The position 4 nucleotide at the 3′ end of the influenza virus neuraminidase vRNA is involved in temporal regulation of transcription and replication of neuraminidase RNAs and affects the repertoire of influenza virus surface antigens

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Introduction
The genome of influenza A virus comprises eight single-stranded RNA segments with negative polarity (Ishihama & Nagata, 1988; Lamb & Choppin, 1983; McCauley & Mahy, 1983). In virions, the genomic RNA segments are associated with RNA polymerase (PB2, PB1 and PA) and nucleoprotein (NP). These four core proteins are essential for expression of influenza virus RNAs (Huang et al., 1990; Kimura et al., 1992; Mena et al., 1994). In virus-infected cells, three different modes of RNA synthesis have been distinguished (Lamb & Choppin, 1983; McCauley & Mahy, 1983): (i) transcription of the negative-sense virion RNA (vRNA) into mRNA; (ii) replication of vRNA into full-length complementary RNA (cRNA); and (iii) replication of cRNA into genomic vRNA. The switch from mRNA into cRNA synthesis involves a requirement for the NP protein (Beaton & Krug, 1986; Krug et al., 1989). The role of each P protein in transcription has been extensively studied (Huang et al., 1990; Nakagawa et al., 1996). Sequence analysis of genomic RNAs of influenza A viruses revealed highly conserved sequences of 12 nt at the 3′ terminus and 13 nt at the 5′ terminus, which are partially complementary to each other and form a panhandle structure (Desselberger et al., 1980; Hsu et al., 1987; Robertson, 1979; Skehel & Hay, 1978; Winter & Fields, 1980). The importance of these conserved sequences in transcription and replication of influenza virus RNAs has been investigated in vivo (Li & Palese, 1992; Neumann & Hobom, 1993; Piccone et al., 1993; Yamanaka et al., 1998). Within the sequence motif conserved at the extreme ends of the influenza virus vRNAs, a unique natural variation, U or C, is observed at position 4 of the 3′ end. To test the role of this nucleotide, two isogenic A/WSN/33 viruses, carrying either C4 or U4 nucleotide at the 3′ end of the neuraminidase (NA) gene, were generated. Compared with the C4 virus, the U4 virus exhibited delayed synthesis of vRNA and stimulation of mRNA synthesis with prolonged accumulation in influenza virus-infected cells. The mRNA/vRNA ratio was increased up to 20-fold by the C4 → U4 substitution suggesting that the U4 nucleotide greatly stimulated transcription of the vRNA template. In isolated virion, the U4 virus had higher NA activity than the C4 virus. In MDBK cells, the U4 virus grew to lower haemagglutination (HA) titres but with higher infectivity than the C4 virus, with a corresponding increase in the ratio of p.f.u./HA units of about 10- to 40-fold. Western blot analysis of isolated virion showed that the ratio of two surface proteins, HA/NA, was greatly decreased in the U4 virus. This suggests that the position 4 nucleotide is a genetic determinant for the repertoire of surface antigens and their ratio could be changed without detrimental effects on virus growth. Results could be used to design genetically engineered influenza virus for vaccination. The observed down-regulation of transcription by C4 nucleotide is consistent with its potential role in segment-specific regulation of influenza virus gene expression, especially PB1, PB2 and PA proteins, during virus infection.
al., 1991; Zheng et al., 1996) and in vitro (Fodor et al., 1994, 1995; Seong & Brownlee, 1992b). Within this conserved sequence motif, a unique natural variation, U or C, is observed at position 4 of the 3' end of the vRNA. Current sequencing data show that, with the potential exception of neuraminidase (NA) and matrix protein (M) genes, in which both nucleotides are documented at this position, all polymerase (PB1, PB2 and PA) genes invariably carry the C residue at this position (Desselberger et al., 1980; Robertson, 1979). Since polymerase proteins are expressed at very low levels in influenza virus-infected cells and are also present at low levels in isolated virions, it could be hypothesized that the C4 nucleotide is involved in down-regulation of transcription. Recently, it has become possible to introduce site-specific mutations into the endogenous influenza virus genome and rescue the mutations in transfected virus (Castrucci & Kawaoka, 1995; Enami et al., 1990). Besides genetic analysis of cis-acting signals in gene replication, this technical development is being actively pursued for functional analysis of influenza virus proteins (Castrucci & Kawaoka, 1993, 1995) and for generating chimeric influenza viruses as genetically engineered vaccine prototypes (Subbarao et al., 1993). Using the classic NA gene rescue system based on the complementation of the host range phenotype of the influenza A/WSN-HK virus (Enami & Palese, 1991; Enami et al., 1990), we have tested various mutations at the 3' end of the NA vRNA. Among these, two isogenic viruses, with a U4 or C4 nucleotide at the 3' end, were successfully generated and maintained in tissue culture. Biochemical characterization of the two isogenic viruses allowed us to test the role of the position 4 nucleotide in the control of transcription and replication of influenza virus RNA.

