

***In Vitro* Recombination of Poliovirus with Coxsackie A Virus Serotype 18 at Downstream Nonstructural Protein-Coding Regions**

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Many genetic recombinations of poliovirus (PV) are to be found in excreted viruses, including viruses from vaccine-associated paralytic poliomyelitis (VAPP) as well as healthy vaccine recipients. Most recombinations were among different serotypes of PVs. However, recombination can also occur between PV and other enteroviruses. It was predicted that the hot spot of the recombination is in the nonstructural protein-coding regions, but the exact site is may be different in each recombination. We have demonstrated that the construct recombinant virus between PV and coxsackie A virus serotype 11 (CAV-11), or with CAV-17 with recombination site in the N-term of 2C-coding region, were viable. However, the recombination of PV with CAV-18 at this site was not viable. To determine if the recombination between PV and CAV-18 can occur at other sites, eight chimeric cDNAs (between PV [isolate PJ156] and CAV-18 [PJ156/CAV-18]), all having different recombination sites (2C-8, 2C-133, 2C-235, 2C-268, 2C-287, 2C-327, 3A-67, 3C-60) were constructed using the long-PCR method. The cDNA was then transcribed *in vitro* and then transfected into the HEP-2 cell-line. As expected, the recombinant virus PJ156/CAV-18, with recombination sites 2C-327, 3A-67, and 3C-60 were viable, while all the others were not. The recombinant viruses displayed a slightly smaller plaque size, but demonstrated quite similar growth as compared to the parental control PJ156. Since analysis for similarity has shown that the homology between PV and CAV-18 was high around these regions, these results supported the copy-choice mechanism of enterovirus recombination.

Key words: poliovirus, CAV-18, recombination

Recombination is integral to the evolution of enteroviruses, including poliovirus (PV). The first evidence of recombination of PV was detected in vaccine-related isolates with chimeric sequences excreted by children exposed to the trivalent oral poliovirus vaccine (OPV) (King 1988; Cammack *et al.* 1988). Similar isolates were later detected in a number of patients with VAPP (Driesel *et al.* 1995; Li *et al.* 1996; Martin *et al.* 2002) as well as in healthy OPV recipients (Cammack *et al.* 1988; Tatem *et al.* 1991; Blomqvist *et al.* 2003). The heterologous sequences of most vaccine-related isolates were derived from the other Sabin OPV strains, with recombinants frequently found among vaccine-related isolates of all serotypes (Cammack *et al.* 1988; Lipskaya *et al.* 1991). A small proportion of vaccine-related isolates have capsid sequences derived from the OPV strains and noncapsid sequences derived from other, nonvaccine viruses. Recombination among the OPV strains is readily detectable because the sequences of the parental vaccine strains are well defined (Toyoda *et al.* 1984).

Recombination also occurs during the circulation of wild-type polioviruses and other enteroviruses. Recombination of OPV and circulating wild-type PV was reported in China (Liu *et al.* 2000; Liu *et al.* 2003), and recombination of OPV and circulating non-polio enteroviruses were reported in the Dominican Republic and Haiti (Kew *et al.* 2002), in Madagascar (Rousset *et al.* 2003), in Egypt (Yang *et al.* 2003), and in the Philippines (Shimizu *et al.* 2004). Sequence analysis showed that the above circulating vaccine-derived polioviruses (cVDPVs) were recombinants between PV and unidentified enterovirus that underwent recombination in the nonstructural protein-coding regions of the genome.

Although the significance and the mechanism of natural genetic recombination are still not understood, it can be suggested that it has a biological role in genetic recombination in PV evolution, especially for the prolonged circulation of OPV-related PV.

Details of the mechanism of PV recombination are not well understood. However, based on current knowledge, the recombination is believed to occur by the 'copy-choice' mechanism with homologous genome templates (Wimmer *et al.* 1993). Generally, for single-stranded RNA viruses such as PV, the mechanism is probably copy choice (template switching during RNA replication) rather than through true recombination i.e. the mechanism is analogous to gene conversion (Kirkegaard and Baltimore 1986). However, it may be different for the different virus recombinations. Furthermore, the hot spot of recombination in the genome of PV is not well defined. We previously demonstrated that chimeric cDNA constructed between PV and CAV-11 or CAV-17, with the crossover site in the N-term. part of 2C-coding region, resulted in viability of the virus (Utama and Shimizu 2005; Utama and Shimizu 2006). However, the virus was not viable when an RNA transcript derived from chimeric cDNA between PV and CAV-18, with the same crossover site, was transfected into the cell-line. In this study, chimeric cDNAs between PV and CAV-18, with various crossover sites, were artificially constructed. The RNA transcripts were then transfected into HEP-2 cell-line. The viability of the virus was analyzed and the viable recombinant viruses were characterized.

