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Expression of Sindbis Virus Structural Proteins via Recombinant Vaccinia Virus: Synthesis, Processing, and Incorporation into Mature Sindbis Virions

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We have obtained a vaccinia virus recombinant which contains a complete cDNA copy of the 26S RNA of Sindbis virus within the thymidine kinase gene of the vaccinia virus genome. This recombinant constitutively transcribed the Sindbis sequences throughout the infectious cycle, reflecting the dual early-late vaccinia promoter used in this construction. The Sindbis-derived transcripts were translationally active, giving rise to both precursor and mature structural proteins of Sindbis virus, including the capsid protein (C), the precursor of glycoprotein E2 (PE2), and the two mature envelope glycoproteins (E1 and E2). These are the same products translated from the 26S mRNA during Sindbis infection, and thus these proteins were apparently cleaved, glycosylated, and transported in a manner analogous to that seen during authentic Sindbis infections. By using epitope-specific antibodies, it was possible to demonstrate that recombinant-derived proteins were incorporated into Sindbis virions during coinfections with monoclonal antibody-resistant Sindbis variants. These results suggest that all the information necessary to specify the proper biogenesis of Sindbis virus structural proteins resides within the 26S sequences and that vaccinia may provide an appropriate system for using DNA molecular genetic manipulations to unravel a variety of questions pertinent to RNA virus replication.

Vaccinia virus, a poxvirus (reviewed in reference 30), has a number of advantages for use as a eucaryotic expression vector. These include the broad host range of the virus, which allows genetic information to be shuttled among a variety of species and cell types; the size of the vaccinia virion and its DNA genome, which accommodates large or multiple foreign inserts (or both) in an infectious virus (47); high-titered stocks of infectious recombinant virus that enable virtually 100% of a cell population to be synchronously infected and to express the foreign gene; the absence of splicing during RNA maturation and cytoplasmic localization of vaccinia gene expression (mediated largely by viral enzymes), that allows proper expression of foreign inserts lacking introns or proper transport signals; and finally, the fact that vaccinia is a relatively safe vaccine strain. By using in vivo marker rescue techniques (18, 32, 57), these attributes have been used in several laboratories to construct a number of recombinants that express influenza hemagglutinin (33, 47), hepatitis B surface antigen (35, 46), herpesvirus thymidine kinase (25, 34) and glycoprotein D antigen (35), the malaria circumsporozoite antigen (45), the rabies virus glycoprotein (21), and the vesicular stomatitis virus G and N proteins (27). Such recombinants may prove useful in the prevention of a variety of human diseases (31, 35, 47), but such applications await further research into vaccinia virus pathogenesis and possible vaccination side effects.

Here we report initial studies on the use of vaccinia as an expression vector to study the production of Sindbis virus-specific proteins and RNA transcripts derived from cloned cDNA. Sindbis virus is the prototype of the alphavirus genus of the family *Togaviridae*. Alphaviruses replicate in the cytoplasm and contain a single-stranded RNA genome with plus strand polarity (reviewed in reference 52). Two species of viral mRNA are found in Sindbis-infected cells: 49S RNA, which is packaged into mature virions as well as serving as message for the nonstructural proteins, and 26S RNA, which encodes the structural polypeptides. In vivo, the 26S RNA is translated into a 130,000-molecular-weight polyprotein (130K polyprotein) which is cotranslationally cleaved and processed into the capsid protein, C, and two glycosylated membrane proteins, E1 and E2 (37; reviewed in reference 39). The following study describes our efforts to produce the Sindbis proteins translated from 26S RNA by using a vaccinia virus vector and to assess the utility of this expression system for investigating basic questions in alphavirus biology.

MATERIALS AND METHODS

Cells, virus, and infections. Vaccinia virus (WR strain) was propagated and titrated as previously described (15). Sindbis virus (HR small plaque strain; or SIN V33/50/23; see below) was grown and titrated with either BHK-21 or primary chicken embryo fibroblast (36) cells. BSC-40 cells, TK^- L cells (LTK^- cells) (14), Chinese hamster ovary (CHO) cells, MDBK cells, and BHK-21 cells (American Type Culture Collection) were maintained at 37°C under 5% CO_2 in Eagle minimum essential medium (Flow Laboratories) supplemented with 10% heat-inactivated fetal calf serum, 2 mM L-glutamine, and 10 μg of gentamicin sulfate per ml. Unless otherwise specified, virus infections and radioactive labeling procedures were carried out precisely as previously described (15).

Recombinant DNA. Plasmid DNA manipulations were carried out essentially as summarized by Maniatis et al. (28). Restriction enzymes, T4 DNA ligase, T4 polynucleotide kinase, and *Escherichia coli* DNA polymerase I were purchased from New England Biolabs; Klenow fragment was purchased from Bethesda Research Laboratories; and calf intestinal alkaline phosphatase was purchased from Boehringer Mannheim Biochemicals. Details of the plasmid con-

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structions are found below and in the legend to Fig. 1. *E. coli* MC1061 (5) was modified by P1 transduction (from strain C600; tn*10*, *recA56*) to tetracycline resistance and a UV-sensitive phenotype (probably *recA56*) and used to propagate recombinant plasmids.

DNA transfection and marker rescue. A 5-μg portion of cesium chloride-purified pVV3S DNA was coprecipitated with 1 μg of wild-type vaccinia DNA (to facilitate marker rescue) and 15 μg of carrier salmon sperm DNA by using the calcium phosphate technique (12). The DNA precipitates were added to monolayers of LTK^- cells (without bromodeoxyuridine selection) which had been infected with wild-type vaccinia at a multiplicity of 0.05 PFU per cell 3 h previously. After 4 h, the cells were washed with serum-free medium and shocked for 40 s with 15% glycerol in HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid)-buffered saline (pH 7.1) (24) to facilitate DNA uptake; normal medium was added, and the infected or transfected monolayers were incubated at 37°C for 72 h. The progeny virions from this initial marker rescue step were harvested and titrated. TK^- virus, including potential recombinants, was amplified by low-multiplicity passage through LTK^- cells in the presence of 25 μg of bromodeoxyuridine per ml. The TK^- vaccinia virus present in the amplified crude stock was then screened for recombinants containing the Sindbis-specific insert of interest by using the nitrocellulose in situ plaque hybridization procedure described by Villarreal and Berg (56). Recombinants of interest were subjected to at least two rounds of plaque purification until 100% of the plaques scored as positive with appropriate radioactive probes.

DNA analyses. To obtain vaccinia DNA, 100-mm dishes of BSC-40 cells were infected with vaccinia virus at a multiplicity of 10 PFU per cell for 24 h. The infected cells were harvested, suspended in 600 μl of phosphate-buffered saline (PBS), and freeze-thawed three times. The crude extract was adjusted to 0.5% Triton X-100, 35 mM β-mercaptoethanol, and 20 mM EDTA, transferred to a 1.5-ml microfuge tube, and centrifuged in a Tomy microfuge (model RC-15A) at 3,000 rpm and 25°C for 2.5 min to pellet the cell nuclei. The supernatant was transferred to a fresh tube and centrifuged at 15,000 rpm and 25°C for 10 min to pellet virus core particles. The pellet was suspended in 100 μl of 10 mM Tris hydrochloride (pH 8)–1 mM EDTA–5 mM β-mercaptoethanol–proteinase K (150 μg/ml)–200 mM NaCl–1% sodium dodecyl sulfate (SDS) and incubated at 50°C for 30 min. After this digestion, the supernatant was twice extracted with Tris-EDTA buffer (10 mM Tris [pH 7.5], 1 mM EDTA), saturated with phenol-chloroform-isoamyl alcohol (25:24:1), and ethanol precipitated. The DNA was pelleted at 15,000 rpm and 4°C for 1 min, air dried, and suspended in 25 μl of Tris-EDTA buffer. Approximately 10 to 20 μg of vaccinia DNA was obtained per dish. The DNA was digested with restriction endonucleases under the conditions suggested by the manufacturer (Bethesda Research Laboratories of New England Biolabs). The digests were electrophoresed in 0.7% agarose (Seakem) gels at 40 V for 12 h in Tris-acetate buffer (42). The DNA bands were visualized by staining with 0.5 μg of ethidium bromide per ml and photographed with a Polaroid MP-4 camera set-up. The DNA was then blotted onto nitrocellulose and hybridized with appropriate nick-translated probes (49).

