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Complementation between Sindbis viral RNAs produces infectious particles with a bipartite genome

(alphaviruses/segmented genomes/virus evolution)

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ABSTRACT **Sindbis virus, the type member of the alphaviruses, is an enveloped virus containing a nonsegmented positive-strand RNA genome. We show that the nonstructural and the structural genes can function to produce infectious virus particles when they are expressed on two different RNA segments. The nonstructural genes are translated from an RNA in which the structural genes have been replaced by the chloramphenicol acetyltransferase gene [Xiong, C., Levis, R., Shen, P., Schlesinger, S., Rice, C. M. & Huang, H. V. (1989) *Science* 243, 1188–1191]. The structural genes are encoded in a defective-interfering RNA but are translated from a subgenomic RNA. Both segments contain the cis-acting sequences required for replication and packaging and are copackaged. This type of genome provides a model for an ancestral intermediate between alphaviruses and the multipartite positive-strand RNA viruses of plants. These different viruses show sequence similarities in their replicative proteins and are thought to have evolved from a common ancestor.**

The genome (49S RNA) of Sindbis virus consists of a single strand of RNA of 11.7 kilobases (kb) that is composed of two domains. The 5′ two-thirds of the genome codes for the viral nonstructural proteins—those proteins required for the replication and transcription of the viral RNAs. The 3′ one-third codes for the viral structural proteins—the capsid protein and the membrane glycoproteins. The nonstructural proteins are translated from genomic RNA, but the structural proteins are translated from a subgenomic 4.1-kb (26S) mRNA. Genomic and subgenomic RNAs are transcribed from genome length negative strands, the latter by internal initiation (reviewed in ref. 1).

The nonstructural genes are expressed and function in the absence of the structural genes. Xiong *et al.* (2) constructed a cDNA that contains those sequences required for the replicative functions of the virus, but the structural genes are replaced by the gene for the bacterial chloramphenicol acetyltransferase (CAT). When RNA (TRCAT RNA, Fig. 1) transcribed from this cDNA is transfected into cells, it is replicated, a subgenomic RNA is transcribed, and the latter is translated to produce CAT protein. The TRCAT genomic RNA is not packaged in the absence of the structural genes. However, if the cells are also infected with Sindbis virus, 49S RNA and TRCAT are found among the progeny particles.

Defective interfering (DI) RNAs derived from the Sindbis genome could provide a way of expressing the viral structural proteins in the absence of infectious virus. We have studied DI RNAs extensively and have defined sequences in these RNAs that are essential for their replication and encapsidation (3, 4). Levis *et al.* (5) reported that a DI RNA containing the CAT gene is translated to produce CAT protein; however translation was not very efficient. High levels of translation might be achieved if a subgenomic RNA, which could serve as an efficient mRNA, were produced. The studies of Levis *et al.* (6), who used DI RNAs to define the promoter for transcription of subgenomic RNAs, point to the feasibility of this approach. A cDNA fragment encompassing 98 nucleotides (nt) upstream and 117 nt downstream from the start of the 26S RNA was inserted into a DI cDNA. Cells transfected with the transcribed DI RNA produce a subgenomic RNA when they are also infected with Sindbis virus. Those subgenomic RNAs did not contain open reading frames and were not translated. We have now constructed a cDNA in which the 26S sequences were inserted downstream of the subgenomic promoter and flanking sequences. The DI RNA [DI(26S)] transcribed from this cDNA is diagramed in Fig. 1. When the DI RNA and TRCAT were transfected into cells, they complemented each other. Both RNAs were replicated and packaged. A significant number of particles contained both RNAs. The two RNAs functioned effectively as a segmented genome giving rise to plaque-forming units (pfu). The segmented genome was stable to plaque purification and continued passaging.

MATERIALS AND METHODS

Transcription and Transfection of Viral RNAs. Transcriptions were carried out using the SP6 DNA-dependent RNA polymerase as described (3). All transcripts were capped during transcription, labeled with [^{3}H]uridine, and analyzed by agarose gel electrophoresis following glyoxal denaturation to verify that they were intact. Lipofection was used for all transfections following the method published previously (4, 8). Cells were transfected with 0.5 μg of each RNA transcript and were incubated overnight at 30°C.

