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# Determination of Influenza Virus Proteins Required for Genome Replication

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**An artificial vaccinia virus vector-driven replication system for influenza virus RNA has been developed. In this system, a synthetic NS-like gene is replicated and expressed by influenza virus proteins supplied through infection with vaccinia virus recombinant vectors. The minimum subset of influenza virus proteins needed for specific replication and expression of the viral ribonucleoprotein was found to be the three polymerase proteins (PB1, PB2, and PA) and the nucleoprotein.**

The orthomyxoviruses are a group of enveloped viruses possessing a negative-strand segmented RNA genome. The influenza A viruses contain eight RNA segments, with more than half of the genome coding for proteins presumed to play active roles in the transcription and replication of the genomic RNA. The viral polymerase consists of a complex of three proteins (PB2, PB1, and PA) along with the nucleoprotein (NP), which is also involved in the assembly of the ribonucleoprotein (RNP) molecule (16). Because of their high-level expression and their presence in the nucleus, which is the site of viral replication (12, 16, 18, 35), it has been suggested that the two virus-encoded nonstructural proteins, NS1 and NS2, play a role in replication. In vitro systems capable of transcribing mRNA from genomic RNP, in use for many years, have shown that the PB2 protein is responsible for binding to the cap structure of host mRNA and the PB1 protein is involved in elongation of mRNA molecules (1, 3, 33). However, the slow development of in vitro replication systems and the limited availability of conditional lethal mutants has greatly hampered our ability to dissect the replication process. Our limited knowledge stems from studies using virus-infected cell extracts (1, 13, 27). From these studies, it has been shown that the presence of the nucleoprotein is required for full-length synthesis of the positive-strand template and the subsequent copying of this template to create genomic vRNA (2, 29).

In recent years, vaccinia virus vectors have been developed to express foreign proteins for immunological studies and to explore structure-function relationships of specific proteins (21, 25, 26). Recombinant vaccinia virus vectors have also been used to express mixtures of viral proteins in an attempt to study virus replication, assembly, and growth (10, 14, 24). In this study, mixtures of recombinant vaccinia viruses expressing individual influenza virus proteins have been used to artificially assemble a functional and specific replication system for influenza virus RNA. Previously, we have described an assay for the transcription, replication, and rescue of a synthetic NS gene-like RNA or an intact viral gene in influenza virus-infected cells (8, 20). In this system, synthetic RNA is added to purified viral polymerase to assemble virus-like RNP molecules (23). These particles are then transfected into influenza virus-infected cells and subsequently replicated by the proteins produced by the superinfecting influenza virus. Although this system is competent for the study of the various replication and packaging signals present in the viral RNA (through site-specific mutagenesis of the synthetic RNA), the need for superinfecting influenza virus makes it difficult to examine the functions of the individual proteins during replication. Here, we describe a completely artificial replication system in which, rather than cells being infected with influenza virus, synthetic RNPs are replicated in cells through the action of influenza virus proteins expressed by recombinant vaccinia virus vectors. In this way we show that the only influenza virus proteins essential for transcription and replication of the template RNP are the three polymerase proteins and the nucleoprotein.

In order to facilitate analysis, a cDNA clone which can produce an influenza virus-like viral RNA molecule coding for a reporter gene was used. This NS-like viral RNA contains the antisense sequence of the coding region of the chloramphenicol acetyltransferase (CAT) gene in place of the antisense coding regions for the nonstructural proteins, NS1 and NS2 (20). This recombinant RNA (IVACAT-1) was incubated with purified influenza virus polymerase complex and used in an attempt to develop a non-influenza virus-dependent replication system. Mouse fibroblast C127 cells were infected with mixtures of recombinant vaccinia viruses (25) and transfected 1 h later with the IVACAT-1 RNP. Mixtures of vectors expressing the three polymerases (PB2-VAC, PB1-VAC, and PA-VAC) and the nucleoprotein (NP-VAC) were used (32). Replication and transcription of the synthetic RNP was assayed by analyzing cells for CAT activity after overnight incubation. Figure 1 shows the CAT activity present in cells initially infected with many of the possible mixtures of the four recombinant vaccinia viruses. A positive control in which the influenza A/WSN/33 virus was used in lieu of the recombinant vaccinia viruses was included (Fig. 1, lane 4). CAT activity was present in this sample as well as in cells infected with all four vaccinia virus vectors (Fig. 1, lanes 8 and 10). Cells expressing any of the subsets of these four proteins did not produce detectable CAT protein (Fig. 1, lanes 5, 6, 7, 9, and 11). In addition, transfected RNA not incubated with the purified polymerase was also negative for CAT expression (20). Thus, the presence of the PB2, PB1, PA, and NP proteins is all that is necessary and sufficient for RNP expression and replication in this system.