MATERIALS AND METHODS

Viral RNA Extraction and Construction of Chimeric cDNA. PJ156 isolated from an acute flaccid paralysis case in

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the Philippines in 2001 (Shimizu *et al.* 2004), and CAV-18 stored in our laboratory, were used as the parental viruses. Viral RNAs were extracted from freeze-thawed lysates of infected cell culture-supernatants using a High-Pure-Viral-RNA Kit (Roche, Germany). Chimeric cDNA constructs between PJ156 and CAV-18 were produced using the long-PCR method, similarly as previously described (Fig 1) (Utama and Shimizu 2005; Utama and Shimizu 2006). Briefly, the 5' and 3' end of the genome of both viruses were separately amplified by the RT-PCR reaction using the Titan One Tube RT-PCR System (Roche). Twenty-five cycles of the PCR reaction were performed after 40 min of the RT reaction. Amplified cDNA fragments were purified with Wizard SV Gel and PCR Clean-Up System (Promega, Madison, USA). These fragments were then fused to each other in a PCR reaction using the Expand™ Long Template PCR System (Roche, Germany) to obtain a target of chimeric full-length cDNA.

RNA Transcription and Transfection. Full-length chimeric cDNAs were produced using *in vitro* transcription by using the RiboMAX™ Large-Scale RNA-Production System (Promega). Five ml aliquots from 20 ml RNA transcript solution were mixed with 100 ml of 0.5 mg·ml⁻¹ DEAE dextran in 1× HeBSS buffer (5 g·l⁻¹ of HEPES, 8 g·l⁻¹ of NaCl, 0.36 g·l⁻¹ of KCl, 0.125 g·l⁻¹ of Na₂HPO₄ 2H₂O, 10 g·l⁻¹ of dextrose), as previously described (Utama and Shimizu 2005; Utama and Shimizu 2006). The transcript-DEAE-dextran mixtures were spread over HEp-2 cells in 24-well plates. The cells were then rocked on a shaker for 30 min at room temperature. The fluids were aspirated off and the cells then incubated at 35 °C in Dulbecco's modified Eagle's medium supplemented with 2% bovine-calf-serum (maintenance medium). Cytopathic effects were observed up to 7 days. HEp-2 cells were subsequently infected with recombinant viruses which showed CPE to confirm the infectivity of the viruses. If CPE was not observed, a 3x blind passage was performed to confirm that no virus had been recovered from the RNA transfection.

Plaque Purification of the Recombinant Virus. All recovered viruses were cloned by plaque-assay on an

HEp-2 cell monolayer (Utama and Shimizu 2005; Utama and Shimizu 2006). A ten-fold serial dilution of viruses, prepared in the maintenance medium, were inoculated in HEp-2 cells using 6-well plates, and incubated at 35 °C for 30 min. The cells were covered with 2 ml of 0.5% (w/v) Agarose-ME in DMEM with 5% (v/v) bovine calf serum. After incubation at 35 °C for 3 days, plates were stained with 2 ml of 0.5% (v/v) neutral-red in maintenance medium containing 0.5% (w/v) Agarose-ME. Plaque size was measured, and plaque numbers were calculated after incubation at 35 °C for a further day.

One-Step Growth-Curve and Temperature Sensitivity Analyses. One-step growth-curve experiments were conducted by infecting a monolayer of HEp-2 cells with viruses at a multiplicity of infection of 10 CCID₅₀ per cell (Utama and Shimizu 2005; Utama and Shimizu 2006). At different times after infection, the cells and supernatant were collected, frozen and thawed three times, and then centrifuged (10 000 x g, 5 min) to remove cell debris. Virus titers in the supernatants were determined by the end-point dilution method in HEp-2 monolayer-cultures in 96-well plates at 35 °C. To test temperature sensitivity, one-step growth experiments were carried out at 35 °C and at 39.5 °C, respectively.

