JOURNAL OF VIROLOGY, Mar. 1984, p. 865–872
0022-538X/84/030865-08$02.00/0
Copyright © 1984, American Society for Microbiology
Vol. 49, No. 3

Common and Distinct Regions of Defective-Interfering RNAs of Sindbis Virus

STEPHAN S. MONROE† AND SONDRA SCHLESINGER*
Department of Microbiology and Immunology, Washington University School of Medicine, St. Louis, Missouri 63110

Received 28 August 1983/Accepted 18 November 1983

Defective-interfering (DI) particles are helper-dependent deletion mutants which interfere specifically with the replication of the homologous standard virus. Serial passaging of alphaviruses in cultured cells leads to the accumulation of DI particles whose genomic RNAs are heterogeneous in size and sequence composition. In an effort to examine the sequence organization of an individual DI RNA species generated from Sindbis virus, we isolated and sequenced a representative cDNA clone derived from a Sindbis DI RNA population. Our data showed that: (i) the 3′ end of the DI RNA template was identical to the 50 nucleotides at the 3′ end of the standard RNA; (ii) the majority (75%) of the DI RNA template was derived from the 1,200 5′-terminal nucleotides of the standard RNA and included repeats of these sequences; and (iii) the 5′ end of the DI RNA template was not derived from the standard RNA, but is nearly identical to a cellular $tRNA^{Asp}$ (S. S. Monroe and S. Schlesinger, Proc. Natl. Acad. Sci. U.S.A. **80**:3279–3283, 1983). We have also utilized restriction fragments from cloned DNAs to probe by blot hybridization for the presence of conserved sequences in several independently derived DI RNA populations. These studies indicated that: (i) a 51-nucleotide conserved sequence located close to the 5′ end of several alphavirus RNAs was most likely retained in the DI RNAs; (ii) the junction region containing the 5′ end of the subgenomic 26S mRNA was deleted from the DI RNAs; and (iii) the presence of $tRNA^{Asp}$ sequences was a common occurrence in Sindbis virus DI RNAs derived by passaging in chicken embryo fibroblasts.

Defective-interfering (DI) particles are a special class of virus mutants with two general characteristics: they contain deletions of the standard virus genome and they interfere with the replication of the standard virus (25). The replication and amplification of DI genomes are dependent on complementation by the standard virus. Many regions of the genome, particularly those involved in coding for *trans*-acting proteins, can be deleted from DI genomes; however, DI genomes should retain those sequences which represent recognition signals for replication and particle assembly. We have been analyzing the genome RNA of DI particles of the alphavirus Sindbis virus with the goal of defining those particular sequences for this virus.

The alphavirus genome is a single-stranded RNA (49S RNA) containing approximately 12,000 nucleotides (38a). During the course of infection, a full-length complementary RNA is made which serves as a template for both the synthesis of new progeny genomic RNA and a subgenomic mRNA (26S RNA). The 26S RNA codes for the structural proteins of the virus (28) and is identical to the 3′ third of the virion RNA (27). Both virion and 26S RNAs are capped at the 5′ end and polyadenylated at the 3′ end. Almost all of the information available about DI particles of alphaviruses comes from studies with two members of this genus, Sindbis virus and Semliki Forest virus (37, 38). The generation of DI particles from these viruses was thought to occur by successive internal deletions until a limit size of 18 to 20S RNA was reached (8, 34). It has become increasingly clear, however, that the structures of the deleted RNA molecules are more complex than can be explained by simple deletions. Pettersson reported that the 5′-terminal sequences of DI RNAs from Semliki Forest virus are heterogeneous and different from the standard virus (26). More recently, sequences obtained from cloned cDNAs derived from DI RNAs demonstrated that the Semliki Forest virus DI RNAs contain repeated segments and rearrangements as well as deletions (15, 16, 33). Our initial sequence analyses of Sindbis virus DI RNAs also indicated that these molecules contain both rearrangements and repeats (5, 18). In addition, they demonstrated that the 5′ termini of two independently derived DI RNAs are almost identical to nucleotides 10 to 75 of a cellular $tRNA^{Asp}$ (19).

The heterogeneity of Sindbis virus DI RNAs made it essential for us to clone cDNA copies to determine the sequence of a unique DI RNA and to enable us to evaluate the pattern of repeats and rearrangements in a single DI RNA species. We report here the sequence of a cDNA copy of a Sindbis virus DI RNA and compare this sequence with that of the standard virion RNA determined by J. and E. Strauss and their colleagues (21–24; J. H. Strauss and E. G. Strauss, personal communication). We have also used isolated DNA fragments from this cloned cDNA to probe for the presence of specific sequences in several other Sindbis virus DI RNA populations.

MATERIALS AND METHODS

Generation of DI particles and purification of viral RNAs. DI-2 particles were generated by 18 undiluted passages of Sindbis virus on chicken embryo fibroblasts (CEF) (5). DI-3 particles were generated from a cloned stock of Sindbis virus by passaging on CEF at a constant multiplicity of 50 PFU per cell. DI-3p7 represents the harvest from the 7th such passage, and DI-3p16 represents the harvest from the 16th passage. DI-4 particles were generated from the same cloned stock, by passaging on baby hamster kidney (BHK) cells at a constant multiplicity of 50 PFU per cell. The yield from the

* Corresponding author.
† Present address: Biophysics Laboratory, University of Wisconsin—Madison, Madison, WI 53706.

15th such passage was used for the preparation of DI RNA. DI-1 was produced by passage on BHK cells (11); DI-5 and DI-6 were obtained by passaging on CEF. Virus growth and purification of virion RNA by phenol-chloroform extraction and oligodeoxythymidylate [oligo(dT)]-cellulose chromatography were described previously (11).

