Nucleotides at the extremities of the viral RNA of influenza C virus are involved in type-specific interactions with the polymerase complex

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Influenza A and C viruses share common sequences in the terminal noncoding regions of the viral RNA segments. Differences at the 5′- and 3′-ends exist, however, that could contribute to the specificity with which the transcription/replication signals are recognized by the cognate polymerase complexes. Previously, by making use of a transient expression system for the transcription and replication of a reporter RNA template bearing either type A or type C extremities, it was shown that a type C RNA template is transcribed and replicated with equal efficiency by either the type A or the type C polymerase complex, whereas a type A RNA template is less efficiently transcribed and replicated by the type C polymerase complex than by the type A complex. To explore the contribution of the nucleotides at the extremities of the RNAs to this type-specificity, the effect of mutations introduced either alone or in combination at nucleotide 5 at the 3′-end and at nucleotides 3′, 6′ or 8′ at the 5′-end of type A or C RNA templates were studied in the presence of either the type A or the type C polymerase complex. The results indicate that the nature of nucleotides 5 and 6′ contribute to type-specificity. Moreover, these results underline the importance of the base pairing between nucleotide 3′ and 8′ at the 5′-end of the RNA. Thus, it could be suggested that the nature of the nucleotides as well as the stability of the secondary structure at the extremities of the viral RNA are important determinants of type-specificity.

Introduction

Influenza A, B and C viruses are members of the *Orthomyxoviridae* family, a group of enveloped negative-sense RNA viruses characterized by their segmented genome. The influenza C virus genome consists of seven single-stranded RNA segments, unlike the influenza A and B virus genomes which consist of eight segments. Sequence analyses of each RNA segment of the influenza A, B and C viruses revealed short conserved noncoding sequences at both the 5′- and the 3′-end (Desselberger *et al.*, 1980; Robertson, 1979). These sequences are partially complementary to each other and have the ability to form a partially double-stranded panhandle, fork or corkscrew structure (Flick *et al.*, 1996; Fodor *et al.*, 1994, 1995; Pritlove *et al.*, 1999).

There are some interesting differences between influenza A and C viral genomic RNAs (vRNAs). First, based on the few existing sequence data available for type C RNA segments (Clern-van Haaster & Meier-Ewert, 1984), 11 nucleotides are conserved at the 3′- and 5′-end of the vRNA on each of the seven segments of influenza C virus, whereas 12 and 13 nucleotides are conserved, respectively, at the 3′- and 5′-end of influenza A virus segments (Robertson, 1979). Despite the high degree of nucleotide conservation, natural variations have been documented at nucleotide 5 from the 3′-end (Desselberger *et al.*, 1980) for influenza C virus RNAs and at nucleotide 4 in the conserved 3′-end region of influenza A virus RNAs (Lee & Seong, 1998*a*). Second, the base-paired duplex region of the type C vRNA is usually shorter than that of type A vRNA (4–5 base pairs in influenza C virus and 4–8 base pairs in influenza A virus). Third, the uridine (U) stretch corresponding to the polyadenylation signal (Poon *et al.*, 1999) usually consists of 5–7 U residues in influenza A virus, whereas in influenza C virus the number of residues is 4–5 and no stretches of 6–7 U are found.

Influenza virus RNAs are complexed with the nucleoprotein (NP) and associated with the polymerase complex, which

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consists of three P proteins (PB1, PB2 and PA for influenza A virus or PB1, PB2 and P3 for influenza C virus), to form the viral ribonucleoproteins (RNPs). From biochemical and genetic data, it has been established that the PB1 subunit displays RNA-dependent RNA polymerase activity and that the PB2 subunit binds to the 5′-cap structure and is involved in the cap-snatching mechanism by which viral mRNA synthesis is initiated, whereas the role of the PA/P3 subunit is still unclear. The NP and P proteins have been shown to be the minimum set of viral proteins that are required for the transcription and replication of vRNA for all three types of influenza viruses (Crescenzo-Chaigne *et al.*, 1999; Jambrina *et al.*, 1997; Mena *et al.*, 1996; Pleschka *et al.*, 1996).

Most of our knowledge about the transcription and replication processes of the influenza virus genome has been derived from studies carried out on type A viruses (Lamb & Krug, 1996). *In vivo* studies showed that the conserved 5′- and 3′-end sequences were sufficient for the expression, replication and packaging of genome segments (Luytjes *et al.*, 1989), although nonconserved sequences were found to modulate the efficiency of transcription and replication (Bergmann & Muster, 1996; Zheng *et al.*, 1996). The nucleotide sequence requirements within the conserved termini controlling influenza virus transcription and replication have been studied extensively by making use of either *in vitro* transcription assays (Fodor *et al.*, 1995; Piccone *et al.*, 1993) or *in vivo* reconstitution of functional RNPs with vRNA-like model templates containing a chloramphenicol acetyltransferase (CAT) reporter gene (Neumann & Hobom, 1995; Pleschka *et al.*, 1996). Although initial *in vitro* studies suggested that the promoter for transcription was entirely contained within the conserved 3′-end sequences, it was later recognized that interaction between the 3′- and 5′-end sequences is required for transcription initiation (Hagen *et al.*, 1994). While the vRNA polymerase strongly binds the vRNA 5′-end (Tiley *et al.*, 1994), it subsequently interacts with the vRNA 3′-end (Li *et al.*, 1998).

