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Evidence for recombination in natural populations of dengue virus type 1 based on the analysis of complete genome sequences

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Recombination events are known to occur in non-segmented RNA viruses like polioviruses or alphaviruses. Analysis of the subgenomic sequences of dengue virus type 1 (DENV-1) structural genes has recently allowed the identification of possible recombination breakpoints. Because DENV is a major human pathogen, this discovery might have important implications for virus pathogenicity, vaccine safety and efficiency, or diagnosis and, therefore, requires clear confirmation. We report the complete sequence determination of one Asian and two African strains of DENV-1 isolated from human patients. Rigorous sequence analysis provided strong evidence for the occurrence of intragenomic recombination events between DENV-1 strains belonging to different lineages. Singapore S275/90 strain appears to be the evolutionary product of a recombination event between viruses belonging to two distinct lineages: one lineage includes an African strain isolated in Abidjan (Ivory Coast) and the other includes isolates from Djibouti and Cambodia. The 'Recombination Detection Program', bootscanning and analysis of diversity plots provided congruent results concerning the existence of a two-switch recombination event and the localization of recombination breakpoints. Thus, the 5′ and 3′ genomic ends of the Singapore S275/90 strain were inherited from a Djibouti/Cambodia lineage ancestor and an internal fragment located in the envelope/NS1 region originated from an Abidjan lineage ancestor.

Introduction

Dengue fever (DF) is a mosquito-borne disease that is widespread in tropical and subtropical areas and now threatens one-third of the total human population. About 60–100 million people become infected with dengue virus (DENV) each year and the outcome is fatal for 20000–30000 of these cases. Death may be due to one of the severe forms of DF, principally dengue haemorrhagic fever (DHF) and dengue shock syndrome (DSS), which occur increasingly in several parts of the world (Gubler & Clark, 1995). Several factors, including uncontrolled urbanization and mobility of human or vector populations, are suspected to be responsible for the presence of DF in regions where it has never been observed previously. The aetiological agent of DF is a flavivirus, a member of the family *Flaviviridae*, with a single-stranded, non-segmented, positive-sense RNA approximately 11 kb in length. Four genetically distinct serotypes of DENV (1–4) exist and now co-circulate in many regions of the world.

Recombination events are known to occur in non-segmented RNA viruses like polioviruses (Cooper *et al.*, 1974) or alphaviruses (Hahn *et al.*, 1988). These events might have major implications for virus evolution, pathogenicity, vaccine safety and efficiency, or diagnosis. Consequently, the identification of possible recombination events concerning a major human pathogen such as DENV-1 (Holmes *et al.*, 1999; Worobey *et al.*, 1999) is an important discovery that clearly

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requires further investigation and confirmation. In the present article, we report the complete sequence determination of one Asian and two African strains of DENV-1 isolated from human patients. This gave us the opportunity to undertake a rigorous sequence analysis and provided strong evidence for the existence of intragenomic recombination events occurring between the different lineages of DENV-1.

Methods

Virus isolation and propagation. All work with infectious virus was carried out in a biosafety level 3 laboratory. Two African strains, Dl/H/IMTSSA-DJIB/98/606 (referred to as 'Djibouti') and Dl/H/IMTSSA-ABID/99/1056 (referred to as 'Abidjan'), were studied. The Djibouti strain was isolated in 1998 from the serum of a French soldier with a febrile illness during a DF outbreak in the region of Djibouti. The Abidjan strain was isolated from the peripheral blood leukocytes of another French soldier who had been infected in 1998 in the city of Abidjan (Ivory Coast) (Durand *et al.*, 2000). A third strain, Dl/H/IMTSSA-CAMB/98/658 (referred to as 'Cambodia'), was isolated in 1998 from the white blood cell fraction of a French tourist returning from Cambodia who was hospitalized for a severe febrile syndrome.

