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Molecular Epidemiology of a Variant of Coxsackievirus A24 in Taiwan: Two Epidemics Caused by Phylogenetically Distinct Viruses from 1985 to 1989

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In order to know the phylogenetic relationship and the route of transmission of a variant of coxsackievirus A24 (CA24v), an agent that caused four sequential outbreaks of acute hemorrhagic conjunctivitis from 1985 to 1989 in Taiwan, the nucleotide sequence variations in the virus-encoded proteinase 3C region (549 nucleotides) were studied with 19 isolates. The prototype strain (EH24/70), four isolates from Japan, and two isolates from Hong Kong were used for comparison. The nucleotide sequences of the Taiwan strains from the 1985–1986 and 1988–1989 epidemics were closely related within each epidemic, while they were more distantly related between strains from two epidemics. Phylogenetic analysis by the unweighted pairwise grouping method of the arithmetic average revealed that the 19 Taiwan isolates had diverged into two groups, 1985–1986 and 1988–1989 groups. The time at which these two groups diverged was estimated to be around May 1982, more than 3 years prior to the first appearance of the CA24v epidemic in Taiwan. On each occasion, the viruses caused a 2-year epidemic and then disappeared. The Taiwan isolates from 1985 to 1986 were closely related to the Japan isolates from 1985 to 1986 and the Taiwan isolates from 1988 to 1989 were phylogenetically close to the 1989 Japan isolates, indicating that Taiwan and Japan had two common-source outbreaks. However, none of the 1988 Taiwan isolates were phylogenetically close to the 1988 Japan or Hong Kong isolates. The evidence revealed that Taiwan has had two repeated but discontinuous introductions of CA24v since its first appearance in Taiwan in 1985. None of the other CA24v strains have been detected so far.

There are two highly contagious enteroviruses that cause acute hemorrhagic conjunctivitis (AHC). One is enterovirus 70 (EV70), which appeared for the first time around 1970 in West Africa and subsequently spread to the whole world (13). The other is the coxsackievirus A24 variant (CA24v), an antigenic variant of coxsackievirus A24, which was isolated for the first time in 1970 in Singapore (21). In contrast to EV70, the prevalence of CA24v has been restricted to Southeast Asia. In October 1985, an outbreak of AHC caused by CA24v first appeared in Taiwan. In June 1986 came a second outbreak, which was followed by two subsequent outbreaks that started in August 1988 and August 1989 (1, 14).

Because it is known that some RNA viruses may evolve at rates approximately 10^6-fold faster than those of the DNA genome (9), it was suggested that the high degree of infectivity of CA24v to the human conjunctiva was conferred by mutations accumulating in the RNA genome and causing an expanded epidemic (15). Serological evidence showed that prior to the 1985–1986 outbreaks, there were high titers of antibody against EV70 in serum among the Taiwanese population, but titers of antibody against CA24v were very low, if present at all (1, 6), suggesting the absence of CA24v in this country. Thus, CA24v is considered to be a useful model for analyzing the mode of introduction of a virus in Taiwan.

Comparison of the nucleotide sequences of viruses provides the basis for reliable analysis for the phylogenetic relationship among them. In the previous study, nucleotide sequence variations of the $3C^{pro}$ region of the CA24v genome were compared by using 32 isolates collected from various parts of the world. The phylogenetic analysis revealed that CA24v appeared at one focal point around 1963, several years before the first isolation of CA24v, and all strains isolated after 1985 were derived from a common progenitor prevalent in 1982 (11). Another study also suggested that Japan had three independent introductions of CA24v during the period from 1985 to 1989 and that Taiwan had at least two introductions from 1985 to 1988 which were synchronized with two of the three outbreaks in Japan (12). To clarify these points, we determined and compared the $3C^{pro}$ nucleotide sequences of 19 isolates from Taiwan from 1985 to 1989, together with 4 Japan and 2 Hong Kong isolates, along with the prototype strain isolated in Singapore.

