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Biochemistry

Functional expression in primate cells of cloned DNA coding for the hemagglutinin surface glycoprotein of influenza virus

(simian virus 40 vector/glycosylation/hemagglutination/surface protein)

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ABSTRACT **We have used simian virus 40 (SV40) DNA as a vector for expression of functional activity of a cloned influenza viral DNA segment in primate cells. Cloned full-length DNA sequences coding for the hemagglutinin of influenza A virus (Udorn/72/[H3N2]) were inserted into the late region of a viable deletion mutant of SV40, and the hybrid DNA was propagated in the presence of an early SV40 mutant (*ts*A28) helper. Infection of primate cells with the hybrid virus produced a polypeptide similar in molecular size to the hemagglutinin of influenza virus, as shown by immunoprecipitation and gel electrophoresis. The polypeptide was glycosylated, as shown by incorporation of radioactive sugars. The putative hemagglutinin exhibited functional activity, as shown by agglutination of erythrocytes. In addition, an indirect immunofluorescence assay showed that the hemagglutinin polypeptide of the hybrid virus could be detected on the surface of infected cells.**

Influenza virus acquires its two virion glycoproteins, hemagglutinin (HA) and neuraminidase, during the final stage of viral maturation at the cell surface. The HA, encoded by viral gene segment 4 (1–4), is a trimeric molecule (HA_0 subunit, M_r 70,000–75,000) that projects from the surface of the virion (5). This surface projection causes erythrocytes to agglutinate (6) and is responsible for the binding of virus to cell surface receptors during the initial stage of infection (7). Immunological response to the HA antigen is a primary factor in determining the host's resistance or susceptibility to the virus (8–10). Alteration in the HA (and to a lesser extent, the neuraminidase) is a prominent feature of new antigenic variants of influenza A virus responsible for pandemics or epidemics (11, 12).

An understanding of the molecular basis of influenza HA is emerging. Analysis of the amino acid sequence suggests that there are defined regions or domains in the HA polypeptide responsible for its specific functional properties and for attachment of glycosyl residues (13–15). Specific changes of amino acids in the protein molecule are found in antigenically drifted strains of influenza virus (11, 12, 16). More recently, the 3Å structure of the HA glycoprotein has been elucidated (17). It is important, however, to obtain direct evidence demonstrating the correlation between the HA polypeptide domains and their proposed functions. Both specific and crossreactive antigenic determinants in the HA molecule that give rise to humoral as well as cellular immunological responses remain to be defined. It is also important to further examine the functional involvement of hemagglutinin in viral infectivity as it relates to virulence and to transmissibility among animal species.

We have recently devised procedures to clone DNA sequences of influenza virus by using cDNA duplexes derived from genomic RNA and cytoplasmic viral mRNA (18). Sequence analysis indicates that several of the DNA clones contain full-length copies of genomic RNA segments (19, 20). Thus, these full-length DNA segments could potentially produce viral transcripts that have all the control signals for viral gene expression in eukaryotic cells. The use of cloned influenza DNA sequences for expression of functional activity should allow us to investigate several of the aspects of influenza virus mentioned above. As a first step, we attempted to produce influenza HA polypeptide in primate cells from the cloned full-length HA DNA inserted in a simian virus 40 (SV40) vector. In this report, we describe the construction of HA–SV40 DNA recombinants and the characterization of functional activities exhibited by the HA polypeptide product.

MATERIALS AND METHODS

Virus and Cells. Influenza virus strain A/Udorn/72 (H3N2) was used for cloning of full-length DNA derived from genomic RNA and cytoplasmic viral mRNA (18). Wild-type SV40 and a derived viable deletion mutant dl *2330* (21) were also used. Primary African green monkey kidney (AGMK) cells were purchased from Flow Laboratories (McLean, VA) and were passaged once or twice in Eagle's minimal essential medium/10% fetal calf serum. Confluent AGMK cells infected with either SV40 or constructed HA–SV40 recombinant virus were maintained in minimal medium/2% fetal calf serum as indicated.

