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Detection of Respiratory Syncytial Virus Genome by Subgroups-A, B Specific Reverse Transcription Loop-Mediated Isothermal Amplification (RT-LAMP)

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Annual seasonal outbreaks of respiratory syncytial virus (RSV) infection occur every winter. Most patients are diagnosed clinically by a rapid detection kit for RSV protein(s) from nasopharyngeal secretion (NPS), but some problems have been reported on the specificity and sensitivity of such rapid detection kits. To ratify these issues, a sensitive, specific, simple, and rapid molecular based diagnostic method is expected to be introduced and we have developed a method to detect the RSV genome of subgroups A and B independently by reverse transcription loop-mediated isothermal amplification (RT-LAMP). We detected the genomic RNA corresponding approximately to 0.1 TCID 50 in the sample by RT-LAMP for both RSV subgroups under isothermal condition within 60 min after extraction of RNA. Specific DNA amplification was monitored by a real-time turbidimeter and the quantity of RNA was calculated. The RSV genome was detected in 47 of 50 NPS by RT-LAMP, and in 42 by nested RT-PCR, whereas virus isolation was positive for 29 and enzyme-linked immunoassay (EIA) for 34. RSV subgroup A was detected in 25 by RSV RT-LAMP A, RSV subgroup B in 23 by RSV RT-LAMP B, and dual infection with RSV subgroups A and B was identified in one case. They were confirmed with digestion with a specific restriction enzyme, Bgl II. The results showed the potential clinical feasibility of RT-LAMP as a useful diagnostic tool for the detection of RSV with high sensitivity similar to nested RT-PCR. ***J. Med. Virol. 77:121–127, 2005.*** © 2005 Wiley-Liss, Inc.

KEY WORDS: respiratory syncytial virus (RSV); subgroups A and B; reverse transcription polymerase chain reaction (RT-PCR); reverse transcription loop-mediated isothermal amplification (RT-LAMP); rapid diagnosis

INTRODUCTION

Respiratory syncytial virus (RSV) is a single-stranded negative-sense RNA virus and one of the most important pathogens for lower respiratory tract infection in infants and children, which belongs to a member of the genus Pneumovirus of the family Paramyxoviridae with two distinct different subgroups A and B. RSV has two major envelope glycoproteins F and G and small hydrophobic protein. The G protein plays an important role in the initial step of viral attachment to the cell and subsequent membrane fusion to allow viral penetration [Collins et al., 2001]. The G protein region is hypervariable and used for the target of genotyping [Johnson and Collins, 1989; Cane and Pringle, 1995; Kamasaki et al., 2001; Seki et al., 2001] but we reported on RT-PCR to detect the N gene from clinical samples [Yui et al., 2003].

RSV is a ubiquitous respiratory virus pathogen and all infants have experienced RSV infection by 2 years of age [Glezen et al., 1986]; reinfection is a common event in older infants and children, as well as adults [Henderson et al., 1979; Hall et al., 1991]. Through repetitious infection by RSV, herd immunity develops

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and the symptoms become milder. RSV occasionally causes serious respiratory infections in premature newborns, infants having cardiopulmonary disorders, and immuno-compromised infants [Anas et al., 1982; Hall et al., 1986]. RSV infection results in the development of serious illness and some infants infected with RSV are required to be hospitalized [Hall et al., 1990]. In these situations, infection-control for the inpatients is always focused in winter season. In young healthy adults, RSV infection usually causes a milder form of illness and often remains undiagnosed [Hall, 2001]. In the elderly, it frequently results in hospitalization with severe or sometimes fatal cardiopulmonary complications [Falsey et al., 1995]. Repeated infections, therefore, occur throughout life, but the significance of RSV infection in the elderly is not fully accepted because of the poor sensitivity of RSV detection kits in the case of lower virus load [Casiano-Colon et al., 2003].

The laboratory diagnosis of RSV infection was performed classically by virus isolation but this method is time-consuming and not appropriate for a clinical setting with a lower sensitivity in comparison with reverse transcription polymerase chain reaction (RT-PCR) [Falsey et al., 2003]. At present, several kinds of simple, specific, sensitive, and rapid diagnostic kits are available, which are based on an enzyme-linked immunoassay (EIA) for the detection of RSV specific antigen(s). The limitation of some kits is their sensitivity and specificity for clinical usage. A low level of RSV load was not detected and false positives resulted in useless triage, long-term isolation, and unnecessary medical cost for infection control. In some virus laboratories, molecular based diagnostic methods are employed such as RT-PCR [Johnson and Collins, 1989; Yui et al., 2003]. A new sensitive method for DNA amplification, termed loop-mediated isothermal amplification (LAMP), was developed by Notomi et al. [2000]. It employs a strand displacement DNA polymerase and a set of specially designed primers recognizing different regions in the targeted DNA sequence without PCR-like cycling reactions of denature, annealing and extension. We developed a new method for the detection of the RSV genome by reverse transcription LAMP (RT-LAMP) and compared its sensitivity with EIA antigen detection kit, virus isolation and nested RT-PCR.

MATERIALS AND METHODS

Clinical Samples

We obtained NPS samples from patients clinically suspected as having RSV infection from December 2002 to April 2003 to compare the sensitivity of rapid diagnostic kits, virus isolation, conventional RT-PCR, and RT-LAMP. NPS samples were obtained by suctioning with a sterile plastic mucus extractor with 5 ml of PBS from both nostrils. All samples were examined instantly to detect the presence of the RSV antigen by EIA using Testpack RSV (Abbot Laboratories, North Chicago, IL). The remaining was kept at −70°C until virus isolation and RNA extraction.

Virus Isolation

A portion (0.4 ml) of the specimen was inoculated onto roller-tube cultures of HEp-2 cells and the appearance of characteristic CPE was observed for 2 weeks. HEp-2 cells were cultivated in Eagle's minimum essential medium (MEM) supplemented with 10% fetal calf serum (FCS), appropriate antibiotics and maintained in MEM with 2% FCS [Yui et al., 2003].

RNA Extraction and RT-PCR

Total RNA was extracted from 200 μl of NPS with a magnetic beads RNA purification kit (TOYOBO Co. Ltd, Osaka, Japan) and the RNA pellet was suspended in 25 μl of distilled water. It was subjected to RT-PCR and RT-LAMP. Nested PCR was targeted for the nucleocapsid (N) gene of RSV [Johnson and Collins, 1989; Yui et al., 2003]. Virus genomic RNA was first converted into cDNA by AMV reverse transcriptase (Life Sciences, Inc., St Petersburg, FL) at 50°C for 1 hr using C-RSN+ (5′-GGGTCGACAATTCACTGGGTTAATACCTAT-3′). The first PCR was performed with a set of primers, RSN-F1+ (5′-GCCCCGGGGAGATAGAATCTAGAAAATCCT-3′) and RSN-B1-(5′-GCGGAGCTCTTTGGGTTGTTCAA-TATATGG-3′). The first PCR was performed using 1.25 U of Ex Taq DNA polymerase (TaKaRa BioMedicals, Tokyo, Japan) and the first five cycles were at 94°C for 30 sec, 50°C for 1.5 min, and 73°C for 1.5 min. They were followed by 30 cycles of denature at 93°C for 1 min, reannealing at 58°C for 1 min, and extension at 72°C for 2.5 min, with a final extension period of 5 min at 72°C. For the nested PCR for the N gene, 460 nucleotides were amplified with a set of RSN-F2+ (5′-CCGGTACCGAA-ATGGGAGAGGAGCTCC-3′) and RSN-B2− (5′-CCGC-ATGCATAAACCCAACAACTTG TTCC-3′) with 1.25 U of Taq DNA polymerase (TaKaRa BioMedicals, Tokyo, Japan), as reported previously [Yui et al., 2003]. PCR products were electrophoresed through 2% agarose gel.

RSV RT-LAMP

LAMP was characterized by auto-cycling strand displacement DNA synthesis performed by a DNA polymerase with high strand displacement activity and by a specially designed set of primers. Diagram of RT-LAMP is shown in Figure 1. We synthesized six primers; two outer primers (F3 and B3), two inner primers, a forward inner primer (FIP) and backward inner primer (BIP), and two additional loop primers (Loops F and B). FIP contains an alignment complementary to F1 linked with the F2 sequence, and BIP contains a B1 sequence linked with a sequence complementary to B2 region. These four primers amplified the targeted DNA and we synthesized two loop primers, F and B located between F1 and F2, between B1 and B2, respectively. The addition of two loop primers enhances the specificity and reactivity [Nagamine et al., 2002]. Genomic RNA is first converted to cDNA with F2 portion of FIP (Fig. 1①). Outer F3 primer anneals to the same genome RNA and begins cDNA synthesis with strand displacement of cDNA-RNA complex formed by FIP primer (Fig. 1②).

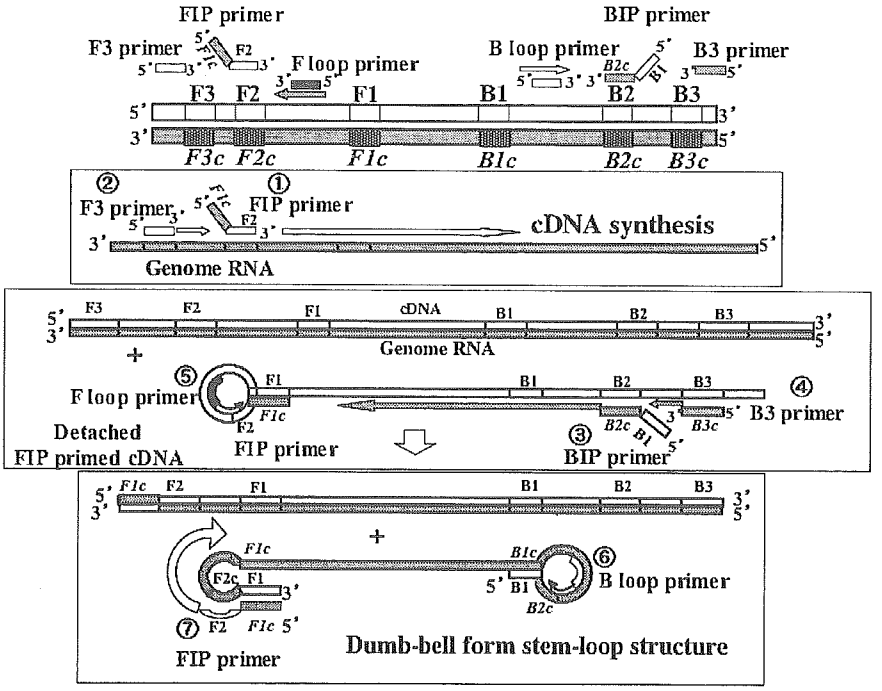


Fig. 1. Diagram of RT-LAMP reaction. Unfilled squares and bars mean positive sense portions and filled dotted squares and bars mean negative sense portions. Arrows show the direction of elongation reaction. cDNA is synthesized by FIP and F3 primers(① and ②). BIP and B3 primers anneal to the FIP primed cDNA (③ and ④) and loop structure is constructed at the 5′ end of FIP primed cDNA (⑤). F loop primer binds the free loop portion and detaches the loop stem (⑤). BIP primed dumb-bell form loop structure is made and B loop and FIP primers bind to the both ends of dumb-bell loop structure (⑥ and ⑦).

