In vitro transcription and polymerase binding studies of the termini of influenza A virus cRNA: evidence for a cRNA panhandle

David C. Pritlove, Ervin Fodor, Baik L. Seong† and George G. Brownlee*

Chemical Pathology Unit, Sir William Dunn School of Pathology, University of Oxford, South Parks Road, Oxford OX1 3RE, UK

An in vitro transcription assay was used to study transcription from synthetic RNA corresponding to the 3' terminus of influenza A virus cRNA. Micrococcal nuclease-treated influenza virus ribonucleoprotein was used as a source of active polymerase complex. Mutations at two regions of the 13 nucleotide-long conserved cRNA 3' terminus were shown to reduce transcription templated by the short added model RNAs. The first region, at positions 1 and 2 from the 3' terminus, was shown to be affected by the exact nature of the dinucleotide primer used in the in vitro transcription reactions and may not be relevant in vivo. The second region, centred on positions 11 and 12, may be involved in base pairing with conserved nucleotides at the 5' terminus of the cRNA. Evidence for this comes from the finding that RNA corresponding to 5' conserved sequences, but mutated to restore the postulated base pairing with the mutated 3' ends, could partly restore transcription. Binding of the influenza virus polymerase complex to a set of 5'-mutated RNAs was investigated using a photochemical cross-linking assay. Specific binding to two regions of the cRNA 5' terminus was demonstrated, at positions 1 to 3 and positions 8 to 10. Together, these observations suggest that a panhandle forms from the termini of the cRNA molecule and that this structure may play a role in transcription to produce virion RNA.

Introduction

The eight negative-stranded virion RNA (vRNA) gene segments of influenza A virus are each transcribed to produce both mRNA and exact copy, full-length cRNA. The cRNA is used as a template to make more vRNA (Krug et al., 1989). vRNA has been extensively studied with regard to sequence elements to which the influenza virus polymerase complex binds and initiates transcription of the two types of positive-stranded RNA. Short conserved sequences are found at both termini of vRNA which display partial inverted complementarity (Robertson, 1979; Desselberger et al., 1980). These sequences are able to base-pair with each other, both in virions and in infected cells, forming a panhandle structure (Hsu et al., 1987). Using RNA-depleted influenza virus polymerase complex preparations capable of transcribing added RNA templates, several groups have investigated the promoter requirements for positive-strand synthesis from vRNA.

Using polymerase purified from virus fractionated by salt gradient centrifugation (Honda et al., 1988), the conserved sequence at the 3' end of the vRNA was found to be sufficient to promote RNA synthesis from an added RNA template in vitro (Parvin et al., 1989). The ability of added 3' end sequences alone to promote transcription was also observed when polymerase prepared by micrococcal nuclease treatment of viral cores was used (Seong & Brownlee, 1992a, b). Similar polymerase preparations were used to identify specific binding sites in the conserved 3' terminus of vRNA (Fodor et al., 1993). Also using the nuclease-treated polymerase derived from influenza virus, it was found that added 3' templates carrying certain mutations, which reduced transcription drastically, could have that activity ‘rescued’ if an RNA corresponding to the 5' arm of the panhandle with mutations designed to restore base-pairing with the mutant 3' arm was added. This rescue of transcription by the conserved vRNA 5' sequences and the observation that the polymerase could bind specifically to the 5' arm of the panhandle suggested that the vRNA panhandle was in fact involved in transcription (Fodor et al., 1994, 1995). These studies further demonstrated that the first 9
and 10 nucleotides at the termini of the vRNA panhandle were not involved in base-pairing but rather formed an open ‘RNA fork’ structure with the adjacent run of nucleotides being base-paired (Fodor et al., 1994, 1995).

Separate studies using polymerase complex derived from recombinant vaccinia virus found that added sequences representing both the 3' and 5' conserved vRNA sequences were obligatory for endonuclease function (Hagen et al., 1994). Additional studies using the recombinant polymerase confirmed that specific binding of polymerase to the vRNA 5' conserved sequences occurred (Tiley et al., 1994) and further suggested that specific binding also occurred to the 5' arm of synthetic RNAs carrying cRNA terminal sequences.