Other Procedures. The plasmid constructions, analysis of RNA, and other procedures have been described previously (3, 4) or are detailed in the figure legends.

RESULTS

Sindbis Virus with a Segmented Genome Produces Plaques. Monolayers of chicken embryo fibroblasts transfected with TRCAT and DI(26S) RNA synthesized four species of RNA [TRCAT RNA, its subgenomic RNA, and DI(26S) RNA and its subgenomic RNA (Fig. 2*a*, lane 1)], demonstrating that some cells were cotransfected and that the enzymes encoded by the TRCAT genome replicated the DI genome and transcribed the subgenomic 26S RNA. Cells transfected with TRCAT alone synthesized only TRCAT RNA and its subgenomic RNA, but cells transfected only with DI(26S) RNAs did not produce any viral RNAs (data not shown).

A sample of medium harvested from cells transfected with TRCAT and DI(26S) RNAs (passage 1 medium) was used to infect new cells that were analyzed for the presence of viral

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Abbreviations: CAT, chloramphenicol acetyltransferase; DI, defective interfering; pfu, plaque-forming unit(s); nt, nucleotide(s); moi, multiplicity of infection.

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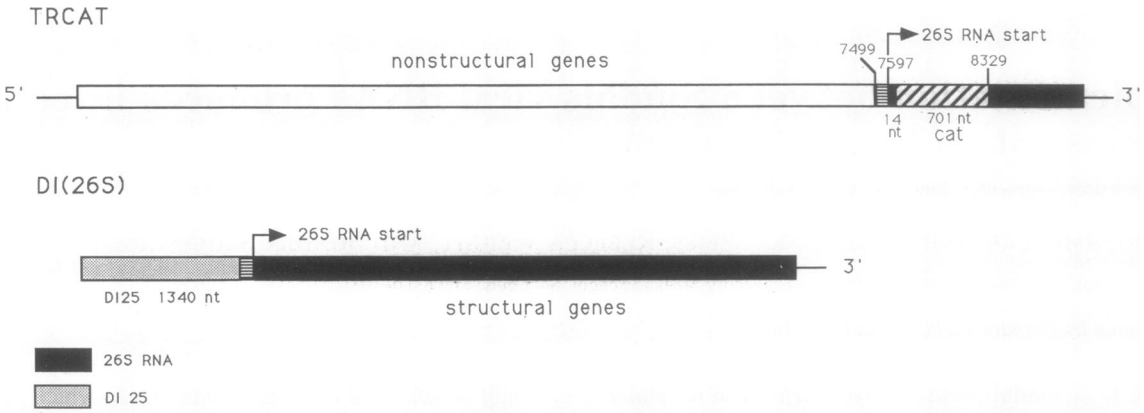

FIG. 1. Diagrams of Sindbis RNAs transcribed from cDNAs. TRCAT has been described (2). It contains 9×10^3 nt. The sequence from the Sindbis genome extends from the 5′ terminus for 7611 nt followed by an *Xba* I linker. At the 3′ terminus there are 616 nt plus the poly(A) tail from the Sindbis genome. The cDNA of DI(26S) was constructed from DI25 (3) and Toto1102 (7). The genome size is 5.6×10^3 nt. It contains 98 nt upstream of the start of 26S RNA (indicated by the horizontally lined region). The minimal sequences that define the promoter for 26S RNA transcription are 18 or 19 nt upstream and 5 nt downstream of the start of 26S RNA (6).

RNAs (Fig. 2*a*, lane 2). TRCAT and the DI(26S) RNAs and their respective subgenomic RNAs were the only viral RNAs detected. In some instances, a minor species of RNA migrating close to the size expected for virion 49S RNA was also observed. This RNA was Sindbis-specific based on blot hybridizations of the RNA after transfer from the gel to nitrocellulose (data are not shown, but see Fig. 2*a*, lane 6, and *Discussion*). No viral RNAs were detected in cells exposed to the extracellular fluids from cells transfected with either TRCAT or DI(26S) RNA alone. These results demonstrated that DI(26S) RNA provided the viral structural proteins for TRCAT as well as for its own packaging.

