Proc. Natl. Acad. Sci. USA
Vol. 84, pp. 2019–2023, April 1987
Medical Sciences

Comparison of the virulent Asibi strain of yellow fever virus with the 17D vaccine strain derived from it

CHANG S. HAHN*, JOEL M. DALRYMPLE†, JAMES H. STRAUSS*, AND CHARLES M. RICE*‡

*Division of Biology, California Institute of Technology, Pasadena, CA 91125; and †Virology Division, U.S. Army Medical Research Institute of Infectious Diseases, Fort Detrick, Frederick, MD 21701-5011

Communicated by Ray D. Owen, November 17, 1986 (received for review October 30, 1986)

ABSTRACT **We have sequenced the virulent Asibi strain of yellow fever virus and compared this sequence to that of the 17D vaccine strain, which was derived from it. These two strains of viruses differ by more than 240 passages. We found that the two RNAs, 10,862 nucleotides long, differ at 68 nucleotide positions; these changes result in 32 amino acid differences. Overall, this corresponds to 0.63% nucleotide sequence divergence, and the changes are scattered throughout the genome. The overall divergence at the level of amino acid substitution is 0.94%, but these changes are not randomly distributed among the virus proteins. The capsid protein is unchanged, while proteins NS1, NS3, and NS5 contain 0.5% amino acid substitutions, and proteins ns4a and ns4b average 0.8% substitutions. In contrast, proteins ns2a and ns2b have 3.0 and 2.3% amino acid divergence, respectively. The envelope protein also has a relatively high rate of amino acid change of 2.4% (a total of 12 amino acid substitutions). The large number of changes in ns2a and ns2b, which are largely conservative in nature, may result from lowered selective pressure against alteration in this region; among flaviviruses, these polypeptides are much less highly conserved than NS1, NS3, and NS5. However, many of the amino acid substitutions in the E protein are not conservative. It seems likely that at least some of the difference in virulence between the two strains of yellow fever virus results from changes in the envelope protein that affect virus binding to host receptors. Such differences in receptor binding could result in the reduced neurotropism and vicerotropism exhibited by the vaccine strain.**

Yellow fever virus belongs to the *Flavivirus* genus of the family Flaviviridae, a group of some 70 closely related viruses many of which cause serious human illness (1). Yellow fever is arthropod-borne, transmitted by mosquitos of the genera *Aedes* and *Haemagogus*. Its natural vertebrate host range is limited to primates in which it is viscerotropic and neurotropic. In man, the virus causes a serious, often fatal, illness marked by liver and kidney involvement and hemorrhage. For several hundred years the virus caused epidemics in the Americas, Europe, and Africa that led to widespread human suffering. With the control of the urban vector of yellow fever *Aedes aegypti* beginning in the early 1900s, epidemic urban yellow fever disappeared. However, the virus remains present in an enzootic cycle in the forests of South America and Africa and causes periodic outbreaks in neighboring human populations.

Reed (2) first proved that yellow fever is transmitted by mosquitos and, shortly thereafter, that the disease agent was filterable. Because there was no recognized, susceptible laboratory host, many years elapsed before the virus responsible was isolated by the Rockefeller Foundation's West Africa yellow fever commission. In 1927, these workers succeeded in isolating a virus from the blood of a young Ghanian named Asibi by monkey/monkey passage (3). This Asibi strain of yellow fever causes an invariably fatal disease when inoculated into rhesus monkeys.

Theiler (4) developed a live, attenuated vaccine strain, which he referred to as 17D, from the Asibi strain. Starting with the Asibi strain that had been passaged 53 times in monkeys, with intermittent passages in *A. aegypti*, the virus was propagated serially in cultures of embryonic mouse tissue (18 passages), minced whole chicken embryo (50 passages), and finally minced chicken embryos without nervous tissue (152 passages). Between the 89th and 114th *in vitro* passage (from the start of the experiment, that is including the passages in embryonic mouse tissue and whole chicken embryos) a marked change in virulence of the virus occurred. The reason for the change in virulence is unknown and attempts to repeat these experiments by virus passage have failed to develop additional avirulent strains. The 17D strain has been widely used as a human vaccine, being safe and highly effective. It causes a mild, generalized infection in humans (or other primates) with involvement of lymphoid tissue and minimal quantities of virus circulating in the blood, and both the viscerotropism and neurotropism of the parental Asibi virus are markedly reduced.

