Identification of an amino acid residue on influenza C virus M1 protein responsible for formation of the cord-like structures of the virus

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Influenza C virus-like particles (VLPs) have been generated from cloned cDNAs. A cDNA of the green fluorescent protein (GFP) gene in antisense orientation was flanked by the 5' and 3' non-coding regions of RNA segment 5 of the influenza C virus. The cDNA cassette was inserted between an RNA polymerase I promoter and terminator of the Pol I vector. This plasmid DNA was transfected into 293T cells together with plasmids encoding virus proteins of C/Ann Arbor/1/50 or C/Yamagata/1/88. Transfer of the supernatants of the transfected 293T cells to HMV-II cells resulted in GFP expression in the HMV-II cells. The quantification of the GFP-positive HMV-II cells indicated the presence of approximately 10^6 VLPs (ml supernatant)^-1. Cords 50–300 μm in length were observed on transfected 293T cells, although the cords were not observed when the plasmid for M1 protein of C/Ann Arbor/1/50 was replaced with that of C/Taylor/1233/47. A series of transfection experiments with plasmids encoding M1 mutants of C/Ann Arbor/1/50 or C/Taylor/1233/47 showed that an amino acid at residue 24 of the M1 protein is responsible for cord formation. This finding provides direct evidence for a previous hypothesis that M1 protein is involved in the formation of cord-like structures protruding from the C/Yamagata/1/88-infected cells. Evidence was obtained by electron microscopy that transfected cells bearing cords produced filamentous VLPs, suggesting the potential role of the M1 protein in determining the filamentous/spherical morphology of influenza C virus.

INTRODUCTION

Reverse genetics, a method for generation of infectious virus particles from cloned cDNAs, has been reported for influenza A virus (Fodor et al., 1999; Hoffmann et al., 2000; Neumann et al., 1999), influenza B virus (Hoffmann et al., 2002; Jackson et al., 2002; Hatta & Kawoaka, 2003) and Thogoto virus (Wagner et al., 2001), which belong to the family Orthomyxoviridae. Data have been reported regarding the generation of virus-like particles (VLPs) for these viruses (Gómez-Puertas et al., 1999; Mena et al., 1996; Neumann et al., 2000; Wagner et al., 2000; Paragas et al., 2001). However, for influenza C virus, also a member of the Orthomyxoviridae, no reports have been documented regarding either reverse genetics or VLP generation.

Influenza A and B viruses have eight RNA segments of negative polarity, whereas the genome of the influenza C virus consists of seven RNA segments, each of which encodes PB2, PB1, P3, haemagglutinin–esterase (HE), nucleoprotein (NP), matrix (M1) protein and CM2, and non-structural proteins (NS1 and NS2) (Lamb & Krug, 2001). PB2, PB1 and P3 are subunits of the RNA polymerase of the virus, HE glycoprotein, which has receptor-binding, acetyl esterase and fusion activities, forms the spike on the virion (Herrler & Klenk, 1991). NP participates in forming ribonucleoproteins (RNP) with virus RNA (vRNA), PB2, PB1 and P3. M1 is abundantly present beneath the envelope, which gives rigidity to the virion. CM2 is the second membrane protein of the virus, and has been demonstrated to possess ion-channel activity (Hongo et al., 2004). Although the function of NS1 remains to be clarified, NS2 is a nuclear export protein of the virus (Paragas et al., 2001).

Smirnov et al. (1991) and Roberts et al. (1998) suggested that the M gene encodes the determinants responsible for the filamentous morphology of influenza A virus. Bourmakina & Garcia-Sastre (2003) indicated by reverse genetics study that amino acid residues 95 and 204 of the M1 protein of A/WSN/33 are critical in determining filamentous virus particle formation. Furthermore, Roberts et al. (1998) and Bourmakina & Garcia-Sastre (2003) described hair-like filaments detected by immunofluorescence on the infected cells producing filamentous virions.
In the case of influenza virus, Nishimura et al. (1990) reported cord-like structures (CLS) >500 μm long protruding from the surface of C/Yamagata/1/88 (YA/88)-infected HMV-II cells. Electron microscopy studies showed that CLS consist of numerous filamentous particles in the process of budding, each of which is covered with a layer of surface projections and aggregated with their long axes. Further analysis of a series of reassortant viruses between YA/88 and C/Taylor/1233/47 (TAY/47), the latter of which is a unique strain incapable of forming CLS, showed that reassortants with the M gene from TAY/47 could not form CLS on infected cells. Comparison of M gene sequences of six strains, including YA/88 and TAY/47, suggested that either or both of amino acid changes at positions 24 and 135 of the M1 protein were responsible for differences in cord-forming ability between the two strains (Nishimura et al., 1994).

