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The Genome Nucleotide Sequence of a Contemporary Wild Strain of Measles Virus and Its Comparison with the Classical Edmonston Strain Genome

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The only complete genome nucleotide sequences of measles virus (MeV) reported to date have been for the Edmonston (Ed) strain and derivatives, which were isolated decades ago, passaged extensively under laboratory conditions, and appeared to be nonpathogenic. Partial sequencing of many other strains has identified ≥15 genotypes. Most recent isolates, including those typically pathogenic, belong to genotypes distinct from the Edmonston type. Therefore, the sequence of Ed and related strains may not be representative of those of pathological measles circulating at that or any time in human populations. Taking into account these issues as well as the fact that so many studies have been based upon Ed-related strains, we have sequenced the entire genome of a recently isolated pathogenic strain, 9301B. Between this recent isolate and the classical Ed strain, there were 465 nucleotide differences (2.93%) and 114 amino acid differences (2.19%). Computation of nonsynonymous and synonymous substitutions in open reading frames as well as direct comparisons of noncoding regions of each gene and extracistronic regulatory regions clearly revealed the regions where changes have been permissible and nonpermissible. Notably, considerable nonsynonymous substitutions appeared to be permissible for the P frame to maintain a high degree of sequence conservation for the overlapping C frame. However, the cause and the effect were largely unclear for any substitution, indicating that there is a considerable gap between the two strains that cannot be filled. The sequence reported here would be useful as a reference of contemporary wild-type MeV.

Key Words: genome nucleotide sequence; contemporary measles virus; comparison with the classical Edmonston strain.

INTRODUCTION

Measles virus (MeV) is an enveloped virus with a nonsegmented, negative sense genome RNA of ~15.9 kb. It is classified in the genus *Morbillivirus* of the family *Paramyxoviridae.* MeV first was isolated in primary human kidney cells (HK) in 1954 (Enders and Peebles, 1954). This strain, Edmonston-Enders, which was passaged 24 times in HK and 28 times in human amnion cells, appears to be the prototype, but its derivatives, including those with extensive passages under laboratory conditions, have been commonly used as a standard Edmonston (Ed) strain (J. S. Rota *et al.,* 1994; for a review, see Griffin and Bellini, 1996). They appeared to be nonpathogenic for monkeys and perhaps for humans (Yamanouchi *et al.,* 1970).

Wild-type MeVs currently circulating in human populations do not grow well in Vero cells, a monkey kidney line. Previously, these cells were most often used to isolate MeV in patients but now appear to have selected a minor population of quasispecies in patients that is not fully pathogenic for monkeys (Kobune *et al.,* 1990). A low-passage seed stock of Ed strain, the so-called wild-type Ed strain, also had been passaged in Vero cells (J. S. Rota *et al.,* 1994). Marmoset B cell lines such as B95a recently have replaced Vero cells as the isolates into these lines retain full pathogenicity for monkeys, producing clinical signs and histopathology very similar to those seen in humans (Kobune *et al.,* 1990). Such a fresh isolate is unable to grow well in Vero cells; however, several blind passages in these cells have been sufficient to select an adapted strain, which is remarkably attenuated in pathogenicity for monkeys (Kobune *et al.,* 1996). Thus, Ed-related strains may not be representative of pathogenic MeV.

At least 15 distinct genetic clusters have been identified for MeV by sequence comparisons of the nucleocapsid protein (N) gene (World Health Organization, 1998). Ed-related strains and most other old isolates belong to the cluster A, whereas many recent isolates, including those in Japan, belong to other clusters such as D_1 to D_5 (Taylor *et al.,* 1991; P. A. Rota *et al.,* 1994; Rima *et al.,* 1995; Jin *et al.,* 1997; Kreis *et al.,* 1997; Yamaguchi *et al.,* 1997). It is unlikely that current viruses are the descendants of the Ed strain or that there was much less diversion around 1954 in view of relative stability of MeV over long periods of time (Rima *et al.,* 1997). Rather,

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different genotypes might have coexisted since early times, and the Ed strain may be a representative of just one genotype.

Despite the above-described pathological and evolutionary diversity, complete genome nucleotide sequences have been reported only for the classical Ed-related strains. One of them even was constructed with several sequence reports of various Ed strain stocks with different passage histories (Billeter *et al.,* 1984; Bellini *et al.,* 1985, 1986; Rozenblatt *et al.,* 1985; Alkhatib and Briedis, 1986; Cattaneo *et al.,* 1989b). The other two are Ed-derived vaccine strains, Ed B (Radecke *et al.,* 1995) and AIK-C strains (Mori *et al.,* 1993). Furthermore, our fundamental understanding of MeV replication and pathogenesis has depended largely upon the studies with Ed-related strains. Likewise, although our knowledge on MeV is expanding by use of a recently established innovative technology to recover infectious virus from cDNA and hence allow reverse genetics (Radecke *et al.,* 1995), the technology is available only for Ed B strain at this moment. Taking into account these issues, there is now an argument for describing at least one reference strain representing contemporary pathogenic MeVs circulating in human populations. Molecular biology of this new reference strain, including the establishment of plasmid-based rescue, will complement Ed-based studies. To this end, we have chosen a well-characterized fresh pathogenic isolate, 9301B, in B95a cells and determined the entire genome nucleotide sequence (Takeda *et al.,* 1998). Here its sequence is compared systematically with that of the classical laboratory standard Ed strain.

The data reveal the presence of extensive synonymous and nonsynonymous substitutions between the two sequences. However, any causes and effects have not been defined for any substitution. On the other hand, attenuation of the 9301B strain through adaptation to growth in Vero cells appeared to be attributable to only several amino acid changes in the polymerase and/or accessory genes (Takeda *et al.,* 1998). Thus, there appeared to be a gap between the classical Ed strain and contemporary 9301B strain that cannot be filled.

