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The RNA Polymerase PB2 Subunit Is Not Required for Replication of the Influenza Virus Genome but Is Involved in Capped mRNA Synthesis

YASUSHI NAKAGAWA,[1] NAOKI KIMURA,[1] TETSUYA TOYODA,[2] KIYOHISA MIZUMOTO,[2,3] AKIRA ISHIHAMA,[2] KINICHIRO ODA,[1] AND SUSUMU NAKADA[1]*

Department of Biological Science and Technology, Science University of Tokyo, Noda, Chiba 278,[1] Department of Molecular Genetics, National Institute of Genetics, Mishima, Shizuoka 411,[2] and School of Pharmaceutical Sciences, Kitasato University, Shirokane, Minato-ku, Tokyo 108,[3] Japan

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An established cell line, clone 64, in which the expression of the RNA polymerase PB1 and PA subunit genes and the nucleoprotein (NP) gene but not the PB2 subunit gene of influenza virus can be induced by the addition of dexamethasone, was used to analyze the replication and transcription machineries of the influenza virus. Both NS-CATc and NS-CATv, the chimeric nonstructural protein chloramphenicol acetyltransferase (NS-CAT) RNAs in the sense and antisense orientations positioned between the 5′- and 3′-terminal sequences of influenza virus RNA segment 8 (the NS gene), respectively, can be transcribed into the corresponding complementary-strand RNA in clone 64 cells only when treated with dexamethasone. Although sense-strand poly(A)$^+$ CAT RNA was detected in the dexamethasone-treated clone 64 cells transfected with NS-CATv RNA, CAT activity was not detected in these cells and the isolated poly(A)$^+$ CAT RNA was inert in an in vitro translation system. However, when the poly(A)$^+$ CAT RNA was capped by using a purified yeast mRNA capping enzyme (mRNA guanylyltransferase), the capped poly(A)$^+$ CAT RNA became translatable in the in vitro translation system. These results indicated that PB1, PA, and NP can support the replication of the influenza virus genome as well as the transcription to yield uncapped poly(A)$^+$ RNA and that PB2 is specifically required for the synthesis of capped RNA.

The influenza A virus has a genome consisting of eight single-stranded RNA segments of negative polarity, altogether encoding at least 10 viral proteins (reviewed in reference 21). Replication and transcription of the influenza virus genome are catalyzed by a virus-encoded RNA-dependent RNA polymerase (reviewed in references 14 and 21). In virions, the ribonucleoprotein cores are composed of genomic RNA, (vRNA), RNA polymerase, and nucleoprotein (NP). The RNA polymerase is composed of three subunits, PB1, PB2, and PA (reviewed in references 14 and 20), which are tightly associated at the double-stranded stem region of the panhandle formed by the 5′ and 3′ termini of each RNA segment (10, 12). RNA polymerase plays an essential role in both replication and transcription.

In virus-infected cell nuclei, three modes of RNA synthesis occur (reviewed in references 20 and 22): (i) transcription of the vRNA into mRNA which contains a cap structure at the 5′ terminus and a poly(A) tail at the 3′ terminus, (ii) the vRNA-directed synthesis of a full-length cRNA without any modifications (the first step of replication), and (iii) the cRNA-directed synthesis of vRNA (the second step of replication). For transcription, cellular RNA with the cap-1 structure is used as a primer (reviewed in reference 20). Biochemical and genetic analyses have revealed that PB1 is involved in transcription initiation and RNA chain elongation, while PB2 recognizes and binds to the cap-1 structure of the cellular mRNA (reviewed in reference 20). PA is present in the RNA polymerase complex together with PB1 and PB2, but its function has not been elucidated. NP, a major component of the ribonucleoprotein cores, is associated at every 15 to 20 nucleotides of the RNA segments (4, 41), and it is required for the efficient elongation of RNA chains (11).

In contrast, little is known about the molecular mechanism of replication. It is believed without conclusive evidence that replication requires all three subunits of RNA polymerase and NP and that it occurs in a primer-independent manner (reviewed in reference 20). In replication systems in vitro using crude extracts prepared from virus-infected cells, the first step of replication has been studied (2, 8, 36). Furthermore, a low level of the second step of replication has been detected by the use of fractionated nuclear extracts (24, 33). However, since all these systems monitored RNA synthesis directed by endogenous templates, the de novo initiation of RNA synthesis and the elongation of RNA chains initiated in infected cells were indistinguishable.