Cloning of double-stranded cDNA. Single-stranded cDNA was synthesized in reactions containing 100 mM Tris-hydrochloride (pH 8.3), 50 mM KCl, 8 mM $Mg(OAc)_2$, 2 mM dithiothreitol, 0.5 mM each of four deoxynucleoside triphosphates, 50 μg of standard or DI-2 RNA per ml, 10 μg of $oligo(dT)_{12-18}$ per ml, and 100 U of avian myeloblastosis virus reverse transcriptase per ml (6). Incubation was at 37°C for 1 h. Alkali digestion of the RNA template, synthesis of the second strand with *Escherichia coli* DNA polymerase I (large fragment), and nuclease S1 treatment of the double-stranded cDNA were carried out as described previously (12). Double-stranded cDNA was chromatographed on Bio-Gel A-5m, and selected fractions were tailed with dCTP (20). Plasmid pUC4, derived from pBR322, contains a single *Pst*I site within a symmetrical M13mp7 polylinker and does not contain a *Pst*I site within the ampicillin resistance gene (39). Linear plasmid DNA was prepared by digestion with *Pst*I and was tailed with dGTP (20). Equimolar amounts of tailed plasmid and cDNA were annealed in 100 mM NaCl–10 mM Tris-hydrochloride (pH 7.5)–1 mM EDTA (35). The DNA was recovered by ethanol precipitation and used to transform competent *E. coli* MC1061 cells by a modification of the method of Kushner (13, 35).

Nuclease digestion of cDNA-RNA hybrids. Uniformly labeled DI-2 RNA was isolated from virus grown in the presence of $^{32}P_i$. This RNA was hybridized to *Pst*I-digested plasmid DNA under conditions favoring RNA-DNA hybrid formation [80% formamide, 0.4 M NaCl, 50 mM piperazine-*N*,*N*′-bis(2-ethanesulfonic acid), pH 6.4, 1 mM EDTA, 50°C] (7). The samples were digested with a mixture of RNases A and T_1, and the extent of protection of the input RNA by various clones was determined by precipitation with trichloroacetic acid. The colinearity of the inserted sequence and the template RNA was determined by hybridization of DI-2 [^{32}P]RNA and clone pSS01 DNA as above, followed by digestion with nuclease S1 and resolution of the protected hybrids on a 1.5% agarose gel.

DNA sequence analysis of clone pSS01 DNA. DNA fragments for sequence analysis were generated by digestion of pSS01 DNA with restriction endonucleases, using standardized conditions (3). Fragments were labeled at their 5′ end with polynucleotide kinase and [γ-^{32}P]ATP (17) or at their 3′ ends with reverse transcriptase and [α-^{32}P]dCTP (32). Individual fragments were resolved by strand separation (17) and were purified by DEAE-cellulose chromatography (32). Sequence analysis was performed according to the procedure of Maxam and Gilbert (17) as modified by Smith and Calvo (32).

Preparation of ^{32}P-labeled hybridization probes. Labeled DNA fragments for blot hybridization analysis were generated by digestion of plasmid DNA under standardized conditions (3). Individual fragments were isolated from native acrylamide gels and purified by DEAE-cellulose chromatography (32). They were subsequently digested for 2 to 5 min at 20°C in 70 mM Tris-hydrochloride–10 mM $MgCl_2$–10 mM dithiothreitol, using 0.1 to 0.25 pmol of 3′ ends and 0.5 U of *E. coli* exonuclease III per μl (31). The digested fragments were then labeled at their 3′ ends by filling in with [α-^{32}P]dCTP and reverse transcriptase as described above.

Blot hybridization analysis of viral RNAs. RNA samples were denatured in 1 M glyoxal–50% dimethylsulfoxide–10 mM sodium phosphate, pH 7, at 50°C for 1 h and resolved on a 1.2% agarose gel (2). The gel was treated for transfer as described before (19), except that the 50 mM NaOH incubation was lengthened to 45 min. The paper was prepared and activated essentially as described by Seed (29). The RNA was electrophoretically transferred to activated paper at 25 V for 6 h in 25 mM sodium phosphate (pH 5.5)–1 mM EDTA (36). The paper was prehybridized at 42°C for 16 h in 50% formamide–5× SSPE (1× SSPE = 0.18 M NaCl, 10 mM sodium phosphate, pH 7.7, 1 mM EDTA)–5× Denhardt solution (4)–1% glycine–125 μg of denatured calf thymus DNA per ml–0.1% sodium dodecyl sulfate (1). Hybridization was at 42°C for 16 to 48 h in the same buffer containing 10% dextran sulfate. After hybridization, the paper was washed twice for 10 min in 2× SSPE–0.1% sodium dodecyl sulfate and then twice for 10 min in 0.1× SSPE–0.1% sodium dodecyl sulfate at room temperature.

Materials. Calf intestinal alkaline phosphatase and *E. coli* DNA polymerase I (large fragment) were obtained from Boehringer Mannheim Biochemicals; restriction enzymes and *E. coli* exonuclease III were from New England Biolabs, Inc.; deoxynucleoside triphosphates, $oligo(dT)_{12-18}$, polynucleotide kinase, and terminal deoxynucleotidyl transferase were from P-L Biochemicals, Inc.; and nuclease S1 was from Sigma Chemical Co. [α-^{32}P]dCTP was obtained from Amersham Corp., and [^{3}H]uridine was from New England Nuclear Corp. [γ-^{32}P]ATP was synthesized from carrier-free $^3H^{32}PO_4$ (New England Nuclear) as described previously (41). Reverse transcriptase from avian myeloblastosis virus was provided by J. Beard, Life Sciences, Inc. The rat genomic clone containing the $tRNA^{Asp}$ gene pRT1dge was kindly provided by S. Nishimura and T. Sekiya (30).