RNA analyses. BSC-40 cells were infected for 5 h in the presence of 100 μg of cycloheximide to amplify early mRNA sequences. Total infected cell RNA was extracted and purified either by the cesium chloride-Sarkosyl method (11) or by isolation of total nucleic acids followed by digestion with DNase I (54). Total RNA was fractionated into $poly(A)^-$ and $poly(A)^+$ fractions with oligo(dT)-cellulose (Collaborative Research, T-3 grade) column chromatography (17). The sizes of the Sindbis-specific transcripts were measured by Northern blotting of the RNA from denaturing formaldehyde gels (28). In S1-nuclease protection studies (2, 7), probes were 5′ end labeled with [γ-^{32}P]ATP (ICN Pharmaceuticals, Inc.) and T4 polynucleotide kinase (New England Biolabs). Annealing conditions and nuclease S1 (PL Biochemicals) concentrations were optimized empirically; both duplex DNAs (10 μg/ml) and denatured carrier DNAs (20 μg/ml) were included during S1 digestion.

Protein synthesis. Monolayers of infected BSC-40 cells in 60-mm dishes were labeled with 5 to 25 μCi of [^{35}S]methionine (1,200 Ci/mM; New England Nuclear Corp.) per ml according to the protocols detailed in the figure legends. The radioactive medium was removed, and the infected monolayers were rinsed gently three times with ice-cold PBS. The cell monolayers were solubilized in 1 ml of 0.5% SDS containing 20 μg of phenylmethylsulfonyl fluoride to inhibit proteases. The extract was pipetted up and down vigorously to shear cellular DNA, frozen and thawed once, sonicated six times for 10 s each, and heated at 90°C for 10 min. Appropriate volumes of extract (50 to 100 μl) were diluted to 1 ml with RIPA buffer (1% [wt/vol] sodium deoxycholate, 1% [vol/vol] Triton X-100, 0.2% [wt/vol] SDS, 150 mM NaCl, 50 mM Tris hydrochloride [pH 7.4]) and mixed with 3 to 5 μl of antiserum; 200 μl of 10% (vol/vol) protein A-Sepharose CL4B beads (Pharmacia Fine Chemicals) in RIPA buffer were added, and the mixtures were gently agitated at 4°C for 1 to 12 h. The beads were then pelleted and washed three times with ice-cold RIPA buffer. The immune complexes bound to the beads were released by adding 50 μl of SDS-polyacrylamide gel electrophoresis sample buffer and heating at 100°C for 3 min. The beads were pelleted in a microfuge, and the supernatants were electrophoresed on 10.8% discontinuous SDS-polyacrylamide gels (53). The gels were then impregnated with 2, 5-diphenyloxazole (3) and exposed on Kodak XAR-5 film at −70°C. The antisera used in these experiments were generated in rabbits with purified Sindbis proteins (38).

Protein transport. Iodinated staphlococcal protein A assays were carried out in a manner similar to that which has been previously described (4). BSC-40 cells were infected in 24-well dishes with Sindbis, wild-type vaccinia, or vaccinia VV3S-7 at a multiplicity of 10 PFU per cell. After 4 h of infection, the medium was removed, the monolayers were rinsed three times with ice-cold PBS–0.5% bovine serum albumin (PBS-A) and then placed on ice; 300 μl of a 1:25 dilution of antiserum in PBS-A was added and allowed to adsorb at 4°C for 1 h. The monolayers were washed three times with ice-cold PBS-A; 200 μl of PBS-A containing 1.0×10^6 dpm of ^{125}I-labeled protein A (8 μCi/μg; New England Nuclear Corp.) was added, and the mixture was incubated at 4°C for 1 h with shaking. The monolayers were then washed four times with ice-cold PBS-A, solubilized with 1 ml of 2% SDS at 60°C, and counted in a gamma counter.

Indirect immunoflorescence. Subconfluent monolayers of BHK-21 cells grown on collagen-coated cover slips were infected with wild-type vaccinia, VV3S-7, or Sindbis virus at a multiplicity of 20 PFU per cell. At 8 h postinfection, the cells were washed twice in PBS and fixed for 2 to 3 min in PBS containing 2% formaldehyde. After several washes in TBS (25 mM Tris hydrochloride, 140 mM NaCl, 5 mM KCl, 1.5 mM Na_2HPO_4, 1 mM $CaCl_2$, 0.5 mM $MgCl_2$, [pH 7.5]),

cells were incubated for 30 min with Sindbis virus E1-specific monoclonal antibody no. 33 (1/100 dilution of ascites fluid in TBS plus 1 mg of gelatin per ml) (40) or E2-specific rabbit immunoglobulin G (IgG) (1/50 dilution of IgG [~5 mg/ml] absorbed against vaccinia-infected BHK monolayers fixed as described above) (38). After several washes in TBS, cells were stained with fluorescein-conjugated goat antibodies to mouse or rabbit IgGs for 30 min. The cover slips were washed with TBS, mounted in 90% glycerol (containing 0.1% phenylene diamine), and viewed with a Leitz phase-epifluorescence microscope, using a ×63 objective.

Phenotypic mixing. Monolayers of chicken embryo fibroblasts were infected with wild-type vaccinia or VV3S-7 at a multiplicity of 10 PFU per cell and labeled with [^{35}S]methionine. After 12 h, the cells were superinfected with either Sindbis virus (HR small plaque strain [50]) or SIN V33/50/23, a Sindbis variant (generously provided by A. L. Schmaljohn) derived from Sindbis strain AR339 by sequential selection with three different epitope-specific neutralizing monoclonal antibodies; no. 33 (E1 specific), no. 50 (E2 specific), and no. 23 (E2 specific). No. 33 and 50 react with the HR small plaque strain. Sindbis virus was harvested after an additional 15 h and purified by both sedimentation velocity and isopycnic centrifugation (36). Immunoprecipitations were done essentially as previously described except that buffers lacked detergent (38). Two microliters of mouse ascites fluid and 10^4 to 10^5 PFU of virus were used for each sample. Washed immunoprecipitates were suspended and quantitated by liquid scintillation counting, and the immunoprecipitation supernatants were assayed for infectivity.

RESULTS

Construction of the vaccinia-Sindbis structural region recombination plasmid. The plasmid pGS20 is a vaccinia virus insertion vector that has been used to construct a number of vaccinia recombinants (26, 48). The construction of a modified insertion plasmid, pVV3, and the recombinant plasmid containing a complete copy of the cDNA corresponding to Sindbis 26S RNA is described in the legend to Fig. 1. The vaccinia-specific sequences in pGS20 from the *Hin*dIII site to the *Xho*I site, which include the 7.5K vaccinia promoter (which drives expression of foreign inserts), were transferred to a smaller ampicillin-resistant pBR322 derivative. Subsequent modifications included destruction of the *Cla*I site in the vaccinia *tk* gene, insertion of a new *Cla*I site replacing the *Bam*HI site downstream from the 7.5K promoter, and insertion of a small polylinker region containing a unique *Sac*I site. The final hybrid recombination plasmid, pVV3S, has the 4.2-kilobase (kb) Sindbis virus 26S cDNA inserted directionally into pVV3 such that the 5′ end of the 26S cDNA was proximal to the vaccinia promoter. This transcriptional unit in turn is flanked by vaccinia virus DNA sequences containing the 5′ and 3′ ends of the vaccinia *tk* gene.

Isolation of a vaccinia recombinant containing Sindbis cDNA. The hybrid insertion plasmid, designated as pVV3S, was coprecipitated with wild-type vaccinia DNA and carrier salmon sperm DNA by the calcium orthophosphate method and adsorbed to vaccinia-infected LTK$^-$ cells at 3 h postinfection. The monolayers were shocked 4 h later with glycerol for 40 s to facilitate DNA uptake, and the infection was then allowed to proceed for 48 h. Recombinant viruses, which should possess a TK$^-$ phenotype by virtue of insertional inactivation of the viral thymidine kinase gene, were amplified by a low-multiplicity passage through LTK$^-$ cells in the presence of bromodeoxyuridine. Plaque hybridization was then used to distinguish recombinants from spontaneous TK$^-$ vaccinia mutants; nick-translated Sindbis 26S cDNA was used as a probe (56). Wild-type vaccinia virus plaques gave no signal, whereas a large number of strongly hybridizing plaques were obvious in the VV3S population. Individual positive plaques were recovered by punching out the corresponding regions from a replica nitrocellulose filter and subjected to at least two cycles of plaque purification until each isolate scored as 100% positive in the plaque hybridization assay. One of the putative positive vaccinia-Sindbis recombinants, VV3S-7, was used for further experiments.