The detached single strand cDNA primed with FIP is produced and 5′ end of F1c portion of FIP binds itself F1 portion, which forms a looped end structure. The B2c portion of the BIP primer attaches to the positive sense cDNA and initiates the elongation process (Fig. 1③). B3 primer anneals to the B3 portion and initiates DNA synthesis with strand displacement of the double strand DNA produced by BIP primer (Fig. 1④). F loop primer attaches to the loop portion and initiates DNA synthesis with displacement of stem of loop structure (Fig. 1⑤) and B loop primer works similarly (Fig. 1⑥). Through this reaction, two types of DNA are produced; double strand DNA from F1c to B3, and BIP-primed negative sense DNA. This BIP-primed single stranded DNA forms dumb-bell stem-loop structure and FIP anneals to the 3′ of F2c and F1 portion to initiate the cycling LAMP reaction (Fig. 1⑦) [Notomi et al., 2000].

From the previous reports, RSV subgroups A and B have different sequence alignment in the N gene region [Johnson and Collins, 1989; Yui et al., 2003] and we designed two independent sets for LAMP primer for RSV A and RSV B. RSV subgroup A (long strain) and RSV subgroup B (B1 strain) were referred from Gene Bank and sequence alignment is shown in Figure 2. The subgroup-specific sets of primers and genome positions are shown in Figure 2. RT-LAMP primer is targeted in the part of the N region similar to the RT-PCR region.

For the reaction of RT-LAMP, a mixture was made in 25 μl of reaction mixture, containing 40 pmol of FIP and BIP, 5 pmol of F3 and B3, 20 pmol of Loop F and Loop B, 1.4 mM of each dNTP, 0.8M betaine (Sigma-Aldrich, Saint Louis), 20 mM Tris-HCL, 10 mM KCL, 10 mM $(NH4)_2SO_4$, 8 mM $MgSO_4$, 0.1% Tween 20, 0.5 U AMV reverse transcriptase (New England Biolabs, Beverly, MA), 8 U Bst DNA polymerase (New England Biolabs), and 5 μl of sample RNA [Nagamine et al., 2001, 2002, Notomi et al., 2000]. The reaction mixture was subjected to real-time turbidimeter LA200 (TERAMECS, Kyoto, Japan) and the RT-LAMP reaction was carried out at 63°C for 60 min [Mori et al., 2004]. As the RT-LAMP reaction progresses, the reaction by-products pyrophosphate ions bind to magnesium ions and form a white precipitate of magnesium pyrophosphate. Thus, measurement of the turbidity closely related to the amplification of DNA [Mori et al., 2004].

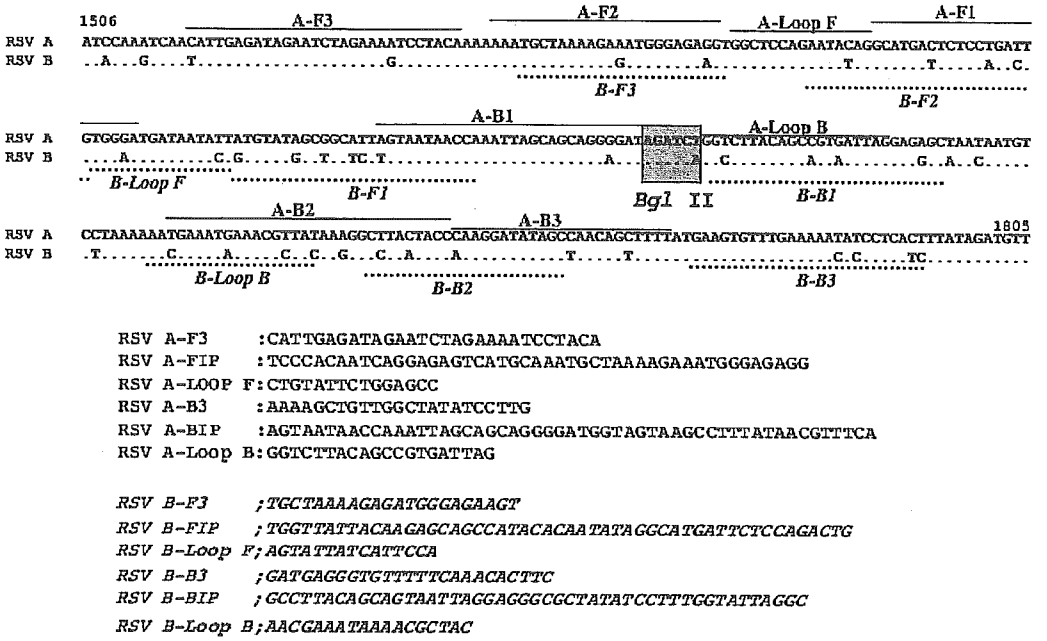
Differentiation of RSV Subgroups A and B

From the results of previous reports, subgroups A and B were classified by restriction fragment length polymorphism (RFLP) assay by Bgl II and Hae III [Yui et al., 2003]. Both RT-LAMP A and B primer sets were designed to avoid the Bgl II restriction site in the targeted region, as shown in Figure 2. RT-LAMP products were digested with Bgl II and electrophoresed.

RESULTS

Sensitivity of LAMP

Laboratory reference (long strain) of RSV subgroup A and a clinical isolate, which was confirmed as subgroup



RSV A-F3 :CATTGAGATAGAATCTAGAAAATCCTACA
RSV A-FIP :TCCCACAATCAGGAGAGTCATGCAAATGCTAAAAGAAATGGGAGAGG
RSV A-LOOP F:CTGTATTCTGGAGCC
RSV A-B3 :AAAAGCTGTTGGCTATATCCTTG
RSV A-BIP :AGTAATAACCAAATTAGCAGCAGGGGATGGTAGTAAGCCTTTATAACGTTTCA
RSV A-Loop B:GGTCTTACAGCCGTGATTAG

RSV B-F3 ;TGCTAAAAGAGATGGGAGAAGT
RSV B-FIP ;TGGTTATTACAAGAGCAGCCATACACAATATAGGCATGATTCTCCAGACTG
RSV B-Loop F;AGTATTATCATTCCA
RSV B-B3 ;GATGAGGGTGTTTTTCAAACACTTC
RSV B-BIP ;GCCTTACAGCAGTAATTAGGAGGGCGCTATATCCTTTGGTATTAGGC
RSV B-Loop B;AACGAAATAAAACGCTAC

Fig. 2. Primer design for the detection of RSV subgroups A and B by RT-LAMP. **Upper panel** shows the sequence alignment of RSV subgroups A and B. Underlined portions represent the primer location of RT-LAMP subgroup A and dotted lines show that of RT-LAMP subgroup B. Square (genome positions 1665–1670) shows the location of Bgl II site in the RSV subgroup A. **Lower panel** shows the primer sequence for RT-LAMP subgroups A and B.

B, were used for the analysis of the sensitivity of RT-LAMP. The sensitivity of nested RT-PCR was reported to detect 0.1 TCID 50 in the previous report [Yui et al., 2003]. We used long strain for RSV subgroup A, which contained $10^{5.5}$ TCID 50/200 μl and the results are shown in Figure 3. Total RNA was extracted from 200 μl of the sample and RNA was suspended in 25 μl, serially diluted at the ratio of 1:10. Five microliters of RNA was subjected to RT-LAMP. The RSV genome was amplified of at least 10^{-6} dilution and genome RNA related to 0.06 TCID 50 of RSV subgroup A was detected by RT-LAMP A.

A clinical isolate of RSV subgroup B was used, which contained $10^{5.0}$ TCID 50/200 μl and RNA was extracted. Similarly, RNA related to 0.02 TCID 50 of RSV subgroup B was detected by RT-LAMP B. Sets of RT-LAMP A and B were specific for RSV subgroups A and B, respectively and they did not show the cross reactivity to each other. RNA genome extracted from influenza virus types A (H1N1 and H3N2) and B containing $10^{6.0}$ TCID 50, was not amplified by RSV RT-LAMP A and B. And there was also no cross reaction with influenza virus types A, B, measles and mumps virus. RSV RT-LAMP A and RSV RT-LAMP B amplified the respective RSV genomes and showed no cross reactivity.

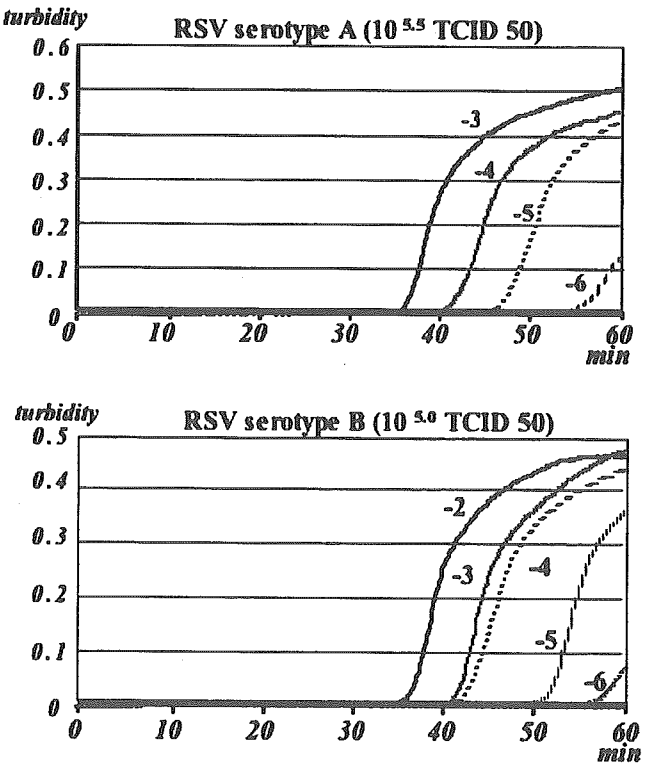


Fig. 3. Sensitivity of RT-LAMP subgroups A and B. **Upper panel** shows the sensitivity of RT-LAMP A for the detection of RSV subgroup A. We used the long strain, which contained $10^{5.5}$ TCID 50/200 μl. Total RNA was extracted from the sample and serial dilutions were subjected to RT-LAMP. **Lower panel** shows the sensitivity of RT-LAMP B for the detection of RSV subgroup B. Clinical isolate of RSV subgroup B was used, which contained $10^{5.0}$ TCID 50/200 μl. The numbers in the figures are the dilution of 10^{-n}.

