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The Influenza A Virus PB2 Polymerase Subunit Is Required for the Replication of Viral RNA

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The transcription and replication of influenza virus RNA (vRNA) were reconstituted in vivo. The experimental approach involved the transfection of plasmids encoding the viral subunits of the polymerase and the nucleoprotein into cells infected with a vaccinia virus recombinant virus expressing the T7 RNA polymerase. As templates, one of two model RNAs was transfected: vNSZ or cNSZ RNA. The RNAs were 240 nucleotides in length, contained the terminal sequences of the NS viral segment, and were of negative or positive polarity, respectively. The accumulation of cRNA and mRNA in cells transfected with vNSZ RNA and the accumulation of vRNA and mRNA in cells transfected with cNSZ RNA were determined by RNase protection assays with labeled vNSZ-L or cNSZ-L probes. The patterns of protected bands obtained indicated that both cRNA replication intermediate and mRNA accumulated when the system was reconstituted with vNSZ RNA. Likewise, both vRNA and mRNA accumulated after reconstitution with cNSZ RNA. The reconstitution of incomplete systems in which any of the subunits of the polymerase or the model RNA were omitted was completely negative for the accumulation of cRNA or vRNA, indicating that the presence of the PB2 subunit in the polymerase is required for replication of vRNA.

The influenza A viruses are orthomyxoviruses whose genome consists of eight molecules of single-stranded RNA of negative polarity. They encode 10 proteins, since the two smallest RNA segments code for two different products by differential splicing (for reviews, see references 26 and 28). The virion RNAs are associated with the nucleoprotein (NP) and the polymerase complex in the form of ribonucleoprotein particles (RNPs) and, once in the infected cells, are first transcribed to yield viral mRNAs. The initiation of mRNA synthesis takes place by a cap-stealing mechanism in which cellular capped hnRNAs are used to generate primers that the viral transcriptase elongates (27). The termination process is mediated by an oligo(U) signal that is present near the 5′ terminus of the viral RNA (vRNA) templates, in conjunction with the RNA panhandle structure (30, 45). Both transcription and replication occur in the nucleus of the infected cells (18, 22). The expression of viral gene products, at least the NP (3), is required for vRNA replication. Positive polarity complementary RNPs (cRNPs) are produced that are full copies of the corresponding vRNPs (17). These serve as templates for the synthesis of progeny vRNPs. The progeny vRNPs are later exported to the cytoplasm and bud from the cell surface as mature virions.

The virus polymerase consists of a heterotrimer formed by the PB1, PB2, and PA proteins (10, 11, 19, 23). Genetic, biochemical, and phylogenetic evidence have shed light about the roles of these subunits in the polymerase. The PB1 protein shares common amino acid motifs with other RNA-dependent RNA polymerases (43). In fact, mutation of these conserved residues abolished the transcriptional activity (4). In addition, extracts from baculovirus-infected cells expressing PB1 protein show some transcriptional activity in vitro (25). The PB2 subunit binds to cap structures (5, 47, 51), and antibodies specific for PB2 protein inhibit the cap-dependent endonuclease activity (29), although this activity requires the cooperation of the other polymerase subunits and the vRNA (7, 16). Moreover, the dependence of a capped-primer for in vitro synthesis is affected by mutations in the PB2 gene (40). These results suggest that PB2 protein is responsible for the transcription initiation step, as proposed earlier (6). By means of a cell line transfected with polymerase cDNAs, Nakagawa et al. concluded that PB2 protein is required for cap-dependent transcription but not for cRNA synthesis (35). The role of PA protein is not understood, but the phenotype of temperature-sensitive virus mutants suggest its involvement in vRNA synthesis (31). The only biochemical activity associated so far with this polymerase subunit is the induction of proteolysis (46), and the relationship between such an activity and vRNA synthesis is still unclear.

The polymerase subunits are associated as soluble complexes in the nucleus of the infected cells (10) and also with the ends of the vRNPs (20, 34). Expression of the P genes from cloned cDNAs leads to the formation of polymerase complexes, both in insect cells (48) and in oocytes (11). In fact, transcription and replication of model RNAs could be reconstituted in vivo by expression of the three polymerase subunits and the NP (8, 21, 24, 32). These systems have relied on the detection of chloramphenicol acetyltransferase (CAT) activity from *cat* gene-containing model vRNAs and do not allow an easy quantitative determination of the different vRNAs synthesized. In this report, we have used model vRNA and cRNA molecules to reconstitute in vivo the influenza virus transcription-replication machinery and have determined the accumulation of mRNA, cRNA, and vRNA by using RNase protection assays. Deletion experiments with such an experimental approach demonstrated that the PB2 subunit of the polymerase is required for replication of vRNA.

