Mutational analysis of the RNA-fork model of the influenza A virus vRNA promoter *in vivo*

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The genome of influenza A virus consists of eight negative-stranded RNA segments which have partially complementary non-coding terminal sequences. Previous transcription studies of the virion RNA promoter *in vitro* have shown that the 5′ terminus forms an integral part of the promoter and an 'RNA-fork' model has been proposed for the initiation of transcription. According to this model part of the promoter is formed by an RNA-duplex which involves complementary residues 10 to 12 of the 3′ end and residues 11′ to 13′ of the 5′ end. With a reverse genetics system, based on the chloramphenicol acetyltransferase (CAT) gene, we have now tested this part of the promoter *in vivo*. Single mutations of the conserved residues at positions 11 and 12 of the 3′ terminus and at positions 12′ and 13′ of the 5′ terminus abolished promoter activity. The introduction of complementary mutations into both termini partially restored activity. On the other hand, mutations at positions 10 of the 3′ terminus and 11′ of the 5′ terminus inhibited activity independently of whether a base-pair was formed or not. Thus, at these positions, the nature of the residues is apparently more important than their ability to form base-pairs. These results extend our previous virion 'RNA-fork' model and are consistent with *in vitro* findings that the 5′ terminus is involved in the initiation of transcription.

Influenza A virus belongs to the segmented negative-strand RNA viruses. The segmented genome is replicated through RNA intermediates by an influenza virus-specific RNA-dependent RNA polymerase. All eight RNA segments have 12 and 13 conserved nucleotides at their 3′ and 5′ ends, respectively (Desselberger *et al.*, 1980; Robertson, 1979), which are partially complementary and form a panhandle structure both in infected cells and in purified virions (Honda *et al.*, 1987; Hsu *et al.*, 1987). Initial *in vitro* transcription studies with synthetic influenza RNA templates suggested that the panhandle structure was not required for the initiation of transcription (Parvin *et al.*, 1989; Seong & Brownlee, 1992*a*), and the 12 conserved nucleotides located at the 3′ end of virion RNA (vRNA) represented the minimal promoter (Seong & Brownlee, 1992*a*). However, there were difficulties in these studies because the RNA polymerase was isolated from virions, which contained residual vRNA 5′ end sequences capable of activating the enzyme (Parvin *et al.*, 1989; Seong *et al.*, 1992).

There is now a growing body of evidence, however, which suggests that the 5′ terminus of vRNA is involved in the initiation of transcription. Both 5′ and 3′ viral ends are required for the endonuclease activity of a recombinant influenza RNA polymerase complex (Hagen *et al.*, 1994). vRNA 3′ end mutants previously shown to be inactive as templates in transcription *in vitro* (Seong & Brownlee, 1992*b*) are activated by mutant vRNA 5′ end sequences which restore base-pairing (Fodor *et al.*, 1994). Furthermore, the vRNA 5′ end directly interacts with the RNA polymerase (Fodor *et al.*, 1994; Tiley *et al.*, 1994). The vRNA 3′ end also interacts with polymerase (Fodor *et al.*, 1993), but with lower affinity than the vRNA 5′ end (Tiley *et al.*, 1994). Taking the role of the vRNA 5′ end into account, an 'RNA-fork' structure was envisaged for the initiation of transcription (Fodor *et al.*, 1994) and this new model (Fig. 1) was further characterized by extensive mutagenesis (Fodor *et al.*, 1995). However, the conclusions were based on an *in vitro* system using short model RNA templates, and there was a need to validate this model *in vivo*.

It is known from mutagenesis studies that the conserved 3′ end nucleotides of vRNA are essential for expression of chloramphenicol acetyltransferase (CAT) reporter gene constructs *in vivo* (Piccone *et al.*, 1993; Yamanaka *et al.*, 1991). However, the effects of mutations in the 5′ strand have only been partly investigated (Neumann & Hobom, 1995) with an emphasis on the proposed single-stranded regions of the

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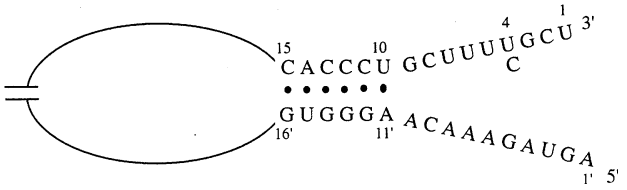