Detection of RSV Subgroups A and B

We obtained 50 NPS samples from patients with upper respiratory infections who were clinically suspected as having RSV infection. We examined EIA, virus isolation, nested RT-PCR, and RT-LAMP, and the results are shown in Table I. Among the 50 samples,

TABLE I. Comparison of Virus Isolation, EIA Rapid Diagnostic Kit, RT-PCR, and RT-LAMP

	Virus isolation +	Virus isolation −	RT-PCR +	RT-PCR −	RT-LAMP +	RT-LAMP −
EIA + (n = 34)	25	9	34	0	34	0
EIA − (n = 16)	4	12	8	8	13	3
V. Iso. + (n = 29)			29	0	29	0
V. Iso. − (n = 21)			13	8	18	3
RT-PCR + (n = 42)					40	2
RT-PCR − (n = 8)					7	1

V. Iso.; Virus isolation.

34 were positive for EIA for the detection of RSV antigen(s) using Testpack RSV. RSV was isolated in 25 of 34 EIA positives and in 4 of 16 EIA negatives. The RSV genome was detected in 34 by nested RT-PCR or by RT-LAMP of 34 EIA positives and, among 16 EIA negatives, it was positive in 8 by nested RT-PCR and in 13 by RT-LAMP. The RSV genome was amplified by both nested RT-PCR and RT-LAMP in 29 isolation positive NPS samples but efficiently amplified by RT-LAMP in 18 of 21 isolation negatives, whereas 13 by nested RT-PCR. Among 42 nested RT-PCR positives, 40 were positive for RT-LAMP and the RSV genome was amplified in 7 of 8 nested RT-PCR negatives. The sensitivity of RT-LAMP was the highest, followed by nested RT-PCR, EIA and virus isolation in this order.

PCR products were confirmed through electrophoresis after digestion with Bgl II and the results are summarized in Table II. Among 50 NPS, RSV subgroup A was confirmed in 22 and RSV subgroup B in 20 by RT-PCR and RFLP. Whereas, RSV RT-LAMP A amplified 25 samples and RSV RT-LAMP B did 23 samples. In NPS samples characterized as RSV subgroups A and B infection by RT-PCR RFLP, the RSV genome was amplified only by the respective RT-LAMP A and B procedure. Among 22 diagnosed as RSV subgroup A infection by RT-PCR RFLP, one case showed the positive results for both RT-LAMP A and B. We supposed that major genome (subgroup A) in the sample would be amplified by RT-PCR but minor genome (subgroup B) might be detected by sensitive RT-LAMP B. One sample each for subgroups A and B identified by RT-PCR RFLP was negative for RT-LAMP. Among eight nested RT-PCR negatives, four were defined as RSV subgroup A infection and three as RSV subgroup B infection by RT-LAMP.

Some of the results of electrophoresis of RT-LAMP products are shown in Figure 4. In samples No. 47 and 174, RSV genome was amplified by RT-LAMP A procedure. LAMP products showed a typical ladder pattern of amplified DNA and they became one major band of 273 bp with minor smaller bands after digestion with Bgl II, specific for RSV serotype A. RSV genome was detected in samples No. 22 and 117 by RT-LAMP B procedure and LAMP products were not cleaved by Bgl II. In sample No. 85, RSV A and B genomes were amplified by both RT-LAMP A and B procedures and the results of electrophoresis after Bgl II digestion are shown in Figure 4.

There was no cross reaction between RT-LAMP A and B procedures and the case No. 85 was thought to be a dual infection because the RSV genome was amplified by both RT-LAMP A and B, although RSV serotype A infection was identified by RT-PCR and RFLP.

DISCUSSION

Rapid diagnosis of RSV in young infants and children has significant benefits for the management of patients with appropriate medication, reducing unnecessary antibiotics, and for infection control to prevent the further expansion in the hospital. Rapid diagnostic kits are based upon the presence of virus specific protein(s). In the case of lower viral load, they cause false negatives [Casiano-Colon et al., 2003]. Conventional nested RT-PCR is time-consuming and requires a cumbersome attention to prevent carryover contamination, although the precautious manipulation is similar. Thus, conventional RT-PCR was limited to special laboratories and not appropriate for clinical bedside application. Therefore, a molecular-based rapid diagnostic method has been strongly anticipated. In this standpoint of view, real time RT-PCR using a light cycler instrument was reported for the detection of RSV [Whiley et al., 2002; Falsey et al., 2003; Gueudin et al., 2003]. The system is based upon PCR and PCR products are monitored by fluorophore-labeled hybridization probes and takes approximately 2 hr to obtain the results, which is extremely minimized in terms of the reaction time in comparison with conventional RT-PCR. Whiley et al. [2002] reported that the sensitivity of real time RT-PCR

TABLE II. Differentiation of RSV Subgroups A and B by RT-PCR RFLP and RT-LAMP

	LAMP A +	LAMP A −	LAMP B +	LAMP B −
RT-PCR RFLP A (n = 22)	21	1	1	21
RT-PCR RFLP B (n = 20)	0	20	19	1
RT-PCR negative (n = 8)	4	4	3	5

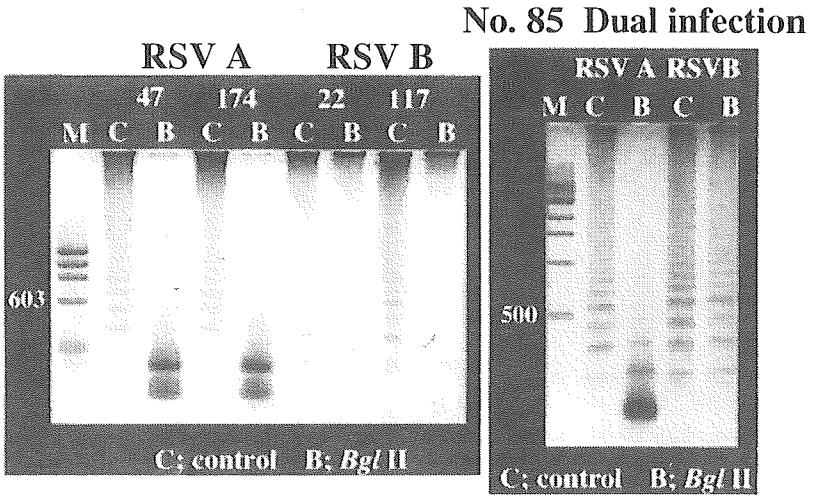


Fig. 4. Electrophoresis of RT-LAMP products after digestion with Bgl II. No. 47 and 174 were diagnosed as RSV subgroup A infection and No. 22 and 117 as RSV subgroup B infection by RT-LAMP. No. 85 was diagnosed as dual infection of RSV subgroups A and B.

was 50 pfμ/ml. However, it requires a special apparatus and chemical reagents and did not differentiate the subgroups A and B.

A more sensitive and specific method for DNA amplification method was developed by Notomi et al. [2000] and termed LAMP. This method employed a DNA polymerase with strand displacement activity and a set of four specially designed basic primers, which recognize six distinct different sequences on the targeted DNA. The first step of the reaction is the formation of stem-loop DNA structure and the inner primers produce the 5′ and 3′ end loop structures. All primers initiate DNA synthesis together with strand displacement, yielding the original stem-loop DNA structure and multiple stem-loop DNA products after repetitive reactions. LAMP has been applied to detect DNA genome for many kinds of infectious diseases [Iwamoto et al., 2003; Kuboki et al., 2003; Maruyama et al., 2003; Ihira et al., 2004]. Recently, RT-LAMP was developed to detect West Nile virus [Parida et al., 2004]. Definite features of LAMP indicate its rapidness, high sensitivity, high specificity, and simplicity, which are required for rapid diagnosis. We developed a new method for the detection of the RSV genome by RT-LAMP and compared the sensitivity with the detection of RSV antigens by EIA, virus isolation and the nested RT-PCR. We detected approximately 0.06 TCID 50 of RSV subgroup A and 0.02 TCID50 of RSV subgroup B within 60 min after extraction of RNA from the samples. The sensitivity of RT-LAMP was ~0.1 TCID50/5 μl RNA sample and it was calculated as 4-12 TCID 50/ml with higher sensitivity as reported by real time RT-PCR [Whiley et al., 2002]. Although the case No. 85 was diagnosed as RSV A infection by RT-PCR RFLP, it was identified as dual infection by RT-LAMP A and B. RT-LAMP was negative in one sample each diagnosed as subgroup A or B by RT-PCR RFLP. These cases were virus isolation negative and RT-PCR was positive and the sample were freeze-thawed several times and we supposed that bad preservation condition would influence the sensitivity. In comparison with conventional nested RT-PCR, RSV RT-LAMP A and B demonstrate high sensitivity. As for the running cost of RT-LAMP, the cost for one sample by RT-LAMP is approximately 80% of that by real-time PCR and the real-time turbidimeter costs less than one third of real-time PCR machine and the size is approximately 25 × 30 × 20 cm. RT-LAMP RSV should be applied in clinic-based or hospital-based rapid diagnosis to manage the patients and to prevent nosocomial infection because of its simplicity, rapidity, sensitivity and specificity. And also it would be available for RSV epidemiological surveillance.

