Journal of General Virology (1996), 77, 2149–2157. Printed in Great Britain

Molecular assembly of the influenza virus RNA polymerase: determination of the subunit–subunit contact sites

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Influenza virus RNA polymerase with the subunit structure PB1–PB2–PA is involved in both transcription and replication of the RNA genome. By transfection of various combinations of cDNA encoding wild-type and serial deletion mutants of each P protein subunit and co-immunoprecipitation with subunit-specific antibodies, the subunit–subunit contact sites on all three of the P proteins were determined. Results indicate that binary complexes are formed between PB1–PB2 and PB1–PA but not between PB2–PA. Therefore, we concluded that PB1 is the core subunit for assembly of the virus RNA polymerase. The C-terminal 158 amino acids of PB1 bound to the N-terminal 249 amino acids of PB2, while the N-terminal 140 amino acids of PB1 bound to the C-terminal two-thirds of PA. PB2–PA binding was not detected when they were expressed in the absence of the PB1 subunit.

Introduction

Influenza virus has a genome of eight negative-sense ssRNA segments and the virion also contains an RNA-dependent RNA polymerase (Lamb, 1989). Influenza virus RNA polymerase catalyses both transcription [the synthesis of plus-strand mRNA containing a host cell-derived cap1 structure at the 5′ terminus and a poly(A) tail at the 3′ terminus] and replication (the synthesis of full-length plus-strand cRNA and the cRNA-dependent synthesis of minus-strand vRNA) (Ishihama & Barbier, 1994; Ishihama & Nagata, 1988; Krug *et al.*, 1989). Besides RNA polymerization, the virus RNA polymerase also performs template-dependent capped RNA cleavage (Kawakami *et al.*, 1983; Plotch *et al.*, 1981) and apparent proof-reading of nascent RNA chains (Ishihama *et al.*, 1986)

The RNA polymerase purified from influenza virus consists of one molecule each of three subunits, PB1, PB2 and PA (Honda *et al.*, 1990). *In vitro* reconstitution studies of enzymatically active RNA polymerase using individual P proteins purified either from baculovirus expressing P protein cDNAs (Kobayashi *et al.*, 1992) or by SDS–PAGE of virions (Szewczyk *et al.*, 1988) confirmed this subunit structure. The function of each subunit has been genetically and biochemically characterized. For instance, PB1 can be cross-linked with nucleotide substrates (Asano *et al.*, 1995; Braam *et al.*, 1983) and insect cell nuclear extracts containing PB1 subunit alone are able to catalyse RNA synthesis using synthetic short RNA templates (Kobayashi *et al.*, 1996). This indicates that PB1 is involved in polymerization of RNA chains. PB2 can be cross-linked *in vitro* with cap1 analogues (Braam *et al.*, 1983; Ulmanen *et al.*, 1981) and RNA synthesized *in vivo* in the absence of PB2 lacks the 5′ cap structure (Nakagawa *et al.*, 1995), together suggesting that PB2 is required for cap-snatching. The role of PA is not clear yet, but temperature-sensitive (ts) mutations in the PA gene affect vRNA synthesis but not mRNA synthesis (Krug *et al.*, 1975; Mahy *et al.*, 1981; Mowshowitz, 1981; Scholtissek & Bowles, 1975; Scholtissek *et al.*, 1976).

Recently, transfection systems using reconstituted ribonucleoprotein complexes have been established (Luytjes *et al.*, 1989; Yamanaka *et al.*, 1991). These allow identification of the minimum and essential requirements for *cis*-acting RNA signals

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(promoters and/or origins recognized by the virus RNA polymerase) and *trans*-acting protein components (RNA polymerase subunits and NP) for the expression and replication of recombinant RNA genomes. For detailed analysis of the *in vivo* function(s) of each P protein in transcription and replication, we transfected P protein cDNAs in various combinations. The results were in agreement with the prediction noted above. For instance, transfection of a recombinant genomic RNA into cells expressing PB1, PA and NP (but not PB2) led to the synthesis of virus mRNA that lacked a cap1 structure (Nakagawa *et al.*, 1995).

These transfection studies were extended to express mutant P proteins in order to map their functional sites. We describe the subunit–subunit contact sites on each P protein as determined by analysis of series of deletion mutants. Results indicate that PB1 is the core subunit of the RNA polymerase.

Methods

■ **Cell culture and transfection.** COS-7 cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Gibco/BRL) containing 5% fetal bovine serum (FBS; JRC Scientific), 100 U/ml penicillin and 100 μg/ml streptomycin. Subconfluent cells in 24-well plates were washed three times with Opti-MEM I (Gibco/BRL) and incubated for 6 h in Opti-MEM I containing 4% Lipofectin (Gibco/BRL) and one or combinations of the P protein expression plasmids. After incubation, media were replaced with DMEM containing 5% FBS and the cells were incubated overnight.

■ **Construction of epitope-tagged plasmid pCMVcHA.** pCMV1 was a gift of M. Stinski (University of Iowa, Ames, Iowa, USA; Andersson *et al.*, 1989). pCMVcHA was constructed by inserting a synthetic oligonucleotide duplex composed of 5′ CACGCGTTATCC-GTATGATGTGCCCGACTATGCGTAAT 3′ and 5′ CTAGATTAC-GCATAGTCGGGCACATCATACGGATAACGCGTGGTACGCTG-AT 3′ into pCMV1 between the *Kpn*I and *Xba*I sites (Fig. 1). pCMVcHA was designed to express fusion proteins with C-terminal haemagglutinin (HA) epitope tags by in-frame insertion at the *Mlu*I site.

■ **Construction of HA-tagged polymerase mutants.** A nested set of cDNAs for PB1, PB2 and PA deletion mutants was constructed by PCR amplification using pAPR206 (accession number J02151), pAPR102 (J02152) and pAPR303 (J02153) as templates and synthetic oligonucleotides as primers (Young *et al.*, 1983). PCR was carried out using 1 μg of template and 1 μM each of the primers for 15 cycles of denaturation at 94 °C for 1 min, annealing at 55 °C for 2 min and extension at 72 °C for 3 min, followed by a final extension at 72 °C for 10 min. The amplified fragments except PB1N200, PB1N300, PB1N399 and PB1N500 were digested with *Kpn*I and *Mlu*I, purified by agarose gel electrophoresis and inserted into pCMVcHA between the *Kpn*I and *Mlu*I sites (Fig. 1). PB1N200, PB1N300, PB1N399 and PB1N500 were digested with *Bam*HI and *Mlu*I, purified by agarose gel electrophoresis and inserted into pCMVcHA between the *Bgl*II and *Mlu*I sites. The sequences of PCR-amplified fragments were checked by dideoxynucleotide sequencing using a BcaBEST sequencing kit (Takara).

■ **Construction of pCMVPB2, pCMVPB1 and pCMVPA.** For the construction of expression plasmids for non-tagged wild-type PB1, PB2 and PA (pCMVPB1, pCMVPB2 and pCMVPA), the cloned PCR fragments were isolated after treatment with *Kpn*I and *Xba*I and inserted into pCMV1 between the *Kpn*I and *Xba*I sites (Fig. 1).