Genomic structure of VV3S-7. The structure of the genome of vaccina recombinant VV3S-7 was probed by restriction mapping and blot analysis (49). At the bottom of Fig. 2 is drawn the *Sal*I M region from wild-type vaccinia DNA which contains the *Hin*dIII J fragment and the *tk* gene. Below that is the DNA structure of the desired recombinant with the 4.2-kb Sindbis cDNA, flanked by the 7.5K promoter and polylinker sequences, residing within the vaccinia *tk* gene. In such a recombinant, the 4.8-kb *Hin*dIII J fragment would be displaced to 9.2 kb, and the 5.4-kb *Sal*I M fragment would be replaced by four fragments having sizes of 5, 2.3, 1.5, and 1.3 kb (8, 51). As expected, the 4.8-kb *Hin*dIII J band disappeared in VV3S-7 and a new band appeared at 9.2 kb. Similarly, the *Sal*I M fragment of VV3S-7 was replaced by four smaller fragments. The DNA from the agarose gel was then transferred to nitrocellulose and probed with either ^{32}P-labeled *Hin*dIII-J DNA or Sindbis 26S cDNA. These results confirm that the new 9.2-kb *Hin*dIII band in VV3S-7 DNA contains J sequences and indicate that the 5- and 1.3-kb *Sal*I bands also contain vaccinia sequences. The Sindbis probe did not hybridize to wild-type vaccinia DNA but did hybridize to the 9.2-kb *Hin*dIII fragment as well as to the 5-, 2.3-, and 1.5-kb *Sal*I fragments from VV3S-7.

Transcription of Sindbis sequences. BSC-40 cells were infected with vaccinia recombinant VV3S-7 in the presence of cycloheximide (100 μg/ml) to amplify early mRNA sequences. At 5 h postinfection, the cytoplasmic RNA was extracted and purified, and the poly(A)$^+$ fraction was obtained by oligo(dT)-cellulose chromatography. This RNA was fractionated by electrophoresis on denaturing formaldehyde gels, transferred to nitrocellulose, and subjected to Northern blot analyses with nick-translated pVV3S as a probe. A 4.7-kb RNA which was transcribed from the vaccinia recombinant hybridized to the probe (Fig. 3, right side). The left side of Fig. 3 shows 26S RNA (4.1 kb) and virion RNA (11.7 kb) from Sindbis-infected cells. These results are consistent with proper transcription of the entire Sindbis insert including the poly(A) tract and termination at the *tk* early transcription-termination region (58). A faint band in the poly(A)$^-$ fraction was detected at about 4.1 kb (data not shown), suggesting that some premature transcription termination may occur near the 3′ end of the Sindbis insert, perhaps at the poly(dA) tail of approximately 35 nucleotides derived during cDNA cloning of the Sindbis RNA.

To determine the 5′ end of the VV3S-7 Sindbis-specific transcripts nuclease S1 mapping procedures were used (Fig. 4). RNA was obtained from cells at 4 h postinfection, which in cells permissive for vaccinia replication corresponds to the period during shift from early to late gene expression. These results show that the Sindbis transcripts, in cells permissive for vaccinia replication (BSC-40 and BHK cells), are being initiated at two distinct sites within the 7.5K promoter region. This is in agreement with data demonstrating two RNA start sites in the 7.5K transcription unit as well

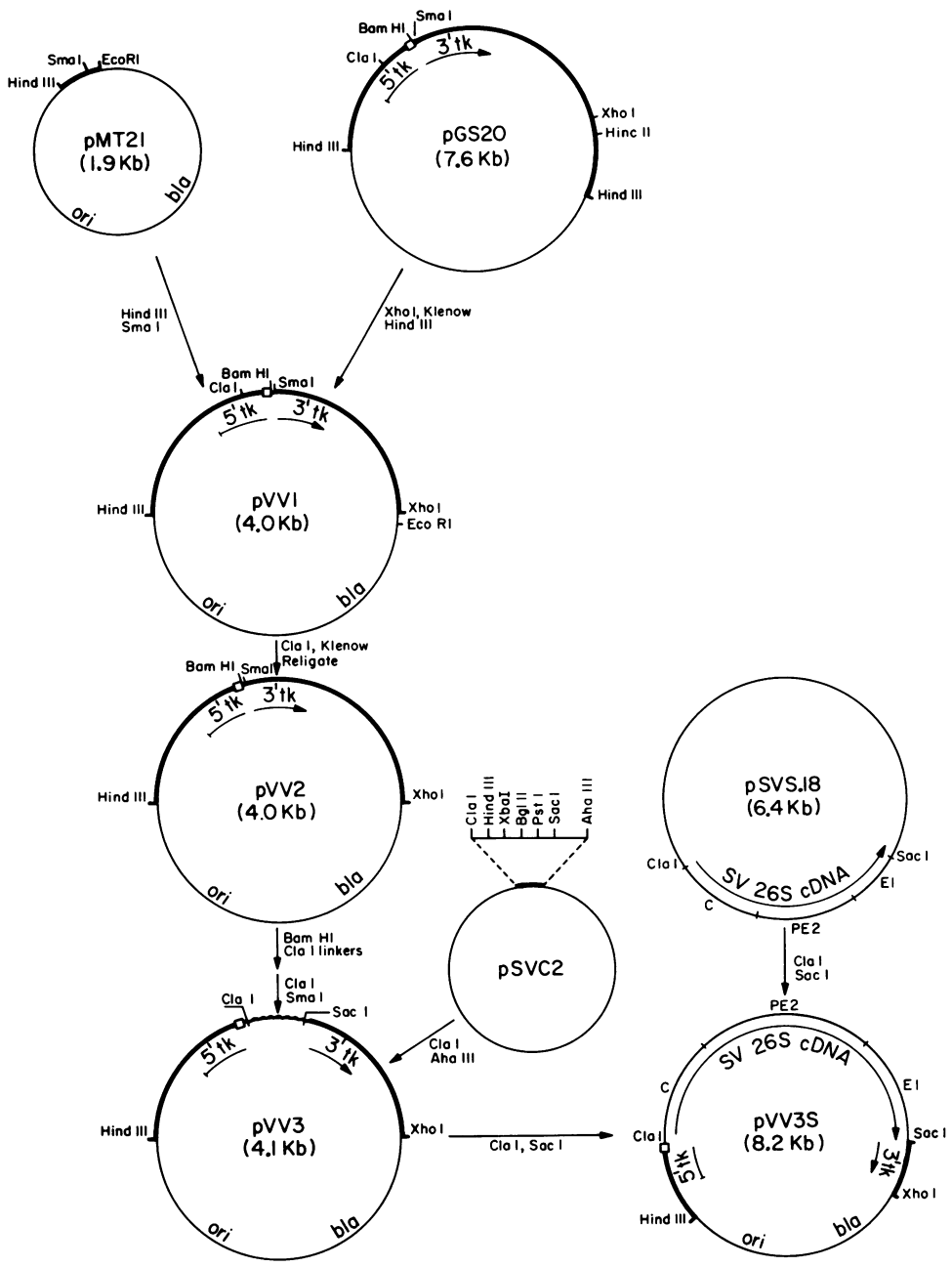

FIG. 1. Construction of pVV3S recombination plasmid. pGS20 was digested with *Xho*I, filled in using the Klenow fragment, digested with *Hin*dIII, and the 1.8-kb fragment containing the 7.5K promoter and *tk* coding region was ligated to pMT21 (a 1.9-kb ampicillin-resistant pBR322 derivative from Henry V. Huang) which had been digested with *Hin*dIII and *Sma*I. The resulting plasmid, pVV1, was digested with *Cla*I, filled in with Klenow, and reclosed, giving rise to pVV2, which therefore lacks a *Cla*I site. The *Bam*HI site of pVV2 was converted to a *Cla*I site by insertion of *Cla*I linkers, and the polylinker region from pSVC2 (C. Rice, unpublished) containing sites for *Cla*I, *Hin*dIII, *Xba*I, *Bgl*II, *Pst*I, and *Sac*I was inserted by directional cloning into the *Cla*I and *Sma*I sites of the modified pVV2 (the blunt end of the polylinker fragment was generated by using an *Aha*III site ~70 nucleotides from the *Sac*I site). This plasmid is designated pVV3. The Sindbis cDNA insert was derived by partial *Ava*II digestion of a plasmid containing the 3'-terminal 5.5 kb of the Sindbis genome, including poly(A) (51; C. Rice, unpublished data) and insertion of *Cla*I linkers. A plasmid containing the *Cla*I site immediately adjacent to the sequences corresponding to the start of 26S RNA was designated pSVS.18. This construct was digested with *Cla*I and *Sac*I [a unique *Sac*I site immediately follows the Sindbis 3'-terminal poly(A) tail] and the 26S cDNA fragment was ligated into the corresponding sites in pVV3. Vaccinia DNA sequences are designated by bold lines, the vaccinia 7.5K promoter is shown as an open box.