**Methods**

**Virus and cells.** Madin–Darby bovine kidney (MDBK) cells were incubated in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% foetal calf serum (FCS). MDBK cells were used for transcription experiments and selection of rescued virus. Influenza A/WSN/33 (H1N1) virus was grown in MDBK cells. Influenza A/WSN-HK (H1N2) virus, a reasortant between A/WSN/33 and A/9/68, was used for the ribonuclease (RNase) transfection experiments (Castrucci & Kawaoka, 1993; Enami et al., 1990). A/WSN-HK virus was propagated in 11-day-old embryonated hen's eggs and titrated in Madin–Darby canine kidney cells. All the viruses and cell lines were a kind gift of Y. Kawaoka of the University of Wisconsin, Madison, WI, USA.

**Construction of plasmids.** A plasmid pT3 WSN (NA15) containing the cDNA of the influenza A/WSN/33 virus NA gene flanked by the Ksp632I site and T3 RNA polymerase recognition sequence was kindly provided by Y. Kawaoka. Mutagenesis of the A/WSN/33 NA 3' non-coding sequence was achieved by PCR using the plasmid pT3 WSN (NA15) as the template (Castrucci & Kawaoka, 1993). For the wild-type, the sequence of primers were: 5' GATGAATTCTCTTCGAGCGAA- AGCGAGACTTAAAT 3' (36-mer; primer A) and 5' GACAGC- TTTATACCCCT 3' (18-mer; primer B). Primer A carries an EcoRI site for cloning purposes and the Ksp632I site for run-off cleavage. All mutants were constructed using primer B and variants of primer A which carry corresponding nucleotide changes. The PCR fragments were digested with EcoRI and HIndIII and ligated into the pUC19 cloning vector. The desired nucleotide changes were verified by dye-dye sequencing of all mutant plasmid constructs.

**RNP transfection and virus rescue.** Influenza virus RNA polymerase was prepared from the X-31 virus as previously described (Seong & Brownlee, 1992a). RNP transfections were performed (Enami & Palese, 1991) using the micrococcal nuclease (MN)-digested influenza virus cores as the source of RNA polymerase and NP (Seong & Brownlee, 1992a). Briefly, plasmids were digested with Ksp632I, filled in with the Klenow enzyme, and transcribed with T3 RNA polymerase in the presence of the MN-digested influenza virus cores. The in vitro reconstituted RNP complex was then transfected into MDBK cells which were infected with A/WSN-HK virus at an m.o.i. of 1. Twenty hours after infection, transfectants in the supernatant were plated in MDBK cells in the absence of trypsin. Plaque purification was repeated three times in the absence of trypsin before biochemical characterization of transfected viruses.

**Sequence analysis of the 3' end of virion RNA.** The 3'-end sequence of the NA gene was determined as follows: RNA (1 µg) extracted from virus was poly(A) tailed by poly(A) polymerase (BRL) in the presence of 0.25 mM ATP. The reaction mixture was extracted with phenol–chloroform and reverse transcribed by murine leukaemia virus reverse transcriptase (BRL) in the presence of the primer, 5' AGCG- AATTCTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTT3'. Reverse transcribed products were amplified by PCR using an additional primer, 5' AGCAAGCTTACGAAACGAGGTITTTGTGAAACAAACTCT3', and cloned into pUC19 cloning vector. The sequence of the cloned gene was verified by dye-dye sequencing.