A 200 µl sample of either a serum or a leukocyte fraction was co-incubated with C6/36 cells (*Aedes albopictus*) grown at 28 °C in Leibowitz L15 medium supplemented with 2% tryptose phosphate broth, 30 U/ml heparin and 5% foetal calf serum (added 1 h after culture). Supernatants were collected on day 6 post-infection after a single passage on C6/36 cells.

Immunofluorescence analysis using serotype-specific monoclonal antibodies (courtesy of Nick Karabatsos, Centers for Disease Control, Fort Collins, CO, USA) was used for the assignment of the three DENV strains to serotype 1.

Preparation of viral RNAs and cDNAs. First passage supernatants of infected cell cultures were used for viral RNA extraction, reverse transcription, PCR amplification and genomic sequencing, according to procedures described previously (Tolou *et al.*, 2000). Briefly, RNA was extracted using the High Pure Viral RNA kit (Roche) and reverse transcribed using Superscript II reverse transcriptase (Gibco/BRL). Specific primers designed from the complete nucleotide sequence of the reference DENV-1 Singapore S275/90 strain (Fu *et al.*, 1992) were used for PCR amplification. A list of the primer sequences is available on request to the corresponding author.

For each strain, 13 to 14 overlapping PCR fragments spanning the whole genome were obtained using AmpliTaq DNA Gold (Perkin Elmer) and directly sequenced using the DNA Sequencing kit (PE Biosystems) and an automatic sequence analyser (ABI PRISM 310, Perkin Elmer). All positions on the genomes were verified in three to five independent sequencing reactions.

Genetic analysis. The nucleotide sequences of strains Djibouti, Abidjan and Cambodia were compared with previously determined complete sequences of DENV-1 [Singapore S275/90 strain (M87512) and Western Pacific Nauru island (U88535)], DENV-2 [Jamaica/N.1409 strain (M20558)], DENV-3 [H87 strain (M93130)] and DENV-4 (Ml4931). Alignments were generated using the CLUSTAL W 1.7 program with default parameters (Thompson *et al.*, 1994). Nucleotide positions were numbered by reference to the sequence of the Nauru island strain and nucleotide and amino acid pairwise distances were calculated using the MEGA program software (Kumar *et al.*, 1993).

Systematic screening for the presence of recombination patterns was achieved using nucleotide alignments and the UPGMA-based Recombination Detection Program (RDP) (kindly provided by Darren Martin, University of Cape Town, South Africa). The RDP applies a pairwise-distance scanning approach combined with an UPGMA-based reconstruction algorithm (Martin & Rybicky, 2000); the probability that the nucleotide arrangement in a recombinant region may have occurred by chance is tested using a binomial distribution [adapted from Rice (1995); Martin & Rybicky, 2000]. Sliding windows ranging from 5 to 100 nt were tested.

Recombination events detected by this program were further investigated by manual bootscanning (Sibold *et al.*, 1999; Santti *et al.*, 1999) using the Jukes–Cantor algorithm for distance calculation and the neighbour-joining method for tree building (implemented in the MEGA package). Bootscanning initially produces a phylogenetic tree from a window of aligned data at one end of the sequence alignment. This window is then incrementally shifted along the alignment and a phylogenetic analysis is carried out on each new window of the sequence alignment. In the present study, sliding windows of 500 nt with a 100–250 nt overlap were used to pinpoint the region where recombinations occurred. Each resulting tree was submitted to 500 replicates of bootstrap replication (Felsenstein, 1985) to test the robustness of the resulting branching patterns. The bootstrap values supporting the grouping of a recombinant strain with either a minor or a major parent were used to draw comparative curves. Phylogenetic trees in the recombinant and non-recombinant regions where constructed using the same algorithms as above.