as in a chimeric 7.5K chloramphenicol acetyltransferase gene (6); the downstream promoter is utilized early in infection (26, 55), and the other (approximately 55 nucleotides upstream) is used late in infection. Thus, the transcription of Sindbis sequences is essentially constitutive during a VV3S-7 infection of permissive cells. In MDBK and CHO cells which are nonpermissive for vaccinia replication (16), the Sindbis-specific transcripts were still produced but were

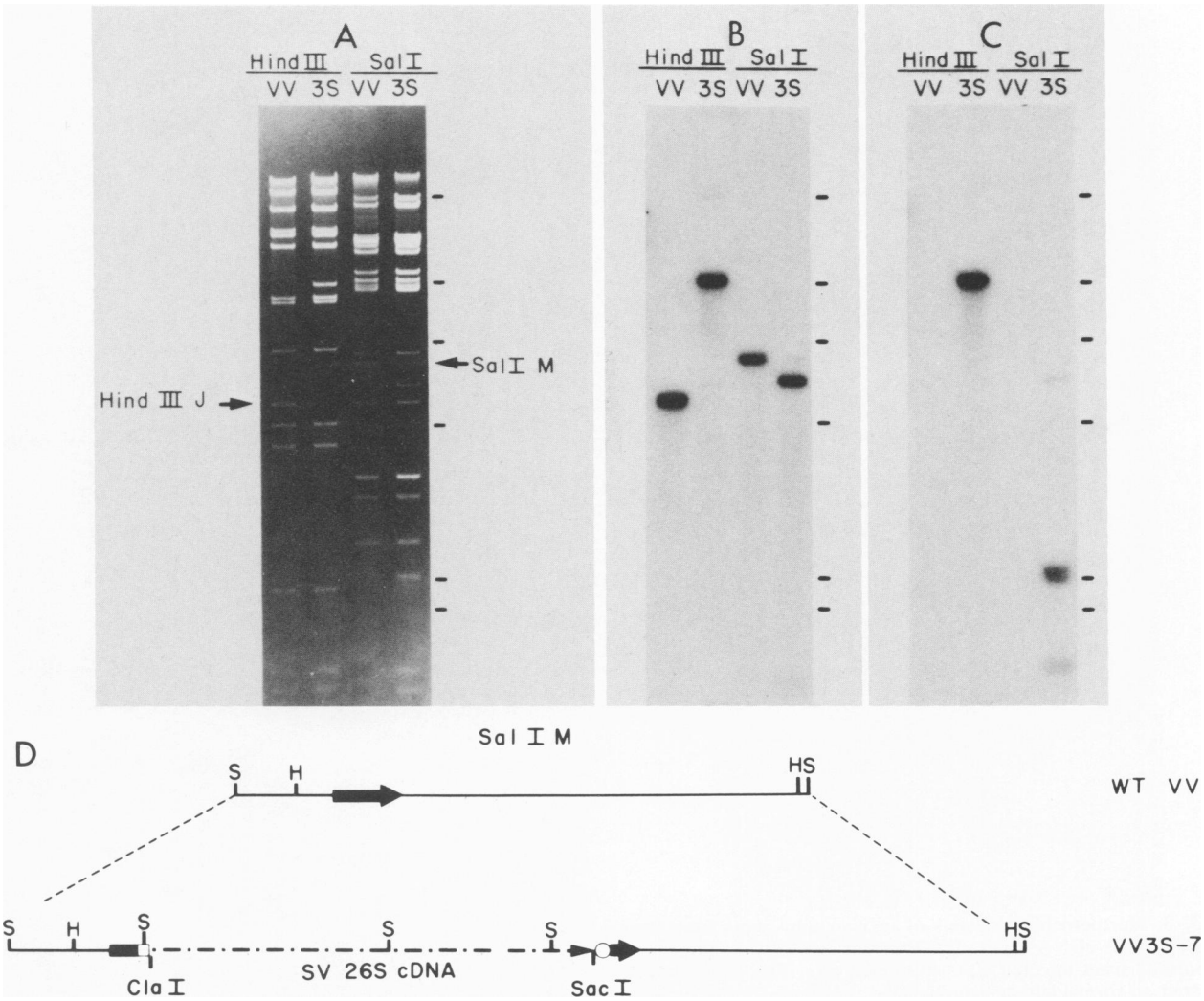

FIG. 2. Analysis of VV3S-7 genome structure. Viral DNA was extracted and purified from monolayers of cells infected with wild-type vaccinia virus (VV) or VV3S-7 (3S). The DNA was digested with restriction endonucleases, fragments were resolved by agarose gel electrophoresis (ethidium bromide-stained pattern is shown in panel A), and Southern blot analyses were carried out from either vaccinia virus nick-translated probes *Hin*dIII-J DNA (B) or Sindbis 26S cDNA (C). Size markers from a λ *Hin*dIII digest (shown at the right) are 23.1, 9.4, 6.6, 4.4, 2.3, and 2.0 kb. The predicted genomic structures of the *Sal*I-M region of wild-type and recombinant vaccinia are diagrammed below.

derived exclusively from the early 7.5K transcription start (Fig. 4). The VV3S-7 Sindbis transcripts have not yet been analyzed to determine if they possess unique or heterogeneous 3′ ends, although the sharp band seen in Fig. 3 indicates that some of the transcripts terminate within a limited region if not at a unique point.

Expression of Sindbis proteins. To determine what protein products were translated in vivo from the Sindbis-specific transcripts, BSC-40 cells infected with wild-type vaccinia virus, vaccinia recombinant VV3S-7, or Sindbis virus were labeled with [^{35}S]methionine. The radioactively labeled proteins were immunoprecipitated with heterospecific anti-Sindbis antisera as well as with monospecific antisera directed against the Sindbis E1, E2, or C protein. The precipitated proteins were separated by electrophoresis on SDS-polyacrylamide gels (Fig. 5). In Sindbis-infected cells (Fig. 5, right side) the heterospecific Sindbis-specific antiserum precipitates precursor glycoprotein PE2, glycoproteins E1 and E2, and capsid protein C, as well as trace amounts of a 108K polyprotein which contains the amino acid sequences of both PE2 and E1. The three monospecific antisera displayed the expected specificities and the control preimmune serum did not cause the precipitation of any labeled protein. None of the antisera reacted specifically with any proteins from vaccinia-infected cells (Fig. 5, left side). In cells infected with the vaccinia recombinant VV3S-7, Sindbis proteins PE2, E1, E2, and C were present (Fig. 5, center lanes). The Sindbis proteins from VV3S-7-infected cells comigrate with authentic Sindbis proteins, which suggests that they have been correctly cleaved and processed. In an attempt to quantitate the amount of Sindbis-specific proteins synthesized in VV3S-7-infected cells, radioactive extracts from cells infected with equivalent multiplicities of VV3S-7 or Sindbis were immunoprecipitated, and the radioactivity was assayed; after 4 or 18 h of infection, VV3S-7-infected cells appeared to contain immunoprecipitable Sindbis proteins at about 10% of the level observed in Sindbis-infected cells (data not shown).

A number of parameters of VV3S-7 expression of Sindbis proteins were analyzed by immunoprecipitation and gel

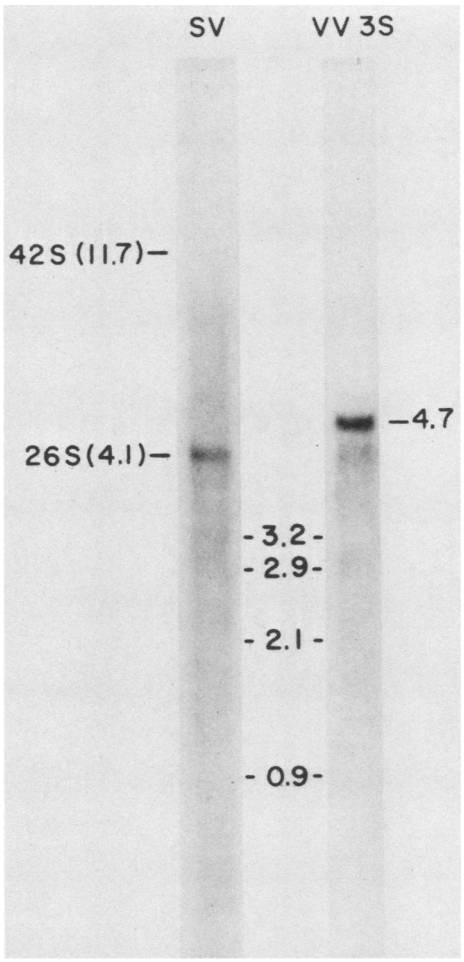

FIG. 3. Northern blot analysis of in vivo transcripts from the VV3S-7 Sindbis cDNA insert. The indicated RNAs were extracted and purified from infected BSC-40 monolayers. The RNAs were separated on formaldehyde-agarose gels, transferred to nitrocellulose and hybridized to a 26S RNA-specific nick-translated probe. SV, total Sindbis RNA; VV3S, poly(A)$^+$ RNA from cells infected with VV3S-7.