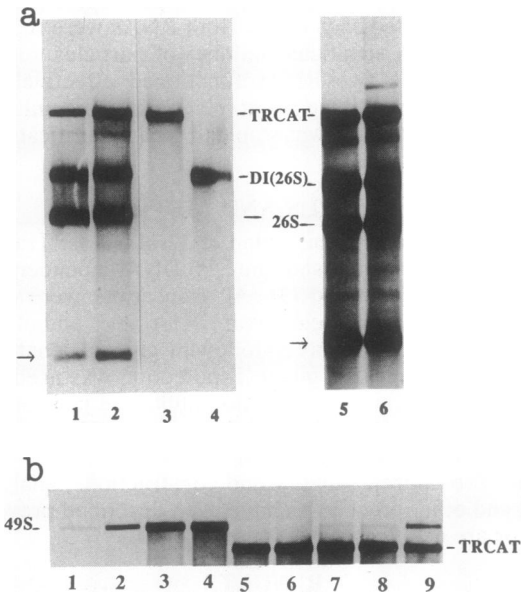

FIG. 2. Synthesis of Sindbis-specific RNAs in chicken embryo fibroblasts. (*a*) RNA analysis in cells transfected with Sindbis RNAs and in cells infected with medium from transfected cells. Cells transfected with TRCAT RNA (0.5 μg), DI(26S) RNA (0.5 μg), or the two together were incubated at 30°C for 16 hr in the presence of 50 μCi of [^{3}H]uridine (1 Ci = 37 GBq) and 1 μg of actinomycin D per ml to label the viral-specific RNAs. The uptake of RNA was enhanced by lipofectin (4, 8). Lane 1 shows the viral RNA pattern from cells transfected with TRCAT and DI(26S) RNA transcripts. Data for transfections with TRCAT and the DI RNAs alone are not shown. Samples from the medium harvested from transfected cells (passage 1 medium) were used to infect new monolayers of chicken embryo fibroblasts and the cells were labeled as described above. Lane 2 shows the viral RNA pattern in cells infected with medium from the TRCAT/DI(26S) transfection. The arrow indicates the position of migration of the subgenomic RNA transcribed from the negative-strand template of TRCAT. Lanes 3 and 4 show the TRCAT and DI(26S) transcripts, respectively. Lanes 5 and 6 show the Sindbis-specific RNAs synthesized in cells infected with plaque-purified virus. Samples of passage 1 medium from cells transfected with TRCAT and DI(26S) RNA were titered. Pieces of agarose containing individual plaques were isolated, and the virus was eluted. Samples from the eluates were used to infect chicken embryo fibroblasts that were labeled as described above. One of the 29 plaques containing only the segmented genome (lane 5) and one of the two plaques containing a 49S-like RNA (lane 6) are shown. Lanes 1–4 are from a different autoradiogram than lanes 5 and 6. The markers run along with lanes 5 and 6 verified that TRCAT, DI(26S), and 26S RNAs migrated at the correct positions. Details of the transcriptions, transfections, and RNA analysis have been described (3, 4). (*b*) Synthesis of viral-specific RNAs in cells infected with wild-type Sindbis virus in the absence or presence of plaque-purified TRCAT/DI(26S) particles. Chicken embryo fibroblasts were infected with wild-type Sindbis virus (lanes 1–4), with plaque-purified TRCAT/DI(26S) particles (lane 5), or with plaque-purified TRCAT/DI(26S) particles and Sindbis virus (lanes 6–9), and the cells were labeled as described above. TRCAT/DI(26S) particles were added at 1.1×10^5 pfu (lanes 5–9); wild-type Sindbis virus was added at 50 pfu (lanes 1 and 6), 250 pfu (lanes 2 and 7), 500 pfu (lanes 3 and 8), and 5×10^3 pfu (lanes 4 and 9). Only the pattern of labeled RNA in the region of the gel where 49S and TRCAT RNA migrate is shown. The other RNA species were identical to those seen in *a*.