The pairwise distance between a putative recombinant and each possible parental strain was calculated using the MEGA package with sliding windows. The first window was 1000 nt in length and corresponded to nt 1–1000 of the nucleotide alignment of a putative recombinant and its possible parental strains. Pairwise distances between the recombinant strain and each parent were calculated independently. The window was then incrementally shifted along the alignment in 1 nt steps (the overlap with the preceding window was therefore 999 nt). Pairwise distances were calculated for each new window until the end of the alignment. These distances were used to draw curves (diversity plots) illustrating the genetic relationship of the recombinant strain with each parental strain along the complete genome.

The sequence identity between a recombinant strain and each of its parents was calculated both inside and outside the recombinant region using the Chi-square test to determine if the sequence identity to a given parent was significantly different.

Results

Sequences

The complete sequences of the Abidjan, Djibouti and Cambodia strains were determined (except for the first and last 14 nt of the genomes) and deposited in the GenBank database under accession numbers AF298807, AF298808 and AF309641, respectively.

The three complete genomes are 10 721 nt in length and include a 10 176 nt open reading frame encoding a 3392 aa viral polyprotein in which the putative cleavage sites are mostly conserved compared with the prototype strain.

Genetic distances

Pairwise distances between DENV strains were calculated and a matrix of the nucleotide and amino acid distances is

Table 1. Nucleotide and amino acid pairwise distances between DENV-1 (Cambodia, Djibouti and Abidjan strains), -2, -3, and -4

Nucleotide pairwise distances (%) are shown above the diagonal and amino acid pairwise distances (%) are shown below the diagonal.

Serotype	Virus strain	DENV-1 strains					DENV-3	DENV-2	DENV-4
		Singapore	Cambodia	Djibouti	Nauru island	Abidjan			
1	Singapore	–	0·036	0·048	0·063	0·071	0·272	0·314	0·331
1	Cambodia	0·021	–	0·041	0·066	0·082	0·271	0·313	0·330
1	Djibouti	0·023	0·017	–	0·067	0·083	0·270	0·315	0·329
1	Nauru island	0·023	0·022	0·023	–	0·077	0·274	0·313	0·332
1	Abidjan	0·024	0·027	0·028	0·025	–	0·275	0·310	0·331
3	DENV-3	0·223	0·220	0·220	0·221	0·222	–	0.312	0·327
2	DENV-2	0·281	0·278	0·281	0·278	0·277	0·281	–	0·326
4	DENV-4	0·323	0·319	0·318	0·322	0·319	0·308	0·314	–

presented in Table 1. It is interesting to notice that the African Djibouti strain is more closely related to the Asian Cambodia and Singapore strains than to the other African Abidjan strain. The latter strain appears to be the most divergent DENV-1 isolate that has been fully described to date at both the nucleotide and the amino acid level.

Analysis of recombinations

Using standard analysis parameters (homology box, 30 nt; sliding window, 50 nt), data from the RDP suggested that the Singapore strain is the evolutionary product of a recombination event between viruses belonging respectively to the Abidjan and the Djibouti/Cambodia lineages. The 5′ and 3′ ends of the Singapore strain genome were inherited from a Djibouti/Cambodia lineage ancestor and an internal fragment located in the envelope/NS1 region originated from an Abidjan lineage ancestor.

Considering the Abidjan and Cambodia strains as the respective minor and major parents of the Singapore strain, the RDP located the recombination between positions 1147 and 2716, with a highly significant P value of $1{\cdot}2\times10^{-12}$. Considering the Abidjan and Djibouti strains as the respective minor and major parents of the Singapore strain, the RDP located the recombination between positions 1024 and 2683, with a slightly lower P value of 6×10^{-8}.

Manual bootscanning using the Jukes–Cantor and neighbour-joining methods confirmed the recombinant origin of the Singapore strain (Fig. 1*a*). Considering the Cambodia strain as the major parent, recombination was localized between positions 1295 ($\pm$50 nt) and 2592 ($\pm$50 nt) [Fig. 1*a*, (i)]. Considering the Djibouti strain as the major parent, recombination was localized between positions 1195 ($\pm$50 nt) and 2592 ($\pm$50 nt) [Fig. 1*a*, (ii)].