■ **Preparation of anti-PB1, anti-PB2 and anti-PA antibodies.** Full-sized PB1, PB2 and PA proteins were expressed in *Escherichia coli* using the T7–RNA polymerase system (Studier *et al.*, 1990). In brief, a *Sma*I–*Bam*HI adapter (Takara) was inserted into the *Bam*HI site of pET3c to make pET3Sc. cDNA carrying the PB1, PB2 or PA genes of influenza virus A/PR8/34 was cloned from pAPR206, pAPR102 or pAPR303 (Young *et al.*, 1983) into pET3Sc at a *Sma*I site located downstream of the T7 promoter. Using synthetic oligonucleotide primers and PCR, these plasmids were modified so as to express non-fusion proteins (without the phage gene 10 protein). The resulting expression plasmids, pETPB1, pETPB2 and pETPA, were transformed into *E. coli* BL21 (λDE3) containing pLysS plasmid. The expression of P proteins was induced by addition of 1 mM-IPTG (Wako). P proteins were purified by 7% polyacrylamide SDS–PAGE using 491 Prep Cell (Bio-Rad). Antibodies against PB1, PB2 or PA were produced in rabbits.

■ **Co-immunoprecipitation of the expressed polymerase proteins.** In order to determine the contact site with PB2 and PA on the PB1 subunit, 3 μg of either pCMVPB2 or pCMVPA were co-transfected onto COS-7 cells in 24-well plates with 3 μg each of either wild-type (pHAPB1wt) or one of the mutant PB1 expression plasmids (pHAPB1N100, pHAPB1N200, pHAPB1N300, pHAPB1N399, pHAPB1N500, pHAPB1N599, pHAPB1N700, pHAPB1C100, pHAPB1C217, pHAPB1C317, pHAPB1C435, pHAPB1C535 or pHAPB1C617). Likewise, for determination of PB1 and PA contact sites on the PB2 subunit, pCMVPB1 or pCMVPA were co-transfected with wild-type (pHAPB2wt) or one of the mutant PB2 expression plasmids (pHAPB2N104, pHAPB2N205, pHAPB2N304, pHAPB2N401, pHAPB2N505, pHAPB2N602, pHAPB2C100, pHAPB2C200, pHAPB2C300, pHAPB2C400, pHAPB2C500 or pHAPB2C600). For determination of PB1 and PB2 contact sites on PA, pCMVPB2 or pCMVPB1 were co-transfected with wild-type (pHAPAwt) or one of the mutant PA expression plasmids (pHAPAN100, pHAPAN200, pHAPAN300, pHAPAN400, pHAPAN500, pHAPAN600, pHAPAC99, pHAPAC199, pHAPAC299, pHAPAC399, pHAPAC499 or pHAPAC599).

After transfection, the cells were pulse-labelled with 1 μCi/ml of [^{35}S]methionine for 0·5, 1, 2, 3, 4 and 6 h in methionine-free MEM. The pulse-labelled cells were washed three times with PBS(−) and lysed with 0·6 ml of TEN(0·5)T buffer (50 mM-Tris–HCl pH 7·6, 0·5 M-NaCl, 1 mM-EDTA, 1% Triton X-100 and 1 mM-PMSF; Sigma). After nuclei were removed by centrifugation at 35000 r.p.m. for 30 min at 4 °C in a TLA-45 rotor (Beckman), the supernatant was diluted to 150 mM-NaCl with TET (50 mM-Tris–HCl pH 7·6, 1 mM-EDTA, 1% Triton X-100 and 1 mM-PMSF; Sigma) and incubated with antibodies against each P protein in the presence of Protein A–agarose (Gibco/BRL) overnight at 4 °C. The antigen–antibody–Protein A complexes were washed three times with the same buffer, resuspended in SDS–sample buffer and subjected to electrophoresis on SDS–polyacrylamide gels (5–15% polyacrylamide gradient or 7·5% polyacrylamide with 4 M-urea). After staining with Coomassie brilliant blue, the gels were treated with Amplify (NEN) and subjected to fluorography using XAR5 film (Kodak) or directly analysed with an image analyser (BAS2000; Fuji Film).

Results

Expression and formation of RNA polymerase

Expression of a single subunit or combinations of the three subunits of influenza virus RNA polymerase was established

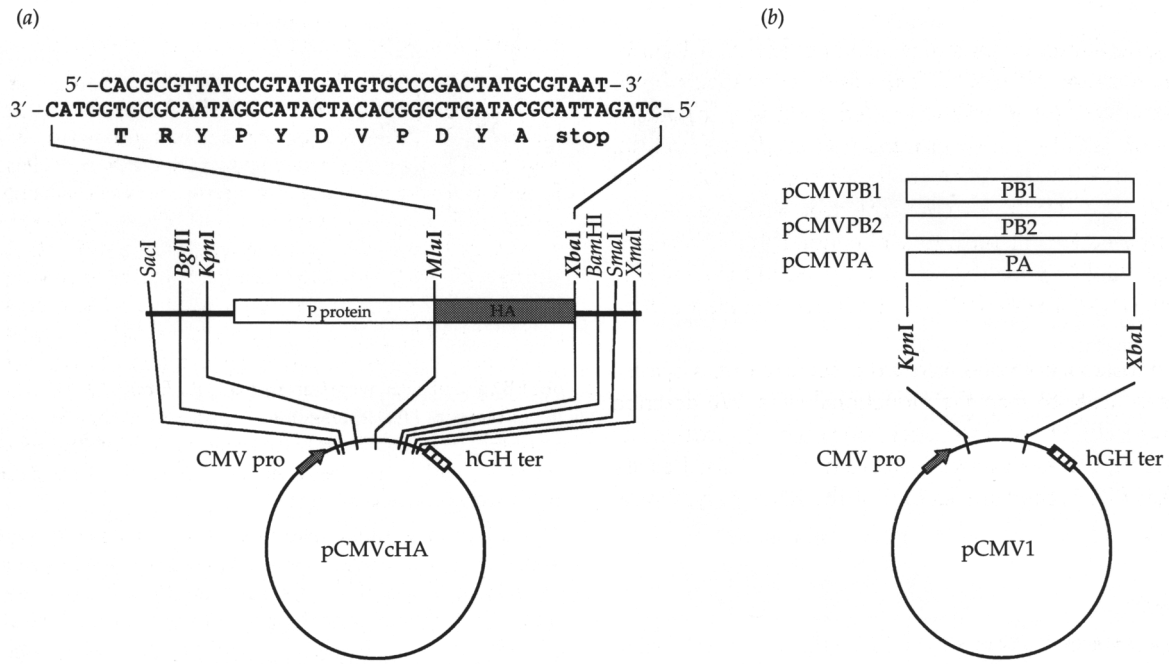

Fig. 1. Construction of wild-type and mutant influenza virus RNA polymerase expression plasmids with or without HA-tag. (*a*) HA-tagged polymerase expression plasmids (pHApol series). Plasmid pCMVcHA was constructed by inserting a synthetic oligonucleotide pair into pCMV1 between the *Kpn*I and *Xba*I sites. The inserted oligonucleotide encodes a HA epitope with the indicated amino acid sequence. pHApol plasmids were constructed by insertion of P protein cDNA into pCMVcHA at the *Mlu*I site to express fusion proteins with a C-terminal HA epitope tag (see Methods). Unique restriction sites are indicated in bold. (*b*) Non-tagged wild-type P protein expression plasmids (pCMVpol series). PCR-amplified wild-type P protein cDNAs were inserted into pCMV1 between the *Kpn*I and *Xba*I sites (see Methods). CMV pro, cytomegalovirus immediate early promoter; hGH ter, human growth hormone terminator.