Construction of HA–SV40 Recombinant Virus. Full-length HA-specific DNA was obtained from *Pst* I digestion of pFV88 that contains HA DNA at the *Pst* I site of pBR322 (18). To position the HA DNA segment into the late region of SV40 for gene expression, form I DNA of SV40 mutant dl *2330* was digested with *Hae* II/*Bam*HI, and the resulting large DNA fragment (A fragment) was isolated (see Fig. 1). Both HA DNA (10 μg) and the *Hae* II/*Bam*HI A fragment of dl *2330* (5 μg) were mixed and digested with nuclease S1 at 45°C for 30 min to remove the protruding terminal nucleotides from the DNA fragments. After phenol extraction and alcohol precipitation, the DNA components were dissolved in 100 μl of ligase reaction buffer (70 mM Tris base, pH 7.6/10 mM $MgCl_2$/1 mM ATP/15 mM dithiothreitol containing gelatin at 20 μg/ml and 5 units of T4 DNA ligase). The ligase reaction was carried out at 4°C overnight. DNA products from the ligase reaction were then used to transfect AGMK cells. SV40 *ts*A28 helper was added to the transfected cells and the co-infected cells were incubated at 40°C, a restrictive temperature for this SV40 mutant. This procedure was similar to the infectious center assay described elsewhere (22). Viral plaques that appeared 12–14 days postinfection were isolated, and virus stock was prepared from each isolate after

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Abbreviations: HA, hemagglutinin; SV40, simian virus 40; P_i/NaCl, phosphate-buffered saline; bp, base pair(s).

further infection of AGMK cells. To assay for the presence of HA-SV40 recombinant virus, small molecular weight DNA from AGMK cells infected with individual virus stocks was prepared according to the Hirt procedure (23), separated on an agarose gel, and blotted onto nitrocellulose paper. HA DNA-specific sequences were detected by hybridization with a ^{32}P-labeled cDNA probe derived by treating influenza genomic RNA with reverse transcriptase (RNA-dependent DNA nucleotidyltransferase) (24). All experiments that require P3 containment according to the National Institutes of Health recombinant DNA research guidelines were performed at the P3 facility of the Georgetown University Division of Molecular Virology and Immunology (Rockville, MD), kindly made available to us by John Gerin.

Radiolabeling and Analysis of Polypeptides. AGMK cells infected with either influenza A virus for 14 hr or HA–SV40 recombinant virus for 72 hr or uninfected control cells were starved for 1 hr in minimal medium lacking methionine or glucose. The infected cells were rinsed with phosphate-buffered saline (P_i/NaCl) and then incubated with either methionine-free minimal medium containing [^{35}S]methionine (100 μCi/ml; 1 Ci = 3.7×10^{10} becquerels) for 6 hr or glucose-free minimal medium containing [^{3}H]mannose, [^{3}H]fucose, and [^{3}H]glucosamine (100 μCi/ml of each) for 2 hr. Extracts were made from labeled cells by using RIPA buffer (1% Triton X-100/1% deoxycholate/0.15 M NaCl/0.1% NaDodSO$_4$/0.1 M Tris base, pH 7.6/1 mM phenylmethylsulfonyl fluoride and 5000 units of Trasylol) and centrifuged at 40,000 rpm for 40 min to remove insoluble cell debris. To 500 μl of a cell lysate, 5 μl of sheep antiserum to purified hemagglutinin (hemagglutination-inhibition titer of 1:512) was added, and the mixture was incubated for 3 hr at 4°C. An equal volume of a slurry of protein A-Sepharose beads (1:1) in 0.1 M phosphate, pH 7, was then added, and the mixture was shaken in an Eppendorf shaker for 20 min. The protein A-Sepharose immune complexes were washed four times with RIPA buffer and suspended in 25 μl of sample buffer (125 mM Tris base, pH 6.8/5% NaDodSO$_4$/20% glycerol/0.002% bromphenol blue). Samples were analyzed on 15% polyacrylamide gels (acrylamide/bis acrylamide, 30:0.8) buffered with 375 mM Tris base, pH 8.3/0.2% NaDodSO$_4$. Autoradiographs were made from gels that were fixed in 10% acetic acid/50% methanol for 60 min and exposed on Kodak X-Omat AR film at −70°C (25).

Agglutination of Erythrocytes. Extracts of AGMK cells infected with either HA–SV40 recombinant virus or SV40 virus were prepared. Briefly, equal numbers of the infected cells were suspended in P_i/NaCl and disrupted with 25 strokes in a Dounce homogenizer. Serial dilution of the extracts was made in microtiter wells. An equal volume of a 0.5% suspension of guinea pig erythrocytes was added to each well, and the agglutination reaction was allowed to take place at room temperature.