MATERIALS AND METHODS

Viruses. Twenty-five CA24v strains isolated in 1985 to 1989 were investigated together with the prototype strain (EH24/70) isolated in 1970 (Table 1). There were 19 Taiwan, four Japan, and two Hong Kong isolates. The Taiwan isolates were collected throughout Taiwan, and nine of them were included in our previous nucleotide sequence analysis (14). HeLa cells were infected with the isolates and incu-

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TABLE 1. CA24v strains

Strain		Collection		Source or reference
No.	Original code	Locality	Yr/mo	
1	EH24/70	Singapore	1970/09	21
2	L001/85	Taiwan	1985/10	14
3	L048/85	Taiwan	1985/10	14
4	L062/85	Taiwan	1985/10	14
5	L077/85	Taiwan	1985/10	14
6	YC-85-134	Japan	1985/10	16
7	1908/85	Taiwan	1985/12	14
8	V053/86	Taiwan	1986/06	14
9	V090/86	Taiwan	1986/07	14
10	V116/86	Taiwan	1986/07	14
11	V150/86	Taiwan	1986/07	14
12	V172/86	Taiwan	1986/07	14
13	YC-86-100	Japan	1986/08	10
14	151/88	Taiwan	1988/05	14
15	465/88	Taiwan	1988/09	14
16	487/88	Taiwan	1988/09	14
17	HK14576	Hong Kong	1988/09	11
18	HK14919	Hong Kong	1988/09	11
19	590/88	Taiwan	1988/09	14
20	88E-340	Japan	1988/09	18
21	380/89	Taiwan	1989/05	This paper
22	722/89	Taiwan	1989/09	This paper
23	89E-398	Japan	1989/09	18
24	740/89	Taiwan	1989/09	This paper
25	804/89	Taiwan	1989/10	This paper
26	865/89	Taiwan	1989/11	This paper

bated at 37°C until a complete cytopathic effect was observed. In order to avoid arbitrary selection of the minor virus populations, the isolates were not plaque purified, the only exception being the prototype strain EH24/70, which was successively plaque purified three times in HeLa cells.

Nucleotide sequence analysis. The culture fluid from infected HeLa cells was treated with proteinase K (100 μg/ml) in the presence of 1% sodium dodecyl sulfate. After incubation at 37°C for 30 min, the mixture was extracted with an equal volume of phenol mixture (phenol-chloroform-isoamyl alcohol, 25:24:1), and RNA was precipitated in ethanol. The nucleotide sequence of primers, amplification of a 674-bp fragment including the entire $3C^{pro}$ region, and molecular cloning with the M13 phage vector have been described previously (11). The nucleotide sequence was determined by the dideoxy chain termination method described by Sanger et al. (19) with Sequenase (United States Biochemical, Cleveland, Ohio). We sequenced two different clones in the reverse direction. If any differences between them were found, the third clone was sequenced.

Construction of a phylogenetic tree. The evolutionary rate was estimated in our previous report (11), and the rate (0.17 nucleotide substitutions of 549 $3C^{pro}$ nucleotides per month, or 3.7×10^{-3} per nucleotide per year) was applied for the analysis.

The phylogenetic tree was constructed as described previously (11). Briefly, by using the nucleotide substitution rate (b) and isolation time of the strain, the observed distance (d_{ij}) was converted to the adjusted distance (d'_{ij}) between the isolates at a fixed time (t). The equation is $d'_{ij} = b(t - t_i) + b(t - t_j) + d_{ij}$, where t_i and t_j are the isolation times of strains i and j, respectively. Taking the d'_{ij} as a genetic distance, the phylogenetic tree was constructed by the unweighted pairwise grouping method of the arithmetic average (UPGMA). The branching time (t_{ij}) was calculated by the equation $t_{ij} = t - (d'_{ij}/2b)$.