RESULTS

Construction Chimeric Virus PJ156/CAV-18. cDNA chimeras between PJ156 and CAV-18, with a crossover site in the N-term. part of 2C-coding region (amino acid no. 8 of 2C), (PJ156/CAV-18(2C-8)) were firstly constructed (Fig 2). After RNA transfection into HEp-2 cells and incubation at 35 °C, no CPE was observed, which implied that the virus was not viable. These results suggested that recombination might be not occurring between PJ156 and CAV-18 at the 2C-8 site. To find out whether recombination can occur at other sites, various chimeric cDNAs between PJ156 and CAV-18 with different crossover sites (2C-8, 2C-133, 2C-235, 2C-268, 2C-287, 2C-327, 3A-67, 3C-60) were constructed using appropriate sets of primers (Table 1). After RNA transfection into HEp-2 cells following by incubation at 35 °C, only the

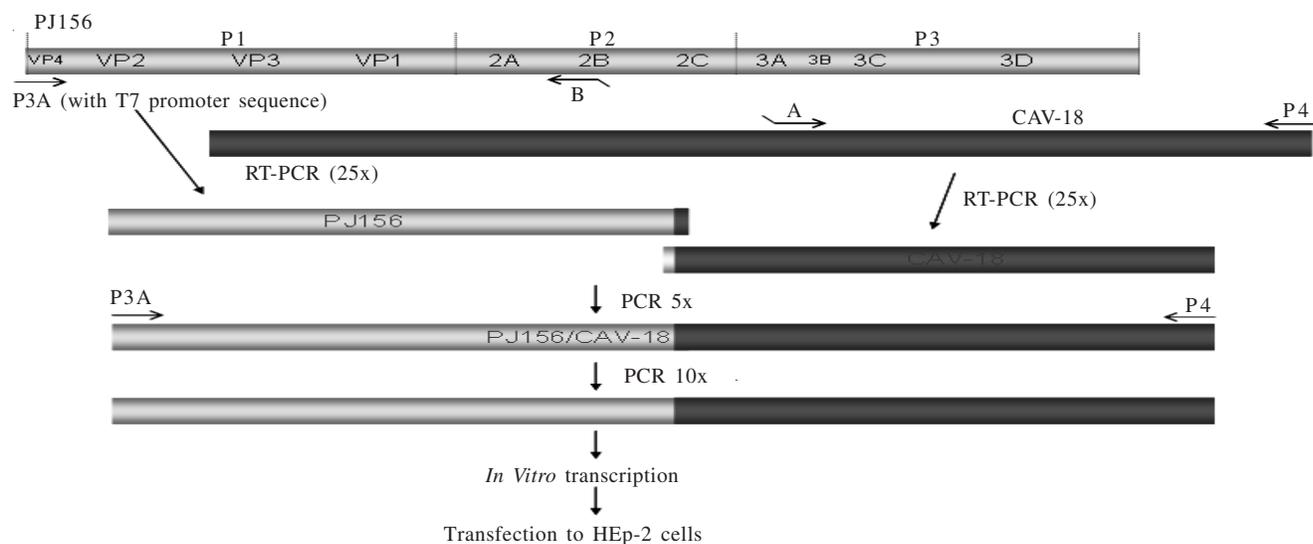


Fig 1 Strategy for construction of chimeric virus PJ156/CAV-18. The detail procedure in Materials and Methods.