We have sequenced the genome of a plaque-purified virus derived from the 17D-204 vaccine strain of yellow fever (5). The 17D-204 strain, supplied by the American Type Culture Collection, had been passed an additional 14 times in chicken embryo tissue culture, for a total of 234 *in vitro* passages. To obtain RNA for cloning and sequencing, the virus was passed twice in chicken embryo fibroblasts in our laboratory, plaque-purified in Vero cells, and passed twice in BHK cells; virus for RNA preparation was then grown in SW-13 cells (5). Thus, the 17D strain sequenced by us had been passed 240 times *in vitro*.

We now report the sequence of virtually the entire genome of the Asibi strain of yellow fever and compare the sequences of the 17D and Asibi strains at both the nucleotide level and the amino acid level. In addition to defining the changes that have occurred during 240 serial passages, this work represents a necessary prelude for studying the biological significance of these changes as related to the different virulence of the two strains.

MATERIALS AND METHODS

Asibi Strain Propagation and RNA Isolation. The Asibi strain of yellow fever virus was obtained from the Yale Arbovirus Research Unit reference collection as viremic monkey serum (supplied by R. Shope). This virus was originally isolated in rhesus monkeys (3) and had undergone ≈45 serial monkey passages consistently producing a fatal illness (6); it is unknown how many of these 45 passages

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

‡Present address: Department of Microbiology and Immunology, Washington University School of Medicine, Box 8093, 660 South Euclid Avenue, St. Louis, MO 63110.

correspond to the monkey passages of the laboratory strain of Asibi used as the starting virus for *in vitro* passages that led to 17D. The virulence of this Asibi strain was demonstrated by inoculation of additional rhesus monkeys, all of which suffered a lethal infection with yellow fever pathology. Virus was isolated in LLC-MK_2 cell culture by direct inoculation of viremic plasma from one of these monkeys, and the infectious cell culture fluids were used to infect cultures of Vero cells. This Vero cell-passaged Asibi strain virus was titered at 7.8×10^7 plaque-forming units/ml and was used as inoculum for all subsequent virus production.

Confluent monolayers of Vero cells in 150-cm^2 flasks were infected at a multiplicity of ≈0.1, and the cultures were incubated at 36°C in Eagle's medium supplemented with 5% (vol/vol) heated fetal bovine serum and antibiotics. Virus-containing culture medium was harvested 4–5 days after infection (depending upon the first evidence of virus induced cytopathology) and concentrated by polyethylene glycol precipitation. Virus was purified by density-gradient centrifugation on potassium tartrate/glycerol gradients followed by rate-zonal centrifugation on linear sucrose gradients, and the RNA was extracted from $NaDodSO_4$-disrupted virions using a phenol/cresol/8-hydroxyquinoline/chloroform mixture (7).

Cloning of Asibi cDNA. A cDNA library from Asibi genomic RNA was constructed as described (5, 8). Ampicillin-resistant colonies from this library were screened by colony hybridization using nick-translated restriction fragments derived from the library of 17D yellow fever clones as probes, as described (9). Colonies with larger inserts (obtained using cDNA size class of 2 kilobases or larger) were screened with a 5′ probe of 2280 base pairs that extends from the 5′ end of 17D yellow fever to the first *Eco*RI site and with a 3′ probe of 2580 base pairs, which extends from nucleotide 8280 in 17D to the 3′ end, or with probes derived from other regions of the 17D genome. To obtain clones containing the extreme 3′ end of the RNA ≈4000 clones from the smaller insert class (derived from Asibi double-stranded cDNA of 0.8–2 kilobases long) were screened with a fragment derived from the extreme 3′ end of 17D. This fragment, 150 nucleotides long, extends from the *Xba* I site at nucleotide 10,708 to the 3′ end of 17D yellow fever. We found three positive clones with this probe, two of which were identical. One of these clones had an unusual structure and probably arose by self-priming; it was used to obtain the Asibi sequence through to the 3′-terminal nucleotide.

Sequence Analysis of Asibi cDNA. Plasmid DNA from selected Asibi clones was sequenced using the chemical method (10, 11) as described (12).