In the present study, we first describe successful generation of influenza C VLPs from transfected 293T cells by expression of an artificial vRNA-like reporter gene and nine virus proteins from cloned cDNAs. Second, we provide evidence that an amino acid at position 24 of the M1 protein is critical in forming CLS, based on a series of transfection experiments with plasmids encoding M1 mutants.

**METHODS**

**Cells, viruses and antibodies.** 293T cells, provided by Dr Y. Kawaoka, were maintained in Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum. The HMV-II line of human malignant melanoma cells was maintained in RPMI 1640 medium with 10% calf serum (Nishimura et al., 1989). Monoclonal antibodies (mAbs) against HE and NP proteins of AA/50 and antiserum against AA/50 virions were prepared previously (Sugawara et al., 1983). Monoclonal antibodies (mAbs) against HE and NP proteins of AA/50 and antiserum against AA/50 virions were prepared previously (Sugawara et al., 1991, 1993; Yokota et al., 1983).

**Determination of the sequences of 5’ and 3’ ends of RNA segment 5.** The vRNA was extracted from egg-grown AA/50 was reverse-transcribed into cDNA using a primer complementary to the 12 nucleotides of the 3’ end of vRNA (Kimura et al., 1997). The open reading frames (ORFs) for PB2, PB1, P3, HE and M1 were PCR-amplified and the products cloned into the following protein expression vectors. PB2, PB1 and P3 ORFs were subcloned into the NotI and XhoI sites of pcDNA3.1 (+) (Invitrogen), and pcDNA/PB2-AA, pcDNA/PB1-AA and pcDNA/P3-AA were obtained, respectively. Plasmid pME18S/HE-AA was obtained by subcloning the HE ORF into the NotI and SpeI sites of transient expression vectors pME18S (Hongo et al., 1998; Takebe et al., 1988), M1 ORF was subcloned into the SacI and XhoI sites of pCAGGS.MCS (provided by Dr Y. Kawaoka), and pCAGGS.MCS/M1-AA was constructed. The construct pCAGGS.MCS/AA was obtained by subcloning the NP ORF with the consensus sequence described above. The NS1 ORF was obtained by reverse transcription (RT)—PCR using vRNA extracted from egg-grown YA/88, and pME18S/NS1-YA was constructed. The Met-CM2 ORF, an ORF with an additional ATG prior to the first amino acid of CM2, was PCR-amplified and pME18S/Met-CM2-YA was constructed. The Met-CM2 ORF, an ORF with an additional ATG prior to the first amino acid of CM2, was PCR-amplified and pME18S/Met-CM2-YA was constructed. The Met-CM2 ORF, an ORF with an additional ATG prior to the first amino acid of CM2, was PCR-amplified and pME18S/Met-CM2-YA was constructed. The Met-CM2 ORF, an ORF with an additional ATG prior to the first amino acid of CM2, was PCR-amplified and pME18S/Met-CM2-YA was constructed. The Met-CM2 ORF, an ORF with an additional ATG prior to the first amino acid of CM2, was PCR-amplified and pME18S/Met-CM2-YA was constructed. The Met-CM2 ORF, an ORF with an additional ATG prior to the first amino acid of CM2, was PCR-amplified and pME18S/Met-CM2-YA was constructed. The Met-CM2 ORF, an ORF with an additional ATG prior to the first amino acid of CM2, was PCR-amplified and pME18S/Met-CM2-YA was constructed. The Met-CM2 ORF, an ORF with an additional ATG prior to the first amino acid of CM2, was PCR-amplified and pME18S/Met-CM2-YA was constructed. The Met-CM2 ORF, an ORF with an additional ATG prior to the first amino acid of CM2, was PCR-amplified and pME18S/Met-CM2-YA was constructed. The Met-CM2 ORF, an ORF with an additional ATG prior to the first amino acid of CM2, was PCR-amplified and pME18S/Met-CM2-YA was constructed. The Met-CM2 ORF, an ORF with an additional ATG prior to the first amino acid of CM2, was PCR-amplified and pME18S/Met-CM2-YA was constructed. The Met-CM2 ORF, an ORF with an additional ATG prior to the first amino acid of CM2, was PCR-amplified and pME18S/Met-CM2-YA was constructed. The Met-CM2 ORF, an ORF with an additional ATG prior to the first amino acid of CM2, was PCR-amplified and pME18S/Met-CM2-YA was constructed. The Met-CM2 ORF, an ORF with an additional ATG prior to the first amino acid of CM2, was PCR-amplified and pME18S/Met-CM2-YA was constructed.

