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The PB1 Subunit Alone Can Catalyze cRNA Synthesis, and the PA Subunit in Addition to the PB1 Subunit Is Required for Viral RNA Synthesis in Replication of the Influenza Virus Genome

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We indicated that the PB1 and PA subunits of RNA polymerase and nucleoprotein (NP) can support replication of the influenza virus genome as well as transcription to yield uncapped poly(A)$^+$-RNA (Y. Nakagawa, N. Kimura, T. Toyoda, K. Mizumoto, A. Ishihama, K. Oda, and S. Nakada, J. Virol. 69:728–733, 1995). To analyze the functions of the PB1 and PA subunits in replication and transcription, YP1N clones in which the PB1 and NP genes can be expressed in response to dexamethasone were established. cRNA was transcribed from model viral RNA (vRNA), but vRNA synthesis from model cRNA was not detected in YP1N clones. Furthermore, poly(A)$^+$-RNA directed from model vRNA was synthesized in YP1N clones. These results indicated that PB1 and NP can support the syntheses of cRNA and poly(A)$^+$-RNA and that the PA subunit, in addition to that of PB1 and to NP, is required for vRNA synthesis. In summary, the PB1 subunit is involved in the catalytic activities of nucleotide elongation, and the PA subunit may act as an allosteric modulator and cause a conformational change from a cRNA- to a vRNA-synthesizing form of the PB1 subunit.

The influenza A virus is a segmented, negative-stranded RNA virus encoding its own RNA-dependent RNA polymerase (reviewed in references 26 and 28). The RNA polymerase is composed of three subunits, PB1, PB2, and PA, which are tightly associated at the double-stranded stem region of the panhandle formed by the 5′ and 3′ termini of the nucleoprotein (NP)-encapsidated viral genome segments (17, 18). These ribonucleoprotein cores are responsible for the replication and transcription of the virus-specific RNAs in the nuclei of infected cells in a highly regulated process.

The molecular mechanism and regulation of transcription have been relatively elucidated by biochemical and genetic analyses (reviewed in references 21, 22, and 26). The PB2 subunit first binds specifically to the cap-1 structure at the 5′ termini of nascent host cell mRNA transcripts (4, 44). Thereafter, the polymerase endonucleolytically cleaves the mRNAs to 10- to 13-mers (8, 14). These short capped RNAs serve as primers for viral mRNA synthesis (15). The polymerase then transcribes viral RNAs (vRNAs) by elongation to nucleotides 17 to 22 from the 5′ ends of the vRNAs, at which point it adds A residues to nascent RNA chains, leading to the formation of viral mRNA with both the cap-1 structure at the 5′ terminus and the poly(A) tail at the 3′ end. The PB1 subunit is thought to be involved in the catalytic activity of nucleotide elongation, since it can be UV cross-linked to 3′-terminal nucleotides of elongating mRNA chains (6). In addition, both PB1 and PB2 are considered to play roles in recognizing the transcription promoter of template vRNAs, because both basic subunits can be cross-linked to synthetic RNAs with the 3′-terminal sequence of vRNAs (12). In contrast, no specific functions in transcription have yet been identified for the PA subunit, except that it is present, with PB1 and PB2, in transcriptive complexes isolated from infected cells (20) and in purified ribonucleoprotein cores (16). The PA subunit may not be involved in transcription, since no temperature-sensitive defects have been detected in PA temperature-sensitive mutants (13, 32, 35).

During the replication cycle, a complete complementary transcript (cRNA) directed by vRNA is synthesized in a primer-independent manner. The cRNA is then used as a template for the synthesis of more vRNA by a similar mechanism (reviewed in references 21, 22, and 26). Both transcripts are probably wrapped in NP (40). In contrast to what is known about transcription, little is known regarding the molecular mechanism of replication, since replication has been analyzed in crude extracts prepared from virus-infected cells or fractionated nuclear extracts (2, 11, 30, 40, 41). Thus, because only RNA synthesis directed by endogenous templates was monitored, the de novo initiation of RNA synthesis was indistinguishable from the elongation of RNA chains initiated in infected cells. Accordingly, it had been believed without any conclusive evidence that replication requires all three subunits of RNA polymerase and NP.