Our previous work (Crescenzo-Chaigne *et al.*, 1999) showed that the influenza A and C virus RNA templates were transcribed and replicated with the same efficiency with the influenza A virus polymerase complex, but that the type A RNA template was amplified by the influenza C virus polymerase with a dramatically reduced efficiency. On the basis of this data, we analysed, in this study, the nucleotides involved in the type-specific interaction of the type A and C polymerase complexes with the viral promoters. By making use of the genetic system described by Pleschka *et al.* (1996) for the reconstitution of functional RNPs and by comparing the ability of the polymerase complex of the influenza A and C viruses to transcribe and replicate either wild-type or mutated type A and C RNA templates, we found that nucleotide 5 at the 3′-end and, to a lesser extent, nucleotide 6′ as well as the 3′:8′ base pair in the stem of the hairpin loop at the 5′-end of the vRNA are important determinants of type-specificity (prime notation is used throughout to distinguish 5′-end

residues from 3′-end residues; Fodor *et al.*, 1994). Furthermore, our results suggest that sequence requirements for the type C polymerase are more stringent that those required for the type A polymerase.

Methods

■ **Plasmids for the expression of influenza A and C virus core proteins.** Plasmids pHMG-PB1, pHMG-PB2, pHMG-PA and pHMG-NP, which express the PB1, PB2, PA and NP proteins, respectively, of influenza A virus A/Puerto Rico/8/34 (A/PR/8/34) under the control of the mouse hydroxymethylglutaryl coenzyme A reductase (HMG) promoter (Gautier *et al.*, 1989) were kindly provided by J. Pavlovic (Institut für Medizinische Virologie, Zurich, Switzerland). Plasmids pHMG-C-PB1, pHMG-C-PB2, pHMG-C-P3 and pHMG-C-NP, which express PB1, PB2, P3 and NP proteins, respectively, of influenza C virus C/Johannesburg/1/66 (C/JHB/1/66) under the control of the same HMG promoter have been described previously (Crescenzo-Chaigne *et al.*, 1999).

■ **Plasmids for the expression of wild-type and mutated type A and C model RNA templates.** The pA/PRCAT and pC/PRCAT plasmids, which direct the expression of model RNA templates derived from the nonstructural (NS) segments of A/WSN/33 and C/JHB/1/66 viruses, respectively, were described previously (Crescenzo-Chaigne *et al.*, 1999). They each contain the CAT gene sequences in an anti-sense orientation flanked by the cDNA sequences corresponding to the 5′- and 3′-end of the NS gene segments inserted at the *Bbs*I site of vector plasmid pPR, which is flanked by the human Pol I promoter and hepatitis delta virus ribozyme sequences, such that exact 5′- and 3′-termini of the model RNA templates are ensured.

Mutations at nucleotides corresponding to the 5′- and/or 3′-end of types A or C model RNA templates were generated by PCR amplification of the CAT gene and NS noncoding sequences in the presence of primers containing appropriate mutations and using either pA/PRCAT or pC/PRCAT, respectively, as a template. The exact sequences of the primers can be obtained from the authors upon request. For PCR amplification, from 10 to 50 ng of plasmid was used as the starting template in 100 μl of 2 mM Tris–HCl, pH 7·5, 10 mM KCl, 0·1 mM DTT, 0·01 mM EDTA and 1·5 mM MgCl₂ in the presence of 2·5 U Expand High Fidelity polymerase (Roche), 0·25 mM dNTPs and 1 μM of oligonucleotide primers. Amplification was for 25 cycles, each consisting of 30 s at 94 °C, 30 s at 45 or 50 °C and 2 min at 72 °C. The amplified products were purified from low-melting-point agarose gels by the QIAquick gel extraction kit (Qiagen) and inserted at the *Bbs*I sites of pPR treated with Klenow enzyme. Positive clones were purified using a QIAfilter plasmid midi kit (Qiagen) and subsequently subjected to phenol–chloroform extraction and ethanol precipitation.

■ **Sequencing.** The presence of mutations was assessed by sequence determination using the Big Dye terminator sequencing kit (Perkin Elmer), according to the supplier's instructions, and analysis on an ABI prism 377 automatic sequencer (Perkin Elmer).

■ **Transfections and CAT assays.** COS-1 cells were grown in Dulbecco's modified Eagle's medium containing 10% foetal calf serum. Subconfluent monolayers of COS-1 cells (3×10^5 in 35 mm dishes) were transfected by using 10 μl FuGENE 6 (Roche) with 1 μg of either pA/PRCAT or pC/PRCAT together with pHMG-PB1 (1 μg), pHMG-PB2 (1 μg), pHMG-PA/P3 (1 μg) and pHMG-NP (4 μg) plasmids. After 48 h of incubation at 37 °C, cells were harvested for lysis and reporter gene assays, as described previously (Crescenzo-Chaigne *et al.*, 1999).

