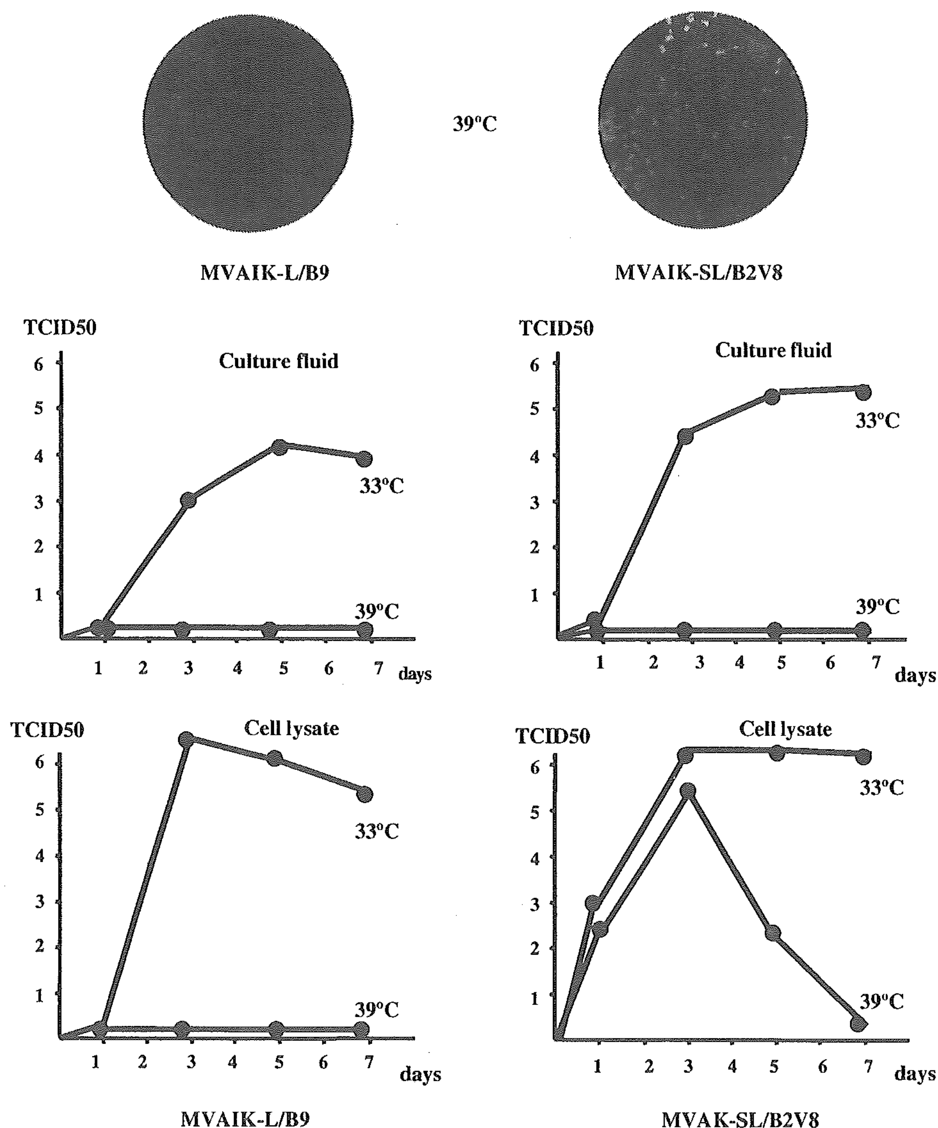

Fig. 2. Virus CPE and virus growth in Vero cells at different temperatures for MVAIK-L/B9 and MVAIK-SL/B2V8. Infectivity was examined in B95a cells.

of the C protein. Three amino acid substitutions, Lys N129 Gln (from Lys to Gln at the position 129 of the N protein), Ala L542 Thr and Gly L2032 Arg, were common in MVAIK-L/B9 and MVAIK-SL/B2V8. Amino acid changes except for the position 2032 of the L gene were all back-mutations to the Edmonston strain. MVAIK-SL/B2V8 had 13 amino acid changes that were the same as the Edmonston strain except for the position L2032. One silent mutation was observed in MVAIK-L/B9 and three in MVAIK-SL/B2V8.