Diversity plots are presented in Fig. 1(*b*). Considering the Cambodia strain as the major parent, recombination was localized between positions 1470 and 2740 [Fig. 1*b*, (i)]. Considering the Djibouti strain as the major parent, recombination was localized between positions 1100 and 2700 [Fig. 1*b*, (ii)].

As expected, the variability inside and outside the recombinant region was not significantly different when the Cambodia and Abidjan strains ($P=0{\cdot}36$) or the Cambodia and Djibouti strains ($P=0{\cdot}77$) were compared. In contrast, the variability inside the recombinant region was significantly higher than that observed outside the recombinant region when the Singapore and Cambodia strains ($P<10^{-7}$) or the Singapore and Djibouti strains ($P<10^{-4}$) were compared. Similarly, the variability inside the recombinant region was significantly lower ($P<5\times10^{-3}$) than that observed outside the recombinant region when the Singapore and Abidjan strains were compared.

In the recombinant region (nt 1295–2592), the grouping of the Singapore strain with the Abidjan strain was supported by a bootstrap value of 100% [Fig. 1*c*, (ii)]. For the 5′ and 3′ flanking fragments, clustering of the Singapore strain within the Cambodia/Djibouti lineage was supported by bootstrap values of 99% and 100% [Fig 1*c*, (i) and (iii)].

Localization of the 5′ breakpoints identified by the different methods is presented in Fig. 2 together with those reported by Worobey *et al.* (1999). As expected, the breakpoints detected in our study are located at the beginning of a region encompassing multiple positions conserved only for the Abidjan and Singapore strains.

Discussion

The identification of natural recombination events between virus strains is important for our understanding of virus evolution. However, the detection of virus sequence recombinations has several prerequisites. In particular, (i) the recombinant region must be long enough to allow relevant