In contrast, relatively little is known about the requirements for transcription of vRNA from the cRNA template. In in vitro transcription assays, the conserved 13 nucleotides at the 3' end of the cRNA were sufficient to promote transcription (Parvin et al., 1989; Seong & Brownlee, 1992a). One in vitro study included a systematic mutagenesis of cRNA 3'-terminal sequences, as part of a longer T7 transcript containing influenza virus sequences flanking chloramphenicol acetyl transferase gene sequences. This approach identified positions 5, 8, 9 and 11 as being most important for transcription but did not investigate any possible influence of cRNA 5'-terminal sequences or investigate polymerase binding to mutated cRNA sequences (Li & Palese, 1992).

The present study uses nuclease-treated polymerase preparations to investigate the effect of mutations in short model RNA templates representing the conserved cRNA 3' terminus. The possible role of 5' conserved cRNA sequences in transcription and polymerase binding is also investigated.

**Methods**

**Preparation of synthetic RNA templates.** Synthetic RNA templates were synthesized chemically using an Applied Biosystems DNA/RNA Synthesizer. The sequences of wild-type templates and the various mutations introduced are given in the corresponding figure legends. Deprotection was achieved by heating the column support containing the RNA at 60 °C in the presence of dry ethanol:ethanolamine (1:1) for 3 h, after which the volume was reduced overnight in a vacuum centrifuge. The resultant viscous material was dissolved in 1 M tetrabutylammonium fluoride in tetrahydrofuran and incubated for 24 h at 30 °C (slightly modified from Lamond & Sproat, 1994). The deprotected RNA was then precipitated in 10 volumes of dry butan-1-ol and collected by centrifugation at 3000 g for 20 min. Before use, the RNA was purified by electrophoresis through 20% polyacrylamide into water by incubation overnight at 4 °C with agitation. Urea and salts were removed by Sephadex G25 chromatography. The RNA was lyophilized, dissolved in water and the concentration determined by spectrophotometry at 260 nm. Solutions were adjusted to 10 μM with water.

**Preparation of influenza virus polymerase.** Active influenza virus polymerase complex, essentially free of endogenously templated transcriptional activity, was obtained as previously described (Seong & Brownlee, 1992a) by micrococcal nuclease treatment of influenza ribonucleoprotein (RNP) purified from sucrose gradient-purified influenza virus. Influenza virus strain X-31 (a reassortant of A/HK/8/68 and A/PR/8/34) was disrupted in detergents and RNP purified by centrifugation through a glycerol step gradient. Gradient fractions were analysed by SDS-PAGE to identify fractions containing nucleoprotein and polymerase but depleted in membrane proteins. These were pooled and treated with micrococcal nuclease (Sigma; 6 units/ml) for 5 h at room temperature in the presence of 1 mM-CaCl2 before inactivating the nuclease by the addition of EGTA to 6 mM.

**Transcription assay.** The transcription assay was modified from a previously reported study (Seong & Brownlee, 1992a). Assays contained 10 pmol of RNA template and 1-5 μl of nuclease-treated RNP (containing 0.5-1 μg influenza virus protein, as judged by Bradford assay) in a 5 μl volume. The reactions also contained 30 mM-Tris-HCl pH 7.4, 50 mM-KCl, 5 mM-MgCl2, 10 mM-DTT, 0.5 mM-APG, 1 mM-ATP, 0.5 mM-GTP and -UTP and 50 μM-CTP including approximately 2 μCi [32P]CTP to give a final sp. act. of around 8 μCi/mmol. All components were mixed directly and incubated at 30 °C for 3 h. Reactions were stopped by addition of 6 μl 90% formamide containing 1 mM-EDTA and electrophoresis dyes. After heating at 95 °C for 5 min, reaction mixtures were loaded directly onto 20% polyacrylamide gels containing 7 M-urea and separated by electrophoresis at 50 W for 3 h. Regions of the gel containing reaction products were located by autoradiography, excised and their 32P content determined by liquid scintillation counting using water-miscible scintillant. Counts were corrected by subtraction of the background count, which was determined from similar reactions containing no added RNA template. Corrections were made for differing [32P]CTP incorporation due to the varying G content of templates. For each experiment, the activity of the added wild-type template was defined as 100% and the background as 0%. Activity of mutants was expressed as a percentage of wild-type and the mean and SD of at least four experiments was calculated for each.