by transfection of PB1, PB2 and/or PA cDNA under the control of the cytomegalovirus promoter (Fig. 1). The synthesis of each P protein was monitored by immunoprecipitation of radiolabelled proteins with mono-specific antibodies. Using this expression system, we first analysed the formation of RNA polymerase complexes after co-transfection of all three P protein expression plasmids and by immunoprecipitation of radiolabelled P proteins with antibodies against each. Addition of the HA tag did not interfere with subunit assembly.

After a 30 min pulse with radioactive methionine, the synthesis of all three P proteins was detected by immunoprecipitation, even though the levels of labelled P proteins were not equal (pre-existing non-radioactive P protein levels were not determined). Both PB1 and PB2 were immunoprecipitated by treatment with either anti-PB2 or anti-PB1 antibodies in 30 min pulse (Fig. 2), and 10 min pulse samples (data not shown). Even after the 30 min pulse, however, radioactive PA was not immunoprecipitated with anti-PB1 or anti-PB2 antibodies. After a 3 h pulse, radioactive PA was co-immunoprecipitated by anti-PB1. These results indicate that the assembly of all three P proteins takes place in the transfected cells using PB1 as a core. Thus, the methods employed were found to be useful for the detection of P protein complexes formed *in vivo*.

Formation of binary P protein complexes

Next, we analysed the formation of binary P protein complexes by co-transfection of two different cDNA plasmids in various combinations. Binary complexes were identified for PB1–PB2 and PB1–PA combinations (Figs 3 and 4) but not for PB2–PA (Fig. 5). Thus, we concluded that both PB2 and PA bind to PB1, and therefore PB1 is the core subunit in the assembly of influenza virus RNA polymerase, but PB2 and PA do not interact with each other.

Mapping of the subunit–subunit contact sites

The P protein expression system was employed to determine the subunit–subunit contact sites on each P polypeptide. For this purpose, the P protein expression plasmids were modified to express mutant P proteins with various deletions from the N and C termini (Fig. 6).

PB1–PB2 contact sites. In order to determine the PB1-binding site on PB2, simultaneous expression of wild-type PB1 and various PB2 mutants with either N- or C-terminal deletions was achieved by co-transfection of wild-type PB1 expression plasmid (pCMVPB1) and the series of expression plasmids for

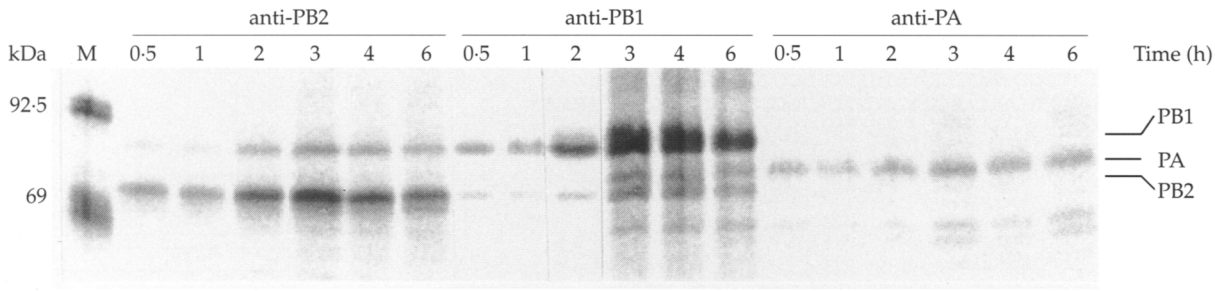

Fig. 2. Time course of RNA polymerase complex formation in P protein-expressing cells. COS-7 cells were transfected simultaneously with pCMVPB2, pCMVPB1 and pCMVPA and pulse-labelled with [^{35}S]methionine for 0·5, 1, 2, 3, 4 and 6 h, as indicated. Cell lysates were prepared and subjected to immunoprecipitation with anti-PB2, anti-PB1 or anti-PA antibodies. The proteins precipitated were separated though SDS–7·5% polyacrylamide gels containing 4 M-urea. The migration positions of molecular mass standards (lane M) are indicated on the left, while the positions of PB1, PB2 and PA are indicated on the right.

