

# Molecular Epidemiology and Evolution in an Outbreak of Fulminant Hepatitis B Virus

Maria Alma Bracho,<sup>1</sup> María José Gosalbes,<sup>1</sup> Francisco González,<sup>2</sup> Andrés Moya,<sup>1</sup>  
and Fernando González-Candelas<sup>1\*</sup>

*Institut Cavanilles de Biodiversitat i Biologia Evolutiva and Departament de Genètica, Universitat de València, 46071 València, Spain,<sup>1</sup> and Direcció General de Salut Pública, Conselleria de Sanitat i Consum, Micer Mascó 26, 46010 València, Spain<sup>2</sup>*

Received 27 July 2005/Returned for modification 14 October 2005/Accepted 31 January 2006

**In order to establish the transmission pathway for two outbreak patients affected by fulminant hepatitis B (FHB) following a shared period of hospitalization, we sequenced the complete genomes of the hepatitis B viruses (HBV) isolated from them as well as from the suspected common source and 11 additional controls. Phylogenetic and statistical analyses of these sequences revealed that the two FHB patients were indeed infected by a common source and that the fatal development of the disease did not appear to be associated with any mutation previously reported to be related to FHB. These data have also allowed us to estimate the extent and distribution of genetic variability along the genomes of HBV genotype D samples from the same source population. As a result of these analyses, we provide an improved statistical method to individualize the assignment of each suspected patient and the source of an outbreak and information on which genome region to analyze in the molecular epidemiological assessment of hepatitis B virus transmission cases.**

Hepatitis B virus (HBV), the prototype member of the *Hepadnaviridae* family, causes acute and chronic liver disease, chronically infecting more than 400 million people worldwide, with an estimated risk of death of 25% for chronically infected persons (15), thus representing one of the most serious public health problems. HBV is one of the smallest DNA viruses infecting humans, and its genome comprises a 3.2-kb linear strand complementary to a shorter (1,700 to 2,800 nucleotides [nt]) strand. The two strands are arranged in a circular, partially double-stranded molecule. The genome contains four partially overlapping open reading frames (P [polymerase], C [core], S [surface], and X proteins), with no noncoding regions and about 50% of its genome involved in two overlapping reading frames. This extremely compact nature of the HBV genome imposes important limits on viral evolution, a situation designated “constrained evolution” (18).

HBV isolates are grouped into eight major genotypes (designated A to H) with different geographic distributions. Genotype D is the most prevalent in southern European countries, whereas genotype A has a worldwide distribution. Although there is no clear relationship between HBV genotype and clinical course, they seem to affect disease profile, with a higher chance of viral clearance and sustained remission for patients infected with genotype A than for those infected with genotype D (15).

Molecular and serological characterizations of HBV have been used to infer chains and sources of infection in several settings, such as nosocomial infections (11, 12, 23), mother-to-infant transmission (13), or infections derived from close familial contact (5, 38). Different strategies have been employed in these analyses, most of them involving the sequencing of a

representative portion of the HBV genome. However, there is no consensus on the best choice of region for this kind of analysis. Several authors have used the core region (11, 38), others have employed the S region (13), and many have used more than one region (12). The complete genome sequences of nine isolates were used to study a nosocomial infection involving cardiac transplant patients (23). Recently, the same approach has been used to look for specific mutations associated with fulminant hepatitis B (FHB) in several donor-recipient pairs (4, 7). There have been conflicting reports on the association between specific mutations in the HBV genome and fulminant hepatitis, especially in the precore (nt 1896) and core (nt 1762 and 1764) promoter regions (2, 16, 22, 28, 31).

Although nosocomial transmissions were soon identified as a risk factor for HBV (17), the frequent report of this type of infection indicates that it still represents a serious and unsolved problem. However, as this is a well-identified risk and prevention measures are known and easy to implement, there is also an increase in the number of plaintiffs seeking financial compensation from infected patients. Hence, establishing the origin of specific HBV infections is frequently required, with implications for public health.

In April 2002, two fulminant hepatitis B cases associated with a public hospital in Castelló de la Plana (Spain) were detected, suggesting a possible case of nosocomial transmission. Both patients had been hospitalized in the same surgery service during a few days in late December 2001, at the same time as a chronic hepatitis B carrier. All three were determined to be affected by HBV genotype D, the most common genotype in Spain (6). Here we report the molecular epidemiological study of the outbreak and several population control samples through sequencing of the complete HBV genome along with a detailed analysis of the molecular evolutionary patterns throughout the different regions of this virus. Furthermore, given the fatal outcome of the disease in the two suspect recipients and the chronic status of the putative donor, this

\* Corresponding author. Mailing address: Institut Cavanilles de Biodiversitat i Biologia Evolutiva, Universitat de València, Apartado 22085, 46071 València, Spain. Phone: (34) 96 3543653. Fax: (34) 96 3543670. E-mail: fernando.gonzalez@uv.es.

information allowed us to analyze the possibility of establishing associations between specific viral mutations and FHB.