Recently, we constructed in vivo replication-transcription systems with established cell lines (clone 76 and clone 64) and nonstructural protein chloramphenicol acetyltransferase chimeric model RNA (NS-CAT RNA) templates and found that the PB2 subunit is not required for replication or transcription to yield uncapped poly(A)$^+$-RNA (24, 25, 33). To elucidate functions of the PB1 and PA subunits of RNA polymerase, we established cloned cell lines YP1N25 and YP1N27, which carry the PB1 and NP genes. cRNA was transcribed from model vRNA exogenously added to these clones after exposure to dexamethasone, but vRNA was not synthesized from model cRNA. In addition, uncapped poly(A)$^+$-RNA was also tran-

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scribed from model vRNA in the dexamethasone-treated YP1N clones.

Expression of the PB1 and NP genes in YP1N clones and the NP gene in the YN30 clone. Monolayers of mouse C127 cells that were 40 to 50% confluent were cotransfected with 5 μg of plasmid pBMSA-PB1, 5 μg of pBMSA-NP, and 1 μg of pSTneoB carrying the neomycin resistance gene (23) by the lipofection procedure (25). Plasmids pBMSA-PB1 and pBMSA-NP contain the PB1 and NP cDNAs of the influenza virus genome, respectively (34), downstream of the mouse mammary tumor virus long terminal repeat, which is the glucocorticoid-inducible promoter. We cotransfected the NP-expressing plasmid with the PB1-expressing plasmid, since NP may play a role in the processivity of the polymerase (17) and suppress the polyadenylation signal by preventing premature termination (3, 40). In addition, the products of replication are probably wrapped in NP during synthesis (40). Fifteen clones in which the PB1 gene was highly expressed in response to dexamethasone were first isolated among G418-resistant clones by the cell enzyme-linked immunosorbent assay (ELISA) described by Rothlein et al. (37). Briefly, cells incubated with 10^{-6} M dexamethasone for 24 h were fixed with 2% formaldehyde in phosphate-buffered saline (PBS) for 5 min at −20°C and treated primarily with anti-PB1 antibody for 2 h. The cells were incubated with biotin-conjugated anti-rabbit immunoglobulin G (heavy plus light chains; Tago) for 30 min, with streptavidin–β-galactosidase (GIBCO-BRL) for 30 min, and then with the substrate (5′-methylumbelliferyl-β-D-galactoside; Sigma), and the optical density at 355 nm was determined with a Titertek Fluoroskan II. Two clones, YP1N25 and YP1N27, which emitted intense signals of PB1 and NP in response to dexamethasone, were selected by Western blot (immunoblot) hybridization with anti-PB1 and anti-NP antibodies, respectively.

To isolate a cell line in which the NP gene alone can be expressed, the pBMSA-PB1 plasmid was omitted from the transfection described above. After G418-resistant colonies were obtained, 14 clones with intense hybridization signals in response to dexamethasone were primary isolated by dot blot hybridization with a cDNA of the NP gene (34). Clone YN30 was finally selected by Western blot hybridization with anti-NP antibody.

We measured the levels of PB1 and NP in clones YP1N25, YP1N27, and YN30 as well as in established clones 76 and 64 (34) expressed in response to dexamethasone by means of a cell ELISA (37) with anti-PB1 and anti-NP antibodies (Fig. 1). Clone 76 expressed all three polymerase subunit genes and the NP gene, whereas clone 64 expressed genes encoding the PB1 and PA subunits and NP but not the PB2 subunit. The uninduced level of PB1 in clone YP1N25 was similar to the induced levels in clones 76 and 64 in the presence of dexamethasone. After clone YP1N25 was incubated with dexamethasone, the expression level of PB1 increased about twofold (Fig. 1A). PB1 was not expressed in clone YP1N27 in the absence of dexamethasone, but it was induced to almost the same level as those in clones 76 and 64 upon its addition (Fig. 1A). The expression level of NP in clone YP1N25 was twofold higher than those in dexamethasone-treated clones 76 and 64 regardless of dexamethasone (Fig. 1B). The induced levels of NP in clones YP1N27 and YN30 incubated with dexamethasone were similar to those in clones 76 and 64, but the expression of NP was not detected in clone YP1N27 and a one-fifth amount of NP was expressed in clone YN30 in the absence of dexamethasone. However, the levels of PB1 and NP induced in these clones were extremely lower than those in virus-infected cells (data not shown).