MATERIALS AND METHODS

Biological materials. The vaccinia virus recombinant vTF7-3 (13), capable of expressing the bacteriophage T7 RNA polymerase, was kindly provided by B. Moss. The COS-1 cell line (14) was a gift from Y. Gluzman. The preparation of plasmids pGPB1, pGPB2, and pGPA, which contain the cDNAs of the polymerase subunits (9, 37) under control of a T7 promoter in the pGEM vector, has been described previously (32). Plasmid pGNPpA was constructed by subcloning

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the open reading frame of the NP gene from plasmid pSVa963 (44) into pGEM vector under control of the T7 promoter. The pIVACAT1-S plasmid (42) was kindly provided by P. Palese.

Preparation of RNA probes. The origin of plasmid pNSZ has been described previously (39). It was generated by digestion of pIVACAT1-S plasmid with *Nco*I and *Sal*I, filling in with Klenow polymerase, and self-ligation. To generate templates for positive-polarity RNAs, a PCR product was produced with plasmid pNSZ and oligodeoxynucleotides specific for the NS ends, one of them fused to the T7 promoter sequence (T7 NS 5′+; -TAATACGACTCACTATAAGCAA AAGCAGGGTGAC-) and the other containing the recognition sequence for a *Ksp*632I site (NS 5′−; -GCGCGCGGATCCCTCTTCAAGTAGAAACAAGG GTGT-). The PCR product was purified and cloned between the *Hin*dIII and *Bam*HI sites of pUC18, generating plasmid pcNSZ. Negative-polarity template RNA (vNSZ RNA -240n-) was synthesized by in vitro transcription of pNSZ plasmid previously digested with *Hga*I endonuclease. Positive-polarity template RNA (cNSZ RNA -240n-) was synthesized by in vitro transcription of pcNSZ plasmid previously digested with *Ksp*632I endonuclease. Transcription conditions were those described for the Ambion Megascript kit. The quantification of the synthesized RNA was done by including [^{3}H]UTP in the reaction mixture, and the quality of the RNA product was checked by denaturing agarose gel electrophoresis. The synthesis of labeled probes for RNase protection assays was carried out as indicated above, except that the pNSZ or pcNSZ plasmids were digested with *Asp* 718 or *Eco*RI endonucleases and [^{32}P]GTP was included in the reaction. Thus, probes vNSZ-L RNA -264n- and cNSZ-L RNA -269n- were synthesized.

Transfections. The conditions for cell culture have been described previously (38). For infection and transfection, cultures of COS-1 cells in 60-mm-diameter dishes were infected with vTF7-3 virus at a multiplicity of infection of 5 to 10 PFU per cell. After virus adsorption for 1 h at 37°C, the cultures were washed with Dulbecco's modified Eagle's medium (DMEM) and transfected with 1.5 μg (each) of the pGPB1 and pGPB2 plasmids plus 150 ng of pGPA and 6 μg of pGNPpA as described previously (32, 33). The DNAs were diluted to 300 μl of DMEM, and, in a separate tube, cationic liposomes (1 to 3 μl per μg of DNA) were diluted to 300 μl in DMEM. The contents of both tubes were mixed, kept at room temperature for 15 min, and added to the culture plates containing 3 ml of DMEM. After 5 h of adsorption of DNA liposomes to the cells at 37°C, the cultures were washed again with DMEM and further transfected with 300 ng of vNSZ or cNSZ RNA as described above and incubated at 37°C for 14 to 16 h.

Isolation of RNAs from cultured cells. Infected and transfected cultures were washed with cold phosphate-buffered saline and lysed in 0.5 ml of a buffer containing 150 mM NaCl–10 mM EDTA–1% sodium dodecyl sulfate–100 mM Tris (pH 7.5). The DNA was sheared by passing through a G21 needle, and the mixture was treated with 100 μg of proteinase K per ml for 30 min at 37°C. After extraction with phenol-chloroform mixture and ether, the nucleic acids were precipitated with ethanol. Nucleic acids were treated with RNase-free DNase (140 U/ml) for 60 min at 37°C. After further extraction with phenol-chloroform mixture and precipitation with ethanol, the poly(A)$^+$ RNA was isolated by two rounds of oligo(dT) cellulose chromatography (1), as described previously (53), except that 1% Sarkosyl substituted for sodium dodecyl sulfate in all buffers and the binding and washing steps were done at 4°C. Retained and not retained RNAs were ethanol precipitated. Poly(A)$^-$ RNA was resuspended in hybridization buffer (80% formamide, 0.4 M NaCl, 5 mM EDTA, 40 mM PIPES [piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); pH 6.4]), heated for 5 min at 85°C, and self-annealed for 2 h at 50°C. After digestion with RNase A as described below for the RNase protection assay, the RNA was treated with proteinase K, extracted with phenol, and recovered by ethanol precipitation.