**Construction of Pol I plasmid containing reporter genes.** The enhanced green fluorescent protein (GFP) (pEGFP-C2; Clontech) and luciferase (pGL3; Promega) genes were PCR-amplified using a pair of primers containing the determined sequences of segment 5 non-coding regions and BsmBI sites. The products digested with BsmBI were inserted in antisense orientation between an RNA polymerase I promoter and terminator of pH21, and pPolI/NP-AA.GFP(−) and pPolI/NP-AA.Luc(−) were obtained.

**Construction of virus protein-expressing plasmid DNAs (Table 1).** The vRNA extracted from egg-grown AA/50 was reverse-transcribed into cDNA using a primer complementary to the 12 nucleotides of the 3’ end of vRNA (Kimura et al., 1997). The open reading frames (ORFs) for PB2, PB1, P3, HE and M1 were PCR-amplified and the products cloned into the following protein expression vectors. PB2, PB1 and P3 ORFs were subcloned into the NotI and XhoI sites of pcDNA3.1 (+) (Invitrogen), and pcDNA/PB2-AA, pcDNA/PB1-AA and pcDNA/P3-AA were obtained, respectively. Plasmid pME18S/HE-AA was obtained by subcloning the HE ORF into the NotI and SpeI sites of transient expression vectors pME18S (Hongo et al., 1998; Takebe et al., 1988), M1 ORF was subcloned into the SacI and XhoI sites of pCAGGS.MCS (provided by Dr Y. Kawaoka), and pCAGGS.MCS/M1-AA was constructed. The construct pCAGGS.MCS/AA was obtained by subcloning the NP ORF with the consensus sequence described above. The NS1 ORF was obtained by reverse transcription (RT)—PCR using vRNA extracted from egg-grown YA/88, and pME18S/NS1-YA was constructed. The Met-CM2 ORF, an ORF with an additional ATG prior to the first amino acid of CM2, was PCR-amplified and pME18S/Met-CM2-YA was constructed. The Met-CM2 ORF, an ORF with an additional ATG prior to the first amino acid of CM2, was PCR-amplified and pME18S/Met-CM2-YA was constructed. The Met-CM2 ORF, an ORF with an additional ATG prior to the first amino acid of CM2, was PCR-amplified and pME18S/Met-CM2-YA was constructed. The Met-CM2 ORF, an ORF with an additional ATG prior to the first amino acid of CM2, was PCR-amplified and pME18S/Met-CM2-YA was constructed. The Met-CM2 ORF, an ORF with an additional ATG prior to the first amino acid of CM2, was PCR-amplified and pME18S/Met-CM2-YA was constructed. The Met-CM2 ORF, an ORF with an additional ATG prior to the first amino acid of CM2, was PCR-amplified and pME18S/Met-CM2-YA was constructed. The Met-CM2 ORF, an ORF with an additional ATG prior to the first amino acid of CM2, was PCR-amplified and pME18S/Met-CM2-YA was constructed. The Met-CM2 ORF, an ORF with an additional ATG prior to the first amino acid of CM2, was PCR-amplified and pME18S/Met-CM2-YA was constructed. The Met-CM2 ORF, an ORF with an additional ATG prior to the first amino acid of CM2, was PCR-amplified and pME18S/Met-CM2-YA was constructed. The Met-CM2 ORF, an ORF with an additional ATG prior to the first amino acid of CM2, was PCR-amplified and pME18S/Met-CM2-YA was constructed. The Met-CM2 ORF, an ORF with an additional ATG prior to the first amino acid of CM2, was PCR-amplified and pME18S/Met-CM2-YA was constructed. The Met-CM2 ORF, an ORF with an additional ATG prior to the first amino acid of CM2, was PCR-amplified and pME18S/Met-CM2-YA was constructed. The Met-CM2 ORF, an ORF with an additional ATG prior to the first amino acid of CM2, was PCR-amplified and pME18S/Met-CM2-YA was constructed. The Met-CM2 ORF, an ORF with an additional ATG prior to the first amino acid of CM2, was PCR-amplified and pME18S/Met-CM2-YA was constructed. The Met-CM2 ORF, an ORF with an additional ATG prior to the first amino acid of CM2, was PCR-amplified and pME18S/Met-CM2-YA was constructed.
constructed using vRNA extracted from the egg-grown TAY/47 as described above. Nucleotide sequences of the primers described in this study will be provided by the authors on request.