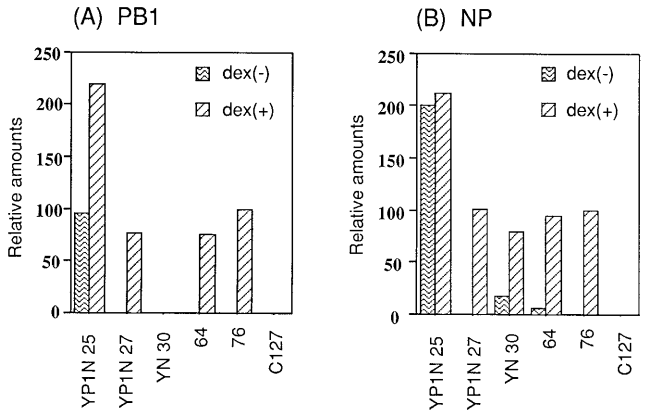

FIG. 1. Relative amounts of PB1 (A) and NP (B) expressed in clones YP1N25, YP1N27, and YN30. Cells incubated in the presence (+) or absence (−) of 10^{-6} M dexamethasone (dex) for 24 h were fixed with 2% formaldehyde in PBS at −20°C. After being washed, the cells were incubated with anti-PB1 or anti-NP antibody and then with biotin-conjugated anti-rabbit immunoglobulin G and streptavidin–β-galactosidase. Subsequently, the cells were exposed to 5′-methylumbelliferyl-β-D-galactoside, and the optical density at 355 nm was determined. The amounts of PB1 and NP are presented relative to those in clone 76 incubated with dexamethasone (100%).

Replication of NS-CAT RNAs in YP1N clones. We indicated, using clone 64, that the PB1 and PA subunits of RNA polymerase and NP can support the replication of the influenza virus genome (33). To analyze the function of PB1 and PA in replication, we transfected the chimeric NS-CAT RNAs into YP1N25 and YP1N27. To detect synthesized cRNA, 100 ng of the NS-CATv (vRNA sense) RNA-DOTAP {*N*-[1-(2,3-dioleoyloxy)propyl]-*N*,*N*,*N*-trimethylammonium-methylsulfate; Boehringer Mannheim} complex was transfected into monolayer cultures of YP1N clones incubated with 10^{-6} M dexamethasone as described previously (24, 25, 33). Six hours later, total cellular RNA was extracted with acid guanidinium thiocyanate-phenol-chloroform (7). Plus sense NS-CATc DNA complementary to NS-CATv RNA was synthesized from total cellular RNA (2 μg) by using avian myeloblastosis virus reverse transcriptase (RT) (Seikagaku Kogyo) and synthetic oligonucleotide primer SN24 (5′-AGTAGAAACAAGGGTGTT-3′), which is not observed in the mRNA sequence. CAT DNA (661 bp) was amplified from NS-CATc DNA by PCR with synthetic oligonucleotides SN202 (5′-GGAGCTAAGGAAGCTAAAA TG-3′), corresponding to the initiation codon and upstream region of the CAT gene, and SN204 (5′-ACTCATCGCAGT ACTGTTGTA-3′), corresponding to positions 15 to 35 from the termination codon of the CAT gene, being used as primers. The cRNAs were detected in both clone YP1N25 and clone YP1N27, as was shown in a typical experiment (one of three independent experiments; Fig. 2A, lanes 1 and 2). The level of cRNA in clone YP1N25 was 30 to 50% of those found in clone 64 (Fig. 2A, lane 4) and clone 76 (lane 5). The intensity of the band derived from cRNA in clone YP1N27 was very faint. Furthermore, a very faint band was detected in clone YP1N25 but not in clone YP1N27 by an RNase protection assay with SN24 being used as a probe (data not shown). It is not clear why cRNA synthesis was undetectable in clone YP1N25 without dexamethasone, whereas the amounts of PB1 and NP in clone YP1N25 were similar to and twofold higher than those in dexamethasone-treated clone YP1N27, respectively. There was no cRNA in clone YN30, in which only the NP gene was induced in response to dexamethasone (Fig. 2A, lane 3). These results indicated that PB1 and NP are sufficient for the synthesis of cRNA.

