

Mutational analysis of influenza virus promoter elements *in vivo*

Gabriele Neumann and Gerd Hobom*

Institut für Mikrobiologie und Molekularbiologie, Frankfurter Strasse 107, D-35392 Giessen, Germany

RNA polymerase I transcription *in vivo* in transiently DNA-transfected cells has been used to express influenza virus vRNA molecules coding for chloramphenicol acetyltransferase (CAT) in an antisense orientation. Influenza virus superinfection provided viral RNA polymerase and other proteins required for transcriptional conversion of minus-strand vRNA into plus-strand viral mRNA molecules expressing CAT activity. This system has been used for analysis of the vRNA sequences which cooperatively constitute the

vRNA promoter structure via nucleotide exchanges as well as deletions and insertions of both terminal segments. Several mutants caused greatly enhanced expression over wild-type levels, which was transmitted during serial passage of progeny virus. The data obtained for the mutations in various promoter elements support a model implicating double-stranded vRNA promoter structures in binding of viral polymerase, and in consecutive steps during initiation of RNA synthesis.

Introduction

The genome of influenza A virus consists of eight different single-stranded viral RNA (vRNA) molecules of negative polarity, which have in common 5'- and 3'-terminal sequences that are also largely complementary to each other. These conserved segments of 13 and 12 nucleotides in length have been observed to form dsRNA panhandle structures (Hsu *et al.*, 1987; Seong & Brownlee, 1992; Fodor *et al.*, 1993) which have recently been analysed *in vitro* in more detail using internally deleted model RNAs (Baudin *et al.*, 1994; Tiley *et al.*, 1994). In the virion, the panhandle formed at the ends of all RNA segments binds specifically to the viral RNA polymerase complexes, while the interiors of the segments associated with viral nucleoprotein (NP) stay single-stranded (Compans *et al.*, 1972; Honda *et al.*, 1988; Martín *et al.*, 1992). Upon infection these viral RNPs initially serve as templates for the synthesis of viral mRNAs by a specific cap-snatching mechanism (Plotch *et al.*, 1979; Braam *et al.*, 1983) and later direct synthesis of full-length complementary RNAs (cRNAs), probably dependent on the absence or presence of newly synthesized NP protein, respectively (Shapiro & Krug, 1988). The plus-strand cRNAs are thereafter used in similar reactions as templates for progeny vRNA synthesis.

The viral RNA polymerase complex, consisting of

proteins PB1, PB2 and PA, is responsible for all three modes of RNA synthesis during the virus replication cycle, involving specific binding to the terminal segments of both vRNA and cRNA templates. Sequence comparisons revealed that the vRNA and cRNA termini have similar, but not identical sequences. For that reason vRNA and cRNA recognition may be dissimilar processes, with structural deviations allowing for asymmetries in the initiation frequencies of plus- and minus-strand RNA synthesis, and possibly in viral RNP packaging, which has also been suggested to be controlled by the panhandle RNA sequence (Hsu *et al.*, 1987).

Recently, we reported an *in vivo* system for the introduction of specific mutations into the genome of influenza viruses. Viral cDNA was inserted in an antisense orientation between the mouse rDNA promoter and terminator sequences. This evolved from *in vitro* transcription experiments utilizing nuclear extracts from Ehrlich ascites cells, which produced transcripts exactly resembling influenza virus vRNA. For a series of *in vivo* studies, the viral coding sequence was replaced by the coding sequence for chloramphenicol acetyltransferase (CAT), with both viral terminal non-coding sequences being retained on the transcript. After transfection of this recombinant DNA template into mouse cells followed by influenza virus infection, CAT activity was detectable. Transfer of supernatants to different cells demonstrated that CAT vRNAs transcribed *in vivo* by cellular RNA polymerase I were not only transcribed by the viral RNA polymerase into plus-strand mRNA and

* Author for correspondence. Fax +49 641 702 9659.

translated into CAT protein, but also were replicated and packaged into infectious progeny virus particles (Zobel *et al.*, 1993; Neumann *et al.*, 1994).

We have used this system for a stepwise introduction of single and multiple mutations into the conserved panhandle RNA sequences, thereby effectively converting the haemagglutinin (HA) vRNA promoter sequence into a HA cRNA promoter sequence and vice versa. For these series of constructs CAT activities have been measured both in transfected and infected B82 cells and, after passaging of B82 supernatants, in secondarily infected MDCK cells. From the results obtained we propose a model for the terminal dsRNA structure recognized by the viral RNA polymerase in consecutive steps of the initiation of viral mRNA synthesis.

Methods

Plasmid constructions. Plasmids with mutated vRNA and/or mutated cRNA promoter sequences are derivatives of pHL926 (Zobel *et al.*, 1993; Neumann *et al.*, 1994). In pHL926 a hybrid CAT cDNA with flanking non-coding sequences derived from influenza virus vRNA segments was inserted in an antisense orientation between the mouse rDNA promoter and terminator sequences. The CAT reporter gene was introduced by replacing exactly the coding sequence for HA, retaining the untranslated viral 5' and 3' sequences of segment 4.

Viral RNA 5' end mutations were created by PCR, using a general primer hybridizing to a position in the flanking rDNA promoter sequence, and a specific primer carrying the desired nucleotide substitution to be introduced in the viral terminal sequence. The PCR products were first digested with the restriction enzymes *Bgl*II and *Spe*I, then inserted into the left boundary position by exchanging the segment between these restriction sites in pHL926; their sequences were confirmed by DNA sequencing.

Generation of vRNA 3' end mutations followed the same general scheme at the right boundary. PCR products were obtained by using a general primer complementary to a CAT gene internal sequence and a specific primer with appropriate nucleotide exchanges. Following digestion with restriction enzymes *Nco*I and *Sca*I, the PCR products were cloned into *Nco*I- and partially *Sca*I-digested plasmid pHL926. Any PCR-derived sequences were investigated by DNA sequencing.

For constructs with both 5' and 3' end mutations, 5' mutant fragments were obtained by *Bgl*II and *Spe*I restriction and inserted into the appropriate 3'-terminal variation plasmid.

Cells and viruses. Influenza virus strain A/FPV/Bratislava was grown in NIH 3T3 cells. For transfection and passaging experiments B82 cells (a mouse L cell line) and MDCK cells were used.

