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The Influenza Virus Nucleoprotein Synthesized from Cloned DNA in a Simian Virus 40 Vector Is Detected in the Nucleus

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We obtained DNA sequences coding for the nucleoprotein (NP) of an influenza A virus by reverse transcription of virion RNA with synthetic oligonucleotide primers. Terminal sequence analysis showed that the cloned gene contained a full-length copy of the virion RNA segment. The NP-specific DNA was inserted into the late region of a simian virus 40 vector, and the DNA recombinant was propagated in the presence of an early simian virus 40 temperature-sensitive mutant helper. Infection of African green monkey kidney cells with the recombinant produced a polypeptide immunoprecipitable with NP-specific antisera. The polypeptide product had a molecular weight of 56,000, identical to that of the nucleoprotein of influenza virus as estimated on polyacrylamide gels. The putative NP was detected in the nucleus of infected primate cells by an immunofluorescence assay. This nuclear localization of NP from recombinant DNA was similar to that seen during influenza virus infection.

Influenza virus nucleoprotein (NP), serologically characterized as a type-specific antigen that distinguishes type A, B, and C viruses, constitutes a major internal component of the virion (18). NP interacts with viral genomic RNA to form helical ribonucleoprotein complexes (5, 7, 15). These complexes, in association with the three viral polymerase proteins, are transcriptionally active, generating viral mRNA species (17). Monocolonal antibodies specific to NP affect mRNA transcription in vitro, and at least three nonoverlapping antigenic determinants on the viral polypeptide have been demonstrated (23). Influenza virus temperature-sensitive mutants defective in the NP gene fail to synthesize mRNA or virion RNA in infected cells at restrictive temperature (11, 20, 21). These data suggest that NP is directly or indirectly involved in transcription and replication. Recent evidence indicates that the nucleus is the site of influenza mRNA transcription and that NP is primarily present in the nucleus of infected cells (3, 6, 9, 10). Studies on another negative-strand virus, vesicular stomatitis virus, suggest that its NP may play a role in the regulation of viral replication through a specific binding to vesicular stomatitis virus RNA (1); however, analogous experiments have not been carried out with influenza virus. Many important questions concerning the structural features and involvement of NP in transcription remain to be elucidated. In this report, we describe cloning of the full-length NP gene and characterization of the viral polypeptide produced in African green monkey kidney (AGMK) cells during infection with the cloned gene inserted into a simian virus 40 (SV40) vector.

Virion RNA was prepared from the egg-grown virus as described previously (12) and used to prepare double-stranded DNA for cloning. As shown in Fig. 1, the first cDNA strand was synthesized by reverse transcription of RNA by using a synthetic dodecanucleotide primer (AGCAAAAGCAGG) complementary to the conserved 3′ terminus. After removal of the virion RNA from the hybrids (0.2 N NaOH at 70°C for 30 min), the cDNA strand was converted to double-stranded DNA by using another dodecanucleotide primer (AGTAGAAACAAG) which corresponds to the 5′-common sequence of virion RNA (12, 16, 26). The DNA duplexes were separated on polyacrylamide gel, and the fifth largest DNA segment was isolated for characterization of the NP gene by restriction enzyme digestion and for cloning in the *Escherichia coli* system. The NP gene was inserted into the late region between the *Hpa*II and *Bam*HI sites of SV40 in an expression vector with a *Bam*HI linker. In this vector, the *Hpa*II site of SV40 was converted to a *Bam*HI site that could be used for insertion, and the *Taq*I site had been converted into an *Xba*I site for joining with pBR322; an *Xba*I site had been introduced previously into pBR322 (4) (kindly given by P. Gruss). The cloned NP DNA was further characterized by digestion with restriction enzymes.

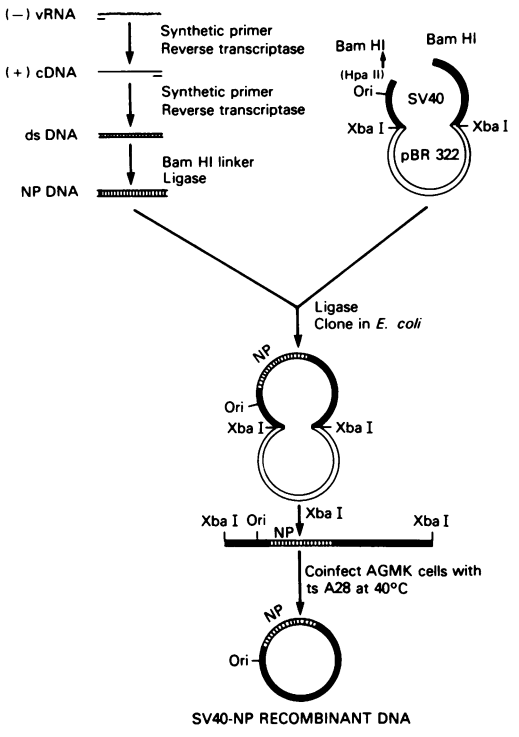

FIG. 1. Scheme for cloning influenza virus NP DNA and construction of SV40-NP recombinant.