Fig. 1. The RNA-fork model for the initiation of vRNA transcription (Fodor *et al*., 1994, 1995). The sequence shown is for segment 8 of influenza virus A/PR/8/34. It postulates a base-paired region and a single-stranded region, involving both the 3′ and 5′ ends of the RNA segment. Numbering of nucleotides is from the 3′ end of the 3′ strand and from the 5′ end of the 5′ strand. 5′ end numbers are distinguished by a prime. Natural variation, U or C, is observed at position 4 of the 3′ end.

RNA-fork. Moreover, no systematic analysis has been conducted *in vivo* on the duplex region of the proposed structure. Using a CAT reporter gene system, we show here that base-pairing in the proposed duplex region is indeed important for CAT expression, except at position 10:11′. This extends our previously proposed RNA-fork model.

Negative-sense CAT RNAs flanked by the non-coding sequence of the influenza A/PR/8/34 virus NS gene were synthesized by transcription of pIVACAT1 (Luytjes *et al*., 1989) or pBXPCAT1 (Li & Palese, 1992) by T7 RNA polymerase. Mutations were introduced either by PCR using mutagenic oligonucleotide primers for pIVACAT, or by restriction fragment replacement for pBXPCAT. Micrococcal nuclease-treated viral cores were used as the source of influenza virus RNA polymerase and nucleoprotein, for reconstitution of ribonucleoprotein (RNP) complexes (Seong & Brownlee, 1992*a*). Monolayers of MDBK cells were infected with helper virus, X-31 or A/WSN/33, prior to transfection with RNP (Enami & Palese, 1991). CAT assays were performed as described previously (Luytjes *et al*., 1989; Seong & Brownlee, 1992*a*). The conversion of chloramphenicol to its acetylated products was estimated by laser densitometry of the exposed X-ray films or by phosphoimage analysis.

We first tested the role of the C12–G13′ base-pair. A single mutation, C12 → A12 (position 12 from the 3′ end of vRNA, see Fig. 1) abolished CAT activity (< 2% of wild-type) (Fig. 2; clone 2). However, the CAT activity was rescued to 36% of that of the wild-type by introducing a complementary G13′ → U13′ mutation which restores an A12–U13′ base-pair (clone 3). Similarly, another mutation at the same position, C12 → U12, also abolished CAT activity (clone 4), but restoration of the base-pair (U12–A13′) by double mutation (C12 → U12; G13′ → A13′) again rescued activity to 31% of the wild-type (clone 5). Neither of the single 5′ end mutants (G13′ → U13′ and G13′ → A13′) were active (clones 6 and 7, respectively). We then tested the C11–G12′ base-pair. The point mutation C11 → A11 was detrimental to CAT activity (< 2% of wild type, clone 8). However, its activity was rescued to 22% of wild-type by an additional G12′ → U12′ mutation in the 5′ end which introduced a A11–U12′ base-pair (clone 9). The single 5′ end mutant (G12′ → U12′) was inactive (clone 10).

Finally, the properties of the U10–A11′ base-pair were investigated. Single mutations, U10 → G10 and A11′ → C11′, significantly decreased CAT activity (clones 11 and 12, 21% and 5% of wild-type, respectively), but both mutants exhibited detectable activity. A combination of the two mutations, U10 → G10 and A11′ → C11′, which introduced a G10–C11′ base-pair, did not stimulate CAT activity further (20% of wild-type; clone 13). These results suggested that the properties of the base-pair at positions 10–11′ are different from those at positions 11–12′ and 12–13′. Therefore, more mutants were generated at positions 10–11′ for further investigation. Single mutations at position U10 in the 3′ end, either U10 → C10 or U10 → A10, led to a marked decrease of CAT activity, but these mutants still showed detectable activities (13% and 5% of wild type, clones 14 and 15, respectively). However, single mutations at the corresponding nucleotide A11′ in the 5′ end abolished CAT activity (A11′ → G11′, clone 16; and A11′ → U11′, clone 17). Restoration of the base-pair by double mutation (C10–G11′ bp by U10 → C10 and A11′ → G11′ or A10–U11′ bp by U10 → A10 and A11′ → U11′) failed to rescue CAT activity (clones 18 and 19). Additional double mutants, U10 → A10 and A11′ → C11′ with an A10 × C11′ mismatch, and U10 → G10 and A11′ → U11′ with a G10:U11′ wobble pair, showed no detectable activity (clones 20 and 21). In summary, the activities of position 10–11′ mutants were decreased relative to wild-type, independently of whether a base-pair formed or not.