A number of other cell lines were then tested as hosts for this vaccinia virus-driven system. These included MDBK, HeLa, 293 (11), and L cells. In each case, CAT activity was

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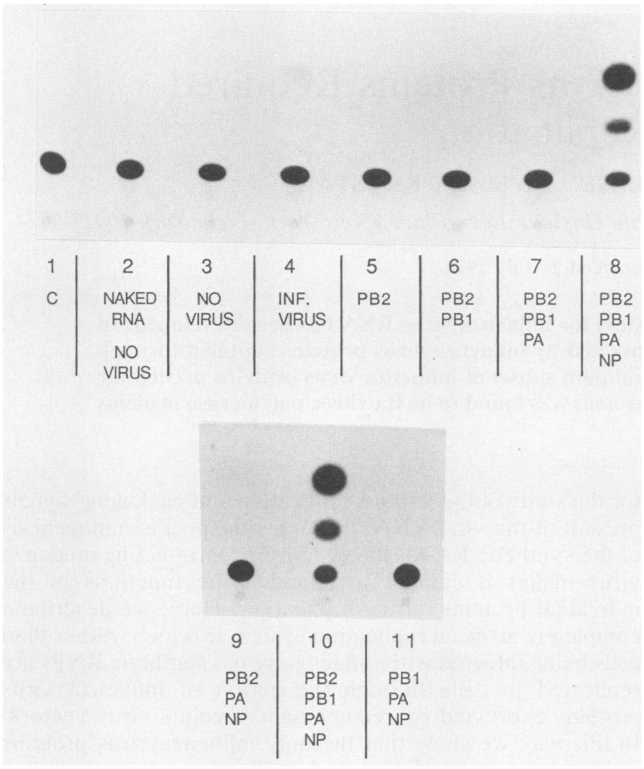


FIG. 1. CAT expression in vaccinia virus-infected IVACAT-1 RNP-transfected cells. Approximately $10^6$ mouse C127 cells in 35-mm-diameter dishes were infected with mixtures of recombinant vaccinia viruses (32) at a multiplicity of infection of 5 to 10 for each vector. After 1.5 h, synthetic IVACAT-1 RNP was transfected into the virus-infected cells as described elsewhere (20). Cells were incubated overnight, harvested, and assayed for CAT activity by standard procedures (9). The assays contained 0.05 μCi of [$^{14}$C]chloramphenicol, 20 μl of 40 mM acetyl coenzyme A (Boehringer Mannheim Biochemicals), and 50 μl of cells extracts in 0.25 M Tris buffer (pH 7.5). Incubation times were approximately 4 h. The labels under the lane numbers indicate the treatment of cells. Lanes: 1, control; 2, naked RNA transfection (no polymerase added), no helper virus infection; 3, RNP transfection, no helper virus; 4, RNP transfection, influenza virus as helper; 5 through 11, RNP transfection, vaccinia virus vectors as helper viruses expressed the indicated influenza virus proteins.

