Nucleotides 9 to 11 of the influenza A virion RNA promoter are crucial for activity in vitro

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The 12 nucleotide conserved sequence at the 3' end of influenza A virion RNA is sufficient to function as a promoter in vitro. By introducing point mutations in all 12 positions of this promoter in model RNA templates and studying the efficiency of RNA synthesis in vitro, we show that only three nucleotides, residues 9, 10 and 11, are crucial for activity, although other nucleotides play a significant but less important role. Additions or deletions within the promoter are tolerated, resulting in either an increase or a decrease in promoter activity, depending on the mutation introduced; in some cases premature termination is caused. Taking these observations into account, a model for RNA polymerase binding and copying of the promoter is discussed.

Introduction

Influenza virus RNA polymerase catalyses three different reactions in the virus replication cycle. These are (i) transcription of the negative-sense virion RNA (vRNA) into mRNA, (ii) replication of vRNA into cRNA and (iii) replication of cRNA into vRNA (reviewed in McCauley & Mahy, 1983; Lamb & Choppin, 1983). The same vRNA template is used both for mRNA synthesis using host-derived cap-containing primer (Bouloy et al., 1978) and for cRNA synthesis, for which there is no requirement for a primer (Hay et al., 1982). Despite the known regulation of transcription at termination (Shapiro & Krug, 1988), there must be regulatory events at the initiation of transcription which determine whether a transcript is destined to become mRNA, in which case it is capped, or cRNA, in which case it lacks a primer. How this is achieved is not known.

The 5' and 3' ends of the vRNA segments of influenza A viruses have 13 and 12 nucleotide conserved sequences, respectively, which are partially complementary to each other, forming a panhandle structure (Robertson, 1979; Desselberger et al., 1980; Hsu et al., 1987). Recently, methods for the in vitro reconstitution of influenza virus ribonucleoprotein (RNP) complexes have been developed which use influenza virus proteins depleted of vRNA either by CsCl gradient centrifugation (Honda et al., 1988; Parvin et al., 1989) or by micrococcal nuclease digestion (Seong & Brownlee, 1992). By analysing model template RNAs in vitro and influenza virus-like chloramphenicol acetyltransferase (CAT) RNA in vivo, the 12 nucleotide conserved sequence at the 3' end of influenza A virus vRNA has been found to be important in transcription (Parvin et al., 1989; Luytjes et al., 1989). Subsequent analysis of a series of deletion mutants showed that these 12 nucleotides are sufficient to function as a promoter for cRNA synthesis and that no other sequence is required. Furthermore, polymerase proteins prepared by the micrococcal nuclease procedure are active, using model RNA templates, in three influenza virus reactions used to mimic transcription and/or replication in vivo. These are cap primer-initiated RNA synthesis (mimicking transcription), primer-independent cRNA synthesis (mimicking replication) and vRNA synthesis using an ApG primer (Seong & Brownlee, 1992; Seong et al., 1992).

We introduced mutations in all 12 positions of the vRNA promoter and analysed the efficiency of RNA synthesis either with globin mRNA as a cap donor, or with or without ApG as a primer, thus assessing the effect of mutations on both transcription and replication. Mutations at most positions had a measurable inhibitory effect, but only those in the internal 5' CUG 3' triplet, positions 9 to 11, were crucial for activity in all three reactions. Surprisingly, some mutations strongly increased promoter activity in the absence of primer, whereas others caused premature termination.