The map positions of restriction enzyme sites, including *Pst*I, *Bgl*II, and *Pvu*I, corresponded with those of NP genes from other influenza viral strains (8, 22, 25). To ascertain that our NP clone contained a full-length copy of the vRNA segment, we sequenced the terminal nucleotides of the DNA segment. At both termini, the conserved nucleotide sequences of virion RNA were completely represented (Fig. 2) (16). Similar to the NP sequence of A/PR/8/34(H1N1) and A/NT/60/68(H3N2), the ATG initiation codon on the sense strand was located at positions 46 to 48 and was followed by an identical coding sequence of eight amino acids in the region sequenced. At the 3′ end of the sense strand, identical sequences were also present in the cloned gene, and a termination codon was therefore inferred from other NP DNA sequences described.

To express NP polypeptide, we used the SV40 vector of Gruss et al. (4) and constructed an SV40-NP recombinant that contained an NP DNA insert in the sense orientation relative to the late transcription of SV40. The SV40 moiety lacked the late region between the *Hpa*II and *Bam*HI cleavage sites; therefore, all the late RNA splice sequences were absent in this vector. The SV40-NP recombinant yielded a predicted fragment of 1262 base pairs after *Pst*I digestion at the unique *Pst*I site in the NP DNA and in the pBR322-SV40 vector. Linear DNA of SV40-NP was prepared from the plasmid after *Xba*I digestion and was subsequently used for infection of AGMK cells in the presence of an early temperature-sensitive mutant of SV40 (*ts*A) (Fig. 1). The construction of SV40-NP recombinant virus was essentially identical to the procedure described previously (19).

AGMK cells infected with the SV40-NP recombinant were analyzed for the production of the NP polypeptide. Two separate approaches were employed: one with polyacrylamide gel electrophoresis to analyze the molecular size of the product and the other with an indirect immunofluorescence assay to detect the intracellular location of the polypeptide. In the first experiment, confluent AGMK cells (2×10^5) were infected with the recombinant for 72 h and labeled with [^{35}S]methionine (20 μCi; specific activity, 1,300 Ci/mmol) for 5 h. Cell lysates were subsequently prepared in 400 μl of RIPA buffer (0.3 M NaCl, 1% deoxycholate, 1% Triton, 0.1% sodium dodecyl sulfate, 0.1 M Tris [pH 7.4], 1 mM phenylmethylsulfonyl fluoride, 5,000 U of trasylol). Immunoprecipitation was carried out with 10 μl (70 ng) of a mixture of

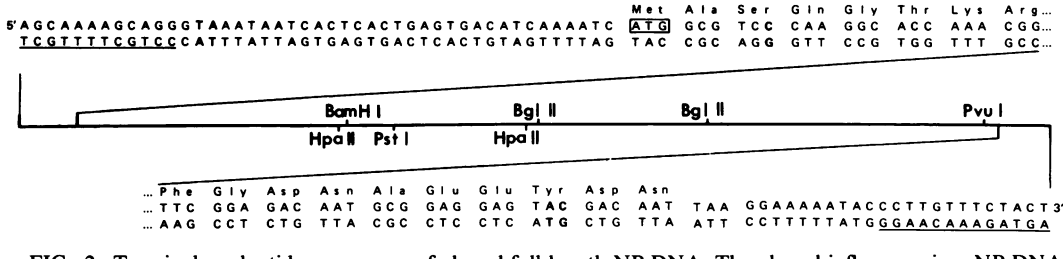

FIG. 2. Terminal nucleotide sequences of cloned full-length NP DNA. The cloned influenza virus NP DNA was mapped by restriction enzyme digestion, and terminal nucleotides were sequenced by the method of Maxam and Gilbert (14). Shown in the linear map are several endonuclease cleavage sites. Both oligonucleotide primers completely represented in the cloned gene are underlined. These sequences are identical to the previously published data from which the predicted amino acid sequences are indicated.

three monoclonal antibodies specific to three distinct epitopes of NP (5/1, 3/1, and 7/3; kindly provided by K. van Wyke) and a 10-μl slurry of protein A-Sepharose in phosphate-buffered saline. The immunoprecipitates were analyzed on sodium dodecyl sulfate-polyacrylamide gels (Fig. 3). All three independent isolates of SV40-NP recombinant yielded a labeled band of 56,000 daltons, equivalent in size to the NP produced during influenza virus infection. No such labeled polypeptide was found in SV40-infected cells (data not shown). Other minor bands were not related to the NP recombinant, since they were also present in the uninfected cell control. The putative NP represented the only polypeptide product of the cloned NP insert in the SV40 expression vector. Our cloned NP gene appeared to contain a complete coding sequence, and translation of NP transcript occurred at the initiation codon at nucleotide positions 46 to 48 of the NP gene, since NP synthesized from SV40-NP recombinant DNA-infected cells was identical in size to that produced during influenza virus infection.