**Isolation of cellular RNAs from influenza virus-infected cells.** Confluent MDBK cells were infected with transfected viruses at an m.o.i. of 1 in 60 mm dishes. After 45 min adsorption at room temperature, the inoculum was removed. The infected cells were washed three times with PBS and 2 ml serum-free DMEM was added (designated the zero time of infection). At various times post-infection (p.i.) at 37°C (shown in Figs 4c and 5a), the medium was removed, and the cells were washed twice with PBS and harvested. Total RNAs were extracted with the RNA isolation kit (Ambion) following the manufacturer's instructions. The RNAs were precipitated with ethanol and the concentration of RNA was measured photometrically. Usually 40 µg RNA was obtained from 5×10⁶ cells per 60 mm dish. Fractionation of mRNA from total RNAs was carried out using the Oligotex mRNA kits (Qiagen). Both the mRNA-enriched fraction and the mRNA-depleted fraction (cRNA + vRNA) were subjected to the RNase protection assay.

**RNase protection assay.** For analysis of the positive-sense influenza virus-specific mRNA and cRNA, negative-sense NA-specific RNA was used as a probe. The probe RNA was 244 nt long and contained a nonviral sequence of 44 nt at the 3' end. For analysis of the negative-sense vRNA, a positive-sense RNA probe, 234 nt in length and containing a nonviral sequence of 34 nt at the 3' end was used (Fig. 4a). The cDNAs for each probe, with upstream T3 RNA polymerase recognition sequences, were cloned into the EcoRI and HindIII site of pUC19. Plasmid DNAs were linearized by EcoRI and transcribed with T3 RNA polymerase in the presence of [α-³²P]CTP (400 Ci/mmol; Amersham). Radioactive transcripts were extracted with phenol–chloroform and precipitated with ethanol. The ³²P-labelled RNA transcript was separated and isolated by denaturing PAGE. Specific activity of the RNA probe was calculated by liquid scintillation counting.
The RNase protection assay was carried out according to the manufacturer’s instructions (Ambion) with or without prior fractionation of mRNA from total RNAs from influenza virus-infected cells. RNA (10 µg), 10 µg of a yeast RNA and 10 µg of a tRNA as a negative control, labelled with [cytosine-32P]-CTP, were mixed together in a total volume of 15 µl. Hybridization buffer (20 µl; 80% deionized formamide, 100 mM sodium citrate pH 6·4, 300 mM sodium acetate pH 6·4 and 1 mM EDTA) was added and the mixture was heated for 3 min at 90 °C and incubated for 12 h at 45 °C. Then, 200 µl of RNase digestion buffer containing 100 U/ml RNase T1 was added, and the reaction mixture was incubated for 30 min at 37 °C. After addition of 300 µl of RNase inactivation/precipitation mixture, the RNAs were precipitated at −20 °C for 1 h, dissolved in 8 µl loading buffer, and analysed on 5% polyacrylamide–8 M urea denaturing gel. The gel was dried, exposed to X-ray film, and the RNA bands were semi-quantified by densitometric scanning (LKB; ULTROSCAN XL Enhanced Laser Densitometer).

**NA assay.** NA activity was assayed as described previously (Aymard-Henry *et al.*, 1973; Castrucci & Kawaoka, 1993). Briefly, serial 2-fold dilutions of influenza virus stock (50 µl) were incubated with the same volume of fetuin (50 mg/ml) in the presence of 0·1 M MES buffer (pH 5·9) and 1·5 mM CaCl$_2$ at 18 h at 37 °C. After addition of N-acetylneuraminic acid released by NA was chemically converted to a pink chromophore, extracted with n-butanol–HCl, and measured photometrically at 549 nm.

**ELISA.** The 96-well microtitre plates were coated with serial 2-fold-diluted influenza virus solution in PBS for 1 h at room temperature and blocked with blocking buffer (1% BSA, 0·02% NaN$_3$, 0·06% diaminobenzidine tetrahydrochloride in TBS–0·2% Tween 20, 4% BSA, pH 7·4) for 1 h. Influenza A/WSN/33 virus-specific MAb 10C9 (specific for NA) or 4B2 [specific for haemagglutinin (HA)] (provided by P. Palese in Mount Sinai School of Medicine, New York, USA) were added to each well. After incubation at room temperature for 1 h, each well was washed and antibody–enzyme-conjugated solution (goat anti-mouse IgG conjugated with alkaline phosphatase) was added. After incubation at room temperature for 1 h, the wells were washed and substrate solution (3 mM p-nitrophenyl phosphate; Sigma) was added. The plate was incubated at room temperature until the yellow colour was visualized. The reaction was stopped by addition of 0·5 M NaOH and $A_{540}$ was determined on an ELISA plate reader.