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The phosphoprotein of attenuated measles AIK-C vaccine strain contributes to its temperature-sensitive phenotype

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Abstract

Measles AIK-C vaccine strain exhibits temperature-sensitivity (*ts*). To identify the structural proteins, which contribute to the *ts* property of AIK-C virus, reverse genetics was used. MV-minigenome RNA was replicated at 32.5, 37, and 39 °C when the plasmids expressing N, P, and L proteins of the Edmonston strain (the parental strain of AIK-C) were used, whereas the minigenome RNA replicated only at 32.5 °C but did not at 37 °C and higher temperature when N, P, and L protein expression plasmids of the AIK-C strain were used. A series of minigenome experiments revealed that the amino acid substitution of leucine at position 439 of the P protein by proline (P439-Pro) contributes to the *ts* phenotype of AIK-C. Four recombinant viruses having various P genes were rescued from the modified AIK-C genome cDNA and *ts*-characteristics were compared in Vero cells by plaque formation assay. The results showed that the P439-Pro of AIK-C virus played a key role in the *ts* phenotype, but the other substitutions in the P gene might have an accessory function in the expression of the phenotype.

Keywords: Measles virus; Temperature-sensitivity; AIK-C vaccine strain

1. Introduction

Measles virus (MV) belongs to the order *Mononegavirales* with a nonsegmented negative-sense RNA genome having 15,894 nucleotides in length and encoding six structural proteins. The nucleoprotein (N), phosphoprotein (P), and large protein (L) associate with the RNA genome to shape the nucleocapsid structure. This ribonucleoprotein complex serves as a template for replication and transcription. Two external glycoproteins of the viral particle, hemagglutinin protein (H) and fusion protein (F), are responsible for viral attachment to the host cell and the fusion of viral and host cell membrane and after this step, the viral nucleocapsid penetrates into the cytoplasm. The matrix protein (M) is thought to contribute to the viral assembly and maturation. The P gene encodes two non-structural proteins; C and V proteins. The C protein is translated from the second AUG codon of the P mRNA [1]. The V protein is translated from V mRNA synthesized through a unique processing found in most *paramyxoviruses*, called RNA editing [2]. Recent studies showed that the C and V proteins were related to the viral replication and pathogenesis in vivo and played important roles to block interferon alfa/beta signal transduction [3–7].

Recently, two methods for direct genetic manipulation of the genome RNA of *Mononegavirales* have been established; the minigenome system and the infectious cDNA clone system. The former uses natural defective interfering (DI) genome RNA or artificial internally deleted genome RNA having reporter gene instead of virus component genes. Transcription and replication of minigenome RNA are driven by viral proteins, which are co-expressed by plasmids or

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helper standard viruses. The latter system is based on a similar technology, but uses the authentic full-size genome cDNA so that the infectious recombinant viruses can be rescued [8–10]. These "reverse genetics" techniques are powerful tools not only for basic study on viral properties, such as viral protein characteristics, the mechanism of replication, transcription and pathogenesis, but also for practical purposes, such as the development of new vaccines and new viral vectors.

MV is a causative agent of measles as an acute illness in children and, in extremely rare incidence, slowly progressive and lethal disease of the central nervous system, known as subacute sclerosing panencephalitis (SSPE). After worldwide acceptance of efficient and safe live attenuated measles vaccines, number of measles cases drastically decreased in developed countries. Therefore, measles has been considered to be eradicable by vaccination [11]. Although the remarkable progress has been made by the Expand Programme on Immunization (EPI) organized by the World Health Organization (WHO), measles still ranks as one of the leading causes of the high mortality among children, especially under 9 months of age in developing countries [12].

The further attenuated live measles AIK-C vaccine strain was developed in 1974 from the Edmonston strain through passage in sheep kidney cells and chick embryo cells [13]. Since then, more than 15 million children have been immunized with AIK-C mainly in Japan and its efficacy and clinical safety have clearly shown [14]. Recently, the AIK-C strain has also been proven to be effective for children at 6 months of age or earlier. Accordingly, the AIK-C strain is one of the candidate vaccines for young infants at 6 months of age for high-risk groups in developing countries [15]. The AIK-C strain exhibits temperature-sensitivity (*ts*) [16]. Although little is known about how the virus becomes attenuated, it is widely accepted that *ts* phenotype related to viral attenuation mechanism. In this study, we identified the viral protein, which contributes to the *ts* phenotype using reverse genetics. Our data would benefit the further understanding of the nature of viral attenuation.

2. Materials and methods

2.1. Cells and viruses

HeLa and chick embryo fibroblast (CEF) cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% fetal calf serum (FCS). Vero cells were grown in minimum essential medium (MEM) supplemented with 5% FCS. The Epstein–Barr virus-transformed marmoset B cell line B95a was cultured in RPMI1640 medium supplemented with 5% FCS. All cells were propagated at 37 °C in 5% CO_2. T7 RNA polymerase-expressing vaccinia virus vTF7-3 was kindly supplied by Dr. B. Moss (NIH, Bethesda, MD, USA) [17]. T7 RNA polymerase-expressing, replication-deficient vaccinia virus, MVAT7 pol, was a generous gift from Dr. G. Sutter (GSF Environment and Health Research Center, FRG) [18]. The AIK-C seed strain for vaccine production was used. AIK-C strain was propagated in CEF or Vero cells. The Edmonston strain was propagated in Vero cells.

2.2. cDNAs and sequence determination

The cDNA spanning the L gene of the AIK-C genome was synthesized by RT-PCR using specific primers, MVLF9239: 5′-gtccaagtggttccccgttatggac (mRNA sense) and MVLR15799: 5′-ccacctagggcaggattagggttcc (genome sense), referred by the results reported by Mori et al. [19]. The cDNA derived from the Edmonston strain (laboratory strain) was constructed by Schmid's method [20]. The 3′- and 5′-end sequences of the MV genome were amplified using the 3′-RACE System for Rapid Amplification of cDNA Ends (Invitrogen, Carlsbad, CA, USA) with MVSR340: 5′-tggagactccacaaataagg (genome sense) and MVSR635: 5′-cgtaaccgcctttgcgagcaag (genome sense) primers and the 5′-full RACE core set (Takara, Tokyo, Japan) with MVLF15752: 5′-gttagtcggatacagtgc (mRNA sense), MVLF15807: 5′-aatcctgccctaggtggtta (mRNA sense), MVLR15610: 5′-agtctagtatcagatagccgg (genome sense), and MVLR 15717: 5′-ggtctccttgactgttacctt (genome sense) primers, respectively. Nucleotide sequence was determined with primers at 300–350 base intervals along the AIK-C sequence using an ABI 377 sequencer (Applied Biosystems, Foster City, CA, USA).

2.3. Plasmids

Three modified plasmids based on pUC19 were used for construction of the full-genome cDNA. Through a 4-step modification, *Xmn*I and *Nar*I sites of pUC19 were deleted and a *Not*I site was added just before the *Eco*RI site of the polylinker site. The resultant plasmid was designated pUC19M-5. *Ase*I and *Sca*I sites were deleted from pUC19M-5 to give pUC19M-7. The polylinker site of pUC19M-7 was exchanged with that of pNEB193 (NEB, Beverly, MA, USA) and the resultant plasmid, which carried *Not*I and *Pac*I sites on the polylinker site, was designated pUC193M-7. pCITE-K was constructed by deletion of the S.Tag, His.Tag sequences from pCITE 4a(+) plasmid (Novagen, Madison, WI, USA). pCITE-K plasmid has the T7 promoter, the Encephalomyocarditis virus internal ribosomal entry site (IRES), a multiple cloning site, a poly (A) signal and T7 terminator sequences. pCITE-K plasmid was used as a vector for construction of N, P, and L protein-expression plasmids.

2.4. Construction of MN, P and L protein-expression plasmids

The N, P and L cDNAs derived from the Edmonston and AIK-C strains were subcloned into pCITE-K plasmid, which was placed downstream of the T7 promoter and IRES sequence. The plasmids carrying the genes originating from

the Edmonston strain were designated pCIN01 (N gene), pCIP01 (P gene), and pCIL01 (L gene) and those bearing the genes derived from AIK-C, pCIAN01 (N gene), pCIAP01 (P gene) and pCIAL01 (L gene), respectively.

2.5. Construction of minigenome cDNA and synthesis of minigenome RNA

A cDNA template that could generate the negative-sense minigenome RNA was constructed. The cDNA, consisting of 1152 nts, contained the 3′-terminal 107 and 5′-terminal 109 nts corresponding to authentic MV genome RNA. These MV terminal sequences flanked a renilla luciferase open reading frame composed of 936 nt (Promega, Madison, WI, USA) [21]. The T7 RNA polymerase promoter and hepatitis delta virus ribozyme [22] are designed such that transcribed RNA created the specific 3′- and 5′-termini of the MV genome. Two T7 terminator sequences were aligned downstream from the ribozyme sequence to stop the RNA transcription efficiently by the T7 RNA polymerase [8]. The constructed cDNA was subcloned into pUC18 plasmid and the resultant plasmid was designated p18MGKLuc01. The negative-sense minigenome RNA was synthesized from the *Hin*dIII-digested p18MGKLuc01 plasmid by in vitro transcription using AmpliscribeTM T7 transcription kits (EPICENTRE TECHNOLOGIES, Madison, WI, USA) according to the supplier's instructions. The synthesized RNA was resuspended in RNase-free water.

2.6. Minigenome system

HeLa cells prepared in 12-well plates (about 1.5×10^5 cells/well) were infected with T7 RNA polymerase-expressing vaccinia virus, vTF7-3, at a multiplicity of infection (MOI) of 2–3. After 1 h adsorption, the cells were washed with Opti-MEM I (Invitrogen, Carlsbad, CA, USA) and then transfected with 0.5 μg of synthesized minigenome RNA and 0.5 μg of MV-N, 0.25 μg of MV-P and 0.1 μg of MV-L protein-expression plasmids using DMRIE-C reagent (Invitrogen, Carlsbad, CA, USA). After incubation at 32.5 °C for 3 h in 5% CO_2, the medium was replaced with fresh 5% FCS-DMEM containing cytosine arabinofuranoside (Ara-C) (40 μg/ml). After incubation at 32.5 or 37 or 39 °C for 40 h in 5% CO_2, the transfected cells were harvested and the assay for Renilla luciferase activity was done using a dual-luciferase assay system (Promega, Madison, WI, USA). The luciferase activity was quantitated by TopCount (Packard Instrument Company, Meriden, CT, USA).