RESULTS

Sequence of Asibi Yellow Fever. The sequencing strategy used to obtain the nucleotide sequence of Asibi yellow fever is diagramed in Fig. 1. The sequence obtained was completely overlapped and was determined for at least two independent clones throughout virtually the entire genome. This allowed the detection of heterogeneity in the cloned cDNA population (either due to heterogeneity in the RNA genomes cloned or due to errors introduced during reverse transcription and subsequent cloning). Since the RNA templates that were used for cloning were not derived from plaque-purified virus, such clonal differences might be expected, and we found six nucleotides that differed between two clones. The sequence obtained totaled 10,848 nucleotides and represents the entire Asibi genomic RNA sequence with the exception of the 5′-terminal 14 nucleotides.

Comparison of Nucleotide Sequences of 17D and Asibi Yellow Fever. All of the nucleotide differences found between Asibi and 17D yellow fever are shown in Fig. 2. Clonal

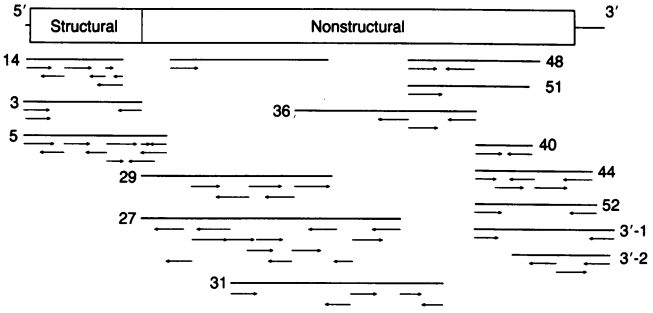

FIG. 1. Sequencing strategy used to obtain Asibi yellow fever sequence from cDNA clones. A representation of the yellow fever genome is shown with the coding region indicated as a box. Below are shown various cDNA clones drawn to scale. The regions of each clone sequenced are indicated. All sequencing was 3′ to 5′ on the cDNA, and the direction of the arrow indicates whether the plus strand (←) or the complementary minus strand (→) was sequenced. Clones 3 and 5 start with the 15th and 17th nucleotide, respectively, from the 5′ end of the yellow fever genome. The 3′-terminal 111 nucleotides were present in only one clone, 3′-1, whose structure is complex and which probably arose by self-priming. In all other regions at least two independent clones were sequenced.

differences found among the Asibi clones are also presented. A total of 68 nucleotide differences were found between the two strains that had been fixed and an additional 6 nucleotides that differed in two different clones of Asibi yellow fever. In each clonal difference detected, one of the nucleotides found at a given position was the same as that found in 17D yellow fever. The sequence of 17D yellow fever that has been reported is a consensus sequence derived from sequencing more than one clone throughout the entire region (5). With a total of 68 changes (0.63% of the genome) there was an average of about 0.27 change fixed per passage. Assuming an effective multiplication of 2^{10} at each passage, this would represent 2×10^{-6} change fixed per nucleotide per generation. Changes presumably occur more frequently than this but do not survive selection pressure during continued passage.

The nucleotide changes are summarized as to transitions and transversions in Fig. 3. Transitions are five times more common than transversions, as expected if most of the changes occur by mispairing during RNA replication.

Nucleotide changes are scattered throughout the genome but occurred more frequently in the envelope protein region (1.0% nucleotide sequence difference), in the ns2a region (1.6% difference), in the ns2b region (1.0% difference), and in the 3′-noncoding region (1.2% difference) (Fig. 4). Changes in the untranslated region may be more frequent because of a relative lack of selective pressure against changes that occur in these regions, although it is of interest that no changes were found in the 5′-untranslated region. Within the coding region, selection against deleterious changes in protein sequence would be expected, and it is notable that approximately half of the nucleotide changes (36 of 68 changes) do not result in a change in a coding assignment. Outside the envelope protein region and the ns2a regions, in which a disproportionate number of the nucleotide changes lead to coding changes, 32 of 47 nucleotide changes do not result in an amino acid substitution.

Changes in Amino Acid Sequence. Amino acid differences between 17D and Asibi yellow fever are given in Fig. 2 and summarized in Fig. 4. Overall, there are 32 amino acid changes (0.94% difference) between the two strains and an additional three clonal differences in the Asibi strain. Thus, there has been an average of 0.12 change fixed per passage, or 4×10^{-6} change per amino acid per generation. As is clear from Fig. 4, these changes are not randomly distributed.