**Photochemical cross-linking assay.** A previously described UV cross-linking method was used to investigate binding of the influenza virus polymerase complex to RNAs (Fodor et al., 1994). RNAs were 5'-end-labelled using T4 polynucleotide kinase and [32P]ATP to a final sp. act. of 1250 Ci/mmol. All components were mixed directly and incubated in a buffer containing 50 mM-Tris-HCl pH 7.4, 50 mM-KCl, 10 mM-NaCl, 5 mM-MgCl2, and 10 mM-DTT. After incubation at 30 °C for 30 min the reaction tubes were boiled and inverted on a transilluminator and UV-irradiated for 8 min at 302 nm. Five μl of 4% SDS, 10% 2-mercaptoethanol, 0.125 mM-Tris–HCl pH 6.8 and electrophoresis dyes were added and the reaction mixtures boiled for 4 min before being separated by 8% SDS-PAGE. Gels were dried and autoradiographed to visualize polymerase proteins which had bound and become cross-linked to labelled RNA.

**Results**

**cRNA promoter mutagenesis**

Fourteen nucleotide-long RNA templates containing single nucleotide substitutions at each of the 13 conserved 3'-terminal nucleotides of influenza A virus cRNAs were...
assayed for ApG-primed transcription (Fig. 1a). Transversions in either of two regions of the promoter, nucleotides 1 and 2 (numbering from the 3' terminus) and positions 10, 11, 12 and to a lesser extent 13, showed marked reductions in activity compared to wild-type. Mutations at positions 3 and 4 stimulated activity two- to fourfold while transversions of single nucleotides between positions 5 and 9 were tolerated without a significant change in activity. Position 12 was most critical, with activity being at or below 10% of wild-type levels when the C was changed to A, G or U. Transversions (to A or G) at position 11 also drastically reduced activity while the transition to C resulted in greater than wild-type levels. Mutations at positions 10 and 13 also reduced transcription levels but not as severely as for positions 11 and 12.

The low activity observed for mutants at positions 1 and 2 was further investigated to take account of the expected requirement for complementarity between the dinucleotide primer ApG and the 3' terminus of the template (3' UpCp-). For position 1 U → G, the use of CpG as primer in place of ApG restored activity to at least that seen for ApG-primed transcription from wild-type template. (Fig. 1b, lane 6). The position 2 mutant 2 C → A was not however rescued using ApU (lane 7) although some restoration of activity was observed when the double mutant 1 U → G, 2 C → A was transcribed using Cpu as primer (lane 10). Transcription could also be detected for the position 2 mutant and ApU if the third nucleotide was mutated to a G (Fig. 1b, lane 9).

Because of the known several fold greater activity of vRNA than cRNA templates in in vitro transcription assays (Parvin et al., 1989; Seong & Brownlee, 1992a), mutant cRNAs representing each individual difference between the vRNA and cRNA 3’ sequences were also tested. These differences are: position 3 is A in cRNA but G in vRNA, position 5 is C in cRNA but U in vRNA, position 8 is U in cRNA and C in vRNA, and position 11 is U in cRNA and C in vRNA. This latter change is referred to as ΔU11 because it is equivalent to the deletion of the U residue at position 11 and the movement of the adjacent C to replace it in the shorter vRNA conserved sequence. Fig. 1(c) shows that the transitions at positions 3 and 5 result in an approximately twofold stimulation (lanes 3 and 4), position 8 does not significantly affect promoter activity (lane 5) while the ΔU11 mutation causes a several fold increase in activity (lane 6), producing levels similar to the wild-type vRNA (lane 7). Further investigation of RNAs carrying various combinations of the point differences between the vRNA and cRNA sequences (data not shown) suggested that ΔU11 is the single most important determinant of the higher transcriptional activity associated with vRNA in the in vitro transcription assay.