Nucleotide sequence accession number. The nucleotide sequence data reported in this paper will appear in the DDBJ, EMBL, and GenBank nucleotide sequence data bases with the following accession numbers: EH24/70, D10294; L001/85, D13269; L048/85, D10304; L062/85, D13270; L077/85, D10305; YC-85-134, D13271; 1908/85, D10306; V053/86, D10308; V090/86, D10309; V116/86, D13272; V150/86, D13273; V172/86, D10310; YC-86-100, D13274; 151/88, D13275; 465/88, D10323; 487/88, D10324; HK14576, D13276; HK14919, D13277; 590/88, D13278; 88E-340, D13279; 380/89, D13280; 722/89, D13281; 89E-398, D13282; 740/89, D13283; 804/89, D13284; 865/89, D13285.

RESULTS

Comparison of $3C^{pro}$ nucleotide sequences among CA24v isolates. On the basis of the nucleotide sequence of the standard strain (20), two synthetic oligonucleotides were designed to amplify a 674-bp target sequence from the 3′ end of 3A to the 5′ end of $3D^{pol}$ (nucleotide residues 5371 to 6044). The 549 nucleotides of $3C^{pro}$ (nucleotide residues 5461 to 6009) of 11 Taiwan isolates and six foreign isolates were newly determined in this study. The primers were successfully used to amplify the sequences of all of the isolates tested. No exception was found among the strains isolated from 1970 to 1989. The 549 nucleotides of $3C^{pro}$ from 26 isolates were easily aligned because there were neither insertions nor deletions in this region (Fig. 1). The nucleotide substitutions were determined with pairwise analysis. When the nucleotide sequence of the prototype was used as a master sequence, 87 nucleotide positions were found to be substituted from the prototype in at least one of the isolates from 1985 to 1989. Of these, 45 nucleotides were substituted in all 25 strains compared, 8 were unique to the 1985–1986 Taiwan and Japan isolates, and 6 were found in the 1988–1989 Taiwan and 1989 Japan isolates. Twenty-eight other substitutions either were found in small numbers or were found in different combinations of the isolates. Thus, on the basis of the alignment of the $3C^{pro}$ sequences, the Taiwan isolates were divided into two groups: the isolates from 1985 to 1986 and those from 1988 to 1989. Taiwan isolates from 1985 to 1986 and 1989 were closely related to the Japan isolates from the respective years, but none of 1988 Taiwan isolates were close to the 1988 Japan and Hong Kong isolates. The genetic distance matrices determined by pairwise comparisons among the strains are shown in Table 2.

Isolates from 1985 to 1986. Ten Taiwan isolates and two Japan isolates from 1985 to 1986 composed a group with zero to four nucleotide differences among them (Table 2). Six of them (strains 2, 3, 6, 7, 8, and 9) showed identical nucleotide sequences. When this group was compared with the prototype, the differences in nucleotides were in the range 58 to 61, of which 8 were group specific, i.e., not found in other groups (Fig. 1).

One of the two Japan isolates, strain 6, from the 1985–1986 epidemic was found to have a sequence entirely identical to those of the five Taiwan isolates from 1985 to 1986, and the other (strain 13) had only one nucleotide different from them. The close relatedness thus observed between the Taiwan and Japan isolates in this group indicated that common-source outbreaks simultaneously occurred in both areas.

Isolates from 1988 to 1989. Nine Taiwan isolates and one Japan isolate from 1988 to 1989 composed a group with zero to three nucleotide differences among them (Table 2). The