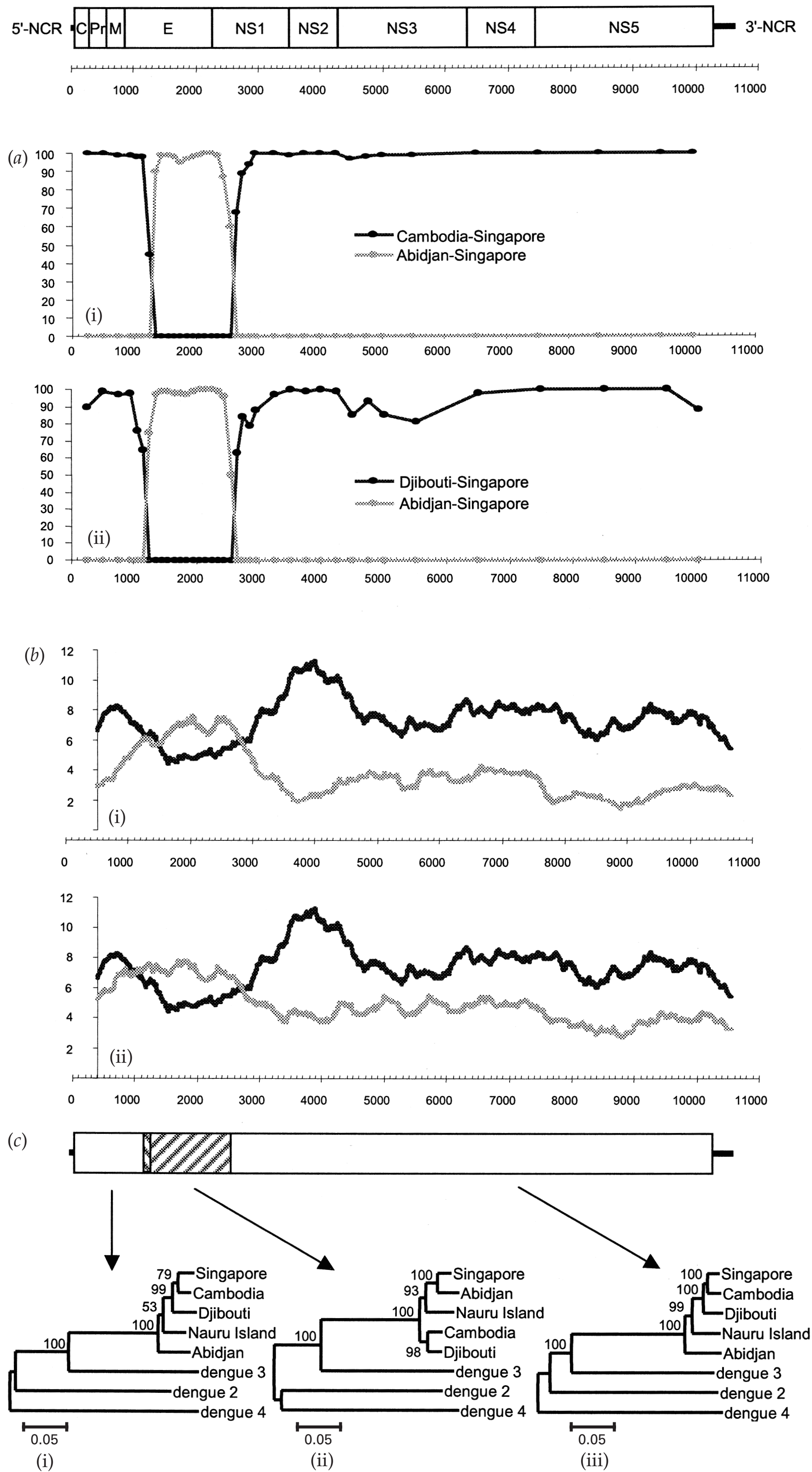

Fig. 1. Analysis of recombination events occurring in the viral genome (top) using the results of manual bootscanning obtained by the Jukes–Cantor and neighbour-joining methods. The *x*-axes represent the nucleotide positions along the viral genome