Recently, we developed the clone 76 cell line, in which all three RNA polymerase genes and the NP gene are integrated in the chromosome and can be expressed in response to dexamethasone (17, 18, 28). Exogenously added model RNAs such as chloramphenicol acetyltransferase (CAT) RNAs in the sense and antisense orientations, positioned between the 5′- and 3′-terminal sequences of segment 8 RNA encoding nonstructural (NS) proteins, can be replicated and transcribed in clone 76 cells incubated with dexamethasone. In an effort to elucidate the function of each polymerase subunit protein, we established another cell line, clone 64, which carries the PB1 and PA subunit genes of RNA polymerase and the NP gene but lacks the PB2 gene (28). In this study, chimeric NS-CAT RNAs in the sense (NS-CATc) and antisense (NS-CATv) orientations were replicated in dexamethasone-treated clone 64 cells. However, CAT activity was undetectable in NS-CATv

* Corresponding author. Present address: Molecular Medicine Research Laboratory III, Yamanouchi Pharmaceutical Co., Ltd., Tsukuba, Ibaraki 305, Japan. Phone: 81-298-52-5111, ext. 3210. Fax: 81-298-52-5412.

RNA-transfected clone 64 cells. In addition, we describe how the poly(A)$^+$ CAT RNA isolated from clone 64 cells incubated with dexamethasone lacks a cap structure at the 5′ end and how it cannot be translated in vitro with a rabbit reticulocyte system. After adding the cap-1 structure, the poly(A)$^+$ CAT RNA was translated into CAT protein. These results indicated that the PB1 and PA subunits of RNA polymerase and NP are sufficient for replication of the influenza virus genome and for transcription to yield uncapped poly(A)$^+$ RNA. The possible involvement of PB2 in cap recognition is discussed.

MATERIALS AND METHODS

Cells. Mouse C127 cells and derivative cell lines, clones 64 and 76 (28), were incubated in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum.

RT-PCR. Monolayer cultures of clones 64 and 76 at 40 to 50% confluence were incubated with 10^{-6} M dexamethasone for 6 h at 37°C, and total cellular RNA was extracted with acid guanidinium thiocyanate–phenol-chloroform (3). Twenty micrograms of total cellular RNA was amplified by reverse transcriptase (RT)-mediated PCR (39) with the synthetic oligonucleotides PB2-SN3 (5′-TACCATGGCCACAATTATTGCTTCG-3′) and PB2-SN1 (5′-ATGGAAAG AATAAAAGAACTAAGAAATC-3′), which correspond to positions 1236 to 1212 and 28 to 55 in the sense orientation of the PB2 gene, respectively, to detect PB2 mRNA. Also, PB1-SN5 (5′-CTGTCGACTCCGGCTTGAATCCCTT-3′) and PB1-SN4 (5′-GATGTCAATCCGACCTTACTTTTCT-3′), which correspond to positions 1418 to 1394 and 28 to 52 in the sense orientation of the PB1 gene, respectively, were used for RT-PCR to detect PB1 mRNA.

Immunofluorescence. Monolayer cultures at 40 to 50% confluence grown on coverslips were incubated with 10^{-6} M dexamethasone for 17 h at 37°C. The cells were washed with phosphate-buffered saline (PBS) and fixed with 2% formaldehyde–PBS at room temperature for 5 min. The cells were incubated with 100 μl of rabbit anti-PB1, anti-PB2, anti-PA (1), or anti-NP antibody at a 1:200 or 1:1,000 dilution for 30 min at room temperature, were washed three times with 0.1% Nonidet P-40–PBS, and were stained with fluorescein isothiocyanate-conjugated goat F(ab′)$_2$ anti-rabbit immunoglobulin G (H + L; Tago) at a 1:200 dilution at room temperature for 30 min. After a washing with 0.1% Nonidet P-40–PBS, the cells were mounted in 100 mM Tris-HCl (pH 8.0)–90% glycerol–1 μg of paraphenylenediamine per ml for observation under a fluorescence microscope (Olympus).

In vitro RNA synthesis. Plasmids pOUMS101 (40) and pT7/NS-CATc (the same as pT7/NS-CATm described in reference 17) were cleaved with *Mbo*II, and the T7 polymerase reaction was carried out by the standard protocol (5) with RNasin (Takara Shuzo) used as a supplement in the presence or absence of [α-^{32}P]UTP (Amersham). Template DNA was removed by DNase I (Takara Shuzo) digestion. These RNA transcripts were used after phenol-chloroform extraction.

