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

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A previous study of the 12 nucleotide-long influenza A virion RNA promoter has shown that three nucleotides, residues 9 to 11, were crucial for transcription in vitro, although other nucleotides play a significant but less important role. A model for polymerase-promoter recognition was proposed, according to which there were two sites: a binding site at residues 9 to 11 and a regulatory site at or near the site of initiation at residue 1. By studying the effect of point mutations in the promoter on the binding efficiency of the polymerase using a photochemical cross-linking assay, we now show that residues 9 to 12 are crucial for binding. In addition residues 4 to 8, though not as important, are involved in binding, possibly by stabilizing the polymerase-promoter complex. Both PB1 and PB2 apparently play an important role during virion RNA promoter recognition and binding.

Introduction

During the influenza virus life cycle three types of RNA transcripts are synthesized. These are mRNA, complementary RNA (cRNA) and virion RNA (vRNA). Influenza virus mRNAs are synthesized using short, capped RNA fragments as a primer originating from host cell mRNAs. They are polyadenylated and are incomplete copies of vRNA because they lack sequences complementary to 17 to 22 nucleotides at the 5' end of vRNA. By contrast, cRNAs are full-length, non-capped, non-polyadenylated copies of vRNA, and serve as templates for the production of more vRNA (Ishihama & Nagata, 1988; Lamb & Choppin, 1983; McCauley & Mahy, 1983). The molecular mechanism and regulation of transcription and replication are poorly understood.

All three reactions, i.e. transcription of negative sense vRNA into mRNA, replication of vRNA into cRNA and replication of cRNA into vRNA, are catalysed by the influenza virus RNA polymerase complex consisting of three virus-encoded proteins, PB1, PB2 and PA. PB1 is involved in the initiation and elongation of mRNA chains (Braam et al., 1983; Ulmanen et al., 1981). Consistent with this, its amino acid sequence contains motifs found in a series of positive- and negative-stranded RNA virus polymerase genes (Poch et al., 1989). PB2 recognizes and binds to the cap structure of mRNAs (Ulmanen et al., 1981) and is probably responsible for the endonucleolytic cleavage of the capped host cell mRNAs (Galarza et al., 1991). No specific function has been assigned to PA, although it may be required for replication since temperature-sensitive mutations of PA affect replication, but not transcription (Mahy et al., 1981; Krug et al., 1975). PA is, however, essential for polymerase activity as it is required for successful assembly of an active polymerase complex along with PB1 and PB2 (Kobayashi et al., 1992).

It is well known that there is a 12 nucleotide (nt)-long highly conserved sequence 5' CCUGCUUUUGCU_{on} 3' at the 3' end of all vRNA segments of influenza A viruses (Robertson, 1979; Desselberger et al., 1980). Recently, we have shown that these 12 conserved nucleotides are sufficient to function as a promoter for mRNA and cRNA synthesis in vitro (Seong & Brownlee, 1992a). Furthermore, by studying the efficiency of RNA synthesis in vitro using model RNA templates with individual point mutations in all 12 positions of the vRNA promoter, we have shown that three nucleotides, residues 9, 10 and 11, within the promoter are crucial for activity, although other nucleotides also play a significant but less important role. We proposed that the promoter has two sites: (i) a polymerase binding site at residues 9 to 11, and possibly 12, and (ii) a regulatory site at residues 1, and possibly 2, 3 and 4 (Seong & Brownlee, 1992b). To test
this model we now investigate the binding of the polymerase complex to the vRNA promoter using a photochemical cross-linking assay. By studying the effects of mutations at all 12 positions in the vRNA promoter, we provide evidence that residues 9 to 12 are crucial for the effective binding of the polymerase complex to vRNA promoter and that they represent a binding site. In addition to its known cap-binding and endonuclease functions (Ulmanen et al., 1981; Galarza et al., 1991), PB2 cooperates with PB1 in promoter recognition and binding.