CAT gene expression was determined using the CAT ELISA kit (Roche), which allowed detection of 0·05 ng/ml CAT.

Results

Construction of mutated influenza A and C virus model RNA templates

We have shown previously that the influenza A virus RNA template was amplified by the influenza C virus polymerase with a dramatically reduced efficiency (Crescenzo-Chaigne *et al.*, 1999). Within the conserved sequences at the 3′- and 5′-end, only four nucleotides differ between the influenza A and C virus RNAs, i.e. nucleotides 3′, 6′ and 8′ at the 5′-end and nucleotide 5 at the 3′-end (Fig. 1). In order to determine to what extent the differences within the conserved sequences of the vRNA extremities may account for the reduced efficiency of recognition of type A RNA by the type C polymerase complex, mutations were introduced at one or several of the four positions that differ between type A and C vRNAs in the corresponding pA/PRCAT and pC/PRCAT plasmids (see Methods). Type A model RNA templates with type C substitutions or type C model RNA templates with type A substitutions were thus obtained. Transcription and replication in the presence of either the type A or the type C polymerase complexes were evaluated by making use of a transient expression system, as initially described by Pleschka *et al.* (1996), which allows the *in vivo* reconstitution of active RNPs from a model RNA template in the presence of the four influenza virus core proteins (PB1, PB2, PA/P3 and NP) (see Methods).

Effect of mutations at positions 5 and/or 6′ within the type A model RNA template

The role in type-specificity of nucleotides 5 and 6′ at the 3′- and 5′-end, respectively, was investigated first. According to the hairpin loop model of vRNA (Flick *et al.*, 1996; Pritlove *et al.*, 1999), these nucleotides are not base-paired and are thus likely to be accessible for interactions with the polymerase complex. Mutations $U_5 \rightarrow C_5$ and $A_{6'} \rightarrow U_{6'}$ were introduced alone or in combination into the type A RNA template, thus resulting in a type A model RNA harbouring type C-specific nucleotides at positions 5 and/or 6′ of the conserved extremities. The level of transcription and replication of the mutated RNA templates was evaluated upon *in vivo* reconstitution of functional RNPs in the presence of either the type A or the type C polymerase complexes by measuring the level of CAT expression.

In the presence of the type A polymerase complex, the levels of CAT for the type A RNA template harbouring single $A_{6'} \rightarrow U_{6'}$ or $U_5 \rightarrow C_5$ mutations were found to be reduced approximately 1·3- to 2-fold, with respect to the type A wild-type RNA template (Fig. 2, lanes 2, 3; $P < 0·05$ and $P < 0·02$ in the Student *t*-test). When both mutations were present, no further reduction of CAT levels was detected, which is

consistent with the CAT level that is observed with the type C wild-type RNA template (Fig. 2, lanes 4, 5). However, in the presence of the type C polymerase complex, the introduction of either of the $A_{6'} \rightarrow U_{6'}$ or $U_5 \rightarrow C_5$ mutations in the type A RNA template resulted in a 4- to 5-fold increase of CAT levels, with respect to the type A wild-type RNA template (Fig. 2, lanes 2, 3; $P < 0·01$ and $P < 0·005$ in the Student *t*-test). When both mutations were present, the level of CAT was found to be increased by up to 7-fold, reaching a level that corresponds to about half of the activity of that measured for the type C wild-type RNA template in the presence of the type C polymerase (Fig. 2, lanes 4, 5). Thus, mutation $A_{6'} \rightarrow U_{6'}$ or $U_5 \rightarrow C_5$ seemed to affect the recognition of the type A model RNA template by the type A polymerase complex to a lesser extent than recognition of the same template by the type C polymerase complex.

### Effect of mutations at positions 5 and/or 6′ within the type C model RNA template

In order to confirm the role of nucleotides at positions 5 and 6′ of the 3′- and 5′-end of the RNA in recognition by the type C polymerase complex, mutations $C_5 \rightarrow U_5$ and $U_{6'} \rightarrow A_{6'}$ were introduced either alone or in combination within the type C model RNA template, thus resulting in a type C model RNA harbouring type A-specific nucleotides at positions 5 and/or 6′ of the conserved extremities.

As shown in Fig. 3, for each of the mutated type C RNA templates, similar or slightly higher levels of CAT activity were obtained with the type C polymerase complex (Fig. 3, lanes 2–4). However, introduction of the $C_5 \rightarrow U_5$ mutation either alone or in combination with the $U_{6'} \rightarrow A_{6'}$ mutation resulted reproducibly in an increase of the level of CAT activity in the presence of the type A polymerase complex and reached a level comparable to that achieved with the cognate type A wild-type RNA template (Fig. 3, lanes 3–5). Thus, whereas mutations $C_5 \rightarrow U_5$ and $U_{6'} \rightarrow A_{6'}$ did not seem to affect recognition of the type C RNA template by the homotypic type C polymerase complex, the $C_5 \rightarrow U_5$ mutation appeared to contribute, to some extent, to the recognition of the heterotypic type C RNA template by the type A polymerase complex, although not statistically significant in this case ($P < 0·1$ in the Student *t*-test).