RNA transfection. For RNA transfection, 150 ng (in 60-mm-diameter dishes for CAT assay) or 100 to 500 ng (in 100-mm-diameter dishes for Northern [RNA] hybridization) of RNA was mixed with 18 μg (for CAT assay) or 36 μg (for Northern blotting) of lipofection reagent {*N*-[1-(2,3-dioleoyloxy)propyl]-*N*,*N*,*N*-trimethylammonium-methylsulfate; DOTAP, Boehringer Mannheim}. The mixture was incubated at room temperature for 15 min; then 2.4 ml (for CAT assay) or 4.8 ml (for Northern blotting) of Dulbecco's modified Eagle's medium containing 0.21% bovine albumin was added. Cells at 40 to 50% confluence were incubated with 10^{-6} M dexamethasone for 24 h at 37°C before transfection with the RNA-DOTAP complexes described above.

Northern hybridization. Cells (in 100-mm dishes) were incubated with 10^{-6} M dexamethasone for 24 h before transfection with 150 ng of the RNA-DOTAP complexes. Six hours later, total cellular RNA was prepared as described above. Poly(A)$^+$ RNA was purified from total cellular RNA by using oligo(dT)$_{30}$-Latex (Takara Shuzo). Total cellular RNA or poly(A)$^+$ RNA was resolved by electrophoresis on 1% formaldehyde-agarose gels and transferred to nylon membranes (23). Northern hybridization was performed with the ^{32}P-labeled synthetic oligonucleotide probes, SN24 (5′-AGTAGAAACAAGGGTGTT-3′, not found in the mRNA sequence) to detect cRNA, SN25 (5′-AGCAAAAGCAGGGT GAC-3′) for vRNA, and the 0.7-kb *Bam*HI CAT fragment from pOUMS101 (40) for mRNA as described previously (32, 38).

CAT assay. Cells (in 60-mm dishes) were incubated with 10^{-6} M dexamethasone for 24 h before transfection with 150 ng of the RNA-DOTAP complexes. Six hours later, 2.5 ml of Dulbecco's modified Eagle's medium containing 0.21% bovine albumin was added and the cells were cultivated for 18 h in the absence of dexamethasone. The medium was changed to fresh Dulbecco's modified Eagle's medium containing 10% fetal calf serum, and the cells were incubated for a further 9 h. Cells were harvested at various times after transfection, and the CAT assay proceeded as described by Gorman et al. (9). About 100 μg of protein was assayed.

In vitro translation. In vitro translation was performed according to the manufacturer's (Amersham) recommendations, using 0.2 μg of poly(A)$^+$ RNAs from clones 64 and 76, as well as from the parental C127 cells described above, or 2 μg of poly(A)$^+$ RNA which was capped in vitro as described below plus L-[^{35}S]methionine and rabbit reticulocyte lysate. Fifty microliters of the reaction mixture was incubated for 1 h at 30°C and for another 10 min at 37°C. After addition of lysis buffer (50 mM Tris-HCl [pH 8.0], 150 mM NaCl, 1 mM EDTA, 0.5% deoxycholate, 1% Triton X-100, 0.1% sodium dodecyl sulfate [SDS], 1 mM phenylmethylsulfonyl fluoride, 1 μg of leupeptin per ml), the cell lysate was centrifuged for 30 min at 15,000 rpm in an Eppendorf centrifuge. The supernatant was incubated first with an anti-CAT antibody (5 Prime → 3 Prime Inc.) for 8 h at 4°C and then with protein A-agarose (Pharmacia) for 15 min at room temperature. Precipitates were loaded onto a 7.5 to 15% gradient SDS-polyacrylamide gel and analyzed with a Fujix BAS2000 (Fuji Film Inc.).

Addition of the cap structure. The capping reaction proceeded essentially as described previously (14). Four micrograms each of poly(A)$^+$ RNAs from clone 64 and 76 cells was incubated with 5 U of the mRNA capping enzyme purified from yeast cells (14) in the presence of [α-^{32}P]GTP (Amersham). After phenol-chloroform extraction and ethanol precipitation, the poly(A)$^+$ RNAs were loaded onto a 3% polyacrylamide gel and exposed to Fuji RX X-ray film.

For in vitro translation, poly(A)$^+$ RNAs were capped and methylated with 5 U of mRNA capping enzyme prepared from vaccinia virus (37) in the presence of 50 μM *S*-adenosyl-L-methionine (Boehringer Mannheim) and GTP. After phenol-chloroform extraction and ethanol precipitation, the poly(A)$^+$ RNAs were used in the in vitro translation system described above.