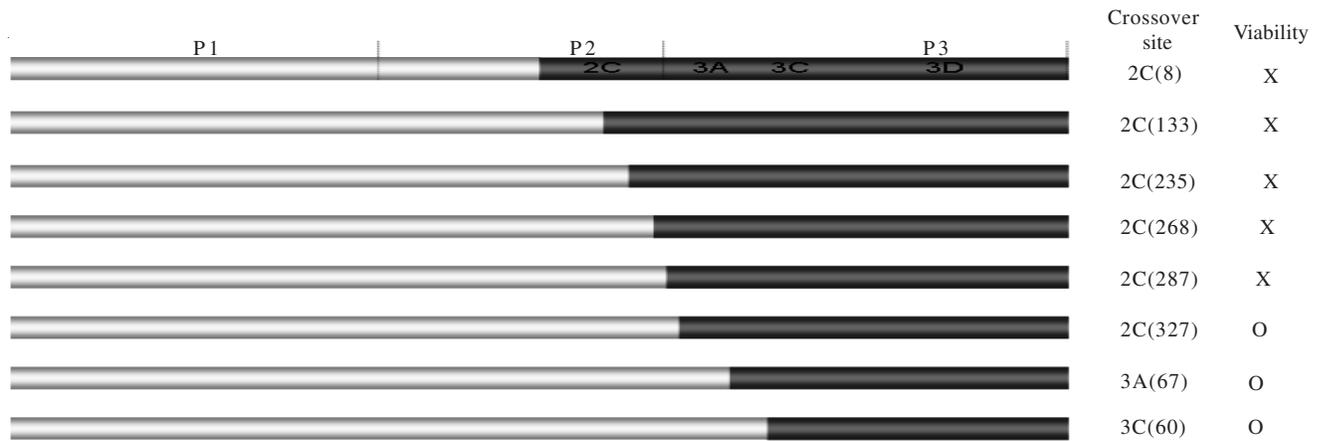


Fig 2 Viability of recombinant viruses derived from chimeric PJ156/CAV-18 cDNA. The number in parentheses describes the number of amino acid in each protein.

Table 1 Primers used for construction of chimeric cDNAs

Recombinant	Crossover site*	Sequence	A/B
PJ156/CAV-18 (2C-8)	2C (8)	5'-GGAGATAGCTGGCTCAAGAAAGTTCACAGAGGCGTGCAATGC-3'	A
		5'-GCATTGCACGCCTCTGTGAACCTTCTTGAGCCAGCTATCTCC-3'	B
PJ156/CAV-18 (2C-133)	2C (133)	5'-GGTGACGGCAGCCAGGGACAGGAAAATCTGTTGCCACCAGC-3'	A
		5'-GCTGGTGGCAACAGATTTTCTGTCCCTGGGCTGCCGTGCACC-3'	B
PJ156/CAV-18 (2C-235)	2C (235)	5'-CACCCCAACTGTGGCTCACAGTGATGCCCTAAGCCGG-3'	A
		5'-CCGGCTTAGGGCATCACTGTGAGCCACAGTTGGGGGTG-3'	B
PJ156/CAV-18 (2C-268)	2C (268)	5'-GGCGATGGCCACTGAAATGTGCAAGGAATGCCCTCAACCAGC-3'	A
		5'-GCTGGTTGAGGGCATTCTTGACATTTTCAGTGCCATCGCC-3'	B
PJ156/CAV-18 (2C-287)	2C (287)	5'-CCCTTGGTGTGTGGCAAGGCCATCCAATTAATGGAC-3'	A
		5'-GTCCATTAATGGATGGCCTTGCCACACACCAAGGG-3'	B
PJ156/CAV-18 (2C-327)	2C (327)	5'-GGAACTGTATGGAAGCTCTGTTCCAAGGACCAATCAGC-3'	A
		5'-GCTGATTGGTCTTGGAACAGAGCTTCCATACAGTTCCC-3'	B
PJ156/CAV-18 (3A-67)	3A (67)	5'-CCTCCAGCTGTCACTACCTTTGCAGCTGTGGCTGG-3'	A
		5'-CCAGCCACAGCTGCAAAGGTAGTGACAGCCTGGAGG-3'	B
PJ156/CAV-18 (3C-60)	3C (60)	5'-GGAGTTGAGGTGTTAGACGCCAAGGCCCTGAAGACCAGGCAGGG-3'	A
		5'-CCCTGCCTGGTCTTCAAGGGCCTTGCGCTTAACACCTCAACCTCC-3'	B
P3A		5'-CATGCTAATACGACTCACTATAGGTTAAAACAGCTCTGGG-3'	**
P4		5'-TTTTTTTTTTTTTTTTTTTTTTTCTCCG-3'	

* () described amino acid no. in each protein, ** T7 promoter sequence is underlined.