**Transfection into 293T cells.** Opti-MEM I medium (Invitrogen) (950 μl) was incubated with 2 μl μg⁻¹ DNA of Trans IT LT-1 (Panvera) for 10 min, then a mixture of plasmid DNAs was added and incubated for 45 min at room temperature. The DNA–LT-1 mixture was added to 1 x 10⁶ 293T cells in a 35 mm Petri dish. The cells were incubated at 37 °C for 24 h and the medium was replaced with fresh Opti-MEM I medium followed by incubation at 34 °C.

**VLP assay.** The culture media of the transfected 293T cells were collected at 48 h post-transfection (p.t.) and clarified by low-speed centrifugation. The resulting supernatant was treated with tosylamide-phenylethyl chloromethyl ketone (TPCK)-treated trypsin (20 μg ml⁻¹) at 37 °C for 10 min followed by addition of soybean trypsin inhibitor. The monolayered HMV-II cells were incubated with the supernatant at 34 °C for 60 min, and subsequently infected with the helper virus (AA/50) at m.o.i. 10 and incubated for 24–48 h. GFP-positive HMV-II cells were observed in a fluorescence microscope (Leica) and photographed. Luciferase activity expressed in the HMV-II cells was measured with Lumat 9507 (Berthold Technologies) according to the manufacturer’s instructions for the luciferase assay system (Promega).

**Immunoblotting.** Transfected 293T cells were resolved by SDS-PAGE on 13% gels containing 4 M urea under reducing conditions. After SDS-PAGE, immunoblotting was carried out as described by Grambas et al. (1992) using the antibodies described above. The virus proteins were detected by an enhanced chemiluminescence Western blotting analysis system (Amersham) according to the manufacturer’s instructions.

**Electron microscopy.** For negative staining of VLPs, the culture media of transfected 293T cells were collected at 48 h p.t., clarified by low-speed centrifugation, and the supernatant was recentrifuged at 35000 r.p.m. for 1 h in a Beckman SW40 TI rotor. The resulting pellet was suspended in 30 μl Opti-MEM I medium, then mixed with equal volume of 2% sodium phosphotungstate pH 7.0. One drop of the mixed suspension was placed on a microgrid with carbon film and dried at room temperature. To analyse the cords, transfected 293T cells in a Petri dish were washed twice with PBS, and the cord-bearing cells were placed on a microgrid with carbon film, negatively stained with 2% sodium phosphotungstate pH 7.0 and dried at room temperature. Alternatively, transfected 293T cells were fixed with 2.5% glutaraldehyde in 0.1 M cacodylate buffer pH 7-4 at 4 °C for 120 min, then post-fixed with 1% osmic acid in the same buffer at 4 °C for 60 min. They were then dehydrated by sequential treatment with 60, 70, 80, 90, 95, 97, 97-5 and 100% ethanol and embedded in an epoxy resin mixture; thin sections were stained with uranyl acetate and lead citrate before observation. Electron micrographs were recorded at 100 kV (Hitachi H-7100 electron microscope) at a total magnification of ×90000.