cRNA panhandle investigation

In the case of vRNA terminal sequences, it had previously been shown that nucleotides at positions 10 and 11 from the 3’ end of vRNA were crucial for promoter activity in the in vitro transcription assay. The activity of RNAs mutated in this critical region could however be restored by introducing complementary base changes at positions 11' and 12' of a second RNA corresponding to the 5' arm of the vRNA panhandle; the (') notation is used to denote sequences of the 5' arm as opposed to 3' arm sequences. These, and subsequent experiments (Fodor et al., 1994, 1995) demonstrated that base pairing occurred between sequences near each end of the vRNA molecule. Positions 10, 11 and 12 in the 3’ arm of vRNA pair with positions 11’, 12’ and 13’ in the 5’ arm, respectively, while positions 1 to 9 remain unpaired and form a forked structure (Fodor et al., 1994, 1995).

To test whether a similar structural requirement occurred in cRNA molecules, attempts were made to rescue the activity of 3’ cRNA molecules mutated at positions 11 or 12 by the addition of mutated RNAs corresponding to 5’ arms of a postulated cRNA panhandle. Several possible mismatched base paired structures are possible with the first 10 or so nucleotides from the cRNA terminus. At the critical region around positions 10 and 11, two possible alignments were considered. Firstly, inspection of the sequences of the model RNAs actually used predicted that, in the critical region, mutants at positions 11 and 12 which result in an approximately twofold increase in activity (lanes 3 and 4) were retested, in the presence of RNAs corresponding to 5’ arms of a postulated cRNA panhandle. The second possibility is based on inspection of longer regions of complementarity found in naturally occurring cRNA molecules which suggest a more stable alignment (when adjacent sequences are included) by pairing, for example, position 11 3’ arm) with position 11’ 5’ arm) to form a base paired structure (Fig. 2a, top). The second possibility is based on inspection of longer regions of complementarity found in naturally occurring cRNA molecules which suggest a more stable alignment (when adjacent sequences are included) by pairing, for example, position 11 3’ arm) with position 11’ 5’ arm) to form a base paired structure (Fig. 2a, top). The second possibility is based on inspection of longer regions of complementarity found in naturally occurring cRNA molecules which suggest a more stable alignment (when adjacent sequences are included) by pairing, for example, position 11 3’ arm) with position 11’ 5’ arm) to form a base paired structure (Fig. 2a, top).
Fig. 1. *In vitro* transcription of mutant cRNAs. Autoradiographs of 20% polyacylamide gels containing 8 M-urea, loaded with dinucleotide-primed transcription reactions (see Methods). (a) Mutagenesis of the cRNA sequences. Lane 1, wild-type cRNA 14mer (3' UCAUCUUUGUUCCG) comprising the conserved 13 nucleotide-long cRNA 3' sequence and an extra G at the 5' end, added to increase label incorporation. Lane 2, no added template. Lanes 3 to 11 are reactions containing single nucleotide transversions at positions 1 to 9 (numbering from the 3' end) as indicated. Lanes 12 to 23 are reactions containing each possible single base change at positions 10, 11, 12 and 13. The bar chart shows the mean percentage activity compared to wild-type. The error bars are 1 SD. (b) Investigation of priming at positions 1 and 2 of cRNA. Lanes 1 to 4, dinucleotide primers with no added template. Lane 5, wild-type cRNA 14mer template (sequence as for a) primed with dinucleotide ApG. Lane 6, position 1 mutant (U → G) primed with CpG. Lane 7, position 2 mutant (C → A) primed with ApU. Lane 8, position 3 mutant (A → G) primed with ApG. Lane 9, double mutant (2 C → A, 3 A → G) primed with ApU. Lane 10, double mutant (1 U → G, 2 C → A) primed with CpU. (c) Mutagenesis investigating the
Fig. 2. Attempted rescue of 3' cRNA mutants by addition of mutated cRNA 5' arms. (a) Possible alignments of sequences at the critical positions 11 and 12 of the 3' cRNA arm with the 5' cRNA arm. Nucleotide numbers followed by (') refer to 5' arms to distinguish them from 3' arm numbering. The n → n' and n → n'-1 alignments show the sequences of the actual wild-type RNAs used in transcription assays. The virus example (segment 8 of influenza A/PR/8/34) is included to show the rationale for the n → n'-1 line up. The sequences in bold type are segment-specific but base paired in a similar manner for all virus segments. Various other mismatched and bulged alignments are possible in the first 10 nucleotides (data not shown). (b) Transcription reactions were set up containing various 3' cRNA arms with or without added 5' cRNA. Lane 1, no added template RNA. Lane 2, wild-type cRNA 14mer 3' arm (3' UCAUCUUUGUCC). Lane 3, wild-type cRNA 3' arm (as lane 2) plus wild-type cRNA 5' arm (5' AGCAAAAGCAGGC). Lanes 4, 5 and 6 contain 3' arm mutated at position 11 (11 U --> A) alone (lane 4) or with added 5' arm mutants: 10' A --> U (lane 5) or 11' G --> U (lane 6). Lanes 7, 8 and 9 contain 3' mutant 12 C --> A alone (lane 7) or with added 5' arm mutant 11' G --> U (lane 8) or 12' G --> U (lane 9). Lanes 10, 11 and 12 contain 3' mutant 12 C --> G alone (lane 10) or with added 5' arm mutant 11' G --> C (lane 11) or 12' G --> C (lane 12). Lanes 13, 14, 15 and 16 contain 3' mutant 11 U --> G alone (lane 13) or with added 5' arm mutant 10' A --> C (lane 14), 10' A --> U (lane 15) or 11' G --> C (lane 16). Lane 17 contains 3' 10 U --> A alone and (lane 18) with added 10' A --> C. Lane 19 contains 3' 10 U --> G alone or (lane 20) with added 10' A --> C.