Table 2 Plaque phenotype and amino acid substitution in determined region of each recombinant virus

Virus	Crossover site*	Plaque size (mm)	Titer (pfu/ml)
PJ156	-	2-6	6.6 x 10 ⁸
PJ156/CAV-18 (2C-327)	2C (327)	1-2	3.5 x 10 ⁸
PJ156/CAV-18 (3A-67)	3A (67)	1-4	3.4 x 10 ⁸
PJ156/CAV-18 (3C-60)	3C (60)	1-4	2.6 x 10 ⁸

* () described amino acid no. in each protein.

recombinant PJ156 and CAV-18 with crossover sites at 2C-327, 3A-67, and 3C-60 (each virus designated as PJ156/CAV-18(2C-327), PJ156/CAV-18(3A-67), and PJ156/CAV-18 (3C-60)) were viable, whereas recombinant viruses with crossover sites at (2C-8, 2C-133, 2C-235, 2C-268, 2C-287 were not viable (Fig 2). We also attempted to recover non-viable viruses by transfecting the cells with the RNA derived from their chimeric cDNAs and incubating at 30 °C. However, no CPE was observed, even though the process was repeated for another two times. These results suggested that recombination could only occur between PJ156 and CAV-18 at downstream of 2C-coding regions, different with recombination of PV and CAV-11 and CAV-17 as previously reported (Utama and Shimizu 2005; Utama and Shimizu 2006).

Plaque Assay of Recombinant Viruses. Plaque assay of recovered recombinant viruses, along with their parental

PJ156 isolate, was performed to analyze the effect of recombination on viral phenotype. The results showed that PJ156/CAV-18 (2C-327) expressed small plaques (1-2 mm in diameter), as compared with PJ156 (2-6 mm in diameter) (Table 2, Fig 3). Two other recombinant viruses, PJ156/CAV-18 (3A-67) and PJ156/CAV-18 (3C-60), showed intermediate-sized plaques (1-4 mm in diameter), but still smaller compared to the parental PJ156. The titer of all recombinant viruses was also varied, namely 3.5 x 10⁸ pfu·ml⁻¹, 3.4 x 10⁸ pfu·ml⁻¹, and 2.6 x 10⁸ pfu·ml⁻¹, respectively for PJ156/CAV-18 (2C-327), PJ156/CAV-18 (3A-67), and PJ156/CAV-18 (3C-60), and was close to half that of the parental PJ156 (6.6 x 10⁸ pfu·ml⁻¹) (Table 2). These findings suggest that recombination between PJ156 and CAV-18 at different crossover sites resulted in only slight or no differences in phenotypes as defined by plaque size and virus titer.

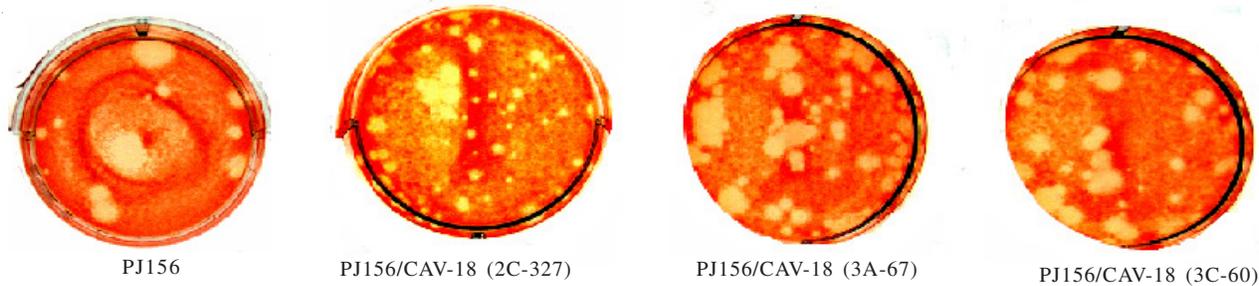


Fig 3 Plaque size of the parental and recombinant viruses. The positions of crossover site are described in parentheses.