Methods

Preparation of RNA templates. RNA templates of 14 to 25 nucleotides in length (as used in Fig. 1 to 5) and carrying point mutations in the 12 nucleotide vRNA promoter were synthesized and purified, and yields of products were measured as before (Seong & Brownlee, 1992) by T7
RNA polymerase transcription of a partial DNA duplex consisting of a double-stranded T7 promoter and a single-stranded template sequence. Chromatography on Qiagen was used as a final purification step to remove unincorporated NTPs. Quantification was by comparison of \([\gamma-^{32}P]ATP\) and T4 polynucleotide kinase labelling of test oligonucleotides with an external standard of a 45 nucleotide synthetic deoxyoligonucleotide as described previously (Seong & Brownlee, 1992). When the quantity of template is not specified (see Results) identical amounts in the range of 2 to 3 pmol of different mutant template were used. The sequence of the wild-type RNA is 5'-GGCCUGCUUUGCU-3' (14 nucleotides) or 5'-GGGAUCCUAAGUACCUGCUUUGCU-3' (25 nucleotides). (The 12 nucleotide vRNA promoter is underlined. Nucleotide positions are numbered 1 to 12 from 3' to 5'). All mutations were transversions (C→A, U→A or G→C) unless otherwise stated. In addition, single base insertion mutants were also synthesized (15 and 26 nucleotides; see Results). The T7 RNA transcripts were heterogeneous in size at their 3' ends (Milligan et al., 1987), with minor products of one nucleotide smaller, or one or two nucleotides greater in size (data not shown). Short RNA templates (11 to 15 nucleotides in length) with different numbers of internal U residues were synthesized chemically using the Applied Biosystem DNA synthesizer (M. Gait, personal communication) (Table 1). After Sephadex G-25 (NAP-10; Pharmacia) column chromatography, the RNA was purified by 20% PAGE in 7 M-urea followed by elution with 1 M-NaCl and purification on a Qiagen column. The concentration of the RNA was estimated from the A260. Synthetic RNAs were >95% pure as analysed by 5' 32p-labelling using T4 polynucleotide kinase and PAGE.

**Reconstitution and transcription.** Influenza A virus RNA polymerase was prepared by digestion of the RNA component of the RNP core (X-31, a reassortant of strains A/HK/8/68 x A/PR/8/34) with micrococcal nuclease followed by EGTA inactivation (Seong & Brownlee, 1992) before reconstitution with RNA templates. Briefly, 1 to 2 μl of polymerase was mixed with RNA template and incubated at 30°C for 30 min. Then, transcription buffer was added [final concentration 50 mM-Tris-HCl pH 7.4, 50 mM-KCl, 1 mM-EDTA (TE buffer) with 6 units RNase at 37°C]. After incubation at 30°C for 3 h, the RNA was phenol-chloroform-extracted and ethanol-precipitated with 10 μg yeast carrier RNA, and analysed on a 16 or 18% polyacrylamide gel in 7 M-urea. Labelled RNA products were estimated quantitatively by densitometric scanning of the exposed X-ray film, and the effects of each mutation were assessed by averaging two or more experiments.

For analyses of the different length mutants and their premature termination products, T4 polynucleotide kinase was added after transcription and incubated at 37°C for 1 h to phosphorylate the transcripts at their 5' end (to improve their resolution by 25% PAGE) with ATP present in the transcription mixture. RNAs were analysed by 25% PAGE in 7 M-urea. This concentrated gel was prepared by mixing two stock solutions of 50% acrylamide (acylamide: bisacylamide, 30:1) and 10 x TBE buffer in the required weight of urea, and finally adjusting the volume with H2O. Labelled RNAs were eluted from the gel by soaking in H2O for 10 h at 4°C and purifying from the aqueous layer by desalting using an NAP-10 column (Pharmacia) followed by freeze-drying. RNase T1 (Sigma) digestion was carried out in 10 mM-Tris-HCl pH 7-4, 1 mM-EDTA (TE buffer) with 6 units RNase at 37°C for 2 h. Digestion with snake venom phosphodiesterase (Boehringer-Mannheim) was carried out at room temperature in a buffer containing 200 mM-Tris-HCl, pH 8-8 and 10 mM-MgCl2 with 0.5 μg enzyme in 10 μl. Aliquots were removed at 10, 30 and 120 min, and were then combined for analysis by 25% PAGE in 7 M-urea.

**Results**

**Dissection of the promoter by mutation**

To assess the relative importance of each of the 12 nucleotides in the vRNA promoter we used the methodology described previously (Seong & Brownlee, 1992). Mutant RNAs carrying transversions, insertions or deletions in the promoter were constructed either by T7 RNA polymerase transcription of short synthetic deoxyoligonucleotides or by direct RNA synthesis (see Methods and Table 1). After reconstitution with micrococcal nuclease-treated influenza virus cores, these short model RNA templates (11 to 26 nt long) were transcribed in the presence of \([\alpha-^{32}P]P\)triophosphates and the labelled RNA transcripts were analysed by PAGE in 7 M-urea (see Methods).