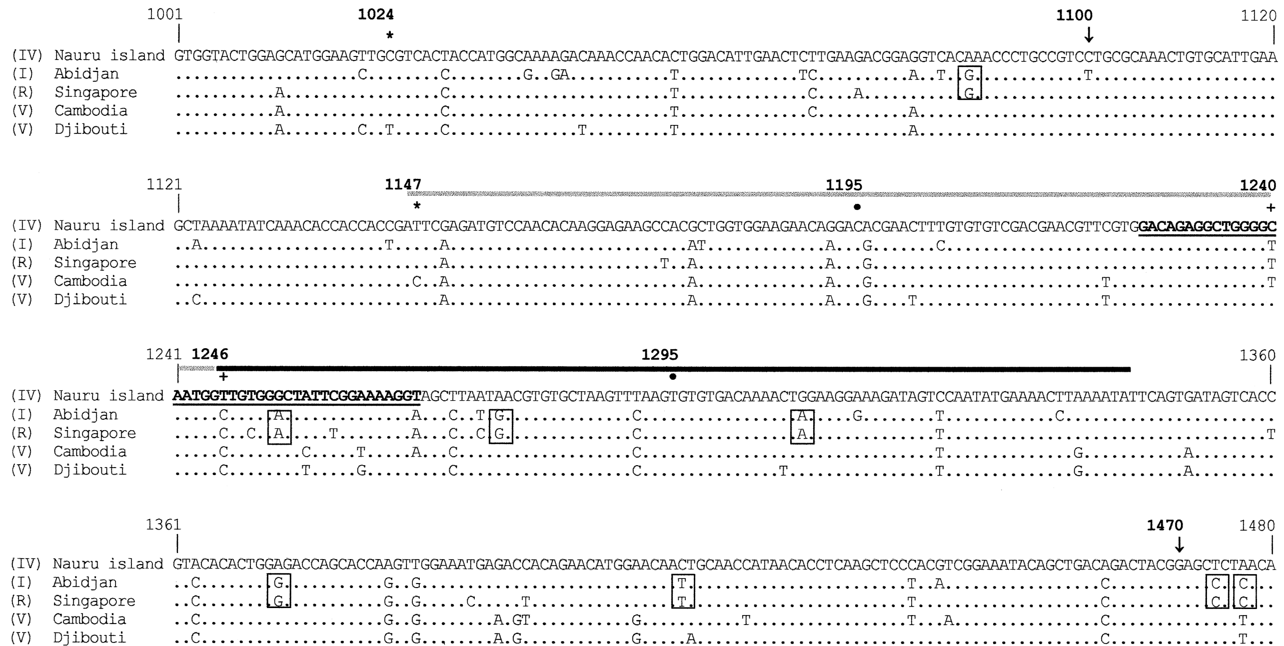

Fig. 2. Nucleotide alignment of the region corresponding to the 5′ breakpoints. Nucleotide positions were numbered by reference to the sequence of the Western Pacific Nauru island strain. The region corresponding to the fusion peptide is bold and underlined. The assignment of the strains to genotypic groups, according to Rico-Hesse (1990), is indicated in parentheses for each strain (R represents a recombinant strain). Breakpoints (*) detected by RDP are indicated. The middle of a 100 nt region including a breakpoint (●), as detected by manual bootscanning, is shown. The Djibouti (black line) or Cambodia (grey line) strains are considered as the major parental strain. The regions of a possible breakpoint detected by diversity plots (↓) and the breakpoints detected by Worobey *et al.* (1999) corresponding to recombinations between groups I and IV strains (+) are also shown. Positions conserved only for the Singapore and Abidjan strains are boxed.

phylogenetic analysis; (ii) the region must be divergent enough in both the minor and the major parents to allow unambiguous identification; and (iii) the recombinant pattern must be maintained during post-recombination evolution. In the case of DENV-1, the identification of recombinations has been hampered by the lack of full-length genomic sequence data. However, analysis of available subgenomic sequences allowed Holmes *et al*. (1999) and Worobey *et al*. (1999) to identify recombinant breakpoints within the structural genes of several DENV-1 isolates. As highlighted by these authors, this finding needed to be confirmed by the analysis of new full-length genomic sequences. We present a study based on the complete genome sequencing and analysis of three new DENV-1 strains isolated from humans.

Three major phylogenetic groups were identified after phylogenetic analysis of five complete DENV-1 sequences. The first group is represented by the African Abidjan isolate, the second by the Western Pacific Nauru island isolate and the third group by the African Djibouti isolate and the Asian Cambodia and Singapore isolates. These phylogenetic groups correspond to the genotypic groups I, IV and V, respectively (Rico-Hesse *et al*., 1990; see below). It is important to note that the African Djibouti isolate is genetically more closely related to the Asian isolates than to the African Abidjan isolate. This supports the hypothesis that DENV strains can be disseminated and maintained far from their original ecological niche (Deubel *et al*., 1993). The simultaneous existence of both 'indigenous' and 'exogenous' DENV strains in the same biotope might constitute the basis for the emergence of recombinant strains.

Genetic analysis of complete DENV-1 sequences showed that the Singapore strain possesses a chimeric sequence. The Singapore strain envelope/NS1 region was inherited from an ancestor belonging to the Abidjan lineage and the rest of the sequence originated from an ancestor belonging to the Cambodia/Djibouti lineage. Further evidence for such a recombination event is consistently found by different

shown above. The *y*-axes represent either the bootstrap values (*a*) or the pairwise distances (*b*) supporting the grouping of the Singapore strain with either minor (grey curve) or major (black curve) parental strains. The closest possible major parental strains are the Cambodia (i) and Djibouti (ii) strains. In both cases, the closest possible minor parental strain is the Abidjan strain. (*c*) Phylogenetic trees in the recombinant and non-recombinant regions using the Jukes–Cantor and neighbour-joining methods. Phylogenetic analyses were performed upstream (i), downstream (iii) and within (ii) the recombinant region. Bootstrap support values are indicated at each fork.