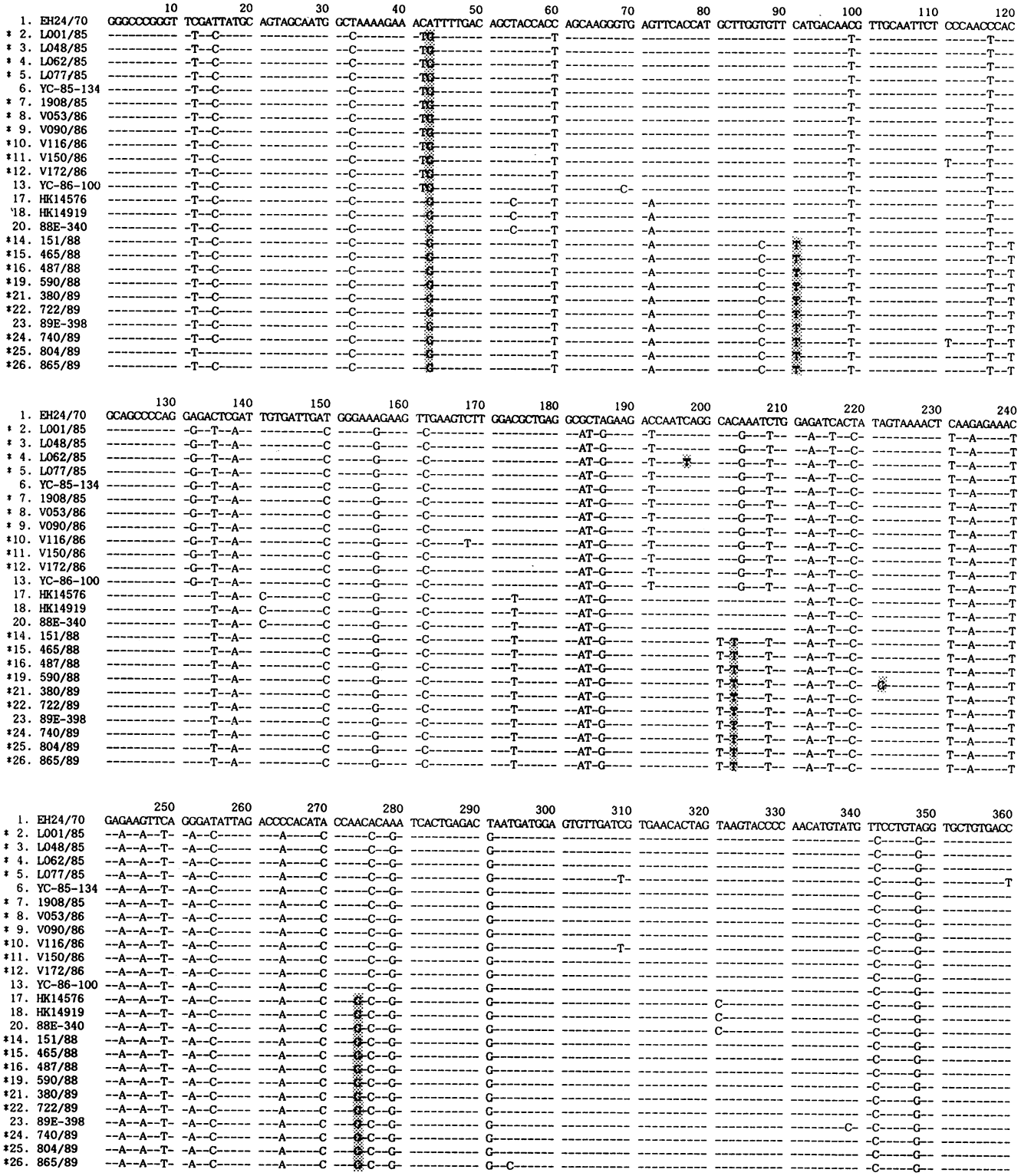

FIG. 1. Comparison of the 549-nucleotide sequences of the $3C^{pro}$ regions of CA24v among the prototype strain EH24/70, 19 isolates from Taiwan (*), 4 isolates from Japan, and 2 isolates from Hong Kong. The nucleotide substitutions associated with amino acid changes are shaded.

numbers of nucleotide differences between the prototype and the isolates were in the range of 60 to 62; of these, 6 were specific for the group (Fig. 1). Four of them (strains 14, 15, 16, and 22) had identical nucleotide sequences. The 1989 Japan isolate (strain 23) differed by only one to three nucleotides from the 1988–1989 Taiwan isolates.