	No	NT	AA
Capsid	304	A G	
	370	C U	
M	854	U C	F L
	883	G A	
	1127	A G	R G
	1140	U C	V A
	1482	U C	V A
	1491	U C	I T
	1572	C A	T K
	1750	U C	
	1819	U C	
Envelope	1870	A G	I M
	1887	U C	F S
	1946	U C	S P
	1965	G A	R K
	2112	G C	R T
	2142	A C	H P
	2219	A G	T A
	2356	U C	
	2687	U C	F L
	2704	G A	
NS1	3274	A G	
	3371	G A	V I
	3613	A G	
	3817	G G,A	
	3860	G A	V M
	3915	U U,A	
ns2a	4007	G A	A T
	4013	U C	F L
	4022	G A	A T
	4054	U C	
	4056	U C	F S
	4289	C A	L I
ns2b	4387	G A	
	4505	C A]	L I
	4507	C U]	
	4612	C U	
	4864	G G,A	
	4873	G U	
	5131	G G,U	M M,I
	5153	G A	V I
NS3	5194	C U	
	5431	U C	
	5473	U C	
	5641	A G	
	6013	U C	
	6023	A G	N D
	6448	U G	
	6529	C U	
ns4a	6758	G A	V I
	6829	C U	
	6876	C U	A V
	7171	G A	M I
ns4b	7571	A C	
	7580	C U	H Y
	7642	C U	
	7701	G A	R Q
	7945	U C	
	8008	C U	
	8629	U C	
NS5	9605	G A	D N
	10075	G G,U	M M,I
	10142	A G	K E
	10243	A G	
	10285	C U	
	10312	G A	
	10316	U U,C	S S,P
	10338	U C	L P
	10367	C U	
3' Non-	10418	C U	
coding	10454	G A	
	10550	C U	
	10800	A G	
	10847	C A	

FIG. 2. Summary of differences between Asibi yellow fever and 17D yellow fever. The changes are grouped by nucleotide number into the various regions of the genome. Nucleotide number (No) is from the 5′ terminus of 17D. To the right is shown the nucleotide (NT) in the 17D genome followed by the nucleotide in the Asibi genome. Where clonal differences were found in Asibi yellow fever, both nucleotides as well as both amino acids if appropriate are shown. If the nucleotide change results in an amino acid substitution, the amino acid (AA) in 17D is shown, followed by the amino acid in Asibi. Note that in every case of clonal differences in the Asibi strain, one of the nucleotides is the same as that found in 17D strain.

Proteins E, ns2a, and ns2b exhibit a disproportionately high rate of change, 2.4% for E, 3% for ns2a, and 2.3% for ns2b. Only 13 changes, or less than half of the total changes found between Asibi and 17D, are found in other regions, an amino acid sequence divergence of only 0.5%.

The function of the various nonstructural proteins in virus replication is unknown. However, NS3 and NS5 probably form components of the viral replicase responsible for replicating RNA. If these proteins do possess enzymatic functions, then amino acid changes might be expected to be deleterious and selected against during continued passage. It is of note that most of the changes found in NS5 occur near the ends of the molecule, with only one change occurring within the central 75% of the protein. It is unknown whether NS1 has enzymatic activity, but it has been postulated that since it is a glycoprotein, it may be involved in virus assembly. The fact that amino acid substitutions in NS1 are rare, as was the case for NS3 and NS5, suggests that whatever role it plays in virus replication, it requires a precise amino acid sequence for that function. NS1, NS3, and NS5 demonstrate a high degree of conservation among different flaviviruses (13).

The small polypeptides ns2a, ns2b, ns4a, and ns4b are hydrophobic in nature and are not highly conserved among flaviviruses (13). Their hydrophobicity profiles are remarkably conserved, however, suggesting that as long as the hydrophobicity profile is unchanged, a large number of amino acid substitutions can be accommodated without affecting the normal function of these proteins. The high frequency of change observed in the ns2 region may simply be a reflection of this; many of the changes that arise may not affect function and are, therefore, not selected against. The amino acid changes found in the ns2 region (Fig. 2) would have only marginal effects upon the hydrophobicity profile.

ASIBI	17D	No	ASIBI	17D	No	ASIBI	17D	No
G	A	11	G	C	0	C	G	1
A	G	17	G	U	1	C	A	2
C	U	20	A	C	4	U	G	3
U	C	14	A	U	1	U	A	0
Transition		62	Pu	Py	6	Py	Pu	6
			Transversion		12			

FIG. 3. Transitions and transversions that have occurred during passage of 17D strain.