RESULTS AND DISCUSSION

Phylogenetic relationship of the strain 9301B with other MeV isolates

Figure 1 illustrates the phylogenetic relationships among MeV strains based upon N gene sequences. Of the 117 strains, 23 sequences including the 9301B were sequenced here. These new sequences were of the isolates from 1977 to 1998 in Japan. Strain 9301B, which was previously shown to be fully pathogenic for monkeys (Takeda *et al.,* 1998), was found to fall in the cluster D_5 along with most other isolates in Japan since 1990 until 1998. These results clearly demonstrate that strain 9301B represents one of those which recently emerged and provide a rationale for using this strain as a reference for contemporary wild-type MeVs.

Comparison of 9301B genome with the Ed strain genome

General features. The genome of the strain 9301B comprises 15,894 nucleotides and thus is identical in length to the classical Ed strain genome. This suggests that the *rule of six* that the genome length of MeV and some other paramyxoviruses should be a multiple of six nucleotides for efficient replication (Calain and Roux, 1993) has stringently operated in MeV evolution. There were 465 nucleotide differences (2.93%) and 114 deduced amino acid changes (2.19%) between the two viral genomes (Fig. 2). However, the transcriptional start and stop signals as well as the intergenic and trailer sequences were perfectly conserved. The editing motifs in the P (phosphoprotein) gene giving rise to V mRNA (Cattaneo *et al.,* 1989a) were also identical between the two. Thus changes are generally nonpermissible for *cis*-acting elements for transcription and replication. The 52-nucleotide-long leader sequence may be exceptional because three nucleotide substitutions were found (Fig. 2).

We calculated synonymous differences per synonymous sites (K_S) using the method established by Miyata and co-workers. This method has been used to compare ~50 pairs of homologous sequences, including α-globin and interferon-α genes (Miyata and Yasunaga, 1980, 1981; Miyata *et al.,* 1980; Miyata and Hayashida, 1982), and for strain comparisons of Newcastle disease virus (NDV), a rubulavirus in the *Paramyxoviridae* (Sakaguchi *et al.,* 1989; Toyoda *et al.,* 1989). These studies demonstrated that synonymous codon changes are neutral to natural selection in evolution and occur with nearly similar rates among many different genes. As shown in Table 1, the K_S values are comparable (0.077–0.098) for the N, M (matrix protein), F (fusion protein), H (hemagglutinin protein), and L (large protein) genes. Thus the two viruses appeared to be equally distant in terms of these ORFs. This further supported the suggestion of lack of recombination between genotypes in the evolutionary process of MeV (Jin *et al.,* 1997; Rima *et al.,* 1997; Yamaguchi, 1997) and other paramyxoviruses (Pringle, 1991; Toyoda *et al.,* 1989). In addition to the P protein, the MeV P gene encodes the C protein and the *trans* V C-terminal region in +1 and −1 frames, respectively, relative to the P frame (Bellini *et al.,* 1985; Cattaneo *et al.,* 1989a). This predicted a lower K_S value with respect to a given overlapping frame in the P gene. Indeed, the K_S value in the entire P frame was already significantly low (0.042) and those in the regions overlapping the other frames were still lower [0.015 and 0.018 for the regions P/C (22–582) and P/V (691–903), respectively] (Table 1).