pfu, in the range of 10^5–10^6 pfu/ml, were detected in passage 1 medium whether or not any RNA the size of 49S RNA was detected in the infected cells. This suggested that the plaques were due to TRCAT and DI(26S) RNAs functioning as a segmented genome. If this were true, the plaques should contain the two RNAs. We analyzed the plaques for CAT and DI RNA sequences by *in situ* plaque hybridization and found that essentially all of them contained both RNAs (Fig. 3). Infectious virus was obtained from individual plaques by cutting out the agarose piece containing the plaque and eluting the virus. A sample of the eluate was then used to infect new cells. For 29 of 31 plaques examined, the only viral RNAs detected in the newly infected cells were TRCAT, the DI RNA, and their appropriate subgenomic RNAs. In the other two cases, in addition to TRCAT and DI RNAs, a small amount of an RNA the size of 49S RNA was observed (Fig. 2*a*, lane 6).

Could the plaques be due to a recombinant 49S RNA that was not detected under the conditions of labeling? To address this question, we infected cells with a sample of plaque eluate containing 1.1×10^5 pfu plus increasing amounts of wild-type Sindbis virus (ranging from 50 to 5000 pfu). At these con-

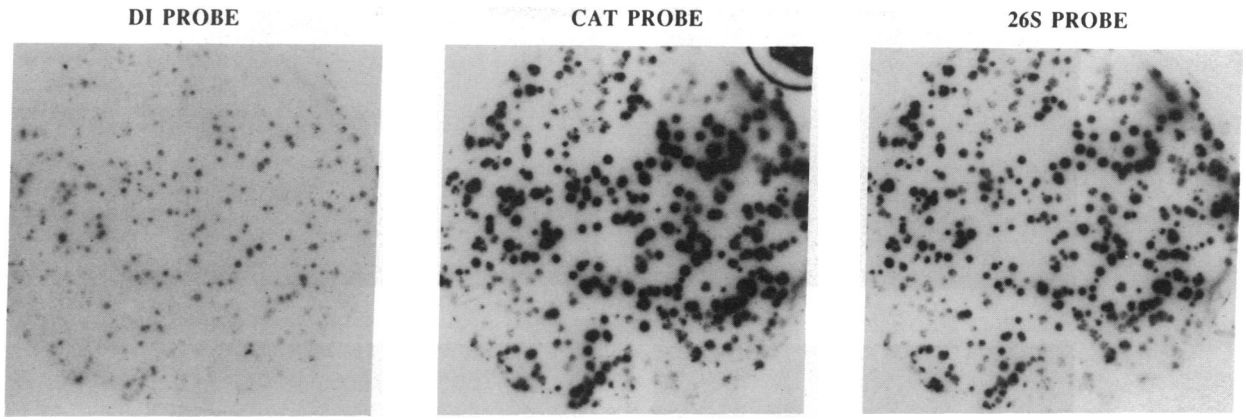

FIG. 3. Blot hybridization of plaques to detect TRCAT and DI(26S) RNA. The procedures for plaque lifts and *in situ* hybridization have been described (9). The DI probe was a 21 nucleotide complementary to the 5′ 21 nt of the DI RNA, which are different from the 5′ end of 49S genomic RNA (10). The CAT probe was a 720-base-pair *Xho* I/*Bam*HI restriction fragment from pCAT. The 26S RNA probe was linearized plasmid DNA containing the entire sequence of 26S mRNA.

centrations of added Sindbis virus, the synthesis of 49S virion RNA was suppressed in cells that were also replicating TRCAT and DI(26S) (Fig. 2*b*). Radioactively labeled 49S RNA could still be detected, however, when 250 pfu of wild-type Sindbis virus was added to the cells. A competent 49S RNA should have been detected if it represented only 0.2% of the particles present in a plaque and it is unlikely that an undetectable level of 49S RNA could be responsible for the formation of a plaque.