**Western immunoblot analysis.** For Western analysis of NA and HA, proteins of the isolated virion were electrophoresed on a 12% SDS–polyacrylamide gel. The separated proteins were then transferred to a nitrocellulose membrane and blocked with 10% skimmed milk dissolved in TBS (100 mM Tris–HCl pH 7·5, 0·9% NaCl). The membrane was washed with TBS containing 0·02% Tween 20 and then incubated for 1 h in the presence of the anti-WSN MAbs 10C9 or 4B2 in TBS-0·02% Tween 20. The membranes were washed and reacted with a goat anti-mouse IgG–horseradish peroxidase conjugate (Promega) in TBS. After washing with TBS-0·02% Tween 20, protein bands were visualized by incubation of substrate (0·06% diaminobenzidine tetrahydrochloride in 0·01M Tris–HCl pH 7·6, 0·03% NiCl and 0·03% H$_2$O$_2$).

**Results**

**Rescue of the C4 and U4 isogenic viruses**

Various mutations were introduced at the 3′ end of the NA vRNA to examine their effects on plaqueing ability in MDBK cells. The mutations tested were single base substitutions at positions 4 and 5 (C4 → A4, U4 and G4; U5 → A5, C5 and G5), single or double base insertions between position 4 and 5 (IC$_4$5, IU$_4$5, IU$_5$4), single base deletion at position 5 (ΔU5), and combinations of base substitution and insertion (C4 → UC and UU) (Fig. 1). Among these mutations, the only virus that was successfully rescued as infectious virus was the C4 → U4 transition mutant. As a control, a transfectant virus carrying the wild-type C4 nucleotide was also generated using the same transfection protocol. The two isogenic viruses were plaque-purified three times in MDMK cells. All biochemical characterizations were carried out in parallel on these two isogenic transfectant viruses. The NA-specific RNA was amplified from purified virions (see Methods) and the desired mutation in vRNA was verified (Fig. 2b). After repeated passage, the whole structural NA gene of the mutant was also sequenced by RT–PCR amplification of the NA RNA. The sequence was identical to that of the published one (Hiti & Nayak, 1982) except for the G → A change at position 533 (Val → Met substitution) and the A → G silent mutation at position 769. Since the same mutations were also found in the wild-type pT3 WSN (NA15) plasmid, any physiological changes observed with the mutant virus could be ascribed to the position 4 nucleotide change. The very fact that the C4 → U4 mutation could be rescued suggests that the mutation is not detrimental to virus growth and the effect of the mutation, if any, should be very subtle. The failure to obtain visible plaques with all other mutations further suggests that these mutations in the 3′ non-coding sequences are detrimental to virus growth (Fig. 1). It should be noted that C4 and U4 are the only nucleotides which vary naturally within the 5′ and 3′ conserved sequences of the influenza virus vRNA (Desselberger *et al.*, 1980; Robertson, 1979).

**Growth characteristics of transfectant viruses**

The growth of the transfectant viruses was characterized in MDBK cells. MDBK cells were infected with the two isogenic viruses at an m.o.i. of 0·01, and maintained in serum-free DMEM. Supernatants were collected at 6, 18, 25, 31, 43, 50, 56
Fig. 2. Sequence analysis of the 3’ end of the NA vRNA. Template virion RNAs were isolated from the purified virus. After poly(A) tailing, RT–PCR products were sequenced and the C4 (a) and U4 (b) nucleotides of the two transfectant viruses were verified. The vRNA sequence was deduced from that of the cDNA (arrowheads indicate the position 4 nucleotide).

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and 68 h p.i. and plaqued in MDBK cells. As shown in Fig. 3, peak yield of viruses was attained 25–30 h after infection. The U4 virus grew to a 2- to 4-fold lower HA titre than the C4 virus (Fig. 3a). However, the plaque forming ability of the U4 virus was about 5- to 10-fold higher than that of the C4 virus (Fig. 3b). Therefore, the p.f.u./HA unit ratio was increased by approximately 10- to 40-fold by the mutation. A similar time-course of the two parameters was reproduced in a separate experiment. The plaque size of the U4 virus was similar to that of the C4 virus in MDBK cells (data not shown).