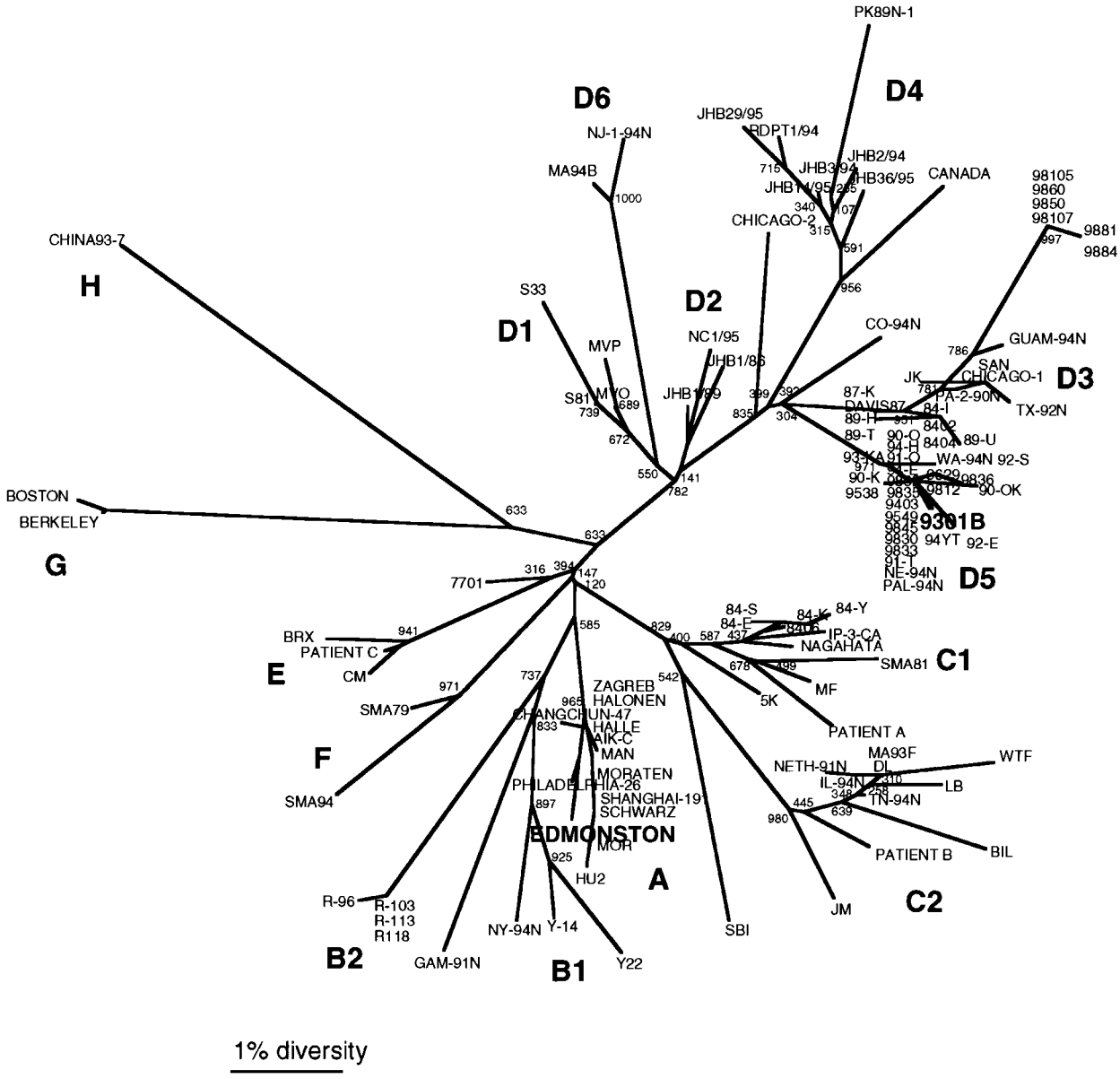

FIG. 1. Unrooted phylogenetic tree drawn by a CLUSTAL W analysis with N gene sequences of 117 MeV strains. The genotypes are indicated in capital letters. The horizontal bar indicates the rate of nucleotide difference. The numbers at the main nodes indicate bootstrap confidence levels obtained by 1000 replicates.

In contrast, if nonoverlapping regions were combined together to a provisional gene P′ [(1–21) + (583–690) + (904–1524)], its K_S was significantly higher (0.069). The entire V (1–903) already displayed a low K_S (0.031), and as expected, the K_S of the *trans* V frame (691–903) overlapping the P frame was still lower (0.022). However, the C frame (1–561) totally overlapping the P frame possessed an unexpectedly high K_S (0.069). This can be explained at least in part by a considerably high flexibility of the P frame (see below).

Table 1 also shows amino acid changing (replacement) sites (K_A) nucleotide differences per site at between each pair of MeV genes. In all the comparisons, K_S is always much larger than K_A, indicating a higher evolutionary rate of synonymous substitution and that the bulk of the nucleotide changes in protein coding regions

FIG. 2. Nucleotide and deduced amino acid differences between 9301B and Edmonston strains. Amino acid differences are shown in parentheses. Open reading frames are shadowed. Greek numbers (I–VI) in the L gene indicate the regions that are conserved among polymerases of nonsegmented negative-strand RNA viruses (Poch *et al.,* 1990).

Region	No.	9301B	Edmonston
leader	26	A	T
	42	A	C
	50	A	G
N	248	C	T
	258	T	C
	260	A	T
	404	C	A
	517	G (137 S)	T (137 I)
	518	C (137 S)	T (137 I)
	617	T	C
	772	G (222 R)	T (222 I)
	773	G (222 R)	T (222 I)
	782	A	G
	793	T	C
	809	G	C
	833	G	A
	839	A	C
	842	G	A
	851	T	C
	918	T	C
	950	C	T
	1010	T	C
	1028	A	G
	1029	G (308 E)	A (308 K)
	1032	A (309 T)	C (309 P)
	1051	T (315 I)	A (315 N)
	1055	A	G
	1124	G	A
	1157	A	G
	1166	T	C
	1187	G	A
	1214	G	A
	1229	G	A
	1247	G	A
	1280	G	A
	1321	G (405 R)	A (405 K)
	1337	C	T
	1357	G	A
	1370	C (421 H)	G (421 Q)
	1394	C	A
	1397	A	G
	1398	G (431 G)	C (431 R)
	1439	G	A
	1466	G	A
	1473	T (456 S)	C (456 P)
	1492	A (462 E)	C (462 A)
	1515	A (470 S)	G (470 G)
	1550	A	C
	1551	G (482 G)	A (482 S)
	1586	C (493 D)	G (493 E)
	1587	G (494 A)	C (494 P)
	1595	C	T
	1605	A (500 T)	G (500 A)
	1621	T (505 L)	C (505 S)
	1625	G	A
	1654	G (516 R)	C (516 T)
	1655	G (516 R)	A (516 T)
	1670	T (521 S)	A (521 R)
	1671	G (522 D)	A (522 N)
	1702	A	G
	1706	T	G

Region	No.	9301B			Edmonston		
		P	V	C	P	V	C
P/V/C	1776	G			C		
	1778	C			T		
	1798	C			T		
	1879	A (25 S)	(25 S)		G (25 G)	(25 G)	
	1885	T			C		
	1891	G (29 V)	(29 V)		A (29 I)	(29 I)	
	1902	C		(25 P)	T		(25 L)
	1944	C (46 D)	(46 D)	(39 T)	G (46 E)	(46 E)	(39 S)
	1954	C (50 R)	(50 R)		T (50 C)	(50 C)	
	1958	A (51 K)	(51 K)	(44 R)	G (51 R)	(51 R)	(44 G)
	2053	T (83 S)	(83 S)		C (83 P)	(83 P)	
	2074	A (90 T)	(90 T)		G (90 A)	(90 A)	
	2095	T (97 S)	(97 S)		C (97 P)	(97 P)	
	2138	C (111 H)	(111 H)		T (111 Y)	(111 Y)	
	2242	G (146 D)	(146 D)		A (146 N)	(146 N)	
	2390	A (195 K)	(195 K)		G (195 R)	(195 R)	
	2401	C			A		
	2406	T			C		
	2455	A (217 N)	(217 N)		G (217 D)	(217 D)	
	2457	T (217 N)	(217 N)		C (217 D)	(217 D)	
	2461	A (219 S)	(219 S)		G (219 G)	(219 G)	
	2476	G (224 A)	(224 A)		T (224 S)	(224 S)	
	2480	A (225 E)	(225 E)		G (225 G)	(225 G)	
	2669	G (288 G)			A (288 E)		
	2703	C	(300 Q)		T	(300 stop)	
	2745	C			T		
	2757	C			T		
	2760	C			T		
	2770	C			T		
	2853	C (353 L)			A (353 I)		
	2965	A (387 N)			G (387 D)		
	2974	C (390 L)			A (390 I)		
	2986	C			T		
	3042	G			A		
	3077	G (424 S)			A (424 N)		
	3120	A			G		
	3142	G (446 S)			A (446 M)		
	3189	A			C		
	3282	C			T		
	3362	T			C		
	3371	T			C		
	3374	T			C		