RESULTS

Sequence of a cDNA clone derived from a population of DI RNAs of Sindbis virus. Our first step in analyzing a cDNA copy of the Sindbis virus DI-2 RNA was to select a cDNA clone representative of a major fraction of the DI RNA species present in the original population. To this end, several clones were examined for the extent to which they protected the total DI RNA population from RNase digestion (Table 1). One of the clones (pSS01) which protected 50% of the original DI RNA was analyzed further to determine whether the insert represented a colinear copy of the DI RNA. This analysis was essential to distinguish between sequence rearrangements present in the original DI RNA and those which might have occurred during the cloning procedures (40). After hybridization of the cDNA with DI-2

TABLE 1. RNase protection of DI-2 RNA by cloned cDNAs

DNA clone	cDNA insert size (kilobases)	RNase resistance (%)[a]
No DNA		5.1
pUC4		6.5
102	1.7	38
104	1.8	14
117	1.9	52
211	2.5	32
225(pSS01)	2.3	54
227	1.4	36

[a] Digestion with RNase is described in the text.

TABLE 2. cDNA restriction fragments used for blot hybridization

Source of DNA (region of the genome)	Restriction fragment	Size (base pairs)
Rat tRNA^{Asp} [a]	*Hae*III	314
pSS01 (B)	*Hin*dIII/*Hpa*II [b]	141
pSS01 (W–X)	*Hpa*II [c]	358
pSindbis 49S (junction)	*Sau*96-I [d]	101

[a] The plasmid carrying the tRNA^{Asp} gene was provided by Nishimura and Sekiya (30).

[b] This fragment is located between nucleotides 138 and 278 in pSS01 DNA and is homologous to nucleotides 130 to 270 of Sindbis virus 49S RNA.

[c] This fragment is located between nucleotides 1,312 and 1,669 in pSS01 DNA and corresponds to nucleotides 2,678 to 3,037 of 26S RNA.

[d] This fragment extends from nucleotide −4 to 97 of Sindbis virus 26S RNA.

[^{32}P]RNA and digestion with nuclease S1, the RNA-DNA hybrids were resolved by agarose gel electrophoresis (Fig. 1). The protected hybrid was approximately the same size as the double-stranded cDNA insert (~2.3 kilobase pairs). This result taken together with the data presented in Table 1 shows that the sequences in the cDNA insert from clone pSS01 were colinear with a major species within the original DI-2 RNA population.

The insert of pSS01 was sequenced by the chemical method of Maxam and Gilbert (17). The strategy used and the sequences obtained are shown in Fig. 2. A diagram of this sequence and that of the 49S virion RNA of Sindbis virus (determined by J. and E. Strauss and their colleagues) are also shown in Fig. 2.

We reported previously that the 5′ terminus of DI-2 RNA is not derived from the standard RNA, but is almost identical to nucleotides 10 to 75 of a cellular tRNA^{Asp} (19). A portion of this tRNA sequence was present at the 5′ end of pSS01 (Fig. 2B). The remaining sequences in the clone were derived from three separate regions of the standard virion RNA. Approximately 75% of the sequences came from the 5′ 1,200 nucleotides of the standard RNA (Fig. 2B, regions B to G). The remaining 25% consisted of the 50 nucleotides at the 3′ terminus (Fig. 2B, region Z) and a 429-nucleotide segment, part of which was duplicated, from within the structural protein coding region (Fig. 2B, regions W and X). The latter sequence was detected in the entire DI-2 RNA population by the dideoxy chain termination method of sequencing and is also present in a DI RNA population derived in BHK cells (DI-1) (18).

Three regions of the DI genome were present in duplicate. Region C was present as a tandem repeat, and regions G and W were also repeated as a unit (Fig. 2B). These repeats decreased the overall sequence complexity of the DI genome. Thus, pSS01 contained only 13% of the sequences present in standard virion RNA, although its physical length was 20% that of the standard. This pattern of deletions, duplications, and rearrangements of the standard RNA resembled that found in DI RNAs derived from Semliki Forest virus (15, 16, 33). We could not detect, however, any significant nucleotide sequence homology between the repeated regions in the Semliki Forest virus-derived DI RNAs (16) and those found in pSS01.

Analysis of Sindbis virus DI RNAs by blot hybridization. We used several fragments from cDNA clones to probe different populations of DI RNAs for the presence of sequences found in pSS01. The four regions examined and the restriction fragments used are described in Table 2. These experiments were undertaken to answer the following questions: (i) How common is the presence of tRNA^{Asp} in Sindbis virus DI RNA populations? (ii) When is the tRNA^{Asp} detected during the evolution of DI RNAs? (iii) Are regions of standard viral sequence, which are conserved in this DI RNA, found in other DI RNAs? and (iv) Is the junction region between the structural and nonstructural genes of the virus deleted from DI RNAs?

The DI-3 RNA was generated in CEF by controlled high-multiplicity passaging. The evolution of this DI RNA population is shown in Fig. 3. The DI RNA obtained from passage 7 has a molecular weight of 2.3 × 10^6, about half the size of standard virion RNA. Although the DI RNA from passage 7 was homogeneous in size, it may represent more than one species. The DI-3 RNAs from passage 16 were heterogeneous in size.