Temporal regulation of influenza virus-specific RNAs in infected cells

An RNase protection assay was adopted to analyse the time-course of expression of the NA-specific RNAs in infected cells. Confluent MDBK cells were infected with viruses at an m.o.i. of 1. At various times p.i., as specified in Fig. 4(c) and Fig. 5(a), RNAs were extracted, hybridized with respective probes, digested with RNase T1 and the protected RNA fragments were analysed by 5% PAGE in 8 M urea. As mRNA lacks the copy of the last 16 nt at the 5’ end of vRNA, the digested product would be shorter than that of cRNA and could be easily resolved on the gel. The rationale for this assay is shown in Fig. 4(a).

Since self-annealing among the mixture of RNAs and hence competition for the probes might affect the assay, a control protection assay was carried out in the presence of various amounts of probe in the reaction mixture. As shown in Fig. 4(b), the intensities of the three influenza virus-specific RNAs were quite similar over the range of probe concentration used, even at one-tenth (Fig. 4b, lanes 4 and 8) of the concentration used in the routine protection assay (Fig. 4b, lanes 1 and 5, respectively). This confirmed that the probe was present in excess over all influenza virus RNAs present in the sample and ensured reliable quantitative data in our experimental setting. The RNase protection assay of total influenza virus-specific RNAs is shown in Fig. 4(c). The RNA bands corresponding to mRNA, cRNA and vRNA were semi-quantified by scanning the exposed X-ray film. As shown in Fig. 4(d), differences were observed in the pattern of temporal regulation of the influenza virus-specific RNAs. The vRNA synthesis of the C4 virus rose sharply at 5–6 h p.i. and continued to increase up to 12 h p.i., whereas that of the U4 virus became apparent at 8–9 h p.i. with about 3–4 h delay as compared to the C4 virus. Synthesis of cRNA was most active at 6 h p.i. in the C4 virus, whereas a lower level of synthesis (about 20–30% of the C4 virus) was observed with the U4 virus in a prolonged period at 6–9 h p.i. These results showed that replication of the influenza virus
genome was reduced and delayed by the U4 nucleotide. However, the mRNA level of the U4 virus was at least comparable to that of the C4 virus and an even higher level of synthesis was observed over a prolonged period of 5–10 h p.i. Within the period of 6–9 h p.i., the ratio of mRNA/vRNA in the U4 virus was about 10- to 20-fold higher than that in the C4 virus. The results indicated that the U4 nucleotide led to higher expression of mRNA and this increase of transcription was not dependent on the vRNA level.

To minimize potential interference among influenza virus-specific RNAs with opposite polarity (see above), a separate protection assay was conducted after separation of mRNA by oligo(dT) chromatography. As shown in Fig. 5(a) and semi-quantified in Fig. 5(b), the pattern of temporal accumulation of mRNA and vRNA was similar to the previous analysis without separation of mRNA (Fig. 4c and d). As previously suggested by the control experiment in Fig. 4(b), this assures that the change in temporal pattern of viral RNAs is a nucleotide-specific effect and not an experimental artifact. In this experimental setting, the cRNA level was below the detection limit even after prolonged exposure of X-ray film and could not be quantified (data not shown). This showed that the transcription event from the vRNA template was greatly stimulated by the U4 nucleotide within the promoter. In these experiments, the culture of influenza virus-infected cells was done in serum-free media. For comparison, the same RNase protection assay was repeated on infected cells in the presence of 2% FCS. We observed essentially the same pattern of temporal regulation of influenza virus-specific RNA synthesis for both viruses (data not shown).

### NA activity associated with isolated virions

Stimulation of NA mRNA synthesis may lead to increased synthesis of NA protein and incorporation of more NA molecules into the virion. To investigate this possibility, a standard NA assay was performed on purified virus using fetuin as substrate (Aymard-Henry et al., 1973; Castrucci & Kawaoka, 1993). Equivalent amounts of each virus (100 HA units) were used as a stock solution and 2-fold serial dilutions were made. The activity profile was compared in Fig. 6. Within the linear range between the amount of virus and absorbance, the U4 virus exhibited consistently higher NA activity (about 2-fold) than the C4 virus. We then performed ELISA assays on HA and NA using purified virus as antigen and MABs against each protein (Table 1). When the ratio of NA/HA was calculated based on $A_{405}$, the U4 virus exhibited consistently higher values (2±1- to 2±6-fold) than the C4 virus. The increase in the ratio of NA/HA was consistent with the higher level of expression of NA mRNA in the U4 virus-infected cells.