RESULTS

Neither the PB2 gene transcript nor PB2 protein was detectable in clone 64 cells. We established two cell lines, clones 64 and 76, in which the expression of the RNA polymerase genes and the NP gene of influenza virus can be induced in response to dexamethasone by transfection into mouse C127 cells with the hormone-inducible expression plasmids pBMSA-PB1, pBMSA-PB2, pBMSA-PA, and pBMSA-NP (28). In cultured cells of clone 64 (a mouse cell line carrying cDNAs for influenza virus RNA segments 2, 3, and 5 [28]), the PB1 and PA subunit genes of RNA polymerase and the NP gene are expressed in response to dexamethasone. On the other hand, the PB2 subunit gene is undetectable in both chromosomal and extrachromosomal fractions of clone 64 cells by Southern hybridization (28). By RT-PCR, the PB2 gene transcript was detected in dexamethasone-treated clone 76 cells (a mouse cell line carrying cDNAs for RNA segments 1, 2, 3, and 5 [28]) but not in clone 64 cells (Fig. 1, lanes 3 and 5), whereas the PB1 gene transcript was found in both clone 64 and clone 76 cells (lanes 2 and 4). Furthermore, in PCR analysis of the genomic DNA, the PB2 gene was undetectable in clone 64 cells but it was present in clone 76 cells (data not shown). We performed RT-PCR and PCR under conditions that allowed mRNA and DNA, respectively, to be detected at a level of one copy per cell. In good agreement with the RNA and DNA analyses, PB2 protein was undetectable in dexamethasone-treated clone 64 cells, but it was revealed in both the nucleus and cytoplasm of clone 76 cells by indirect immunofluorescence analysis (Fig. 2, left-hand column). PB1, PA, and NP proteins were observed in the nuclei and cytoplasm (Fig. 2).

Replication of NS-CAT RNAs in clone 64 cells. The chimeric NS-CATv RNA (vRNA sense) was transfected into both clone 64 and clone 76 cells that were incubated with dexamethasone. Total cellular RNAs were isolated 6 h after transfection, and the level of NS-CAT cRNA (cRNA sense) was analyzed by Northern hybridization. As shown in Fig. 3A, cRNAs were detected at almost the same level in clones 64 and 76. Next, the chimeric NS-CATc RNA (cRNA sense) was transfected into both cell lines and NS-CAT vRNA synthesis was examined. Northern hybridization revealed that vRNA was also synthesized from the cRNA at the same level in both cell lines (Fig. 3B). Furthermore, to confirm cRNA and vRNA syntheses from vRNA and cRNA, respectively, RT-PCR and RNase protection assays using clone 64 cells transfected with NS-CAT RNAs were performed. RT-PCR using oligonucleotides SN24

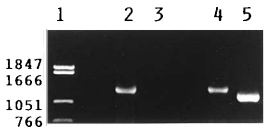

FIG. 1. Detection of PB2 and PB1 mRNAs by RT-PCR. Total cellular RNAs were prepared from clones 64 and 76 incubated with 10^{-6} M dexamethasone for 6 h. Twenty micrograms of total cellular RNAs (clone 64 cells [lanes 2 and 3] and clone 76 cells [lanes 4 and 5]) were amplified by RT-PCR using PB2-specific primers (PB2-SN3 and PB2-SN1 [lanes 2 and 4]) or PB1-specific primers (PB1-SN5 and PB1-SN4 [lanes 3 and 5]). RT-PCR conditions were as described in Materials and Methods. Lane 1, DNA size markers (sizes are indicated in base pairs on the left).

(to detect cRNA) and SN25 (to detect vRNA) and RNase protection assays using NS-CATv RNA (to detect cRNA) and SN-CATc RNA (to detect vRNA) revealed that the cRNA was synthesized from vRNA and vRNA was synthesized from cRNA in clone 64 cells (data not shown). These results indicated that exogenous viral RNA can be replicated in clone 64, as it is in clone 76.

The nature of poly(A)$^+$ RNA in NS-CATv RNA-transfected clone 64 cells. Since it is believed that the PB2 subunit is involved in generation of capped primers for viral mRNA synthesis (20), the nature of plus-stranded RNA in clone 64 cells transfected with the NS-CATv RNA was analyzed in detail. Total cellular RNAs were isolated from clones 64 and 76 6 h after transfection with the NS-CATv RNA and were then fractionated into poly(A)$^+$ RNAs with oligo(dT)$_{30}$-Latex. Poly(A)$^+$ CAT RNAs were detected in both cell lines (Fig. 3C). To negate the possibility that a little cRNA was present in the poly(A)$^+$ RNA fraction, Northern hybridization using the SN24 oligonucleotide probe was performed. No cRNA was detectable in the poly(A)$^+$ RNA fractions prepared from clones 64 and 76 (data not shown). These results indicated that clone 64 cells can synthesize poly(A)$^+$ CAT RNA from exogenous vRNA, as can clone 76. However, CAT activity was not detected in clone 64 cells transfected with NS-CATv RNA (Fig. 4, lanes 6 to 8). In separate experiments, no faint bands were evident in the NS-CATv-transfected clone 64 cells and faint bands such as those in Fig. 4, lanes 6 and 7, were sometimes detected in the negative control (data not shown). Thus, the faint bands in lanes 6 and 7 seem to be artifacts. In the NS-CATv RNA-transfected clone 76 cells, CAT activity was detected 21 h after transfection, reaching a maximum level after 27 h and slightly decreasing after 33 h (Fig. 4, lanes 2 to 4). These results indicated that the poly(A)$^+$ CAT RNA in clone 64 cells is not translatable.