2.7. Construction of a full-length cDNA genome corresponding to the AIK-C RNA genome

To construct a full-length cDNA genome corresponding to the complete 15,894 nucleotides of the AIK-C RNA genome, the 8 AIK-C cDNA fragments spanning the entire viral genome were combined. First, we added a *Not*I site/T7 promoter sequence just upstream of the leader sequence and a hepatitis delta virus ribozyme sequence immediately downstream of the trailer sequence by a PCR extension method using specific primers. Then the T7 terminator sequence/*Hin*dIII site was added behind the ribozyme sequence. These additional sequences were designed to generate the antigenomic RNA with the correct 3′- and 5′-termini of MV genome RNA under control of the T7 RNA polymerase. The 8 AIK-C cDNA fragments including a *Not*I/T7 promoter-leader and trailer-ribozyme-T7 terminator/*Hin*dIII sequences were ligated at appropriate restriction endonuclease sites. The plasmid having a full-length AIK-C cDNA genome was designated pMVAIK-C.

2.8. Rescue of the recombinant virus from cloned cDNA

B95a cells prepared in 12-well plates were infected with a vaccinia virus MVAT7 pol that expresses T7 RNA polymerase at an MOI of 10. MVAT7 pol was derived from a highly attenuated and host range restricted vaccinia virus, strain Ankara that is unable to propagate in human and most other mammalian cell lines [18]. After 1 h adsorption, the cells were washed with Opti-MEM and transfected with 0.5 μg of pCIAN01, 0.25 μg of pCIAP01, 0.1 μg of pCIAL01, and 1.5 μg of pMVAIK-C or derivatives of pMVAIK-C carrying various P genes using CellFectin transfection reagent (Invitrogen, Carlsbad, CA, USA). After incubation at 32.5 °C for 3 h, the medium containing transfection reagent/plasmid complex was replaced with fresh 5% FCS-DMEM. The tranfected cells were incubation at 32.5 °C in 5% CO_2 until cytopathic effect (CPE) could be observed in the transfected cells (about 5 days). The rescued viruses were passaged twice in B95a cells at 32.5 °C and the supernatants were harvested and stocked at −70 °C. The P genes of the four rescued viruses were amplified by the RT-PCR and were ascertained the nuclotide substitutions by direct sequence.

2.9. Evaluation of the ts phenotype of recombinant viruses

Replication of rescued recombinant viruses and the AIK-C vaccine strain and Edmonston strain were determined by plaque forming assay at 32.5, 36, 37, 38, 39 and 40 °C with Vero cells. Vero cells were prepared in 6-well plates and infected with serially diluted viruses. After incubation at permissive temperature (32.5 °C) for 1 h, the infected cells were washed with PBS twice, overlaid 2 ml of the 0.5% agarose contained 1% CS-MEM and then further incubated at various temperature indicated above for 6 days. Then, the cells were fixed with 2 ml of 0.5% Glutaraldehyde for 2 h. After removed the agar, the fixed cells were immunostained using anti-MV antibody to identify and enumerate the plaque by ABC staining using Vecta Stain elite ABC kit (Vector Laboratories, Burlingame, CA, USA).

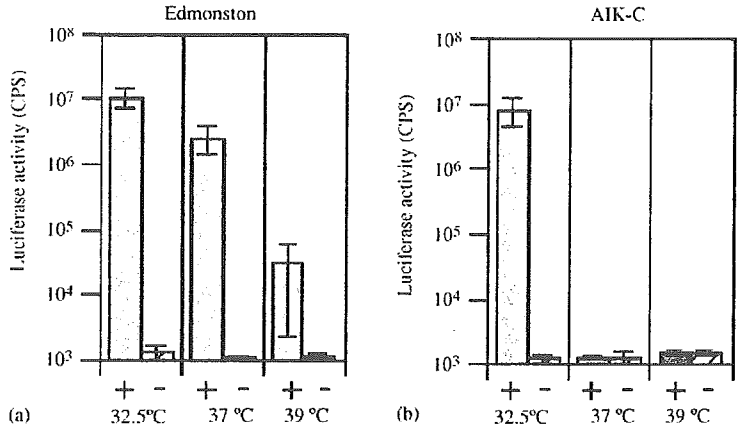

Fig. 1. The minigenome experiments supported by the Edmonston-N, P, and L protein expression plasmids or the AIK-C-N, P, and L protein expression plasmids at 32.5, 37, or 39 °C. HeLa cells prepared in 12-well plates were infected with vaccinia virus vTF7-3 at an MOI of 2–3 and then transfected with 0.5 μg of negative-sense minigenome RNA holding the luciferase gene and (a) the set of pCIN01 (0.5 μg), pCIP01 (0.25 μg), and pCIL01 (0.5 μg) plasmids derived from the Edmonston strain or (b) the set of pCIAN01 (0.5 μg), pCIAP01 (0.25 μg), and pCIAL01 (0.5 μg) plasmids derived from the AIK-C strain: (+), tranfected with minigenome RNA and the set of plasmids; (−), transfected with only minigenome RNA as a negative control. The transfected cells were maintained at 32.5, 37, or 39 °C in 5% CO_2 for 40 h and lysed to measure luciferase activity. The luciferase activity (counts per second, CPS) was counted by Top Count (Packard Instrument Company, Meriden, CT, USA). The experiments were carried out at 3 times and the mean values and ±1.0 S.D. were indicated.

3. Results

3.1. Minigenome replication supported by the set of N, P, and L protein-expression plasmids derived from Edmonston and AIK-C strains at various temperatures

The MV vaccine strain, AIK-C, exhibits the *ts* phenotype (shut off temperature at 39 °C), whereas the Edmonston strain, the parental strain of AIK-C, does not [16]. Recently, the *ts* propertiy of AIK-C was reported not to be attributable to either of the viral envelope proteins [23]. We assumed that viral RNA synthesis-related genes or proteins contribute to the *ts* phenotype of AIK-C and the minigenome system is helpful to analyze the phenotype. Our laboratory Edmonston strain which has been propagated in Vero cells does not show *ts* phenotype (shut off temperature at >40 °C). Thus, we attempted to identify the *ts*-related mutation in AIK-C strain using the minigenome system with the cDNAs derived from our Edmonston strain and from vaccine seed AIK-C strain. Nucleotide sequences of the leader, N gene, P gene, L gene and trailer regions were determined. The leader and trailer sequences of the Edmonston strain were identical to those of AIK-C strain [19]. This implies that these *cis*-element sequences for viral transcription/replication are not responsible for *ts* phenotype of AIK-C strain. Nucleotide differences that caused the amino acid substitutions between Edmonston and AIK-C strains were observe in 3 for the N gene, 5 for the P gene (including C and V genes) and 15 for the L gene.

When the negative-sense minigenome RNA containing the luciferase gene was transfected with the Edmonston-derived N, P and L proteins expression plasmids and incubated at the 32.5, 37, and 39 °C, high level of luciferase activity was observed at any temperature conditions, but considerable reduction at 39 °C (Fig. 1a). In contrast, when the AIK-C-derived N, P and L expression plasmids were used, the luciferase activity was detected only at 32.5 °C. No activity was detected even at 37 °C (Fig. 1b). These results suggested that AIK-C-derived protein(s) responsible for viral replication and/or transcription are involved in the *ts* phenotype. In addition, the temperature point for *ts* was shifted to lower temperature in the minigenome system, suggesting that the system may be more sensitive to temperature as compared with the infectious viruses. As the *ts* property seemed much highlighted at 37 °C rather than at 39 °C in the minigenome system (Fig. 1a and b), further study using minigenome system was performed at 32.5 and 37 °C.

3.2. Identification of the protein of strain AIK-C that contributes to the ts phenotype

To identify the protein that contributes to the *ts* phenotype of AIK-C, minigenome experiments were carried out with various combinations of the N, P, and L expression plasmids which are derived from either the Edmonston or the AIK-C strain (Fig. 2). All combinations of the plasmids supported high level of luciferase expression at 32.5 °C, suggesting that the minigenome RNA was replicated and transcribed. In contrast, when minigenome experiments were carried out at 37 °C using pCIAP01 plasmid, the AIK-C derived P expression plasmid, as expression partner, no luciferase activity was observed. The other combinations excluding the pCIAP01 plasmid could support the luciferase expression at 37 °C. These results indicate that the proteins derived from the AIK-C P gene functioned at 32.5 °C, but not at 37 °C in the minigenome system, suggesting the proteins expressed from P gene of AIK-C might contribute to the *ts* phenotype.

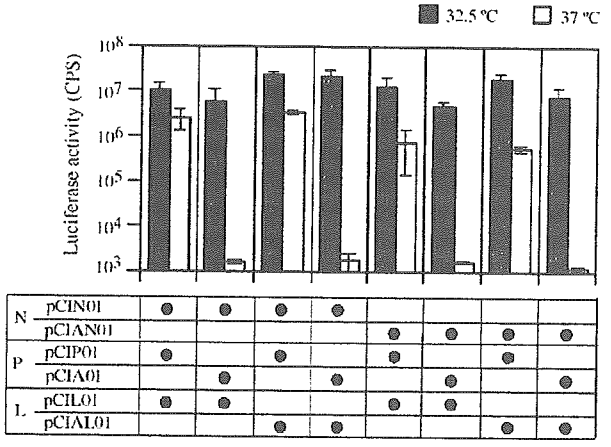

Fig. 2. Luciferase activity in the minigenome experiments supported by the various combinations of N, P, and L protein-expression plasmids at 32.5 or 37 °C. The vTF7-3 virus-infected HeLa cells were transfected with the minigenome RNA and various combinations of the pCIAN01 or pCIN01, pCIAP01 or pCIP01, and pCIAL01 or pCIL01 plasmids and incubated at 32.5 (dark gray bar) or 37 °C (light gray bar) for 40 h. After cell lysis, luciferase activity (CPS) was measured. Dots in the table under the figure indicate the plasmid employed in the minigenome experiments. The experiments were carried out at 3 times and the mean values and ±1.0 S.D. were indicated.