5' but not by 11' G --> U (lane 6). The activity of mutant 11 U --> G (lane 13) was not significantly increased in the presence of 10' A --> C or 10' A --> U (lanes 14 and 15) although this is thought to be due to the reduced efficiency with which these particular 5' end mutants bind the polymerase complex in the photochemical cross-linking assay (see below; data not shown). The addition of 11' G --> C to 11 U --> G (lane 16) resulted in a further reduction in activity of this mutant, possibly due to the introduction of a stable G-C base pair which would cause the incorrect line up of the RNAs. Examination of the possible cRNA panhandle sequences shows that no pairing is predicted for position 10 under the n → n'-1 model. The only feasible pairing for position 10 is to 10', but 10' is already paired to position 11. Addition of 10' A --> U to 10 U --> A or 10' A --> C to 10 U --> G (lanes 17 to 20) both failed to stimulate transcription, suggesting that the predicted pairing of 11

differences between cRNA and vRNA sequences. Lane 1, no added template. Lane 2, 13mer cRNA containing only the conserved sequence (3' UCAUCUUUGUCC). Lane 3, wild-type cRNA sequence except at position 3 (A changed to G as found in vRNA). Lane 4, cRNA except at position 5 (C changed to U as found in vRNA). Lane 5, cRNA except at position 8 (U changed to C as found in vRNA). Lane 6, cRNA but with U at position 11 deleted as found in vRNA (this particular mutant is still 13 nucleotides long due to addition of an extra G at the 5' end, the signal was equivalent when a 12mer was used instead; data not shown). Lane 7, wild-type vRNA 12mer conserved sequence (3' UCGUUUUCGUGC).
Fig. 3. Photochemical cross-linking of labelled RNA to the polymerase complex. 8% SDS-polycrylamide gels were used to resolve the crosslinked products (see Methods). (a) Cross-linking to mutant 5' cRNA 13mer molecules. Lane 1, wild-type cRNA 5' end (5' AGCAAAAG-CAGGC) cross-linked to polymerase proteins. Lanes 2-13, cross-linking using RNAs mutated at each of the conserved 12 nucleotides of the cRNA 5' arm, as follows: Lane 2, position 1' A->U. Lane 3, position 2' G->C. Lane 4, position 3' C->G. Lane 5, position 4' A->U. Lane 6, position 5' A->U. Lane 7, position 6' A->U. Lane 8, position 7' A->U. Lane 9, position 8' G->C. Lane 10, position 9' C->G. Lane 11, position 10' A->U. Lane 12, position 11' G->C. Lane 13, position 12' G->C. (b) Competition of binding to labelled wild-type cRNA 5' arm by unlabelled mutant cRNA 5' arms. Lane 1, wild-type cRNA 13mer with no added competitor (a). Lanes 2-14, labelled wild-type cRNA 13mer plus a 20-fold excess of unlabelled competitor as follows: lane 2, wild-type competitor. Lane 3, position 1' A->U. Lane 4, position 2' G->C. Lane 5, position 3' C->G. Lane 6, position 4' A->U. Lane 7, position 5' A->U. Lane 8, position 6' A->U. Lane 9, position 7' A->U. Lane 10, position 8' G->C. Lane 11, position 9' C->G. Lane 12, position 10' A->U. Lane 13, position 11' G->C. Lane 14 position 12' G->C.