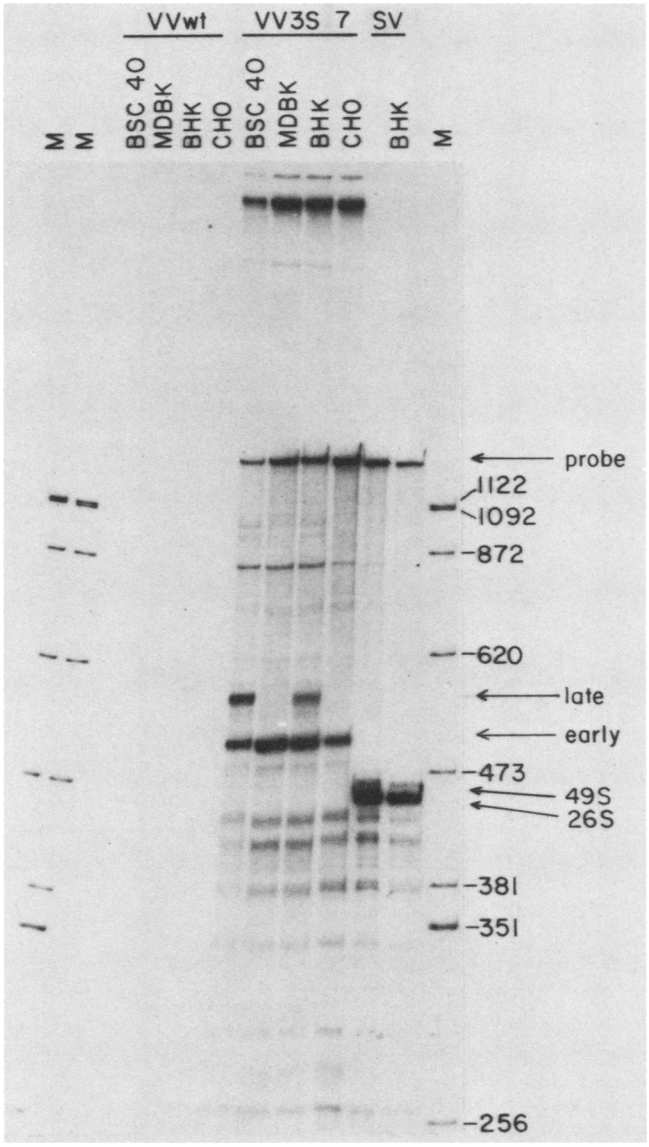

FIG. 4. S1 analysis. RNA was isolated at four hours postinfection from either wild-type vaccinia (VV wt), VV3S-7, or Sindbis (SV) virus-infected cell monolayers as indicated. Equal proportions of these samples were annealed with an excess of a 5′ end-labeled probe derived from pVV3S, digested with S1, and protected fragments were denatured and separated on 4% acrylamide-urea sequencing gels. For the Sindbis-infected RNA sample, 5% (right lane) or 10% (left lane) of the protected material was loaded. The probe was the 1530 nucleotide *Hin*dIII-*Nco*I fragment of pVV3S, 5′ end-labeled at the *Nco*I site which is 445 nucleotides 3′ to the start of the 26S cDNA insert. Two protected fragments were found in Sindbis virus-infected cells corresponding to 26S RNA (445 nucleotides) and 49S RNA (454 nucleotides). Protected fragments in VV3S-7-infected cells corrsponded to the early 7.5K promoter start (504 nucleotides) and, in permissive cells, to the late 7.5K promoter start (~560 nucleotides, seen only in the VV3S-7-infected BSC-40 and BHK lanes). The sizes of the end-labeled DNA markers (M) are indicated.

electrophoresis (Fig. 6). BSC-40 cells infected with vaccinia VV3S-7 were continuously labeled with [^{35}S]methionine, and the accumulation of Sindbis proteins was examined (Fig. 6a). Sindbis proteins were not evident at 1 h postinfection, but they became evident at 2 h postinfection and continued to accumulate throughout the 24 h of infection. This would suggest that the Sindbis proteins are relatively stable in vaccinia-infected cells. Note that a vaccinia protein that migrates very close to PE2 and that immunoprecipitates nonspecifically for unknown reasons is present in the long labels and complicates the interpretation of the pattern.

A second series of experiments which involved pulse-labeling is shown in Fig. 6b. Three important features of VV3S-7 expression of Sindbis proteins are seen. First, the Sindbis proteins are synthesized throughout the infection cycle. Second, there appear to be two peaks of expression, one at 2 h and one later in infection. This may be due to the shift from early to late transcription by the 7.5K promoter, but it could involve posttranscriptional processes as well. Finally, it can be seen that during a pulse no E2 is produced, but rather the precursor to E2 is present. The pulse-chase studies shown in Fig. 6c show that PE2 can be chased into E2. It is interesting to note that PE2-to-E2 processing kinetics appear to occur at the same rate in Sindbis-and VV3S-7-infected cells. It also appears (Fig. 6c) that all three Sindbis proteins turn over in these experiments and that these proteins are more labile in the VV3S-7-infected cells

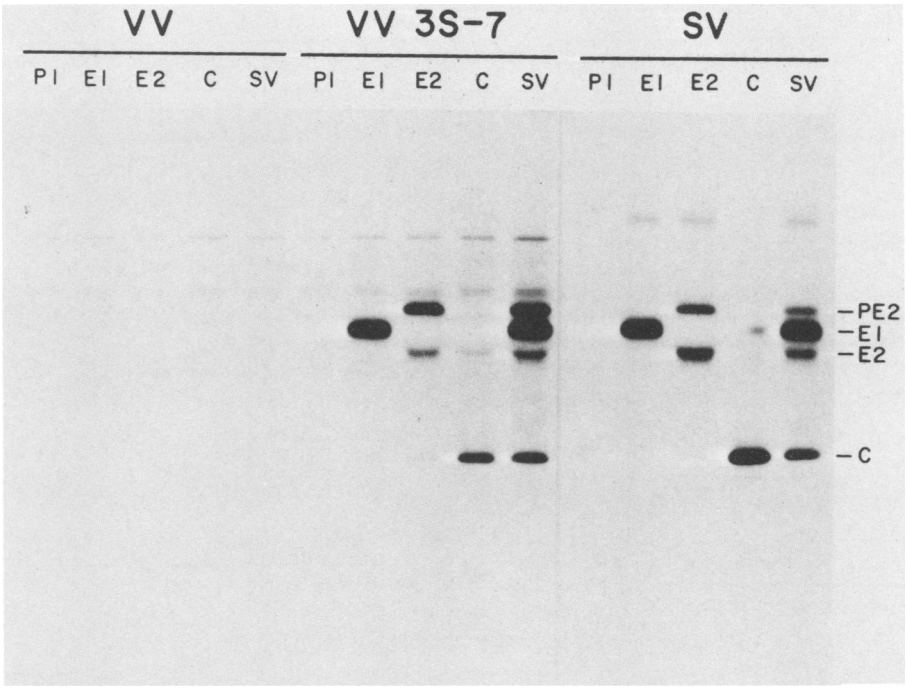

FIG. 5. Expression of Sindbis proteins. Proteins labeled from 3 to 4.5 h postinfection with [^{35}S]methionine (25 μCi/ml in methionine-free medium) were prepared from vaccinia (VV), VV3S-7, or Sindbis (SV) virus-infected cells and immunoprecipitated with preimmune serum (PI), heterospecific anti-Sindbis serum (SV), or monospecific antisera directed towards Sindbis envelope (E1 or E2) or capsid (C) proteins. The immune precipitates were then subjected to SDS-polyacrylamide gel electrophoresis and autoradiography. Sindbis-specific proteins are identified in the right margin.

than in Sindbis-infected cells. Note also that the 108K polyprotein is present in small amounts in Sindbis-infected cells (where it turns over rapidly) but is not seen in VV3S-7-infected cells.