Effect of mutations at positions 3′ and/or 8′ within the type A model RNA template

In vitro and *in vivo* studies have shown base pairing occurring between nucleotides 2′:9′ and 3′:8′ at the 5′-end of the RNA hairpin loop structure (Flick & Hobom, 1999; Pritlove *et al.*, 1999). In order to determine to what extent the hairpin loop structure is implicated in type-specific interactions with the polymerase complex and since nucleotides at positions 3′ and 8′ are different in the type A and C RNA templates, a series of mutations were introduced at these positions at the 5′-end
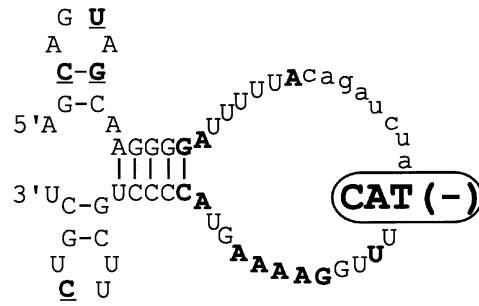
Type A model RNA
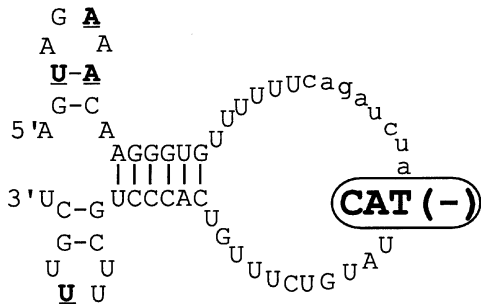
Type C model RNA

Fig. 1. Nucleotide sequences and predicted (Flick *et al.*, 1996) hairpin loop structures of the model RNA templates. The nucleotide sequences of the type A and C model RNAs transcribed from plasmids pA/PRCAT and pC/PRCAT are those of the NS genome segment (uppercase) of the A/WSN/33 and C/JHB/1/66 strains of influenza virus. Nucleotides shown in bold differ between the type A and C model RNAs. The nucleotides studied in this paper are underlined. Unrelated nonviral sequences are shown in lowercase.
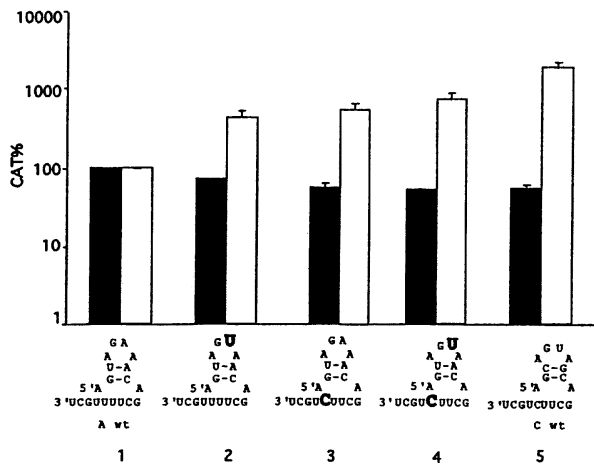

Fig. 2. Effect of mutations at positions 5 and/or 6′ of the 3′- and 5′-end of the type A RNA template on CAT expression by the influenza A and C virus polymerase complexes. Mutations (indicated in bold) were introduced into the plasmid pA/PRCAT at the nucleotides corresponding to positions 5 and/or 6′ of the vRNA. COS-1 cells were transfected with the four pHMG plasmids encoding the core proteins of A/PR/8/34 (filled bars) or C/JHB/1/66 (open bars), together with 1 μg of pA/PRCAT-derived plasmids expressing wild-type (lane 1) or mutated negative-sense model RNA templates (lanes 2–4) or with 1 μg of plasmid pC/PRCAT (lane 5). At 48 h after transfection, cell extracts were prepared and tested for CAT expression as described in Methods. CAT levels are expressed as percentage values of those measured with wild-type pA/PRCAT in the presence of plasmids expressing either the type A (100% = 4347 ng/ml CAT) or the type C (100% = 208 ng/ml CAT) core proteins. The mean ± SD of three independent experiments is shown.