**Methods**

Preparation of micrococcal nuclease-treated viral core. The micrococcal nuclease-treated viral core was prepared as described previously (Seong & Brownlee, 1992a). Briefly, the viral core isolated from X-31 (a recombinant of influenza viruses A/HK/68 × A/PR/8/34) by Triton X-100 and lysosomatin disruption and glycerol gradient centrifugation was treated with micrococcal nuclease (Boehringer) followed by EGTA inactivation.

Preparation of RNA templates. Model RNA templates, wild-type (14 nt long) or with point mutations (14 nt long) or with an insertion of a 3′ residue between positions 4 and 5 (15 nt long) in the 12-nt-long vRNA promoter, were synthesized by T7 RNA polymerase transcription of a partial DNA duplex as before (Seong & Brownlee, 1992b) in the presence of [γ-32P]ATP (800 Ci/mmol). The sequence of the wild-type model RNA template is 5′ GGCUCUGCUUUUGCUoM 3′ (the 12-nt-long vRNA promoter is underlined). Where mutant sequences were used, this is indicated in the Results. Full-length products of T7 polymerase transcription were purified by 18% PAGE in 7 M-urea followed by elution from the gel with QO buffer (100 mM-NaCl, 50 mM-MOPS pH 7.0, 15% ethanol) overnight at 4°C and purification on a Qiagen column. The concentration of template RNA was estimated from the radioactive yield (measured by scintillation counting) assuming that it had the same specific activity as the starting labelled triphosphate (800 Ci/mmol). Wild-type 12 nt-long vRNA promoter 5′ CCUCUGCUUUUGCUoM 3′ and U15 sequence were chemically synthesized using an Applied Biosystems DNA synthesizer (Model 380B) and after deblocking (B. Sprott, personal communication) were purified by Sephadex G-25 (NAP-10, Pharmacia) column chromatography and 20% PAGE in 7 M-urea, followed by elution with H2O and purification on Qiagen column. Yields were measured spectrophotometrically at A260. The synthetic oligonucleotides were 5′ end-labelled (for cross-linking studies) with [γ-32P]ATP and T4 polynucleotide kinase followed by phenol-chloroform extraction and purification on a Qiagen column.

Reconstitution and photochemical cross-linking. About 4 μl of micrococcal nuclease-treated viral core protein was mixed with 0.01 to 0.05 pmol of 32P-labelled RNA template (see above) and 1 unit of human placental ribonuclease inhibitor (Amersham) in transcription buffer (50 mm-Tris–HCl pH 7.8, 50 mM-KCl, 10 mM-NaCl, 5 mM-MgCl2, 1 mM-DTT) and the volume was adjusted to 10 μl with H2O. After 1 h incubation at 30°C, the mixture (in a 0.5 ml open Eppendorf tube) was carefully inverted onto the surface of a transilluminator (Model TM-36, Ultraviolet Products) and u.v.-irradiated (302 nm) for 10 min at room temperature. The cross-linked products were analysed immediately, or after immunoprecipitation (see below), by 8% PAGE. Urea (4 M) was included in polyacrylamide gels to improve the resolution of PA and PB2 subunits of the polymerase complex (Lamba & Choplin, 1976). Gels were dried and the cross-linked products were revealed by autoradiography.