Our analysis of different mutants at positions 10–11′ supports the view that the nature of nucleotides is more important than the base-pairing at these positions. It should be noted that some restrictions in sequence were previously observed at these positions *in vitro* (Fodor *et al*., 1995). For example, the activity of mutants carrying complementary mutations at positions 10–11′ was always much lower than that observed for complementary mutants at positions 11–12′ or 12–13′. We now observe (this paper), *in vivo*, that none of the base-pair mutants at positions 10–11′, i.e. A10–U11′, C10–G11′, G10–C11′ and G10:U11′, could rescue the activity of the single base mutants. Instead, the activity of mutants was critically related to the nature of the nucleotide at position 11′. Thus, all mutants at positions 10–11′ with the wild-type A11′ showed low but detectable activities varying from 5–21% of wild-type (i.e. G10 × A11′, C10 × A11′ and A10 × A11′; clones 11, 14 and 15, respectively). With the exception of the A10 × C11′ double mutant (clone 20), low but detectable activities (5–20% of wild-type) were observed with the C11′ mutants (i.e. U10 × C11′ and G10–C11′; clones 12 and 13, respectively). However, all mutants with U11′ lacked activity (i.e. U10 × U11′, A10–U11′ and G10:U11′, Fig. 2; clones 17, 19 and 21, respectively). Overall, these *in vivo* results emphasize the importance of A11′, confirming previous *in vitro* transcription (Fodor *et al*., 1995) and cross-linking studies (Fodor *et al*., 1994). Moreover, this nucleotide has been shown to be involved in polymerase binding (Tiley *et al*., 1994).