Fig. 3. Effect of mutations at positions 5 and/or 6′ of the 3′- and 5′-end of the type C RNA template on CAT expression by the influenza A and C virus polymerase complexes. Legend is as in Fig. 2, except that the mutations were introduced into the plasmid pC/PRCAT. Wild-type (lane 1) or mutated (lanes 2–4) pC/PRCAT or wild-type pA/PRCAT (lane 5) plasmids were transfected with the four pHMG plasmids encoding either the type A (filled bars) or the type C (open bars) core proteins. CAT levels are expressed as percentage values of those measured with wild-type pC/PRCAT in the presence of plasmids expressing either the type A (100% = 1168 ng/ml CAT) or the type C (100% = 2192 ng/ml CAT) core proteins. The mean ± SD of five independent experiments is shown.

of the type A model RNA template and their transcription and replication were studied in the presence of either the type A or the type C polymerase complexes. As shown in Fig. 4, mutation $U_{3'} \rightarrow C_{3'}$, which resulted in the disruption of the $U_{3'}:A_{8'}$ base pair, completely abolished transcription and replication of RNA with the type A polymerase complex, whereas a very low level (5%) of CAT expression was detected with the type C polymerase complex (Fig. 4, lane 2). When a noncanonical $U_{3'}:G_{8'}$ base pair was created within the hairpin
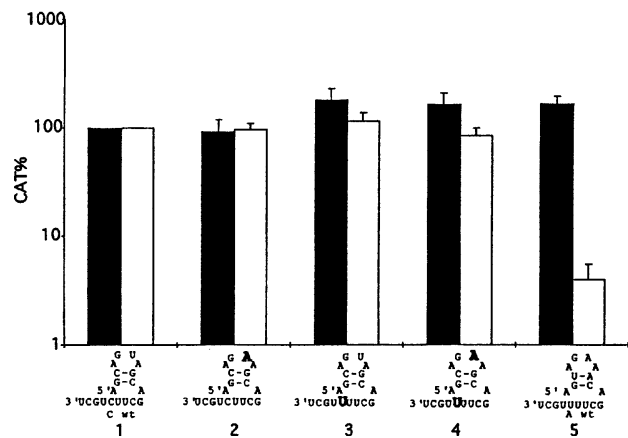
loop of the vRNA template, a low, albeit significant, level of CAT activity was observed with both the type A and the type C polymerase complexes (Fig. 4, lane 3). In contrast, when either a $G_{3'}:C_{8'}$ or a $C_{3'}:G_{8'}$ base pair was restored, and in the presence of type A polymerase complex, the level of CAT expression reached 70% of that measured with the type A wild-type RNA template. This was comparable to the CAT levels achieved with the type C wild-type RNA template harbouring a $C_{3'}:G_{8'}$ base pair at its 5′-end (Fig. 4, lanes 4–6). In addition, in the presence of the type C polymerase complex, the level of CAT was found to be increased 3-fold as compared to that observed with the type A wild-type RNA template when a $G_{3'}:C_{8'}$ base pair was introduced at the 5′-end (Fig. 4,
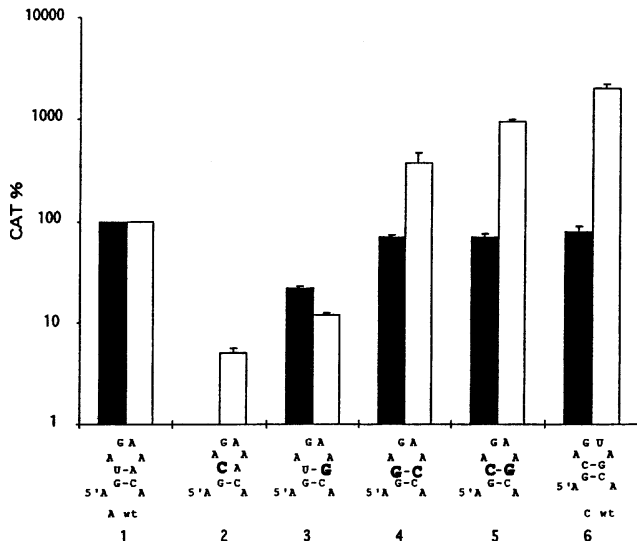
Fig. 4. Effect of mutations at positions 3′ and/or 8′ of the 5′-end of the type A RNA template on CAT expression by the influenza A and C virus polymerase complexes. Legend is as in Fig. 2, except that mutations were introduced into the plasmid pA/PRCAT at nucleotides corresponding to positions 3′ and/or 8′ of the vRNA. Wild-type (lane 1) or mutated (lanes 2–5) pA/PRCAT or wild-type pC/PRCAT (lane 6) plasmids were transfected with the four pHMG plasmids encoding either the type A (filled bars) or the type C (open bars) core proteins. CAT levels are expressed as percentage values of those measured with wild-type pA/PRCAT in the presence of plasmids expressing either the type A (100% = 3450 ng/ml CAT) or the type C (100% = 182 ng/ml CAT) core proteins. The mean ± SD from one representative experiment in duplicate (out of four) is shown.