Indirect Immunofluorescence Assay. AGMK cells grown on microtiter slides and infected with HA–SV40 recombinant virus and SV40 *ts*A28 helper or SV40 alone were incubated at 40°C for 72 hr. AGMK cells infected with influenza A virus were incubated for 14 hr at 37°C. Cells were fixed in cold acetone for 10 min and air dried. Sheep hemagglutinin antiserum or normal sheep serum was adsorbed to the cells for 30 min at room temperature and the excess was rinsed off with P_i/NaCl. Fluorescein isothiocyanate-conjugated rabbit anti-sheep IgG serum was then adsorbed for 30 min at room temperature and again the excess conjugate was rinsed off with P_i/NaCl. Cells treated in this manner were designated as "fixed" cells; infected cell cultures that were not fixed with acetone but washed with P_i/NaCl to remove medium and stained in a manner similar to the "fixed" cells were designated "unfixed" cells.

RESULTS

Construction of HA–SV40 Hybrid Virus. SV40 DNA has been used as a vector to introduce cloned DNA species into mammalian cells (26, 27). Regions of the SV40 genome responsible for various viral functions are well characterized, and the entire genomic DNA sequence of SV40 is known. Furthermore, the SV40 genome contains transcriptional and posttranscriptional signals that can be used to generate stable RNAs in infected cells. It would therefore be an attractive vector to introduce cloned complete influenza DNA into mammalian cells. AGMK cells infected with a recombinant virus of SV40 carrying cloned influenza DNA segments could yield influenza viral RNAs that specify influenza viral polypeptides. Furthermore, synthesis of influenza viral RNA sequences generated in this host system are potentially useful for reassortment of viral gene segments and for genetic rescue of defective influenza mutants during co-infection of AGMK cells with a recombinant SV40 virus that contains the corresponding functional influenza gene.

We chose a viable deletion mutant of SV40, dl *2330*, for insertion of the full-length HA DNA into the late region of the SV40 genome (21). This mutant lacks ≈240 nucleotides in the small tumor antigen region and another 180 nucleotides in the late leader region. Thus, this mutant could provide an additional 420-nucleotide packaging capacity not normally available in wild-type SV40. Coupled with the excision of the *Hae* II to *Bam* HI segment of the late region of dl *2330*, this vector should accommodate the entire HA DNA, which is ≈1850 base pairs, including the deoxyguanosine/deoxycytosine joining segments (18). The procedure for construction of the HA–SV40 recombinant virus is outlined in Fig. 1. From one plaquing experiment, 18 viral plaques were isolated. Subsequent analysis

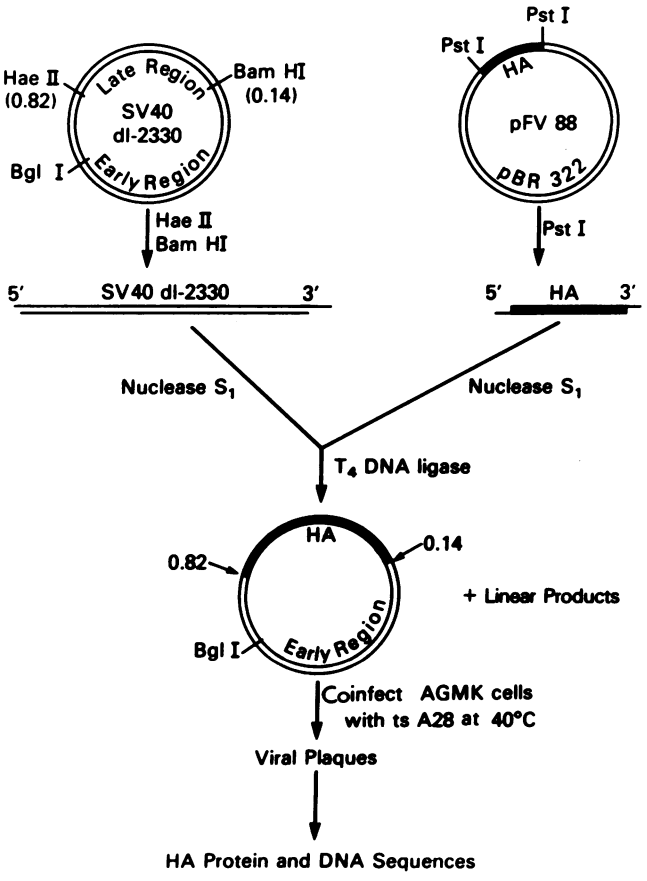

FIG. 1. Construction and isolation of HA–SV40 hybrid virus.