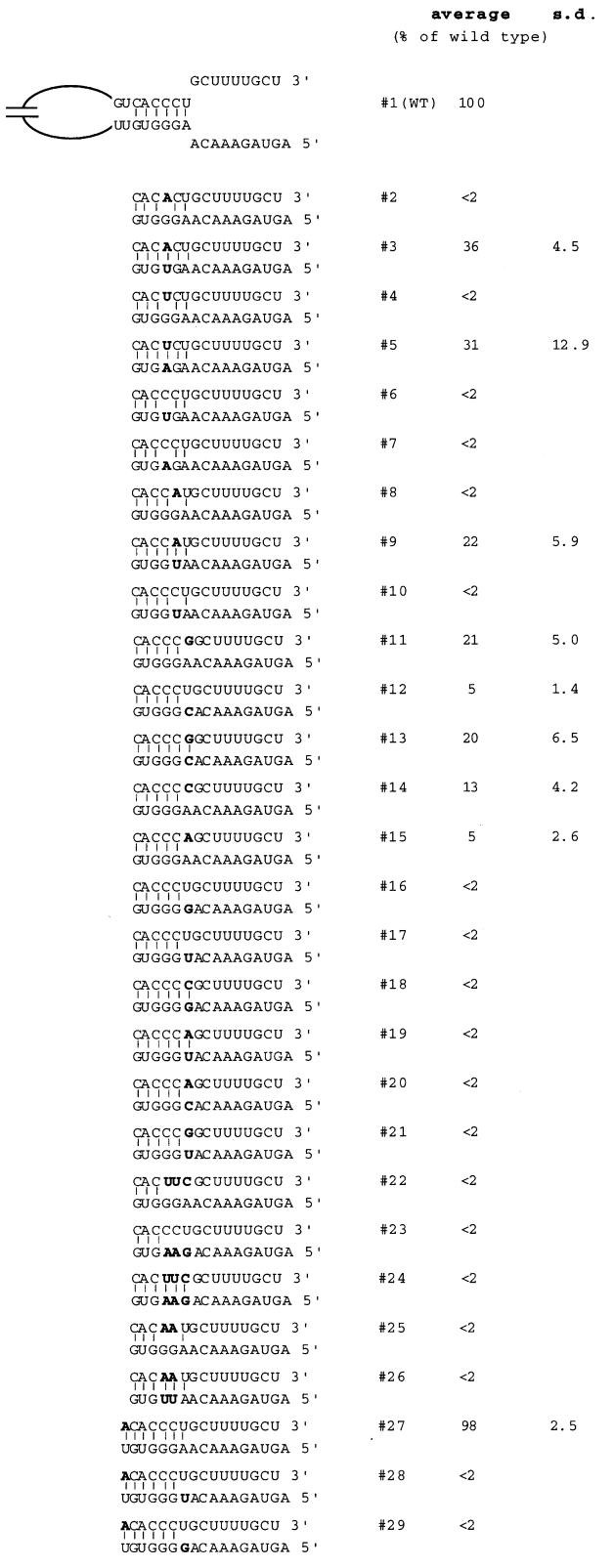

Fig. 2. CAT expression levels following RNP transfection with mutant RNAs. The non-coding terminal sequences are shown base-paired according to the RNA-fork model (Fig. 1). Mutations are indicated by bold letters. Percentage conversion of chloramphenicol to its acetylated products is presented relative to wild-type. For all mutants which showed detectable CAT activities, transfection was repeated at least three times. For 'dead' (< 2%) mutants, the lack of activity was confirmed by two or three independent transfection assays. All plasmids are derived from pIVACAT1 (clones 2, 3, 6; 8 to 21; and 25 to 29) or pBXPCAT1 (clones 4, 5, 7, and 22 to 24), which contain the CAT gene flanked by the non-coding sequences of the NS gene of the influenza A/PR/8/34 virus (Li & Palese, 1992; Luytjes *et al.*, 1989). pBXPCAT1 is a modified form of pIVACAT1 (Li & Palese, 1992). Desired mutations of all recombinant plasmids were verified by sequencing both the 3′ and 5′ ends of the CAT gene. s.d., standard deviation.

Mutations which disrupt the U10–A11′ base-pair could, conceivably, exert their effect by destabilizing the entire duplex region of the RNA-fork. To test this, we added an extra A–U base-pair at the end of the RNA-duplex (A16–U17′) by introducing a U16 → A16 mutation. This mutant (clone 27) has seven base-pairs instead of the usual six in the duplex region of the RNA-fork, yet showed comparable activity (98%) to wild-type. When this mutation (clone 27) was coupled with A11′ → U11′ or G11′ mismatch mutations, the resultant mutants (clones 28 and 29, respectively) both showed negligible activity. This suggests that the detrimental effect of point mutations at the 10–11′ base-pair is unlikely to be due to its effect on the stability of the entire duplex region of the fork.

Previously we showed, by means of *in vitro* transcription assays, that triple mutants at positions 10–12 in the 3′ end could be rescued by a complementary triple mutation which restored base-pairing in the proposed double-stranded region of the RNA-fork (Fodor *et al.*, 1995). We therefore tested triple mutants either at the 3′ end (U10C11C12 → C10U11U12) or at the 5′ end (A11′G12′G13′ → G11′A12′A13′) *in vivo*. As expected, neither mutant was active *in vivo* (clones 22 and 23). However, even if a triple mutation at both 5′ and 3′ ends, which restored base-pairing between 10–12 and 11′–13′, was tested no activity was observed (clone 24). We presume this lack of stimulation is due either to mutation of the crucial nucleotides at positions 10–11′ of the RNA-fork, or to a cumulative effect of base-pair substitutions elsewhere, or is a result of both phenomena. To distinguish these possibilities, we generated constructs which carried mutations at the 11–12′ and 12–13′ positions only. As expected, the double mismatch mutant (C11C12 → A11A12; clone 25) was inactive. We then added the complementary mutations at the 5′ end to generate a double base-pair mutant (C11C12G12′G13′ → A11A12U12′U13′; clone 26) which replaced the wild-type C–G base-pairs at positions 11 and 12 with A–U base-pairs. Clone 26 failed to show detectable activity, suggesting that the sum of the effects of the individual base-pair replacements in clones 3 and 9 was cumulative.