Fig. 5. Effect of mutations at positions 3′ and/or 8′ of the 5′-end of the type C RNA template on CAT expression by the influenza A and C virus polymerase complexes. The legend is as in Fig. 3, except that mutations were introduced into the plasmid pC/PRCAT at nucleotides corresponding to positions 3′ and/or 8′ of the vRNA. Wild-type (lane 1) or mutated (lanes 2–5) pC/PRCAT or wild-type pA/PRCAT (lane 6) plasmids were transfected with the four pHMG plasmids encoding either the type A (filled bars) or the type C (open bars) core proteins. CAT levels are expressed as percentage values of those measured with wild-type pC/PRCAT in the presence of plasmids expressing either the type A (100% = 1288 ng/ml CAT) or the type C (100% = 3353 ng/ml CAT) core proteins. The mean ± SD of three independent experiments is shown.

lane 4; $P < 0.05$ in the Student t-test). The level of CAT expression further increased up to 9-fold when the type C-like $C_{3′}:G_{8′}$ base pair was introduced, reaching a level of about 50% of that achieved with the type C wild-type RNA template (Fig. 4, lanes 5, 6). Thus, in agreement with previous reports (Flick & Hobom, 1999; Pritlove et al., 1999), base pairing between the nucleotides at positions 3′ and 8′ seems to be absolutely required for the efficient transcription and replication of type A vRNA by either the type A or the type C polymerase complexes. Whereas the nature of the 3′:8′ base pair at the 5′-end had only a marginal effect on the efficiency with which the type A vRNA was transcribed and replicated by the type A polymerase complex, a marked preference for a type C-like $C_{3′}:G_{8′}$ base pair was revealed to be required for the transcription and replication of type A model RNA by the type C polymerase complex ($P < 0.05$ in the Student t-test, when comparing the $C_{3′}:G_{8′}$ and $G_{3′}:C_{8′}$ mutants; Fig. 4, lanes 4, 5).

Effect of mutations at positions 3′ and/or 8′ within the type C model RNA template

To analyse the importance of the 3′:8′ base pair within the potential hairpin loop structure at the 5′-end of the type C vRNA, similar mutations were introduced at these positions in the type C model RNA template and their transcription and
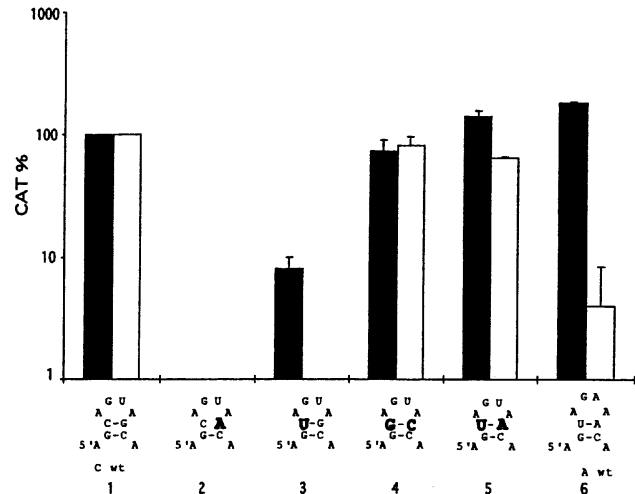
replication were studied in the presence of either the type A or the type C polymerase complexes. As shown in Fig. 5, like that for the type A RNA template, disruption of the 3′:8′ base pair ($C_{3′}:A_{8′}$ mutant; Fig. 5, lane 2) completely abolished transcription and replication of the type C RNA template by the type A polymerase complex, whereas the introduction of a noncanonical $U_{3′}:G_{8′}$ base pair resulted in a low level of CAT expression (Fig. 5, lane 3). For both single mutations at the 3′ and 8′ positions, however, transcription and replication of the type C RNA were below detectable levels in the presence of the type C polymerase complex (Fig. 5, lanes 2, 3). When either the $G_{3′}:C_{8′}$ or the $U_{3′}:A_{8′}$ base pair was restored at positions 3′:8′ of the hairpin loop, the level of CAT expression increased dramatically and reached levels corresponding to 82 and 64%, respectively, of that measured for the type C wild-type RNA in the presence of the type C polymerase complex (Fig. 5, lanes 4, 5). In the presence of the type A polymerase complex, restoration of base-paired nucleotides at positions 3′:8′ within the type C RNA template also resulted in high levels of CAT expression, which, in the case of the type A-like $U_{3′}:A_{8′}$ base pair, reached a level corresponding to about 80% of that achieved for the type A wild-type RNA in the presence of the type A polymerase complex (Fig. 5, lanes 5, 6). Thus, as for the type A vRNA, base pairing between the nucleotides at positions 3′ and 8′ was found to be required for efficient transcription and replication of the type C vRNA by both the type C and the type A polymerase complexes. In addition, a slight preference for a type A-like $U_{3′}:A_{8′}$ base pair over a

$G_{3'}:C_{8'}$ base pair ($P < 0·05$ in the Student t-test) was observed for efficient transcription and replication of the type C RNA template by the type A polymerase complex.