The data analyzing DI-2 RNA, the early- and late-passage DI-3 RNA, and one of the DI RNAs populations generated in BHK cells are shown in Fig. 4 and 5. The results are summarized in Table 3, which also includes information about several other DI RNAs. Figure 4 shows the amount of each RNA sample loaded on the gel based on staining with

TABLE 3. Summary of blot hybridizations to determine the presence or absence of particular sequences in DI RNAs of Sindbis virus

DI	Source	cDNA probe used [a]			
		tRNA^{Asp}	B	W–X	Junction
1	BHK	−	+	+	ND [b]
2	CEF	+	+	+	−
3p7	CEF	−	+	−	−
3p16	CEF	+	+	−	−
4	BHK	−	+	+	−
5	CEF	+	ND	ND	ND
6	CEF	−	ND	ND	ND

[a] See Table 2 for a more complete description of the probe.

[b] ND, Not done.

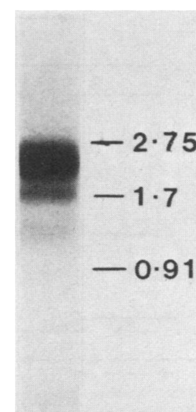
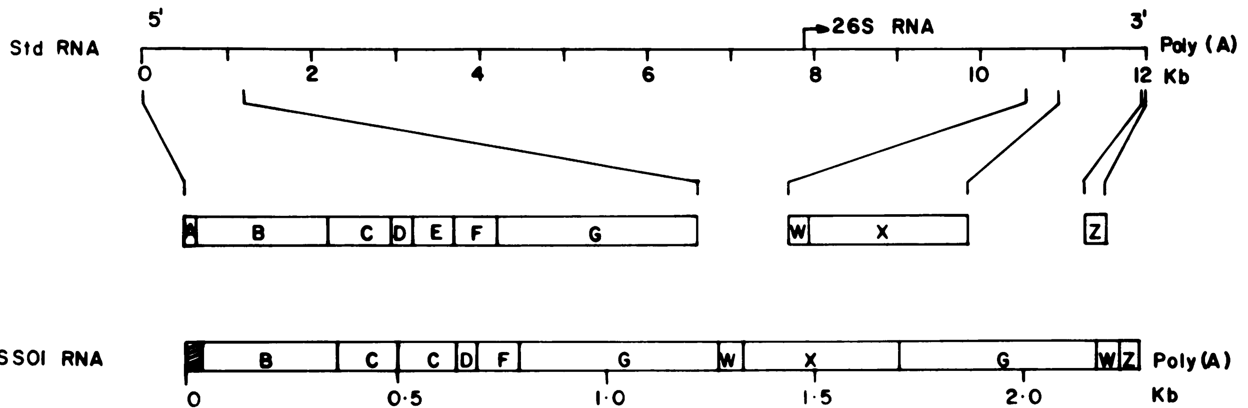


FIG. 1. Agarose gel electrophoresis of nuclease S1-resistant RNA-DNA hybrids. DI-2 [^{32}P]RNA and pSS01 DNA were hybridized and subsequently digested with nuclease S1. The samples were resolved on a 1.5% agarose gel run at 0.75 V/cm for 15 h. The positions of restriction fragment marker bands, identified by ethidium bromide staining, are indicated.