We also examined several other cell lines, both permissive and nonpermissive for vaccinia replication, for the production of Sindbis proteins after infection by VV3S-7, and some of the results are shown in Fig. 6d. CHO, MDBK, BHK, and chicken embryo fibroblast cells infected with VV3S-7 all produced the same series of Sindbis proteins as infected BSC-40 cells. Thus, even in cell lines nonpermissive for vaccinia growth (CHO and MDBK cells), the expression of the Sindbis-specific transcripts and protein products is efficient, and proteolytic processing of the polyprotein precursor occurs.

Posttranslational modifications of Sindbis proteins. Sindbis E1 and E2 proteins are glycosylated membrane proteins. The PE2, E1, and E2 proteins found after infection with Sindbis or vaccinia VV3S-7 comigrated in one-dimensional polyacrylamide gels, which is suggestive evidence for similar states of glycosylation. Two additional experiments which support this observation were conducted. First, the effect of tunicamycin, a drug which inhibits N glycosylation, on Sindbis and VV3S-7 protein expression was assayed (Fig. 7). Sindbis or VV3S-7 proteins that were synthesized in either the presence or the absence of the drug comigrated. The nonglycosylated forms of PE2 and E1, synthesized in the presence of the drug, comigrated, which implies that the polypeptide backbones of the Sindbis proteins and the VV3S-7 proteins are identical. Thus, the fact that the glycosylated forms of Sindbis and VV3S-7 proteins also comigrate implies that they are glycosylated in a similar, if not identical, manner. Second, the VV3S-7-derived PE2, E1, and E2 proteins were shown to be specifically labeled (as opposed to the capsid protein) with the radioactive sugar [^{3}H]glucosamine (Fig. 7).

Transport of Sindbis virus proteins. During a Sindbis virus infection, the virus envelope proteins are core glycosylated during insertion into the endoplasmic reticulum, and the core oligosaccharides are modified during the translocation of the envelope proteins from the site of their synthesis to the cell surface. The VV3S-7-derived Sindbis proteins appear to be modified in the same way as are authentic Sindbis proteins, which implies that these proteins are also being transported to the cell surface. To assay this directly we used a radioimmune assay in which anti-Sindbis antibodies were adsorbed to the surface of infected cells, followed by incubation with ^{125}I-labeled protein A from *Staphylococcus aureus*. The amount of ^{125}I-labeled protein A bound is a measure of immunoreactive Sindbis proteins present at the cell surface (Table 1). Uninfected cells or cells infected with vaccinia wild-type, vaccinia VV3S-7, or Sindbis virus were adsorbed with preimmune serum, anti-Sindbis E1 + E2 antiserum, or anti-Sindbis C protein antiserum. The anti-capsid antiserum serves as a partial control for the integrity of the cells. It has been reported that late in Sindbis infection, the capsid protein is partially accessible at the cell surface to anti-capsid antibodies (44), and we did find an increase in binding of anti-capsid antibody to cells infected with Sindbis virus.

There was no specific binding of antibodies to either mock-infected or vaccinia wild-type-infected cells. Vaccinia VV3S-7-infected cells did not react with preimmune serum but showed significant reactivity with anti-Sindbis E1 + E2 antiserum, indicating that Sindbis E1 or E2 or both are present on the surface. Furthermore, by comparing the radioactivity bound to these cells with that bound to Sindbis-

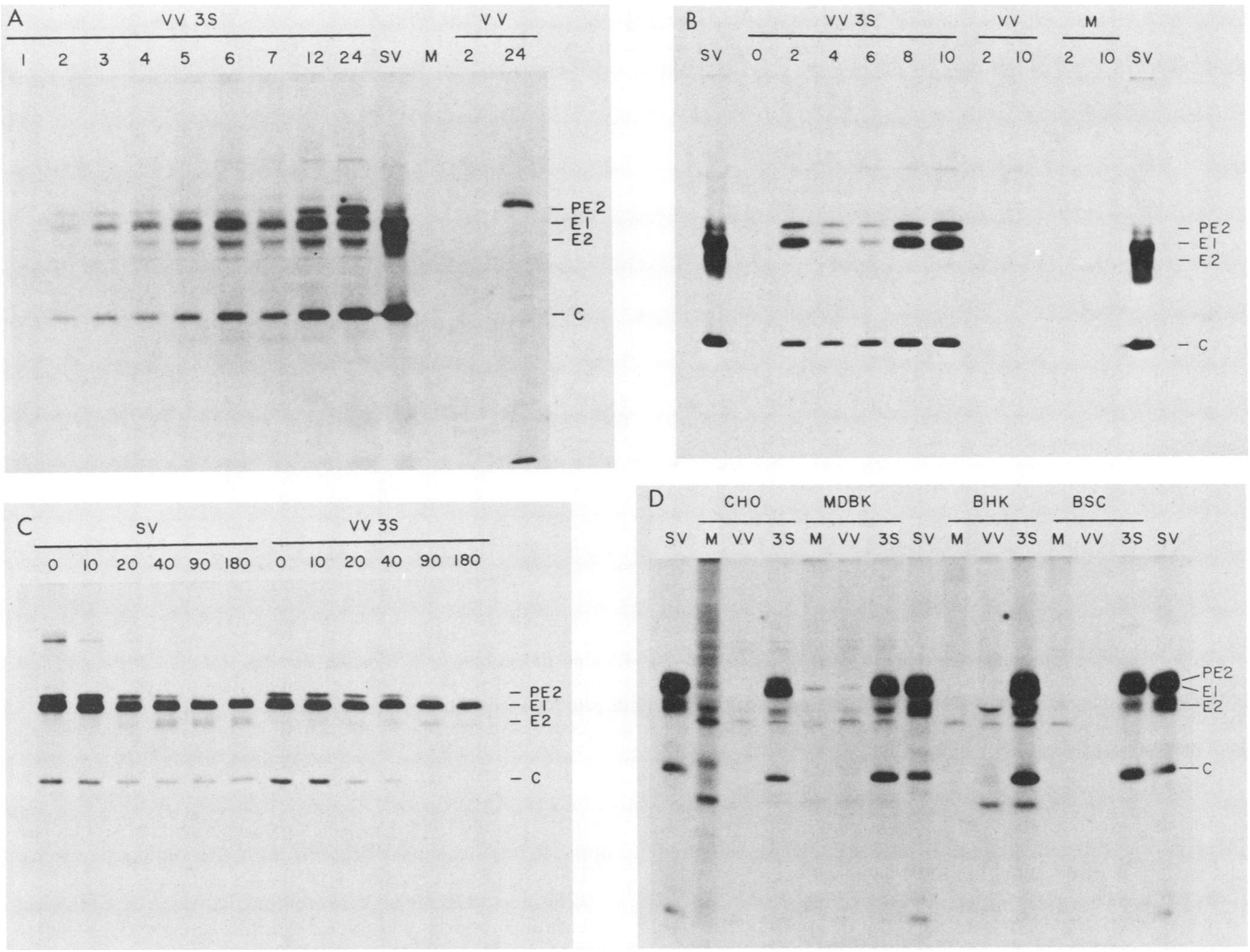

FIG. 6. Kinetics of Sindbis protein expression in VV3S-7-infected cells. Infected or mock-infected BSC-40 cell monolayers were labeled with [^{35}S]methionine in medium containing 1/10 of the normal concentration of methionine (unless otherwise indicated) according to the regimens described below. The radioactive proteins were analyzed by immunoprecipitation with polyspecific anti-Sindbis antisera and followed by SDS-polyacrylamide gel electrophoresis. Sindbis-specific proteins are identified in the right margins. Abbreviations: VV, wild-type vaccinia; VV3S, recombinant vaccinia VV3S-7; SV, Sindbis virus; M, mock-infected cells. (A) Continuous label. [^{35}S]methionine was present from the time of infection until the indicated hours postinfection. SV, M, and VV extracts were labeled for 24 h. (B) Pulse-label. [^{35}S]methionine was added in methionine-free medium for 30 min at the indicated hours postinfection, and then extracts were prepared immediately. The Sindbis markers were from the continuous labeling experiment. (C) Pulse-chase. Monolayers were pulse-labeled with [^{35}S]methionine in methionine-free medium for 10 min at 5 h postinfection. The radioactive label was then replaced with medium containing 100× methionine (non-radioactive) and 75 μg of cycloheximide per ml for the indicated number of minutes. (D) Continuous label in various cell types. [^{35}S]methionine was present from 2 h postinfection until the preparation of the extracts at 24 h postinfection.

infected cells (Table 1), it would appear that these proteins are present at 12% the amount found in Sindbis-infected cells.