Lipofectamin DNA transfection and influenza virus helper infection. For DNA transfection 10⁷ B82 cells were used. Plasmid DNA (5 µg) was mixed with 60 µg of Lipofectamin (Gibco BRL) in serum-free medium and incubated at room temperature for 10–15 min. This mixture was added to the cells which were then washed twice with serum-free medium. The incubation with Lipofectamin and DNA was continued for 1 h. After further incubation with Dulbecco's modified Eagle's medium (DMEM) for 1 h the transfected B82 cells were infected with influenza virus strain A/FPV/Bratislava at a m.o.i. of 0.01 to 1 for another 30–60 min. Further incubation was performed with DMEM.

Passaging of virus-containing supernatants. Under standard conditions, 8 h after influenza virus infection (at a m.o.i. of 0.1 to 1) cells

were harvested for CAT assays and supernatants were collected and spun at 1200 r.p.m. for 5 min to remove cell debris.

Aliquots of virus containing cleared supernatants were used for plaque tests and another aliquot was adsorbed to 10⁷ MDCK cells for 30–60 min for further passaging. Again, 8 h after infection the CPE was verified and cells and supernatants were collected and treated as before.

CAT assay. Cell extracts were prepared as described by Gorman *et al.* (1982). CAT assays were done with [¹⁴C]chloramphenicol or fluorescently labelled chloramphenicol (borondipyrromethane difluoride fluorophore; FLASH CAT kit, Stratagene) as substrates.

For [¹⁴C]chloramphenicol the assay mixture contained 0.1 µCi [¹⁴C]chloramphenicol, 20 µl 4 mM-acetyl CoA, 25 µl 1 M-Tris-HCl pH 7.5 and 50 µl of cell lysate in a total volume of 150 µl. The assay mixture for the fluorescently labelled substrate contained (in a final volume of 80 µl) 10 µl 0.25 M-Tris-HCl pH 7.5, 10 µl 4 M-acetyl CoA, 10 µl fluorescently labelled chloramphenicol and 50 µl of cell lysate. After incubation for 16 h the reaction products were separated by chromatography and either autoradiographed or visualized by UV illumination and photography.

Results

Mutational analysis of vRNA 3'-terminal sequences

Influenza A virus vRNA 5' and 3' ends have almost, but not exactly, complementary sequences with nucleotide mismatches at positions 3, 5 and 8, and an additional unpaired, 'bulged' nucleotide present in the 5' region. Nevertheless, both vRNA termini hybridize into a double-stranded panhandle structure made up of 12 and 13 nucleotides common to all eight RNA segments, plus, on average, three additional base pairs specific for each of the vRNA molecules. Due to the deviations mentioned the cRNA or plus-strand terminal structures are different from the vRNA structures; however, both are recognized by viral RNA polymerase and are used for initiation of RNA synthesis, i.e. they constitute a promoter structure. Even if in the initial recognition and binding of RNA polymerase the dsRNA panhandle structure, as observed in virion RNPs (Hsu *et al.*, 1987), is the likely substrate of primary promoter interaction, the initiation step of transcription at the 3'-terminal region requires a partially single-stranded 'forked' structure (Fodor *et al.*, 1994). In the second phase of vRNA promoter recognition RNA polymerase may be predicted to continue its binding interactions with both the remaining double-stranded segment and the single-stranded 3' template segment, as well as the 5' single-stranded end (Tiley *et al.*, 1994); the double-stranded segment comprises nucleotides 10 to 15 and $\bar{1}\bar{1}$ to $\bar{1}\bar{6}$, and the single-stranded template segment comprises nucleotides $\bar{1}$ up to $\bar{9}$ (overlined nucleotides referring to positions in the vRNA 3' end; e.g. position $\bar{2}$ designates the penultimate nucleotide). Introduction of mutations at specific positions in either the 5' or 3' segment may therefore alter these two (or more) consecutive vRNA promoter structures, panhandle and fork, in different ways; in addition such nucleotide

Table 1. Terminal sequences of vRNA molecules constructed via their murine rDNA/viral cDNA templates, and reporter gene expression rates observed after DNA transfection and influenza virus infection of B82 cells, 8 h post-infection

Plasmid	Structure*	CAT expression	Plasmid	Structure*	CAT expression	Plasmid	Structure*	CAT expression
pHL926	3' - <u>UCG</u> UUU <u>UCG</u> · <u>UCC</u> 5' -AGUAGAAAC _A AGG	+	pHL1183	3' - <u>UC</u> A <u>U</u> C <u>UUU</u> G· <u>UCC</u> 5' -AGCAGAAAC _A AGG	++	pHL1182	3' - <u>UC</u> A <u>U</u> UUU <u>U</u> G· <u>UCC</u> 5' -AGCAGAAAC _A AGG	-
pHL1098	3' - <u>UC</u> A <u>U</u> UUU <u>UCG</u> · <u>UCC</u> 5' -AGUAGAAAC _A AGG	-	pHL1185	3' - <u>UC</u> A <u>U</u> C <u>UUU</u> G· <u>UCC</u> 5' -AGUAAAAC _A AGG	-	pHL1184	3' - <u>UC</u> A <u>U</u> UUU <u>U</u> G· <u>UCC</u> 5' -AGUAAAAC _A AGG	-
pHL1099	3' - <u>UCG</u> U <u>C</u> UU <u>UCG</u> · <u>UCC</u> 5' -AGUAGAAAC _A AGG	-	pHL1150	3' - <u>UC</u> A <u>U</u> C <u>UUU</u> G· <u>UCC</u> 5' -AGUAGAAGC _A AGG	++++	pHL1186	3' - <u>UC</u> A <u>U</u> UUU <u>U</u> G· <u>UCC</u> 5' -AGUAGAAGC _A AGG	++
pHL1100	3' - <u>UCG</u> UUU <u>U</u> G· <u>UCC</u> 5' -AGUAGAAAC _A AGG	-	pHL1147	3' - <u>UC</u> A <u>U</u> C <u>UUU</u> G· <u>UCC</u> 5' -AGCAAAAC _A AGG	-	pHL1187	3' - <u>UC</u> A <u>U</u> UUU <u>U</u> G· <u>UCC</u> 5' -AGCAAAAC _A AGG	++
pHL1101	3' - <u>UC</u> A <u>U</u> C <u>UUU</u> G· <u>UCC</u> 5' -AGUAGAAAC _A AGG	-	pHL1148	3' - <u>UC</u> A <u>U</u> C <u>UUU</u> G· <u>UCC</u> 5' -AGCAGAAAGC _A AGG	+++	pHL1125	3' - <u>UC</u> A <u>U</u> UUU <u>U</u> G· <u>UCC</u> 5' -AGCAAAAGC _A AGG	-
pHL1102	3' - <u>UC</u> A <u>U</u> UUU <u>U</u> G· <u>UCC</u> 5' -AGUAGAAAC _A AGG	+++	pHL1149	3' - <u>UC</u> A <u>U</u> C <u>UUU</u> G· <u>UCC</u> 5' -AGUAAAAGC _A AGG	-	pHL1140	3' - <u>UC</u> A <u>U</u> C <u>UUU</u> GU <u>UCC</u> 5' -AGUAGAAACAAGG	++
pHL1103	3' - <u>UCG</u> U <u>C</u> UU <u>U</u> G· <u>UCC</u> 5' -AGUAGAAAC _A AGG	-	pHL1126	3' - <u>UC</u> A <u>U</u> C <u>UUU</u> G· <u>UCC</u> 5' -AGCAAAAGC _A AGG	++	pHL1152	3' - <u>UC</u> A <u>U</u> C <u>UUU</u> GU <u>UCC</u> 5' -AGUAGAAAC _A AGG	-
pHL1104	3' - <u>UC</u> A <u>U</u> C <u>UUU</u> G· <u>UCC</u> 5' -AGUAGAAAC _A AGG	++++	pHL1124	3' - <u>UCG</u> UUU <u>UCG</u> · <u>UCC</u> 5' -AGCAAAAGC _A AGG	-	pHL1164	3' - <u>UC</u> A <u>U</u> C <u>UUU</u> GU <u>UCC</u> 5' -AGUAGAAAC _A AGG	-