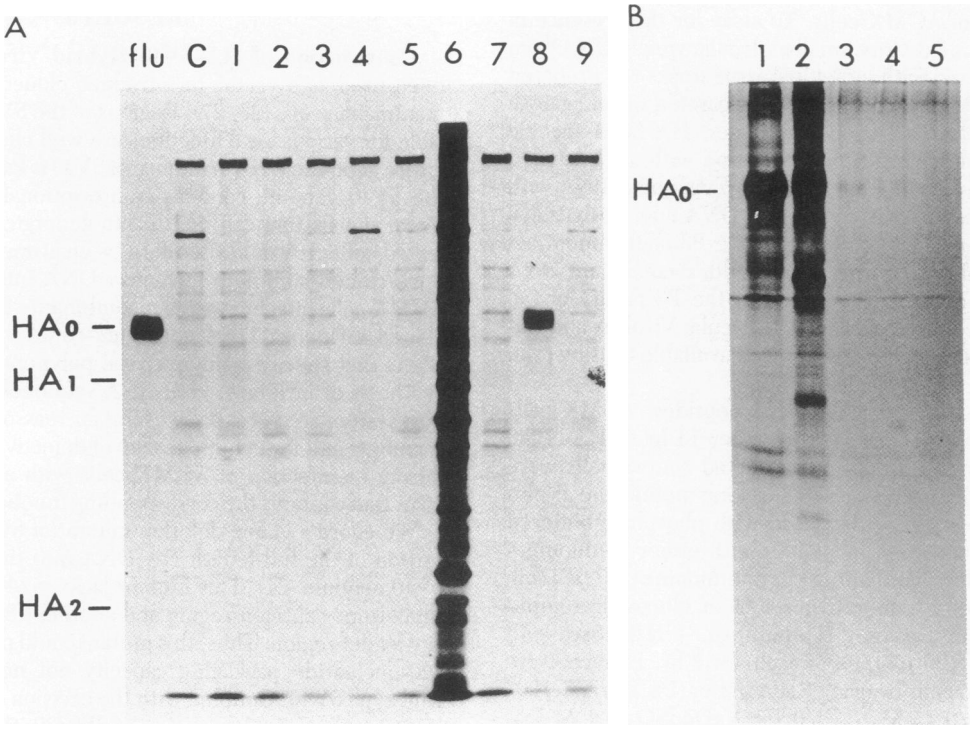

FIG. 2. Analysis of intracellular polypeptides. Plaque isolates of HA–SV40 hybrid virus were screened for HA production in infected cells. (*A*) Cells were labeled with [^{35}S]methionine and lysates prepared in RIPA buffer were immunoprecipitated and analyzed by NADodSO$_4$/polyacrylamide gel electrophoresis. Lanes: 1–9, virus isolates that were positive for HA DNA sequences; flu, HA immunoprecipitated from AGMK cells infected with influenza A virus; C, lysate from uninfected cells. Lane 6 was unintentionally overloaded. (*B*) Cells infected with influenza A virus,, hybrid virus isolate 8, or SV40 were labeled with a mixture of [^{3}H]mannose, [^{3}H]fucose, and [^{3}H]glucosamine, and lysates were analzyed as in *A*. Lanes: 1, influenza virus infection labeled with [^{35}S]methionine; 2, [^{35}S]methionine-labeled HA-SV40; 3, [^{3}H]sugar-labeled influenza virus HA; 4, [^{3}H]sugar-labeled HA-SV40; 5, [^{3}H]sugar-labeled SV40.

showed that 9 of these isolates contained influenza viral DNA sequences (data not shown). These viral isolates therefore contained SV40–influenza HA hybrid genomes.