**Immunoprecipitation of polymerase proteins.** Rabbit polyclonal antibodies specific for influenza virus polymerase proteins PB1, PB2 and PA and prepared against bacterially expressed fusion proteins were used. The antisera directed against PB1 and PB2 were provided by S. C. Ingalls (Digard et al., 1989) and the anti-PA antiserum by I. M. Jones (Jones et al., 1986). For immunoprecipitation of the polymerase proteins with the specific antisera, the polymerase complex (after cross-linking to RNA) was disrupted with 1% SDS at 95°C for 2 min, followed by dilution in immunoprecipitation buffer A (50 mM-Tris–HCl pH 7.4, 100 mM-NaCl, 2 mM-EDTA, 1% NP40, 0.5% sodium deoxycholate, 0.2 mM-PMPSF, 1 μM-leupeptin (Boehringer), 1 μM- pepstatin (Boehringer)) to 0.1% SDS and 5 μl of specific antiserum was added. After incubation on ice for 1 to 2 h, 100 μl of 10% Protein A-Sepharose (Pharmacia) in immunoprecipitation buffer A was added to the antibody-cross-linked protein mixture, and the tubes were rotated at 4°C for 1 h. The Sepharose-bound material was centrifuged and washed twice in 1 ml of immunoprecipitation buffer B (50 mM-Tris–HCl pH 7.4, 100 mM-NaCl, 5 mM-MgCl2, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS). The immunoprecipitates were boiled in 15 μl of SDS–PAGE sample buffer for 5 min. Proteins in the supernatant were separated by 8% PAGE in 4 M-urea and, after drying, detected by autoradiography.

**Results**

All three subunits of the polymerase complex are covalently cross-linked to the vRNA promoter by u.v.-irradiation

Photochemical cross-linking is a powerful method for studying protein–nucleic acid interactions (Schimmel & Budzik, 1977). We decided to use this method as an assay for the specific binding of the influenza A virus RNA polymerase complex to its vRNA promoter. Before testing the effect of point mutations in the promoter on polymerase binding efficiency, it was first necessary to establish conditions for polymerase binding to the wild-type promoter sequence. We used short 32P-labelled model RNA templates and micrococcal nuclease-treated viral cores (see Methods) as previously used in our in vitro study of transcription and replication (Seong & Brownlee, 1992a).

Fig. 1 (lane 1) shows the proteins cross-linked to the 14 nt-long 32P-labelled vRNA promoter-containing sequence. At the expected positions of the PB1, PB2 and PA polymerase proteins (between 82K and 86K) two major labelled bands (marked A and C) and two minor bands (A’ and B) were observed. The bands were absent in a control experiment in which, instead of the 14 nt-long vRNA promoter-containing sequence, a U15 sequence was used in reconstitution and cross-linking (Fig. 1, lane 2). This strongly suggested that the four labelled bands (A, A’, B and C) were specific for the vRNA promoter. The other major labelled product observed in both lanes was the nucleoprotein (NP) which is the most abundant protein of the viral core and binds non-
specifically to RNA very efficiently, as expected (Kingsbury et al., 1987). Compared to the virus-specific non-cross-linked protein controls (e.g. PB1, NP) separated on the same gel and stained with Coomassie blue, minor shifts of the protein–RNA complexes to higher $M_r$ were observed. Treatment of the $^{32}$P-labelled cross-linked products with RNase A resulted in a significant loss (> 80%) of the $^{32}$P label from the RNA–protein complexes and a slight but definite shift in mobility consistent with the loss of nucleotides (data not shown).

Competition experiments with the homologous 12 nt-long vRNA promoter and the heterologous $U_{15}$ sequence and phenylalanine tRNA were carried out to provide further evidence for the specificity of binding of the presumptive polymerase complex proteins to the promoter sequence. The proteins of the micrococcal nuclease-treated viral core were cross-linked to the 5' $^{32}$P-labelled 12 nt-long synthetic vRNA promoter sequence in the absence or presence of increasing amounts of unlabelled competitors (Fig. 2). Homologous competitor (100 pmol; 12 nt-long synthetic vRNA promoter sequence) out-competed the labelled template completely (lane 6), while in the presence of 100 pmol of $U_{15}$ (lane 12) or 200 pmol of phenylalanine tRNA (lane 14) some cross-linking between labelled RNA and the presumptive polymerase proteins was still observed. We conclude that the polymerase proteins preferentially cross-link to the promoter RNA.