Discussion

The precise structure of the promoter that is required for the initiation of transcription and replication of the vRNA segments has been the subject of numerous studies, mainly for influenza A virus. This promoter structure is formed by the interaction of the 5′- and 3′-terminal sequences of the vRNA (Hsu *et al.*, 1987), which are involved in a stepwise interaction with the vRNA polymerase complex (Li *et al.*, 1998). Based on *in vitro* studies of RNA transcription (Fodor *et al.*, 1995; Li & Palese, 1992; Piccone *et al.*, 1993; Pritlove *et al.*, 1998, 1999; Seong & Brownlee, 1992*b*) and on *in vivo* studies of transcription and replication of model RNA templates (Flick & Hobom, 1999; Flick *et al.*, 1996; Kimura *et al.*, 1993; Neumann & Hobom, 1995; Neumann *et al.*, 1994; Seong & Brownlee, 1992*a*; Yamanaka *et al.*, 1991), site-directed mutagenesis studies demonstrated that the promoter is made up of two distinct elements: (i) a distal base-paired element that consists of long-range interactions of residues 10–15 of the 3′-end with residues 11′–16′ of the 5′-end of the vRNA; and (ii) a proximal element that consists of an essentially open structure made up of the extreme-terminal sequences of the vRNA. The proximal and distal elements are separated by an angular nucleotide, $A_{10'}$, which has no counterpart in the 3′ vRNA sequence. Short-range base pairing between residues 2′–3′ and 9′–8′ within the 5′-arm of the vRNA and between residues 2–3 and 9–8 within the 3′-arm of the vRNA results in hairpin loop structures at both segment ends, thus forming the so-called 'corkscrew' structure (Flick *et al.*, 1996). It has been established clearly that the 5′ hairpin loop is required for mRNA but not for cRNA synthesis (Pritlove *et al.*, 1999), whereas the 3′ hairpin loop is not necessary for mRNA synthesis but seems to be required at some stage of RNA replication either for cRNA synthesis or, as a complementary structure at the 5′-end of the cRNA, for vRNA synthesis (Flick & Hobom, 1999; Flick *et al.*, 1996; Pritlove *et al.*, 1999).

The conserved sequences of the extremities of the RNA segments of influenza A, B and C viruses (Stoeckle *et al.*, 1987) are all compatible with the formation of a corkscrew structure on both the vRNA and the cRNA, which is not the case for Thogoto virus, a related orthomyxovirus, for which the sequence at the 5′-end of the cRNA is not compatible with the formation of a hairpin loop (Leahy *et al.*, 1998). Differences between the influenza A, B and C virus promoters are localized in both the proximal and the distal elements. Limited sequence requirements (i.e. position 10:11′) were identified within the distal promoter element of influenza A virus, although base pairing as well as the length of the distal element, which differs between all three types of influenza virus, were found to be critical for the efficient transcription and replication of vRNA

(Flick *et al.*, 1996; Fodor *et al.*, 1994, 1995, 1998; Kim *et al.*, 1997; Lee & Seong, 1998*b*; Tiley *et al.*, 1994). Within the proximal element of the promoter, differences between influenza A, B and C viruses are located at the tip of the tetraloop structures of the 3′- and 5′-end of the vRNA (nucleotides 5 and 6′) as well as at the second base pair (nucleotides 3′:8′) of the stem of the hairpin loop structure at the 5′-end. Site-directed mutagenesis of the influenza A virus promoter has shown that nucleotides 5 and 6′ can be substituted without significantly affecting transcription and replication of the vRNA (Flick *et al.*, 1996; Fodor *et al.*, 1994; Kimura *et al.*, 1993; Poon *et al.*, 1998). It has, therefore, been suggested that these residues are not involved in binding the type A polymerase complex (Fodor *et al.*, 1994). Indeed, as shown here, a substantial activity was retained when nucleotide 6′ was substituted. However, when a $U_5 \rightarrow C_5$ mutation was introduced at the 3′-end of the type A RNA, a 2-fold reduction of CAT levels was observed. This was in agreement with the data reported by Neumann & Hobom (1995), but in contrast to data reported by Flick & Hobom (1999), within the context of an 'up'-promoter, which harbours additional substitutions at positions 3 and 8 at the 3′-end. This observation suggests that nucleotide 5 may contribute, to some extent, to the efficient recognition of the type A promoter by the type A polymerase complex. Consistent with this interpretation was the fact that the introduction of the reciprocal mutation, i.e. type A-like, $C_5 \rightarrow U_5$, within the type C promoter resulted in a reproducible, albeit not statistically significant ($P < 0·1$), increase of the efficiency with which the type C RNA was transcribed and replicated by the type A polymerase complex. Similarly, we have shown here that substitution of nucleotides 5 and/or 6′ within the type C vRNA promoter did not significantly affect the efficiency of transcription and replication of type C RNA by the type C polymerase complex. However, when type C-like nucleotides were introduced at these positions within the type A promoter, the result was a substantial increase of the efficiency of transcription and replication of type A RNA by the type C polymerase complex. Thus, subtle differences that may not show up when using optimal promoter sequences may be revealed within the context of a less active heterotypic promoter sequence. Overall our observations suggest that for both influenza A and C viruses, nucleotide 5 contributes to specific recognition of the promoter by the polymerase complex and, furthermore, that nucleotide 6′ may also participate, to some extent, in the efficient recognition of the promoter.