#### MATERIALS AND METHODS

**Fulminant hepatitis B outbreak patients.** Two FHB cases were declared in the Spanish city of Castelló de la Plana in early spring 2002. The first case (patient 15) was a 63-year-old male who had been intervened of rectum neoplasia on 26 December 2001 at Hospital General de Castelló. He had received no blood transfusion, chemotherapy, or radiotherapy after surgery. The second case (patient 16) was a 61-year-old male who had gone through emergency surgery at the same hospital on 29 December 2001 due to mesenteric ischemia. Prior to surgery, he had received two chemotherapy sessions due to pulmonary neoplasia, and he attended three further sessions afterwards. He also received seven blood transfusions in the next month after surgery. During their stay at the hospital, the two patients occupied different rooms on the same floor and different surgery rooms were used for their interventions.

**Epidemiologic investigation.** Upon detection of the outbreak, an epidemiologic investigation of all patients and medical personnel who used or attended the same facilities at the hospital during the stay of the two FHB cases was undertaken. The search revealed that a 52-year-old woman who was suffering from a lymphoma and was a chronic HBV carrier had stayed on the same floor from 30 December to 1 January to get a Hickman catheter implanted. She received no transfusions during this stay at the hospital, and her chemotherapy sessions were not coincidental with the stay of the two FHB patients. She was operated on in a different surgery room than the two FHB patients. No cases positive for hepatitis B surface antigen were detected among the medical personnel of this hospital.

**Samples.** Serum samples were obtained from 14 patients infected with HBV from the Hospital Universitari La Fe (València, Spain) and Hospital General (Castelló de la Plana), including the three patients involved in the outbreak (patients 07, 15, and 16). All outbreak patients were positive for hepatitis B surface antigen, hepatitis B e antibody, and hepatitis B core antibody and negative for hepatitis B e antigen, hepatitis C virus, hepatitis delta virus, and human immunodeficiency virus. Except for the two FHB patients associated with the outbreak, all samples were obtained from chronic hepatitis B patients.

**DNA genome amplification and sequencing.** Viral DNA was purified from sera with a high pure viral nucleic acid kit (Roche Diagnostics GmbH, Mannheim, Germany) by following the protocol as described by the manufacturer. Purified DNA was stored at  $-80^{\circ}\text{C}$  until used. Viral DNA was amplified and sequenced essentially as described previously (34), with minor modifications of this strategy for complete genome amplification. PCR products were purified using a high pure PCR product purification kit (Roche Diagnostics GmbH, Mannheim, Germany). The purified DNA fragments were directly sequenced by the dideoxy method, using a BigDye Terminator v3.0 cycle sequencing ready reaction kit, and analyzed with an ABI PRISM 3700 sequencer (Applied Biosystems, Foster City, CA). Sequence chromatogram files were analyzed using the Staden package (30). Nucleotides were numbered in accordance with the system of Stuyver et al. (34).

**Phylogenetic analysis.** Based on the full nucleotide sequences, genetic analysis of the 14 HBV isolates was undertaken. Virus genotypes were established by comparison to existing sequences from public databases. Complete genome sequences were aligned using CLUSTAL W (36) and were refined further by visual inspection. Phylogenetic trees were constructed using the neighbor-joining algorithm (27) and maximum likelihood with heuristic search as implemented in PAUP\* (35). Both analyses were based on the best-fitting model for nucleotide substitution by use of Akaike's information criterion (1) according to the strategy implemented in Modeltest 3.1 (24). The tree topology was evaluated by bootstrap analysis with 2,000 replicates.

Different tree topologies corresponding to alternative hypotheses of the relationships between each outbreak sample and the controls were specified previously (9), and their likelihoods were compared to the one derived without topological constraints by means of a Shimodaira-Hasegawa test (29) as implemented in PAUP\* (35) after 1,000 bootstrap pseudoreplicates.

**Analysis of polymorphism.** Estimates of the number of synonymous (SY) and nonsynonymous (NSY) substitutions among sequences were obtained using the Nei-Gojobori method (19), as implemented in the MEGA program (14). Polymorphic sites were evaluated using DnaSP version 4.0 (26) with a 100-nt-wide sliding window and 1-nt steps.

**Nucleotide sequence accession numbers.** The sequences of the 14 new HBV complete genomes have been deposited in the EMBL database with accession numbers AJ627215 to AJ627228.

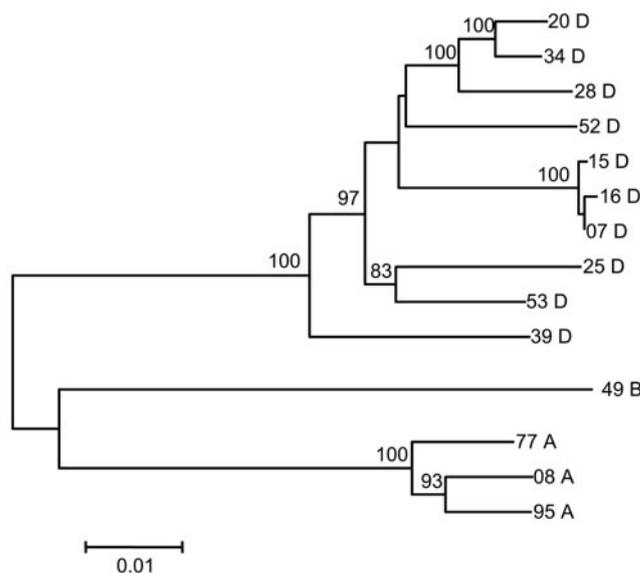


FIG. 1. Phylogenetic tree constructed with the complete genome sequences of 14 HBV isolates. Phylogenetic robustness as estimated by a bootstrap analysis is shown at each node. The patient number and genotype for each sequence are indicated. The scale bar represents genetic distance (substitutions per nucleotide).