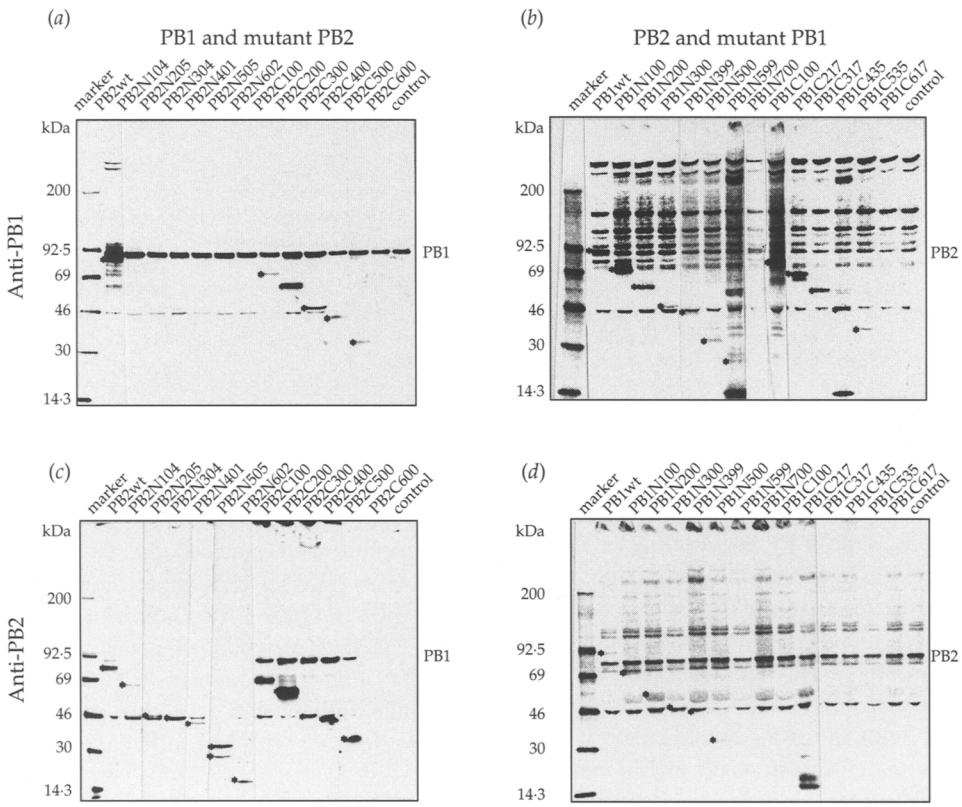

Fig. 3. Formation of PB1–PB2 complexes. Intact PB1 expression plasmid (pCMVPB1) was co-transfected with each of the mutant PB2 expression plasmids (pHAPB2 series) or the vector pCMVcHA (*a*, *c*). Intact PB2 expression plasmid (pCMVPB2) was co-transfected with each of the mutant PB1 expression plasmids (pHAPB1 series) or pCMVcHA (*b*, *d*). Whole-cell extracts with 0·5 M-NaCl lysis buffer were immunoprecipitated with anti-PB1 (*a*, *b*) or anti-PB2 antibodies (*c*, *d*). The bands of mutant P proteins on the SDS gels are indicated by asterisks. PB2N505 expressed two fragments. The migration positions of molecular mass markers are indicated on the left, while the positions of PB1 and PB2 are indicated on the right.

N-terminal deletion mutants (PB2N104, PB2N205, PB2N304, PB2N401, PB2N505 and PB2N602) or C-terminal deletion mutants (PB2C100, PB2C200, PB2C300, PB2C400, PB2C500 and PB2C600). In the 4 h pulse-labelled sample, wild-type PB2 and the C-terminal deletion PB2 mutants, except PB2C600, were co-immunoprecipitated with wild-type PB1 by anti-PB1 antibodies (Fig. 3*a*). None of the N-terminal deletion mutants were co-immunoprecipitated with wild-type PB1, indicating that PB2 binds to PB1 at its N-terminal domain.

On the other hand, when immunoprecipitation was carried out by anti-PB2 antibodies, wild-type PB1 was co-precipitated with five species of PB2 C-terminal deletion mutants, PB2C100,

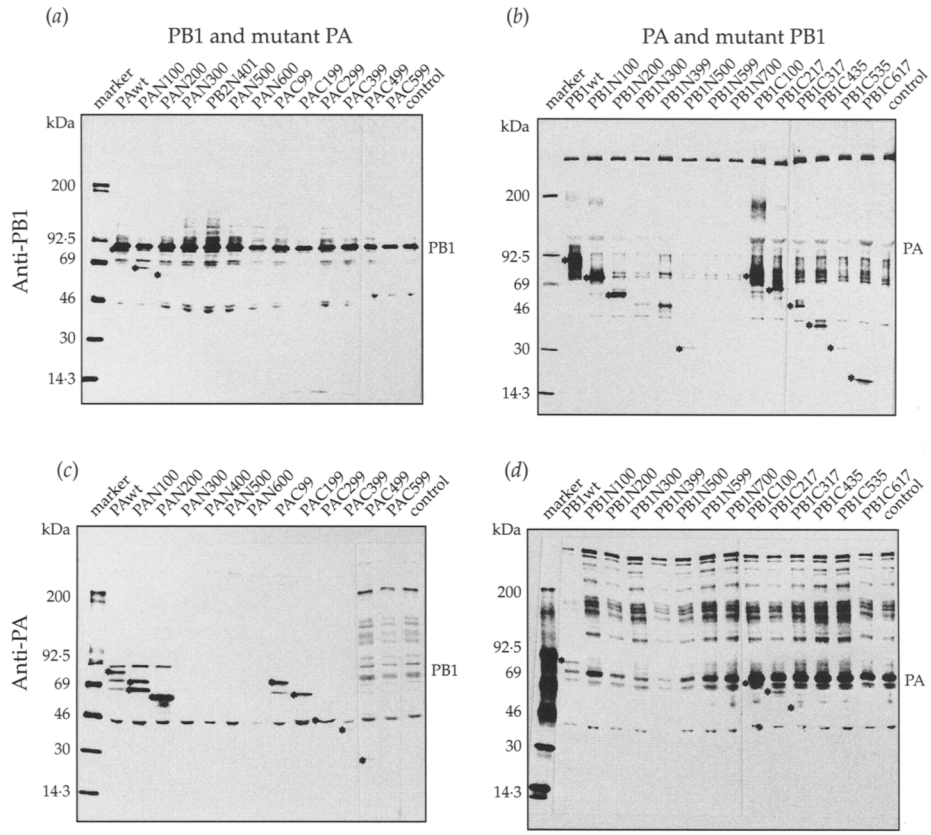

Fig. 4. Formation of PB1–PA complexes. Intact PB1 expression plasmid (pCMVPB1) was co-transfected with each of the PA mutant expression plasmids (pHAPA series) (*a*, *c*). Intact PA expression plasmid (pCMVPA) was co-transfected with each of the PB1 mutant expression plasmids (pHAPB1 series) (*b*, *d*). Whole-cell extracts with 0·5 M-NaCl lysis buffer were immunoprecipitated with anti-PB1 (*a*, *b*) or anti-PA antibodies (*c*, *d*). The bands of mutant polymerase subunits on SDS gels are indicated by asterisks. PAN100 formed two bands. The last three lanes of (*c*) were exposed longer. The migration positions of molecular mass markers are indicated on the left, the positions of PB1 and PA are indicated on the right.

PB2C200, PB2C300, PB2C400 and PB2C500 (Fig. 3*c*). Again PB2C600 was not immunoprecipitated, but the expression of PB2C600 might have been below the detection level. Thus we concluded that the PB1 contact site on PB2 is located upstream from residue 249.

Next, the PB2-binding site on the PB1 subunit was determined by co-expression of wild-type PB2 and the series of PB1 N- or C-terminal deletion mutants. The expression or solubility of PB1 deletion mutants in 0·5 M-NaCl solution was poor when compared with that of PB2 and PA deletion mutants (Fig. 3*b*, *d*). In the 4 h pulse-labelled sample, wild-type PB1 and the N-terminal deletion mutants (PB1N100, PB1N200, PB1N300, PB1N399, PB1N500 and PB1N599) were all co-immunoprecipitated with wild-type PB2 subunit by anti-PB1 antibodies (Fig. 3*b*). When the immunoprecipitation was done with anti-PB2 antibodies, wild-type PB1 and the PB1 N-terminal deletion mutants (PB1N100, PB1N200, PB1N300, PB1N399 and PB1N500) were co-immunoprecipitated. In contrast, the C-terminal deletion mutants (PB1C100, PB1C217, PB1C317, PB1C435 and PB1C535) were not co-immunoprecipitated with PB2 by anti-PB2 antibodies (Fig. 3*d*). The expression of PB1N700 and PB1C617 was not detected. Thus, we concluded that the PB2-binding site on PB1 is located on the C-terminal region downstream from residue 600.