RNase protection assays. The RNase protection assay was carried out as described previously (52). In brief, aliquots of either poly(A)$^+$ RNA or self-annealed poly(A)$^-$ RNA were mixed with labeled probe (5×10^4 to 10×10^4 cpm) in a hybridization buffer containing 80% formamide, 0.4 M NaCl, 5 mM EDTA, and 40 mM PIPES (pH 6.4). The RNAs were denatured for 5 min at 85°C and hybridized for ~20 h at 50°C. Samples were diluted into 0.3 M NaCl–5 mM EDTA–10 mM Tris-HCl (pH 7.5) and digested for 1 h at 37°C with 40 μg of RNase A per ml and 300 U of RNase T_1. For size analysis, RNAs were diluted in gel loading buffer, boiled for 3 min, and run on 4% polyacrylamide–urea sequencing gels prepared under RNase-free conditions. The gels were dried, and the signals were detected by autoradiography, usually by exposure for 5 to 20 h at −80°C with intensifying screens.

RESULTS AND DISCUSSION

Characterization of vRNAs in a transcription-replication system reconstituted in vivo from viral cDNAs. An influenza virus transcription-replication system was reconstituted in vivo in a way analogous to that described previously (32), by transfection with a mixture of pGEM plasmid derivatives able to express the three subunits of the polymerase and the NP into cells previously infected with vaccinia virus recombinant virus vTF7-3 (13). We used deletion-containing versions of the NS-CAT construct present in pIVACAT1-S plasmid (42) to generate model templates of negative and positive polarity. The details for these constructs are indicated in Materials and Methods, and a diagram is shown in Fig. 1. In this way, it is possible to measure the accumulation in vivo of cRNA and mRNA in cells in which a negative polarity template (vNSZ RNA -240n-) had been transfected. Similarly, the accumulation of vRNA and mRNA can be detected in cultures in which a positive polarity template (cNSZ RNA -240n-) had been transfected. A diagram of the experimental strategy used is indicated in Fig. 2.

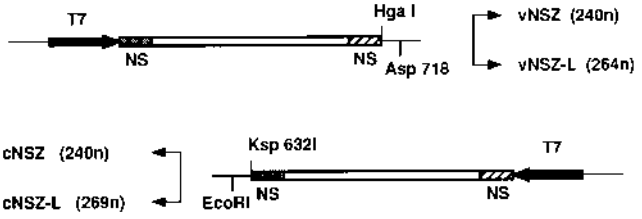

FIG. 1. Structure of the templates and probes. The figure shows a diagram of the relevant regions of plasmids pNSZ (top line) and pcNSZ (bottom line), including the T7 promoter (black arrows) directly fused to the 5′-terminal sequence of the NS segment in negative polarity (dotted box; top line) or positive polarity (tagged box; bottom line). Digestion of the plasmids with either *Hga*I or *Ksp*632I nucleases allows the synthesis of model vRNAs (vNSZ or cNSZ). Digestion with either *Asp* 718 or *Eco*RI leads to the synthesis of elongated probes (vNSZ-L or cNSZ-L) for RNase protection assays. Length in nucleotides (n) is given in parentheses.

Total cell RNA was isolated from cells infected with vTF7-3 virus, transfected with plasmids encoding the polymerase subunits and the NP, and further transfected with vNSZ RNA.