Synthesis of HA Glycoprotein. As one of our objectives for constructing the HA-SV40 hybrids was the synthesis of functional HA in eukaryotic cells, we examined polypeptide production in AGMK cells infected with the nine HA–SV40 isolates. Proteins from cells labeled with [^{35}S]methionine were immunoprecipitated with HA antiserum and analyzed by NaDodSO$_4$/polyacrylamide gel electrophoresis (Fig. 2*A*). Influenza virus infection of AGMK cells in the absence of added trypsin produced a labeled protein band of 70,000–75,000 daltons corresponding to the uncleaved HA (HA$_0$). Two faint but definite bands representing HA$_0$ cleavage products, HA$_1$ (45,000 daltons) and HA$_2$ (30,000 daltons), were also discernible. One of the nine HA–SV40 hybrids tested (gel lane 8) showed a prominent labeled band equivalent in molecular size to that of HA$_0$. In addition, this labeled band exhibited a broad migration pattern that is characteristic of many glycosylated polypeptides in NaDodSO$_4$/polyacrylamide gels. Other labeled polypeptides on the gel were nonspecifically precipitated, as they were also present in the uninfected cell control. In gel lane 8, cleaved HA$_1$ and HA$_2$ were not detected. These findings indicate that one of nine HA–SV40 hybrid viruses produced a putative HA polypeptide in infected AGMK cells. Because the broadness of the immunoprecipitate suggested that the putative hemagglutinin was glycosylated, we tested this possibility by labeling the infected cells with radioactive saccharides, including mannose, fucose, and glucosamine (Fig. 2*B*). The putative HA produced in the HA–SV40 infected AGMK cells was specifically labeled with radioactive sugars. In this regard, it resembled the HA produced during influenza virus infection. These findings demonstrate that infection of AGMK cells with the hybrid HA-SV40 virus produced a glycosylated HA$_0$ similar to the HA$_0$ polypeptide product of influenza virus.

Analysis of HA–SV40 DNA. After it was shown that a plaque isolate of HA–SV40 virus directed the synthesis of a putative HA polypeptide, we were interested in ascertaining the genomic DNA structure of the hybrid virus that produced this polypeptide. An analysis of the hybrid DNA population that contained HA-specific sequences was carried out by using the Southern blotting technique. Linear DNA from a *Bam*HI cleavage of form I DNA from the viral isolate was separated on an agarose gel, blotted onto nitrocellulose paper, and hybridized to ^{32}P-labeled cDNA derived from treatment with reverse transcriptase of influenza virion RNA. Two species of HA–SV40 DNA were present in the viral isolate; a major species [4400 base pairs (bp) long] and a minor one (4800 bp long) in a ratio of ≈30:1. Each *Bam*HI-cleaved linear DNA population was further cloned in the unique pBR322 *Bam*HI site. AGMK cells transfected with the cloned HA–SV40 DNA were subsequently tested by immunofluorescence for the synthesis of the HA polypeptide (see below). These experiments (data not presented) showed that only the 4800-bp species of HA–SV40 DNA, belonging to the minor population of the original isolate, produced the HA polypeptide.

To further confirm the completeness of HA DNA and its strand orientation with respect to late SV40 transcription, we carried out endonuclease cleavage of the HA polypeptide-producing HA–SV40 recombinant DNA (Fig. 3). *Bgl* I cleaves once in the SV40 vector at the origin of DNA replication and once in the HA DNA at 100 bp from the 3′ terminus of the (+) DNA strand (ref. 28; unpublished results). *Bgl* I digestion of the HA–SV40 plasmid (with HA–SV40 inserted at the pBR322

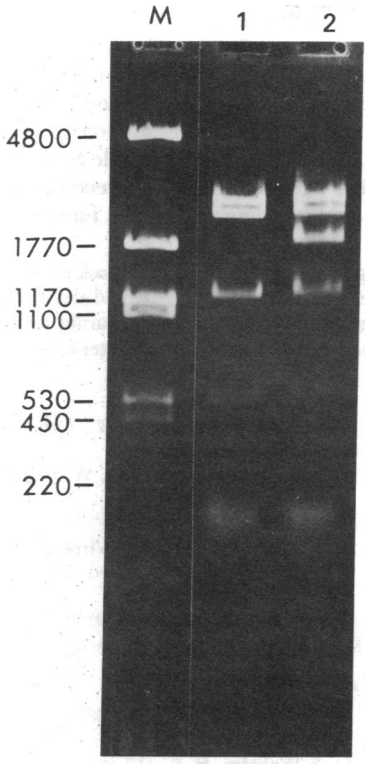

FIG. 3. Restriction endonuclease cleavage of HA–SV40 DNA cloned in pBR322. Plasmid DNA was digested with either *Bgl* I (lane 1) or *Bgl* I/*Bgl* II (lane 2) and the digests were separated on a 1.4% agarose gel. DNA fragments were visualized by ethidium bromide staining. M, SV40 DNA fragments used as size markers.