Amino Acid Changes Within the Structural Protein Region. No changes were found in the capsid protein, and only one change was found in prM (this change occurs within M). There are, however, a large number of changes in the envelope protein (Figs. 2 and 4). Furthermore, 15 nucleotide changes in the envelope gene have led to 12 amino acid substitutions (in contrast to the rest of the coding region where 53 nucleotide changes lead to 20 amino acid substitutions), suggesting that in the E protein, some of the amino acid substitutions may have been positively selected for during passage, rather than simply being neutral in effect. Although it is impossible to predict the effect of any particular amino acid alteration without detailed knowledge of the three-dimensional structure and function of the protein, five nonconservative amino acid substitutions are likely candidates to significantly alter envelope protein structure and function. These are Gly-52 to Arg, Thr-173 to Ile, Lys-200 to Thr, Pro-320 to Ser, Thr-380 to Arg, and Pro-390 to His. It is of note that the last two of these changes occur within a conserved domain of the E protein. The E protein sequences from flaviviruses representing the three serological sub-

REGION	TOTAL NT/AA	CHANGE NT/AA	% CHANGE NT/AA
5' Noncoding	118 / -	0 / -	0 / -
Capsid	363 / 121	2 / 0	0.55% / 0
prM (-M)	267 / 89	0 / 0	0 / 0
M	225 / 75	2 / 1	0.89% / 1.89%
Envelope	1479 / 493	15 / 12	1.01% / 2.43%
NS1	1227 / 409	5 / 2	0.41% / 0.49%
ns2a	501 / 167	6 / 5	1.20% / 2.99%
ns2b	390 / 130	4 / 2	1.03% / 2.31%
NS3	1869 / 623	9 / 2	0.48% / 0.32%
ns4a	861 / 287	6 / 3	0.70% / 0.78%
ns4b	336 / 112	2 / 1	0.60% / 0.89%
NS5	2715 / 905	11 / 4	0.40% / 0.44%
3' Noncoding	511 / -	6 / -	1.17% / -
TOTAL	10862 / 3411	68 / 32	0.63% / 0.97%

FIG. 4. Summary of the differences between Asibi and 17D strains at the nucleotide and the amino acid levels. The number of changes and the percent change at both nucleotide and amino acid levels are shown for various regions of the genome.

groups of the mosquito-borne flaviviruses are aligned in Fig. 5; the domain from Pro-369 to Gly-448 shows a high degree of conservation among flaviviruses. In this region there are the following three amino acid changes between Asibi and 17D: Thr-380 to Arg, Pro-390 to His, and Ala-416 to Thr. It is remarkable that in all three cases, 17D changes resulted in the same amino acid as in the Murray Valley encephalitis/West Nile/St. Louis encephalitis subgroup of flaviviruses. In one case the amino acid is also present in dengue-2 virus (Thr-416), and in a second case (His-390) there is also an aromatic amino acid (phenylalanine) at the equivalent position in dengue-2 virus. It seems unlikely, therefore, that these alterations in the yellow fever E protein sequence are due to random events, and it is tempting to speculate that these substitutions alter receptor affinities. In the Murray Valley encephalitis subgroup, birds are an important natural host, and 17D was selected for efficient multiplication in chicken cells. It should also be kept in mind that only a limited number of amino acid substitutions might be tolerated.

The 3′-Untranslated Region. Of the six nucleotide changes found in the 3′-untranslated region, two occur within the predicted secondary structure at the 3′ end of the RNA. One of these would result in an extra A·U base pair in the 3′-terminal 88 nucleotides, while the second results in the loss of an A·U base pair; thus these two changes should have only a minor effect on the stability of the proposed secondary structure.

There are three repeated-sequence elements in the 3′-untranslated region that are 42 nucleotides long, each of which differs from the others by 4 or 5 nucleotides. These repeats are shown for Asibi RNA in Fig. 6. Note that within this repeat, there are 16 contiguous nucleotides that are repeated identically. In 17D RNA there has been an adenosine to guanosine transition at nucleotide 10,454 that results in a mismatch within one copy of this perfect repeat. The significance of this change is unknown.