PB1–PA contact sites. The PB1-binding site on PA was determined by co-transfection of pCMVPB1 with expression plasmids for the series of PA N- or C-terminal deletion mutants. Labelled PA did not form complexes with wild-type PB1 until 2 h of pulse labelling (Fig. 2). After 3 h of pulse labelling, wild-type PB1 and PB1 derivatives became co-immunoprecipitable with PA. Wild-type PA, PAN100 and PAN200 were co-immunoprecipitated with wild-type PB1 by both anti-PB1 and anti-PA antibodies (Fig. 4*a*, *c*). On the other hand, PAC99, PAC199, PAC299, PAC399 and PAC499 were not co-immunoprecipitated with PB1. Thus, we tentatively concluded that the PB1-binding site is located downstream from residue 201. We did not focus further on the PB1-binding site because the expression of PAN300, PAN400, PAN500, PAN600 and PAC599 was too low to detect immunoprecipitates.

The PA-binding site on PB1 was determined by co-

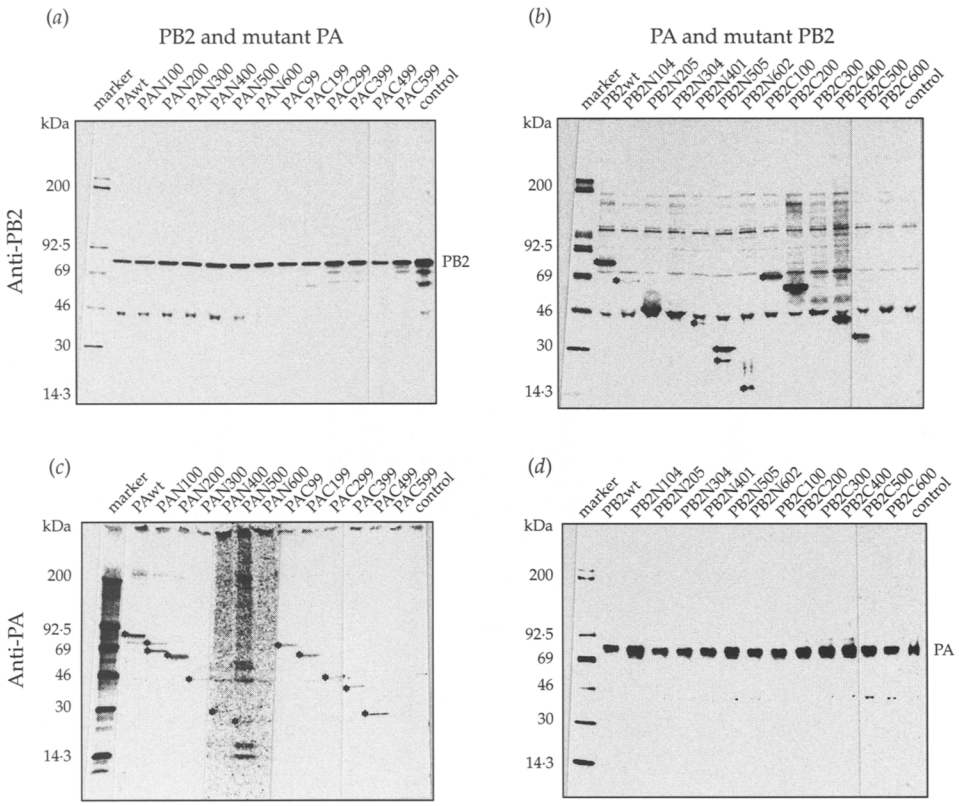

Fig. 5. Formation of PB2–PA complexes. Intact PB2 expression plasmid (pCMVPB2) was co-transfected with each of the PA mutant expression plasmids (pHAPA series) (*a*, *c*). Intact PA expression plasmid (pCMVPA) was co-transfected with each of the PB2 mutant expression plasmids (pHAPB2) (*b*, *d*). Whole-cell extracts with 0·5 M-NaCl lysis buffer were immunoprecipitated with anti-PB2 (*a*, *b*) or anti-PA antibodies (*c*, *d*). The bands of mutant polymerase subunits are indicated by asterisks. Lanes PAN400, PAN500 and PAN600 in (*c*) were exposed longer. The migration positions of molecular mass markers are indicated on the left, the positions of PB2 and PA are indicated on the right.

expression of wild-type PA and the series of PB1 N- and C-terminal deletion mutants. Again, the expression or solubility of PB1 deletion mutants in 0·5 M-NaCl solution was poor when compared with that of PB2 and PA (Fig. 4*b*, *d*). The expression of PB1N300, PB1N599 or PB1N700 was not detected by immunoprecipitation. Wild-type PB1 and all the C-terminal deletion derivatives (PB1C100, PB1C217, PB1C317, PB1C435, PB1C535 and PB1C617) were co-immunoprecipitated with PA by anti-PB1 antibodies (Fig. 4*b*). Wild-type PB1, PB1C100, PB1C217 and PB1C317, on the other hand, were all immunoprecipitated using anti-PA antibodies (Fig. 4*d*). Thus, we concluded that the PA-binding site on PB1 is located upstream from residue 140.

PB2–PA contact sites. The formation of PB2–PA complexes in co-expressing cells was examined by the same procedure as above (Fig. 5). Both wild-type and mutant PB2 and PA proteins except PAN600, PAC599 and PB2C600 were expressed by simultaneous transfection. However, under the conditions employed (using RIPA buffer or extraction buffers containing 0·5, 0·15, 0·1 or 0·05 M-NaCl) co-immunoprecipitation of these two subunits was not detected by either antibody.

Discussion

Subunit–subunit linkage within the influenza virus RNA polymerase

Prokaryotic RNA polymerase provides a good model of subunit assembly. The RNA polymerase core enzyme of *E. coli* with a subunit composition of $\alpha_2\beta\beta'$ is assembled *in vitro* and *in vivo* in a sequential manner: $\alpha \rightarrow \alpha_2 \rightarrow \alpha_2\beta \rightarrow \alpha_2\beta\beta'$ (Ishihama, 1981). Dimerization of the α subunit is the first step in this assembly pathway and thus the α subunit plays a key role in RNA polymerase formation. The α–α, α–β and α–β' contact sites on the α subunit were recently identified in the N-terminal assembly domain by deletion, insertion and mutation analysis (Kimura & Ishihama, 1995, 1996; Kimura *et al.*, 1994). Among eukaryotic RNA polymerases, influenza virus RNA polymerase may provide a model for protein–protein assembly and interaction because it consists of three components and each carries a specific and unique function(s). The *in vitro* assembly systems are not very useful for this purpose at present due to the low solubility of isolated individual P proteins and the low level of reconstitution.