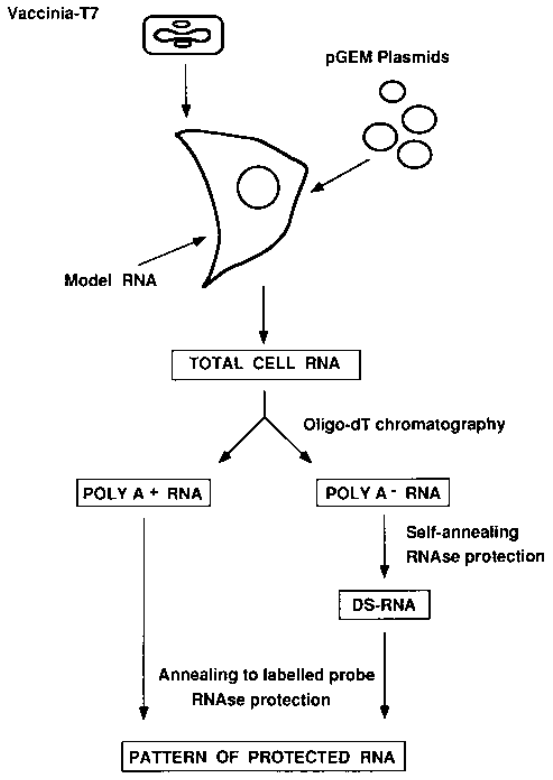

FIG. 2. Diagram of the experimental approach used in this study. (see text for details). DS-RNA, double-stranded RNA.

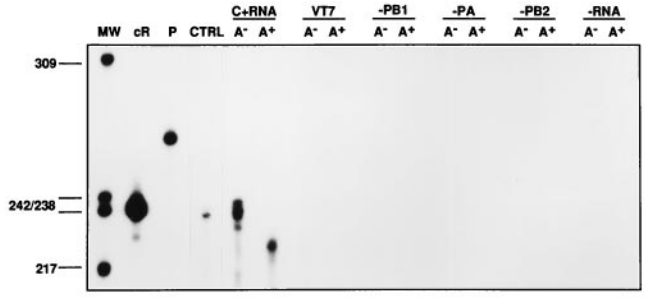

FIG. 3. Accumulation of cRNA and mRNA in a transcription-replication system reconstituted with vRNA template. COS-1 cell cultures were infected with vTF7-3 virus and transfected with either pGEM3 plasmid (VT7) or a mixture of the pGPB1, pGPB2, pGPA, and pGNPpA plasmids (C+RNA). Alternatively, the transfection mixtures were deficient in one of the plasmids, as indicated. After transfection of vNSZ RNA, total cell RNA was isolated, fractionated on oligo(dT) columns, and purified as indicated in Materials and Methods. Aliquots of each RNA sample (equivalent to a 10^6 infected and transfected cells) were assayed by RNase protection with the ^{32}-P-vRNA-L probe, as indicated in Materials and Methods. The protected RNAs were analyzed in a 4% polyacrylamide–urea gel. Slot C+RNA contains the complete system. cR represents marker cRNA generated by in vitro transcription with T7 RNA polymerase, and P indicates the undigested ^{32}-P-vRNA-L probe. CTRL represents the protection obtained with a self-annealed mixture of vRNA and cRNA synthesized in vitro with T7 RNA polymerase. A^- and A^+ denote the results obtained with poly(A)$^-$ or poly(A)$^+$ RNA, respectively. The numbers to the left indicate the length of the molecular weight markers (MW) in nucleotides.

After separation by chromatography on oligo(dT) cellulose, the poly(A)$^+$ and poly(A)$^-$ fractions were analyzed by RNase protection assays. Since the latter RNA fraction contains a large excess of unspecific RNA, it was first self-annealed and treated with RNase A to select for the vRNA-cRNA hybrids present in the sample. The results of the protection assay when ^{32}-P-vNSZ-L probe was used are presented in Fig. 3. As a positive control, a mixture of vNSZ RNA and cNSZ RNA was self-annealed, treated with RNase A, and included in the assay (Fig. 3, CTRL). Such a control sample gave rise to a protection band of approximately the same size as the cRNA marker (Fig. 3, cR). When the RNAs isolated from cells in which the complete system had been reconstituted were analyzed in the same assay, the protection bands obtained matched the predictions; i.e., poly(A)$^-$ RNA led to the same protection band as the vRNA-cRNA control (Fig. 3; compare CTRL with C+RNA), having the same size as the cRNA marker. Other minor protected species were also detected, which had lengths a few nucleotides longer or shorter than that of the cRNA marker. They were also present in the control and can be interpreted as the result of digestion of the probe at sensitive sites before or after the major one. On the other hand, poly(A)$^+$ RNA produced a protection band some 10 to 20 nucleotides smaller. These results indicate that cRNA and mRNA are produced in the reconstituted system.