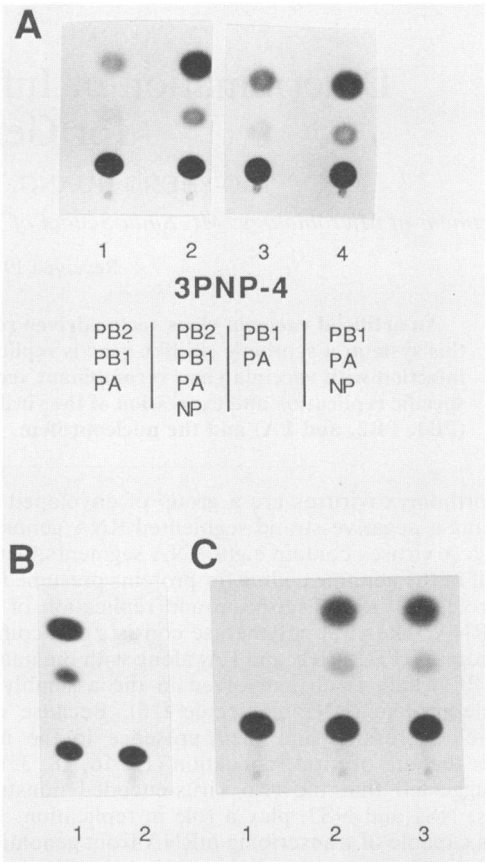


FIG. 2. (A) 3PNP-4 cells were infected with vaccinia virus vectors expressing the PB2, PB1, PA, and NP proteins (lane 2) or various subsets of the four vectors, transfected with IVACAT-1 RNP, and examined for CAT activity as described in the text. The influenza virus proteins expressed in each sample are listed under each lane. (B) 293 cells (11) were infected with the four required vaccinia virus vectors and transfected with synthetic RNP made by using IVACAT-1 (lane 1) or IVACAT-2 (lane 2) RNA. After overnight incubation, cells were harvested and CAT assays were performed. (C) Recombinant vaccinia virus-infected C127 cells were transfected with synthetic RNP examined for CAT activity after overnight incubation. Lane 1, IVACAT-9 RNP transfected into uninfected cells; lane 2, IVACAT-9 RNP transfected into cells infected with the four recombinant vaccinia vectors; Lane 3, IVACAT-1 RNP transfected into cells infected with the four recombinant vaccinia virus vectors.

observed only when cells were infected with vectors that expressed the three polymerase proteins and NP (data not shown). Previously, a cell line (designated 3PNP-4) which constitutively expresses low levels of the PB2, PB1, and PA proteins and high levels of the NP protein was constructed. These cells can complement the growth of *ts* mutants mapping either to the PB2 or NP gene segments (17, 19). Since replication through recombinant vaccinia virus vectors is dependent only on these proteins, it was conceivable that this cell line is able to amplify and express the synthetic CAT-RNP in the absence of any virus infection. However, when this experiment was attempted, no detectable CAT activity was obtained (data not shown). In order to investigate the reasons this cell line did not support replication, mixtures of recombinant vaccinia viruses were used to infect 3PNP-4 cells. As expected, the addition of the four polymerase proteins supported the expression of CAT (Fig. 2A, lane 2). However, CAT activity was also obtained in cases when less than the full complement of vaccinia virus recombinants was used. Indeed, the minimum mixture of vectors needed to obtain CAT activity in 3PNP-4 cells were those expressing only the PB1 and PA proteins (Fig. 2A, lane 2). Therefore, the steady-state levels of PB2 and NP proteins in 3PNP-4 cells are sufficient but the levels of PB1 and PA are below the threshold for CAT expression in the absence of helper virus. This correlates with the complementation phenotype exhibited by these cells, since only the growth of PB2 and NP mutants and not PB1 and PA mutants is significantly complemented at nonpermissive temperature (19).

Since the synthetic IVACAT-1 RNA is of negative polarity, CAT can be synthesized only via transcription off the RNP molecule. Theoretically, detectable levels of CAT can be produced either through transcription off the transfected input RNP (equivalent to primary transcription) or through transcription off amplified RNA (necessitating RNP replication). Previous work using influenza virus infection to drive the expression of the CAT protein showed that detectable

expression occurred only if the input CAT-RNP was replicated (20). This was shown by the use of a second CAT-RNA, IVACAT-2, which contains three mutations within the 12 bases at the 5′ end of the viral RNA (20). This 12-base region is conserved among all eight gene segments in all influenza A viruses (7). This synthetic IVACAT-2 RNP is transcribed in vitro by the influenza virus polymerase, but it is not replicated, and when it is transfected into influenza virus-infected cells, CAT activity remains undetected (20). Therefore, primary transcription off the input RNA does not produce detectable levels of protein in influenza virus-infected cells.