*Bam*HI site) yielded a DNA fragment of 2270 bp corresponding to the proximal portion of the late SV40 region and almost the entire HA DNA except for the small 3'-terminal segment. The *Bgl* I DNA fragment, as expected, was cleaved by *Bgl* II to yield DNA subfragments of 1828, 292, and 150 nucleotide bp (18). These experiments confirm that the cloned HA DNA was inserted (most likely in its entirety) into the late region of SV40 in an orientation that directs the synthesis of influenza viral HA mRNA transcripts using the late SV40 transcription control signals.

Agglutination of Erythrocytes. The HA of influenza virus binds to the sialic acid receptors on the cell surface and causes erythrocytes to agglutinate (6, 7). To demonstrate the functional activity of the putative HA produced from the HA–SV40 hybrid virus, we tested an extract perpared from HA–SV40-infected AGMK cells for hemagglutinating activity. Extracts from wild-type SV40-infected AGMK cells were used as controls. Guinea pig erythrocytes were added to serially diluted cell extracts and allowed to sediment at room temperature (Fig. 4). The extract from AGMK cells infected with the HA–SV40 hybrid consistently exhibited a HA titer of 1:4–1:8 whereas the SV40-infected extracts were negative. The HA was specific since it was inhibited by a 1:128 dilution of sheep H3 HA antiserum but not by a 1:8 dilution of sheep H1 HA antiserum. This indicates that the HA polypeptide product encoded by the HA DNA in the constructed HA–SV40 hybrid exhibited antigenic specificity of the H3 subtype of influenza virus.

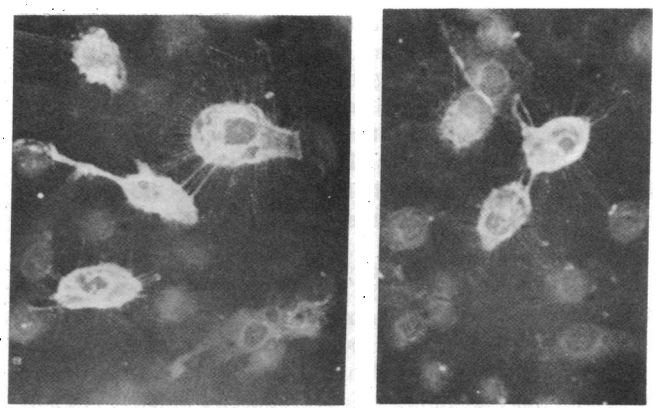

FIG. 5. Indirect immunofluorescence assay of HA in infected cells. AGMK cells were infected with either HA–SV40 hybrid virus for 72 hr or influenza A virus for 14 hr and treated as described in *Materials and Methods*. (*Left*) Cells infected with HA–SV40 virus and fixed with acetone. (*Right*) Cells infected with HA–SV40 virus but not fixed with acetone.

Indirect Immunofluorescence Assay. It is believed that the influenza A virus HA is synthesized in the cytoplasm of infected cells and subsequently transported to and anchored on the cell surface where the final stage of influenza virion maturation takes place (14, 29). To determine sites of HA production and location, an immunofluorescence assay of "fixed" and "unfixed" cells was performed. This assay should differentiate cytoplasmic proteins from cell surface proteins. Both fixed and unfixed AGMK cells infected with HA–SV40 were stained intensely and with the same frequency by specific antibody to HA, indicating that the HA polypeptide specified by the cloned HA DNA sequences was present on the cell surface as well as in the cytoplasm (Fig. 5). In a separate experiment, we also carried out immunofluorescence staining of the nuclear SV40 large-tumor antigen on fixed and unfixed cells infected with SV40. The results showed that SV40 large-tumor antigen fluorescence was seen in fixed cells but not in unfixed cells (data not shown). In addition, AGMK cells were transfected with the cloned HA–SV40 DNA (4800 bp) and doubly stained with fluorescein-