DISCUSSION

The 17D strain of yellow fever virus is one of the safest and most effective live virus vaccines ever developed, especially after stabilization of seed lots (for a study of the different seed lots see ref. 17). In this report we detail the differences in nucleotide and amino acid sequence between this vaccine strain and the parental Asibi isolate from which it was derived. The change or changes responsible for the altered virulence or attenuation of 17D cannot be determined at the current time. Indeed, the situation is complicated by the fact that the 17D vaccine contains a mixture of variants that differ in a number of biological properties (17, 18). It is unclear which of these variants was sequenced to obtain the 17D sequence, since the vaccine strain was plaque-purified before amplification and sequencing. It is unknown if this mixture of variants is important for the vaccine properties of the 17D strain; however, reversion to virulence has never been documented. It is known that a change occurred in the virulence of yellow fever between the 89th and 114th passage; that this change, once it occurred, was stable; and that comparable changes were not obtained when the passage history of the 17D strain was repeated. The Asibi strain sequenced was not plaque purified and a number of clonal differences were found.

Attenuation of a virus by propagation in tissue culture presumably results from selection for variants better adapted for replication in tissue culture and, conversely, less-well adapted for replication in their natural hosts, although accumulation and fixation of nonselected mutations may also be important. In particular, variants might be selected that bind more readily to receptors on cultured chicken cells, leading to more rapid attachment and penetration. Such an alteration in receptor binding could, as a consequence, lead to less-efficient binding to receptors found in hepatocytes or neurons in primates, and thus to an alteration in tissue tropism and reduction in virulence. The large number of changes found in the envelope protein is consistent with such an hypothesis. Changes elsewhere in the genome may also be important, however, particularly if they affect the efficiency of virus replication. Because of the stability of the vaccine strain, it is likely that more than one change is important for its avirulence. If it becomes possible to rescue infectious virus from a cloned cDNA copy of yellow fever, as has been done for several RNA viruses (19–21), it will be possible to test the effect of the individual changes found on the virulence of the virus. Such an approach has been used with the Sabin and