Region	No.	9301B	Edmonston
M	3506	T	C
	3527	A	G
	3566	T	C
	3569	T	C
	3587	C	T
	3629	T	C
	3680	T	C
	3695	A	G
	3701	G	A
	3702	G (89 E)	A (89 K)
	3845	T	C
	3862	A (142 N)	G (142 S)
	3875	A	G
	3884	G	C
	3887	C	T
	3959	C	T
	4010	A	G
	4037	T	A
	4062	G (209 A)	A (209 T)
	4274	T	C
	4286	A	G
	4310	C	T
	4316	G	A
	4450	C	T
	4464	A	G
	4485	T	C
	4497	A	G
	4524	A	G
	4530	T	C
	4575	T	C
	4577	G	A
	4582	T	C
	4583	G	A
	4592	A	C
	4607	C	T
	4611	T	C
	4631	T	C
	4639	C	T
	4642	G	A
	4680	T	C
	4681	C	T
	4687	G	C
	4690	C	T
	4691	A	C
	4694	G	A
	4707	T	C
	4712	T	C
	4713	C	A
	4715	A	C
	4716	G	A
	4720	T	C
	4729	G	A
	4742	C	T
	4750	G	C
	4760	C	T
	4761	T	C
	4763	C	T
	4766	C	T
	4771	G	A
	4793	A	G
	4851	C	T

Region	No.	9301B	Edmonston
F	4898	G	A
	4925	A	G
	4937	C	A
	4945	A	C
	4979	C	T
	5001	C	G
	5016	C	A
	5032	G	A
	5036	C	T
	5097	T	C
	5113	T	C
	5139	G	A
	5147	G	A
	5148	T	C
	5172	C	T
	5176	G	A
	5195	G	C
	5197	A	G
	5243	C	G
	5281	T	C
	5283	G	A
	5284	G	A
	5292	T	C
	5293	A	G
	5303	A	G
	5307	C	T
	5312	C	T
	5320	G	A
	5361	G	A
	5383	T	C
	5397	G	A
	5409	C	T
	5427	C	T
	5449	G	A
	5488	C (11 L)	T (11 F)
	5568	G	A
	5619	G	A
	5703	A	T
	5805	T	A
	5913	A	G
	5937	G	A
	6063	A	C
	6147	C	T
	6180	T	C
	6297	A	C
	6372	T	C
	6414	T	C
	6423	G	A
	6477	G	A
	6546	C	T
	6639	C	T
	6723	G	A
	6738	T	C
	6750	G	A
	6771	G	A
	6777	G	T
	6834	G	A
	6840	G	A
	6879	A	G
	6945	T	C
	6972	G	A
	7022	C (522 T)	A (522 N)
	7023	C (522 T)	T (522 N)
	7025	G (523 R)	A (523 K)
	7035	G	A
	7077	A	G
	7080	G	A
	7177	T	C
	7202	G	A
	7210	C	T