Although there is no direct functional assay currently available for influenza virus NP, many earlier studies indicated that NP is found inside the nuclei of infected cells (2, 13, 24). To obtain further information concerning the functional activity of NP from SV40-NP recombinant, we tested whether the NP product is also detected in the nuclei of infected cells. Monolayers of AGMK cells were infected with SV40 alone, with influenza virus A/Udorn/72(H3N2), or with the SV40-NP recombinant for a period prescribed. Infected cells were fixed with acetone, reacted with the monoclonal antibody mixture to increase the sensitivity of detection, and stained with a fluorescein conjugate of goat anti-mouse serum. As a control, no immunofluorescence was detected in SV40-infected cells. On the other hand, as early as 1.5 h after infection with influenza virus, cells showed intense nuclear fluorescence, indicating that NP was predominantly detected in the nucleus; little or no NP was found in the cytoplasm (Fig. 4A). The appearance of NP during infection with the SV40-NP recombinant was followed kinetically. At 36 to 60 h, NP was largely detected in the nucleus, and the pattern of nuclear fluorescence was identical to that seen during influenza virus infection (Fig. 4B). Despite a low multiplicity of infection (1% of cells infected), detection of NP in the nucleus was observed repeatedly. However, later after infection (72 to 96 h) with the recombinant, immunofluorescent staining of NP became diffuse and was detected in the cytoplasm as well. One interpretation is that the accumulation of NP in the cytoplasm is a result of cellular dysfunction, since SV40 infection is cytopathic at this late stage. These results indicate that the NP product from the cloned gene exhibits the same nuclear localization observed during influenza virus infection. Since no other influenza viral polypeptides or virion RNA segments are present in the infected cell, the specific influx into the nucleus is likely a functional

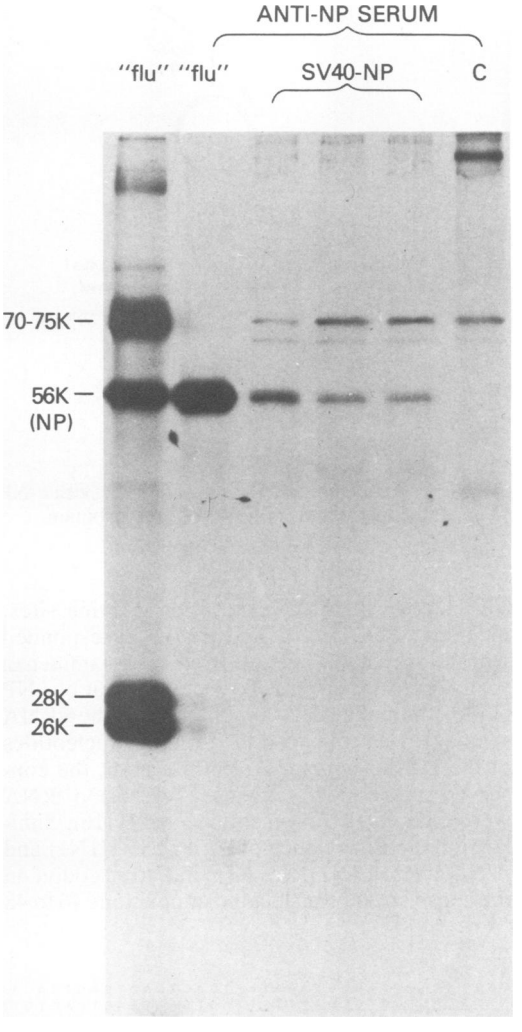

FIG. 3. Polyacrylamide gel analysis of influenza virus nucleoprotein. Lysates were prepared from [^{35}S]methionine-labeled AGMK cells previously infected with influenza virus A/Udorn(H3N2) ("flu") or three separate isolates of SV40-NP recombinant virus. Uninfected cell control (C) lysate was prepared in a similar manner. Immunoprecipitation was carried out with a mixture of three monoclonal antisera specific for NP (5/1, 3/1, and 7/5), and the labeled precipitate was analyzed on sodium dodecyl sulfate-15% polyacrylamide gels (acrylamide:bisacrylamide = 30:1). Molecular size markers of influenza viral proteins are indicated.