* Paired bases have a line between them and mutated bases are indicated with a dot.

exchanges will also result in variations of the corresponding cRNA promoter structure(s).

In investigating the importance of the three mismatch positions, specific single, double or triple nucleotide exchanges were first introduced into the vRNA 3' end sequence at positions 3, 5 and 8, thereby approaching in a step-wise manner a fully double-stranded vRNA promoter structure. At the same time the vRNA 3' template sequence would become equivalent to the cRNA 3' end in these positions, but would not have the additional bulged nucleotide present in the 5' vRNA sequence (Table 1; Fig. 1). Single nucleotide exchanges (pHL1098, pHL1099, pHL1100) abolished the promoter activity and no CAT activity was observed, as has been reported before using a different method (Luo *et al.*, 1991). Two of the double mutant constructs (pHL1101, pHL1103) also gave negative results (Fig. 1*a, b*).

In contrast, for pHL1102 (G3A, C8U; G3A represents a guanosine to adenosine change at position 3) significant CAT activity was detected (Fig. 1*a, b*). This was distinctly higher than for the corresponding wild-type construct (pHL926) which under the conditions applied here (8 h after infection) resulted in rather low levels of CAT expression. This increase in activity was further enhanced for the final construct of the series, pHL1104, carrying the triple exchange G3A, U5C and C8U (Fig. 1*a, b*); transfection of pHL1104 DNA followed by influenza virus infection resulted in a very high level of CAT expression, considerably above the results with pHL1102.

These results have been repeated using various conditions of transfection and infection as well as different times of infection. While the pHL1104 variant was always far superior to any wild-type construct the expression was variable and difficult to quantify (between 20-fold and 100-fold increase). Relatively short infection cycles of 8 h, as prevalently used here, appear to put faster replicating molecules, i.e. mutant sequences, at a further advantage over RNA polymerase I-created wild-type molecules. This was particularly true for comparative analyses of pseudo-vRNA CAT expression molecules after packaging, i.e. during passage of virus progeny, collected 8 h after infection (Fig. 1*b*). Levels of CAT expression were increased for wild-type and related constructs after DNA transfection and 12 h of infection before passage (Neumann *et al.*, 1994). Only minor variations in CAT expression were observed in individual experiments using the higher expression constructs after 8 or 12 h of infection.

Mutational analysis of vRNA 5'-terminal sequences

We also investigated whether the unexpectedly high viral mRNA expression of pHL1104 was the consequence of a stabilized panhandle double-stranded structure or more specifically attributed to the point mutations introduced into the vRNA 3' sequence, e.g. when being recognized as a single-stranded template segment in the forked structure.