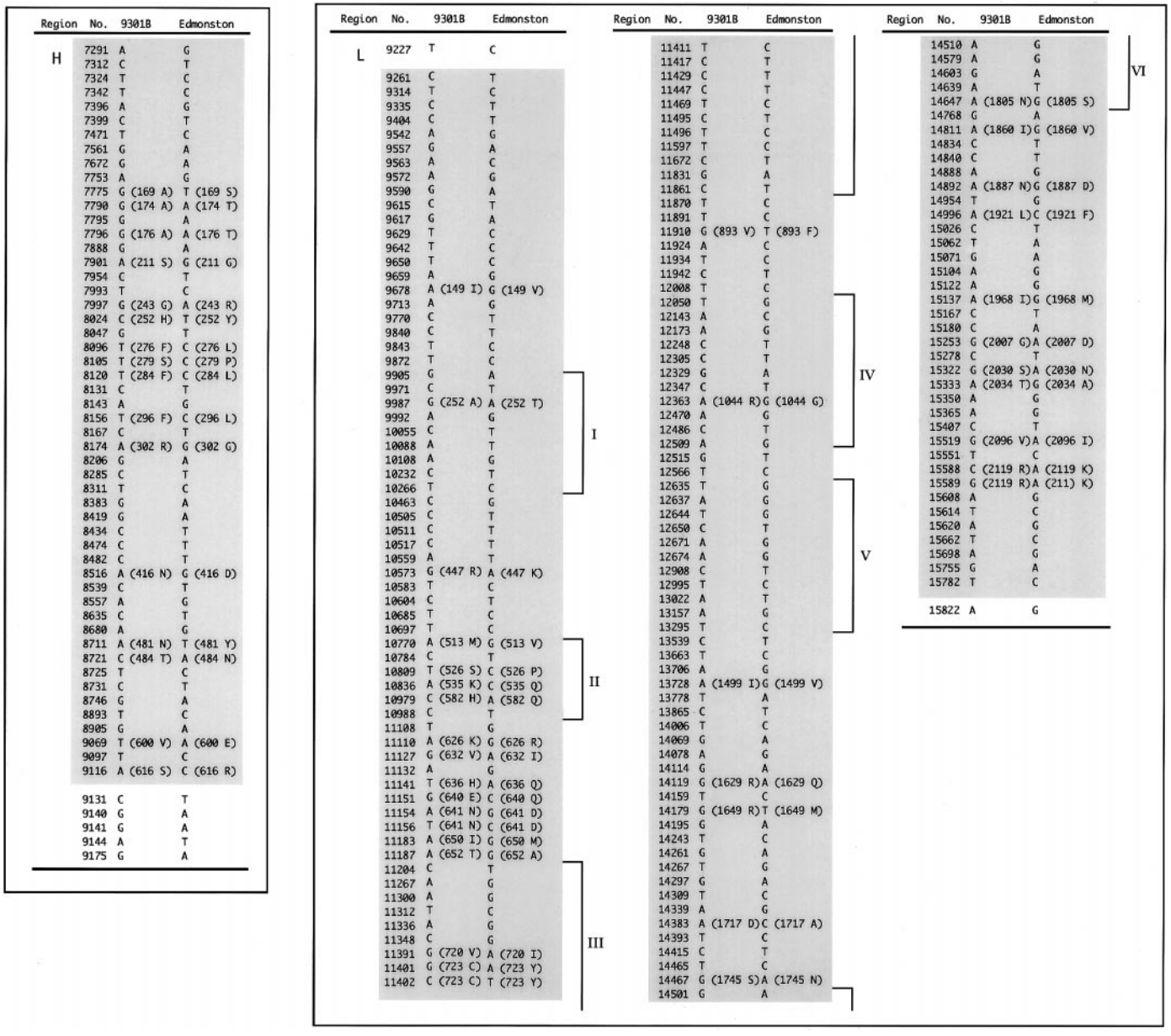

FIG. 2—*Continued*

TABLE 1

Ks and KA Values between 9301B and Edmonston Strains

Gene or gene segment	Nucleotides[a]	K_S	K_A	K_A/K_S
N				
N	1–1578	0.098	0.017	0.173
N1	1–1200	0.092	0.007	0.076
N2	1201–1578	0.114	0.049	0.430
P				
P	1–1524	0.042	0.018	0.429
P/C	22–582	0.015	0.023	1.533
P/V	691–903	0.018	0.006	0.333
P′	(1–21) + (583–690) + (904–1524)	0.069	0.017	0.246
C	1–561	0.069	0.007	0.101
V	1–903	0.031	0.022	0.759
V *trans*	691–903	0.022	0.000	0.000
M	1–1008	0.087	0.004	0.046
F	1–1653	0.077	0.002	0.026
H	1–1854	0.086	0.011	0.128
L				
L	1–6549	0.095	0.006	0.064
LI-VI	(649–1224) + (1483–1797) + (1957–2628) + (2779–3276) + (3385–4128) + (5260–5493)	0.081	0.004	0.049
Lr	(1–648) + (1225–1482) + (1798–1956) + (2629–2778) + (3277–3384) + (4129–5259) + (5494–6552)	0.108	0.009	0.083

[a] The first nucleotide of the initiation codon for each ORF is taken as 1.

have been synonymous. This confirms that the synonymous changes have beenlargely free from selective pressures and occurred at a nearly constant rate. In contrast to K_S values that were relatively consistent, K_A values varied greatly among different genes. The lower frequency of amino acidchanges in F and M proteins than in H or N proteins was reported previously (Baczko *et al.,* 1991; Rota *et al.,* 1992). In our analysis, K_A values were extremely low for the F and M ORFs (0.002 and 0.004, respectively) (Table 1), also indicating the strongest structural constraint for these proteins. On the other hand, the constraint was suggested to be low for the N and P proteins by their high K_A values. When the N ORF was divided to N- and C-terminal halves, the latter (1201–1578) displayed a K_A value of 0.049, one of the highest of all protein coding regions (Table 1), whereas the K_A of N-terminal half (1–1200) was as low as 0.007. Thus, nonsynonymous substitutions have accumulated selectively in the C-terminal half, which is in agreement with the previous view that the N C-terminal region is highly variable (Taylor *et al.,* 1991; P. A. Rota *et al.,* 1994; Rima *et al.,* 1995; Jin *et al.,* 1997).

Evolutionary features of the P gene. K_A and K_S calculations appear to disclose interesting evolutionary features of the P gene containing overlapping frames. In contrast to the other ORFs whose K_S values are generally higher than the respective K_A values, the K_S (0.015) of the P frame overlapping the C frame [P/C (22–582)] was lower than the K_A (0.023). On the other hand, the K_A of the entire C (1–561) totally overlapping the P frame was remarkably low (0.007), whereas its K_S was quite high (0.069). The third letters of C codons are dictated as the first letters of P codons. The high K_S of C thus appears to be explained at least in part by the structural flexibility of the overlapping P frame. On the other hand, the third letters of P codons correspond to the second letters of C codons. The extremely low K_S of P/C suggests inflexibility of the C protein.

The RNA-dependent RNA polymerase is highly error prone because of the lack of proofreading and repair functions and gives rise to heterogeneous mixed progeny populations. Amplification of genome subsets with better fitness is involved in survival of RNA viruses (Domingo *et al.,* 1997). The very low rates of accepted nonsynonymous point mutations in the M, F, and L genes (Table 1) certainly limit the proportion of viable progeny. Thus neutral synonymous changes are maintained more readily than nonsynonymous changes. When an RNA viral gene uses overlapping frames, the opportunity of synonymous changes must be reduced unless the two frames or at least one of them were able to tolerate changes to some extent. In the case of MeV P gene, the C frame appears to have to be conserved, and a high variability of the P frame appears to guarantee the C protein sequence conservation and virus survival under error-prone conditions. In the subfamily *Paramyxovirinae,* the P protein is the most variable of all gene products. C proteins are encoded by the members of the genera *Respirovirus* and *Morbillivirus*. Their sequences are highly conserved at least within a genus, though highly

divergent between the two genera (Nagai, 1998). Thus, at the virus species level, the C protein cannot be so variable. It is likely that the overlapping of variable P and invariable C frames in a single P gene is also a contrivance for respiro- and morbilliviruses to persist in nature.