A

```
ACGCGGGAGA CCGGGGTTCG GTTCCCCGAC GGGGAGCCAA ACAGCCGACC AATTGCACTA CCATCACAAT GGAGAAGCCA GTAGTAAACG TAGACGTAGA   100
CCCCCAGAGT CCGTTTGTCG TGCAACTGCA AAAAGCTTCC CGCAATTTGA GGTAGTAGCA CAGCAGGTCA CTCCAAATGA CCATGCTAAT GCCAGAGCAT   200
TTTCGCATCT GGCCAGTAAA CTAATCGAGC TGGAGGTTCC TACCACAGCG ACGATCTTGG ACATAGGCAG CGCACCGGCT CGTAGCATGT TTTCCGAGCA   300
CCAGTATCAT TGTGTCTGCC CCATGCGTAG TCCAGAAGAC CCGGACCGCA TGATGAAATA TGCCAGTAAA CTGGCGGAAA AAGCGTGCAA GATTACAAAC   400
AAGAACTTCC ATGAGAAGAT TAAGGATCTC CGGACCGTAC TTGATACGCC GGATGCTGAA ACACCATCGC TCTGCTTTCA CAACGATGTT ACCTGCAAAT   500
ATGCCAGTAA ACTGGCGGAA AAAGCGTGCA AGATTACAAA CAAGAACTTG CATGAGAAGA TTAAGGATCT CCGGACCGTA CTTGATACGC CGGATGCTGA   600
AACACCATCG CTCTGCTTTC ACAACGATGT TACCTGCAAC ATGCGTGCCG AATATTCCGT CATGCAGGAC GTGTATATCA ACGCTCCCGG AACTACAACA   700
CCAACTGGGC CGACGAGAAA GTCCTTGAAG CGCGTAACAT CGGACTTTGC AGCACAAAGC TGAGTGAAGG TAGGACAGGA AAATTGTCGA TAATGAGGAA   800
GAAGGAGTTG AAGCCCCGGT CGCGGGTTTA TTTCTCCGTA GGATCGACAC TTTATCCAGA ACACAGGCCA GCTTGCAGAG CTGGCATCTT CCATCGGTGT   900
TCCACTTGAA TGGAAAGCAG TCGTACACTT GCCGCTGTGA TACAGTGGTG AGTTGCGAAG GCTACGTAGT GAAGAAAATC ACCATCAGTC CCGGGATTCA  1000
CGGGAGAAAC CGTCGGATAC GCGGTTACAC ACAATAGCGA GGGCTTCTTG CTATGCAAAG TTACTGACAC AGTAAAAGGA GAACGGGTAT CGTTCCCTGT  1100
GTGCACGTAC ATCCCGGCCA CCATATGCGA TCAGATGACT GGTATAATGG CCACGGATAT ATCACCTGAC GATGCACAAA AACTTCTGGT TGGGCTCAAC  1200
CAGCGAATTG TCATTAACGG TAGGACTAAC AGGAACACCA ACACCATGCA AAATTACCTT CTGCCGATCA TAGCAAAAAT CAAATGCTGC GGCTCCTTGG  1300
AATGTCAGCC GGCCGCTCAT GCAGACTACC TGCAAGGTCT TCGGAGGGGT CTACCCCTTT ATGTGGGGAG GAGCGCAATG TTTTTGCGAC AGTGAGAACA  1400
GCCAGATGAG TGAGGCGTAC GTCGAATTGT CAGCAGATTG CGCGTCTGAC CACGCGCAGG CGATTAAGGT GCACACTGCC GCGATGAAAG TAGGACTGCG  1500
TATAGTGTAC GGGAACACTA CCAGTTTCCT AGATGTGTTC GTGAACGGAG TCACACCAAG AACGTCTAAA GGCCTGAAAG TCATAGCTGG ACCAATTTCA  1600
GCATCGTTTA CGCCATTCGA TCATAAGGTC GTTATCCATC GCGGCCTGGT GTACAACTAT GACTTCCCGG AATATGGAGC GATGAAACCA GGAGCGTTTG  1700
GAGGAAGAAG GAGTTGAAGC CCGGGTCGCG GGTTTATTTC TCCGTAGGAT CGACACTTTA TCCAGAACAC AGGCCAGCTT GCAGAGCTGG CATCTTCCAT  1800
CGGTGTTCCA CTTGAATGGA AAGCAGTCGT ACACTTGCCG CTGTGATACA GTGGTGAGTT GCGAAGGCTA CGTAGTGAAG AAAATCACCA TCAGTCCCGG  1900
GATCACGGGA GAAACCGTGG GATACGCGGT TACACACAAT AGCGAGGGCT TCTTGCTATG CAAAGTTACT GACACAGTAA AAGGAGAACG GGTATCGTTC  2000
CCTGTGTGCA CGTACATCCC GGCCACCATA TGCGATCAGA TGACTGGTAC TAATGGCCAC GGATATATCA CCTGACGATG CACAAAAACT TCTGGTTAAA  2100
CTCAACCAGC GAATTGTCAT TAACGGTAGG ACTAACAGGA ACACCAACAC CATGCAAAAT TACCTTCTGC CGATCCTAGC AAAAATCAAA TGCTGCGGCT  2200
CCTTGGAATG TCAGCCGGCC GCTCATGCAG ACTACTTTTA TTATTTCTTT TATTAATCAA CAAAATTTTG TTTTTAACAT TTC                   2283
```

B



C

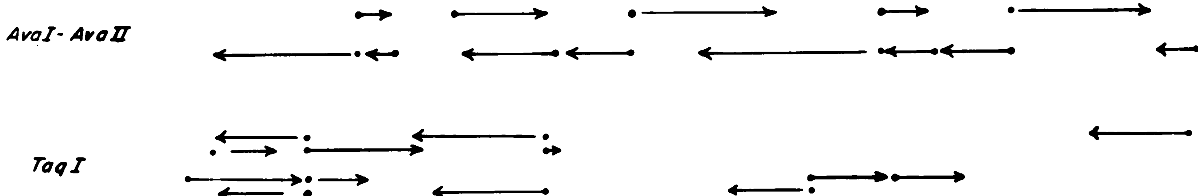


FIG. 2. Strategy used for sequencing pSS01 insert and sequences obtained. (A) Nucleotide sequence of the cDNA insert in plasmid pSS01. The sequence is presented in the 5′ to 3′ direction of the RNA template. (B) A comparison between the deduced sequence of pSS01 RNA and that of standard Sindbis HR virus RNA. The top line represents the total 12 kilobases (kb) of standard RNA; the regions of homology with the DI RNA expanded below. Regions A to G are derived from the 5′ two-thirds of the standard genome which codes for the nonstructural proteins. Regions W to Z are derived from the 3′ one-third of the standard genome which codes for the structural proteins. Region A, not present in the DI RNA, represents the 31 nucleotides at the 5′ end of standard RNA. The hatched region at the 5′ end of the DI RNA represents the 39 necleotides in the clone which are derived from a cellular tRNA (19). Poly (A), Polyadenylated. (C) Sequencing strategy for pSS01 DNA. The dots represent *Ava*I/*Ava*II (upper) or *Taq*I (lower) sites used for labeling. The lengths of the arrows indicate the extent to which each fragment was sequenced.