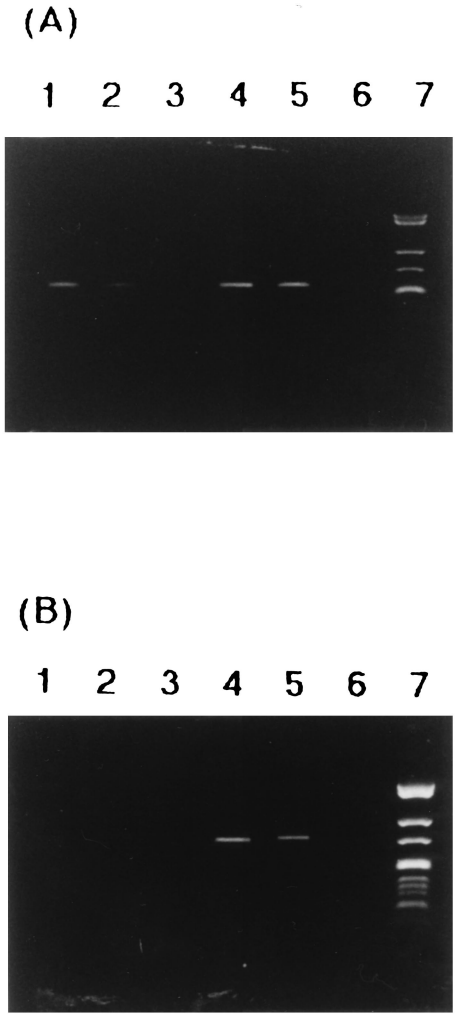

FIG. 2. Detection of cRNA (A) and vRNA (B) syntheses in YP1N clones by RT-PCR. Clones YP1N25 (lanes 1), YP1N27 (lanes 2), YN30 (lanes 3), 64 (lanes 4), and 76 (lanes 5) and parental C127 cells (lanes 6) were incubated with 10^{-6} M dexamethasone for 24 h and then transfected with NS-CATv RNA (model vRNA) (A) or NS-CATc RNA (model cRNA) (B) for 6 h. Total cellular RNAs (2 μg) prepared from these cells were reverse transcribed with avian myeloblastosis virus RT and synthetic oligonucleotide primer SN24, which can hybridize to the 3′ terminus of cRNA but not mRNA (A), or primer SN202, which corresponds to the initiation codon and upstream region of the CAT gene (B). NS-CATc DNA (A) and NS-CATv DNA (B) were amplified by PCR with synthetic oligonucleotide primers SN202/SN204 (A) and SN202/SN24 (B), respectively. Lanes 7, DNA size markers (1,845, 1,666, 1,050, 766, 543, 525, 517, 506, 396, 344, 298, 237, 221, and 220 bp).