Stringency of both the *trans* V and P/V frames, as indicated by low K_S and K_A values, may be possible because the overlapping region is relatively short (213 nucleotides). The *trans* V frames of 9301B and Ed strains differed from each other only in that the latter terminated one codon earlier. Termination codons were excluded in our K_S/K_A computation, and thus the K_A in this case was zero (Table 1). The V protein is encoded by all three genera of the *Paramyxovirinae*. Extraordinarily high sequence conservation has been recognized for the *trans* V frame; in particular; the cysteine-rich C-terminal portion (Nagai, 1998). By using V knockout Sendai virus (Kato *et al.,* 1997a,b) and MeV (Schneider *et al.,* 1997; Valsamakis *et al.,* 1998; Tober *et al.,* 1998), evidence that suggests that the V proteins are critically required in *in vivo* settings, though totally dispensable for replication in cell cultures, has been accumulated. In view of these facts, strict conservation of the *trans* V frame between 9301B and Ed strains appears to be reasonable.

Nontranslated regions. A feature unique to morbillivirus genome is the presence of unusually long nontranslated regions (NTRs) at the 3′ region of M mRNA and at the 5′ region of F mRNA. The 5′ NTR of morbilivirus F mRNA is G-C-rich and predicted to form an extensive secondary structure (Richardson *et al.,* 1986; Buckland *et al.,* 1987). The long 5′ NTR of 573 nucleotides and its G-C-rich feature are conserved in the 9301B strain; GC contents are 64.6 and 63.7% for 9301B and Ed strains, respectively. Within cells, the 5′ NTR enhanced translation of F, whereas in *in vitro* translation this region was inhibitory (Evans *et al.,* 1990). Other reports suggested that this NTR of MeV was not important in cell culture replication (Radecke *et al.,* 1995) but critical for *in vivo* pathogenicity (Valsamakis *et al.,* 1998). On the other hand, as many as 72 nucleotide differences (7.27%) were found in the 3′ M mRNA and 5′ F mRNA NTRs (991 nucleotides in total) between the 9301B and Ed strains (Fig. 2). A high degree of variation in these regions also was noted between not only lytic viruses but also SSPE (subacute sclerosing panencepharitis)-related, persistently infecting viruses (Baczko *et al.,* 1991; Billeter and Cattaneo, 1991). Such a high variability was not found in other noncoding regions. Thus, these two NTRs were subject to a most rapid change in MeV genome but their lengths as well as GC-rich nature appear to have been strictly maintained.

In the F mRNA of Ed strain, there are two potential initiation codons at nucleotide positions 574 and 583. Rota *et al.* (1992) have reported that in current MeV isolates with Vero cells in the United States the first AUG was replaced with GUG. Cathomen *et al.* (1995) demonstrated that the second AUG was the preferential initiation site for the Ed strain. Thus the same replacement, first AUG to GUG, observed also in 9301B strain would not be critical for phenotypic differences between Ed and 9301B strains.

The H gene. Rota *et al.* (1992) reported a temporal accumulation of nucleotide changes resulting in amino acid substitutions in the H genes of the current MeV strains, which were isolated in Vero cells. Our data demonstrated a moderate degree of K_A (0.011) of H gene. Upon expression on cell surface, Ed strain H proteins can adsorb African green monkey erythrocytes, whereas those of recent isolates lack such a hemoadsorbing capacity (Shibahara *et al.,* 1994; Lecouturier *et al.,* 1996). The strain 9301B also appeared to lack this activity. Lecouturier *et al.* (1996) reported that the two amino acid residues, valine and tyrosine at positions 451 and 481 (V451 and Y481), respectively, in the Ed H protein, were important for hemadosorption. These residues also appear to be associated with the downregulation of CD46 (Lecouturier *et al.,* 1996), the receptor for Ed and related strains (Dõrig *et al.,* 1993; Naniche *et al.,* 1993a,b). While V451 was conserved in the 9301B H protein, the Y481 was replaced with asparagine (N481) (Fig. 2). Thus, Y481 of Ed H protein appears to be more important for hemadsorption than V451, or both are required.

There are several reports that suggest that MeV attenuation through adaptation to growth in laboratory cell lines including Vero cells were associated with amino acid substitutions in the H protein (Lecouturier *et al.,* 1996; Tanaka *et al.,* 1998). However, a pair of wild-type, 9403B, and Vero cell-adapted and attenuated form, 9403V, displayed no amino acid substitutions in the H proteins (Takeda *et al.,* 1998). Thus, it remains to be defined whether or not the Ed H protein would indeed contribute to the attenuated phenotype of this strain. What also remains to be established is whether or not current strains including 9301B use CD46 as the receptor.

The L gene. The L protein, the catalytic subunit of RNA-dependent RNA polymerase, was relatively well conserved in amino acid sequence as was indicated by its low K_A value (0.006). In this protein, there are six regions (I–VI) that apparently are conserved in other nonsegmented negative-strand RNA viruses, including vesicular stomatitis virus (VSV), rabies virus (RV), Sendai virus (SeV), and NDV (Poch *et al.,* 1990). The K_A of the entire conserved regions ($L_{I\text{-}VI}$) (3039 nucleotides) was 0.004, whereas that of the remaining regions (Lr) (3510 nucleotides) was more than twice as much (0.009) (Table 1). The K_S values were roughly comparable (0.081 and 0.108) between the two. These data may argue for the suggested importance of the six regions for polymerase function of nonsegmented negative-strand RNA viruses. The region II in other nonsegmented negative-strand RNA viruses is featured by a rhythmically spaced [KEKE-

(hydrophobic)K] motif and thought to be a template recognition site (Poch *et al.* 1990). However, the corresponding sequence of MeV Ed strain was different (QEKE at positions 535–538). This exception was solved by the wild-type 9301B strain, which possessed 535K in place of 535Q (Fig. 2). The Ed's motif may be exceptional even within MeV isolates. The pentapeptide QGDNQ (positions 771–775) in the region III, as expected, was conserved, which contains the GDN corresponding to the GDD of the strictly conserved Asp–Asp element of RNA polymerases and is probably important for template recognition and/or phosphodiester bond formation (Poch *et al.,* 1990).