3.3. Identification of the ts point(s) in the p gene

The P gene of MV coded three proteins, P, C, and V proteins. Four nucleotide differences were found between the AIK-C and Edmonston P genes. Those nucleotide substitutions led to three amino acid substitutions in the P protein (aspartic acid to tyrosine at position 110; P110-Tyr, cysteine to tyrosine at 275; P275-Tyr and leucine to proline at 439; P439-Pro), one substitution in the V protein (aspartic acid to tyrosine at 110; V110-Tyr), and one substitution in the C protein (serine to tyrosine at 134; C134-Tyr) (Table 1). The V protein was not expressed from the P plasmid in the transfected cells due to a lack of RNA editing activity of the T7 RNA polymerase. Thus, the V110-Tyr did not attribute to lack of luciferase expression at 37 °C in the minigenome system. To identify *ts* point(s) in the P or C protein, 8 chimera P plasmids were constructed using appropriate restriction endonuclease sites (Fig. 3a and b), which were designed to express the chimera P or C proteins between the AIK-C and Edmonston strains. Following construction, the nucleotide sequences of the chimera P genes were entirely determined to verify the constructed P gene.

The minigenome experiments using the 8 chimera P plasmids were conducted at 32.5 and 37 °C. When the experiments were performed at 32.5 °C, all the chimeric P proteins induced the high level of luciferase activity, indicating that all chimeric plasmids expressed the functional P proteins (Fig. 4a and b). These chimeras, however, showed the significant differences when the experiments were held at 37 °C. A high level of luciferase expression was observed when Edmonston-P gene backbone plasmids (pCIP01, 03, 04 and 05) were used, but it was reduced dramatically when the pCIP02 was used, which had a proline residue instead of leucine at position 439 of the P protein (Fig. 4a). Conversely, the chimera plasmids originated from the AIK-C P gene except pCIAP02 failed the luciferase expression. The pCIAP02 which had a leucine instead of proline at position 439 of the P protein, restored the luciferase expression at 37 °C (Fig. 4d). Those results indicated that the proline residue at amino acid 439 of the P protein (P439-Pro) plays a key role in the *ts* phenotype of strain AIK-C.

3.4. Replication of rescued recombinant AIK-C viruses containing various P proteins at various temperatures

To confirm the results obtained from the minigenome experiments, the recombinant viruses having the various P genes were rescued using infectious cDNA clone system and tested their temperature-sensitivity. The Edmonston or chimeric P genes were excised at relevant endonuclease site from the pCIP01, pCIP02 and pCIAP02 plasmids and exchanged with the AIK-C P gene of the AIK-C genome cDNA (pMVAIK-C). These chimera genome cDNAs were designated pMVED-P (exchanged with pCIP01), pMVAIK/P439-Leu (pCIAP02) and pMVED/P439-Pro (pCIP02), respectively (Fig. 5). The full-genome cDNAs were transfected into MVAT7 pol-infected B95a cells together with pCIAN01,

Table 1
Nucleotide comparison of the P genes of the Edmonston and AIK-C strains and deduced amino acid substitutions in the P, V, and C proteins

protein	Nuclotide positon	virus Edmonston	virus AIK-C	Amino Acid position	virus Edmonston	virus AIK-C
P	**2134**	**G**	**T**	**110**	**D**	**Y**
	2229	C	A	141	L	L
	2630	**G**	**A**	**275**	**C**	**Y**
	3122	**T**	**C**	**439**	**L**	**P**
V	**2134**	**G**	**T**	**110**	**D**	**Y**
	2229	C	A	141	L	L
	2630	G	A	275	V	V
C	2134	G	T	102	V	V
	2229	**C**	**A**	**134**	**S**	**Y**

Nuclotide substitutions result in amino acid changes.

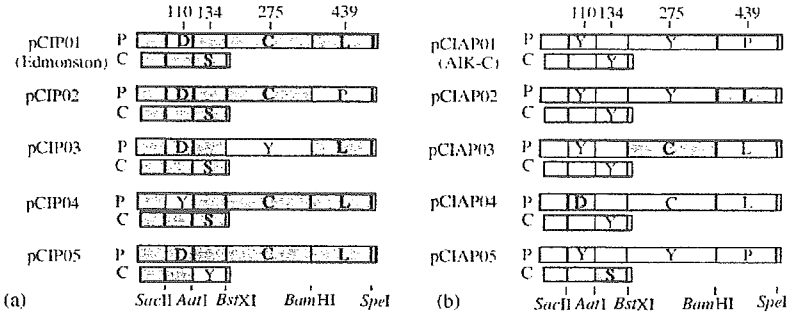

Fig. 3. Construction of chimera P genes. Eight chimera P gene plasmids were constructed. (a) pCIP02, 03, 04, and 05 were originated from pCIP01, the plasmid bearing the Edmonston P gene. In these plasmids, some regions of pCIP01 were replaced with the corresponding region of pCIAP01, the plasmid having the AIK-C P gene, using appropriate restriction endonuclease sites as shown in figure. The resultant plasmids expressed the Edmonston P protein bearing a mutation at amino acid 439 (pCIP02), 275 (pCIP03), and 110 (pCIP05) and the C protein with a mutation at position 134 (pCIP04). (b) pCIAP02, 03, 04, and 05 were originated from pCIAP01 and were changed in some regions to the corresponding region of pCIP01. The pCIAP02, 03 and 04 plasmids expressed the AIK-C P protein having a mutation at amino acid 439 (pCIAP02), 275 (pCIAP03), and 110 (pCIAP05) and the C protein with a mutation at position 134 (pCIAP04). Numbers indicate the amino acid position of the proteins.

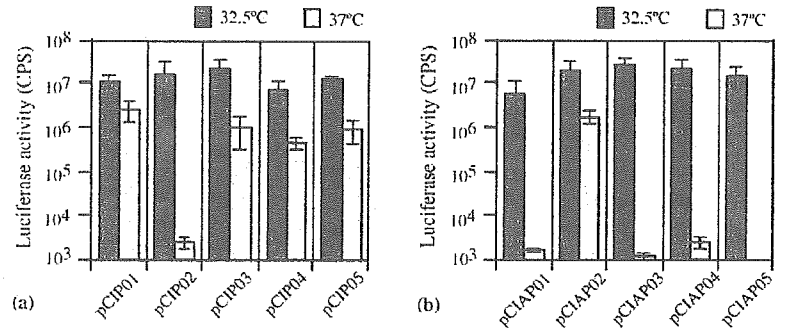

Fig. 4. Luciferase activity in the minigenome experiments using the chimera P plasmids. The vTF7-3 infected HeLa cells were transfected with minigenome RNA, pCIAN01, pCIAL01, and one of the chimera P plasmids. (c) pCIP01 and the derivative plasmids. (d) pCIAP01 and the derivative plasmids. The transfected cells were incubated at 32.5 (dark gray bar) or 37 °C (light gray bar) for 40 h and lysed to measure the luciferase activity. The luciferase activity (CPS) was counted by TopCount (Packard Instrument Company, Meriden, CT, USA). The experiments were carried out at 3 times and the mean values and ±1.0 S.D. were indicated.

pCIAP01 and pCIAL01 plasmids at 32.5 °C and the recombinant viruses were recovered. The P genes of the rescued viruses were amplified by RT-PCR and ascertained by direct sequencing. Each recombinant virus had the P gene corresponding to the transfected AIK-C genome cDNA. The recovered viruses were designated recAIK-C virus (derived from pMVAIK-C), recAIK/ED-P virus (pMVED-P), recAIK/P439-Leu virus (pMVAIK/P439-Leu), and recAIK/ED/P439-Pro virus (pMVED/P439-Pro), respectively.

To examine whether the P gene, especially P439-Pro, contributed to *ts* phenotype of AIK-C strain, the *ts* level of four recombinant AIK-C viruses were tested by plaque formation assay at 32.5, 36, 37, 38, 39 and 40 °C with Vero cells (Table 2). The recAIK-C virus showed the equal level of

Table 2
Efficiency of plaque formation in Vero cells at 32.5, 36, 37, 38, 39 and 40 °C

Virus (plasmid)	Amino acid at 439 position of the P protein	Virus titer (log_{10} PFU/ml) at an indicated temperature (°C)						Shut-off temperature (°C)
		32.5	36	37	38	39	40	
recAIK-C (pMVAIK-C)	Pro	5.6	5.4	5.3	4.7	<0.4	<0.4	39
recAIK/ED-P (pMVED-P)	Leu	6.7	6.7	6.6	6.4	6.2	5.4	>40
recAIK/P439-Leu (pMVAIK/P439-Leu)	Leu	6.6	6.5	6.3	6.2	5.8	<0.4	40
recAIK/ED/P439-Pro (pMVED/P439-Pro)	Pro	6.7	6.2	6.2	6.0	<0.4	<0.4	39
vacAIK-C	Pro	6.9	6.7	6.6	6.2	<0.4	<0.4	39
Edmonston	Leu	5.8	5.7	5.5	5.5	5.1	4.5	>40

Shut-off temperature is defined as the lowest temperature at which a 10^4-fold or greater reduction in titer observed.

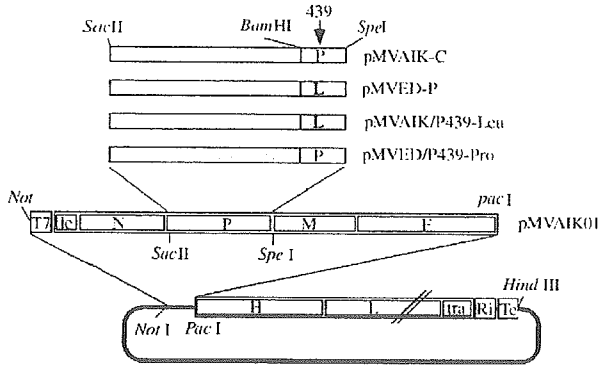

Fig. 5. Strategy for the construction of the recombinant AIK-C genome cDNAs that have various P genes. To rescue of the recombinant AIK-C viruses carrying various P proteins, three chimera cDNAs based on the AIK-C cDNA genome (pMVAIK-C) were constructed. To exchange the P gene of the full-genome cDNA, the *NotI-PacI* fragment of pMVAIK-C was cloned into pUC193M-7 to give pMVAIK01. pMVAIK01 had unique restriction endonuclease sites, *SacII* and *SpeI* sites, located at both ends of the P gene. The pCIP01 *SacII* and *SpeI* fragment having the Edmonston P gene, pCIP02 *SacII* and *SpeI* fragment having the Edmonston P gene containing a mutation that causes substitution of proline for leucine at position 439 of the P protein, and pCIAP02 *SacII* and *SpeI* fragment having part of the AIK-C P gene with proline replaced by leucine at position 439 were inserted into the pMVAIK01 *SacII* and *SpeI* site. The resultant plasmids were digested by *NotI* and *PacI* restriction endonuclease and ligated into *NotI-PacI* digested pMVAIK-C plasmid. These plasmid constructs were designated pMVED-P, pMVAIK/P439-Leu and pMVED/P439-Pro, respectively. Numbers indicate the amino acid position of the P protein: T7, T7 promoter; Ri, hepatitis delta virus ribozyme; Te, T7 terminator; le, leader sequence; tra, trailer sequence.

temperature-sensitivity with the vaccine AIK-C (vacAIK-C). In contrast, the recAIK/ED-P virus having Edmonston-P gene lost the *ts* phenotype, like Edmonston strain (shut off temperature at >40 °C), indicating that the P gene of AIK-C strain contribute to the *ts* phenotype. Further, recAIK/ED/P439-Pro (carrying Edmonston-P gene with P439-Pro) virus also exhibited the equal level of *ts* with recAIK-C and vacAIK-C (shut off temperature at 39 °C). These results showed that P439-Pro is crucial to the *ts* phenotype of strain AIK-C. In addition, recAIK/P439-Leu virus (carrying AIK-P gene with P439-Leu) showed the moderate level of *ts* phenotype (shut of temperature at 40 °C), suggesting that other amino acid substitutions found in the P gene of AIK-C strain might modulate the expression of the phenotype.