Accordingly, we used this mutant RNA to examine whether the vaccinia virus vector-expressed influenza proteins induce CAT activity solely through primary transcription of the input RNP or whether they allow for amplification through replication and subsequent transcription. 293 cells (which routinely express the highest level of CAT activity) were infected with the recombinant vaccinia viruses and then transfected with either IVACAT-1- or IVACAT-2-generated RNPs. Low levels of CAT activity were detected in cells transfected with IVACAT-2 RNP (Fig. 2B, lane 2). When quantitated, 0.5 to 1% of the chloramphenicol was converted to an acetylated form, compared with 0.2 to 0.4% in mock-transfected lanes (not shown). However, much greater levels of activity were present in cells transfected with IVACAT-1 RNP (lane 1; routinely 15 to 50% conversion of chloramphenicol), indicating that amplification was occurring in these cells. Additional evidence for RNP replication comes from the use of another synthetic CAT molecule, designated IVACAT-9 (W. Luytjes, unpublished data). The RNA contains, in 5′-to-3′ order, the entire 5′ noncoding region of the NS gene, the CAT-coding region in the plus sense, a short linker sequence, and the entire 3′ noncoding region of the NS gene. Although transcribed in vitro off a separate cDNA, this RNA represents the cRNA replicative intermediate of IVACAT-1 RNA. This RNA can theoretically express CAT by direct ribosome translation, when it is packaged into RNP and transfected into C127 cells, no activity is detected (Fig. 2C, lane 1). However, when this RNA is transfected into cells in which viral proteins have been supplied with the mixture of recombinant vaccinia viruses, CAT activity is induced (Fig. 2C, lane 2). This implies that the influenza virus polymerase proteins can amplify the expression of CAT, presumably by the continued replication of this cRNA template and subsequent transcription of the vRNA. These data together provide strong evidence that the recombinant vaccinia virus-driven system is sequence specific and that the RNPs are undergoing replication.

In the experiments described here, neither the NS1 nor NS2 protein was required for RNP replication. Although the function of these proteins is not known, it has been speculated that they play a major role in replication, because both proteins are synthesized in large amounts and are present in the nucleus (12, 16, 18, 35). Therefore, whether the addition of one or both of these proteins would have an effect on the levels of CAT expressed from the synthetic NS-like IVACAT-1 was tested. When vaccinia virus vectors expressing either the NS1 or NS2 protein (or the two together) were added to the functional mixture of the four vaccinia virus vectors, the level of CAT gene expression was not significantly altered, indicating that in this system these proteins have little if any effect on IVACAT-1 replication and expression (Fig. 3). Since the above-described experiment now requires the coinfection of up to six separate vaccinia virus