#### RESULTS

**Molecular epidemiology and outbreak analysis.** We have obtained the complete genome sequences of 14 HBV isolates. Ten of these corresponded to genotype D, including those from the outbreak patients; three were assigned to genotype A; and one to genotype B. All genotype D sequences were 3,182 nt long, identical in length to the reference strain HPBAYW (EMBL accession no. J02203) (8, 33). The three genotype A sequences had different lengths, of 3,209, 3,212, and 3,221 nt, with variation from the reference strain HBVADW2 (EMBL accession no. X02763, 3,221 nt long) (33, 37), located around positions 40 to 54, in the genome portion coding for P and pre-S proteins. The genotype B sequence had a total length of 3,215 nt, identical to the reference sequence for this genotype, HPBADW1 (EMBL accession no. D00329) (21).

The general time-reversible model with a proportion of invariable sites equal to 0.4726 and a gamma distribution shape parameter equal to 0.7439 was chosen as the best model of nucleotide substitution for the 15 complete genome sequences (another genotype B sequence from the database was included in the analysis) by use of Akaike's information criterion (1, 24). Based on this evolutionary model, a phylogenetic tree was constructed by maximum likelihood and neighbor joining from the corresponding pairwise distance matrix. The topology was evaluated by bootstrap, and it is shown in Fig. 1. HBV sequences grouped according to their genotype with high bootstrap support, and most groups within genotypes were also well supported. Sequences derived from the three outbreak isolates grouped in a single cluster with 100% bootstrap support, a first indication of their very close relatedness.

To further determine whether the HBV sequences from the outbreak patients were related, we tested three alternative tree topologies (Fig. 2) for genotype D sequences, each represent-

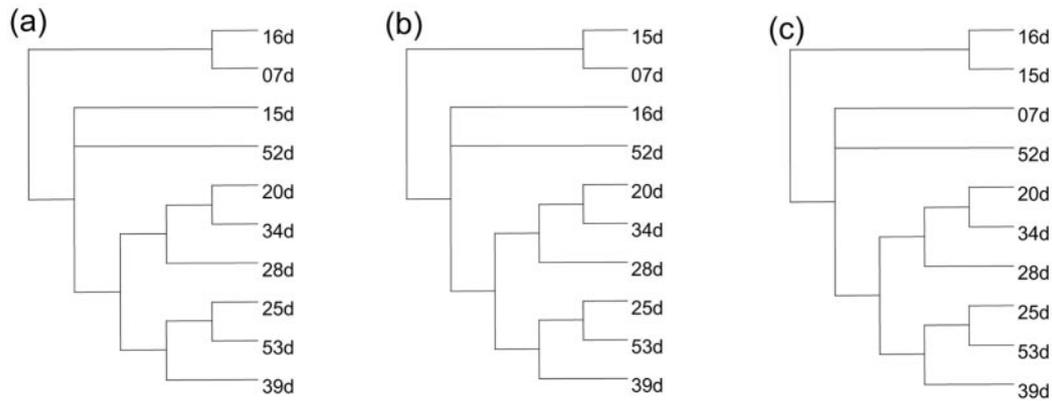


FIG. 2. Phylogenies corresponding to testing whether each sequence related to the outbreak is significantly related to the other sequences in this group or to the general population. The patient number and genotype for each sequence are indicated. (a) Sequence from the suspected source, patient 07. (b) Sequence from patient 15. (c) Sequence from patient 16.

ing the possibility that a particular outbreak sequence actually grouped with the control samples rather than with the two other samples presumably from the outbreak (9). Table 1 shows a summary of the results obtained with the Shimodaira-Hasegawa test. The three alternative topologies were significantly worse than the one shown in Fig. 1, which supports the grouping of the three outbreak isolates in a cluster of closely related sequences. Hence, the molecular sequence data provided further support to the epidemiological enquiry and confirmed that the three patients were involved in a common transmission chain.

#### Distribution of genetic variability along the HBV genome.

Although using a complete genome sequence provides the maximum possible amount of information at the sequence level, usually it is not necessary to sequence the complete genomes of different isolates, especially for population and epidemiology studies. However, in these cases it is necessary to use a genome region with the adequate amount of sequence divergence for the problem being studied (10). Hence, we obtained the distribution of genetic variability along the complete HBV genome sequences of genotype D isolates (Fig. 3). Genetic variation was not uniformly distributed throughout the HBV genome, with differences larger than 10-fold between regions with the highest and lowest levels of variation. Although there was a trend for the most-variable regions to be located in

genome areas coding for only one gene product (e.g., within the P gene), there were exceptions in both directions. The region coding for the 3' end of the S gene, which also codes for the central portion of the polymerase, presented a variability level similar to that found in the nearby downstream region coding for the polymerase only. In contrast, the genome portion flanked by these two regions, which codes for the polymerase only, was one of the most conserved stretches in the entire HBV genome, showing variability levels similar to those found in areas coding for only one gene product, such as the core protein or the amino terminal of the polymerase.