On the other hand, one Japan and two Hong Kong isolates in 1988 composed a unique group. They were clearly distant

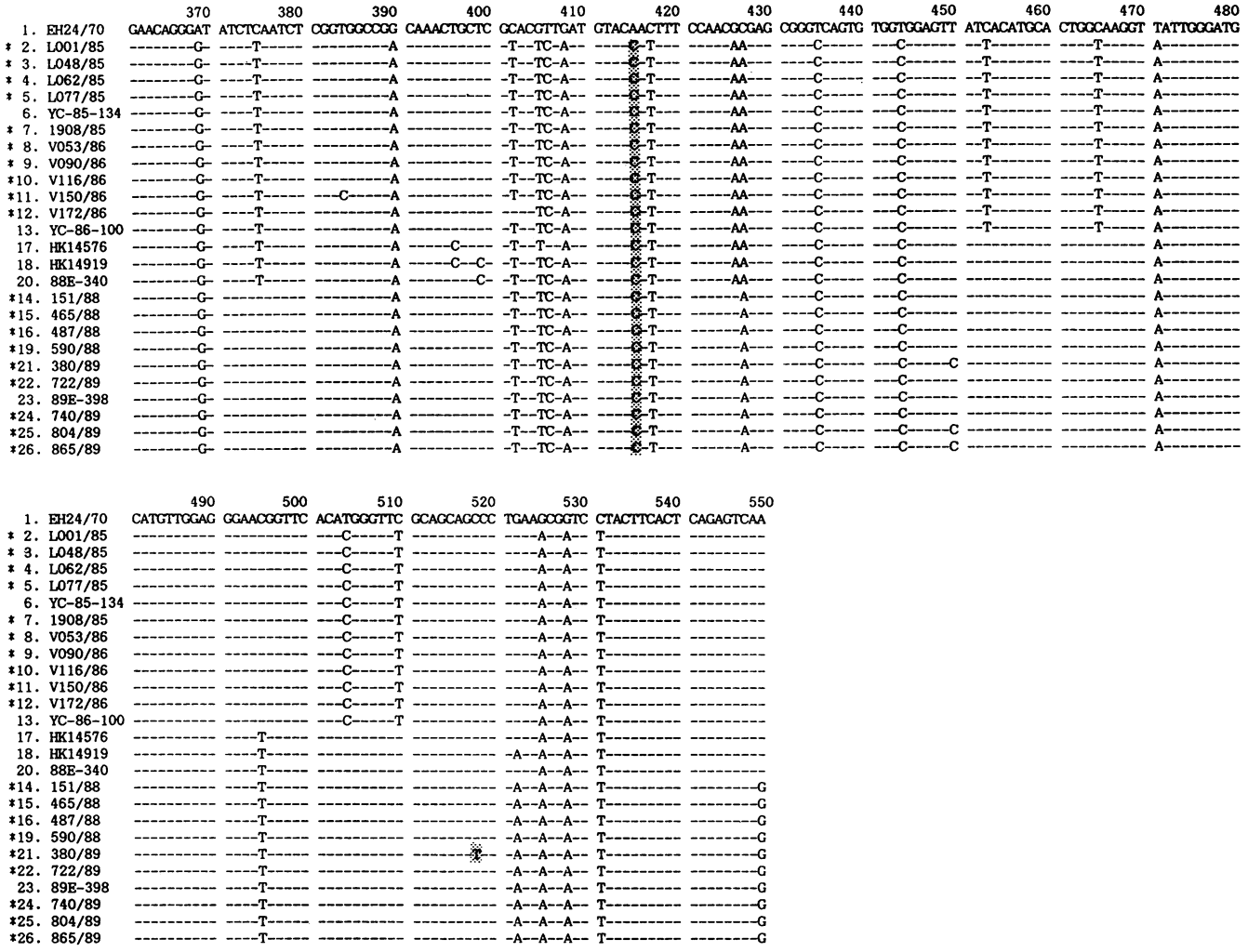

FIG. 1—*Continued.*

from 1988–1989 Taiwan isolates, with 14 to 17 nucleotide differences. They shared three group-specific nucleotides and differed in two to three nucleotides.