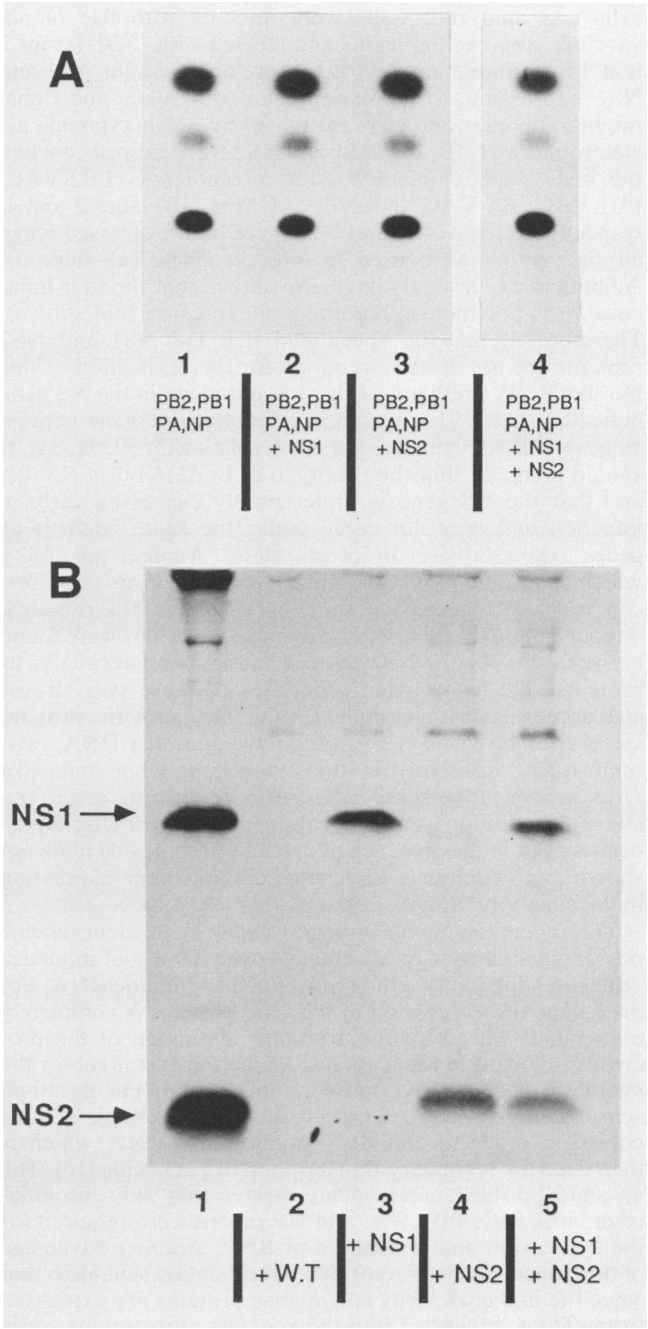


FIG. 3. NS1 or NS2 proteins do not alter expression levels of the NS-like CAT construct. (A) C127 cells were infected with mixtures of vaccinia virus vectors, transfected with IVACAT-1 RNP, and assayed for CAT activity after overnight incubation. Cells were infected with vectors that expressed the influenza virus proteins listed below each lane. (B) Polyacrylamide gel electrophoresis analysis of the expression of NS1 and NS2 in vector-infected cells. C127 cells in 35-mm$^2$ plates were infected with VAC-PB2, VAC-PB1, VAC-PA, VAC-NP, and VAC-NS1, VAC-NS2, or the wild-type WR vaccinia virus strain. Cells were analyzed for NS1 or NS2 expression by immunoprecipitation and electrophoresis on a 10% polyacrylamide gel. Lane 1, Influenza virus infection control. Other lanes show the four vaccinia virus vectors plus WR strain (lane 2), VAC-NS1 (lane 3), VAC-NS2 (lane 4), and VAC-NS1 and VAC-NS2 (lane 5).

recombinants in a single cell, protein expression in these cells was analyzed. Cells were infected with five or six vaccinia virus recombinants and labeled with [$^{35}$S]Met for 3 h at 3 h postinfection. Extracts were analyzed for NS1 and NS2 expression by immunoprecipitation with polyclonal rabbit antibodies and were examined by polyacrylamide gel electrophoresis (10, 28). NS1 and NS2 were expressed when the cells were coinfected with recombinants PB2-VAC, PB1-VAC, PA-VAC, and NP-VAC (Fig. 3B, lanes 3 and 4, respectively), and NS1 and NS2 were both expressed when all six vectors were used to infect a single cell (lane 5). Additional experiments have also shown that the four influenza virus polymerase proteins are expressed (not shown). Therefore, it may be speculated that the NS1 and NS2 proteins are not directly required for the replication of this NS-like RNP. Previous work with mutations in the NS gene indicated that NS1 may function in late viral-gene expression, viral RNA synthesis, or RNA splicing (5, 30, 31, 34). It should be noted that the genotype of IVACAT-1 is NS-like and that the NS gene is preferentially expressed early in infection and may not come under the same controls as genes expressed later in infection (28). Another possibility which cannot be ruled out is that a vaccinia virus protein(s) can replace a necessary function of these NS proteins, although upon inspection, no obvious similarities were found between the NS1 or NS2 protein and known vaccinia virus proteins. The contrasting properties of these two viruses also argues against a complementing vaccinia virus protein, since vaccinia virus is a large double-stranded DNA virus replicating exclusively in the cytoplasm, while influenza virus is a negative-sense RNA virus replicating in the nucleus. In addition, the replication of the synthetic RNPs occurs even in the presence of cytosine arabinoside (data not shown), an inhibitor of replication and late gene expression in vaccinia virus (6, 15, 22).

This recombinant vaccinia virus vector-dependent scheme possesses a number of advantages over the use of influenza virus infection to drive the replication of synthetic RNA. For one, since the expression of the viral proteins is completely artificial, it will allow for a precise dissection of the processes involved in replication. Replication first involves the synthesis of a positive-sense template from the negative-sense genomic RNA. This positive-sense cRNA is then copied in order to amplify genomic-sense RNP, which is then used for secondary transcription or packaging (16). The system described herein demonstrates that only the influenza virus PB2, PB1, PA, and NP proteins are required for the expression and replication of RNP. Another advantage of this vaccinia virus vector-driven replication scheme is that since the influenza virus polymerase proteins are expressed from cDNA integrated into the vaccinia virus, mutagenesis of the polymerase proteins becomes a feasible and powerful method of further analyzing structure-function relationships of the viral polymerase proteins. Also, we are currently attempting to rescue infectious influenza virus through the transfection of mixtures of reconstituted viral RNPs and the use of vaccinia virus vectors as helper viruses.

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