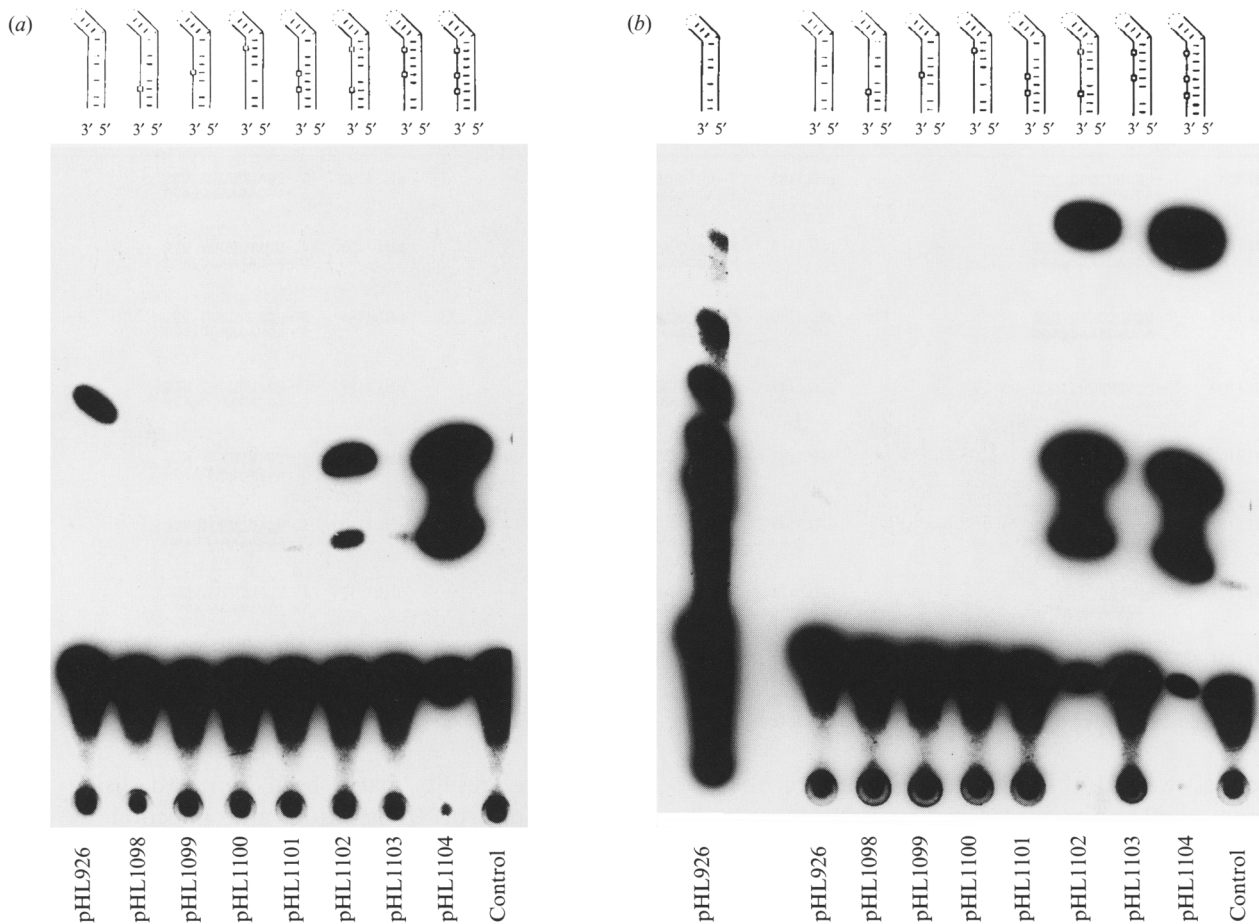


Fig. 1. Nucleotide exchanges in the vRNA 3'-terminal segment. (a) CAT expression in vRNA expression construct (vDNA)-transfected and influenza virus-infected cells. B82 cells (10^7) were transfected with 5 μ g of the indicated plasmid construct DNA in 60 μ g Lipofectamin for 1 h. Following incubation with DMEM for 1 h, the cells were infected with influenza virus A/FPV/Bratislava (m.o.i. of 1). At 8 h post-infection, the cells were harvested and assayed for CAT activity. (b) CAT activity after passaging of virus progeny. Supernatants of the indicated plasmid-transfected and virus-infected cells were collected 8 h post-infection, after sedimentation at 1200 r.p.m. for 5 min. Aliquots of these supernatants were adsorbed to 10^7 MDCK cells for 30–60 min. The infected MDCK cells were assayed for CAT activity 8 h post-infection. Control experiments used mock-transfected and mock-infected cells. The leftmost lane in (b) containing pHL926 was exposed for longer periods than the regular pHL926 lane. Diagrams of the panhandle structures of each plasmid are included. Points of mutation and the predicted presence or absence of base pairings within the panhandle structure are shown. For detailed sequence information see Table 1.

For this purpose we constructed pHL1124, with three complementary point mutations introduced at the 5' end of the vRNA sequence, again at positions 3, 5 and 8 (U3C, G5A, A8G). Together with a wild-type vRNA 3' end these variations again result in a panhandle structure free of mismatches. Therefore pHL1124 was equivalent to pHL1104 but was different in the sequence of its template and non-template strands. No significant CAT expression was detected for pHL1124 (data not shown). We concluded that the increased CAT activity of pHL1104 was not a consequence of the stabilized double-stranded structure itself, but at least in part was a consequence of the individual nucleotide exchanges at positions 3, 5 and 8 at the 3' end of the vRNA sequence, in either single- or double-strand recognition.

Mutational analyses of concerted exchanges at both ends of the vRNA sequence

In order to determine in detail the influence of single, double and triple exchanges at the vRNA 5' end on CAT expression we used the improved vRNA 3' end sequences of pHL1104 and pHL1102 as starting points, rather than the corresponding wild-type sequence. From the series of experiments using constructs related to pHL1104 (Fig. 2) and the equivalent series with pHL1102 (Table 1) it can be concluded that retaining a G residue in position 5 is the most important single feature. A single exchange to an A residue at position 5 as in pHL1185 (Fig. 2a, b) rendered the promoter entirely inactive; single exchanges in positions 3 or 8, as well as a double exchange in these