Although the K_S of the region (1798–1956) in the L frame was 0.084 and hence almost equal to those of most other ORFs, its K_A was extremely high or even the highest (0.057) of all ORFs. The corresponding regions of five other viruses described above are remarkably variable in both length and sequence. They are very short for rhabdoviruses (RV and VSV) and though relatively long, are variable in paramyxoviruses (SeV, NDV, and MeV) (Poch *et al.,* 1990). Poch *et al.* suggested that this region would lie on the surface of L protein because it is very hydrophilic. Probably some immunological pressure has operated preferentially on this presumable surface region, leading to accumulation of nonsynonymous changes.

In summary, we determined the entire genome nucleotide sequence of a contemporary wild strain, 9301B, of MeV. Its comparison with that of the classical Ed strain isolated four decades earlier revealed a large number of synonymous and nonsynonymous substitutions. As shown elsewhere recently (Takeda *et al.,* 1998), attenuation of 9301B through adaptation to growth in Vero cells were associated with only several nonsynonymous changes in the polymerase and accessory proteins. None of these changes were shared with the strain Ed, which is also Vero cell-adapted and attenuated. Low passage seeds of the Ed virus were sequenced and also did not vary greatly from the standard Ed virus and Ed-derived vaccine viruses (Rota *et al.,* 1994). Thus, most changes found between 9301B and Ed strains did not appear to be due to different culture and passage conditions. No other particular nature of selection can be conceptualized for any substitution between the two viruses. Rather, this recent isolate did not appear to be a direct descendant of Ed strain but has come from a distinct lineage. Despite numerous substitutions in other regions, *cis*-acting elements for replication and transcription, including the trailer, transcription start, transcription termination, and intergenic sequences, were perfectly conserved between the two strains. Certain ORFs, including those for V, F, and M, were also well conserved. A stringent structural constraint therefore has operated on these regions beyond different lineages. The P gene containing overlapping frames appeared to display a unique evolutionary pattern that is not seen for monocistronic genes.

MATERIALS AND METHODS

Genome nucleotide sequencing

The MeV strain, 9301B, was isolated with B95a cells from the throat swab of a child diagnosed as having typical acute measles in Japan in 1993. When passaged twice in B95a cells and inoculated subcutaneously into cynomolgus monkeys, this strain caused rash, leukopenia, and bodyweight loss as described previously (Takeda *et al.,* 1998). To obtain sufficient stocks for following analyses, a further three to five passages in B95a cells were done. Using the RNA isolation reagent RNAzol B (Biotex Laboratories, Houston, TX), genomic and antigenomic RNAs were extracted from the 9301B stock virions, which had been purified by sucrose gradient centrifugation. Fifteen cDNA fragments (nucleotide positions, 1 to 830 • 82 to 1721 • 1674 to 2480 • 1777 to 3350 • 3000 to 4462 • 4261 to 4795 • 5449 to 7130 • 7000 to 8042 • 7261 to 9144 • 9001 to 10239 • 10001 to 11240 • 11197 to 12663 • 12501 to 13900 • 13857 to 14797 • 14755 to 15894) were amplified from the genomic and antigenomic RNA templates by reverse transcription (RT)–polymerase chain reaction (PCR) using reverse transcriptase (Superscript II, Gibco-BRL, Gaithersburg, MD); *Taq* DNA polymerase (Ex Taq, TaKaRa, Seta, Japan) and the 15 specific primer pairs. These primer pairs were pMv1f, 5′-^{1}ACCAAACAAAGTTGGGTAAG^{20}-3′ (sense) and pMv1r, 5′-^{830}CTTGTATTCCAGGATTAGAG^{811}-3′ (antisense); pMv2f, 5′-^{82}CAAGAGCAGGATTAGGGATA^{101}-3′ (sense) and pMv2r, 5′-^{1721}TAGGCGGATGTTGTTCTGGC^{1702}-3′ (antisense); pMv3f, 5′-^{1674}CTTCTAGACTAGGTGCGAGA^{1693}-3′ (sense) and pMv3r, 5′-^{2480}TCGGCAGTGCTGGCCCTACT^{2461}-3′ (anti-sense); pMv4f, 5′-^{1777}CACTCCCACGATTGGAGCCG^{1796}-3′ (sense) and pMv4r, 5′-^{3350}GCAGGTAAGTTGAGCTGTAG^{3331}-3′ (antisense); pMv5f, 5′-^{3000}AGGCAGAGATTCAGGCCGAG^{3019}-3′ (sense) and pMv5r, 5′-^{4462}TTGCTGGGCACTACGGTCTA^{4443}-3′ (antisense); pMv6f, 5′-^{4261}GGTTCAAGAAGACCTTATGT^{4280}-3′ (sense) and pMv6r, 5′-^{4795}TGTGGTTCGGTTGTGGAGTT^{4776}-3′ (antisense); pMv7f, 5′-^{5449}ATGTCCATCATGGGTCTCAA^{5468}-3′ (sense) and pMv7r, 5′- ^{7130}GTTTCAAGAGTTGTAGAGGA^{7111}-3′ (antisense); pMv8f, 5′-^{7000}GTTGCTGCAGGGGGCGTTGT^{7019}-3′ (sense) and pMv8r, 5′-^{8042}CTACTTCAAACACTCGGTGC^{8023}-3′ (antisense); pMv9f, 5′-^{7261}ATCATCCACAATGTCACCAC^{7280}-3′ (sense) and pMv9r, 5′-^{9144}AGATTGGTTCACTAGCAGCC^{9125}-3′ (antisense); pMv10f, 5′-^{9001}CTGGTGCCGTCACTTCTGTG^{9020}-3′ (sense) and pMv10r, 5′-^{10239}AATCTAGAGCTTCAATTAAC^{10220}-3′ (antisense); pMv11f,5′-^{10001}AAGAGTCAGATACATGTGGA^{10020}-3′ (sense) and pMv11r, 5′-^{11240}AAGGCAGTACTTCTTGAGAT^{11221}-3′ (antisense); pMv12f, 5′-^{11197}CAGTCAGTGCATTTATCACG^{11216}-3′ (sense) and pMv12r, 5′-^{12663}TATGGCTTCTTAGAGCTCTC^{12644}-3′ (antisense); pMv13f, 5′-^{12501}ATGAGGAAGGGGGGTTTAAC^{12520}-3′ (sense) and pMv13r, 5′-^{13900}AGT-