## RESULTS AND DISCUSSION

**Reporter gene expression in transfected 293T cells**

We transfected various amounts of pCAGGS.MCS/NP-AA into 10⁶ 293T cells to determine the efficiency of transfection. Transfection of 5 μg of the plasmid DNA resulted in detection of the maximum amount of NP by immunoblotting with anti-NP mAb (data not shown). Therefore in our system the total amount of plasmids for transfection was determined to be 5 μg per dish. Next, to determine the optimal amounts of protein-expressing plasmids required for maximum luciferase expression from 0-5 μg of pPolI/NP-AA.Luc(−), we modulated the expression of PB2, PB1, P3 and NP by reducing the amount of each plasmid in a stepwise manner. As a result, in all subsequent experiments we used 0-125 μg pcDNA/PB2-AA, and 0-25 μg pcDNA/PB1-AA, pcDNA/P3-AA and pcCAGGS.MCS/NP-AA. When 0-5 μg pPolI/NP-AA.GFP(−) was transfected under this condition, approximately 30% of the transfected 293T cells were positive for GFP expression at 48 h p.t. (data not shown), a finding which is comparable to that of Neumann et al. (2000) for influenza A virus.

**Transfection of plasmids for VLP generation and characterization of the VLP generation system**

To generate VLP, 0-5 μg pPolI/NP-AA.Luc(−) was transfected into 10⁶ 293T cells together with the nine protein-expressing plasmids shown in Table 1, and the supernatant of the culture fluids collected at 48 h p.t. was transferred to HMV-II cells as described under Methods. The amount of each plasmid that gave the most abundant expression of luciferase in the HMV-II cells was determined to be as follows: 1-25 μg pME18S/HE-AA; 0-3 μg pCAGGS.MCS/M1-AA; 0-0875 μg pME18S/Met-CM2-YA; 0-7 μg pME18S/NS1-YA; 1-0 μg pME18S/NS2-YA. Similarly 0-5 μg pPolI/NP-AA.GFP(−) was transfected into 293T cells together with the plasmids in Table 1, and the supernatant was transferred to HMV-II cells followed by infection with the helper virus. GFP-positive HMV-II cells were detected (Fig. 1a) and the supernatants were determined to contain approximately 10⁶ VLPs ml⁻¹ by quantification of the GFP-positive HMV-II cells. Under these transfection conditions, the haemagglutination titre of the supernatants was 8 HAU ml⁻¹ (data not shown). Neumann et al. (2000) and Wagner et al. (2000) reported the presence of 10⁴ influenza A VLPs per dish and 7 × 10³ Thogoto VLPs ml⁻¹, respectively. For influenza B VLP, Paragas et al. (2001) found the haemagglutination titre of the supernatants to be 32 HAU. Thus it is likely that VLP formation of influenza C virus occurs as efficiently as those of influenza A and B viruses, as long as the RNPVs contain a vRNA derived from one segment.