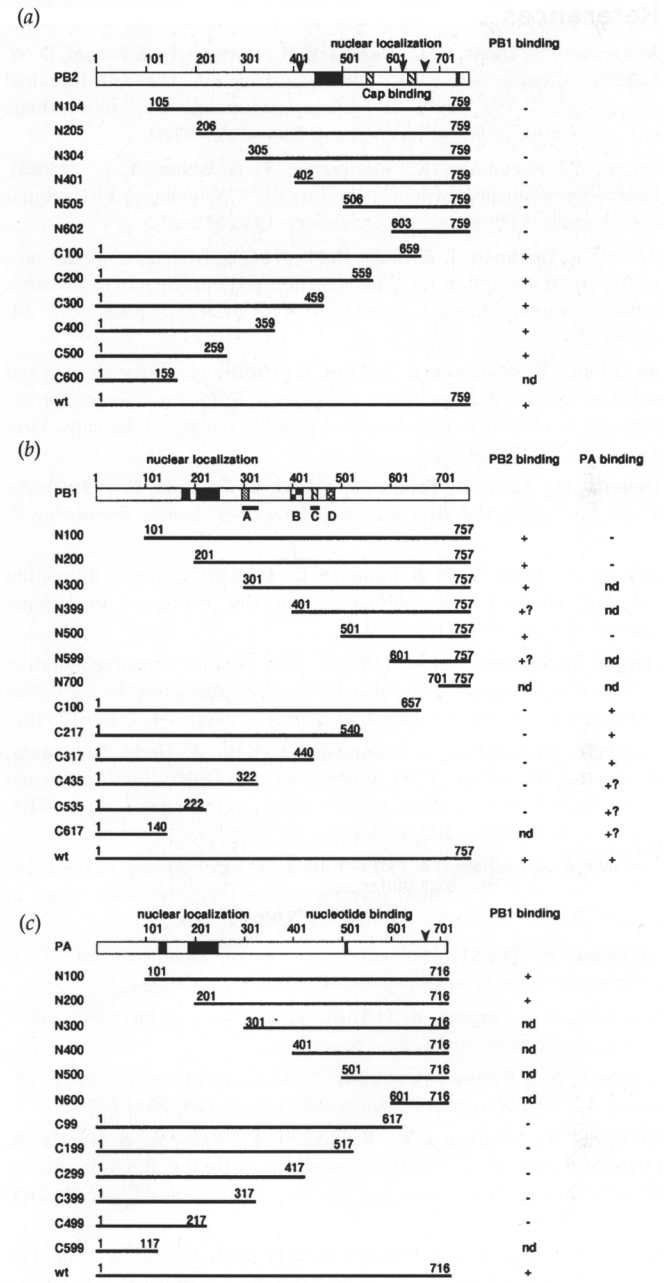

Fig. 6. Construction and assembly activities of the deletion mutants. Molecular maps of the deletion mutants of PB2 (*a*), PB1 (*b*) and PA (*c*) are shown. Numbers on the bars indicate the first and the last amino acid position. The results of co-immunoprecipitation experiments are indicated on the right. For immunoprecipitation of PB1 protein, we used two antibody preparations (see text). The fragments which were co-immunoprecipitated by only one of these antibodies are indicated by '+?' and those that were not detected are indicated by 'nd'. The functional map of each subunit is shown above (for details see Fig. 7). (■) Nuclear localization signals (Jones *et al.*, 1986; Mukaigawa & Nayak, 1991; Nieto *et al.*, 1994); (▨) cap-binding sequences of PB2 (*a*) (de la Luna *et al.*, 1989). The sites of mutations so far determined are indicated by arrowheads (*a*) (Subbarao *et al.*, 1993; Yamanaka *et al.*, 1990). The RNA polymerase consensus motifs of PB1 are indicated as A, B, C and D (*b*) (Delarue *et al.*, 1990; Poch *et al.*, 1989). (*c*) The mutation points of *ts*263 are indicated by arrowheads (Herget & Scholtissek, 1993) and the nucleotide-binding motif of PA (de la Luna *et al.*, 1989) is shown (▮).

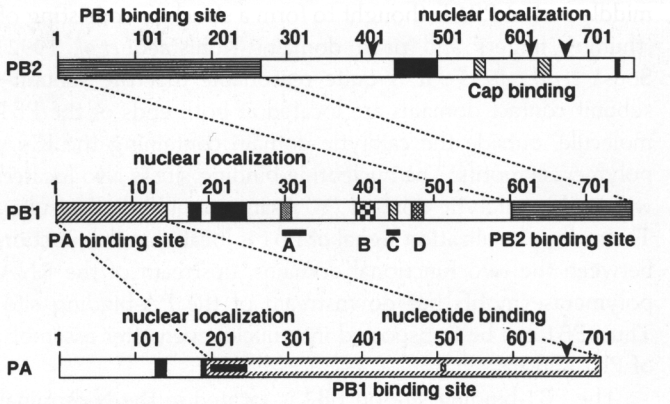

Fig. 7. Functional map of influenza virus RNA polymerase. The subunit–subunit contact sites on each of the RNA polymerase subunits are indicated, together with the functional maps so far identified (for details see Fig. 6). (▤) PB1–PB2 contact sites; (▨) PB1–PA contact sites.

Using the P protein expression system, we demonstrated that binary complexes were formed *in vivo* between PB1–PB2 and PB1–PA. However, we failed to demonstrate the binding of PB2 and PA. Our observations are consistent with a previous analysis using mRNA injection into *Xenopus* oocytes (Digard *et al.*, 1989). The expressed PB2 and PA were not co-immunoprecipitated in the absence of PB1. However, we cannot exclude direct interaction between PB2 and PA in the assembled three protein complex. In the case of *E. coli* RNA polymerase, neither α nor β form stable complexes with β', but $\alpha_2\beta$ complex is capable of forming complexes with β' (Ishihama, 1981).

Subunit–subunit contact sites on each P protein

The results of deletion analysis demonstrated that the PB2 and PA subunits both associate with a single molecule of PB1 but at different domains. The PB2-binding site is located within the C-terminal 158 amino acids while the PA-binding site is located within the N-terminal 140 amino acids (Fig. 7). Pérez & Donis (1995) mapped the PA-binding site on PB1 within the N-terminal 48 amino acids using a two hybrid system, which is consistent with our results.

From sequence comparisons, PB1 is considered to be the catalytic subunit of influenza virus RNA polymerase (Delarue *et al.*, 1990; Poch *et al.*, 1989). In agreement with this prediction, NTPs are specifically cross-linked to the PB1 subunit (Asano *et al.*, 1995; Braam *et al.*, 1983) and nuclear extracts containing PB1 alone can catalyse RNA synthesis from short RNA templates (Kobayashi *et al.*, 1996). The consensus sequence motifs of RNA-dependent RNA polymerases exist in the central portion of PB1 protein (Fig. 7). Therefore, in analogy to the crystallographic structures of T7 RNA polymerase and human immunodeficiency virus reverse transcriptase, the

middle part of PB1 is thought to form a structure consisting of 'thumb', 'fingers' and 'palm' domains (Kohlstaedt *et al.*, 1992; Sousa *et al.*, 1993). It is quite reasonable that the subunit–subunit contact domains are located at both ends of the PB1 molecule, outside the catalytic domain containing the RNA polymerase motifs. The nucleotide-binding site is also located within this catalytic domain (Y. Asano, unpublished results). The nuclear localization signal of PB1 is located in the junction between the two functional domains, upstream of the RNA polymerase motifs but downstream of the PA-binding site. Thus, PB1 can be transported into nuclei even after assembly of PB2 and PA.