Nucleotide and amino acid substitutions among Taiwan isolates. Among 549 nucleotides of CA24v $3C^{pro}$, the differences between the 1985–1986 and 1988–1989 Taiwan isolates ranged from 21 to 25, while the differences between these two Taiwan groups and 1988 Japan and Hong Kong strains ranged from 17 to 21 and 14 to 17, respectively (Fig. 1).

Compared with the nucleotide sequence substitution, the amino acid sequence was quite conserved. Two amino acid-associated nucleotide changes from the prototype, nucleotide positions 43 (I→V) and 415 (N→H), occurred among all recent isolates tested. One additional amino acid change at nucleotide 274 (A→G) was common to the 1988–1989 isolates, including the 1988 Japan and Hong Kong isolates, while the other two changes at nucleotides 91 (H→Y) and 203 (T→I) were specific to 1988–1989 Taiwan and 1989 Japan isolates. In addition, three single amino acid changes were found at nucleotides 197 (C→T) in strain 4, 222 (I→M) in strain 19, and 518 (A→V) in strain 21.

Analysis by phylogenetic tree. The phylogenetic relationship between isolates was analyzed by constructing a UPGMA tree (Fig. 2) by using the previously estimated substitution rate as described in Materials and Methods. For calculation of the UPGMA, the isolates with entirely identical nucleotides or with only one different nucleotide were grouped together.

From the tree, the point of divergence between the 1985–1986 and 1988–1989 isolates was estimated to be May 1982, about 3.5 years prior to the first outbreak in Kaohsiung, Taiwan, and Okinawa, Japan (16). The 1988–1989 Taiwan and 1989 Japan isolates diverged from the 1988 Japan and Hong Kong strains in April 1985, 3 years before the third outbreak in Taiwan. Hence, the divergence probably occurred outside of Taiwan.

DISCUSSION

Regular surveillance conducted by Kaohsiung Medical College since 1980 has revealed three conjunctivitises, AHC, pharyngoconjunctival fever, and epidemic keratoconjunctivitis. Our previous study (6, 17) indicated that the causative agents from 1980 to 1984 were mostly EV70 and adenoviruses. AHC due to CA24v had never been reported in Taiwan until the first outbreak of CA24v (5) in 1985, although several outbreaks associated with CA24v were reported in the Indian subcontinent and Southeast Asia (5, 7). Our

TABLE 2. Number of nucleotide differences among CA24v isolates

Strain[a]		No. of nucleotide differences in strain																								
No.	Code	1	2	3	4	5	6	7	8	9	10	11	12	13	17	18	20	14	15	16	19	21	22	23	24	25	26
1	EH24/70		59	59	60	61	59	59	59	59	61	61	58	59	57	60	58	60	60	60	61	62	60	61	61	60	62
2	L001/85			0	1	2	0	0	0	0	2	2	1	0	18	19	17	21	23	21	22	23	21	22	22	23	23
3	L048/85				1	2	0	0	0	0	2	2	1	0	18	19	17	21	23	21	22	23	21	22	22	23	23
4	L062/85					3	1	1	1	1	3	3	2	1	19	20	18	22	24	22	23	24	22	23	23	24	24
5	L077/85						2	2	2	2	2	4	3	2	20	21	19	23	25	23	24	25	23	24	24	25	25
6	YC-85-134							0	0	0	2	2	1	0	18	19	17	21	23	21	22	23	21	22	22	23	23
7	1908/85								0	0	2	2	1	0	18	19	17	21	23	21	22	23	21	22	22	23	23
8	V053/86									0	2	2	1	0	18	19	17	21	23	21	22	23	21	22	22	23	23
9	V090/86										2	2	1	0	18	19	17	21	23	21	22	23	21	22	22	23	23
10	V116/86											4	3	2	20	21	19	23	25	23	24	25	23	24	24	25	25
11	V150/86												3	2	20	21	19	23	25	23	24	25	23	24	22	25	25
12	V172/86													1	19	20	18	22	24	22	23	24	22	23	23	24	24
13	YC-86-100														18	19	17	21	23	21	22	23	21	22	22	23	23
17	HK14576															3	3	15	17	15	16	17	15	16	16	17	17
18	HK14919																2	14	16	14	15	16	14	15	15	16	16
20	88E-340																	14	16	14	15	16	14	15	15	16	16
14	151/88																		2	0	1	2	0	1	1	2	2
15	465/88																			2	3	2	2	3	3	0	2
16	487/88																				1	2	0	1	1	2	2
19	590/88																					3	1	2	2	3	3
21	380/89																						2	3	3	2	2
22	722/89																							1	1	2	2
23	89E-398																								2	3	3
24	740/89																									3	3
25	804/89																										2
26	865/89																										