To detect vRNA, the chimeric NS-CATc RNA (cRNA sense) was transfected into YP1N clones, and minus sense NS-CATv DNA complementary to NS-CATc RNA was reverse transcribed from total cellular RNA (2 μg) prepared with primer SN202 6 h after transfection. The truncated NS-CAT DNA (811 bp) was also amplified from NS-CATc DNA by PCR with SN24 and SN202 being used as primers. The vRNA was not detected in either YP1N clone, as was shown in a typical experiment (one of three independent experiments; Fig. 2B, lanes 1 and 2). In contrast, vRNAs were synthesized from cRNA in clones 64 and 76 (Fig. 2B, lanes 4 and 5). These results suggested that the PA subunit, in addition to PB1 and NP, is required for vRNA synthesis. To confirm this conclusion, monolayer cultures of cells incubated with 10^{-6} M dexamethasone for 24 h were simultaneously transfected with 100 ng of NS-CATc RNA and 3 μg of transducing plasmid pCMV-PA containing the PA gene of influenza virus linked to the cytomegalovirus promoter (5, 42) by lipofection. Seven hours later, 1 μg of total cellular RNA was amplified by RT-mediated PCR with SN202 and SN24 being used as primers as described above. As can be seen from Fig. 3 (lanes 1 and 2), vRNA was synthesized in both YP1N clones, confirming this notion.

Transcription of NS-CATv RNA in YP1N clones. PB2 is only involved in capped mRNA synthesis in transcription, and PB1, PA, and NP are sufficient for capless poly(A)$^+$-RNA synthesis as described previously (33). To analyze the function of PB1 and PA in transcription, monolayer cultures of cells incubated with 10^{-6} M dexamethasone for 24 h were transfected with 150 ng of the NS-CATv RNA-DOTAP complexes. Six hours later, total cellular RNA was prepared and poly(A)$^+$-RNA was purified from total cellular RNA with Oligotex-$(dT)_{30}$ super (Takara Shuzo). Poly(A)$^+$-RNA (2.4 μg) was resolved by electrophoresis on a 1% formaldehyde–agarose gel and transferred to a nylon membrane (29). Northern (RNA) hybridization was performed with the ^{32}P-labeled 0.7-kb *Bam*HI CAT fragment from pOUMS101 (45) being used as a probe (38, 43). Poly(A)$^+$-RNAs were found in YP1N25 and YP1N27 clones (Fig. 4, lanes 1 and 2) but not in clone YN30 (lane 3). However, the intensity of poly(A)$^+$-RNA bands prepared from YP1N25 and YP1N27 clones were weaker than those from clones 64 and 76 (Fig. 4, lanes 4 and 5). These results indicated that PB1 and NP are essential for poly(A)$^+$-RNA synthesis but insufficient for full transcriptional activity.

Concluding remarks. Reverse genetic studies with vaccinia and simian virus 40 recombinant viruses expressing the RNA polymerase subunit and NP genes (9, 19) have indicated that the PB1, PB2, and PA subunits of RNA polymerase are involved in the transcription and replication of influenza virus genome RNA. However, these methods could not define the function of individual polymerase subunits in viral transcription and replication, since CAT activity was examined after the transfection of chimeric NS-CAT RNA in cells that simultaneously expressed all three polymerase subunits and NP. The activity was not observed in cells that did not express any of these proteins.

In this report, we indicated that PB1 and NP can support

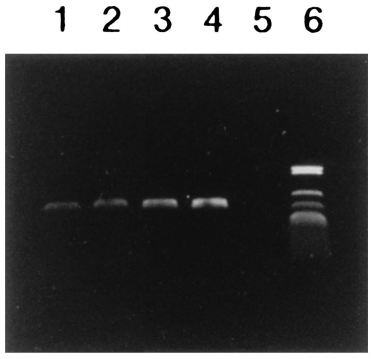

FIG. 3. Rescue of vRNA synthesis in YP1N clones transfected with PA-expressing plasmid pCMV-PA by RT-PCR. Clones YP1N25 (lane 1), YP1N27 (lane 2), 64 (lane 3), and 76 (lane 4) and parental C127 cells (lane 5) were incubated with 10^{-6} M dexamethasone for 24 h and then were simultaneously transfected with NS-CATc RNA and the pCMV-PA plasmid. After 7 h, total cellular RNA was prepared. One microgram of total cellular RNA was amplified by RT-PCR as described in the legend to Fig. 2. Lane 6, DNA size markers (1,845, 1,666, 1,050, 766, 543, 525, 517, and 506 bp).