MVAIK-SL/B2V8 had one amino acid change at the position 338 of the H protein region and three at the positions 278, 453, and 494 of the F protein region. The change at position 278 of the F protein region, which was critical for plaque size, was noted soon after large plaque cloning. MVAIK-SL/B2V2 had Phe at position 278 of the F region. We constructed the F and H expression plasmids from genomic RNA of MVAIK-SL/B2V8 and seven plasmids for F gene and five for H genes showed the same mutations as demonstrated in Table 1. These were expressed under the control of T7 RNA polymerase with no difference in the extent of cell fusion, which was demonstrated at 39 °C as well as at 33 °C (data not shown). Thus, the loss of *ts* was not related to the mutation in the F and H regions.

We cloned P gene region from genomic RNA of MVAIK-SL/B2V8 and all 11 clones showed the same mutations as shown in Table 1. Seven clones of the P gene from MVAIK-SL/B2V4 also showed the same mutations at the positions of C134, P275 and P439.

4. Discussion

The currently available further attenuated live measles vaccine strains, Moraten, Schwarz, Edmonston-Zagreb, and AIK-C, were developed from the Edmonston strain through extensive passages in different cells from susceptible primary

Table 1
Nucleotide differences and deduced amino acid changes of MVAIK-L/B9 and MVAIK-SL./B2V8 in comparison with the AIK-C vaccine strain and parental Edmonston strain

NT position (AA position)	Virus strain			
	AIK-C	Edmonston	MVAIK-L/B9	MVAIK-SL/B2V8
492 (N 129)	A (Lys)	C (Gln)	C (Gln)	C (Gln)
2229(C 134)	A (Tyr)	C (Ser)	*	C (Ser)
2630 (P 275)	A (Tyr)	G (Cys)	*	G (Cys)
3122 (P 439)	C (Pro)	T (Leu)	*	T (Leu)
6291 (F 278)	A (Leu)	C (Phe)	*	C (Phe)
6815 (F 453)	T (Leu)	C (Ser)	*	C (Ser)
6937 (F 494)	T (Cys)	A (Ser)	*	A (Ser)
8282 (H 338)	A (Thr)	C (Pro)	*	C (Pro)
10344 (L 371)	T (Trp)	A (Arg)	A (Arg)	*
10857 (L 542)	G (Ala)	A (Thr)	A (Thr)	A (Thr)
11158 (L 642)	G (Arg)	A (Gln)	*	A (Gln)
I1711 (L 826)	G (Ser)	A (Ser)	*	A (Ser)
12671 (L 1146)	A (Ala)	G (Ala)	G (Ala)	G (Ala)
14651 (L 1806)	A (Lys)	G (Lys)	*	G (Lys)
15039 (L 1936)	T (Tyr)	C (His)	*	C (His)
15327 (L 2032)	G (Gly)	G (Gly)	C (Arg)	C (Arg)
15622 (L 2130)	C (Thr)	T (Ile)	*	T (Ile)

(*) Represents no nucleotide change and underline shows the silent mutation.

cells used for virus isolation [16]. Differences in nucleotides of the Edmonston-derived vaccine strains were reported but the molecular basis of the attenuation has not been well defined as yet [17]. The AIK-C strain was developed through small plaque cloning in sheep kidney cells and chick embryonic cells and has two biological markers; small plaques in Vero cells and extremely low or no virus growth in culture at 39–40 °C. We reported that Leu at 278 position of the AIK-C virus F gene is related to the small plaques in the Vero cells and Phe at 278 of the F gene induces large plaques [4]. RNA virus has a nature of quasispecies and the AIK-C seed virus induced a mixture of small and medium sized plaques in Vero cells, having F278Leu (small plaque-type) and F278Phe (large plaque-type). The characteristics of temperature sensitivity depend upon the P protein, especially Pro at 439 of the P protein [8]. No infectious measles particle was detected in the culture medium of Vero cells at 39 °C culture infected with MVAIK-SL/B2V8 but infectious virus was obtained in cell lysate. It suggested that *ts* phenotype was lost during the passage in Vero cells (MVAIK-SL/B2V8). This was also found for the recombinant virus which the P protein of the AIK-C strain was replaced by that of the Edmonston strain [8]. Recombinant virus strains (MVAIK-SL/B2V8, MVAIK-Edm-P, and MVAIK-AK/Edm-P) produced infectious particles in the cell lysate but not in supernatants. We could not identify the reason why these strains have cell-associated characteristics at 39 °C culture.