[a] Strains 2 through 5, 7 through 12, 14 through 16, 19, 21, 22, and 24 through 26 were Taiwan isolates.

previous serological study (6) revealed that the prevalence rate of CA24v antibody in Kaohsiung before 1981 was as low as 5.3% on average. However, the prevalence rate of EV70 antibody in the same area before 1981 was significantly higher than that of CA24v in most age-groups, with an average of 34.0%. These findings indicated that EV70 had been prevalent since the introduction in 1971, but CA24v had not endemically persisted in Taiwan before 1985, the first outbreak of CA24v in Taiwan.

In the present study, nucleotide sequences of the $3C^{pro}$ region of the 19 isolates from Taiwan in four outbreaks, 1985, 1986, 1988, and 1989, were compared, together with the prototype strain (Singapore) in 1970, the 2 Japan strains in 1985 to 1986, 2 Hong Kong strains from 1988, and 1 of each of the 1988–1989 Japan strains. The phylogenetic tree of CA24v was consistent with that previously reported (11).

The phylogenetic tree of CA24v previously constructed (11) indicated that most of the lineages of the tree contained Singapore strains. This observation is consistent with the fact that an AHC epidemic due to CA24v occurs periodically almost every 5 years in Singapore (8). Therefore, it is possible that the virus has been circulating endemically in Singapore and surrounding areas and has occasionally appeared in other areas such as Taiwan and Japan.

The Taiwan and Japan isolates from 1985 to 1986 were closely related, indicating simultaneous introduction of the virus into both areas. Also, the 1988–1989 Taiwan and 1989 Japan isolates belonged to the same lineage, suggesting another simultaneous introduction of the virus into both areas. However, the 1988 Japan and Hong Kong isolates belonged to a different lineage unrelated to any Taiwan isolate.

Thus, the present study makes it clear that Taiwan has had two repeated but discontinuous introductions of CA24v since its first appearance in Taiwan. On each introduction, the virus was prevalent for two successive years and then disappeared. The fact that only a few nucleotide differences were found among strains within each epidemic indicates that a single or very few viruses were introduced as a common source in a very short period. It is noteworthy that no strain other than these two groups was found in Taiwan, even though the Taiwan isolates used in this study were selected at random from over 300 isolates collected throughout this country. The results also coincided with evidence that AHC due to CA24v had been confined to Southeast Asia and the Indian subcontinent until 1985 and then suddenly and explosively spread to other noncontiguous areas, including Taiwan (1, 14), Japan (12, 15, 16, 18), Central America (3, 4), and Africa (2).