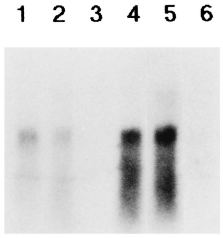

FIG. 4. Northern blot of transcription of YP1N clones transfected with NS-CATv RNA. Clones YP1N25 (lane 1), YP1N27 (lane 2), YN30 (lane 3), 64 (lane 4), and 76 (lane 5) and parental C127 cells (lane 6) were incubated with 10^{-6} M dexamethasone for 24 h and then transfected with NS-CATv RNA. Six hours later, total cellular RNAs were prepared, and then poly(A)$^+$-RNA was purified with Oligotex-$(dT)_{30}$ super. Poly(A)$^+$-RNA (2.4 μg) was resolved by electrophoresis on a 1% formaldehyde–agarose gel and transferred to a nylon membrane. Northern hybridization was performed with the ^{32}P-labeled 0.7-kb CAT fragment being used as a probe.

syntheses of cRNA and uncapped poly(A)$^+$-RNA and that PA in addition to PB1 and NP is required for vRNA synthesis with YP1N clones, which carry cDNAs for the PB1 and NP genes because of transfection with NS-CAT chimeric RNAs. The presence of a unique Asp-Asp sequence flanked by hydrophobic residues on either side and the strand-loop-strand (the β-hairpin) structure in PB1 are thought to be structural landmarks of vRNA-dependent RNA polymerase, according to sequence alignments (10). Furthermore, Asano et al. (1) found that the nucleotide binding site is located on PB1 by photoaffinity labeling with 8-azido GTP being used as a probe. These results also indicate that PB1 is involved in the catalytic activities of nucleotide elongation. In summary, the PB1 subunit is a multifunctional enzyme, performing various activities required for transcription and replication: (i) recognition and binding of promoters (transcription) and origins (replication) on vRNA segments; (ii) the binding of nucleoside triphosphate (transcription and replication); (iii) de novo initiation and elongation of RNA synthesis (transcription and replication); (iv) addition of a poly(A) tail to nascent mRNA (transcription); and (v) read-through of poly(A) signals during cRNA synthesis (replication).

Observations with temperature-sensitive mutants with mutations in the PA gene suggest that the PA subunit is involved in vRNA synthesis (27, 31, 36). Furthermore, no transcription defects in the temperature-sensitive mutants with mutations in the PA gene have been detected (13, 32, 35). These results and our results indicate that the PA subunit has two functions in contributing to the RNA polymerase complex: to achieve full activity in cRNA and mRNA syntheses and to perform vRNA synthesis. The PB1 subunit may be properly folded up with the PA subunit in the intrinsic activity of RNA polymerization. In the case of vRNA synthesis, PA acts as an allosteric modulator and causes a conformational change from a cRNA-synthesizing form to a vRNA-synthesizing form of the PB1 subunit. However, Sanz-Ezquerro et al. (39) recently reported that overexpression of PA induced a general decrease in the steady-state levels of the influenza viral or cellular proteins that were coexpressed. It remains unknown whether PA expression induces cellular proteases or the PA protein has intrinsic proteolytic activity. No significant homologies with any previously sequenced proteins, including cellular and viral proteases, could be found. Accordingly, the possibility that protease activity derived from PA may affect the function of PB1 has not been ruled out.

The possible functions of the RNA polymerase subunits in the replication and transcription of the influenza virus genome are summarized in Table 1.

TABLE 1. Requirement for RNA polymerase subunits in transcription and replication and their functions in the influenza virus[a]

Subunit	Transcription	Replication		Function(s)
		cRNA synthesis	vRNA synthesis	
PB2	+	−	−	Recognition of cap structure of host cell RNA; endonucleolytic cleavage of host mRNA?
PB1	+	+	+	Catalyzes nucleotide addition
PA	±	±	+	Allosteric modulator?

[a] +, required; −, not required; ±, PA subunit is not required for basic activities in transcription and cRNA synthesis of replication but is required for maximal activities.

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