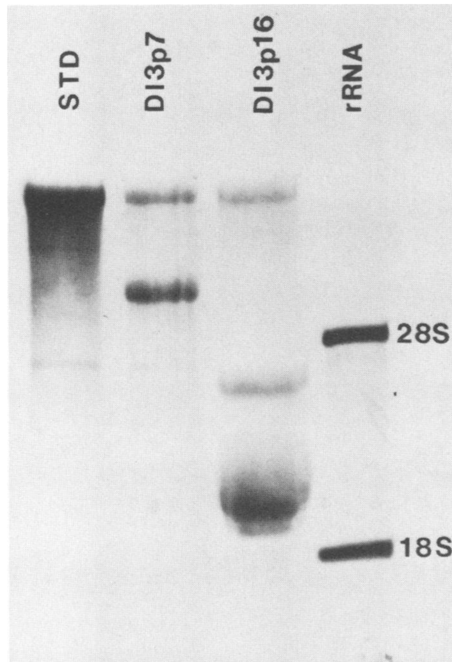


FIG. 3. Analysis of DI-3 RNAs by agarose gel electrophoresis. Sindbis virus was passaged at a multiplicity of infection of 50 on CEF to generate DI-3 particles. Samples of [^{3}H]uridine-labeled RNAs from passage 7 and 16 particles were denatured in glyoxal and analyzed on a 1% agarose gel (2). The gel was processed for fluorography as described previously (14).

ethidium bromide. These data provide an approximation of the amount of RNA present in each lane before transfer to the activated paper. Each of the RNAs of interest gave a positive signal with at least one probe, demonstrating that the amount of material was sufficient to be detected if the appropriate sequences were present.

Based on the data in Fig. 5, only DI-2 and DI-3p16 gave a positive signal with the tRNA^{Asp} probe. All of the DI RNA species of DI-3p16 contained tRNA^{Asp} sequences including the largest species, which was present at a concentration similar to DI-3p7 RNA (Fig. 4, Fig. 5A). The latter RNA did not contain the tRNA^{Asp} sequences. DI-4 RNA, which was present in an amount equivalent to DI-2, was also negative (Fig. 5A). Three other DI RNAs have been analyzed for these sequences, only one of which gave a positive signal (Table 3).

Figure 5B shows the hybridization obtained with the B-region probe. Region B contains a 51-nucleotide sequence highly conserved in all alphaviruses (23), and sequences in this region were also detected in the five DI RNAs. The region marked WX (Fig. 2B), found in both DI-1 and DI-2 (18), was absent from both DI-3 RNA populations, but was present in DI-4 RNA (Fig. 5C). Thus, this sequence is a common but nonessential sequence in DI RNAs. None of the DI RNAs shown in Fig. 5 were detected with the junction region probe, which did, however, detect the standard 49S and 26S RNA species (Fig. 5D). The data shown in Fig. 5 were obtained by analyzing four different blots which were obtained from quadruplicate samples analyzed on a single gel. We have obtained identical results by using different probes with the same blot and reversing the signal between hybridizations.

DISCUSSION

Two approaches have been taken to establish which sequences in the alphavirus genome are essential for replication and packaging. One of them has been to identify the sequences conserved among different alphaviruses (21–24) and the other has been to examine the sequences retained by DI RNAs derived from these viruses (8, 16, 18). The work of the Strausses and their colleagues identified several regions of conservation among alphaviruses, the most striking being at the 3′ terminus. Ou et al. demonstrated a high degree of conservation of the first 19 nucleotides adjacent to the 3′-terminal polyadenylated tract in 10 different alphavirus genomic RNAs (22, 24). Our sequence analysis of Sindbis virus DI RNAs (18), as well as that reported for DI RNAs of Semliki Forest virus (16), establish that 50 to 100 nucleotides of the 3′ standard sequence are present in all alphavirus DI RNAs. Taken together, these results provide strong support for the conclusion that the 3′ sequences represent an important recognition site for alphavirus RNA replication (22).

A second region which is conserved among alphavirus genomic RNAs is the 51-nucleotide sequence located close to the 5′ end and within the nonstructural protein coding sequence (23). Based on an analysis of codon usage, Ou et al. concluded that it is the nucleotide sequence rather than the translated amino acid sequence which is conserved. This stretch of nucleotides can be arranged into two stable hairpins which could serve as recognition structures for alphavirus RNA synthesis or nucleocapsid assembly. This region was present in the cDNA copy (pSS01) of DI-2 RNA.

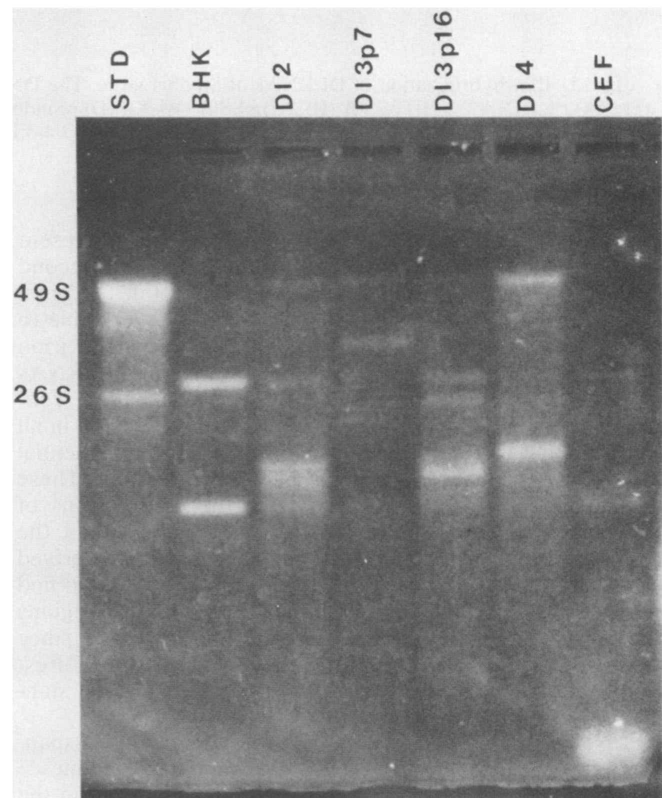


FIG. 4. Analysis of DI RNAs by agarose gel electrophoresis. RNAs from different preparations of Sindbis virus containing DI particles were denatured in glyoxal and analyzed on a 1.2% agarose gel (see text). The gel was stained with ethidium bromide.