Despite the relatively large number of $3C^{pro}$ nucleotide differences between the prototype and 1985–1986 isolates, only two amino acid differences were commonly identified in the isolates after 1985. Three additional amino acid changes were found in isolates after 1988, of which two were specific for the 1988–1989 Taiwan and 1989 Japan isolates, indicating that these substitutions occurred very recently. It was noticed that clinical signs and symptoms of patients with virologically confirmed AHC in 1985 and 1986 were more severe than those of the 1988–1989 patients (unpublished observation). However, the role of amino acid substitution in modifying clinical symptoms, if any, remains to be solved. The $3C^{pro}$ regions compared in the present study deal with only a part of the noncapsid region in virus-encoded proteins (183 of 2,194 amino acids). Therefore, a similar or even greater number of amino acid substitutions are thought to have occurred in other genomic regions and might have consequently resulted in the appearance of new viruses harboring different antigenicities or pathogenicities that

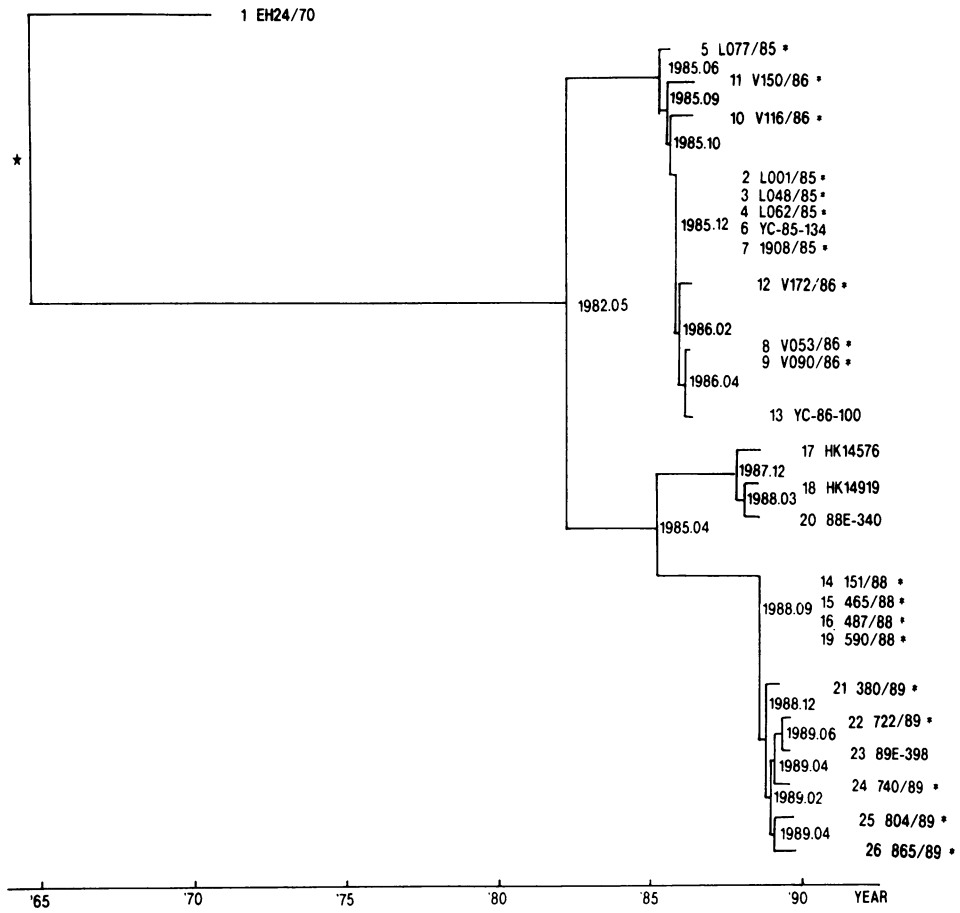

FIG. 2. Phylogenetic analysis of 26 CA24v isolates from Taiwan (*), Hong Kong, and Japan by phylogenetic tree inferred by UPGMA. Times of branching between isolates (shown by year and month in the tree) were calculated by determining the genetic distance between isolates, the time of their isolation, and evolutionary rate as described in Materials and Methods. ★, hypothetical ancestral virus.

would shorten the clinical course of the disease and/or cause milder illness.

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