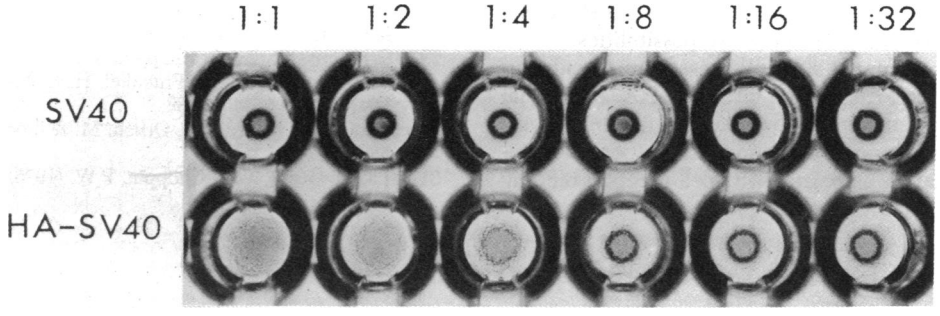

FIG. 4. Hemagglutination by the HA produced from the HA–SV40 hybrid virus. Extracts made from AGMK cells infected with SV40 or HA–SV40 virus were serially diluted as shown. A suspension of 0.5% guinea pig erythrocytes was added to each dilution, and the agglutination reaction was performed at room temperature.

conjugated anti-sheep serum (directed against the globulins in the sheep H3 HA antiserum) and rhodamine-conjugated anti-hamster serum (directed against SV40 large-tumor antiserum). These cells showed coordinate expression of the HA and large-tumor antigen, indicating the specificity of the observed immunofluorescent reactions. Therefore, we conclude that the HA synthesized in HA–SV40-infected AGMK cells is expressed on the cell surface in the absence of influenza virus infection.

DISCUSSION

We have described the construction of a recombinant viral genome consisting of an SV40 vector and a cloned full-length DNA coding for the HA protein of influenza virus. Infection of AGMK cells with this recombinant virus produced a putative HA polypeptide that was immunoprecipitable with HA antiserum. The polypeptide showed a molecular size corresponding to that of uncleaved influenza HA and it was glycosylated, as shown by incorporation of radioactive labeled sugars. Furthermore, the putative HA product appeared to be functionally active; extracts from cells infected with the HA–SV40 recombinant exhibited specific hemagglutination not seen in control cell extracts. The HA product of HA–SV40 exhibited, in the absence of other influenza viral functions, properties characteristic of a surface glycoprotein. These observations suggest that the HA product of HA–SV40 is similar to the HA synthesized in cells infected with influenza virus. This is expected as our cloned HA DNA contains full-length sequences that code for the entire polypeptide sequence. Analysis of amino acid sequences suggests that the HA molecule includes three functional domains: an NH_2-terminal prepeptide signal for transport of the polypeptide from the cytoplasm to cell membranes, a COOH-terminal hydrophobic peptide for anchorage of the polypeptide in the cell membrane, and an internal "hinge" peptide region necessary for activation of viral infectivity through specific cleavage (30, 31. Our finding of HA synthesis demonstrates that amino acid sequences encoded by cloned HA DNA are sufficient for expression of the HA on the surface of eukaryotic cells. We did not observe, however, cleavage of the HA_0 into the HA_1 and HA_2 subunits during infection with the HA–SV40 viral recombinant. This was not surprising as cleavage of HA_0 occurred at a low level in AGMK cells without added trypsin during a productive infection of these cells with influenza A virus (Fig. 2A).

Only one of the nine HA–SV40 isolates examined synthesized HA. There are several possible explanations for the failure of other isolates to produce the polypeptide. Some of the HA–SV40 recombinants may have sustained deletion of DNA sequences that are required for transcription or translation. Also, some HA–SV40 hybrids may contain HA DNA inserted in an opposite orientation so that the sense (+(HA RNA strand is not transcribed. In the latter case, the (−) HA RNA strand that is present in genomic RNA would be synthesized. Analysis of nucleotide sequences at the junctures of SV40 and HA DNA should help to differentiate between these possibilities.

Our HA DNA recombinant should be useful in elucidating several interesting properties of the influenza HA. Individual domains that specify polypeptide functions can be rigorously tested through introduction of deletions or site-specific mutations. The regions that are associated with cell-receptor binding and, similarly, the separate antibody-binding sites that are defined by classes of monoclonal antibodies can be dissected at the molecular level. Experiments involving phenotypic mixing should answer the question of whether hemagglutinin coded for by cloned DNA is expressed normally on the surface of viral particles. If so, it would then be possible to seek evidence for complementation between HA–SV40 recombinant DNA and influenza viral mutants defective in HA function.

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