methods. The screening of putative recombination events between DENV-1 isolates was performed with an RDP specifically designed for screening purposes by Martin & Rybicki (2000) and used previously for the identification of recombination in members of the *Circoviridae* family (Biagini *et al*., 2001). RDP detection of a highly probable recombination between the Abidjan and Cambodia/Djibouti lineages was confirmed by different phylogenetic methods based on alternative algorithms for distance calculation (Jukes–Cantor) and for phylogenetic reconstruction (neighbour-joining). Importantly, phylogenetic analyses provided evidence for the existence of two recombination breakpoints, corresponding to the substitution of the 3′ end of the envelope gene and 5′ end of the NS1 gene of a Cambodia/Djibouti related isolate by the homologous sequence from an Abidjan-related isolate. Localization of these breakpoints was consistently confirmed by diversity plot analysis. All methods used suggested that the 5′ breakpoint is located at position 1195 or 1295 depending on whether the Djibouti strain or the Cambodia strain was considered as the major parent of the Singapore strain. In both cases, the 3′ breakpoint is consistently located at position 2592. However, the precise localization of breakpoints is hampered by (i) the low genetic diversity between the closest possible parental strains (the true parental strains being unavailable) and (ii) the natural post-recombination evolution that occurred in both parental and recombinant strains. This situation differs intrinsically from the situation observed when a recombination event occurs between a cellular mRNA and a viral RNA, as reported for pestiviruses (Meyers & Thiel, 1996). In that case, the sequence of cellular mRNA is not subject to significant evolution and low homology between the viral and cellular RNA sequences permits the exact identification of the nucleotide position of recombination breakpoints.

In a previous study that was based on the phylogenetic analysis of structural genes using the maximum-likelihood method and Monte Carlo simulation, Worobey *et al*. (1999) reported several putative recombination events between DENV-1 isolates and, in particular, suggested the existence of a recombination breakpoint at position 1250 for the Brazil BR/90 and French Guyana FGA/89 strains. The closest possible parental strains belonged in both cases to genotypic groups I and IV, according to the Rico-Hesse classification (Rico-Hesse, 1990). It is interesting to note that this localization is very close to the 5′ breakpoint identified in our study, despite resulting from the analysis of strains belonging to different genotypic groups by totally different methods. Together, these data suggest that this part of the DENV-1 envelope gene is a possible 'hot spot' for recombination. As shown in Fig. 2, irrespective of the method used, the 5′ breakpoints are consistently located within or nearby the sequence encoding the putative fusion peptide of flaviviruses (envelope protein aa 98–111) (Roehrig *et al*., 1989). However, whether this coincidence has biological signification is uncertain. The mechanisms leading to the localization of breakpoints in that particular region are probably more dependent on the characteristics of the virus replication machinery, the nucleotide composition and the RNA structure of that part of the genome than on the selection of a phenotypic character. Furthermore, although the sequences corresponding to the fusion peptide of the Singapore, Abidjan and Cambodia strains differ at the nucleotide level, the encoded peptide sequence is conserved and, therefore, no selective advantage in the fusion process can result from its substitution.

In the study by Worobey *et al*. (1999), the lack of available non-structural sequences did not allow the identification and localization of a 3′ breakpoint. Our results suggest that this 3′ breakpoint does exist and is located in the 5′ end of the NS1 region.