Fig. 1 shows the results for \([^{32}P]CTP\)-labelled transcripts of transcription mutants at all 12 positions as well as one C insertion mutant between positions 4 and 5 (IC4,5) analysed in the absence of added primer (Fig. 1a), with globin mRNA as a primer (Fig. 1b), or with ApG as a primer (Fig. 1c). The major products were of the expected size (see Methods and legend to Fig. 1), i.e. 14 nucleotides for the primer-independent and ApG-dependent reactions, and 35 to 38 nucleotides for the globin-primed reaction. All transcripts showed some degree of microheterogeneity in size. This is probably due to heterogeneity of T7 RNA transcripts (see Methods), because the ApG-primed reaction with >95% pure synthetic RNA gave full-length transcripts of predominantly one size (Fig. 1b). Additional microheterogeneity of cap-primed products (Fig. 1b) may be due to imprecise cleavage of cap RNA by influenza virus-associated endonuclease (Bouloy et al., 1978; Kawakami et al., 1983). The following conclusions emerge from the analyses of these particular mutants (12 transversions and one insertion, see Fig. 1). (i) Mutations at only three nucleotides (positions 9, 10 and 11, lanes 10 to 12) resulted in a significant (<10% of the wild-type activity) inhibition of activity in all three assays. (ii) The
Fig. 1. Analysis of RNA transcripts of promoter mutants. Transcription was carried out either in the absence of primer (a), or in the presence of globin mRNA (b) or ApG (c). About 10 (a), 5 (b) or 2 to 3 pmol (c) RNA was used for reconstitution (see Methods). The lengths of mutant RNA templates used were 14 (a and c) and 25 (b) nucleotides, except for mutant IC4,5, which was 15 (a and c) and 26 (b) nucleotides. The reason for the anomalous mobility of the C2→A2 mutant in (a) lane 2 is unknown; its mobility corresponds to a 16 nucleotide product (two residues longer than the wild-type sequence) and could arise by reiterative copying of the mutant template. We interpret the slightly slower mobility of the G3→C3 mutant (lane 3) as a sequence-specific effect caused by the greater retardation of G residues in dense acrylamide gels (Simoncsits et al., 1977). [α-32P]CTP-labelled RNA transcripts were analysed by 16% PAGE in 7 M-urea. Lanes 1 to 13, U1→A1, C2→A2, G3→C3, U4→A4, IC4,5, U5→A5, U6→A6, U7→A7, C8→A8, G9→C9, U10→A10, C11→A11, C12→A12, lane 14, wild-type and (lane 15) a control without RNA.

mutation at position 12 (lane 13) inhibited promoter activity partially (30 to 50% of wild-type activity) in all three assays. (iii) The effect of all other mutations was variable depending upon the primer. (iv) More specifically, the effect of mutations at positions 4 to 8 (lanes 4 to 9) was not significantly different from that of wild-type promoter in both ApG- and globin RNA-primed reactions. However, for individual reactions there was weak but statistically significant inhibition caused by mutation at positions 7 and 8 (lanes 8 and 9) when the globin primer was used. There was also weak but statistically significant inhibition caused by mutation at positions 4 to 6 (lanes 4 to 7) when ApG was used as a primer. (v) Primer-independent RNA synthesis varied widely among mutants. Both the mutation at position 3 (G→C; lane 3) and a single C insertion between positions 4 and 5 (IC4,5; lane 5) strongly up-regulated primer-independent initiation (on average, a 24-fold and a 10-fold increase, respectively). (vi) Mutations at positions 1 and 2 (lanes 1 and 2) had different effects according to the primer used. The C→A (position 2) mutant was on average 60% as active as the wild-type promoter in the globin cap-primed reaction, whereas in the ApG-primed reaction it was inactive (<5%). Further experiments were then carried out to amplify points (ii) to (vi).

Analysis of position 1 and 2 mutants primed with different dinucleotides

In theory, in the ApG-primed reaction mutations at positions 1 and 2 will affect the base-pairing between the RNA template and the primer, and the efficiency of transcription could depend on the nature of the primer
used. To assess this, we tested three different RNA templates (the wild-type, and the U1→A1 and C2→A2 mutants) with three different primers, ApG, UpG and ApU, in all possible combinations (Fig. 2).