Since CAT activity was undetectable in the NS-CATv RNA-transfected clone 64 cells despite the sufficient production of poly(A)$^+$ CAT RNA, we attempted to translate in vitro poly(A)$^+$ RNA prepared from the NS-CATv RNA-transfected clone 64 cells. Poly(A)$^+$ RNAs prepared from clone 64 and

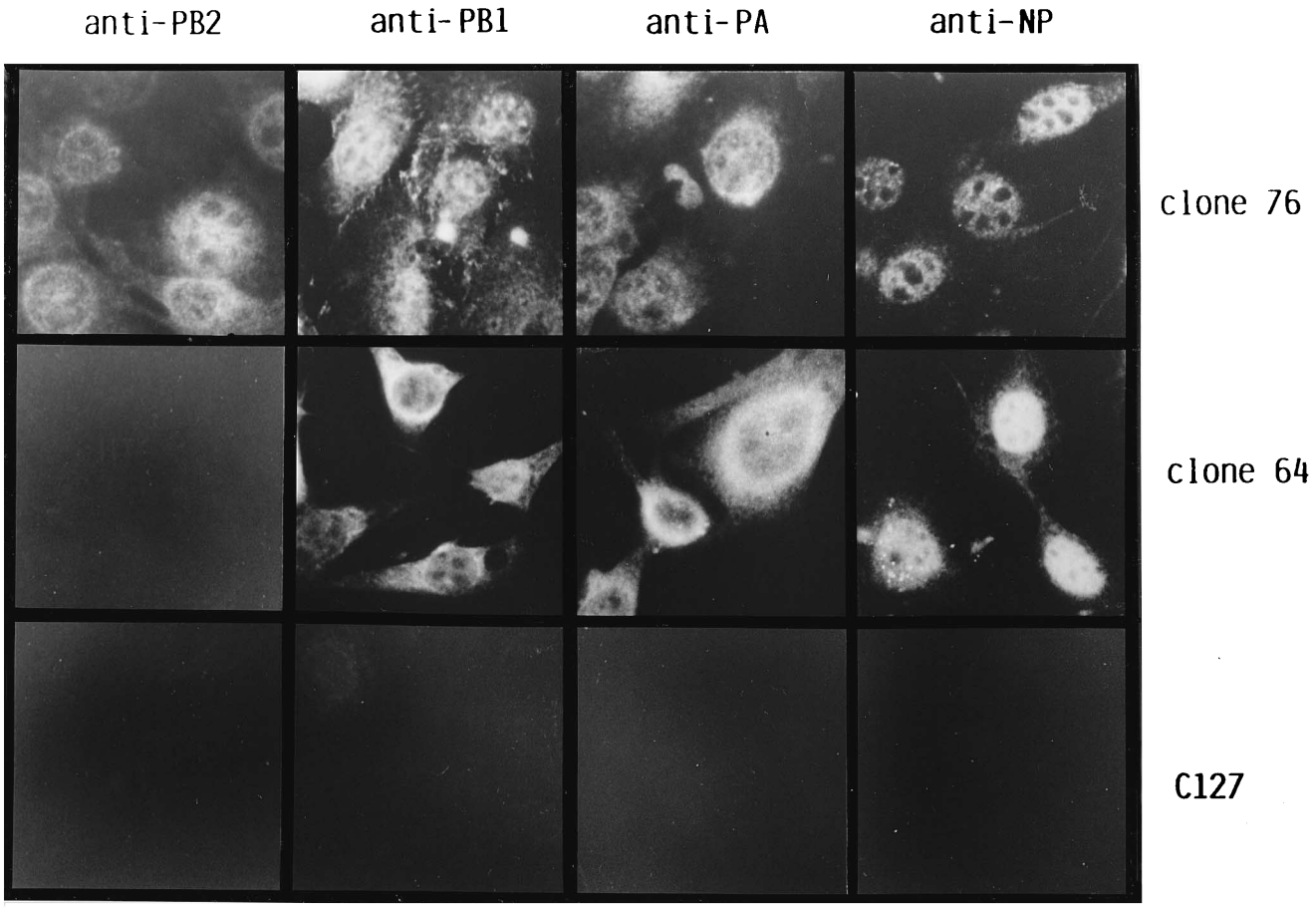

FIG. 2. Expression of RNA polymerase subunit genes and NP gene in clone 64, clone 76, and parental C127 cells. Cells cultured in the presence of 10^{-6} M dexamethasone for 17 h were incubated with anti-PB2, anti-PB1, anti-PA, or anti-NP antibodies (as indicated above the columns) for indirect immunofluorescence. The cell lines are indicated at the right of the rows.

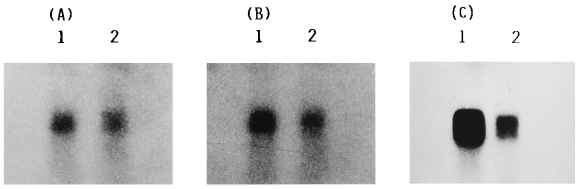

FIG. 3. Northern blots of NS-CAT RNAs in clones 64 and 76. Clones 64 and 76 incubated with 10^{-6} M dexamethasone were transfected with NS-CATv (antisense orientation) (A and C) or NS-CATc (sense orientation) (B) RNA. Twenty micrograms of total cellular RNAs (A and B) or 1.3 μg of poly(A)$^+$ RNA (C) prepared 6 h after transfection was resolved by electrophoresis on 1% formaldehyde-agarose gels. The cRNA (A), vRNA (B), and poly(A)$^+$ CAT RNA (C) transcripts were analyzed by using ^{32}P-labeled SN24 (complementary to the 3′ end of cRNA), SN25 (complementary to the 3′ end of vRNA), and CAT cDNA probes, respectively, by Northern hybridization. Lanes 1, clone 64 cells; lanes 2, clone 76 cells.

clone 76 cells were incubated in rabbit reticulocyte lysate containing [^{35}S]methionine for 60 min, and the labeled proteins were immunoprecipitated with rabbit anti-CAT antibody and analyzed by SDS-polyacrylamide gel electrophoresis. We detected a discrete band with an apparent molecular weight of 30,000 to 32,000 in immunoprecipitates from clone 76 cells, which corresponded to the size of the fusion CAT protein