The PB1-binding site on PB2 is located in the N-terminal 249 amino acids, while the nuclear localization signals and the cap-binding signals are both located in the C-terminal region (Fig. 7; de la Luna *et al.*, 1989; Jones *et al.*, 1986; Mukaigawa & Nayak, 1991). Lawson *et al.* (1992) isolated ts mutants carrying mutations in the PB2 gene. Some of them mapped to amino acid positions 65, 100, 112 and 174 within the PB1-binding region. These mutations might affect the assembly of RNA polymerase.

The PB1-binding site on PA maps to the C-terminal two-thirds (Fig. 7). Even though fine mapping has not yet been performed, the *ts*263 mutation of PA, which affects genome replication, has been mapped to this region (amino acid position 671; Herget & Scholtissek, 1993).

The α subunit of *E. coli* RNA polymerase plays an key role in the assembly of core enzyme with the subunit structure $\alpha_2\beta\beta'$. Within the N-terminal subunit assembly domain, the β and β' subunits bind to the upstream and downstream region, respectively (Igarashi & Ishihama, 1991; Kimura & Ishihama, 1995, 1996; Kimura *et al.*, 1994). Thus, the organization of subunit–subunit contact sites is similar in PB1 of influenza virus RNA polymerase and the α subunit of *E. coli* RNA polymerase. In the case of *E. coli* RNA polymerase, the core enzyme subunits are assembled sequentially in the order: $\alpha \rightarrow \alpha_2 \rightarrow \alpha_2\beta \rightarrow \alpha_2\beta\beta'$. The mechanism of subunit assembly of influenza virus RNA polymerase is not known but our preliminary observations indicate that it is also assembled sequentially (Fig. 2; T. Toyoda, unpublished results). Besides the subunit assembly function, the α subunit of *E. coli* RNA polymerase carries the contact domain with *trans*-acting regulatory proteins and DNA signals. The function of the PB1 subunit is modified from transcriptase in the early phase of virus infection to replicase in the late stage. Involvement of an additional protein factor(s) has been proposed in this conversion (Nagata *et al.*, 1989; Toyoda *et al.*, 1994). The putative factor for switching may interact with the PB1 subunit.

We thank Dr M. Stinski for pCMV1. D.M.A. was supported by a fellowship from Monbusho, Japan. This work was supported by Grants-in-Aid from the Ministry of Education, Science, Sports and Culture of Japan, the Naito Foundation and the Uehara Memorial Foundation.

References

Andersson, S., Davis, D. L., Dahlbäck, H., Jörnvall, H. & Russel, D. W. (1989). Cloning, structure, and expression of the mitochondrial cytochrome P-450 sterol 26-hydroxylase, a bile acid biosynthetic enzyme. *Journal of Biological Chemistry* **264**, 8222–8229.

Asano, Y., Mizumoto, K., Maruyama, T. & Ishihama, A. (1995). Photoaffinity labeling of influenza virus RNA polymerase PB1 subunit with 8-azido GTP. *Journal of Biochemistry* **117**, 677–682.

Braam, J., Ulmanen, I. & Krug, R. M. (1983). Molecular model of a eukaryotic transcription complex: function and movements of influenza virus P proteins during capped RNA-primed transcription. *Cell* **34**, 609–618.

de la Luna, S., Martinez, C. & Ortín, J. (1989). Molecular cloning and sequencing of influenza virus A/Victoria/3/75 polymerase genes: sequence evolution and prediction of possibly functional domains. *Virus Research* **13**, 143–156.

Delarue, M., Poch, O., Tordo, N., Moras, D. & Argos, P. (1990). An attempt to unify the structure of polymerases. *Protein Engineering* **3**, 461–467.

Digard, P., Blok, V. C. & Inglis, S. C. (1989). Complex formation between influenza virus polymerase proteins expressed in *Xenopus* oocytes. *Virology* **171**, 162–169.

Herget, M. & Scholtissek, C. (1993). A temperature-sensitive mutation in the acidic polymerase gene of an influenza A virus alters the regulation of viral protein synthesis. *Journal of General Virology* **74**, 1789–1794.

Honda, A., Mukaigawa, J., Yokoiyama, A., Kato, A., Ueda, S., Nagata, K., Krystal, M., Nayak, D. P. & Ishihama, A. (1990). Purification and molecular structure of RNA polymerase from influenza virus A/PR8. *Journal of Biochemistry* **107**, 624–628.

Igarashi, K. & Ishihama, A. (1991). Bipartite function map of the *E. coli* RNA polymerase α subunit: involvement of the C-terminal region in transcription activation by cAMP-CRP. *Cell* **32**, 319–325.

Ishihama, A. (1981). Subunit assembly of *Escherichia coli* RNA polymerase. *Advance in Biophysics* **14**, 1–35.

Ishihama, A. & Nagata, K. (1988). Viral RNA polymerases. *Critical Reviews in Biochemistry* **23**, 27–76.

Ishihama, A. & Barbier, P. (1994). Molecular anatomy of viral RNA-directed RNA polymerases. *Archives of Virology* **134**, 235–258.

Ishihama, A., Mizumoto, K., Kawakami, K., Kato, A. & Honda, A. (1986). Proofreading function associated with the RNA-dependent RNA polymerase from influenza virus. *Journal of Biological Chemistry* **261**, 10417–10421.

Jones, I. M., Reay, P. A. & Philpott, K. L. (1986). Nuclear location of all three influenza polymerase proteins and a nuclear signal in polymerase PB2. *EMBO Journal* **5**, 2371–2376.

Kawakami, K., Mizumoto, K. & Ishihama, A. (1983). RNA polymerase of influenza virus, IV. Catalytic properties of the capped RNA endonuclease associated with the RNA polymerase. *Nucleic Acids Research* **11**, 3637–3649.

Kimura, M. & Ishihama, A. (1995). Functional map of the alpha subunit of *Escherichia coli* RNA polymerase: insertion analysis of the amino-terminal assembly domain. *Journal of Molecular Biology* **248**, 756–767.

Kimura, M. & Ishihama, A. (1996). Functional map of the alpha subunit of *Escherichia coli* RNA polymerase: *in vivo* analysis of the amino-terminal assembly domain. *Genes to Cells* **1** (in press).

Kimura, M., Fujita, N. & Ishihama, A. (1994). Functional map of the alpha subunit of *Escherichia coli* RNA polymerase: deletion analysis of the amino-terminal assembly domain. *Journal of Molecular Biology* **242**, 107–115.