### Western blot analysis

Western blot analysis of the NA and HA proteins was performed after viral proteins were resolved by 12% SDS–PAGE and transferred to a nitrocellulose membrane (Fig. 7). The control lane of the C4 virus (lane 1) showed, as expected, one single NA band using the 10C9 anti-NA MAb. Using the 4B2 anti-HA MAb, two bands of different molecular mass, probably the uncleaved and cleaved form of HA, were observed (lane 2). In the presence of both antibodies, all three bands of NA and HA were present (lane 3). When a similar analysis was done with the U4 virus (lane 4), a higher level of NA and a much lower level of HA were observed (compare with the C4 virus in lane 3). Based on the intensity of each band, the ratio of HA/NA was at least 20:1 for the C4 virus and about 0:5:1 for the U4 virus (Fig. 7, lanes 3 and 4, respectively), with a corresponding increase of the NA/HA ratio by about 40-fold by C4 → U4 substitution. Similar Western blot analysis on serially diluted viral antigen using anti-NP antibody as a control gave similar values (data not shown). The experiments confirmed a great increase in the
Fig. 4. For legend see facing page.
ratio of NA/HA by the U4 nucleotide and the increment was even more dramatic than that observed in the ELISA assay (Table 1) or in the NA assay (Fig. 6). The whole coding region of the HA of the transfectant virus was sequenced and the sequence was identical to that of the published one (Hiti et al., 1981) except for the A → G single base substitution at position 547 with a corresponding Asp → Gly change (data not shown). Similar natural variation was previously observed in the A/HK/68 virus and is not part of the known antigenic site of HA molecule (Wiley & Skehel, 1987).
Fig. 5. (a) RNase protection assay after separation of influenza virus-specific mRNA and vRNA. At various times p.i., RNAs were isolated from the infected cells and fractionated by oligo(dT)-column chromatography. Both the mRNA-enriched fraction and the mRNA-depleted fraction were subjected to the RNase protection assay in the presence of excess probe (see Fig. 4(b)). The protected RNAs were analysed on a 5% polyacrylamide–8 M urea gel. (b) Temporal accumulation of virus-specific RNAs. The amounts of RNAs corresponding to vRNA and mRNA were estimated by densitometric scanning of the exposed film. The cRNA level was below the detection limit within the experimental setting (data not shown).
Influenza virus RNA transcription and replication

Fig. 6. Comparison of NA activities associated with isolated virions. The highest concentration of virus stock is 100 HA units. Serial 2-fold dilutions of virus stock were incubated with fetuin in the presence of 0.1 M MES buffer (pH 5.9). The absorbance of chemically converted final products was measured at 549 nm. □, C4 virus; ○, U4 virus.

Discussion

The highly conserved sequences at the 5′ and 3′ ends of the influenza virion RNA have been shown to provide pivotal signals for its replication and transcription (Kim et al., 1997; Li & Palese, 1992, 1994; Neumann & Hobom, 1993; Piccone et al., 1993). The only variation within the conserved element observed in natural influenza virus isolates is the U and C nucleotide at position 4 of the 3′ end of vRNA. In the present report, we generated two transfectant viruses which carried a C4 or U4 nucleotide at the 3′ end at the NA vRNA. Other base substitution mutations at position 4, e.g. A4 or G4, or at position 5, could not be rescued as transfectant virus, suggesting that the effect of these nucleotides was detrimental to the virus life-cycle. In previous in vitro analysis, apparent activation of transcription was observed by insertions between positions 4 and 5 at the 3′ end of vRNA (Seong & Brownlee, 1992b). None of these mutations, either alone or in combination with insertions and base substitutions in neighbouring nucleotides were rescued as transfectant virus. The very fact that only the two C4 and U4 isogenic viruses could be obtained by transfection suggests that the effect of the position 4 nucleotide is subtle and that this subtle effect is compatible with the virus life-cycle. In this report, we compared throughout the experiments two independent isogenic viruses which were generated by the same transfection methodology (Enami et al., 1990) and differ at this single nucleotide. The NA gene of the A/WSN/33 virus has been documented to have a C residue at position 4 (Hiti & Nayak, 1982). However, data were not based on direct sequencing of the viral RNA. It should be mentioned that direct sequencing by RT–PCR showed a U4 residue at the 3′ end of the WSN NA gene (P. Palese, personal communication). Since both C and U nucleotides have been documented at position 4 of the NA gene in different influenza A virus isolates (Desselberger et al., 1980; Robertson, 1979), it may be possible that, especially for the NA gene, both