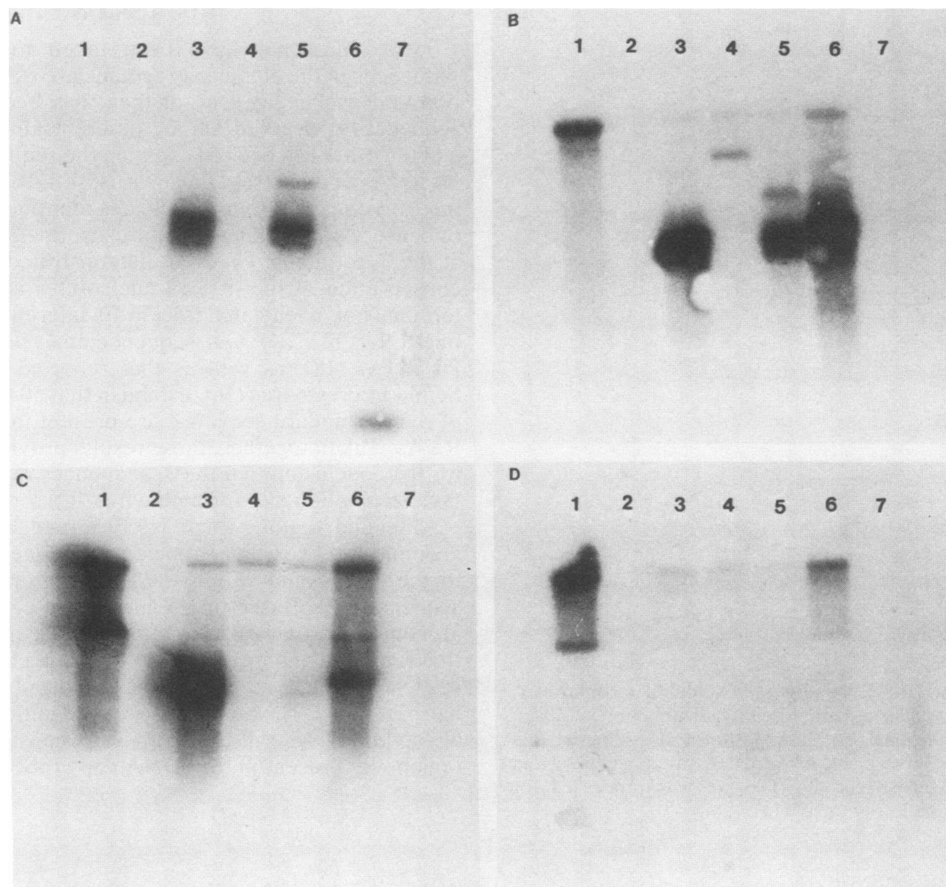


FIG. 5. Blot hybridization of DI RNAs of Sindbis virus. The DNA probes used are described in Table 2. The probes used in each panel were (A) rat $tRNA^{Asp}$, (B) pSS01 (B), (C) pSS01 (W–X), (D) pSindbis 49S (junction). Lanes: 1, standard viral RNA; 2, rRNA from BHK; 3, DI-2 RNA; 4, DI-3p7 RNA; 5, DI-3p16 RNA; 6, DI-4 RNA; 7 4-5S RNA from CEF.

The homologous segment in Semliki Forest virus is present once in one of the DI clones and three times in a second clone (16). This sequence lies between nucleotides 155 and 205 in Sindbis virus 49S RNA. The probe we were able to obtain extended from nucleotides 130 to 270 of the virion RNA. It is likely that the region of the four DI RNAs detected with this probe included the 51-nucleotide conserved segment. The strict conservation of this region in all standard and DI RNAs would be consistent with an essential role for this sequence in alphavirus RNA replication. These 3′ and 5′ conserved sequences were the only regions of nucleotide sequence homology we detected between the Sindbis virus-derived DI RNA and the DI RNAs derived from Semliki Forest virus (16). It was not surprising to find some regions of homology, which may be the regions important in specific recognition for RNA replication, since DI particles derived from either Sindbis or Semliki Forest virus are capable of interfering with the heterologous standard virus (42).

Two other conserved regions are the 21 nucleotides spanning the start of the 26S RNA (21) and the extreme 5′ terminus (23). The former region may be recognized in the full-length negative strand for the initiation of the transcription of 26S RNA (21). This sequence was not present in those Sindbis virus DI RNAs we tested including the DI-3 RNA from passage 7, which represented one of the earliest and largest DI RNAs we have detected. The conserved junction region was also not found in either of the two Semliki Forest virus DI cDNA clones (16). The absence of this junction sequence supports the proposal that it is one of the first regions deleted in the formation of alphavirus DI RNAs (34). The deletion of this region could provide a selective advantage for a DI RNA, the negative strand of which would serve only as a template for replication and not for transcription of a subgenomic mRNA.

The 5′ termini of alphaviruses are not as highly conserved as the three regions described above, although several of the 5′ sequences can be arranged in similar secondary structures (23). The 5′ termini of DI RNAs are distinct from those of the standard viruses. Pettersson found that the 5′ termini of DI RNAs from Semliki Forest virus are heterogeneous (26). Our previous results showing that nucleotides 10 to 75 of a $tRNA^{Asp}$ are at the 5′ terminus of two Sindbis virus D1 RNAs not only established that the standard 5′ end was not essential for replication of the RNA, but also raised the possibility that this tRNA may be involved in the replication of Sindbis virus RNA (19). Our blot analysis of several other DI RNAs shows that the presence of $tRNA^{Asp}$ is a common feature in DI RNAs of Sindbis virus generated in CEF but did not occur during generation of two different DI RNA populations in BHK cells. Furthermore, tRNA sequences were not found in the DI-3 RNA species which had accumu-

lated by passage 7. They were present, however, on all of the DI RNAs isolated after nine additional passages.