To identify formally the presumptive P protein cross-linked bands, immunoprecipitation with anti-PB1, anti-PB2 and anti-PA antisera was carried out (see Methods). Before immunoprecipitation the cross-linked products were treated with 1% SDS to disrupt the polymerase complex. Under these conditions, anti-PB1, anti-PB2 and anti-PA antisera precipitated the PB1–RNA, PB2–
RNA and PA–RNA complexes specifically (Fig. 3, lanes 1, 2, 3) with no apparent cross-reaction with the other P proteins. In the PB1 immunoprecipitate a doublet was observed (Fig. 3, lane 1, PB1, PB1'), which was also present in the original cross-linked products (Fig. 1, A, A'). PB1' presumably represents a proteolytic cleavage product of PB1 or the additional form of PB1 observed by Akkina et al. (1991) in infected cells.

To characterize the interaction between the polymerase subunits and the vRNA promoter further, increasing concentrations of NaCl up to 2 M were used during reconstitution and cross-linking (Fig. 4a). Above 0·6 M-NaCl (lane 5) there was nearly complete suppression of binding of all proteins except PB1 and PB2. The identity of PB1 and PB2 in the cross-linked products at high salt concentration (0·4 M-NaCl) was confirmed by immunoprecipitation with specific anti-PB1 and anti-PB2 antisera (Fig. 4b, lanes 2, 3). No labelled protein was immunoprecipitated by anti-PA antiserum at 0·4 M-NaCl (Fig. 4b, lane 4). The ability of PB1 and PB2 to bind is not significantly affected in the range of 0·04 to 1·5 M-NaCl (lanes 1 to 7), whereas there is no detectable binding of PA above 0·2 M-NaCl (lane 3). Similar results were observed when KCl was used instead of NaCl (data not shown). These results suggest that the binding of PB1 and PB2 subunits to the promoter is sequence-specific, and the binding of PA may be sequence-independent (but see Discussion). During complex formation between the polymerase complex and the vRNA promoter at low ionic strength, however, all three subunits are in close contact with RNA allowing photochemical cross-linking.

Cross-linking of the polymerase complex proteins to the vRNA promoter carrying mutations

To test the hypothesis that residues 9 to 11 and possibly 12 of the vRNA promoter represent the binding site of the promoter (Seong & Brownlee, 1992b) we used the cross-linking assay described above. Mutant RNAs carrying individual transversions at each of the 12 positions of the promoter, or an insertion of a C residue between positions 4 and 5 (IC4,5), were synthesized by T7 RNA polymerase transcription of the corresponding DNA duplex in the presence of [α-32P]CTP (see Methods). The RNA templates were reconstituted with micrococcal nuclease-treated influenza viral cores and u.v.-irradiated at low ionic strength (50 mM-KCl, 10 mM-NaCl) before reconstitution and cross-linking.
PB2 (10 to 30 %) as compared to the wild-type. The most
obvious effect was that mutations at positions 9 to 11
(lanes 11 to 13) completely suppressed the binding of
PB1 (< 5 % of the wild-type as estimated by laser
densitometry); however, very low levels of PB2 were
detected (< 10 %). The mutation at position 12 (lane 14)
resulted in suppression of PB1 binding nearly as great
as that arising from mutations at positions 9 to 11,
although some residual PB1 binding remained (6 %).
The binding of PB2 with the position 12 mutant was
comparable to that achieved with mutants at positions
9 to 11 (i.e. < 10 %). In addition, we tested all three
possible mutations at position 3 (G3 → A3, G3 → C3,
G3 → U3) because of the strong upregulatory effect of
mutant G3 → C3 in in vitro transcription in the absence
of primer (Seong & Brownlee, 1992b). In our binding
assay all three mutants bind both PB1 and PB2 to the
same extent as wild-type (data not shown).

Taken together, these observations showed that (i)
mutations at only four positions (9 to 12) resulted in
severe inhibition of PB1 and PB2 binding (< 10 % of the
wild-type), (ii) mutations at positions 7 and 8 produced
decreased binding of both PB1 and PB2 (10 to 30 % of
the wild-type) and mutations at positions 4 and 5 affected
only PB1 (35 % of the wild-type), and (iii) mutations at
the remaining positions (positions 1, 2, 3 and 6) or an
insertion of a C residue between positions 4 and 5 caused
no detectable inhibition of the binding of PB1 and PB2.