The discrepancy between the results of the previous *in vitro* and the present *in vivo* experiments with respect to the triple mutations and to the mutations at positions 10–11′ probably reflects the requirement of the RNA-fork for functions other than the initiation of transcription, such as polyadenylation (Li & Palese, 1992; Luo *et al.*, 1991; Robertson, 1979) and vRNA synthesis (Pritlove *et al.*, 1995). It must be emphasized that

every mutation introduced into the vRNA promoter will also cause a corresponding change on the cRNA promoter and could affect replication. Alternatively, the 10–11′ base-pair in particular may be unstable and melting would occur at elevated temperatures. It should be recalled that the previous *in vitro* analysis was conducted at 30 °C whereas the present *in vivo* assay was carried out at 37 °C. The detrimental effect of point mutations at positions 10 and 12 is also consistent with earlier reports (Piccone *et al*., 1993; Yamanaka *et al*., 1991).

It should be noted that when studying the triple mutants (clones 22–24) the CAT RNAs originated from pBXPCAT1 (Li & Palese, 1992), rather than from pIVCAT1 which was used for most of the other mutations studied here (see Fig. 2). pBXPCAT1 differs in sequence from pIVCAT1 in that two U residues at positions 18 and 19 of the 3′ end were replaced by AGC residues. The question thus arises as to whether these nucleotide changes in the non-conserved, non-coding region could have affected CAT activity of the triple or other mutants studied here. This seems unlikely, firstly because Li & Palese (1992) reported similar CAT activities for this mutant and wild-type CAT RNA after transfection into cells infected with influenza A/WSN/33 virus. Secondly, our results are consistent with Li & Palese (1992) in that where we studied single or double mutants based on the different parent plasmids, we observed similar CAT activities (compare clones 2 and 4; 3 and 5; 6 and 7).

A recent mutational analysis (Neuman & Hobom, 1995) of the proposed single-stranded region of the RNA-fork *in vivo* showed that a significant increase in promoter activity could be achieved by introducing three point mutations at positions 3, 5 and 8 of the 3′ end in order to stabilize a double-stranded structure between positions 1 to 9 and 1′ to 9′ of the RNA-fork. However, further mutational analysis (Neuman & Hobom, 1995) showed that the above effect was specific for the mutated nucleotides and therefore was unlikely to be caused simply by the formation of an RNA-duplex. These results are consistent with our RNA-fork model that, in this region of the promoter, signals other than an RNA-duplex are important.

In conclusion, because of the somewhat artificial nature of previous *in vitro* assays and the lack of a previous systematic analysis of the importance of 5′ nucleotides *in vivo*, it was desirable to test if the vRNA-fork model was valid *in vivo*. The results presented here are consistent with the role of a vRNA-fork in the initiation of transcription and in particular provide further evidence that the 5′ end of vRNA segments plays an important role as part of the promoter. Mutational analysis of the conserved base-pairs in the proposed double-stranded region of the RNA-fork confirms the results of our previous *in vitro* transcription studies that base-pair replacements at positions 11–12′ and 12–13′ are compatible with promoter activity. It should be noted, however, that these mutations *in vivo* were partially inhibitory, while *in vitro* full activity was observed (Fodor *et al*., 1994). Therefore, it will be particularly interesting to test if these same mutations could be introduced into the genome of infectious influenza viruses (Enami *et al*., 1990). Some base-pair mutations, e.g. U12–A13′, were previously incorporated into the neuraminidase (NA) gene resulting in an attenuated phenotype (Luo *et al*., 1992; Muster *et al*., 1991), although it was difficult to assess the effect of this particular base-pair mutation due to the multiple nature of the mutations introduced. On the other hand, the nature of nucleotides at positions 10–11′ appeared to be more critical *in vivo* than *in vitro*, since restoration of base-pairs by introducing complementary mutations into the 5′ end did not stimulate activity. These results lend support to the view that the nature of nucleotides is more important than the base-pairing ability at this position. Although our *in vivo* results provided no evidence that alternative base-pairs could be substituted for the A10–U11′ base-pair, in the light of our *in vitro* results (Fodor *et al*., 1994, 1995), it seems likely that the A10–U11′ base-pair is maintained in the RNP complex.

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