When pCAGGS.MCS/M1-TAY, which expresses the M1 protein of TAY/47, was used for transfection together with pPolI/NP-AA.GFP(−) and the other eight plasmids expressing PB2, PB1, P3, HE, NP, CM2, NS1 and NS2, GFP-positive HMV-II cells were detected (Fig. 1b), indicating that the TAY/47 M1 protein was functional in reporter gene transfer by the generated VLP. In these experiments a trace amount of GFP was observed in the HMV-II cells after inoculation of the supernatant containing VLPs without subsequent superinfection by the helper virus, and GFP expression was enhanced by helper virus superinfection (data not shown). Therefore RNP incorporated
into influenza C VLPs. 293T cells were transfected with 0.5 μg pPolI/NP-AA.GFP(−) together with the nine protein-expressing plasmids listed in Table 1 (a, d, e), nine plasmids including pCAGGS.MCS/M1-TAY instead of pCAGGS.MCS/M1-AA (b), eight plasmids without pCAGGS.MCS/NP-AA (c) or eight plasmids without pCAGGS.MCS/M1-AA (f). Clarified supernatants collected at 48 h p.t. were treated with TPCK-trypsin then incubated with PBS (a, b, c, f); anti-HE mAb Z2 (d); or anti-HE mAb J14 (e). HMV-II cells were inoculated with the resulting supernatants and subsequently infected with helper virus (C/Ann Arbor/1/50) at m.o.i. 10. The GFP-positive HMV-II cells were detected at 24 h p.i. by fluorescence microscopy (magnification × 200).

Fig. 1. Generation of influenza C VLPs. 293T cells were transfected with 0.5 μg pPolI/NP-AA.GFP(−) together with the nine protein-expressing plasmids listed in Table 1 (a, d, e), nine plasmids including pCAGGS.MCS/M1-TAY instead of pCAGGS.MCS/M1-AA (b), eight plasmids without pCAGGS.MCS/NP-AA (c) or eight plasmids without pCAGGS.MCS/M1-AA (f). Clarified supernatants collected at 48 h p.t. were treated with TPCK-trypsin then incubated with PBS (a, b, c, f); anti-HE mAb Z2 (d); or anti-HE mAb J14 (e). HMV-II cells were inoculated with the resulting supernatants and subsequently infected with helper virus (C/Ann Arbor/1/50) at m.o.i. 10. The GFP-positive HMV-II cells were detected at 24 h p.i. by fluorescence microscopy (magnification × 200).

Inhibition of reporter gene expression by treatment of supernatants with anti-HE mAb

We attempted to neutralize the VLPs in order to verify that they infect cells via HE glycoprotein. Clarified supernatants were incubated with anti-HE mAb J14 or Z2 (only the former of which has neutralization activity; Sugawara et al., 1993) for 60 min before transfer to HMV-II cells. GFP expression was drastically reduced in the J14-treated sample (Fig. 1e), whereas incubation of the supernatants with Z2 did not affect the GFP expression at all (Fig. 1d), indicating that the VLPs, like authentic influenza C viruses, infect HMV-II cells via the HE glycoprotein.

Detection of VLP by electron microscopy

To obtain further evidence that the transfected 293T cells produce influenza C VLPs, culture media of the cells were concentrated and examined by electron microscopy after negative staining. As shown in Fig. 2(a), in the supernatant of the 293T cells transfected with pPoll/ NP-AA.Luc(−) and nine plasmids listed in Table 1 spherical VLPs with a diameter of 80–100 nm were detected. In addition, filamentous VLPs ≥500 nm long with spikes on their surface were observed frequently (Fig. 2a). The VLPs generated from 293T cells transfected with pPoll/NP- AA.Luc(−) and nine plasmids including pCAGGS.MCS/ M1-TAY instead of pCAGGS.MCS/M1-AA were also examined (Fig. 2b). The packing of surface glycoproteins in regular hexagonal arrays (Fig. 2b, triangles), a striking feature of influenza C virus preparations as reported previously (Waterson et al., 1963; Flewett & Apostolov 1967; Compans et al., 1977; Herrler et al., 1981), was observed, and almost all the VLPs detected were roughly spherical. Taken together, these findings suggest that the M1 protein affects the morphology of the influenza C VLP.

