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

Tetsuya Toyoda,1,2 Djanybek M. Adyshev,† Makoto Kobayashi,3 Akira lwata4 and Akira Ishihama1

1 Department of Molecular Genetics, National Institute of Genetics, Mishima, Shizuoka 411, Japan
2 Department of Virology, Kurume University School of Medicine, Kurume, Fukuoka 830, Japan
3 Department of Biology, Jichi Medical School, Tochigi 329-04, Japan
4 Nippon Institute for Biological Science, Ohme, Tokyo 198, Japan

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 (Sewczyk 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.
(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' CACGCGTTATCCGTATGATGTCGGGCACATCATACGGATAACGCGTGGTACGCTG-GCATAGTCGGGCACATCATACGGATAACGCGTGGTACGCTG-3' into pCMV1 between the KpnI and XbaI sites (Fig. 1). pCMVcHA was designed to express fusion proteins with C-terminal haemagglutinin (HA) epitope tags by in-frame insertion at the MluI 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 PCR206 (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 5 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 KpnI and MluI, purified by agarose gel electrophoresis and inserted into pCMVcHA between the KpnI and MluI sites (Fig. 1). PB1N200, PB1N300, PB1N399 and PB1N500 were digested with BamHI and MluI, purified by agarose gel electrophoresis and inserted into pCMVcHA between the BamHI and MluI 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 KpnI and XhoI and inserted into pCMV1 between the KpnI and XhoI 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 Smal–BamHI adapter (Takara) was inserted into the BamHI site of pET3c to make pET3c. 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 pET3c at a Smal 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, pETPB2, and pETPA, were transformed into E.coli BL21 (DE3) containing pLyS5 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, pHAPB1N399, pHAPB1N700, pHAPB1C100, pHAPB1C17, pHAPB1C317, pHAPB1C435 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, pHAPB2N500, pHAPB2N602, pHAPB2C100, pHAPB2C200, pHAPB2C300, pHAPB2C400, pHAPB2C500 or pHAPB2C600). For determination of PB1 and PB2 contact site on PA, pCMVPB2 or pCMVPA 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 [35S]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 0.06 ml of TNE(0.01 M Tris–HCl pH 7.6, 0.5 mM-NaCl, 1 mM EDTA, 1% Triton X-100 and 1 mM-PMSF; Sigma). After nuclei were removed by centrifugation at 35 000 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
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.

Formulation of binary P protein complexes

Next, we analysed the formulation 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 [35S]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 through 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 PBI–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. 3a). 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,
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. 4a, 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 PAN100, PAN400, PAN500, PAN600 and PAC599 was too low to detect immunoprecipitates.

The PA-binding site on PBI was determined by co-
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 $\alpha$ subunit is the first step in this assembly pathway and thus the $\alpha$ subunit plays a key role in RNA polymerase formation. The $\alpha-\alpha$, $\alpha-\beta$ and $\alpha-\beta'$ contact sites on the $\alpha$ 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.
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 αβ 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 & Nakay, 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 ts263 mutation of PA, which affects genome mutations, 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 α2β’β. 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: α → α2 → αβ → αβ’. 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.

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