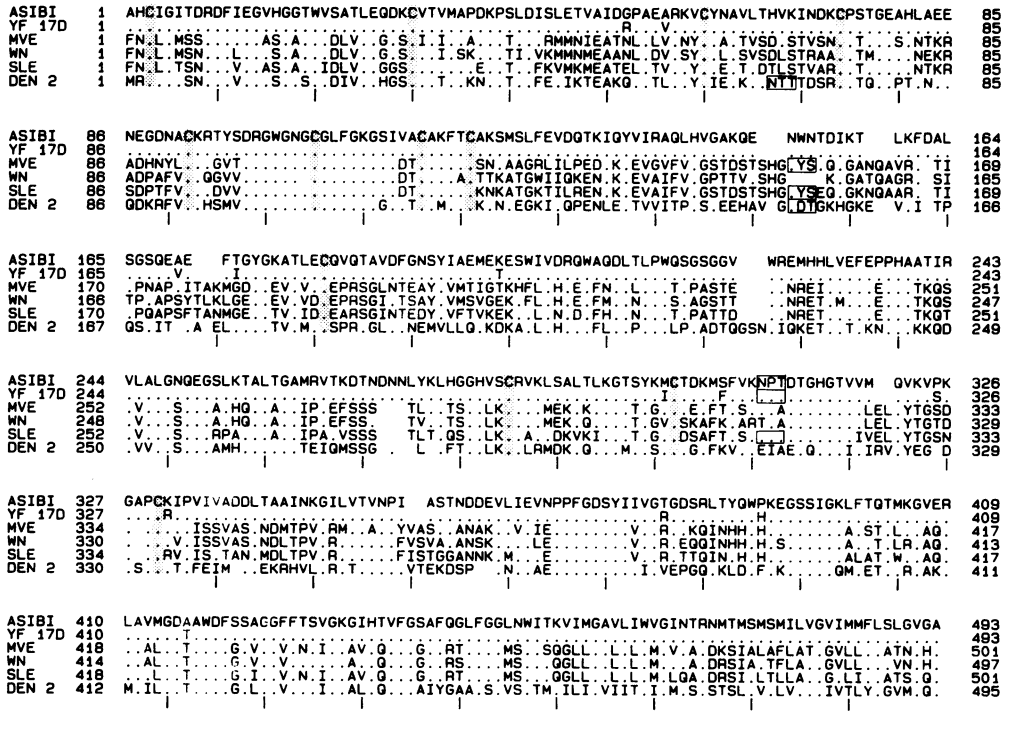

FIG. 5. Envelope protein sequences of five flaviviruses. The aligned amino acid sequence of five flavivirus E proteins are shown in the single-letter amino acid code. Dots indicate that the amino acid is the same as in Asibi yellow fever strain. Murray Valley encephalitis (MVE) (14), West Nile (WN) (15), and St. Louis encephalitis (SLE) (16) viruses belong to a separate serological subgroup of mosquito-borne flaviviruses. Dengue 2 (DEN 2) virus is a member of a third subgroup (Y. S. Hahn, R. Galler, J.M.D., J.H.S., and E. G. Strauss, unpublished data). Cysteine residues are shaded, and potential carbohydrate addition sites are boxed.

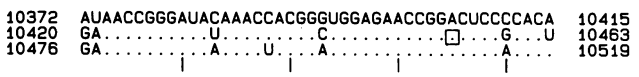

FIG. 6. Repeated sequences in the 3'-untranslated region. The sequences of three repeats in the 3'-untranslated region of Asibi RNA are aligned. Nucleotide 10,454 is a guanosine in the 17D strain, which introduces a mismatch in a 16-nucleotide perfect repeat.

Mahoney strains of poliovirus type I, with the finding that several changes within the genome are involved in the change in virulence between the two strains (22, 23).

Studies by Schlesinger *et al.* (24) have shown that monoclonal antibodies against the envelope protein of 17D yellow fever are often able to discriminate between 17D and Asibi yellow fever. Interestingly, some of these antibodies neutralize Asibi virus but not 17D virus. This suggests that changes in the E proteins are important for determining the structure of E and/or are found in regions important for antibody binding. These authors also found that E proteins of 17D were present in both glycosylated and nonglycosylated forms, whereas Asibi E protein was present in only one form (presumably nonglycosylated from its mobility relative to that of 17D E protein, since we show here that the two proteins are identical in size). No change in the single potential glycosylation site was found between 17D and Asibi, although the change of Phe-305 to Ser is found just upstream of this site. Presumably this change, or other changes upstream that affect the folding of the proteins, leads to changes in accessibility of the site to the glycosylation enzymes.

This study is also of interest because it represents a detailed study of differences that have arisen during 240 passages in which the passage history is known with some clarity. RNA replicases lack proofreading activity and the error frequency of these enzymes is estimated to be on the order of about 10^{-4} per nucleotide per generation, based upon theoretical considerations of the free energy of an A·U or G·C base pair, or upon measured mutation frequencies in a number of virus systems (25, 26). However, measurements in other systems have yielded lower estimates of mutation frequency. Parvin *et al.* (27) found a substitution frequency in the NS gene of influenza of 4×10^{-6} substitution per nucleotide per generation and $<5 \times 10^{-7}$ substitution per generation in the poliovirus VP1 gene. We report here that yellow fever underwent 2×10^{-6} substitution per nucleotide per generation during passage of the 17D strain. The difficulty in relating the various mutation rates observed comes in estimating the fraction of nucleotide substitutions that are capable of survival under the conditions used, which will differ with the protein domain under consideration (some domains or even entire proteins tolerate changes more than others) and with the method of selection [simple ability to persist in a population and form a plaque, as was used by Parvin *et al.* (27), or ability to compete head to head with other viruses in the population, as was the case with yellow fever 17D]. Domingo *et al.* (28) have shown with bacteriophage Qβ that in direct competition, the RNA population consists of an average nucleotide sequence that is maintained during passage, but in which variants arise at high frequency that are subsequently selected against during continued passage. Since many nucleotide changes studied were almost certainly silent changes, the RNA secondary structure of Qβ appears to be important for rapid growth and even silent changes may be selected against, a situation that may well prevail with other RNA viruses and especially with plus-strand RNA viruses. Thus it is unclear whether even the incidence of silent change can be used to assay the inherent mistake frequencies of the viral RNA polymerases. In the case of 17D yellow fever, those alterations that have been fixed presumably represent mutations that are truly neutral (or possibly fixed during plaque purification) as well as positive changes that lead to more efficient growth in tissue culture.

We thank Ms. E. M. Lenches for expert technical assistance. This work was supported by a grant from The World Health Organization and in part by Grants AI 20612 and AI 10793 from the National Institutes of Health and Grant PCM 83 16856 from the National Science Foundation.