Depending on the number of base pairs formed between the RNA template and the primer, the combinations can be divided into three groups. The first includes those combinations in which two base pairs are formed, i.e. wild-type template and the ApG primer (Fig. 2, lane 1), the U1→A1 mutant template and the UpG primer (lane 5), and the C2→A2 mutant template and the ApU primer (lane 9). The wild-type template was the most efficient in transcription, the U1→A1 mutant template was much less efficient (25%) and the C2→A2 mutant template showed 70% of the efficiency of the wild-type template. This suggests that the U1→A1 mutation renders the promoter intrinsically less active because the activity was not rescued by the complementary primer UpG. By contrast, the activity of the C2→A2 mutant could be rescued to at least 70% activity by the complementary primer ApU, implying that position 2 is less important for activity than position 1. This suggests that the low yield of transcript in the ApG-primed reaction with the C2→A2 mutant (Fig. 1c, lane 2) is an artefact due to inefficient base-pairing between ApG and the mutant template, rather than an intrinsic inability to promote transcription.

The second group includes those combinations in which only one base pair is formed between template and primer (Fig. 2, lanes 2, 3, 4 and 7), either an A-U pair at the first position, which lowered activity dramatically (lanes 3 and 7), or a G-C pair at the second position, which lowered activity by 30 to 35% (lanes 2 and 4).

The third group includes those combinations in which no base pairs are formed, and transcripts were undetectable (< 5%; lanes 6 and 8). We conclude that the number of base pairs formed is important and that, if there is only one base pair, a G-C pair is better than an A-U pair, presumably owing to its greater stability. These results are consistent with previous studies showing that base-pairing is an important factor for dinucleotide-primed reactions (Honda et al., 1986).

**The effect of RNA concentration on transcription of mutants at positions 1, 2 and 12**

To assess the effect of mutations at positions 1, 2 and 12 on transcription, globin cap-primed RNA synthesis was tested over a 125-fold range in template RNA concentration. The activity of mutants is shown in Fig. 3 after quantification (see Methods) of the major group of transcripts. The C2→A2 mutant had on average 60% (four experiments) of the activity of the wild-type at the highest RNA concentration tested. By contrast, the U1→A1 mutant had on average only 22% (four experiments) of the activity at the highest RNA concentration. Thus the U1→A1 mutation renders the
promoter very inefficient, whereas the C2→A2 mutation affects cap-dependent RNA synthesis less. The C12→A12 mutant had on average 39% (four experiments) of the activity of the wild-type at the highest RNA concentration tested.

Analysis of a position 5 to 7 triple mutant in the U-rich central region

The effect of mutants with mutations at positions 4 to 8 of the template was not significantly different from that of wild-type template in both ApG- and globin RNA-primed reactions (see above); therefore we investigated the role of this central region of the promoter further. A triple mutant (U5U6U7→A5A6A7) was synthesized and its activity compared with the wild-type promoter in a ApG-primed reaction (Fig. 4). Significant inhibition of activity was observed (< 25% of wild-type), although this inhibition is less than that caused by point mutations at positions 9 to 11 (Fig. 1).

Analyses of up-regulatory mutants

Since both the G3→C3 and the IC4,5 mutants strongly up-regulated the primer-independent initiation (Fig. 1a), we investigated further point and insertion mutants (Fig. 5). All three possible point mutations at position 3, and an insertion of a U residue (IU4,5) were studied. Of the position 3 mutants, only the G3→C3 mutation increased RNA synthesis in the absence of the primer (Fig. 5a, lower section, lane 3). The G3→A3 or G3→U3 mutants were indistinguishable from wild-type (Fig. 5a, lower section, lanes 1, 2 and 4). This showed that up-regulation was nucleotide-specific for mutations of position 3. Single base insertion mutants, with either a U or a C insertion between positions 4 and 5, also resulted in strong up-regulation (Fig. 5a, lower section, lanes 4 to 6). We conclude, at least for primer-independent initiation, that the insertional up-regulation is not nucleotide-specific. In the case of the insertion of a C between positions 4 and 5, cap-initiated products were efficiently synthesized (Fig. 1b, lane 5).