In a second experiment, the Sindbis proteins present on the surface of BHK cells infected by vaccinia VV3S-7 were visualized by indirect immunofluorescence. At 8 h postinfection, the cells were fixed and incubated with either E1 or E2 monospecific antibodies (Fig. 8). Although wild-type vaccinia-infected cells showed a weak and diffuse pattern of immunofluorescent staining, both E1 and E2 (or PE2) could be specifically detected in the surface of VV3S-7-infected cells. The recombinant-derived glycoproteins had a patchy, focal appearance over the entire cell surface. This was similar to the pattern observed for Sindbis virus-infected cells or cells coinfected with both Sindbis virus and wild-type vaccinia virus (data not shown), but the VV3S-7-infected cells had a significantly lower level of fluorescent staining. Thus, these qualitative levels of immunofluorescence are in agreement with the results of the radioimmune assays mentioned above.

Biological activity of Sindbis proteins. The proteins encoded in the 26S cDNA Sindbis insert in vaccinia VV3S-7 have structural, as opposed to enzymatic, functions in the Sindbis life cycle. We performed several sets of experiments to determine whether the recombinant-derived polypeptides are capable of functioning in virus assembly. Preliminary experiments showed that in mixed infections of Sindbis virus and vaccinia virus (at high input multiplicities), the time

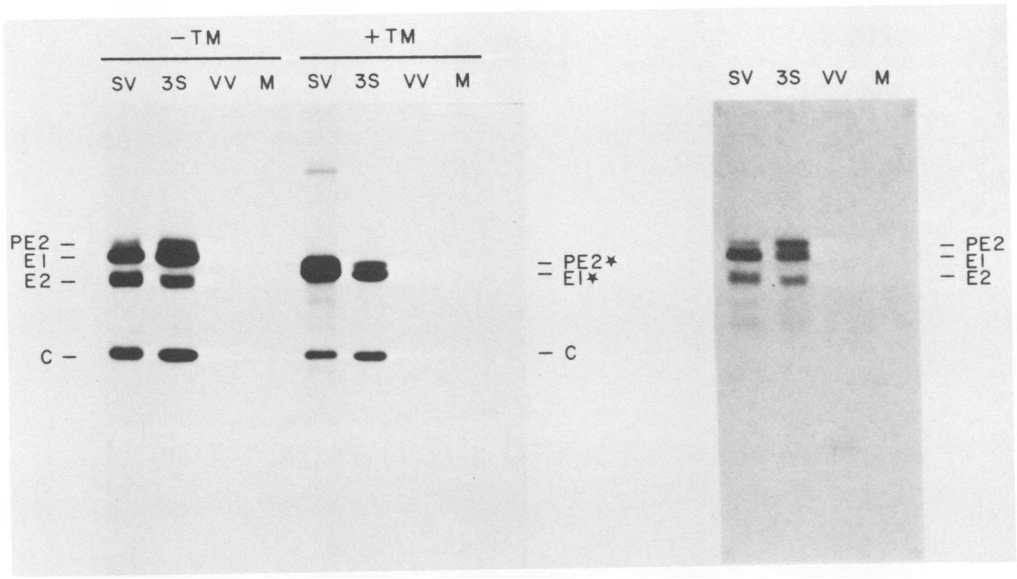

FIG. 7. Processing of Sindbis proteins in VV3S-7-infected cells. The left panel shows the effects of carrying out infections in the presence (+TM) or absence (−TM) of 1 μg of tunicamycin per ml. The proteins were continuously labeled for 4 h. The positions of the nonglycosylated Sindbis virus proteins (PE2* and E1*) are indicated. The right panel shows the results of infections in the presence of [^{3}H]glucosamine. In both experiments the radioactive proteins were immunoprecipitated with anti-Sindbis antiserum, separated on SDS-polyacrylamide gels, and fluorographed.

course of Sindbis infection and the yield of infectious virus were essentially unaffected (data not shown). In addition, chicken embryo fibroblast cultures which had been infected with vaccinia virus for 12 h could be superinfected with Sindbis virus with only a 5- to 10-fold loss in Sindbis yield. A number of complementation studies were undertaken with VV3S-7 and various Sindbis RNA$^+$ temperature-sensitive mutants defective in each of the structural protein genes. In these experiments, we examined the yield of Sindbis virus (assayed at the permissive temperature) produced from mixed infections of these mutants and VV3S-7 at the nonpermissive temperature. Monolayers were either infected with both viruses simultaneously or superinfected with Sindbis virus after 12 h of vaccinia infection; mixed infections with wild-type vaccinia virus were used as controls. Under these conditions, no significant increase in the yield of progeny Sindbis virus was detected in any of the mixed infections with VV3S-7.

As an alternative approach, we examined the ability of the vaccinia-encoded Sindbis glycoproteins to be incorporated into mature Sindbis virions. A number of glycoprotein-specific monoclonal antibodies capable of neutralizing Sindbis virus (AR339 strain) have been used to sequentially select resistant variants (40; A. L. Schmaljohn, unpublished data). A triple variant designated SIN V33/50/23, which is resistant to monoclonal antibody no. 33 (E1 specific) as well as monoclonal antibody no. 50 (E2 specific), was used to superinfect cells which had been previously infected with either wild-type vaccinia or VV3S-7. The vaccinia-encoded Sindbis proteins in VV3S-7 were derived from the HR small plaque strain of Sindbis virus, which reacts with both of these monoclonal antibodies. The progeny Sindbis virions in the medium were purified by both sedimentation velocity and equilibrium density centrifugation and tested by immunoprecipitation with the above-mentioned epitope-specific antisera capable of distinguishing between the Sindbis glycoproteins encoded by VV3S-7 and the input Sindbis virions. The data in Table 2 indicate that VV3S-7-derived proteins are capable of being incorporated into mature Sindbis virions which are infectious (as determined by removal of infectivity from immunoprecipitation supernatants and neutralization assays; data not shown) and implies, but does not rigorously prove, that they are biologically active. Similar results were obtained in BHK cells (data not shown). VV3S-7 proteins are incorporated specifically into Sindbis virions, as neither E1, E2, or C could be detected in mature VV3S-7 virions (data not shown).

TABLE 1. Presence of Sindbis glycoproteins at the infected cell surface[a]

Infecting virus	Reactivity (cpm) with the following antiserum[b]		
	PI	αC	αE1 + αE2
None (Mock infection)	1,090	6,100	3,600
Vaccinia	940	3,600	3,000
VV3S-7	920	4,700	11,800
Sindbis	1,000	31,700	72,200

[a] Iodinated staphylococcal protein A test for the presence of surface proteins which are reactive with anti-Sindbis antisera.
[b] PI, preimmune; αC, anti-Sindbis capsid; αE1 + E2, anti-Sindbis E1 and E2.

DISCUSSION

We have constructed a vaccinia virus recombinant which contains a complete cDNA copy of Sindbis virus 26S RNA inserted into the vaccinia *tk* gene. This insert is expressed as a 4.7-kb poly(A)$^+$ transcript which is translated into Sindbis PE2, E1, E2, and C proteins which are correctly cleaved, glycosylated, and transported. This demonstrates that the 26S sequences specify all the necessary catalytic activities or recognition signals required to ensure the proper synthesis and maturation of the encoded polypeptides. A similar conclusion was reached by Huth et al. (19), who microinjected the 26S mRNA of Semliki Forest virus into *Xenopus* oocytes and found that the capsid protein and the glycoproteins were formed and that the glycoproteins were