Plaque-purified virus samples containing the segmented genome have been passaged several additional times giving titers of about 2×10^7 pfu/ml. The two RNAs retained their original size and no 49S RNA was detected.

Copackaging of TRCAT and DI(26S) RNAs. If TRCAT and DI(26S) RNA function as a segmented genome to give rise to plaques, either the two RNAs must be copackaged or the concentration of particles containing one of the RNAs must be high enough to permit coinfection. Copackaging was first suggested by the observation that plaque titrations were linear at high dilutions and that the same number of plaques was obtained when samples were titered on 10^6 or 10^7 cells (Table 1). These data could only be the result of infection by two independent particles if both particles were present at a concentration 500-fold greater than the pfu [to give a multiplicity of infection (moi) of $1–2 \times 10^{-3}$ on 10^7 cells].

We used immunofluorescence microscopy to determine the number of cells infected with particles containing TRCAT RNA and the number infected with TRCAT and DI(26S) RNA. Three classes of fluorescent-positive cells were identified: those positive for CAT, those positive for the Sindbis glycoprotein E1, and those positive for both (Table 2 and Fig. 4). Six percent of the cells were positive for the Sindbis glycoprotein, which, based on the Poisson distribution, is the percent calculated to be infected at a moi of 0.06 pfu per cell. The cells positive for CAT and negative for the Sindbis structural protein (119 − 47 = 72) should be ones infected with particles containing only the TRCAT genome. Of the total number of cells counted, 150 were infected with TRCAT (see Table 2). A moi of 0.12 would be required to infect this number, 13% of the total. Based on these calculations, the concentration of particles able to produce CAT protein in infected cells is twice that of the particles able to give rise to pfu. If the presence of the two RNA segments in the same cell were due to coinfection rather than copackaging, DI particles would have to be present at a moi of 0.5 for 6% of the cells to be coinfected.

The immunofluorescence data did not give any information about the level of particles containing DI RNAs since these particles, if they infected cells by themselves, would be inactive. DI particles present in excess should be rescued by the addition of Sindbis virus to the samples. Dilutions of a virus stock containing TRCAT and DI(26S) particles were used to infect cells with or without added wild-type Sindbis virus (Fig. 5). In the absence of added Sindbis virus, TRCAT genomic RNA and its subgenomic RNA were still clearly detected when cells were infected with 6×10^3 pfu (lane 2). At this moi, the DI genomic and 26S subgenomic RNAs were only barely visible. In the presence of Sindbis virus, DI(26S) RNA was now detected at the same input pfu (lane 6, 6×10^3 pfu) as TRCAT and its subgenomic RNA had been in the absence of Sindbis virus. These data show that Sindbis virus was able to rescue the DI RNA but only at a dilution similar

Table 1. Plaque titrations provide evidence for copackaging of the segmented genome of Sindbis virus

Dilution of virus	No. of cells	Plaques counted
5×10^5	10^6	34, 36, 42, 42, 35, 47
	10^7	34, 38
10^6	10^6	16, 15, 21, 13, 15, 24
	10^7	18, 18
5×10^6	10^6	7, 4, 4, 3, 4, 5

Plaque titrations on 10^6 cells were performed in six-well 35-mm cell culture dishes and those on 10^7 cells were in 100-mm dishes. Samples of the diluted virus were added to the dishes for a 1-hr absorption period. The inocula were removed and the cells were overlaid with minimal essential medium containing 0.75% agarose. Plaques were visualized after staining with crystal violet.

Table 2. Immunofluorescence analysis to determine the number of cells expressing the CAT protein and the Sindbis structural protein E1

Inoculum	No. of cells counted	Positive for Sindbis	Positive for CAT	Positive for both
TRCAT/DI(26S)*	1131	78 (6%)	119 (11%)†	47
Sindbis virus	280	20 (7%)	0	0

The methods are described in the legend to Fig. 4. A moi of 0.06 pfu per cell was used.

*The TRCAT/DI(26S) virus was the same as that used to obtain the data in Fig. 2*b*. No 49S RNA was detected when cells were labeled with [^{3}H]uridine.