4. Discussion

Further attenuated live MV vaccines are widely used due to their high effectiveness and low incidence of adverse reactions and play a central role for prevention of measles infection in the world. Most of them, such as Edmonston–Zagreb, Moraten, Schwarz and AIK-C strains were attenuated by the classical and empirical approach from the Edmonston strain but genetic basis of the attenuation of those vaccines has not been well understood.

Strain AIK-C exhibits the *ts* property (shut off temperature at 39 °C). This property is unique to AIK-C strain among MV vaccines. There is little direct evidence that *ts* phenotype of AIK-C contributes for its attenuation, due to lack of an appropriate experimental animal model to evaluate MV pathogenesis. However, since the *ts* mutant is less vigorous in their growth at higher temperature than the parental virus, it is conceivable that this phenotype plays a part in viral attenuation. In fact, *ts*-mutant of some negative strand RNA viruses, such as Influenza A, human parainfluenza type 3 (HPIV 3) and respiratory syncytial virus (RSV) were evaluated as attenuated viruses in animal experimental model [24–27]. Thus, we considered that the *ts*-related mutation of AIK-C might involve attenuation of AIK-C strain. This study is the first report to identify the *ts* related-mutation of currently available MV vaccines at molecular level using reverse genetics.

Although the AIK-C stain propagated well at 37 °C, the minigenome system conducted with the AIK-C protein expression plasmids did not work at 37 °C. The reason of the discrepancy was not analyzed well at present. The minigenome system was considered to represent measles virus replication and transcription process, but not identical to the ordinary processes, because, at least, T7 RNA polymerase-expressing vaccinia virus was co-existed in order to express the N, P and L proteins in the system. Furthermore, due to high transcriptase activity of T7 RNA polymerase, amount and ratio of N, P and L proteins could be different from measles virus replication. It is mere speculation that these differences might make the minigenome system more sensitive to temperature than ordinary virus replication.

We identified the P439-Pro as the *ts* point of AIK-C strain. Recently, the nucleotide sequences of five Edmonston-derived vaccine strains including AIK-C, Moraten, Schwarz, Rubeovax and Edmonston–Zagreb were reported and compared with that of low-passage, wild-type Edmonston virus to analyze the genetic bases of attenuation of MV vaccine strains [28,29]. Some common amino acid changes among all vaccines were considered to play important roles for viral attenuation. Additional vaccine specific substitutions were considered to determine the property or attenuated level of individual strains. The P439-Pro was reported to be a unique substitution to AIK-C strain among other MVs, suggesting the molecular basis of peculiar phenotype to AIK-C strain.

The mechanism how P439-Pro contributes to the phenotype has not been analyzed. The P protein is considered to be multifunctional protein. It acts for viral RNA synthesis as a unit of RNA polymerase together with L protein as well as viral RNA encapsidation in company with N protein [30]. Thus, the P protein appears to play a central part of viral RNA synthesis. On the other hand, the P protein also regulates the N protein localization in cell [31]. The C-terminal region (including the position 439) of the P protein is essential for the interaction of N protein together with the extreme amino-terminal residue of the P protein, P–P self-interaction and L protein interaction [32–34]. These interactions are considered to be important for viral RNA synthesis. Thus, this

region is significant for exhibition for P function. It is mere conjecture that the substitution from Leu to Pro at position 439 of P protein might reduce the ability to bind these proteins at higher temperatures, and consequently, impair viral genome replication and/or mRNA synthesis.

The recAIK/P439-Leu virus showed a moderate *ts* level (shut-off temperature at 40 °C) (Table 2). This suggested the possibility that the other *ts* related-amino acid substitutions might exist in the P gene of AIK-C, besides P439-Pro. Sequence comparison of the various MV P gene showed that the P275-Tyr, P439-Pro, and C134-Tyr substitutions were characteristic to AIK-C strain. Conversely, P/V110-Tyr was conserved in all MVs as well as other moribilliviruses including canine distemper virus (CDV) [35], rinderpest virus (RV) [36] and Phocid distemper virus (PDV) [37]. The P/V110-Asp seems to be a peculiar mutation of our laboratory Edmonston strain, suggesting that P/V110-Tyr might have no contribution to the *ts* phenotype. Recently, the roles of C protein have been scrutinized using the reverse genetics. The recombinant MV defective in C protein showed that the C protein was dispensable for growth in Vero cells [38], but essential in human peripheral blood cells [39], played some roles in MV pathogenesis in vivo [4,5], and inhibited the type I interferons response [6]. Some mutations in the C protein of MV increase the RNA synthesis [40] and may relate to cell tropism [41]. These results indicate that the C protein is involved in the process of viral RNA synthesis and with a representation of virus phenotype. Thus, it is possible that the amino acid substitution in the C protein of AIK-C may affect the viral character at higher temperature. Anyhow, recombinant viruses recovered from modified AIK-C genome cDNAs might be necessary to examine the contribution of other mutations found in AIK-C P gene to *ts* phenotype.

We elucidated the mutation related to the *ts* characteristic, and probably having some connections with attenuation of AIK-C vaccine strain in this study. Other *moribilliviruses*, such as CDV, PDV, and RV are formidable pathogens in animals like a MV in human. Among these viruses, the P439-Leu and the surrounding sequence are well conserved. This implies it would be possible to add a *ts* phenotype (and probably an attenuation phenotype) to those viruses by changing leucine to proline at that position in the P protein. Current reverse genetics technology of negative strand RNA virus will facilitate to introduce the mutation on the RNA genome. Recovering viruses will be candidates for live virus vaccine.

The nucleotide sequence data reported in this paper is available in DDBJ/EMBL/GenBank nucleotide sequence databases with accession no. AB046113 (AIK-C genome cDNA), AB046218 (Edmonston-P gene), AB052821 (Edmonston-N gene), and AB052820 (Edmonston-L gene).

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Passage in Vero cells alters the characteristics of measles AIK-C vaccine strain

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Abstract

Objective of this study is to evaluate the feasibility of measles vaccine production in Vero cell culture. We constructed the full-length cDNA, pIC-MVAIK-F278Leu (small plaque-type) and pIC-MVAIK-F278Phe (large plaque-type) from the AIK-C measles vaccine strain attenuated from the Edmonston wild-type. MVAIK-S/B2 was rescued from pIC-MVAIK-F278Leu after two passages in B95a cells and MVAIK-SL/B2V1 was obtained through large plaque cloning in Vero cells. MVAIK-SL/B2V8 was obtained after eight passages in Vero cells. It produced large plaques in Vero cells, grew well at 39 °C, and thus the characteristics of the AIK-C vaccine strain were lost. Thirteen amino acid changes were observed; one in the N, two in the P, one in the C, three in the F, one in the H, and five in the L protein regions. Twelve of these changes excluding one in the L gene were back mutated to the Edmonston strain. Change from Leu to Phe at position 278 of the F protein was an early event during adaptation to Vero cells and the P gene was back-mutated to the Edmonston wild-type. As for the control, MVAIK-L/B9 strain was obtained after passages in B95a cells from pIC-MVAIK-F278Phe (large plaque-type). It maintained the same temperature sensitivity as the AIK-C vaccine strain and only four amino acid changes, one in the N and three in the L protein region, were observed without any mutations in the P, C, M, F, and H genes. The passage of the measles vaccine AIK-C strain in Vero cells lost the characteristics of small plaque inducibility and temperature sensitivity (*ts*) phenotype.

Keywords: Measles virus; Temperature sensitivity; Measles vaccine; Vero cells; Plaque assay; Reverse genetics

1. Introduction

Measles virus is a member of the genus Morbillivirus of the family Paramyxoviridae and order Mononegavirales and consists of 15,894 nucleotides, coding six structural proteins. The nucleoprotein (N), phosphoprotein (P), large protein (L) and genome RNA constitute the ribonucleoprotein complex (RNP). Two glycoproteins of hemagglutinin (H) and fusion (F) are present as envelope spikes and they work in the process of virus attachment and cell fusion. The M protein acts in virus assembly and maturation [1]. The establishment of a rescue system of the infectious virus from cDNA of measles virus was reported [2,3]. We developed the rescue system of infectious virus from cDNA of the AIK-C measles vaccine strain [4]. Recent developments in reverse genetics of Mononegavirales have contributed to better understanding of the mechanisms of virus replication, transcription, and pathogenesis [5].

The measles vaccine strain AIK-C was developed from the Edmonston strain through plaque cloning in sheep kidney cells and chick embryonic cells at 33 °C [6,7]. It shows optimal growth at 33 °C with small plaques but extremely poor or no growth at 39–40 °C, demonstrating temperature sensitivity (*ts*). We reported that Leu at position 278 of the F protein was responsible for small plaque formation in Vero

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cells and Phe at 278 of the F protein of the Edmonston strain induced large plaques in Vero cells [4]. The AIK-C strain was a mixture of two virus genomes, having Leu or Phe at position 278 of the F protein gene although it did not influence the *ts* characteristics. The P protein of the AIK-C strain was responsible for the *ts* characteristics and Pro at position 439 of the P gene significantly contributed to the *ts* phenotype from the results of minigenome and reverse genetic assays [8]. Recently, wild measles virus strains were isolated in B95a cells and showed no CPE in Vero cells [9]. Measles virus isolated in B95a cells had full pathogenicity in cynomolgus monkeys and no pathogenicity after adaptation in Vero cells [10]. During the adaptation process, several amino acid changes occurred: two amino acid changes in the P and V regions, one in the C protein, three in the H region, and two in the L region, but the critical regions responsible for pathogenesis were not identified [10].