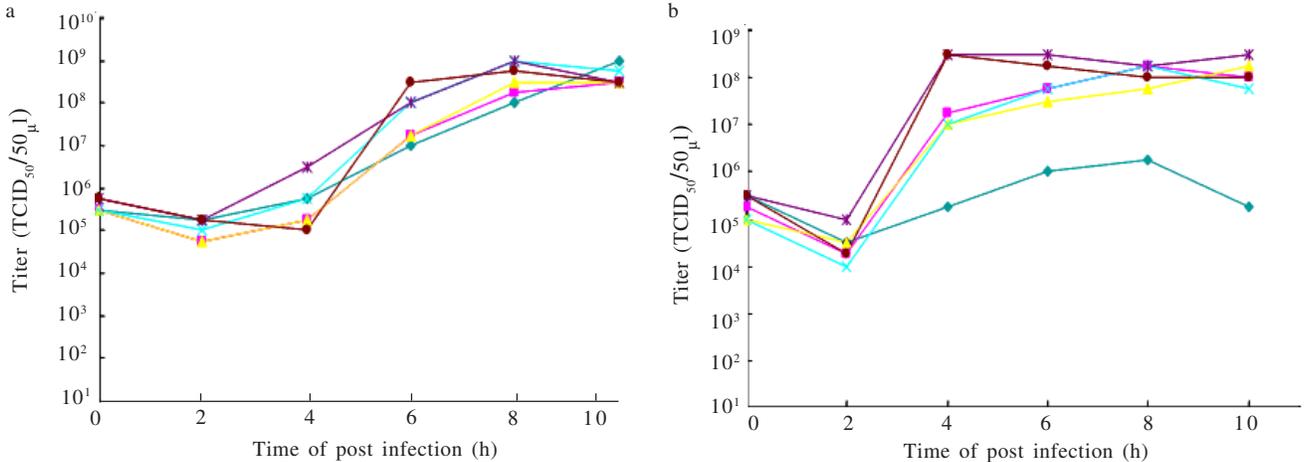


Fig 4 One-step growth of viruses at 35 °C (a) and 39.5 °C (b). —●— Sabin 1, —■— Mahoney PJ156, —▲— PJ156, —×— 156/CA 18(2C-327), —*— 156/CA 18(3A-67), —●— 156/CA 18(3C-60).

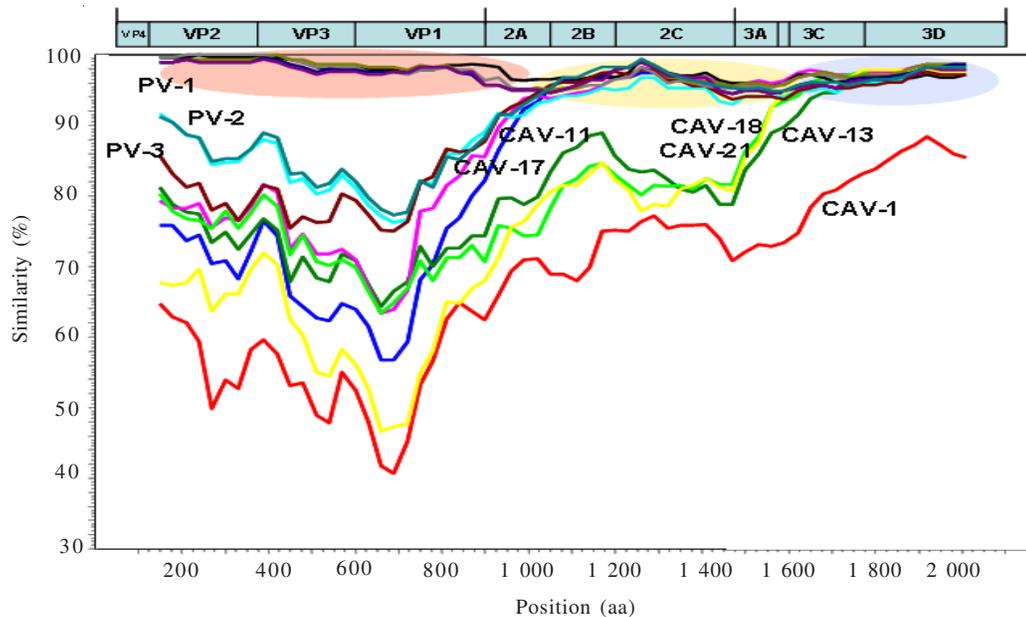


Fig 5 Similarity analysis between PV and some coxsackei A viruses based on the entire amino acids using SimPlot software version 3.5.1. PV-1, poliovirus serotype 1; PV-2, poliovirus serotype 2; PV-3, poliovirus serotypy 3; CAV-1, coxsackie A virus serotype 1; CAV-11, coxsackie A virus serotype 11; CAV-13, coxsackie A virus serotype 13; CAV-17, coxsackie A virus serotype 17; CAV-18, coxsackie A virus serotype 18; CAV-21, coxsackie A virus serotype 21.