with an extra 19 amino acids of the N-terminal region of NS1 protein (40) (Fig. 5A, lane 1). Another band at the position corresponding to a molecular weight of 25,000 to 26,000 seemed to be the authentic CAT protein initiated from the initiation codon of the CAT gene. However, no detectable band was observed with poly(A)$^+$ RNA from clone 64 cells (lane 2) or from the parental C127 line (lane 3). These results also indicated that the poly(A)$^+$ CAT RNA in clone 64 cells cannot be translated.

Capping of poly(A)$^+$ RNA from NS-CATv RNA-transfected clone 64 cells. One possible explanation is that the poly(A)$^+$ CAT RNA in clone 64 cells does not contain a cap structure at the 5′ end. To test this possibility, the poly(A)$^+$ RNAs prepared from clones 64 and 76 were incubated with purified yeast mRNA capping enzyme in the presence of [α-^{32}P]GTP. If the cap structure is absent in the poly(A)$^+$ CAT RNA and if the RNA molecules carry either pppN or ppN at the 5′ termini, the cap structure can be added in vitro to these termini, as the purified yeast mRNA capping enzyme carries both RNA 5′-triphosphatase and RNA guanylyltransferase activities (15). After the capping reaction, the products were analyzed by electrophoresis on a 3% polyacrylamide gel containing 7 M urea. A discrete ^{32}P-labeled band was identified from clone 64 cells at a position corresponding to that for a sequence of 890

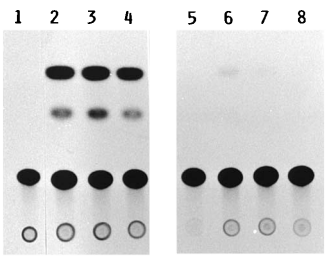

FIG. 4. Expression of NS-CATv RNA transfected in clones 64 and 76. NS-CATv RNA was transfected into dexamethasone-treated clones 76 (lanes 2 to 4) and 64 (lanes 6 to 8). The cells were harvested at 21 (lanes 2 and 6), 27 (lanes 3 and 7), or 33 (lanes 4 and 8) h after transfection. The CAT assay was performed with crude cell extracts. Lanes 1 and 5, mock-transfected clone 76 and clone 64 cells, respectively.