Table 1. The NA/HA ratio in ELISA

Isolated viruses were used as antigens for ELISA. Results are based on A_{549}.

<table>
<thead>
<tr>
<th>Serial dilution of virus</th>
<th>C4 virus</th>
<th>U4 virus</th>
<th>U4 virus/C4 virus</th>
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<tr>
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nucleotides would be compatible with the virus life-cycle without detrimental effect on virus growth.

The nature of the position 4 nucleotide was associated with significant changes in temporal regulation in influenza virus RNA synthesis. Most prominently, a 10- to 20-fold increase in transcription was achieved per vRNA template. Since mRNA synthesis is different from cRNA synthesis at both initiation and termination steps, the stimulation could be either at the level of initiation or polyadenylation. None of the current models for polyadenylation postulates the importance of the 3’ end nucleotides. For example, the stuttering model involves the base-paired RNA duplex region and the poly(U) sequence (Li & Palese, 1994), whereas the other model postulates the binding of polymerase to the 5’ end of vRNA during transcription (Fodor et al., 1994; Tiley et al., 1994). Therefore, activation of mRNA synthesis is probably at the initiation step. How the same vRNP complex commits itself either to the transcription mode or the replication mode is poorly understood. Transcription of influenza virus mRNA is a complex process which involves three polymerase proteins (PB1, PB2 and PA) and NP (Huang et al., 1990), and the temporal regulation of the switch from transcription to replication would influence the relative populations of influenza virus-specific RNAs. Except for the involvement of the NP protein in the anti-termination process, the role of individual polymerase proteins in the switch process is largely unknown. Presumably, the nature of the nucleotide within the RNA promoter and/or its influence on the panhandle structure (Fig. 1) may effect potentially differential contact with the polymerase complex during transcription. The position 4 nucleotide was shown to interact with polymerase by cross-linking studies (Fodor et al., 1993), although the relative affinity to various nucleotides has not been investigated. Recently, the structure of the influenza virus vRNA panhandle was investigated by NMR (Cheong et al., 1996), and the potential effect of the position 4 nucleotide on the structure merits further investigation.

As well as the dramatic effect on transcription, we also observed a change in replication: delayed synthesis of vRNA and reduced synthesis of cRNA. It is uncertain whether the effect is at the level of vRNA → cRNA synthesis or cRNA → vRNA synthesis or both. With regard to the replication of cRNA into vRNA, it should be remembered that substitution of C4 → U4 at the 3’ end of the vRNA would result in a corresponding change of G4 → A4 at the 5’ end of the cRNA. It has been reported that the 5’ end of the vRNA constitutes an integral part of the vRNA promoter (Fodor et al., 1994, 1995; Kim et al., 1997). If the 5’ end of the cRNA is similarly involved in the synthesis of vRNA (Prilove et al., 1995), this particular nucleotide change may also be involved in temporal regulation of vRNA synthesis.