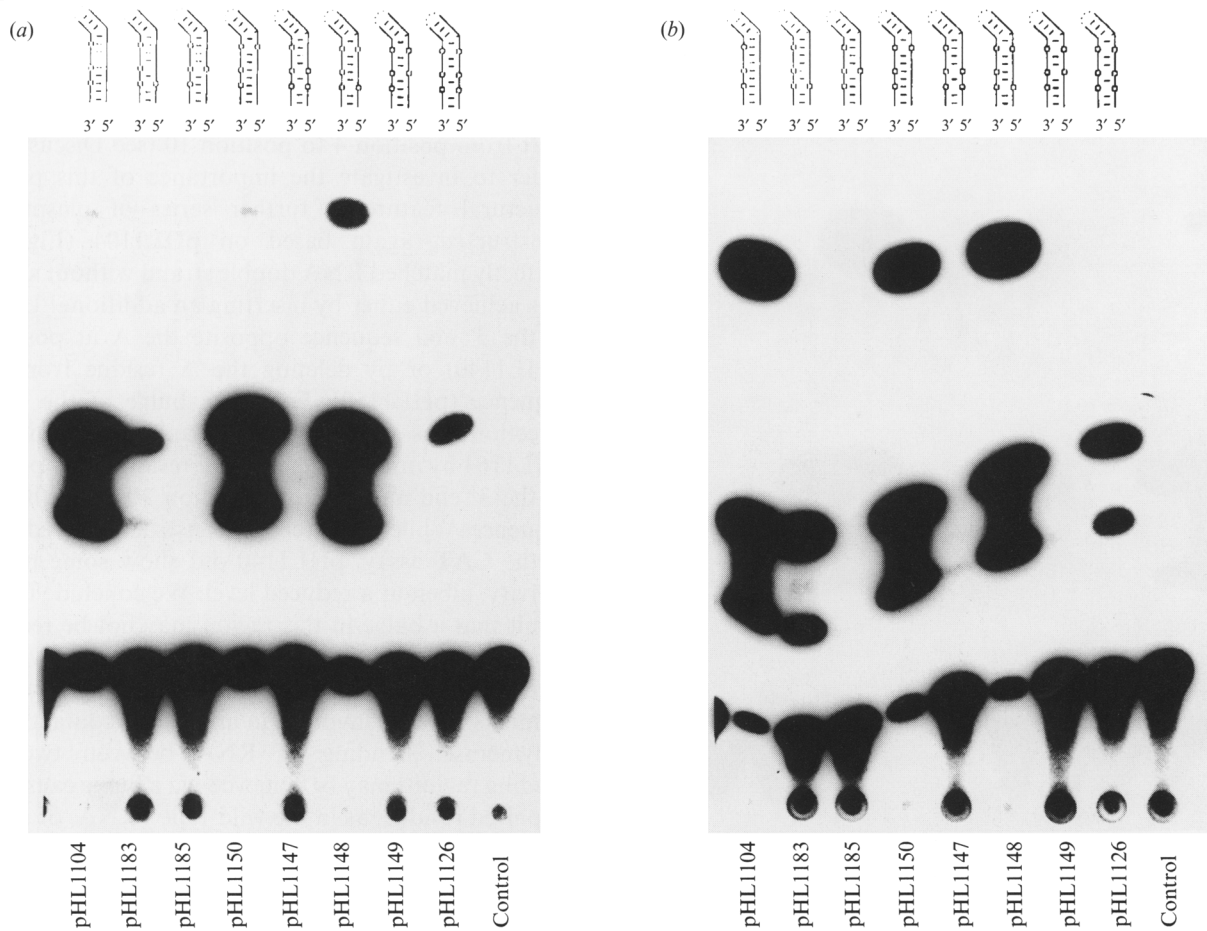


Fig. 2. Nucleotide exchanges in the vRNA 5'-terminal segment. Series of constructs based on the 3'-terminal sequence present in mutant pHL1104-derived vRNA transcripts. (a) CAT expression in vDNA-transfected and influenza virus-infected cells. (b) CAT activity after passaging of virus progeny. For experimental details see Fig. 1. Control experiments used mock-transfected and mock-infected cells.

positions (Fig. 2*a, b*; pHL1183, pHL1150 and pHL1148), retained considerable promoter activity even if this was slightly reduced from the level observed for pHL1104. While the G5A nucleotide substitution, opposite nucleotide C5 in the 3' terminus, resulted in the loss of 1 base pair (in the panhandle context) within the pHL1104 series, a base pair was gained by exactly the same G5A exchange within the pHL1102 series, opposite the U5 residue present in the pHL1102 vRNA 3' end. Since the G5A exchange in pHL1184 resulted in loss of promoter function despite gaining 1 base pair, we conclude that the G at position 5 within the 5' non-template strand is important for RNA polymerase binding rather than being part of a panhandle double-stranded structure. The importance of a G residue at this position has been observed earlier in a single-step mutational analysis (Li & Palese, 1992), while non-template strand binding of RNA polymerase has been recently studied *in vitro* (Tiley *et al.*, 1994). In contrast to the deleterious effect of an exchange at position 5,

variations at positions 3 and in particular 8, are of minor importance. The series of 5' nucleotide exchanges opposite the pHL1104 and the pHL1102 versions of the vRNA 3' end gave the same patterns of results, although expression of pHL1102 derivatives was reduced fivefold (data not shown), as was characteristic for the parental construct. The only result in both series not quite in agreement with a uniquely important role for a G residue in position 5 was the triple exchange in pHL1126, which retained low promoter activity in spite of an A residue in the position (Fig. 2*a, b*). Owing to six concerted exchanges in positions 3, 5 and 8 and $\bar{3}$, $\bar{5}$ and $\bar{8}$, the pHL1126 vRNA terminal structure is nearly equivalent to a wild-type cRNA double-strand, with the exception of an unpaired A at position 10 of the 5' end in pHL1126 and an unpaired U at position $\bar{10}$ of the 3' end in the wild-type cRNA structure. This similarity suggests that the cRNA-like structure of pHL1126 may compensate for the A in place of the G at position 5, at a reduced level of activity. In the parallel pHL1102 series, the

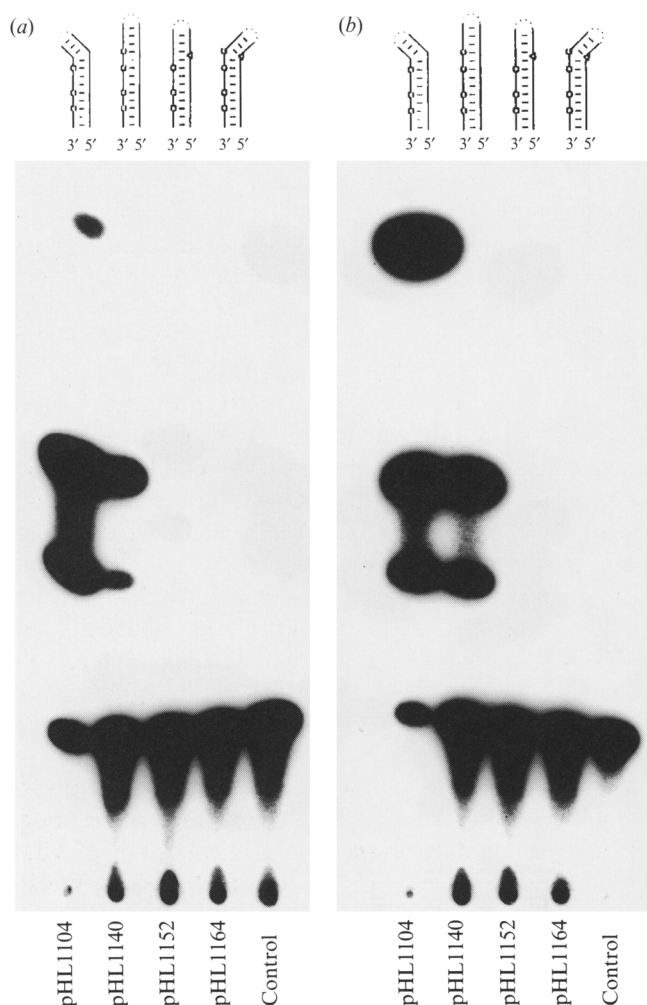


Fig. 3. Nucleotide insertions and deletions related to the bulge at position 10 in the vRNA panhandle. The various constructs are again based on the 3'-terminal sequence of mutant pHL1104 vRNA transcripts. (a) CAT expression in vDNA-transfected and influenza virus-infected cells. (b) CAT activity after passaging of virus progeny. For experimental details see Fig. 1. Control experiments used mock-transfected and mock-infected cells.

corresponding triple exchange clone pHL1125 did not show any promoter activity. Because of its deviation at position 5 it may not resemble the double-stranded structure present in wild-type cRNA as closely.