M1 protein is involved in the formation of CLS on transfected 293T cells

It was observed that cords 50–300 μm long protruded from approximately 10% of the 293T cells transfected with pPoll/ NP-AA.Luc(−) and the nine plasmids listed in Table 1 (Fig. 3a). Nishimura et al. (1994) reported that 62 influenza C virus strains examined, with the exception of TAY/47, are capable of forming CLS on infected cells. The cords observed in this study appeared to be equivalent to CLS, as adsorption of chicken erythrocytes to the cords was observed (data not shown), a phenomenon described by Nishimura et al. (1990). To verify that the cords from transfected 293T cells are similar to CLS, we examined the cords by electron microscopy after negative staining or thin sectioning. Negative staining indicated that the cords consisted of several filamentous structures in a close parallel arrangement (data not shown). In thin sections the cords appeared to be composed of bundles of filamentous VLPs, as a layer of clearly spaced projections (Fig. 4a, arrowheads) and a hexagonal arrangement of spikes (Fig. 4a, triangles) were observed. In cross-sections
Fig. 2. Electron micrographs of VLPs. 293T cells were transfected with 0.5 μg pPolI/NP-AA.Luc(−) together with the nine plasmids listed in Table 1 (a) or the nine plasmids including pCAGGS.MCS/M1-TAY instead of pCAGGS.MCS/M1-AA (b). At 48 h p.t. the VLPs generated from the 293T cells were concentrated from the culture media, negatively stained, then examined under an electron microscope. Arrowheads and triangles denote spikes on the VLPs and packing of surface glycoproteins in regular hexagonal arrays, respectively. Bars, 100 nm.

Fig. 3. Cord formation on transfected 293T cells. 293T cells were transfected with 0.5 μg pPolI/NP-AA.Luc(−) together with the nine plasmids listed in Table 1 (a) or the nine plasmids including pCAGGS.MCS/M1-TAY instead of pCAGGS.MCS/M1-AA (b). At 48 h p.t. the cells were observed under a light microscope (magnification ×200). Cords protruding from cells are indicated by triangles.
Fig. 4b) The cords were demonstrated to be filamentous VLPs, the spikes of which bind to each other to form a bundle. Therefore we conclude that the cords from transfected 293T cells are similar in morphology to the CLS described by Nishimura et al. (1990).

Nishimura et al. (1994) also proposed that the M gene is the key determinant of cord-forming ability of the virus, and that the M1 protein is responsible for this ability based on comparisons of the nucleotide sequences between five CLS-producing strains (AA/50; Yamagata/64; Aomori/74; Aichi/1/81; YA/88) and a non-producing strain (TAY/47). To obtain direct evidence that the M1 protein is involved in CLS formation, pCAGGS.MCS/M1-TAY, which encodes the M1 protein of TAY/47, was constructed and transfected into 293T cells together with pPolI/NP-AA.Luc(−) and the other eight protein-expressing plasmids. No CLS were observed from the transfected cells (Fig. 3b), although the...
The M1 protein of TAY/47 was expressed equivalently in the transfected cells, as confirmed by immunoblotting (data not shown). This finding clearly indicates that M1 protein is the key determinant for CLS formation, whereas no cords were observed when AA/50 M1 protein was expressed alone in 293T cells (data not shown).

**An amino acid at residue 24 of the M1 protein is critical for CLS formation**

Both the M1 proteins of AA/50 and TAY/47 consist of 242 amino acids, and there were two amino acid substitutions at positions 24 and 133 between the two strains (Nishimura et al., 1994). On the other hand, there were three amino acid differences at residues 24, 67 and 133 between the M1 cDNA clones of AA/50 and TAY/47 we selected (Table 2). This discrepancy is likely to represent a difference between our viruses and those sequenced by Nishimura et al. Therefore we focused on the two amino acid residues at positions 24 and 133, and constructed pCAGGS.MCS/M1-AA-A24T and pCAGGS.MCS/M1-AA-D133N, plasmids encoding M1 mutants of AA/50 in which Ala 24 and Asp 133 were changed to Thr and Asn, respectively, in order to determine the amino acid responsible for CLS formation. Transfection of pCAGGS.MCS/M1-AA-A24T together with pPolI/NP-AA.Luc(−) and the other eight protein-expressing plasmids resulted in no CLS formation (Table 2), suggesting that the amino acid at position 24 of M1 is involved in CLS formation. To examine further whether the replacement of Thr 24 of the TAY/47 M1 protein with Ala can restore CLS formation, pCAGGS.MCS/M1-TAY-T24A was constructed and transfected together with pPolI/NP-AA.Luc(−) and the other eight protein-expressing plasmids. CLS were observed on approximately 10% of the transfected cells (Table 2). Thus the amino acid at residue 24 of the M1 protein was demonstrated to be critical for CLS formation.