The prerequisite for recombination between two different viral genomes is the simultaneous infection of a single cell by two different virus strains. The most probable mechanism of recombination is the so-called 'copy-choice' mechanism (Cooper *et al*., 1974) in which recombination results from a template switch of the viral RNA polymerase during genome replication. To date, virtually all studies of recombination in RNA viruses have supported this model. The existence of two distinct breakpoints implies two template switches, a situation that is reminiscent of the recombination between cellular mRNAs and the genomic RNA of pestiviruses (family *Flaviviridae*). The different aspects of recombination in pestiviruses have been extensively investigated by H.-J. Thiel and collaborators and summarized in the aphorism 'Start with one, leave for a second, return to the first: a mechanism resembling a well-known love story' (for a review see Meyers & Thiel, 1996). The polymerase copies the first part of the major parent genome and then leaves this template, reinitiates polymerization at a second template (corresponding to the minor parent) and then finally returns to the major parent genome template. The precise mechanisms of the template switches are unknown, but it must be noted that, in the present case, recombination led to the exact replacement of a major parent sequence by the homologous sequence from the minor parent. No additional insertion, duplication or deletion was noted and the genome length is identical both in the recombinant strain and in each of the parental strains.

Recombination can occur during synthesis of the negative- or positive-strand of the viral genome. However, in the case of viruses with a single-stranded RNA of positive-polarity, the high concentration of positive-stranded RNA (which represents the acceptor template) renders recombination more probable during negative-strand synthesis. In particular, this is the case in polioviruses (Jarvis & Kirkegaard, 1992) and pestiviruses (Meyers & Thiel, 1996).

The epidemiological and clinical implications of recombinations among DENV-1 strains are unknown. The fact that the Singapore strain was isolated from a human case of DF indicates that recombinant strains can be pathogenic and raises the question of the possible influence of recombination events

on virus virulence. In this regard, recombination could be of great relevance for the use of attenuated multivalent DENV vaccines in endemic regions.

The identification of recombinant strains might also be important for genetic classification of DENV strains. Rico-Hesse (1990) and collaborators proposed a classification system that is based on the analysis of genetic distances between partial nucleotide sequences (Western Pacific Nauru island strain nt 2282–2521). This classification was based on the hypothesis that intramolecular recombination does not occur in this region of the DENV genome. Unfortunately, the part of the genome used for classification is located within the recombinant region that is described in this study. According to the classification of Rico-Hesse (1990) (genetic distance within a given genotypic group $< 6\%$), the Djibouti and Cambodia strains are assigned to group V and the Singapore and Abidjan strains are assigned to group I. The assignment of the Singapore strain to group I is consistent with the fact that the envelope/NS1 region of this strain was inherited from the Abidjan lineage, but does not take into account the fact that the rest of the genome is more closely related to those strains belonging to group V.

It is difficult to determine the geographical region in which recombination occurred. However, it must be noted that (i) the recombinant strain was isolated in south-east Asia and that its more closely related strain is the south-east Asian Cambodia isolate and (ii) the topology of the phylogenetic tree suggests that the Djibouti strain is the African progenitor of the Cambodia strain. These data are consistent with the hypothesis that recombination occurred in south-east Asia between a virus closely related to the Cambodia strain and another strain derived from the Abidjan African lineage. This supports the fact that at least some lineages of DENV-1 are of African origin, implying that DENV has been present in Africa for a long time. Despite the fact that the four serotypes of DENV have been detected in Africa, DF is not frequently diagnosed in Africans and clinical symptoms are generally mild. The occurrence of either DHF or DSS has rarely been documented in Africa and outbreaks are generally reported in expatriate populations, while native populations appear to be less affected. Although low reporting of DF in native humans might be a bias due to insufficient interest in this diagnosis, a long history of mutual adaptation of viruses and humans is also possible and compatible with the hypothesis of the African origin of DENV.

The recognition of recombination as a mechanism driving the evolution of DENV-1 emphasizes the importance of collecting full-length genomic data for strains from various geographical origins. This will allow the improvement of phylogenetic analysis models and further the understanding of the natural history that leads to the genetic diversity of DENV-1 populations (an important point to investigate the epidemiology of DF and the pathogenesis of virus strains). Finally, very little is known about the precise molecular mechanisms of recombination between DENV isolates. A better understanding of this point requires the development of experimental models for co-infection and the generation of recombinant strains.

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