unrooted trees created by phylogenetic analyses of the 358 bases between nt 908 and 1256 using the program DNA ML (maximum likelihood) (a) and the 214-amino-acid sequence using the programs ProtDist and Neighbor (b).

code for a 63-amino-acid protein. Subsequently, Bichko and coworkers also reported a correspondingly novel form of HDAg detectable in HDV-transfected cell cultures and in infected woodchucks (1). In the same region and reading frame the African HDV isolates also contain one or two additional termination codons within the sequence corresponding to ORF-K and predicting a potential coding capacity of 35- or 30-amino-acid polypeptides, respectively. This could indicate the existence of different variants of the protein. However, the significance and function of this protein are still unclear. It might even be a translation product without any biological function created by frameshifting due to insertion or deletion events in the ∂Ag open reading frame.

It has been stated that the characteristic 8-bp RNA structure surrounding the editing site pair is required for maximum RNA editing efficiency (3). The AU base pair (positions 578 and 1014) in the predicted RNA editing structure was found to be a GC pair in the two African isolates. Earlier experiments have shown that a change of the AU at positions 578 and 1014 into a GC pair resulted in a reduction to 75% editing efficiency compared with that of the wild type (3). It would be interesting to see whether this variation in the RNA editing structure has any impact on HDV replication in vivo and in vitro.

The region from amino acids 79 to 107 of HDAg has been identified as a domain responsible for HDV RNA binding mainly from investigations with the prototype strain (26). Several of our HDV isolates showed variations of one to four amino acids in this region. This naturally existing variation indicates that the amino acid sequence requirement for the RNA binding function may not be that strict.

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