The distribution of synonymous and nonsynonymous substitutions among outbreak and genotype D sequences along the viral genome is shown in Fig. 4. Substitutions were not distributed at random in overlapping and nonoverlapping coding regions for the 10 genotype D sequences. Most synonymous substitutions appeared in the nonoverlapping fragments of the polymerase gene (*pol*), as expected from the lower restriction for these changes, since they affect only one protein product and do not alter the encoded amino acid. Conversely, the central portion of this gene, which overlaps the S gene, harbored substantially more nonsynonymous than synonymous substitutions. The core gene is the least overlapping with *pol*, and it displayed a markedly different pattern of substitutions. In both sections of the core gene, overlapping and nonoverlapping with *pol*, there were more nonsynonymous than synonymous substitutions: 39 NSY and 36 SY and 7 NSY and 5 SY substitutions, respectively. This was also the case for the X gene, again in both overlapping (14 NSY and 5 SY) and nonoverlapping (13 NSY and 5 SY) regions. Finally, the S gene, which fully overlaps with *pol*, presented a different pattern, with fewer nonsynonymous than synonymous substitutions (40 NSY and 54 SY).

**Mutations associated with FHB.** We have tested for the presence of mutations associated with FHB in the patients included in this study, because the two patients that prompted our analysis died shortly after nosocomial infection with HBV. Table 2 presents a presence/absence analysis of mutations potentially associated with FHB in the sequences included in this study. The analysis shows that the majority of HBV isolates (from both the outbreak and the nonoutbreak patients) har-

TABLE 1. Tests for the alternative hypotheses of each isolate from the outbreak not belonging to it<sup>a</sup>

Isolate excluded	Ln <sup>b</sup>	$\Delta^c$	P
None	7,167.342	Best	
07	7,290.979	123.636	<0.001
15	7,287.886	120.544	<0.001
16	7,290.979	123.636	<0.001

<sup>a</sup> A one-tailed Shimodaira-Hasegawa test with RELL (1,000 replicates) was performed on the topology represented in Fig. 1 for genotype D sequences and the three alternative topologies (Fig. 2), where each outbreak isolate sequence was presumed to group with control samples and not with the two other outbreak isolates.

<sup>b</sup> Ln, log likelihood.

<sup>c</sup>  $\Delta = \text{Ln}(\text{H1}) - \text{Ln}(\text{H0})$ , where H0 is the topology shown in Fig. 1 for genotype D sequences, and H1 is an alternate topology considering each outbreak-related isolate as grouping with control samples.

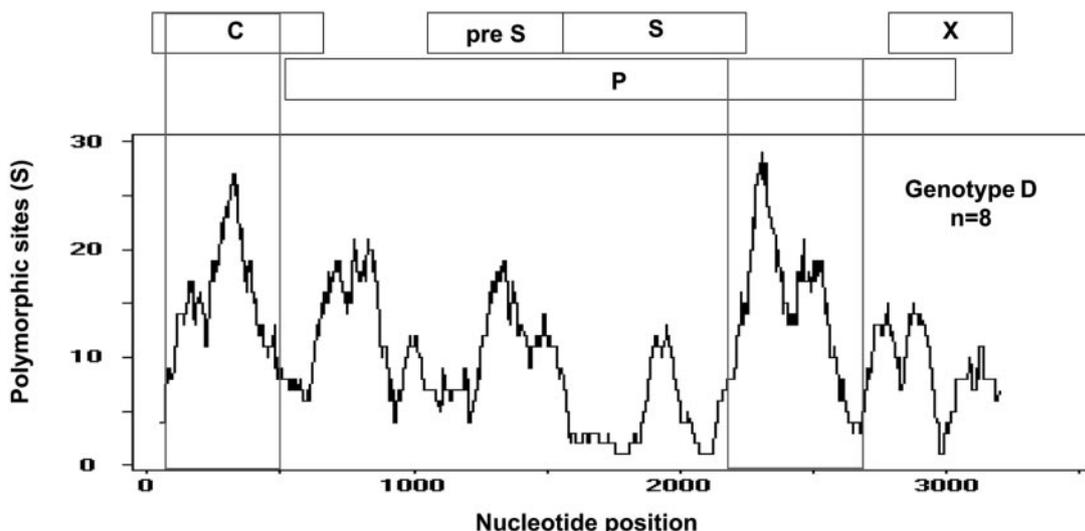


FIG. 3. Nucleotide diversity along the HBV genome. The number of polymorphic sites in 100-nt-wide windows among eight complete genotype D genomes sequenced in this study is represented (only one genome sequence from the outbreak was included in this analysis).

bored one or more mutations presumably linked to FHB. Nevertheless, only the two outbreak patients suffered a rapid, fatal development of the disease despite the fact that viruses isolated from these two persons had potential FHB mutations

identical to those of the virus from the infection source, a chronically infected patient who did not progress to FHB.