Kobayashi, M., Tuchiya, K., Nagata, K. & Ishihama, A. (1992). Reconstitution of influenza virus RNA polymerase from three subunits expressed using recombinant baculovirus system. *Virus Research* **22**, 235–245.

Kobayashi, M., Toyoda, T. & Ishihama, A. (1996). Influenza virus PB1 protein is the minimal and essential subunit of RNA polymerase. *Archives of Virology* **141**, 525–539.

Kohlstaedt, L. A., Wang, J., Friedman, J. M., Rice, P. A. & Steitz, T. A. (1992). Crystal structure at 3·5 Å resolution of HIV-1 reverse transcriptase complexed with an inhibitor. *Science* **256**, 1783–1790.

Krug, R. M., Ueda, M. & Palese, P. (1975). Temperature-sensitive mutants of influenza WSN virus defective in virus-specific RNA synthesis. *Journal of Virology* **16**, 790–796.

Krug, R. M., Alonso-Caplen, F. V., Julkunen, I. & Katze, M. G. (1989). Expression and replication of the influenza virus genome. In *The Influenza Viruses*, pp. 89–152. Edited by R. M. Krug. New York: Plenum Press.

Lamb, R. A. (1989). Genes and proteins of the influenza viruses. In *The Influenza Viruses*, pp. 1–87. Edited by R. M. Krug. New York: Plenum Press.

Lawson, C. M., Subbarao, E. K. & Murphy, B. R. (1992). Nucleotide sequence changes in the polymerase basic protein 2 gene of temperature-sensitive mutants of influenza A virus. *Virology* **191**, 506–510.

Luytjes, W., Krystal, M., Enami, M., Parvin, J. D. & Palese, P. (1989). Amplification, expression and packaging of a foreign gene by influenza virus. *Cell* **59**, 1107–1113.

Mahy, B. W. J., Barrett, T., Nichol, S. T., Penn, C. R. & Wolstenholme, A. J. (1981). Analysis of the functions of influenza virus genome RNA segments by use of temperature-sensitive mutants of fowl plague virus. In *The Replication of Negative Stranded Viruses*, pp. 379–387. Edited by D. H. L. Bishop and R. W. Compans. New York: Elsevier.

Mowshowitz, S. L. (1981). RNA synthesis of temperature-sensitive mutants of WSN influenza virus. In *The Replication of Negative Stranded Viruses*, pp. 317–323. Edited by D. H. L. Bishop and R. W. Compans. New York: Elsevier.

Mukaigawa, J. & Nayak, D. P. (1991). Two signals mediate nuclear localization of influenza virus (A/WSN/33) polymerase basic protein 2. *Journal of Virology* **65**, 245–253.

Nagata, K., Takeuchi, K. & Ishihama, A. (1989). *In vitro* synthesis of influenza viral RNA: biochemical complementation assay of factors required for influenza virus replication. *Journal of Biochemistry* **106**, 205–208.

Nakagawa, Y., Kimura, N., Toyoda, T., Mizumoto, K., Ishihama, A., Oda, K. & Nakada, S. (1995). The RNA polymerase PB2 subunit is not required for replication of the influenza virus genome but is involved in capped mRNA synthesis. *Journal of Virology* **69**, 728–733.

Nieto, A., de la Luna, S., Bárcena, J., Portela, A. & Ortín, J. (1994). Complex structure of the nuclear translocation signal of influenza virus polymerase PA subunit. *Journal of General Virology* **75**, 29–36.

Pérez, D. R. & Donis, R. O. (1995). A 48-amino acid region of influenza A virus PB1 protein is sufficient for complex formation with PA. *Journal of Virology* **69**, 6932–6939.

Plotch, S. J., Bouloy, M., Ulmanen, I. & Krug, R. M. (1981). Initiation of influenza viral RNA transcription by capped RNA primers: a unique cap (m7GpppXm)-dependent virion endonuclease generates 5′ terminal RNA fragments that prime transcription. *Cell* **23**, 847–858.

Poch, O., Sauvaget, I., Delarue, M. & Tordo, N. (1989). Identification of four conserved motifs among the RNA-dependent polymerase encoding elements. *EMBO Journal* **8**, 3867–3874.

Scholtissek, C. & Bowles, A. L. (1975). Isolation and characterization of temperature-sensitive mutants of fowl plague virus. *Virology* **67**, 576–587.

Scholtissek, C., Harms, E., Rhode, W., Orlich, M. & Root, R. (1976). Correlation between RNA fragments of fowl plague virus and their corresponding gene functions. *Virology* **74**, 322–344.

Sousa, R., Chung, Y. J., Rose, J. P. & Wang, B.-C. (1993). Crystal structure of bacteriophage T7 RNA polymerase at 3·3 Å resolution. *Nature* **354**, 593–599.

Studier, W. F., Rosenberg, A. H., Dunn, J. J. & Dubendorff, J. W. (1990). Use of T7 RNA polymerase to direct the expression of cloned genes. *Methods in Enzymology* **185**, 60–89.

Subbarao, E. K., London, W. & Murphy, B. R. (1993). A single amino acid in the PB2 gene of influenza A virus is a determinant of host range. *Journal of Virology* **67**, 1761–1764.

Szewczyk, B., Laver, W. G. & Summers, D. F. (1988). Purification, thioredoxin renaturation and reconstituted activity of the three subunits of the influenza A virus RNA polymerase. *Proceedings of the National Academy of Sciences, USA* **85**, 7907–7911.

Toyoda, T., Kobayashi, M. & Ishihama, A. (1994). Replication *in vitro* of the influenza virus genome: selective dissociation of RNA replicase from virus-infected cell ribonucleoprotein complexes. *Archives of Virology* **136**, 269–286.

Ulmanen, I., Broni, B. A. & Krug, R. M. (1981). The role of two of the influenza virus core P proteins in recognizing cap 1 structures (M7gpppnm) on RNAs and in initiating viral RNA transcription. *Proceedings of the National Academy of Sciences, USA* **78**, 7355–7359.

Yamanaka, K., Ogasawara, N., Ueda, M., Yoshikawa, H., Ishihama, A. & Nagata, K. (1990). Characterization of a temperature-sensitive mutant in the RNA polymerase PB2 subunit gene of influenza A/WSN/33 virus. *Archives of Virology* **114**, 65–73.

Yamanaka, K., Ogasawara, N., Yoshikawa, H., Ishihama, A. & Nagata, K. (1991). *In vivo* analysis of the promoter structure of the influenza virus RNA genome using a transfection system with an engineered RNA. *Proceedings of the National Academy of Sciences, USA* **88**, 5363–5373.

Young, J. F., Desselberger, U., Graves, U., Palese, P., Shatzman, A. & Rosenburg, M. (1983). Cloning and expression of influenza virus genes. In *The Origin of Pandemic Influenza Viruses*, pp. 129–138. Edited by W. G. Laver. New York: Elsevier.

Received 8 February 1996; Accepted 29 April 1996