Discussion

Recently we proposed a model for the recognition of the
vRNA promoter of influenza A virus (Seong & Brownlee,
1992b). According to this model the vRNA promoter is
bipartite consisting of a binding site and a regulatory site
separated by a U-rich spacer sequence. In this paper, by
studying the effects of mutations in the vRNA promoter
on the binding efficiency of the polymerase complex, we
provide evidence that there is a binding site at the
promoter region as proposed previously. The effect of
mutations on binding efficiency is summarized in Table
1.

Only mutations at positions 9 to 12 (group 1) inhibited
the binding of PB1 and PB2 dramatically which
confirms their importance for polymerase binding. In
our previous study, when we investigated the effects of
point mutations within the promoter on the efficiency of
in vitro transcription, a similar result was observed.
Mutations at positions 9 to 11 caused a significant
(> 90 %) inhibition in all three transcription reactions
(primer-independent, globin mRNA-primed and ApG-
primed). The mutation at position 12 had an intermediate
effect on the transcription efficiency (Seong & Brownlee,
1992b), whereas it showed a strong inhibition of binding
in the binding assay performed here (Table 1), suggesting

NaCl, equivalent to conditions somewhere between those
for lanes 1 and 2, Fig. 4a), under conditions where all
three polymerase subunits were cross-linked (see
Methods and above). Because the separation of PA from
PB2 was poor and it was not possible to estimate the
binding of PA by laser densitometry scanning, we
assessed the polymerase binding by measuring the levels
of PB1 and PB2 bands in the cross-linking assay.

Fig. 5 shows that the point mutations at positions 1 to
6 (lanes 2, 3, 4, 5, 7, 8) or an insertion of a C residue
between positions 4 and 5 (lane 6) did not affect the
polymerase binding dramatically compared with the
wild-type promoter (lane 1), as determined by the levels
of PB1 and PB2 bands. Nevertheless, some changes
occurred. Although PB2 remained apparently unaffected
by these mutations, mutations U4 → A4 (lane 5) and
U5 → A5 (lane 7) resulted in a decreased binding of
PB1 (about 35 % of the wild-type as estimated by laser
densitometry scanning). Mutations at positions 7 and 8
(lanes 9 and 10) caused low levels of binding of PB1 and
PB2 (10 to 30 %) as compared to the wild-type. The most

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Fig. 5. Effect of point mutations or insertions on polymerase-RNA
complex formation. Lane 1 (wild-type), lane 2 (U1 → A1), lane 3
(C2 → A2), lane 4 (G3 → C3), lane 5 (U4 → A4), lane 6 (IC4,5), lane 7
(U5 → A5), lane 8 (U6 → A6), lane 9 (U7 → A7), lane 10 (C8 → A8),
lane 11 (G9 → C9), lane 12 (U10 → A10), lane 13 (C11 → A11) and lane
14 (C12 → A12). Mutant G3 → C3 (lane 4) shows decreased intensity of
cross-linked proteins due to underloading and not to the inhibitory
effect of this mutation. In repeated experiments (not shown) this mutant
showed a pattern comparable to the wild-type promoter. The positions
of the cross-linked PB1, PB2, PA and NP bands are indicated. NP
was expected to cross-link the wild-type and mutant RNA species (14
or 15 nt long) equally well as NP is known to bind to RNA non-
specifically (Kingsbury et al., 1987) and 14 nt-long oligoribonucleotides
are known to bind purified recombinant NP in a gel shift assay (P. G.
Murray, V. Knott & G. G. Brownlee, unpublished). Bands with
mobility faster than NP may represent proteolytic fragments of NP.
The identity of the other products is unknown.
Table 1. Summary of effects of promoter mutations on polymerase binding