Most polymorphic sites appearing in the outbreak-related sequences were located in the *pol* gene (10 of 11). Of these, six

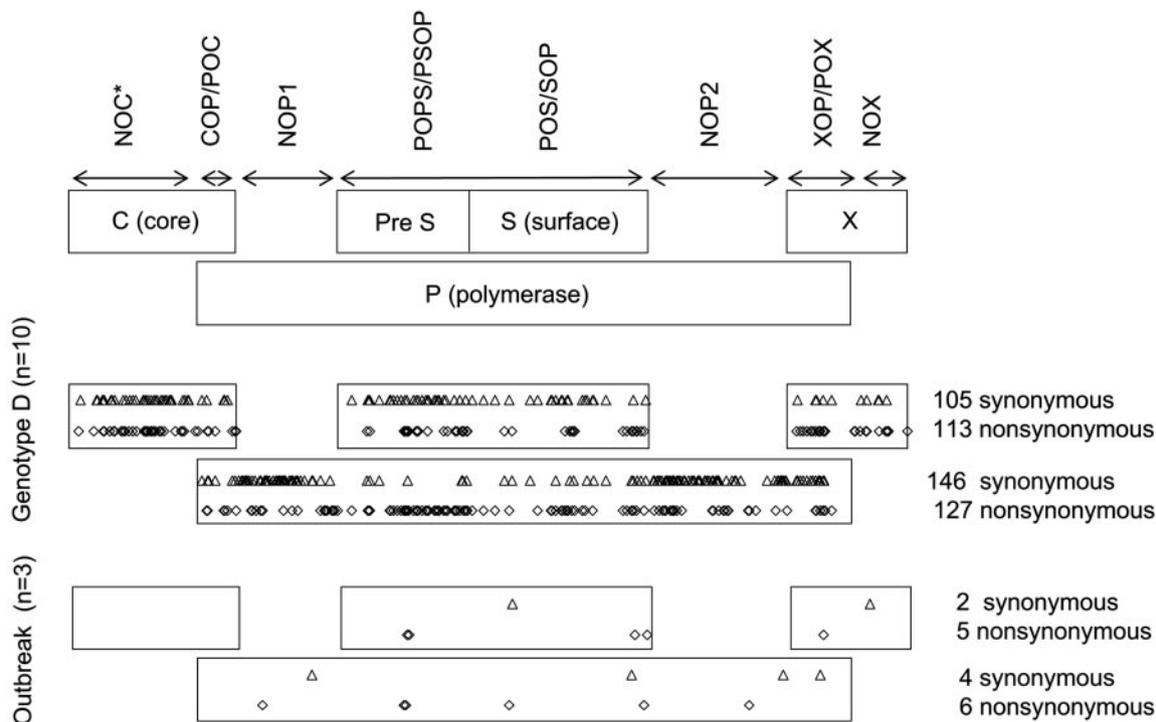


FIG. 4. Locations of SY ( $\Delta$ ) and NSY ( $\diamond$ ) substitutions in the complete genomes for outbreak (3 sequences) and genotype D (10 sequences) samples, considering the different overlapping and nonoverlapping gene regions. \*, no substitutions were found in the 25 nt overlapping core and X genes. These nucleotides have been considered a nonoverlapping part of the core gene. Genome/gene regions are abbreviated as follows. NOC, nonoverlapping core gene; COP, core gene overlapping *pol*; POC, *pol* overlapping core gene; NOP1, nonoverlapping *pol*, part 1; POPS, *pol* overlapping pre-S; PSOP, pre-S overlapping *pol*; POS, *pol* overlapping S, SOP, S overlapping *pol*; NOP2, nonoverlapping *pol*, part 2; XOP, X overlapping *pol*; POX, *pol* overlapping X; NOX, nonoverlapping X.

TABLE 2. Mutations proposed to be associated with FHB in the samples analyzed in this study

Patient <sup>a</sup>	Presence/absence <sup>b</sup> of mutation by region										
	Enhancer I/X promoter		NRE <sup>c</sup>		Enhancer II/core promoter						Precore
	C/G1249T <sup>d</sup>	T1250C	A/G1633 <sup>d</sup>	A/G1634 <sup>d</sup>	C1653T	A1752C	T1753C/A/G <sup>d</sup>	C1754T	A1762T	G1764A	G1896A
07	+	+	-	-	+	+	-	+	-	-	+
15	+	+	-	-	+	+	-	+	-	-	+
16	+	+	-	-	+	+	-	+	-	-	+
20	-	-	-	-	-	-	-	+	-	-	-
34	-	-	-	-	-	-	-	+	-	-	-
28	-	+	-	-	-	-	+	+	+	+	+
25	+	+	+	-	-	-	-	+	-	-	+
52	-	-	-	-	+	+	-	+	-	+	+
53	-	-	-	-	-	-	-	+	-	-	+
39	+	-	-	-	-	+	-	-	-	-	+
08	-	-	+	-	-	-	+	+	+	+	-
95	-	-	+	-	+	-	-	+	+	+	-
77	-	-	+	-	-	-	+	+	-	+	+
49	-	-	+	+	-	-	-	+	-	-	+

<sup>a</sup> Patients 15 and 16 correspond to the two outbreak patients affected by FHB, and patient 07 was their source of infection. The remaining patients in the study were chronic HBV carriers.