Analysis of the 3′:8′ base pair of the stem of the 5′ hairpin loop structure allowed us to confirm the importance of this structure for recognition of the type A wild-type promoter by the type A polymerase, as shown previously *in vitro* (Pritlove *et al.*, 1999) or *in vivo* in the context of the 'up'-promoter (Flick & Hobom, 1999; Flick *et al.*, 1996). In addition, short-range interactions within the 5′-arm of the vRNA were also found to be essential for recognition of the promoter by the type C

polymerase complex. In contrast to the type A polymerase, for which a weakly interacting base pair seemed to be preferred both in the context of either the type A or the type C promoter, a strongly interacting base pair, with an additional preference for C:G over G:C at positions $3':8'$ was required, particularly in the context of the type A vRNA, for optimal transcription and replication by the type C polymerase. However, influenza B virus RNA, which harbours a type A-like $U_{3'}:A_{8'}$ base pair as the only difference with type C vRNA within the proximal promoter element, is transcribed and replicated by the type C polymerase as efficiently as the homotypic type C RNA (Crescenzo-Chaigne *et al.*, 1999). In this case, contribution of the distal promoter element, the length of which is extended in the case of type B (9 bp) as compared to either the type C (5 bp) or the type A (6 bp) vRNA, remains to be determined. It is of interest to note in this context that the type B promoter is the only one which is efficiently recognized by all three types of influenza virus polymerase complexes (Crescenzo-Chaigne *et al.*, 1999) and also the only heterologous orthomyxovirus promoter which can be used, to some extent, by the Thogoto virus polymerase (Weber *et al.*, 1998). Comparison of the amino acid sequences of the type A and C polymerase proteins, which share 27–39% identity, did not reveal any obvious clues concerning the specificity of their recognition of the vRNAs (Yamashita *et al.*, 1989).

Overall, our results show that, as for type A, a hairpin loop structure at the 5′-end of the vRNA is required for efficient recognition of the viral promoter in the case of influenza C virus. Whether a similar hairpin loop structure at the 3′-end of the vRNA and/or at the 5′-end of the cRNA is also required at some stage of replication of the type C RNA, as suggested for the type A RNA (Flick & Hobom, 1999), remains to be determined. Experiments are in progress to address this question. Furthermore, our results emphasize the importance of nucleotides 5 and, to a lesser extent, 6′, for type-specific recognition of the promoter sequence by either the type A or the type C polymerase complexes. A sequence-specific contribution of the $3':8'$ base pair was also found, in agreement with other reports. More stringent sequence requirements within the proximal promoter element seem to prevail for the type C compared with the type A polymerase, which may account for the fact that the type C promoter could be used quite efficiently by the type A polymerase, but not in reverse (Crescenzo-Chaigne *et al.*, 1999). Whether or not similar sequence requirements would be found within the proximal promoter element for the type B polymerase remains to be determined. Thus, it may be suggested that residues 5 and 6′ of the tetraloop structures at the 3′- and 5′-end of the vRNA are involved in binding of the polymerase complex. The experimental approach used here did not, however, allow us to determine if specific interactions are required both at the level of initiation of transcription and of replication. The involvement of residues of the tetraloop structures in type-specific interactions is further suggested by the overall conservation of the nucleotide sequence of the extremities of the vRNA segments for each of the three influenza virus types. Although sequence data for the extremities of the vRNA segments are limited for type A and even more so for type B or type C influenza virus isolates, such conservation contrasts with the substantial exchangeability of the promoter nucleotide sequence determined experimentally. By making use of reverse genetics, an influenza A virus mutant with a $U_5 \rightarrow C_5$ substitution at the 3′-end of NA-coding segment 6 could be rescued as infectious virus (Bergmann & Muster, 1995). Interestingly, for this virus the overall level of segment 6 vRNA was found to be reduced 3-fold as compared to wild-type virus, in agreement with the 2-fold reduction of the level of transcription and replication observed here. However, a similar $U_5 \rightarrow C_5$ mutant could not be rescued as infectious virus when present in the context of a promoter harbouring a C rather than a U at position 4 at the 3′-end (Lee & Seong, 1998*a*). Furthermore, among all possible substitutions at position 4, the only position of the conserved 3′-end of type A vRNA in which a unique and natural U or C variation is observed, the only mutant that could be rescued as viable virus was the $C_4 \rightarrow U_4$ mutant (Lee & Seong, 1998*a*). It was thus postulated that the residue at position 4 of the promoter is involved in segment-specific temporal regulation of transcription and replication of the vRNA. The U_4 virus exhibited a higher infectivity than the C_4 virus, which may account for the fact that the $U_5 \rightarrow C_5$ virus could be rescued in the context of the U_4 virus but not in that of the C_4 virus. Attempts to rescue mutants with substitutions of the $U_{3'}:A_{8'}$ base pair should help to determine to what extent the stem of the hairpin loop might also be involved in sequence-specific interactions with the polymerase complex.

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