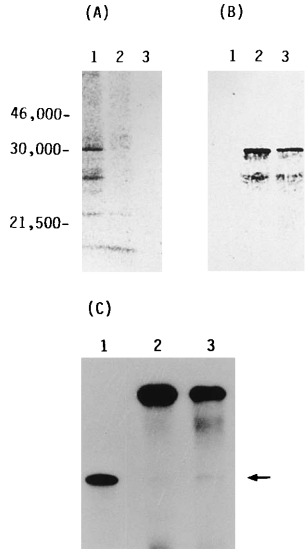

FIG. 5. In vitro translation of NS-CAT-specific poly(A)$^+$ RNA before (A) or after (B) in vitro capping. (A) Poly(A)$^+$ RNAs (0.2 μg) prepared from clone 76 (lane 1), clone 64 (lane 2), or C127 (lane 3) cells were translated in a rabbit reticulocyte lysate containing [^{35}S]methionine. (B) Poly(A)$^+$ RNAs from clone 64 (lane 2) or clone 76 (lane 3) were capped and methylated with vaccinia virus capping enzyme and were then translated in the rabbit reticulocyte lysate containing [^{35}S]methionine. The translation products were immunoprecipitated with anti-CAT antibody and were loaded onto a 7.5 to 15% gradient SDS-polyacrylamide gel. Lane 1, uncapped clone 64 poly(A)$^+$ RNA. The positions of size markers are shown at the left. (C) In vitro capping of poly(A)$^+$ CAT RNA prepared from clone 64 cells. Poly(A)$^+$ RNAs prepared from clone 64 (lane 3) and clone 76 (lane 2) cells were incubated with yeast mRNA capping enzyme and [α-^{32}P]GTP prior to analysis by electrophoresis on a 3% polyacrylamide gel containing 7 M urea. Lane 1, RNA size marker of 890 nucleotides.

bases (Fig. 5C, lane 3). This size exactly corresponded to that of the poly(A)$^+$ CAT RNA. Other bands indicating sequences shorter than 890 bases (cRNA replication intermediates) were not evident. For poly(A)$^+$ RNA from clone 76 cells, a weaker band was detected at the same position (lane 2). The capping enzyme isolated from vaccinia virus also produced similar results (data not shown). These results suggested that the level of uncapped RNA is higher in poly(A)$^+$ CAT RNA from clone 64 cells than that from clone 76.

To confirm this conclusion, a cap structure was added in vitro to the 5′ end of poly(A)$^+$ CAT RNA prepared from clone 64 cells, and its translatability was examined in vitro. Poly(A)$^+$ RNA from clone 64 cells was incubated with the capping enzyme prepared from vaccinia virus, which contains mRNA (guanine-7-)methyltransferase activity in addition to RNA phosphatase and guanylyltransferase activities, in the presence of *S*-adenosylmethionine, because a methylated cap structure is required for efficient translation (26, 27). When capped and methylated poly(A)$^+$ RNA from clone 64 cells was translated in the reticulocyte lysate, a discrete band was detected at the same position as the fusion CAT protein observed with poly(A)$^+$ RNA from clone 76 cells (Fig. 5B). These results indicated that the poly(A)$^+$ CAT RNA from clone 64 cells lacks the cap structure but that it can be capped in vitro.

DISCUSSION

In clone 76 cells, all three polymerase subunit genes and the NP gene of influenza virus can be expressed in response to dexamethasone. Under induced conditions, this cell line can complement the growth of all three types of temperature-

sensitive polymerase mutants at nonpermissive temperatures (28). Furthermore, exogenous NS-CAT chimeric RNAs can be replicated and transcribed in response to dexamethasone (17, 18).

For transfection of negative-stranded viral RNA, Huang et al. (13) have designed an artificial vaccinia virus vector-driven replication system and de la Luna et al. (7) also have described an artificial simian virus 40 vector-driven replication system. In both systems, CAT activity was detected after transfection of negative-sense CAT RNA in cells that simultaneously expressed all three polymerase subunit proteins (PB1, PB2, and PA) and NP protein. The activity was, however, not observed in cells that did not express any of these proteins.