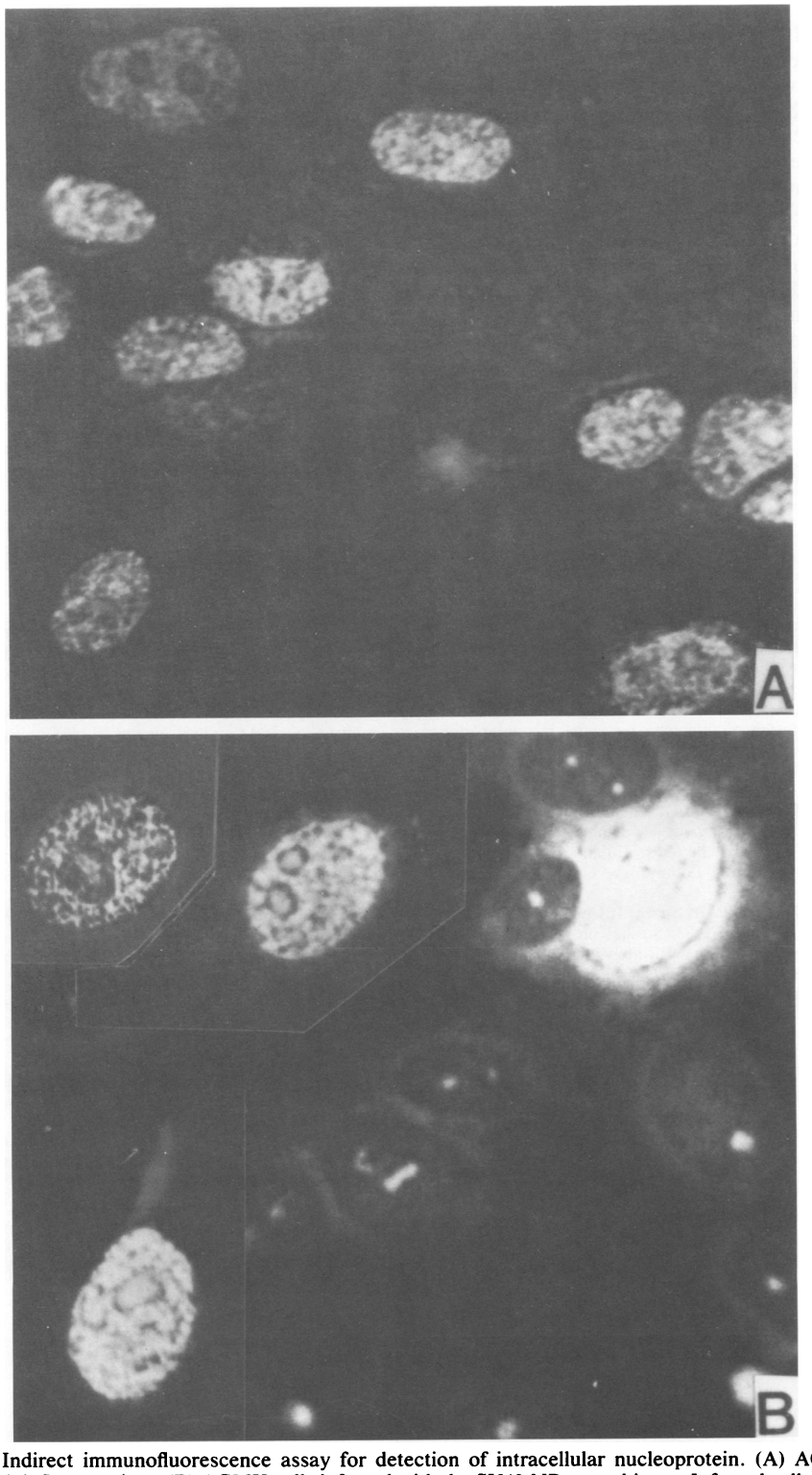

FIG. 4. Indirect immunofluorescence assay for detection of intracellular nucleoprotein. (A) AGMK cells infected with influenza virus. (B) AGMK cells infected with the SV40-NP recombinant. Infected cell monolayers were fixed with acetone and incubated with a mixture of three monoclonal antibodies specific to NP and a fluorescein conjugate of goat anti-mouse serum. Infected cell nuclei are intensely stained. The nucleoli of uninfected cells were brightened by staining with ethidium bromide to locate the nuclear contour.

property of NP. The specific detection of the NP polypeptide in the nucleus is markedly different from the previous observations concerning influenza virus hemagglutinin that accumulates at the cell surface (19). In summary, the present study permits the following conclusions. First, when inserted into an SV40 expression vector, the cloned full-length NP DNA produced an immunoprecipitable polypeptide identical in molecular size to influenza virus NP. Second, the putative NP detected in the nucleus early after recombinant DNA infection was analogous to the NP produced during influenza virus infection, suggesting that the NP product exhibits at least one of its many functional characteristics.

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