As for the vaccine production, passages are limited to five times from the seed virus to prevent the accumulation of mutations. Most RNA viruses are considered to be a mixture of heterogeneous viruses because of their high incidence of mutation in the RNA genome in nature [18]. The seed of the AIK-C strain was established through plaque purification but may consist of heterogeneous viruses. In contrast, MVAIK strains rescued from the cloned cDNA are thought to be rather homogenous single genome species even after two passages in B95a cells. To investigate the genetic stability of recombinant measles virus rescued from the cloned cDNA, virus was passaged in Vero cells and B95a cells. In Vero cell passage, MVAIK-SL/B2V8 had a total of 13 amino acid differences; one in the N protein, two in the P, one in the C, three in the F, one in the H, and five in the L genes. The significance of the amino acid positions of 278 of the F protein and 439 of the P protein were elucidated in this report. In B95a cell passage, MVAIK-L/B9 had four amino acid differences; one in the N protein and three in the L gene. Four mutations observed for the MVAIK-L/B9 were common in the MVAIK-SL/B2V8 and all mutations were back mutations to the Edmonston strain except at position 2032 of the L protein region. Mutations have a fixed tendency of back-mutation to the parental wild-type Edmonston strain, not showing random mutation and the rate of mutation was higher through passages in Vero cells than in B95a cells.

The establishment of further attenuated live vaccines is thought as a selection of a mutant virus that can adapt to grow in different cell cultures from susceptible hosts. Vaccine seed might have a mixture of quasispecies of genomes and an extremely low proportion of minority genome was present as their past dominance. In biological adaptive systems, evolving viral quasispecies possessed a molecular memory in the form of minority components that populate their mutant spectra [18–20]. These were demonstrated in the selection of biological clones of foot-and mouth disease virus (FMDV) resistant to a monoclonal antibody [19]. Domingo et al. [18] reported that memory was a property of the quasispecies as a whole, and not the result of an inherent tendency of the dominant genomes to mutate to the memory genomes.

In this study, we started to generate a single genome construct from the vaccine seed strain having representative biological characteristics; introducing small plaques in Vero cells and *ts* phenotype. Infectious virus was recovered after two passages and was further propagated in Vero cells or B95a cells. After eight passages, it showed mutations in the fixed direction to the original Edmonston strain as a memory of a past attenuation process through passages at lower temperatures and different cells. Passage in B95a cells showed to induce less mutations and the genome soon after the rescue of the virus seems to be homogeneous. Large plaques were produced at the first passage in Vero cells and we selected a large plaque generated from the small plaque type genome. Thus, early genomic changes were supposed to be a mutation rather than selection. From the results of cloning experiments of the F and P gene, the critical mutations were demonstrated at early passage history. We speculate that the genome had a memory of the attenuation process during the passage in different cell lines. The mutated positions were genetically unstable and could be easily back-mutated in Vero cells.

Infectious virus was recovered in B95a cells and additional eight passages in B95a cells did not influence the biological phenotype without mutation in the P genes related to the *ts* phenotypes. However, in the Vero cells large plaques were observed at the first passage and cloned virus had Phe at position 278 of the F gene.

In summary, recombinant virus recovered from infectious cDNA clone was back-mutated to the parental Edmonston strain, losing *ts* phenotypes and small plaque inducibility after passages in Vero cells and we speculated that the genome had past mutation memory.

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