<sup>b</sup> +, presence; -, absence.

<sup>c</sup> NRE, negative regulator element.

<sup>d</sup> Nucleotide depends on genotype.

were nonsynonymous (four overlapping to the S gene and two in the nonoverlapping regions of *pol*) and four were synonymous (two in the nonoverlapping regions and one each overlapping the S and X genes). A summary of some relevant features of these mutations is presented in Table 3. None has been postulated to be associated with FHB, and since none was present in both infected patients and absent in the source it is not possible to invoke any of these mutations as the only cause of the fatal development of the disease in those two persons.

## DISCUSSION

Our analyses have revealed substantial genetic variation in the genome sequences of HBV genotype D isolates from the same population and with different degrees of relatedness, from the very closely related isolates recently derived from a common source to the more distant, epidemiologically unrelated isolates. This has allowed us to establish a clear link between the two outbreak case patients and the putative infection source, reinforcing the epidemiological analysis which, without molecular evidence, also gave a strong indication about this transmission case. The complete genome sequences

derived from the source and the two infected patients present a small number of differences (eight and nine differences for patients 15 and 16, respectively, from a total of 3,218 nt), many of which correspond to polymorphic sites in the source that are resolved in the recipients (five cases). Similar or even higher numbers of differences (ranging from 0 to 43) have been found by full-genome comparisons between HBV isolates from source and recipient pairs in several analyses (4, 7, 25, 32). Our statistical analyses provide strong, additional support for a link between the three full genome sequences considered in the outbreak and additional full genome sequences obtained from HBV-infected individuals from the same population. The epidemiological and molecular phylogenetic analyses have led us to conclude that there was a common origin for HBV in the two outbreak patients and that this common source was patient 07, with whom both had been in contact in a public hospital during two days at the end of 2001.

Our study has revealed that genetic variation is not evenly distributed along the HBV genome (Fig. 3). Despite the presence of extensive overlapping of coding regions, we found high levels of genetic variation within genotype D sequences ob-

TABLE 3. Analysis of variant sites among the three outbreak-related sequences

Source or location of sequence	Polymorphism <sup>a</sup> at nt:										
	289	765	814	1221	1356	1500	1679	2547	2738	3064	3069
<b>Patients</b>											
07	Y	R	W	R	C	T	A	A	C	G	R
15	T	A	A	G	C	C	A	A	Y	A	A
16	C	G	T	A	T	T	C	G	C	G	G
<b>Genes</b>											
<i>pol</i>	NSY	SY	NSY	NSY	SY	SY		NSY	SY	NSY	NSY
Other	SY (S)	NSY (S)	NSY (S)			NSY (X)	SY (X)			NSY (S)	NSY (S)

<sup>a</sup> Standard ambiguity codes for nucleotide polymorphism are used. Most sites are located in the *pol* gene; for those that are not, the relevant gene is reported in parentheses. The nature of the polymorphism is indicated (SY or NSY).

tained from the same population, which provides ample opportunities for the application of molecular sequence analysis to epidemiological analysis of HBV. Similar patterns of heterogeneity in genetic variation along the viral genome have been documented previously (3, 7, 20). In general, the genome region of choice for outbreak analyses depends on the problem in question and its associated time frame, because an adequate level of variation for discriminating among the different alternatives under consideration must be provided (10). From the data presented here, it is recommended to analyze the most rapidly evolving HBV genomic regions, such as the nonoverlapping stretches in the core and *pol* genes, for the study of outbreaks or transmission chains or for studies involving sequences recently derived from a common ancestor. This does not necessarily contradict the "constrained evolution" model for HBV (18), since different evolutionary forces act at different time scales. Among very closely related isolates, drift and hitchhiking selection, for instance, may contribute to temporary polymorphism in positions that will most likely be lost from the population and will not contribute to differentiation among more distantly related isolates.

Transient polymorphisms, such as those detected in the source but resolved in the outbreak patients, may also explain why some mutations have been associated with fulminant hepatitis B even though they appear only in some fatally affected patients. We present evidence of such a lack of association in the three patients involved in the analyzed outbreak. However, other reasons may also explain the recurrent presence of this kind of mutation associated with FHB. For instance, it may be that two or more mutations in different positions of the HBV genome are required to produce the fatal effects or that these depend not only on the virus itself but also on other host factors.

Another remarkable feature is the high frequency of non-synonymous mutations among the variants detected. Half of the six mutations appearing in regions where two genes overlap were nonsynonymous for both genes. However, it is possible that they have negative effects on viral fitness and will eventually be removed from the population by purifying selection. Nevertheless, their low frequency prevented their identification as strongly negatively selected codons. Evidently, more studies are needed to completely understand the reasons why such different clinical outcomes should characterize patients affected by very similar viruses.