We also tested U4→C4 because this is the only natural variation observed in the influenza A virus vRNA promoter (Robertson, 1979; Winter & Fields, 1980; Allen et al., 1980; Desselberger et al., 1980). We observed
significant up-regulation of primer-independent initiation (Fig. 5b, lower section, lane 3) which was nucleotide-specific, the U4→A4 mutant failing to show a similar increase (Fig. 1a, compare lanes 4 and 14). The extent of up-regulation, although significant, was less than that observed with the G3→C3 mutant (see Table 2 for summary). However, cap-initiated transcription was indistinguishable from wild-type (Fig. 5c, lane 2). In all these up-regulatory mutants (G3→C3, U4→C4, IC4,5 and IU4,5) there was no significant difference in the yield of mutant transcripts compared to that of wild-type transcripts in the ApG-dependent reaction (Fig. 5a and b, upper sections).

Analysis of mutants of different length

Mutations in positions 4 to 8 either had no effect or only weakly inhibited synthesis in the ApG-primed reaction (Fig. 1c) and therefore we increased or decreased the length of the U-rich (U4-7) central region of the promoter in order to examine the extent to which the promoter could accommodate different lengths of internal sequence. A series of mutant RNAs with different numbers of internal U residues (Table 1) was synthesized chemically (see Methods) and tested in transcription with ApG over a 125-fold range of RNA concentration (Fig. 6a). Decreasing the number of U residues by one nucleotide (−1U mutant) or increasing the length by three nucleotides (+3U mutant) caused a significant decrease in promoter activity. Increasing the length by one or two nucleotides (the +1U and +2U mutants) only weakly inhibited activity (30 to 90% of wild-type).

Interestingly, either increasing or decreasing the number of U residues resulted in the appearance of transcripts (Fig. 6b, arrowed) shorter than wild-type. These were four to eight nucleotides long (see below) depending on the mutant studied. The absence of such short transcripts derived from the IC4,5 mutant (data not shown) suggested that this phenomenon is associated with runs of U residues. In theory, these short transcripts could be due either to premature termination or to internal initiation within the mutant promoter. Initiation with ApG at a potential internal site, U7C8, would yield transcripts of the same size (six nucleotides) in all length mutants. Since the length of short transcripts varied (Fig. 6b), internal initiation seemed unlikely. Furthermore, if internal initiation were occurring the product 5' pAGCAp*Gp*G 3' should be present [asterisk marks...
Influenza A virus promoter

Fig. 7. (a) T1 RNase digestion of short RNA transcripts. A 12 nucleotide wild-type transcript (lane 1) isolated from Fig. 6(b), lane 2, and shorter transcripts (lanes 3 and 4) isolated from Fig. 6(b), lanes 3 and 4, respectively, were treated with RNase T1 (lanes 2, 5 and 6; see Methods) or untreated (lanes 1, 3 and 4). Two labelled fragments transcribed from the wild-type promoter were assigned CAAAAGp* (asterisk) and pAGp* (arrowhead). (b) Digestion of the premature termination products derived from the -1U, +1U and +2U mutants (Fig. 6b) (lanes 1, 2 and 3, respectively) with snake venom phosphodiesterase. The shortest RNA is three nucleotides long (pApGp*CoH) and is shown with an asterisk. Undigested products are indicated by arrowheads.

We conclude that short transcripts are due to premature termination after initiation at the correct ApG site.

To characterize the premature termination products further, they were eluted and analysed with RNase T1 (see Methods) with a control full-length 12 nucleotide transcript. A fragment, comigrating with pApGp* from the control wild-type 12 nucleotide transcript (which generated two marker-labelled products, pAGp* and CAAAAGp*, in an [α-32P]CTP transcription reaction), was present in the T1-RNase digest of the various short transcripts (Fig. 7a, lanes 2, 5 and 6). Partial digestion of the premature termination products with snake venom phosphodiesterase (a 3’ exonuclease releasing nucleoside 5’-monophosphates) produced a series of shorter RNAs (Fig. 7b). Because the shortest labelled RNA (shown with an asterisk) must be three nucleotides long (pApGp*CoH), we deduced from the number of labelled products in the partial venom phosphodiesterase digest that the sizes of the major premature termination products are four nucleotides in the -1U mutant (lane 1), six nucleotides in the +1U mutant (lane 2) and seven nucleotides in the +2U mutant (lane 3) and, by extrapolation, eight nucleotides in the +3U mutant (Fig. 6b, lane 5). This shows that, regardless of the number of U residues inserted or deleted in the U-rich region, a major termination site occurs at precisely the same distance (seven nucleotides) from the 5’ end of the template RNA. This site corresponds to the junction between U5 and U6 in the wild-type template (see Table 3).