Mutational analysis of the bulged double-stranded vRNA structure

An extra unpaired residue within the 5'-terminal sequence is a specific feature of the proposed panhandle structure of influenza virus vRNA. It is expected to cause a major bulge in the structure together with adjacent non-complementary residues, and may be part of a specific recognition element for the viral RNA polymerase. In several earlier structural interpretations the A

at position 10 was proposed to be unpaired, but in an *in vitro* analysis of protein-free model vRNAs the A at position 4 was shown to be exposed (Baudin *et al.*, 1994). However, on RNA polymerase binding the bulge may shift from position 4 to position 10 (see Discussion). In order to investigate the importance of this particular structural feature, a further series of plasmids was constructed, again based on pHL1104 (Fig. 3). A perfectly matched RNA double strand without any bulge was achieved either by inserting an additional U residue in the 3' end sequence opposite the A at position 10 (pHL1140) or by deleting the A residue from the 5' sequence (pHL1152). Finally, a bulge of the opposite direction was created in the panhandle structure of pHL1164 by inserting an extra U residue after position 9 of the 3' end and deleting position 10 from the 5' end sequence. While the latter two constructs proved inactive in the CAT assay, pHL1140 did show some promoter activity, albeit at a reduced level. We conclude from this result that a bulge in this region may not be recognized directly by the viral RNA polymerase, but may serve as a flexible joint between two more rigid structural elements that are involved in immediate contact with the polymerase. Bending of RNA between two major binding regions may be achieved via a bulge caused by an unpaired residue, as in the wild-type vRNA, or similarly but less efficiently by an AA:UU base-paired structure, such as in pHL1140; the other two structures mentioned would not permit the correct interaction with RNA polymerase (see Discussion).

Serial passaging of influenza virus carrying promoter mutations

All experiments described above consisted of an initial measurement of viral mRNA synthesis and CAT expression in DNA-transfected, virus-infected B82 cells, followed by a second measurement of reporter gene expression in infected MDCK cells, after passaging of progeny virus-containing supernatants. CAT expression following virus passage requires packaging of pseudo-viral vRNAs and new rounds of viral mRNA synthesis in infected cells. All virus promoter mutants analysed and found to be active in transfected and helper-infected B82 cells also resulted in CAT expression after transfer, and consistently resulted in equivalent patterns of activity. Therefore packaging cannot yet be correlated with any specific element in the vRNA promoter structure and does not appear to be a limiting factor in constructing influenza virus mutants in this system. While CAT expression following virus passage generally appeared to be increased over the levels before passage this might simply be due to different cells being used in the two

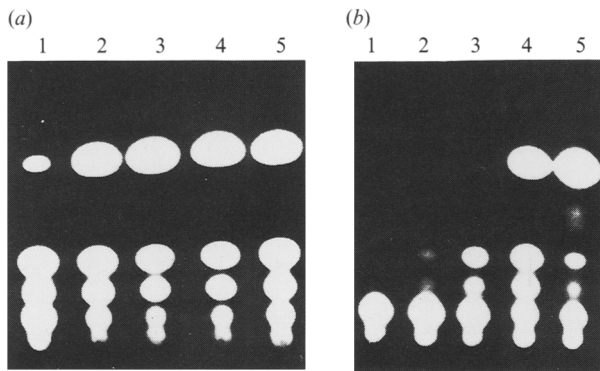


Fig. 4. Serial passaging and CAT expression. B82 cells (10^7) were transfected with 5 μ g of pHL1104 DNA (in 60 μ g Lipofectamin) and infected 2 h later with influenza virus A/FPV/Bratislava (m.o.i. of 1). At 8 h post-infection the cells were assayed for CAT activity (lane 1). After sedimentation an aliquot of the supernatant was adsorbed to 10^7 MDCK cells. Further rounds of passaging were done similarly by harvesting the cells 8 h after infection for assaying CAT activities and using one aliquot of the supernatant to infect fresh MDCK cells. (a) CAT activities as observed in cell extracts, undiluted. (b) CAT activities as observed in cell extracts, diluted 1:100. Lanes 1, CAT activities measured in vDNA-transfected and virus-infected cells; lanes 2, CAT activities measured after the first round of passaging; lanes 3, passage 2; lanes 4, passage 3; lanes 5, passage 4.

steps, with MDCK cells being superior to B82 cells in influenza virus mRNA synthesis and in progeny yields. Therefore, several experiments of serial passage in MDCK cells were performed using pHL1104- and pHL926-derived influenza virus supernatants. In these serial passages, always done using aliquots of supernatants harvested 8 h after infection, a stepwise increase of CAT expression was observed for mutant derivatives, in accordance with their initially increased expression rates (Fig. 4), but not for wild-type constructs. The superior performance of viral RNA promoters carrying sequence deviations, such as pHL1104 or pHL1102, is not only true for viral mRNA synthesis, but also for viral RNA replication, and in similar ratios. Therefore, these mutant viral RNAs accumulate and are effectively selected in further passaging, whereas packaging appears to be a neutral event, at least for the variants analysed here.