The AIK-C strain is one of the candidate vaccine strains for the Expanded Programme on Immunization (EPI) to overcome maternally conferred immunity, resulting in a high sero-conversion rate in young infants at 6 months of age [11–13]. A vaccine manufacturer intended to produce the AIK-C measles vaccine in large scale using Vero cells for intra-nasal usage but we have no information on the stability of the characteristics of the AIK-C through passage in Vero cells.

In this study, we investigated the genetic stability of infectious virus recovered from cDNA with Leu at position 278 of the F protein, which induced small sized plaques, and purified large plaques by plaque cloning in Vero cells. After several passages in Vero cells the original *ts* phenotype was lost. For comparison, infectious virus was rescued from cDNA having Phe at position 278 of the F protein and propagated in B95a cells. We compared the characteristics of infectious virus rescued from the full-length cDNA after different passage histories in Vero cells or B95a cells.

2. Materials and methods

2.1. Measles virus strains

The AIK-C seed strain was used for vaccine production. The AIK-C strain was attenuated from the wild Edmonston virus kindly provided by the late Prof. Enders [6,7].

2.2. Construction of full-length cDNA of AIK-C and recovery of the recombinant virus

We constructed two full-length cDNA plasmids of the AIK-C vaccine seed strain as previously reported [4,14]. Briefly, we constructed two parts; the first half consisted of AIK-C cDNA from the leader sequence to the *Pac* I site at nucleotide position 7238 of the AIK-C genome and the second half of the AIK-C cDNA consisted of the H and L regions from the 7238 *Pac* I site to the trailer sequence. To change the amino acid at position 278 from Leu to Phe, a fragment of the recombinant plasmid derived from the Edmonston strain was used. The construct was designated as pIC-MVAIK-F278Phe and the original AIK-C cDNA as pIC-MVAIK-F278Leu. For the recovery of the recombinant measles virus, B95a cells were infected with MVAT7 pol., nonreplicative vaccinia virus expressing T7 RNA polymerase kindly provided by Dr. G. Sutter, and then pAIK-N, pAIK-P, pAIK-L and pIC-MVAIK-F278Leu (small plaque-type) or pIC-MVAIK-F278Phe (large plaque-type)were transfected, as reported previously [4]. B95a cells were cultured at 32.5 °C in 5% CO_2 and through two blind passages in B95a cells, typical cytopathic effect (CPE) was observed. The strains rescued from the respective cDNA were designated as MVAIK-S/B2 from pIC-MVAIK-F278Leu and MVAIK-L/B2 from pIC-MVAIK-F278Phe.

MVAIK-S/B2 was infected into Vero cells and the wells were overlayed with 0.5% agar in minimum essential medium (MEM) supplemented with appropriate antibiotics and 5% fetal calf serum (FCS). After 7 days of culture at 33 °C, three large plaques were cloned and passaged in Vero cells. After eight passages in Vero cells at 33 °C, MVAIK-SL/B2V8 was obtained. MVAIK-L/B2 was further propagated in B95a cells and through seven additional passages at 33 °C, and MVAIK-L/B9 was obtained (as shown in Fig. 1).

2.3. Plaque and infectivity assays

Monolayers of Vero cells were inoculated with measles virus or virus strains rescued from full-length cDNA and were overlayed with 0.5% agar in Eagle's MEM supplemented with antibiotics and 5% FCS, as previously reported [14]. After 7 days of incubation in 5% CO_2 at 37 °C, the cells were fixed with 0.25% glutaraldehyde. Hyperimmune rabbit antibodies to measles virus were added to the plate, followed by, after vigorous washing, biotinylated goat antibodies to rabbit IgG. The plates were stained with an VECTASTAIN Elite ABC kit (VECTOR Labo., Burlingame, CA). To examine the growth of infectious virus, monolayers of Vero cells were infected with the AIK-C, Edmonston, MVAIK-L/B9 and MVAIK-SL/B2V8 (approximately m.o.i. = 0.01). The culture fluid and infected cells were obtained at days 1, 3, 5 and 7 of culture and the infectivity in B95a cells was expressed as TCID50/ml.

2.4. Cloning of the P, F, H gene, sequence analysis, and expression experiments

Coding regions of the N, P, M, F, H, and L proteins were divided into several fragments which were amplified from genomic RNA using positive sense primers at the RT step of RT-PCR [15] and sequenced by a dye terminator method using the ABI 377A sequencer (Applied Biosystems, Foster City, CA, USA). The F and H gene expression plasmids were constructed from cDNA synthesized from genomic RNA of MVAIK-SL/B2V8 and expression experiment was carried

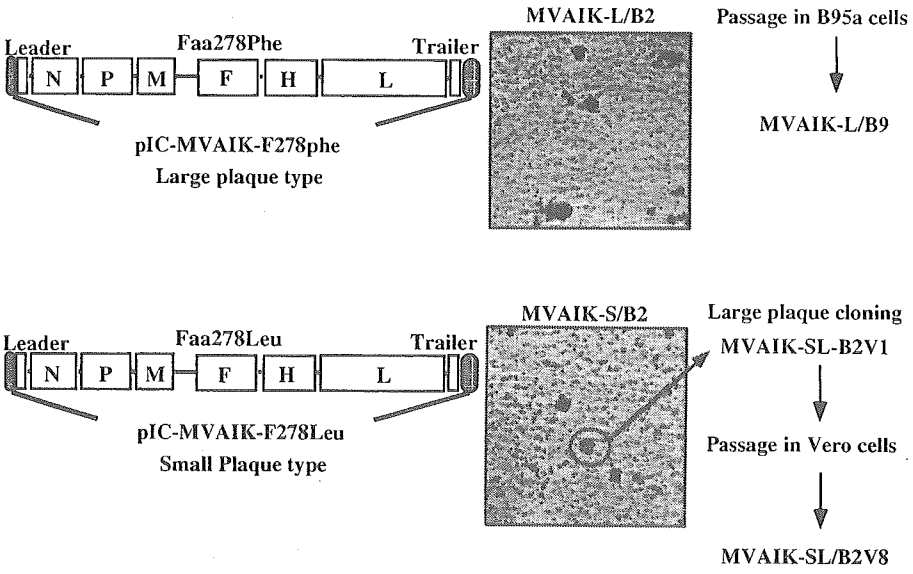

Fig. 1. Scheme of construction of recombinant viruses and passage history of rescued virus strains.

out under the control of T7 RNA polymerase [4,14]. PCR products of the P gene amplified from genomic RNA of MVAIK-SL/B2V8 and MVAIK-SL/B2V4 were cloned and sequenced.

3. Results

3.1. Construction of infectious clone and propagation of viruses

We constructed two infectious cDNAs having Leu (pIC-MVAIK-F278Leu) or Phe (pIC-MVAIK-F278Phe) at position 278 of the F protein gene. After two passages in B95a cells, infectious viruses were recovered and designated as MVAIK-S/B2 and MVAIK-L/B2, respectively (Fig. 1). MVAIK-L/B2 induced large plaques in Vero cells and MVAIK-L/B9 was propagated after seven additional passages in B95a cells. MVAIK-S/B2 induced mainly small or medium sized plaques in Vero cells and three large plaques were isolated and propagated independently in Vero cells (MVAIK-SL/B2V8).

3.2. Temperature sensitivity and plaque formation

The original AIK-C measles vaccine strain had *ts* and did not grow at 39–40 °C but grew well at 33 °C with small and medium size plaques in Vero cells. We reported that Leu at position 278 of the F protein was responsible for small plaques [4]. Plaque formation assay and virus growth analysis at different temperatures were carried out in Vero cells and the results are shown in Fig. 2. MVAIK-L/B9 induced large plaques in Vero cells. MVAIK-S/B2 induced mainly small plaques but some large plaques were demonstrated as shown in Fig. 1. MVAIK-SL/B2V1 was isolated through large plaque cloning in Vero cells and MVAIK-SL/B2V8 was propagated after additional eight passages in Vero cells. MVAIK-L/B9 and MVAIK-SL/B2V8 were inoculated in Vero cells at the m.o.i. of 0.01 and culture fluid was collected at days 1, 3, 5 and 7 of culture. Cells were freeze-thawed with 400 μl of MEM supplemented with 5% FCS and after sonication they were stocked at −70 °C. Infectivity was assayed in B95a cells and the results are shown in Fig. 2. MVAIK-L/B9 grew well at 33 °C but no virus grew at 39 °C in the culture fluid. Infectious particles were detected at 33 °C but none at 39 °C in the cell lysate. Large plaques were produced at 33 °C but not at 39 °C. MVAIK-SL/B2V8 grew well at 33 °C but no infectious virus was detected in the supernatant at 39 °C. Cell-associated infectious virus was recovered at 39 °C as well as 33 °C on day 3 of culture but reduced after day 5 of culture at 39 °C. MVAIK-SL/B2V8, which was propagated through eight passages in Vero cells, lost *ts* and produced large plaques in Vero cells at 39 °C as well as at 33 °C.

3.3. Nucleotide differences in MVAIK-L/B9 and MVAIK-SL/B2V8 strains in comparison with the original infectious clones (pIC-MVAIK-F278Leu and pIC-MVAIK-F278Phe)

Nucleotide sequences of six coding regions, N, P, M, F, H, and L genes, were investigated and the deduced amino acids that differed from the original cDNA of AIK-C are depicted in Table 1. MVAIK-L/B9 had five nucleotide changes with four amino acid differences; one at amino acid position 129 of the N protein (N129), and three in the L gene (L 371, L 542 and L 2032). MVAIK-SL/B2V8 had 16 nucleotide changes with 13 amino acid differences; one in the N protein, two in the P, one in C, three in the F, one in the H, and five in the L. Three nucleotide changes were observed in the P gene region with two amino acid changes at the P protein and with one