#### ACKNOWLEDGMENTS

We thank Eddie Holmes, Mario A. Farés, and F. Xavier López-Labrador for comments and suggestions to improve the manuscript.

This work was supported by Conselleria de Sanidad, Generalitat Valenciana, and project Grupos03/204 from Agencia Valenciana de Ciencia y Tecnología.

#### REFERENCES

1. Akaike, H. 1974. A new look at the statistical model identification. *IEEE (Inst. Electr. Electron. Eng.) Trans. Automatic Control* **19**:716–723.
2. Baumert, T. F., S. A. Rogers, K. Hasegawa, and T. J. Liang. 1996. Two core promoter mutations identified in a hepatitis B virus strain associated with fulminant hepatitis result in enhanced viral replication. *J. Clin. Investig.* **98**:2268–2276.
3. Bollyky, P. L., and E. C. Holmes. 1999. Reconstructing the complex evolutionary history of hepatitis B virus. *J. Mol. Evol.* **49**:130–141.
4. Chen, Y., K. Michitaka, H. Matsubara, K. Yamamoto, N. Horiike, and M. Onji. 2003. Complete genome sequence of hepatitis B virus (HBV) from a patient with fulminant hepatitis without precore and core promoter mutations: comparison with HBV from a patient with acute hepatitis infected from the same infectious source. *J. Hepatol.* **38**:84–90.
5. Dumpis, U., E. C. Holmes, M. Mendy, A. Hill, M. Thursz, A. Hall, H. Whittle, and P. Karayiannis. 2001. Transmission of hepatitis B virus infection in Gambian families revealed by phylogenetic analysis. *J. Hepatol.* **35**:99–104.
6. Echevarria, J. M., A. Avellón, and L. O. Magnius. 2005. Molecular epidemiology of hepatitis B virus in Spain: identification of viral genotypes and prediction of antigenic subtypes by limited sequencing. *J. Med. Virol.* **76**:176–184.
7. Friedt, M., P. Gerner, P. Wintermeyer, and S. Wirth. 2004. Complete hepatitis B virus genome analysis in HBsAg positive mothers and their infants with fulminant hepatitis B. *BMC Gastroenterol.* **4**:11.
8. Galibert, F., E. Mandart, F. Fitoussi, P. Tiollais, and P. Charnay. 1979. Nucleotide sequence of the hepatitis B virus genome (subtype ayw) cloned in *E. coli*. *Nature* **281**:646–650.
9. González-Candelas, F., M. A. Bracho, and A. Moya. 2003. Molecular epidemiology and forensic genetics: application to a hepatitis C virus transmission event at a hemodialysis unit. *J. Infect. Dis.* **187**:352–358.
10. González-Candelas, F., and A. Moya. 2005. Time and rate of evolution are the key to establish transmission cases. *AIDS* **19**:1552–1553.
11. Harpaz, R., L. Von Seidlein, F. M. Averhoff, M. P. Tormey, S. D. Sinha, K. Kotsopoulou, S. B. Lambert, B. H. Robertson, J. D. Cherry, and C. N. Shapiro. 1996. Transmission of hepatitis B virus to multiple patients from a surgeon without evidence of inadequate infection control. *N. Engl. J. Med.* **334**:549–554.
12. Kidd-Ljunggren, K., E. Broman, H. Ekvall, and O. Gustavsson. 1999. Nosocomial transmission of hepatitis B virus infection through multiple-dose vials. *J. Hosp. Infect.* **43**:57–62.
13. Komatsu, H., A. Inui, Y. Morinishi, T. Sogo, and T. Fujisawa. 2001. Sequence analysis of hepatitis B virus genomes from an infant with acute severe hepatitis and a hepatitis B e antigen-positive carrier mother. *J. Med. Virol.* **65**:457–462.
14. Kumar, S., K. Tamura, I. B. Jakobsen, and M. Nei. 2000. MEGA: molecular evolutionary genetics analysis, version 2.0b3. [Online.] <http://www.megasoftware.net>.
15. Lai, C. L., V. Ratzu, M. F. Yuen, and T. Poynard. 2003. Viral hepatitis B. *Lancet* **362**:2089–2094.
16. Liang, T. J., K. Hasegawa, N. Rimon, J. R. Wands, and E. Ben Porath. 1991. A hepatitis B virus mutant associated with an epidemic of fulminant hepatitis. *N. Engl. J. Med.* **324**:1705–1709.
17. Löfgren, B., E. Nordenfelt, T. Lindholm, and B. Lindergård. 1982. A ten-year follow-up of a hepatitis B epidemic in a dialysis unit. *Scand. J. Infect. Dis.* **14**:165–169.
18. Mizokami, M., E. Orito, K. Ohba, K. Ikeo, J. Lau, and T. Gojobori. 1997. Constrained evolution with respect to gene overlap of hepatitis B virus. *J. Mol. Evol.* **44**:83–90.
19. Nei, M., and T. Gojobori. 1986. Simple methods for estimating the numbers of synonymous and nonsynonymous nucleotide substitutions. *Mol. Biol. Evol.* **3**:418–423.
20. Nordner, H., A. M. Courouce, and L. O. Magnius. 1994. Complete genomes, phylogenetic relatedness, and structural proteins of six strains of the hepatitis B virus, four of which represent two new genotypes. *Virology* **198**:489–503.
21. Okamoto, H., M. Imai, M. Shimozaki, Y. Hoshi, H. Iizuka, T. Gotanda, F. Tsuda, Y. Miyakawa, and M. Mayumi. 1986. Nucleotide sequence of a cloned hepatitis B virus genome, subtype ayr: comparison with genomes of the other three subtypes. *J. Gen. Virol.* **67**:2305–2314.
22. Omata, M., T. Ehata, O. Yokosuka, K. Hosoda, and M. Ohto. 1991. Mutations in the precore region of hepatitis B virus DNA in patients with fulminant and severe hepatitis. *N. Engl. J. Med.* **324**:1699–1704.
23. Petzold, D. R., B. Tautz, F. Wolf, and J. Drescher. 1999. Infection chains and evolution rates of hepatitis B virus in cardiac transplant recipients infected nosocomially. *J. Med. Virol.* **58**:1–10.
24. Posada, D., and K. A. Crandall. 2001. Selecting the best-fit model of nucleotide substitution. *Syst. Biol.* **50**:580–601.
25. Rokuhara, A., E. Tanaka, S. Yagi, M. Mizokami, Y. Hashikura, S. Kawasaki, and K. Kiyosawa. 2000. De novo infection of hepatitis B virus in patients with orthotopic liver transplantation: analysis by determining complete sequence of the genome. *J. Med. Virol.* **62**:471–478.
26. Rozas, J., J. C. Sanchez-DelBarrio, X. Messegue, and R. Rozas. 2003. DnaSP, DNA polymorphism analyses by the coalescent and other methods. *Bioinformatics* **19**:2496–2497.
27. Saitou, N., and M. Nei. 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol. Biol. Evol.* **4**:406–425.
28. Sato, S., K. Suzuki, Y. Akahane, K. Akamatsu, K. Akiyama, K. Yunomura, F. Tsuda, T. Tanaka, H. Okamoto, Y. Miyakawa, and M. Mayumi. 1995. Hepatitis B virus strains with mutations in the core promoter in patients with fulminant hepatitis. *Ann. Intern. Med.* **122**:241–248.
29. Shimodaira, H., and M. Hasegawa. 1999. Multiple comparisons of log-likelihoods with applications to phylogenetic inference. *Mol. Biol. Evol.* **16**:1114–1116.