Adjusting for the differing number of labelled C residues in the transcripts of the length mutants, we estimate that the molar ratio of short to full-length transcripts is 5:1 for the +1U and +2U mutants, 12:1 for the +3U mutant and 26:1 for the -1U mutant. This ratio indicates that most products terminate prematurely. Therefore, the much lower activity of either the +3U or -1U mutant compared with wild-type (Fig. 6b) in the synthesis of full-length transcript is, at least in part, due to premature termination. The relatively high activity of +1U and +2U mutants in the synthesis of full-length transcripts (30 to 90% of that of wild-type; see Fig. 6a) despite premature termination suggests that RNA polymerase transcribes the initial six or seven nucleotides of these length mutants more efficiently than those of the wild-type promoter.

Table 3. Mapping the premature termination site in the length mutants

<table>
<thead>
<tr>
<th>Sequence*</th>
<th>Mutant</th>
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<tbody>
<tr>
<td>CCUGCUUUUGCU</td>
<td>-1U</td>
</tr>
<tr>
<td>CCUGCUUUUUGCU</td>
<td>Wild-type</td>
</tr>
<tr>
<td>CCUGCUUUUUUGCU</td>
<td>+1U</td>
</tr>
<tr>
<td>CCUGCUUUUUGCU</td>
<td>+2U</td>
</tr>
<tr>
<td>CCUGCUUUUUGCU</td>
<td>+3U</td>
</tr>
</tbody>
</table>

* Arrowhead indicate the major site of premature termination on the vRNA promoter. The open arrowhead on the wild-type promoter indicates the proposed pause site (see Discussion). The crucial triplet is underlined.

Discussion

We describe in this paper a dissection of the influenza A virus vRNA promoter in vitro using short model RNA promoter mutants. Table 4 summarizes the effect of the point mutations on promoter activity, classifying them into groups 1 to 3 according to the extent of inhibition of...
activity compared to the wild-type promoter. A further group, 4, comprises mutations in which stimulation of promoter activity was observed. Only mutations at positions 9 to 11 (group 1) significantly inhibited transcription in all assays, suggesting that these nucleotides in the promoter (and possibly the adjacent position 12) form a three to four nucleotide long contiguous RNA polymerase-binding site. Definitive evidence for this site would require more complete mutational analysis (e.g. introducing different natural nucleotides, different modified nucleotides and sugars). Nevertheless, the fact that transversions of contiguous residues 9 to 11 caused a significant (> 90%) inhibition in all three assays strongly argues for their importance. Presumably this binding site positions the 3'-terminal U of the promoter at the active site of the polymerase complex to initiate transcription and replication.

The fact that mutations of group 3 (positions 4 to 8) are only weakly or insignificantly inhibitory suggests that these central residues of the promoter make either only weak or no contact with the polymerase complex. It is not certain whether the significant inhibitory effect of the triple mutation (U5U6U7→A5A6A7) in this central region was due to the loss of these weak contacts or to the introduction of new unfavourable interactions by mutation. We propose that these ‘U-rich’ central residues may, in part, function as a ‘spacer’ between the major polymerase binding site, the 5' CUG 3' triplet, and possibly with the adjacent C residue (position 12) of the promoter. It is separated by a U-rich spacer from a second regulatory site (site 2), interacting with the 3'-terminal U residue and possibly residues 2, 3 and 4 (see text).

mutant is usually more inhibitory than a position 2 mutant (see Fig. 1 and 3, and Table 4).