to 10' is correct and that position 10 remains unpaired. Thus, the n-n'-1 model (Fig. 2a) is supported.

Cross-linking to the cRNA 5' arm

The 5' arm of the postulated cRNA panhandle was investigated for polymerase binding using the photochemical cross-linking protocol used previously to investigate the vRNA panhandle (Fodor et al., 1993, 1994). Radiolabelled 14 nucleotide-long RNAs based on the 5'-terminal sequence of cRNA, including transversion mutations at each position, were incubated with nuclease-treated RNP at 30°C for 30 min. UV irradiation was then used, at previously determined doses, to form covalent linkages between individual polymerase proteins and the labelled RNA. Following SDS-PAGE and autoradiography, signals at the positions of the polymerase proteins due to bound radiolabelled RNA indicated that the particular mutant tested had retained the ability to be recognized. The presumed identity of the PB1 band and the PB2/PA doublet was confirmed by immunoprecipitation of cross-linked products using antisera raised to individual polymerase proteins as described for the vRNA studies (data not shown; Fodor et al., 1994). The specificity of the binding was confirmed by heterologous competition using UI5 and yeast tRNA (data not shown) and by homologous and mutant competition in the presence of a 20-fold excess of unlabelled competitor RNA (Fig. 3). The non-specific binding of the probe RNA by nucleoprotein served as an internal control for probe addition and cross-linking efficiency.

Fig. 3 (a) shows that transversions at positions 1', 2', 3', and at 8' and 9' caused loss of polymerase binding (lanes 2, 3, 4, 9 and 10) with reduced but detectable binding being observed for position 10' (lane 11). The remaining positions (4'-7' and 11' and 12') showed clear cross-linking but with an altered (and reproducible) pattern of cross-linking compared to wild-type. This is particularly evident for position 6' and 7' where, in contrast to the wild-type pattern, very strong PB1 binding occurred compared to the PB2/PA doublet (lanes 7 and 8 compared to lane 1). Similar alterations in binding profile were observed for vRNA mutants (Fodor et al., 1994).

The specificity of binding to mutant cRNA 5' ends is demonstrated in Fig. 3 (b) by competition between labelled wild-type probe and an excess (20-fold) of each of the mutant RNAs. Mutant RNAs that were unable to bind polymerase did not compete with the labelled wild-type probe, while mutants which retained binding ability did compete and prevented the probe from binding. Thus, the pattern seen when unlabelled mutant RNAs were used in competition was the inverse of the pattern seen when labelled mutant RNAs were tested alone (compare Fig. 3a lanes 2 to 13 with Fig. 3b lanes 3 to 14).