The accumulation of mRNA, cRNA, and vRNA in vivo is dependent on PB2 protein expression. To determine which viral elements are required for the synthesis in vivo of cRNA and mRNA, poly(A)$^+$ and poly(A)$^-$ RNAs were isolated from cells in which a deficient system had been reconstituted, lacking each of the single elements that constitute the complete system. These RNAs were analyzed by RNase protection assay as described above. None of the incomplete combinations led to the accumulation of detectable cRNA or mRNA (Fig. 3). This lack of biological activity in the incomplete systems cannot be explained by instability of the proteins expressed under these conditions, since each of the polymerase subunits is stable when expressed independently (15, 33, 39, 40, 46, 54). It was expected that elimination of either PB1 or vRNA would abolish any transcription and replication, because no template or polymerase activity would be available, but the requirement of PB2 for synthesis of cRNA contrasts with the results reported by Nakagawa et al. (35), who found that a cell line expressing only the PB1 and PA subunits of the polymerase, but not PB2 protein, was able to direct the synthesis in vivo of cRNA and noncapped mRNA upon transfection of model vRNA.

If the PB2 subunit of the polymerase were indeed required for replicative synthesis, it would be predicted that the accumulation of vRNA should also need the presence of PB2 protein in the reconstituted system in vivo. To test such a prediction, a viral replication system was reconstituted in vivo in a way analogous to that described above but with the replicative intermediate cRNA used as the template. Under these conditions, the reconstituted system should be able to direct the synthesis of progeny vRNA and, eventually, the production of mRNA (secondary transcription). Thus, cultures of COS cells were infected with vaccinia virus vTF7-3 virus, transfected with the recombinant plasmids encoding the polymerase and the NP, and further transfected with cNSZ RNA. Total cell RNA was isolated, fractionated on oligo(dT) cellulose, and analyzed by RNase protection assay. Two different probes were used: vNSZ-L for detection of mRNA in the poly(A)$^+$ fraction and cNSZ-L for detection of vRNA in the poly(A)$^-$ fraction. The results are presented in Fig. 4 and 5. The protection of the ^{32}-P-vNSZ-L probe (Fig. 4, P) with poly(A)$^+$ RNA (Fig. 4, C+RNA) lead to a band 10 to 20 nucleotides shorter than the cRNA marker (Fig. 4, cR), in agreement with the predictions. For detection of vRNA, the poly(A)$^-$ fraction RNA was self-annealed and treated with RNase as indicated above (see Materials and Methods and Fig. 2) to eliminate unspecific RNA and then was used for protection of ^{32}-P-cRNA-L probe (Fig. 5, P). As a control sample, a mixture of vNSZ and cNSZ RNAs transcribed with T7 RNA polymerase was also self-annealed and included in parallel in the experiments. Protection with this control sample (Fig. 5, CTRL) gave a band with a size identical to that of the cRNA marker (Fig. 5, cR). Likewise, protection with the RNA isolated from the

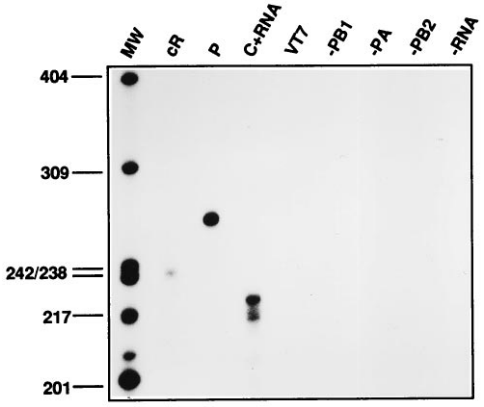

FIG. 4. Accumulation of mRNA in a transcription-replication system reconstituted with cRNA template. Cultures of COS-1 cells were infected and transfected as indicated in the legend to Fig. 3. All transfected cultures were further transfected with cNSZ RNA, except one (−RNA). Total cell RNA was isolated, and the poly(A)$^+$ RNA was purified on oligo(dT) columns as indicated in Materials and Methods. Aliquots of each RNA sample were assayed by RNase protection with ^{32}-P-vRNA-L probe. The protected RNAs were analyzed with a 4% polyacrylamide–urea gel. All symbols are as described in the legend to Fig. 3.