The amino acid at position 24 of M1 is located within a hydrophobic region consisting of 10 consecutive uncharged amino acids (residues 23–32) (Hongo et al., 1994; Tada et al., 1997; Yamashita et al., 1988). The X-ray crystal structure of the N-terminal portion of the influenza A virus M1 protein has been determined, and the hydrophobic surface opposite the RNA-binding region is hypothesized to be involved in membrane interaction (Sha & Luo, 1997). Therefore amino acid 24 of the influenza C virus M1 protein is possibly involved in membrane association.

Although we have provided direct evidence that the M1 protein is involved in CLS formation, the mechanism by which CLS are formed remains to be clarified. Nishimura et al. (1990) observed by electron microscopy that most of the long filaments projecting from infected cells were devoid of nucleocapsids, and hypothesized that in influenza C virus-infected cells a pinch-off mechanism comes into action very ineffectively, probably due to a smaller amount of nucleocapsids in the cells or very inefficient incorporation of the nucleocapsids into the virions. It is therefore possible to assume that CLS are formed due to very inefficient incorporation of nucleocapsids into VLPs. As the M1 protein is generally believed to play a central role during virion assembly by bridging the envelope and the nucleocapsid, investigations of the possible difference in association between nucleocapsids and the M1 proteins mutated at position 24 would be of help in understanding the mechanism of CLS formation.

Comparison of the M gene nucleotide sequences of the representative 34 influenza C virus strains isolated between 1947 and 1998 showed that all strains, except TAY/47, had Ala at position 24 of the M1 protein (Matsuzaki et al., 2002; Tada et al., 1997). This finding is consistent with the fact that 62 influenza C virus strains examined are capable of forming CLS on infected cells, with the exception of TAY/47 (Nishimura et al., 1994). Therefore the ability to form CLS can be considered to be a general characteristic of influenza C viruses, but the role of CLS-forming ability in virus replication remains to be clarified.

In this report we provide evidence that 293T cells expressing the M1 protein of TAY/47 produced mainly spherical VLPs, and those expressing the M1 protein of AA/50 produced filamentous VLPs in addition to spherical ones. This finding suggests that influenza C virus M1 protein has a potential role in determining the morphology (spherical/filamentous) of the virion. As shown in Fig. 1(b) and 2(b), TAY/47 M1 protein was functional in VLP formation as well as reporter gene transfer. Furthermore, all three M1 mutants in Table 2 (AA-A24T; AA-D133N; TAY-T24A) were also functional in reporter gene transfer (data not shown), suggesting that the Ala/Thr mutation at position 24 of the M1 protein would be tolerated during influenza infection.

### Table 2. M1 proteins expressed in 293T cells and cord formation on the cell

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<tr>
<th>M1 protein*</th>
<th>Amino acid residue†</th>
<th>Cord formation</th>
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<td>AA-A24T</td>
<td>T</td>
<td>P</td>
</tr>
<tr>
<td>AA-D133N</td>
<td>A</td>
<td>P</td>
</tr>
<tr>
<td>TAY-T24A</td>
<td>A</td>
<td>A</td>
</tr>
</tbody>
</table>

*The cDNAs for these M1 proteins were cloned into the expression vector pCAGGS.MCS and the resulting plasmid DNAs were transfected into 293T cells together with pPolI/NP-AA.Luc(−) and the other eight protein-expressing plasmids.

†Both the M1 proteins of AA/50 and TAY/47 consist of 242 amino acids; differences in the amino acids between the two strains are shown.

‡The difference at this residue was not reported by Nishimura et al. (1994).
C virion formation. Therefore future reverse genetics studies of recombinant influenza C virus with mutations at position 24 of the M1 protein will give us greater insight into the question of whether M1 is the key determinant for generation of filamentous virions.

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