Unlike NA or M genes, in which natural variation of C or U has been documented, all three polymerase (PB1, PB2 and PA) genes sequenced so far invariably carry a C4 nucleotide (Desselberger et al., 1980; Robertson, 1979). This strong conservation, without any exception, strongly supports its role in down-regulation of transcription of polymerase genes. Since only catalytic amounts of polymerase proteins are present in influenza virus-infected cells and isolated virions (Inglis & Mahy, 1979; Lamb & Choppin, 1976), down-regulation would be important for economizing viral protein synthesis. It is also possible that the non-coding region of vRNA segments may also play a role in down-regulation, although it is difficult to find consensus elements in these heterogeneous sequences. In fact, a number of studies have investigated the potential role of non-coding nucleotides using an in vitro reporter gene system (Kim et al., 1997; Li & Palese, 1992; Neumann & Hobom, 1993; Piccone et al., 1993; Yamanaka et al., 1991), an in vitro transcription system (Fodor et al., 1993, 1994; Seong & Brownlee, 1992b), or using transfected viruses which carry mutations in the endogenous RNA genome (Bergmann & Muster, 1996; Luo et al., 1992; Zheng et al., 1996). It has been shown that genetic determinants of replication control also involve non-conserved nucleotides of the terminal sequences (Bergmann & Muster, 1996; Luo et al., 1992; Zheng et al., 1996). Multiple mutations had dramatic effects on replication and also affected mRNA synthesis (Bergmann & Muster, 1996; Zheng et al., 1996). Therefore, transcription and replication control is achieved by nucleotides both within and outside the conserved sequences. The natural variation in the NA gene is allowed, we suggest, because NA does not play an obligatory role in the virus life-cycle (Bo and Nayak, 1986; Breuning et al., 1987; Breuning & Scholtissek, 1986; Pattnaik et al., 1986; see below) and therefore the expression level could vary. The M protein is one of the most abundantly expressed proteins and yet C4 variation is documented (Desselberger et al., 1980). However, translation of the M1 protein is greatly stimulated by the influenza virus NS1 protein (Enami et al., 1994), and we suggest that translational control plays an important role in M gene expression. The dramatic effect on overall balance of mRNA, cRNA and vRNA levels as shown in this study suggests that the influenza virus has adopted the position 4 single base substitution within the RNA promoter as a strategy to control segment-specific gene regulation.

The increase in virion-associated NA activity in the U4 virus is probably due to the stimulation of NA protein synthesis in infected cells and incorporation of more NA molecules in the virion. Moreover, the U4 virus exhibited a much reduced amount of HA in the virus, resulting in a dramatic decrease in the ratio of HA/NA in the virion. The decrease in HA is also reflected in a great increase (about 10- to 40-fold) in the p.f.u./HA ratio in the U4 virus. The function of NA is the removal of receptors from the HA during the later stage of infection, and this prevents self-aggregation and promotes the release of virus from the infected cell. The virus expressing more NA and less HA protein would promote the release of virus particles from virus aggregates (Liu et al., 1995) and may increase the p.f.u. We have sequenced the whole coding region of the HA in this transfectant and failed to detect
any significant changes in coding sequences which would account for the observation. One possibility is that overexpression of the NA molecule effectively saturates the ER/Golgi transport apparatus, and the transport of HA molecules to the membrane of the infected cell could be hampered. The results suggest that the ratio of the two major surface proteins could be changed without detrimental effects on the virus life-cycle. The result is also consistent with previous reports that in influenza A viruses the presence of NA or HA at the plasma membrane is not necessarily obligatory for virus assembly and budding (Bos & Nayak, 1986; Breuning et al., 1987; Breuning & Scholtissek, 1986; Patnaik et al., 1986). Since both HA and NA proteins are involved in protective immunity against influenza virus infection (Ada & Jones, 1986), it is conceivable that the transfected viruses with substitution of this particular nucleotide may elicit different repertoires of antibodies against each of the proteins. The change in the relative contribution towards protective immunity may be usefully applied for vaccination with genetically engineered influenza viruses.

In conclusion, we have shown that two different processes of influenza virus gene expression could be temporally regulated by a single base change within the RNA promoter. Unlike DNA viruses, in which replication and transcription are controlled independently by DNA polymerase and RNA polymerase, respectively, a unique challenge in influenza virus gene regulation is that the same vRNA template is used for transcription and replication by the same RNA-dependent RNA polymerase. To accommodate this requirement, the influenza virus RNA promoter may not be an optimal, but a unique control device to ensure an optimal level of synthesis of virus growth. It is likely that the influenza virus utilizes this unique control device to ensure an optimal level of synthesis of virus-specific proteins.

We gratefully acknowledge Professor Y. Kawaoka in the University of Wisconsin in Madison for viruses, plasmids and cell lines in the initial period of this work. We are grateful for Dr P. Palese in Mount Sinai School of Medicine for monoclonal antibodies against HA and NA and personal communications regarding unpublished work. This work was supported by the Ministry of Health and Welfare in Korea.

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Received 5 March 1998; Accepted 6 April 1998