30. **Staden, R., K. Beal, and J. Bonfield.** 1999. The Staden package, 1998, p. 115–130. *In* S. Misener and S. Krawetz (ed.), *Computer methods in molecular biology*. Humana Press, Inc., Totowa, N.J.
31. **Sterneck, M., S. Gunther, T. Santantonio, L. Fischer, C. E. Broelsch, H. Greten, and H. Will.** 1996. Hepatitis B virus genomes of patients with fulminant hepatitis do not share a specific mutation. *Hepatology* **24**:300–306.
32. **Sterneck, M., T. Kalinina, S. Otto, S. Gunther, L. Fischer, M. Burdelski, H. Greten, C. E. Broelsch, and H. Will.** 1998. Neonatal fulminant hepatitis B: structural and functional analysis of complete hepatitis B virus genomes from mother and infant. *J. Infect. Dis.* **177**:1378–1381.
33. **Stuyver, L. J., S. A. Locarnini, A. Lok, D. D. Richman, W. F. Carman, J. L. Dienstag, and R. F. Schinazi.** 2001. Nomenclature for antiviral-resistant human hepatitis B virus mutations in the polymerase region. *Hepatology* **33**:751–757.
34. **Stuyver, L., S. De Gendt, C. Van Geyt, F. Zoulim, M. Fried, R. F. Schinazi, and R. Rossau.** 2000. A new genotype of hepatitis B virus: complete genome and phylogenetic relatedness. *J. Gen. Virol.* **81**:67–74.
35. **Swofford, D. L.** 2002. PAUP\*: phylogenetic analysis using parsimony (\* and other methods), version 4.0beta. Sinauer Associates, Sunderland, Mass.
36. **Thompson, J. D., D. G. Higgins, and T. J. Gibson.** 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, positions-specific gap penalties and weight matrix choice. *Nucleic Acids Res.* **22**:4673–4680.
37. **Valenzuela, P., M. Quiroga, J. Zaldivar, P. Gray, and W. J. Rutter.** 1980. The nucleotide sequence of the hepatitis B viral genome and the identification of the major viral genes. Academic Press, New York, N.Y.
38. **Zampino, R., S. Lobello, M. Chiaramonte, C. Venturi-Pasini, U. Dumpis, M. Thursz, and P. Karayiannis.** 2002. Intra-familial transmission of hepatitis B virus in Italy: phylogenetic sequence analysis and amino-acid variation of the core gene. *J. Hepatol.* **36**:248–253.