Discussion

The study of cRNA 3' mutants presented here identifies two regions essential for in vitro transcription and shares some features similar to findings reported previously for the vRNA 3' arm (Seong & Brownlee, 1992b) and for a cRNA study (Li & Palese, 1992).

Mutations at positions 1 and 2 caused a drastic reduction of transcription but, to some extent, this
reduction was due to the dinucleotide priming requirement of the *in vitro* transcription assay. In this study the cRNA position 1 lesion could be fully recovered using a complementary dinucleotide primer, whereas additional mutations at position 1 or 3 were required for the position 2 mutation before activity could be detected using cognate dinucleotide primers (Fig. 1b). The inability of dinucleotide ApU to prime transcription from the position 2 C → A mutant (starting 3' UAC-) may be due to the predicted weak interaction of the primer with the template (two A-U base pairs compared to the usual one A-U and one G-C pair), although for vRNA, ApU was able to rescue transcription from a position 2 mutant to 70% of wild-type levels (Seong & Brownlee, 1992b). Since position 3 is G in vRNA but A in cRNA, the effect of a double mutation (2 C → A, 3 A → G) was investigated using ApU as a primer. Significant activity was observed above background levels (Fig. 1b, lane 9) but this was substantially lower than for the wild-type template (lane 5) and even lower than for the 3 A → G mutation alone (lane 8). The reason that ApU failed to prime transcription from the position 2 C → A mutation but primed weakly off the double mutant 2 C → A, 3 A → G is unknown. The double mutant 1 U → G, 2 C → A was examined in order to see if the introduction of a G-C pair could stabilize priming. Fig. 1(b) lane 10 shows a signal, but of aberrant mobility 1 nucleotide longer than expected, perhaps with the U residue pairing with the G at position one. No further characterization of this product was performed. Overall we conclude that the down-regulation of transcription observed for mutations at positions 1 and 2 is affected by the dinucleotide primer used in the *in vitro* transcription assay and may not be relevant *in vivo*.

The low activity and rescue by complementary dinucleotide primer of the position 1 mutant occurred in another study, although no inhibition of transcription was observed when position 2 was mutated (Li & Palese, 1992). One possible explanation for this discrepancy is that, due to the longer transcription products analysed in that study, misprimed products of aberrant length may have occurred. The down-regulation at positions 1 and 2 is at least in part due to the requirement for dinucleotide priming in the *in vitro* assay and is of questionable significance to actual viral transcription, which is not thought to involve dinucleotide primers.

For the vRNA 3' sequences, an early study reported unprimed transcription using micrococcal nuclease-treated enzyme (Seong & Brownlee, 1992a). Such specific unprimed transcription was not observed when the present cRNA templates were tested in the absence of primer (data not shown).

The stimulation of transcription at positions 3 and 4 was also noted for the published cRNA study and position 3 stimulation also occurs for vRNA sequences (Li & Palese, 1992; Seong & Brownlee, 1992a). The sequences between position 5 and 9 showed little effect on transcription while position 10, 11 and 12 drastically reduced activity. The observation that the 11 U → C mutation (Fig. 1a, lane 17) increased activity (in contrast to all mutations at this or the adjacent nucleotides which all reduced activity) is probably due to the creation of a vRNA-type promoter by introducing a mutation equivalent to the ΔU11 mutation investigated in Fig. 1(c), lane 6. This finding is entirely consistent with our previous finding of a requirement for base pairing between the termini of the vRNA molecule which involves this region (Fodor et al., 1994, 1995).