Discussion

In influenza virus vRNA synthesis the parental negative-strand vRNA is copied into plus strand cRNA, which is then copied into progeny vRNA, from the first to the last nucleotide. However, this amplification of viral RNAs proceeds in an inherently asymmetrical way, since vRNA molecules are synthesized in excess over cRNA molecules. This is consistent with the idea that cRNA carries a promoter structure that is more active than

vRNA in binding viral RNA polymerase and in initiating RNA synthesis. Initially the 3' ends of single-stranded vRNA and cRNA templates had been implicated as viral promoter sequences. But the detection of double-stranded panhandle structures involving both ends of the vRNA sequence in virions (Hsu *et al.*, 1987) suggested more complicated substrates for RNA polymerase binding and initiation of daughter-strand synthesis. A slightly different, but nevertheless definite RNA panhandle structure has also been observed with model vRNA molecules in the absence of viral proteins *in vitro* (Baudin *et al.*, 1994). Originally, several RNA polymerase-vRNA-binding experiments *in vitro* appeared to show recognition of 3'-terminal oligonucleotides only. But, by using recombinant viral polymerase free of residual RNA oligonucleotides instead of enzyme preparations from virions, this has since been shown to be an artefact. Under these conditions RNA polymerase binding to viral RNA, as well as endonucleolytic cleavage of cellular mRNAs by subunit PB2, was observed to depend on vRNA 5'- plus 3'-terminal sequences, with even higher affinity for the 5' non-template segment (Hagen *et al.*, 1994; Tiley *et al.*, 1994).

In contrast to the employment of both vRNA and cRNA promoter structures in RNA replication, only vRNA promoters will also serve in initiation of viral mRNA synthesis, by utilizing the cap-snatching mechanism (Plotch *et al.*, 1979; Braam *et al.*, 1983). While it has been claimed that cRNA promoters do not have the capacity to act according to this scheme (Tiley *et al.*, 1994), the failure to observe viral antisense mRNA molecules may simply reflect the lack of cRNA molecules early in infection in the absence of surplus viral NP protein; small amounts of such antisense transcription products might also have gone undetected. In this report we describe a mutational analysis of the vRNA promoter structure *in vivo* which, in approaching the structure of the cRNA promoter via three nucleotide exchanges (and improving the RNA double-stranded format), shows considerably enhanced activity in viral mRNA synthesis over the wild-type vRNA promoter. The continuing increase of viral CAT mRNA expression during consecutive steps of viral passage proves that the same vRNA promoter mutants show higher activity in both cRNA and mRNA synthesis, and suggests that the cRNA promoter structure might be stronger than the vRNA promoter in either capacity.

Additional variation of the 5'-terminal sequence indicated the importance of a G residue in position 5, with some further improvement seen using a complementary C residue in position 5 at the 3' end. The important role of this G residue has been observed before in a serial mutation analysis (Li & Palese, 1992).

Based on these data, 5' G residue 5 in the single-stranded configuration may be involved in binding of RNA polymerase to 5' single-stranded end, as has been observed for the non-template strand terminal segment (nucleotides involved unspecified; Tiley *et al.*, 1994). While panhandle-type double-stranded structures are likely to constitute the initial RNA polymerase-binding substrate, a partial separation of template and non-template strands is expected to take place consecutively, resulting in a forked structure such as proposed by Fodor *et al.* (1994). Specific binding of RNA polymerase to single-stranded regions in this structure may be predominantly oriented towards sequence elements in the non-template strand, since following its initiation at the 3' end, the growing point of RNA synthesis will have to move along the entire template strand. It is therefore possible that such a binding interaction survives most or all of the individual rounds of mRNA synthesis, as has been proposed (Tiley *et al.*, 1994).

The triple nucleotide exchanges introduced into vRNA molecules derived from pHL1104 templates create three additional base pairs which would be able to stabilize the panhandle structure. More specifically they favour a bulged A at position 10 over the bulged A at position 4 conformation which was observed for the wild-type RNA sequence *in vitro* (Baudin *et al.*, 1994). Since the changes introduced lead to a considerable enhancement of gene expression, we propose that a 'bulged 10' conformation may result from viral polymerase binding to a 'bulged 4' conformation of wild-type vRNA; binding would be more readily achieved with a preformed mutant vRNA structure such as pHL1104 (Fig. 5). The interpretation given is also consistent with data from other promoter variants analysed here, which are intermediate in expression rates and in potential stabilization of a bulged 10 conformation.

The bulged A residue 10 (which may be exchanged for other nucleotides; R. Flick & G. Hobom, unpublished results) may constitute a kind of flexible joint or angular kink which in turn suggests two major, structurally stable binding sites to the left and right of this element. One of these sites has to be the double-stranded sequence element of, on average, 6 base pairs extending from positions 11 to 16 and $\bar{1}0$ to $\bar{1}5$, respectively. While the distal 3 base pairs for the various RNA segments deviate in sequence, but not in base-pairing capacity, constant base pairs 12- $\bar{1}1$ and 13- $\bar{1}2$ have been shown experimentally to be variable in double exchanges (R. Flick & G. Hobom, unpublished results). At least 4 consecutive base pairs are required to retain some degree of promoter function (Luo *et al.*, 1991). With all of these data it seems clear that the main recognition element in this region is a stable RNA double strand. While it is possible that one or more residues are also recognized

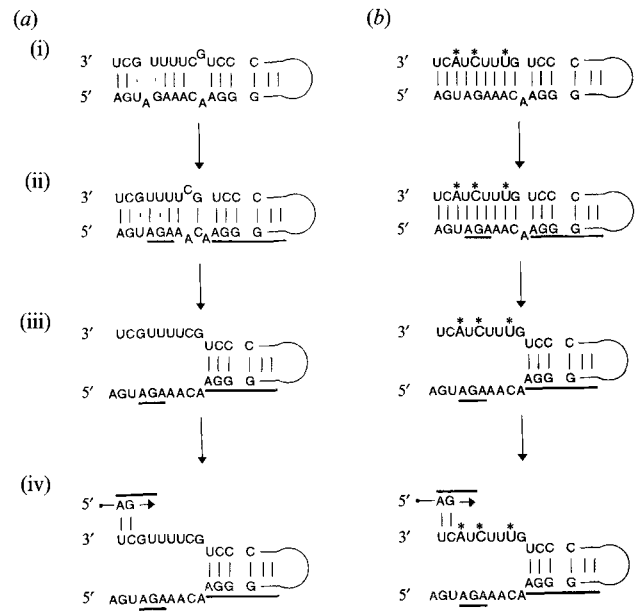


Fig. 5. Proposed scheme of consecutive conformational steps occurring prior to initiation of viral mRNA synthesis in wild-type influenza virus vRNA (a) and pHL1104-derived mutant sequences (b). Positions of the three mutations in pHL1104 vRNA are indicated by an asterisk. The steps are: (i) free RNA panhandle structure, bulged at position 4 (wild-type vRNA; Baudin *et al.*, 1994) or at position 10 (mutant vRNA); (ii) position 10 bulged panhandle structures after binding of viral RNA polymerase (the proposed protein binding positions are underlined); (iii) forked structures of partial strand separation; (iv) initiation of viral mRNA synthesis via hybridization of capped primer oligonucleotide.