Confirmation of the greater importance of position 1 compared to position 2 derives from the fact that the activity of a position 1 mutant cannot be rescued by complementary dinucleotide primer whereas a position 2 mutant can be rescued to 70% of wild-type activity (see Fig. 2 and Results). However, further mutational studies with different nucleotides are required to prove this point definitely. Although the promoter activity is not strictly dependent on the sequence of the spacer separating sites 1 and 2, down-regulation caused by a one nucleotide deletion in the spacer and strong up-regulation caused by insertion of one nucleotide suggest an important role for the length of this central spacer region in promoter regulation. We speculate that, after binding of the polymerase complex at site 1, the 3' end of the promoter must be properly (and differentially) positioned at the catalytic site of the polymerase, for
termination at a more or less discrete site, producing a
dissociation from the RNA template as no premature
transcription to position 5 and then pause briefly (without
the natural RNA, the influenza virus polymerase may
clearance' disengages the polymerase from the promoter
downstream positions 9, 10 and 11 (site 1). In the case of
component of it (either PB1, PB2 or PA) bound to
could be caused by a downstream block to polymerase
movement, caused by the polymerase complex itself or a
interaction of the polymerase with runs of U residues
abutting the panhandle
of the mRNA, possibly by slippage and reiterative
of the mutant RNA templates the polymerase dissociates
reading through this region. This reiterative copying
polymerases (Munson & Reznikoff, 1981; Moroney &
steric block is cleared. If true, this could be one of the
rate-limiting steps in transcription and a potentially
important step for its control. A similar brief pausing
RNA polymerase at an initiation step, before 'promoter
disengages the polymerase from the promoter
for full-fledged elongation, occurs in Escherichia coli RNA polymerase (McClure, 1985). The influenza virus
polymerase is known to add the poly(A) tail to the 3' end
of the mRNA, possibly by slippage and reiterative
copying of runs of U residues abutting the panhandle
structure (Luo et al., 1991). We speculate that the
interaction of the polymerase with runs of U residues
may be intrinsically weak, and may even cause dissociation of the polymerase in a certain sequence context.

Recent in vitro analysis of the influenza virus promoter
using a CAT RNA construct has been carried out mostly
with double mutants, and cannot be compared directly
with our present in vitro analysis of point mutants. However, it is interesting that a double mutation at
positions 10 and 11 (Yamanaka et al., 1991) affected
promoter activity in vitro most dramatically. It is
surprising that previous mutational analysis of the viron
RNA promoter in vitro (Parvin et al., 1989) failed to
demonstrate the importance of nucleotides 9 to 11. We
can only speculate that this may be due to the different
methods used for the preparation of the polymerase
(Seong et al., 1992) or the more limited nature of their
mutational analysis. It also remains to be tested whether
the weaker activity of the related influenza A virus
cRNA promoter (Parvin et al., 1989; Seong & Brownlee,
1992; Seong et al., 1992) is due to the variation of the
CUG triplet sequence or to differences in length and
sequence in other positions. It is interesting to note that
Li & Palese (1992) have observed recently that the same
position 3 and 4 point mutants in the cRNA promoter
up-regulate transcription, as observed here with the
vRNA promoter. However, there was no evidence for
the very significant inhibition of transcription observed
when mutations at nucleotides 9 to 11 were studied.

The significance of the various promoter up-regulatory
mutants (Table 2) is difficult to understand, although it
clearly demonstrates that the promoter is not 'optimal'
for cRNA synthesis, presumably to ensure the balanced
synthesis of mRNA and cRNA from the same promoter.
In vivo, both mRNA and cRNA are synthesized from the
same vRNA promoter at the same time (Hay et al., 1977)
so that any up-regulation of cRNA synthesis could result,
by competition, in a decrease in mRNA synthesis, which
could be detrimental depending upon the severity of its
effect. It also implies that, in the natural vRNA
promoter, replication is down-regulated, and positions 3
and 4 are important for this regulation. Interestingly,
the U4→C4 mutant significantly up-regulated replication.
Since a U→C mutation at position 4 is the only natural
variation in the influenza A virus promoter in RNA
segments 1 to 3 and sometimes 7 (Robertson, 1979;
Winter & Fields, 1980; Allen et al., 1980; Desselberger
et al., 1980), this effect could be important for segment-specific regulation of transcription and replication
(Smith & Hay, 1982; Hatada et al., 1989; Varich et al.,
1990).

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