†The Sindbis structural proteins can only be expressed in cells also replicating TRCAT. Some suppression of CAT protein synthesis must occur in cells also producing the viral structural proteins. The total number of cells infected with TRCAT was 150, the sum of the cells positive for the CAT protein (119) and those positive for only the Sindbis structural protein (78 − 47 = 31).

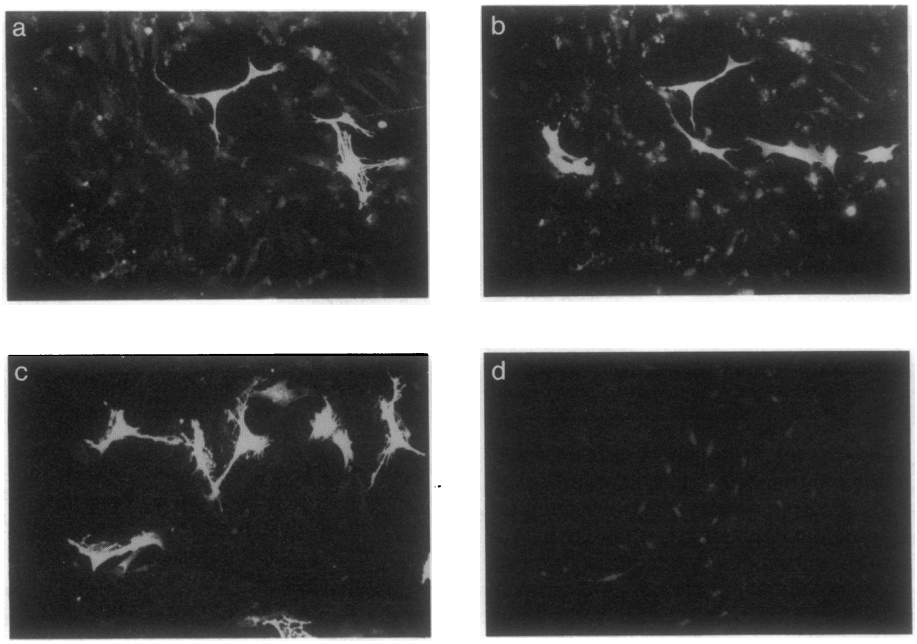

FIG. 4. Immunofluorescence microscopy of cells infected with a TRCAT/DI(26S) plaque eluate. Subconfluent monolayers of chicken embryo fibroblasts on coverslips were infected with either TRCAT/DI(26S) particles (*a* and *b*) or Sindbis virus (*c* and *d*) at a moi of 0.06 pfu per cell. A minimum time of 6 hr after infection was required to detect a positive signal for CAT. The cells were incubated in the presence of a Sindbis virus E1-specific mouse monoclonal antibody (1:500 dilution of ascites fluid) (11) to inhibit spread of infection. At 6 hr after infection the cells were washed with phosphate-buffered saline, fixed with 4% glutaraldehyde, and then permeabilized with 0.5% Triton X-100. The cells were treated with rabbit anti-CAT antibody (from 5 Prime → 3 Prime, Inc.) and with the monoclonal E1-specific antibody followed by fluorescein-conjugated goat anti-rabbit antibody and rhodamine-conjugated goat anti-mouse antibody. (*a* and *c*) Staining of the Sindbis E1 glycoprotein with the fluorescein-tagged antibody. (*b* and *d*) Staining of the CAT protein with the rhodamine-tagged antibody. The original pictures were taken in color to show the differences in labeling.

to the one at which TRCAT was still detected in the absence of helper virus. These results suggest that particles containing DI RNA were not present in a significant excess over those containing TRCAT RNA.

Taken together, the linearity of the plaque titrations, the immunofluorescence data, and the RNA analysis are all most consistent with copackaging of the two RNAs. They do not rule out the possibility that some of the plaques were due to coinfection.