GAAGGACCATGGATAGG[13881]-3′ (antisense); pMv14f, 5′-[13857]TTCTGGCATTGTGGTATTAT[13876]-3′ (sense) and pMv14r,5′-[14797]GTGACTTCGGGCCTCCCGTT[14778]-3′ (antisense); and pMv15f, 5′-[14755]GTAATATTGTCAAAGTGCTC[14774]-3′ (sense) and pMv15r, 5′-[15894]ACCAGACAAAGCTGGGAATA[15875]-3′ (antisense). The cDNAs spanning the noncoding region between the M and F ORFs were difficult to obtain, but this difficulty was overcome using the Superscript one-step RT-PCR System (Gibco-BRL) and a gene-specific primer pair, 5′-[4735]CCAGCAACTGGAAGGGCCC[4753]-3′ and 5′-[5578]CACTTCCTATCCCTACCACC[5559]-3′. The cDNAs of leader and trailer regions were amplified using 5′ RACE (the rapid amplification of cDNA ends) system (Gibco-BRL). Using reverse transcriptase (Superscript II; Gibco-BRL) and gene-specific primers, 5′-[470]GGTACCTCTTGATGCGAAGG[451]-3′ (for leader) and 5′-[15181]CTTCACCTGGACTTATAGGT[15200]-3′ (for trailer), first-strand cDNAs were synthesized from genome RNA for trailer and antigenome RNA for leader. After first-strand cDNA synthesis, template RNAs were removed by RNase H. Oligo dC sequences then were added to 3′ ends of cDNAs using terminal deoxynucleotide transferase (TdT) and dCTP. After the tailing reaction, PCR amplification was performed using *Taq* DNA polymerase (Ex Taq, TaKaRa), the 5′ RACE anchor primer, 5′-CUACUACUACUAGGCCACGCGTCGACTAGTACGGGIIGGGIIGGGIIG-3′, and nested gene-specific primers, 5′-[53]AGTGCACTAGAAGATGATCA[34]-3′ (for leader) and 5′-[15268]GATATCAATCCTACTCTGA[15286]-3′ (for trailer). All amplified PCR fragments were cloned into the TA cloning vector pCR2.1 (Invitrogen, Carlsbad, CA), and three independent clones of each fragment were sequenced using an A.L.F. DNA sequencer II (Pharmacia, Uppsala, Sweden).

Sequence data analyses

The assembly of the obtained nucleotide sequences was performed using the GENETYX-MAC program (Software Development Co., Ltd., Tokyo, Japan). The assembled nucleotide sequence of 9301B genome, which is available with Accession No. AB012948, then was compared with that of Ed strain, which was obtained from DDBJ, EMBL, GenBank nucleotide sequence databases with Accession Nos. KO1711 and X16565. The sequence was revised according to the previous report (Radecke and Billeter, 1995); deletion of the A residue at nucleotide position 30 and insertion of one A residue into the polyadenylation signal of P gene. A neighbor-joining (NJ) phylogenetic tree (Saitou and Nei, 1987) was constructed using the 385-bp sequences (nucleotide positions 1302–1686) in the N gene of 117 MeV strains. Twenty-two of the sequences were determined in this study and are available with Accession Nos. AB019696–AB019703 and AB021997–022010. The previously reported sequences were obtained from DDBJ, EMBL, GenBank nucleotide sequence databases. The analysis was aided by CLUSTAL W program (Thompson *et al.,* 1994). Divergences were estimated by the two-parameter method (Kimura, 1980). The phylogenetic tree was generated using the TERRVIEW 1.4 program (Page, 1996). The bootstrap test was applied to estimate the confidence of the branching patterns of NJ tree (Felsenstein, 1985). The nomenclature of genetic subtypes were according to the standardization by the World Health Organization (1998). K_S and K_A in coding regions were calculated as described previously for another paramyxovirus, NDV (Sakaguchi *et al.,* 1989; Toyoda *et al.,* 1989). Briefly, at each position in the codon, three different single base changes are always possible, and of these, a fraction (f) results in synonymous substitution, whereas the remainder (1 − f) led to replacement substitutions. In the codon UUU, for example, the first two positions are replacement sites (f = 0), whereas for the last position one-third of changes correspond to synonymous sites (f = 1/3). Thus the codon UUU contains 0.333 (= 0 + 0 + 1/3) synonymous sites and 2.667 (= 1 + 1 + 2/3) replacement sites. Calculation of numbers of synonymous and replacement sites (NS and NA) and the numbers of synonymous and amino acid substitutions (MS and MA) was done by comparing a pair of nucleotide sequences in each coding region. K_S and K_A represent, respectively, MS/NS and MA/NA. Details of this procedure and its application for various genes have been described previously (Miyata and Yasunaga, 1980, 1981; Miyata *et al.,* 1980; Miyata and Hayashida, 1982).

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