<table>
<thead>
<tr>
<th>Group</th>
<th>Nucleotide position</th>
<th>Effect</th>
<th>Binding activity (%)*</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>9, 10, 11, 12</td>
<td>Strong inhibition</td>
<td>&lt; 10</td>
<td>PB1 and PB2</td>
</tr>
<tr>
<td>2</td>
<td>4, 5, 7, 8</td>
<td>Intermediate inhibition</td>
<td>10 to 60</td>
<td>U4→A4 and U5→A5 only PB1, U7→A7 and C8→A8 PB1 and PB2</td>
</tr>
<tr>
<td>3</td>
<td>1, 2, 3, 6</td>
<td>Weak to insignificant inhibition</td>
<td>60 to 100</td>
<td>PB1 and PB2</td>
</tr>
</tbody>
</table>

*Wild-type binding was taken as 100%.

that the C residue at position 12 of the promoter is involved in polymerase binding.

In addition, mutations at positions 4, 5, 7 or 8 (Table 1, group 2) resulted in reduced binding of PB1 or both PB1 and PB2. Nevertheless, both polymerase subunits associated with the promoter to some extent. In the transcription assay (Seong & Brownlee, 1992b) the individual mutations at these central residues (positions 4 to 8) showed weak or insignificant effects on transcription, but a triple mutation (U5U6U7→A5A6A7) in this central region caused a significant inhibition of the ApG-primed reaction. Taking these previous results and the results of the cross-linking assay together, we deduce that residues 4 to 8 are probably also important (although not crucial) for efficient binding, possibly because they help to stabilize the structure of the polymerase-promoter complex.

In contrast, mutations at positions 1, 2, 3 or 6 or an insertion of a C residue between positions 4 and 5 in the promoter (group 3) caused no detectable inhibition of PB1 and PB2 binding. In our previous transcription assay (Seong & Brownlee, 1992b) mutations at positions 1 and 2 inhibited transcription at intermediate level, whereas a mutation at position 3 (G3→C3) or an insertion of a C residue between positions 4 and 5 caused strong upregulation of primer-independent reaction. Thus these mutations in the proposed regulatory site apparently do not affect binding of the polymerase.

The efficient cross-linking of PB1 and PB2 to the wild-type vRNA promoter, even at high ionic strength, suggests that both polymerase subunits specifically bind to the promoter. The cross-linking of PA to the wild-type promoter, however, was abolished if the salt concentration was increased above 0.2 M NaCl. This may suggest that the interaction between PA and RNA lacks the sequence specificity characteristic of PB1 and PB2. However, we cannot exclude the alternative, but less likely, explanation that PA binds to the promoter specifically, but with lower affinity only at low salt levels. Nevertheless, it is clear that PA binding properties differ from those of PB1 and PB2. In the light of these results, it seems likely that both PB1 and PB2 play an important role during polymerase recognition and binding, but PA is not involved directly in this process. We cannot exclude, however, that an interaction of PA with PB2 or more likely with PB1 (Digard et al., 1989) is necessary for the proper conformational arrangement of the RNA binding domains in PB1 and PB2. Most mutations (see Table 1) inhibited the binding of PB1 and PB2 equally suggesting that both bind to approximately the same region of the promoter. The binding of PB1 and PB2 to the promoter may be cooperative, positioning the RNA between PB1 and PB2.

A computer search of PB1 and PB2 for an RNA-binding consensus (Bandzilius et al., 1989), present in many RNA-binding proteins, revealed two regions of PB1 of influenza A viruses (residues 249 to 256 RGFVVFYVE and 499 to 503 YGFVA) which closely resembled the K/RGF/YG/AFVXF/Y octapeptide of the RNA-binding consensus. Experiments with mutated PB1 would be required to test whether these candidate regions are involved in RNA binding. No RNA-binding consensus was found in the PB2 of influenza A viruses.

In summary, we show here in cross-linking studies that both PB1 and PB2 components of the polymerase complex are involved in recognition of the vRNA promoter and specifically bind to residues 9 to 12 of the promoter. This confirms our previously hypothesis of a binding site in the vRNA promoter (Seong & Brownlee, 1992b).

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