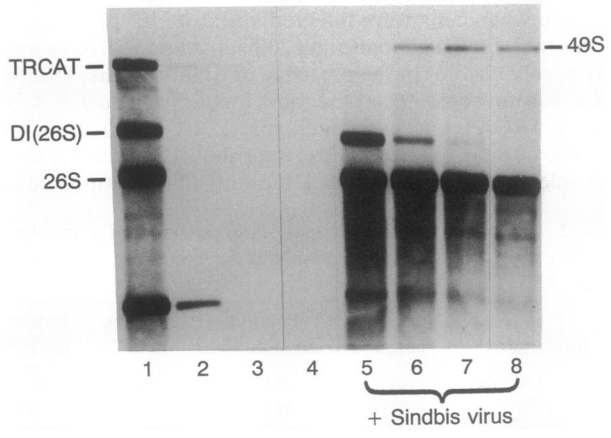

FIG. 5. Rescue of DI(26S) RNA in cells infected with TRCAT/DI(26S) particles and Sindbis virus. Monolayers of chicken embryo fibroblasts were infected with decreasing amounts of a stock of a TRCAT/DI(26S) plaque eluate in the absence (lanes 1–4) or presence (lanes 5–8) of Sindbis virus, added at a moi of 20 (10^7 pfu) to ensure that all cells would be infected. The cells were labeled with [^{3}H]-uridine in the presence of actinomycin D for 7 hr at 37°C. TRCAT/DI(26S) particles were added as follows: lanes 1 and 5, 6×10^4 pfu; lanes 2 and 6, 6×10^3 pfu; lanes 3 and 7, 6×10^2 pfu; lanes 4 and 8, 60 pfu.

DISCUSSION

These studies show that Sindbis virus can be stably propagated in cultured chicken embryo fibroblasts when its genome is divided into two segments: one containing the nonstructural genes essential for replication of the genome, the other containing the structural genes. In some experiments we also detected an RNA the size of the Sindbis genomic 49S RNA, suggesting that recombination had occurred. We have isolated this RNA free of the segmented genomes and established that it is an infectious recombinant genome distinct from the wild-type 49S RNA (B.W. and S.S., unpublished data). Recombinants that arise in cells replicating TRCAT and DI(26S) probably do not have a significant selective advantage over the segmented genomes and would be suppressed as were the low levels of wild-type Sindbis virus RNA in coinfection experiments.

Copackaging of the two segments in the same particle may also be a factor in perpetuating the segmented genome. Coinfection should also lead to transmission of the segmented genome, but, if the two RNAs were in separate particles, high multiplicities of infection would be essential for continued survival. Animal viruses whose genomes are naturally segmented do package the segments within the same particle. The mechanisms of spread of these viruses in nature probably would make a requirement for coinfection an inefficient means for survival. Plant viruses, however, do package their RNA segments in separate particles. These viruses are produced in large amounts and this may ensure that the different particles all enter the same cell.

The ability of the two domains of the Sindbis genome to function effectively as two segments may be relevant to some of the ideas about the evolution of RNA viruses. A comparison of the protein sequences of different members of positive-strand RNA virus families has grouped these viruses into two large superfamilies, one referred to as Sindbis or alpha-

virus-like and the other referred to as picorna-like (12–14). The alpha-like superfamily includes the plant viruses: the tobamoviruses, bromeviruses, cucumoviruses, ilarviruses, and tobraviruses. The latter four have segmented genomes. The sequence similarity among the alphavirus-like family is in their replicative proteins (15, 16). In contrast, they show no relatedness among their structural proteins. The similarity of some regions and diversity of others led to the concept of a modular construction of viral genomes (12). Each module could evolve independently. New viruses would arise by reassortment of segments or by intermolecular recombination. Both phenomena occur in RNA viruses, although reassortment among segments would be expected to occur at a higher frequency than recombination. Sequence similarities between the replicative proteins of Sindbis virus and the plant viruses led to the hypothesis that they have evolved from a common ancestor (15, 16). A virus with a segmented genome, such as the one described here, provides a model that might be representative of an ancestral intermediate between alphaviruses and the multipartite positive-strand RNA viruses of plants.

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