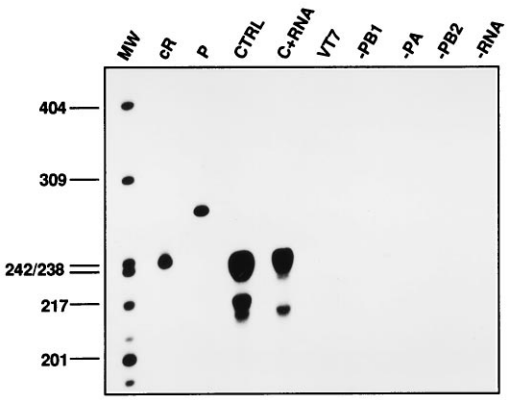

FIG. 5. Accumulation of vRNA in a transcription-replication system reconstituted with cRNA template. Cultures of COS-1 cells were infected and transfected as indicated in the legend to Fig. 3. All transfected cultures were further transfected with cNSZ RNA, except one (−RNA). Total cell RNA was isolated and the poly(A)$^-$ RNA was purified on oligo(dT) columns and self-annealed as indicated in Materials and Methods. Aliquots of each RNA sample were assayed by RNase protection with ^{32}-P-cRNA-L probe. The protected RNAs were analyzed with a 4% polyacrylamide–urea gel. All symbols are as described in the legend to Fig. 3.

complete system (Fig. 5, C+RNA) produced a band with mobility identical to that of the control sample and the cRNA marker. These results indicated that the system reconstituted with cNSZ as the template is capable of replication to yield vNSZ RNA and, furthermore, is able to use the progeny RNA for transcription. Therefore, the system reconstituted with cNSZ RNA as a template can be used to assay which viral genes are needed for the second step of the replication process (i.e., the synthesis of vRNA from cRNA).

Deficient systems in which any single viral element was lacking were reconstituted in vivo. Poly(A)$^+$ and poly(A)$^-$ RNAs were isolated from these cultures and were analyzed by RNase protection assay as indicated above. The results are presented in Fig. 4 and 5. The elimination of any subunit of the polymerase or the template RNA led to no detectable accumulation of either mRNA (Fig. 4) or vRNA (Fig. 5). These results are in contrast with those from a recent publication by Nakagawa et al. (36). Nakagawa et al. established cell lines transfected with PB1- and NP-expressing plasmids and transfected them with positive- or negative-polarity model RNAs. Based on reverse transcription (RT)-PCR analyses, they concluded that PB2 protein expression is dispensable for either cRNA or vRNA synthesis. The reasons for the discrepancies between Nakagawa et al.'s results and those presented in this report are not known at present. It is possible that, given the sensitivity of the RT-PCR technique, they have detected a residual activity of deficient polymerase complexes not seen by RNase protection assays. The expression system used in the present report was optimized for activity (33) and allows the reconstitution of highly active polymerase. Under these conditions, it is possible to detect very readily the accumulation of mRNA, cRNA, and vRNA by RNase protection assays. However, no signal was observed when PB2 protein expression was omitted. We cannot exclude that a minimal activity escaped detection. However, if that were the case, we feel it would not be relevant for influenza virus infection.

Concluding remarks. The results presented in this report indicate that the presence of the PB2 subunit in the influenza virus RNA polymerase is required for the replication of vRNA, both in the first step (synthesis of cRNA from a vRNA template [Fig. 3]) and in the second step (synthesis of vRNA from a cRNA template [Fig. 5]). However, the body of evidence in the literature indicates that PB2 protein is involved in transcription initiation, because this subunit can be cross-linked to cap analogs (5, 47, 51), antibodies against PB2 protein affect transcription initiation (2) or endonuclease activity (29) in vitro, and mutations in the PB2 gene alter the usage of capped primers in transcription reactions in vitro (40). Therefore, the requirement of PB2 protein for replication may be indirect; i.e., the presence of PB2 in the complex may alter the conformation of either PB1 or PA proteins or may affect their interaction. In this context, it is worth mentioning that all available evidence indicates that the subunits of the polymerase interact to form a trimeric structure (11, 19, 49). The interactions among the subunits in the complex have been studied by several groups. Direct binding of the PB2 and PA proteins to the PB1 subunit has been detected (11), and the regions involved in such interactions have been mapped (15, 40, 41, 50, 55). Although no direct interaction between PB2 and PA proteins has been detected (11, 54) and the binary interactions PB1-PB2 and PB1-PA can take place in the absence of the third component of the complex (15, 41, 50, 55), it is still unclear whether the interactions of PB2 and PA proteins with PB1 are independent. In addition, it has been reported that PB2 protein can be cross-linked to vRNA (12). Such interaction may be involved not only in transcription but also in replication of vRNA.

In summary, an experimental approach has been reported that allows the detection of all types of influenza virus RNAs in a system reconstituted from cloned viral proteins. Because of its sensitivity and versatility, it is especially suited to study the phenotype of mutants affected in the polymerase subunits and the NP.

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