One-Step Growth of Recombinant Viruses. A one-step growth experiment of the recombinant viruses, along with their parental viruses at 35 and 39.5 °C, was conducted to analyze the effect of recombination on viral growth and temperature sensitivity. PJ156/CAV-18 (2C-327), PJ156/CAV-18 (3A-67) and PJ156/CAV-18 (3C-60), PJ156/CAV-17 showed a similar growth pattern compared to the parental PJ156 at

35 °C (Fig 4a). Other reference viruses such as Sabin 1 and Mahoney strains showed a similar pattern at this temperature. All the PJ156/CAV-18 recombinant viruses also demonstrated a similar growth to the PJ156 and Mahoney at 39.5 °C (Fig 4b), which means that all the viruses were equally temperature sensitive. Moreover, the Sabin 1 virus was sensitive to this temperature. These results imply that recombination between

PJ156 and CAV-18 at different crossover site in nonstructural protein-coding region result in recombinant viruses which have similar temperature-sensitivity characteristics.

DISCUSSION

Recombination has been shown to occur in PV, either among vaccine strains (Cammack *et al.* 1988; Lipskaya *et al.* 1991) or between vaccine strains and wild-type PV (Liu *et al.* 2000; Liu *et al.* 2003) and other unidentified non-polio enteroviruses (Rousset *et al.* 2003; Yang *et al.* 2003; Shimizu *et al.* 2004). We have shown that PV could recombine with CAV-11 or CAV-17 at the N-term. part of the 2C-coding region (Utama and Shimizu 2005; Utama and Shimizu 2006). However, the CPE was not apparent when RNA derived from chimeric cDNA of PJ156/CAV-18, with the crossover site at N-term. part of 2C-coding region, was transfected into the HEp-2 cell-line. Similarity analysis has demonstrated that homology was high between PV and CAV-18 at the 3 region (represents 3A, 3B, 3C, and 3D regions) (Utama and Shimizu 2006) (Fig 5). Therefore, it is predicted that PV can recombine with CAV-18 in these regions. However, there is no direct evidence to prove this hypothesis. By using the long-PCR method, eight chimeric cDNAs between PJ156 and CAV-18, each with different crossover sites, were constructed. The chimeric cDNAs were respectively designated PJ156/CAV-18 (2C-8), PJ156/CAV-18 (2C-133), PJ156/CAV-18 (2C-235), PJ156/CAV-18 (2C-268), PJ156/CAV-18 (2C-287), PJ156/CAV-18 (2C-327), PJ156/CAV-18 (3A-67), and PJ156/CAV-18 (3C-60). After *in vitro* transcription, each transcript was transfected into HEp-2 cell-line. As expected, PJ156/CAV-18 (2C-327), PJ156/CAV-18 (3A-67), and PJ156/CAV-18 (3C-60) showed CPE in the cell-line, while others did not (Fig 2). These results imply that recombination can occur at a site in which the homology is high between both viruses. Based on this evidence, it is suggested that the recombination between PV and CAV-18 occur by a 'copy-choice' mechanism.

PJ156/CAV-18 (2C-327) showed smaller plaque size (1-2 mm) compared to PJ156/CAV-18 (3A-67) and PJ156/CAV-18 (3C-60) (1-4 mm), and compared with the parental PJ156 (2-6 mm). The titer of the recombinant viruses also varied from 2.6×10^8 to 3.5×10^8 pfu·ml⁻¹, and was close to half that of the parental PJ156 strain (6.6×10^8 pfu·ml⁻¹) (Table 2). These findings suggest that the recombination between PJ156 and CAV-18 at different crossover sites resulted in only very slight or no differences in phenotypes for plaque sizes and virus titer, but different phenotype compared to the parental PJ156. Also all of the PJ156/CAV-18 recombinant viruses demonstrated a similar growth pattern both at 35 °C (Fig 4a) and at 39.5 °C (Fig 4b). Thus, all the recombinant viruses were similarly temperature resistant (Fig 4b). This is the same as for the parental PJ156 and Mahoney strains, which are highly pathogenic strains (Fig 4b), suggesting that recombination between PV and CAV-18 may result in viruses which have characteristics similar with highly pathogenic viruses. To elucidate to pathogenic characteristic of the

recombinant viruses, however, the neurovirulence test using PV receptor-transgenic mice has to be performed.

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