individually within the structure, such sequence specificities remain undetermined. A major binding element for RNA polymerase on the other side of position 10 is less evident, but is most likely located in the 'debulged' region around positions 4 and 5. Direct contacts are suggested here because of the specific requirement for a G residue in position 5, and because of an initial (hypothetical) debulging interaction involving position 4 (as proposed above). This region of the forked single strand may be expected to interact with the viral polymerase at a distance of nearly one helical turn below the double-stranded polymerase-binding element. While an extra A residue at position 10 may be optimum for creating a correctly shaped bulge or kink between both binding sites, other structures are possible (e.g. pHL1140, Table 1; R. Flick & G. Hobom, unpublished results), which excludes direct interactions between RNA polymerase and residues constituting the bulge.

In summary we propose the following model, shown in Fig. 5, consisting of a series of consecutive steps of interaction between a vRNA promoter structure (wild-type as opposed to mutant pHL1104) and viral RNA polymerase: a free RNA panhandle structure, bulged at position 4, becomes bulged at position 10 after binding of viral RNA polymerase to the double-stranded element

at positions 11–16/10–15 and the G residue at position 5. Partial strand separation then occurs to result in a forked structure, which will allow viral mRNA synthesis to be initiated from the free 3' end of template vRNA.

We thank C. Beckort, N. Pohl and U. Ruppert for expert technical assistance. Influenza virus strain A/FPV/Bratislava (mouse-adapted) was kindly provided by P. Staeheli. Helpful suggestions of R. Rott and C. Scholtissek are gratefully recognized. This work was supported by the Deutsche Forschungsgemeinschaft.

References

- BAUDIN, F., BACH, C., CUSACK, S. & RUIGROK, R. W. H. (1994). Structure of influenza virus RNP. I. Influenza virus nucleoprotein melts secondary structure in panhandle RNA and exposes the bases to the solvent. *EMBO Journal* **13**, 3158–3165.
- BRAAM, J., UHMANEN, I. & KRUG, R. (1983). Molecular model of a eukaryotic transcription complex: functions and movements of influenza P proteins during capped RNA-primed transcription. *Cell* **34**, 609–618.
- COMPANS, R. W., CONTENT, J. & DUESBERG, P. H. (1972). Structure of the ribonucleoprotein of influenza virus. *Journal of Virology* **10**, 795–800.
- FODOR, E., SEONG, B. L. & BROWNLEE, G. G. (1993). Photochemical cross-linking of influenza A polymerase to its virion RNA promoter defines a polymerase binding site at residues 9 to 12 of the promoter. *Journal of General Virology* **74**, 1327–1333.
- FODOR, E., PRITLOVE, D. C. & BROWNLEE, G. G. (1994). The influenza virus panhandle is involved in the initiation of transcription. *Journal of Virology* **68**, 4092–4096.
- GORMAN, M., MOFFAT, L. & HOWARD, B. (1982). Recombinant genomes which express chloramphenicol acetyl-transferase in mammalian cells. *Molecular and Cellular Biology* **2**, 1044–1057.
- HAGEN, M., CHUNG, T. D. Y., BUTCHER, J. A. & KRYSTAL, M. (1994). Recombinant influenza virus polymerase: requirement of both 5' and 3' viral ends for endonuclease activity. *Journal of Virology* **68**, 1509–1515.
- HONDA, A., UEDA, K., NAGATA, K. & ISHIHAMA, A. (1988). RNA polymerase of influenza virus. Role of NP on RNA chain elongation. *Journal of Biochemistry* **104**, 1021–1026.
- HSU, M., PARVIN, J. D., GUPTA, S., KRYSTAL, M. & PALESE, P. (1987). Genomic RNAs of influenza viruses are held in a circular conformation in virions and in infected cells by a terminal panhandle. *Proceedings of the National Academy of Sciences, USA* **84**, 8140–8144.
- LI, X. & PALESE, P. (1992). Mutational analysis of the promoter required for influenza virus virion RNA synthesis. *Journal of Virology* **66**, 4331–4338.
- LUO, G., LUYTJES, W., ENAMI, M. & PALESE, P. (1991). The polyadenylation signal of influenza virus RNA involves a stretch of uridines followed by the RNA duplex of the panhandle structure. *Journal of Virology* **65**, 2861–2867.
- MARTÍN, J., ALBO, C., ORTÍN, J., MELERO, J. A. & PORTELA, A. (1992). *In vitro* reconstitution of active influenza virus ribonucleoprotein complexes using viral proteins purified from infected cells. *Journal of General Virology* **73**, 1855–1859.
- NEUMANN, G., ZOBEL, A. & HOBOM, G. (1994). RNA polymerase I-mediated expression of influenza viral RNA molecules. *Virology* **202**, 477–479.
- PLOTCH, S., BOULOY, M. & KRUG, R. M. (1979). Transfer of 5'-terminal cap of globin mRNA to influenza viral complementary RNA during transcription *in vitro*. *Proceedings of the National Academy of Sciences, USA* **76**, 1618–1622.
- SEONG, B. L. & BROWNLEE, G. G. (1992). Nucleotides 9 to 11 of the influenza A virion RNA promoter are crucial for activity *in vitro*. *Journal of General Virology* **73**, 3115–3124.
- SHAPIRO, G. & KRUG, R. (1988). Influenza virus RNA replication *in vitro*: synthesis of viral template RNAs and virion RNAs in the absence of an added primer. *Journal of Virology* **62**, 2285–2290.
- TILEY, L. S., HAGEN, M., MATTHEWS, J. T. & KRYSTAL, M. (1994). Sequence-specific binding of influenza virus RNA polymerase to sequences located to the 5' ends of the viral RNAs. *Journal of Virology* **68**, 5108–5116.
- ZOBEL, A., NEUMANN, G. & HOBOM, G. (1993). RNA polymerase I catalysed transcription of insert viral cDNA. *Nucleic Acids Research* **21**, 3607–3614.

(Received 7 November 1994; Accepted 24 February 1995)