In the 5' sequences examined for polymerase binding by the photochemical cross-linking assay, two clear regions were involved in binding (Fig. 3). Mutations at positions 1', 2' and 3' and positions 8', 9' and to a lesser extent position 10', show decreased cross-linking to the polymerase proteins. This profile is similar but not identical to the observations made for the vRNA 5' end where positions 1' to 3', 9' and possibly 7', 8' and 11' were found to be most crucial for polymerase binding (Fodor et al., 1994). A recent study, which used RNA modification to interfere with binding of vRNA and cRNA sequences to a recombinant polymerase complex, also reported differences between the pattern of binding to the two types of RNA (Tiley et al., 1994). These authors reported for cRNA that modification of positions 1', 3', 4', 5', 9' and 10' in the 5' terminal sequences reduced polymerase binding, as determined by gel shift assay, with positions 3' and 9' being most important. The importance of positions 4' and 5' noted by these authors was not confirmed in the present study, presumably due to the different methods used. These authors also reported no interference in polymerase binding when sequences in the 3' arm of the cRNA panhandle were modified. This is consistent with our inability to demonstrate specific binding to the 3' arm of cRNA using the photochemical cross-linking assay (data not shown). The observed reduced binding at position 10' in this study probably accounts for the inability of 10' A → C or 10' A → U to rescue 11 U → G (Fig. 2b, lanes 13 to 16).

The observation of partial rescue of the position 10 and 11 cRNA mutants by 5' arms mutated to restore base pairing demonstrates that the cRNA promoter can function as a panhandle. The question as to whether a cRNA panhandle is obligatory for transcription is less certain, since cRNA 3' arms alone are competent transcription templates in the *in vitro* transcription reaction and the addition of wild-type cRNA 5' ends does not stimulate transcription further (Fig. 2b, lanes 2 and 3). Unlike the case for added vRNA 3' templates,
which may be transcribed via 5' arms endogenous to the RNA polymerase preparations, no cRNA 5' arms are expected to be present in polymerase preparations derived from virus particles. Nevertheless, we have not ruled out the possibility that short 5' cRNA molecules are present in our enzyme preparations or are synthesized in the transcription reactions from fragments of vRNA which survived nucleicase treatment. The presence of such fragments may explain the activity of cRNA 3' ends which occurred in the absence of added cRNA 5' end. Furthermore, if these endogenous fragments are in excess, they may account for the failure of added wild-type 5' cRNA to stimulate transcription further. An alternative explanation, that a panhandle is not obligatory for transcription, cannot be excluded. The absence of stimulation of wild-type vRNA 3' arm by added 5' vRNA sequences has also been observed (Fodor et al., 1994). The possibility that vRNA 5' arms present in the RNA polymerase preparation are mediating transcription of the wild-type cRNA sequences is not likely, due to the properties of the predicted perfect duplex of a cRNA 3' and vRNA 5' arm. Firstly, such duplexes bind polymerase poorly (Tiley et al., 1994). Secondly, if equimolar amounts of 3' cRNA and 5' vRNA synthetic templates are added to the in vitro transcription assay, transcription is severely down-regulated (data not shown).

RNA polymerase preparations derived from virus particles, as used in this study, may be deficient in cellular or additional viral factors which may play a role in transcription from the cRNA promoter during infection. At least one cellular factor has been identified which interacts with nucleoprotein (O'Neill & Palese, 1995). It is therefore possible that the cRNA panhandle which functions in the in vitro transcription assay is overridden or modified during infection by factors yet to be identified.

For vRNA, the requirement of the 5' arm of the panhandle for the endonuclease function, which provides capped primers for synthesis of viral mRNA, suggests a role for the vRNA panhandle in mRNA (but not necessarily cRNA) production (Hagen et al., 1994; Fodor et al., 1994). This hypothesis is also supported by the observed correlation of mRNA production with the proportion of vRNA molecules found in a panhandle configuration in infected cells (Hsu et al., 1987). The requirement of the panhandle for polyadenylation (Luo et al., 1991) is also consistent with mRNA production from a panhandle structure. Given these data, it is tempting to speculate that cRNA production (which does not require the endonuclease function) occurs without the need for a panhandle structure. The logical extension of this argument is that cRNA templates, which only produce vRNA transcripts and do not utilize endonuclease-cleaved capped primers, would not use a panhandle. However, the demonstration in this paper that mutant cRNA 3' arms can be rescued by appropriately mutated 5' cRNA arms provides direct evidence that the cRNA molecule can function in a panhandle configuration. This finding is corroborated by the specific binding of the influenza virus polymerase complex to the 5' end of cRNA.

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References


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