1. Monath, T. P. (1986) in *The Togaviridae and Flaviviridae*, eds. Schlesinger, S. & Schlesinger, M. J. (Plenum, New York), pp. 375–440.
2. Reed, W. (1901) *Med. Rec.* **60,** 201–209.
3. Stokes, A., Bauer, J. H. & Hudson, N. P. (1928) *Am. J. Trop. Med.* **8,** 103–164.
4. Theiler, M. (1951) in *Yellow Fever*, ed. Strode, K. (McGraw-Hill, New York), pp. 39–136.
5. Rice, C. M., Lenches, E. M., Eddy, S. R., Shin, S. J., Sheets, R. L. & Strauss, J. H. (1985) *Science* **229,** 726–733.
6. Theiler, M. & Anderson, C. R. (1975) *Am. J. Trop. Med. Hyg.* **24,** 115–117.
7. Repik, P. M., Dalrymple, J. M., Brandt, W. E., McCown, J. M. & Russell, P. K. (1983) *Am. J. Trop. Med. Hyg.* **32,** 577–589.
8. Rice, C. M., Dalgarno, L., Galler, R., Hahn, Y. S., Strauss, E. G. & Strauss, J. H. (1987) in *Molecular Trends in Virology*, Proceedings of the International Symposium, June 1986, eds. Bauer, H., Klenk, H. D. & Scholtissek, C. (Springer, Giessen, F.R.G.), in press.
9. Maniatis, T., Fritsch, E. F. & Sambrook, J. (1982) *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY).
10. Maxam, A. M. & Gilbert, W. (1980) *Methods Enzymol.* **65,** 499–560.
11. Smith, D. R. & Calvo, J. M. (1980) *Nucleic Acids Res.* **8,** 2255–2274.
12. Strauss, E. G., Rice, C. M. & Strauss, J. H. (1984) *Virology* **133,** 92–110.
13. Strauss, J. H. & Strauss, E. G. (1987) in *RNA Genetics*, eds. Domingo, E., Holland, J. & Ahlquist, P. (CRC, Boca Raton, FL), in press.
14. Dalgarno, L., Trent, D. W., Strauss, J. H. & Rice, C. M. (1986) *J. Mol. Biol.* **187,** 309–323.
15. Wengler, G., Castle, E., Leidner, U., Nowak, T. & Wengler, G. (1985) *Virology* **147,** 264–274.
16. Trent, D. W., Kinney, R. M., Johnson, B. J. B., Vorndam, A. V., Grant, J. A., Deubel, V., Rice, C. M. & Hahn, C. (1987) *Virology*, in press.
17. Monath, T. P., Kinney, R. M., Schlesinger, J. J., Brandriss, N. W. & Bres, B. (1983) *J. Gen. Virol.* **64,** 627–637.
18. Liprandi, F. (1981) *J. Gen. Virol.* **56,** 363–370.
19. Taniguchi, T., Palmieri, M. & Weissmann, C. (1978) *Nature (London)* **274,** 223–228.
20. Racaniello, B. R. & Baltimore, D. (1981) *Science* **214,** 916–919.
21. Ahlquist, P., French, R., Janda, M. & Loesch-Fries, L. S. (1984) *Proc. Natl. Acad. Sci. USA* **81,** 7066–7070.
22. Omata, P., Kohara, M., Kuge, S., Komatsu, T., Abe, S., Semler, B. L., Kameda, A., Itoh, H., Arita, M., Wimmer, E. & Nomoto, A. (1986) *J. Virol.* **58,** 348–358.
23. LaMonica, N., Meriam, C. & Racaniello, V. R. (1986) *J. Virol.* **57,** 515–525.
24. Schlesinger, J. J., Brandriss, N. W. & Monath, T. P. (1983) *Virology* **125,** 8–17.
25. Holland, J., Spindler, K., Horodyski, F., Grabau, E., Nichol, S. & VandePol, S. (1982) *Science* **215,** 1577–1585.
26. Steinhauer, D. A. & Holland, J. J. (1986) *J. Virol.* **57,** 219–228.
27. Parvin, J. D., Moscona, A., Pan, W. T., Leider, J. M. & Palese, T. (1986) *J. Virol.* **59,** 377–383.
28. Domingo, E., Sabo, D., Taniguchi, T. & Weissmann, C. (1978) *Cell* **13,** 735–744.