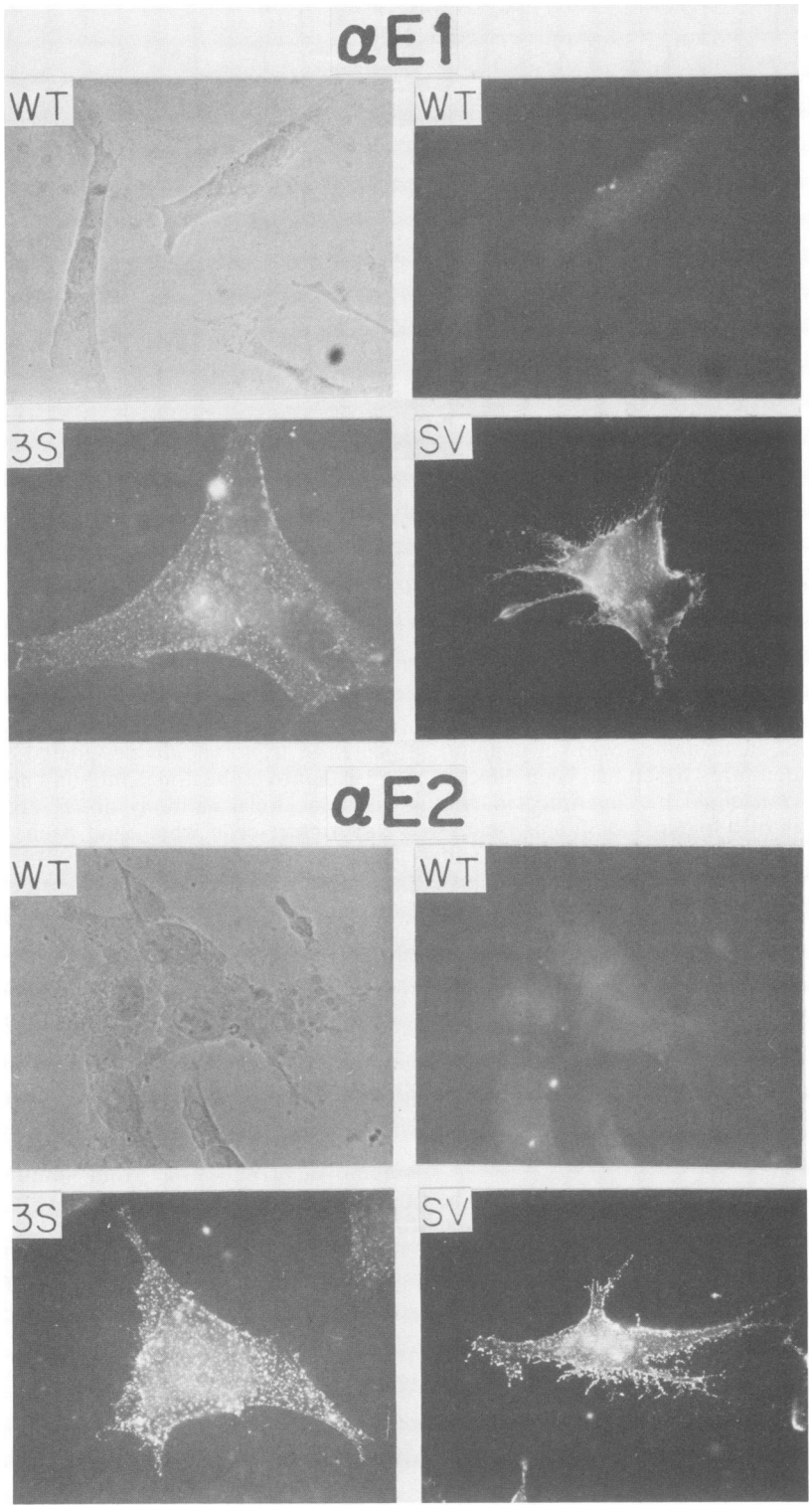

FIG. 8. Immunofluorescence microscopy of VV3S-7-infected cells. BHK cells infected for 8 h with either wild-type vaccinia virus (WT), VV3S-7 (3S), or Sindbis virus (SV) were fixed, incubated with E1-specific (αE1) or E2-specific (αE2) antibodies and stained with fluorescein-conjugated goat antisera as described in the Methods section. Immunofluorescence photographs are shown for all of these samples with phase contrast photographs included for wild-type vaccinia-infected cells only.

transported to the plasma membrane. We have also shown here that the Sindbis proteins produced from the vaccinia recombinant are incorporated into infectious virions in cells coinfected with antigenically distinguishable Sindbis virus, implying that these proteins are biologically active.

The processing of the alphavirus structural proteins re-

TABLE 2. Incorporation of vaccinia-encoded Sindbis glycoproteins into infectious Sindbis virus[a]

Mixed infection	% of Sindbis progeny reacting with the following antiserum[b]		
	Nonimmune	No. 33	No. 50
Sin V33/50/23 plus wild-type vaccinia	1	1	2
Sin V33/50/23 plus VV3S-7	2	32	56
Sin HRsp plus wild-type vaccinia	0	85	78

[a] See the text for experimental protocol.
[b] Expressed as percent of input cpm.

quires several proteolytic cleavages. The results presented here as well as earlier results show that these cleavages can occur in the absence of viral RNA replication or expression of the nonstructural proteins; thus, nonstructural proteases or virus nucleocapsids are not required for processing of the structural proteins. The cleavage of the capsid protein from the nascent polyprotein precursor is believed to be catalyzed by an autoproteolytic activity that resides within the capsid protein itself (1, 13, 41, 43). The two cleavages that separate PE2 and E1 from one another (and give rise to a small polypeptide, termed 6K protein, encoded between PE2 and E1 [59]) may be catalyzed by signalase (37). Such a mode of cleavage would imply that the C terminus of PE2 is found in the lumen of the rough endoplasmic reticulum, at least transiently, for signalase is known to be active only on the lumenal sides of the endoplasmic reticulum and not within the cytoplasm. Both cleavages occur after alanine, one of the preferred substrates for signalase. If, alternatively, the C terminus of PE2 is cytoplasmic, cleavage must be catalyzed by a currently unknown protease, which we believe unlikely, or by a virus protease present in the structural protein domain, of which the only one known at present is the capsid protease. The last cleavage in the processing of the alphavirus structural proteins, that of PE2 to E2 and E3 (20, 29, 59), has been postulated to be catalyzed by a Golgi protease during transit to the plasma membrane (9, 37). The expression experiments reported here as well as other expression experiments using Semliki Forest virus (19) show that this processing can occur in the absence of virus budding.

Previous attempts to express cloned copies of alphavirus 26S RNA have used DNA copies under the control of simian virus 40 promoters microinjected into nuclei (10, 22, 23). In one construction, incorrect splicing apparently led to incorrect processing and transport of one of the glycoproteins, whereas a second construction gave correct processing and transport. We have also attempted to use simian virus 40 promoters in an attempt to express Sindbis genes with unsatisfactory results (C. M. Rice, unpublished data). The use of vaccinia virus as a vector obviates the difficulties encountered by spurious splicing, since replication occurs in the cytoplasm. In addition, the use of an infectious vaccinia recombinant to express alphavirus genes has the obvious advantage that large numbers of diverse cell types can be synchronously infected with virtually 100% efficiency, a result that cannot be achieved by microinjection or calcium phosphate uptake of DNA. Finally, the biology of vaccinia and that of alphaviruses are compatible, allowing alphaviruses to replicate to high titers in cells infected with vaccinia. On the other hand, because both viruses possess a rather wide host range, cells can be chosen in which one virus will replicate well while being refractory to the replication of the other virus. Thus, this system of alphavirus gene expression is flexible and can be adapted to the purposes of the particular experiment.

The experiments demonstrating incorporation of the vaccinia-encoded Sindbis proteins into virions in mixedly infected cells imply that these proteins are properly processed and transported and possess biological activity. Unfortunately, we were unable to demonstrate complementation of VV3S-7 with RNA$^+$ *ts* mutants as a more stringent test of biological activity. This indicates either that the Sindbis cDNA clone used in this study is defective, which seems unlikely in view of the data presented here as well as extensive nucleotide sequence analysis (51), or, more likely, that the vaccinia recombinant may express insufficient quantities of alphavirus structural proteins to rigorously demonstrate their activity by complementation. In this event, it may be possible to obtain complementation by developing vaccinia expression systems that utilize more efficient promoters. In any event, this system is ideally suited to the use of site-specific mutagenesis to study the processing, transport, and immunogenicity of alphavirus glycoproteins. Improvements in the methodology for isolation of recombinant viruses, further characterization of vaccinia promoters and transcription termination signals, and development of rapid transient expression assays for preliminary analysis of recombinant plasmid constructions would greatly facilitate the use of vaccinia for such studies. These areas are currently being pursued in this and other laboratories.

Finally, the use of vaccinia virus to express foreign virus genes for potential vaccine development is under investigation in a number of laboratories. The results here show that vaccinia is also potentially useful for the development of vaccines against alphaviruses. Studies to test the efficiency of the recombinant VV3S-7 in producing immunity to Sindbis virus in experimental animals are under way.

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