We established the clone 64 cell line by transfection into mouse C127 cells with expression plasmids pBMSA-PB1, pBMSA-PB2, pBMSA-PA, and pBMSA-NP, in which the PB1 and PA subunit genes and the NP gene can be expressed in response to dexamethasone (28). The induced levels of the PB1, PA, and NP genes in clone 64 cells are almost the same as those in clone 76 cells (28). Since they lack PB2 protein, clone 64 cells cannot complement the growth of three polymerase temperature-sensitive mutants at nonpermissive temperatures (28). In this study, we examined the replication and transcription of an exogenous chimeric RNA of influenza virus in the clone 64 cells. Highly sensitive DNA detection methods failed to reveal the PB2 gene in clone 64 cells (28). We tried to detect the PB2 mRNA, the PB2 gene, and PB2 protein in clone 64 cells by RT-PCR, PCR, and indirect immunofluorescence analysis, respectively, but all failed. Thus, we concluded that the PB2 subunit gene is absent from clone 64 cells.

Indirect immunofluorescence showed that the proteins expressed in clone 76 (PB2, PB1, PA, and NP) and in clone 64 (PB1, PA, and NP) were localized not only in the nuclei but also in the cytoplasm (Fig. 2), even though there are intrinsic nuclear localization signals in PB1 (1, 29, 34), PB2 (16, 25, 34), PA (30, 34), and NP proteins (6). The different profiles of polymerase subunit localization and NP protein localization of the cells may reflect a difference in the nuclear transport systems.

Both cRNA synthesis from vRNA and that of vRNA from cRNA were detected in clone 64 cells incubated with dexamethasone, as they were in clone 76 (Fig. 3A and B). These results indicated that clone 64 carries an apparatus that can replicate the exogenous viral RNA and that the PB1 and PA subunit genes as well as the NP gene are sufficient for the construction of this machinery. In parallel, we examined the transcription level of exogenous NS-CATv RNA. Northern hybridization revealed that poly(A)$^+$ CAT RNA was synthesized in clone 64 cells at almost the same level as that in clone 76. Although Fig. 3C shows a higher level of poly(A)$^+$ CAT RNA in clone 64 than in clone 76, the higher level of poly(A)$^+$ CAT RNA in clone 64 was not constant. However, the poly(A)$^+$ CAT RNA in clone 64 cells could not be translated (Fig. 4 and 5), because the poly(A)$^+$ CAT RNA does not have the cap structure at the 5′ terminus. In fact, it was capped in vitro by capping enzymes isolated from yeast cells and vaccinia virus (Fig. 5C). Furthermore, after the in vitro capping with the enzyme prepared from vaccinia virus, which contains mRNA (guanine-7-)methyltransferase activity in addition to RNA phosphatase and guanylyltransferase activities, this poly(A)$^+$ CAT RNA was translated in vitro into CAT protein (Fig. 5B). Furthermore, when clone 64 cells were infected with the PB2-expressing vaccinia recombinant virus, PB2-VAC (34), CAT activity was detected after an incubation with dexamethasone (data not shown). These results indicated that PB1, PA, and NP are sufficient for the synthesis of poly(A)$^+$ RNA but that PB2 is required for cap recognition. Our results are consistent with observations that PB2 recognizes and binds to the cap structure of the cellular mRNA (for a review, see reference 20). Figure 6 shows a schematic illustration of the roles of RNA polymerase subunits in the replication and transcription of the influenza virus genome.

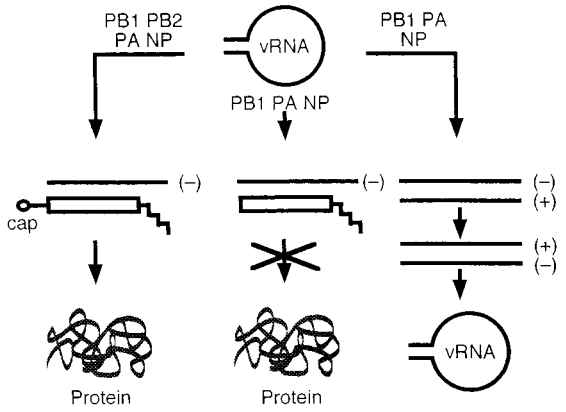

FIG. 6. Roles of RNA polymerase subunits and NP in replication and transcription of the influenza virus genome. PB1 and PA subunits of RNA polymerase and NP are sufficient for replication of the influenza virus genome (right-hand column) and transcription to yield uncapped poly(A)$^+$ RNA (middle column). The PB2 subunit is required for transcription to form capped poly(A)$^+$ RNA (left-hand column). −, vRNA; +, cRNA.

The role(s) of each subunit of RNA polymerase has been studied in vitro by using reconstituted RNA polymerase (19, 31, 35). To analyze the in vivo functions of each RNA polymerase subunit, however, our in vivo replication-transcription system using established cell lines and the model templates may be useful.

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