The probable initiation site for Sindbis virus nonstructural protein synthesis is located at nucleotides 60 to 62 in the 49S RNA (23). This site lies within region B of SS01 RNA. There is, however, in SS01 RNA a single-nucleotide deletion within the 22nd codon which shifts the reading frame and results in two tandem termination codons. Two Semliki Forest virus DI clones also retain the putative initiation codon, but both contain frameshifts leading to truncated peptides 17 or 131 residues long (16). The lack of significant translation of DI RNAs could be a factor in providing them with a selective advantage. The 49S RNA molecules must be divided between translation and replication, whereas the DI RNAs would be involved only in the latter process.

The generation of alphavirus DI RNA molecules must involve a complex series of events. The first alphavirus DI RNAs generated are larger than those which accumulate later (8, 34; and Fig. 3), and the 18 to 20S DI RNAs which eventually become the predominant species contain repeats and rearrangements of standard sequences (16; Fig. 2B). There is also evidence that continued evolution can lead to DI RNAs with an increased size due to further duplications of the repeated sequences (9). Thus, there may be selection for molecules in which certain regions are present in multiple copies. With the availability of cDNA clones and appropriate expression vectors it should be possible to construct specific DI molecules and identify precisely the sequence and structural requirements for alphavirus RNA replication and packaging. This type of analysis may provide insight into why the peculiar structures of the alphavirus DI RNAs give them a selective replicative advantage.

ACKNOWLEDGMENTS

This work was supported by Public Health Service grant AI 11377 from the National Institute of Allergy and Infectious Diseases. S.S.M. was supported, in part, by a RHO Scholarship.

We thank David Kingsbury II for his photographic work and for technical assistance, Manuel Tsiang for isolating DI-5 and DI-6, and S. Nishimura and T. Sekiya for plasmid pRT1dge. We especially thank Jim and Ellen Strauss for providing us with their sequence of Sindbis virion RNA.

LITERATURE CITED

1. **Alwine, J. C., D. J. Kemp, B. A. Parker, J. Reiser, J. Renart, G. R. Stark, and G. M. Wahl.** 1979. Detection of specific RNAs or specific fragments of DNA by fractionation in gels and transfer to diazobenzyloxymethyl paper. Methods Enzymol. **68**:220–242.
2. **Carmichael, G. G., and G. K. McMaster.** 1980. The analysis of nucleic acids in gels using glyoxal and acridine orange. Methods Enzymol. **65**:380–391.
3. **Davis, R. W., D. Botstein, and J. R. Roth.** 1980. Advanced bacterial genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
4. **Denhardt, D.** 1966. A membrane-filter technique for the detection of complementary DNA. Biochem. Biophys. Res. Commun. **23**:641–646.
5. **Dohner, D., S. Monroe, B. Weiss, and S. Schlesinger.** 1979. Oligonucleotide mapping studies of standard and defective Sindbis virus RNA. J. Virol. **29**:794–798.
6. **Efstratiadis, A., and L. Villa-Komaroff.** 1979. Cloning of double-stranded cDNA, p. 15–36. *In* J. K. Setlow and A. Hollaender (ed.), Genetic engineering, principles and methods, vol. 1. Plenum Publishing Corp., New York.
7. **Favaloro, J., R. Treisman, and R. Kamen.** 1980. Transcription maps of polyoma virus-specific RNA: analysis by two-dimensional nuclease S1 gel mapping. Methods Enzymol. **65**:718–749.
8. **Guild, G. M., and V. Stollar.** 1977. Defective interfering particles of Sindbis virus. 5. Sequence relationships between SV_{STD} 42S RNA and intracellular defective viral RNAs. Virology **77**:175–188.
9. **Kääriäinen, L., R. F. Pettersson, S. Keränen, P. Lehtovaara, H. Söderlund, and P. Ukkonen.** 1981. Multiple structurally related defective-interfering RNAs formed during undiluted passage of Semliki Forest virus. Virology **113**:686–697.
10. **Kennedy, S. I. T.** 1976. Sequence relationships between the genome and the intracellular RNA species of standard and defective-interfering Semliki Forest virus. J. Mol. Biol. **108**: 491–511.
11. **Kennedy, S. I. T., C. J. Bruton, B. Weiss, and S. Schlesinger.** 1976. Defective-interfering passages of Sindbis virus: nature of the defective virion RNA. J. Virol. **19**:1034–1043.
12. **Kurtz, D. T., and C. F. Nicodemus.** 1981. Cloning of the alpha-2-mu globulin cDNA using a high efficiency technique for the cloning of trace messenger RNAs. Gene **13**:145–152.
13. **Kushner, S. R.** 1978. An improved method for transformation of *Escherichia coli* with ColE1 derived plasmids, p. 17–23. *In* H. W. Boyer and S. Nicosia (ed.), Genetic engineering. Elsevier, Amsterdam.
14. **Lasky, R. A.** 1980. The use of intensifying screens or organic scintillators for visualizing radioactive molecules resolved by gel electrophoresis. Methods Enzymol. **65**:363–371.
15. **Lehtovaara, P., H. Söderlund, S. Keränen, R. F. Pettersson, and L. Kääriäinen.** 1981. 18S defective interfering RNA of Semliki Forest virus contains a triplicated linear repeat. Proc. Natl. Acad. Sci. U.S.A. **78**:5353–5357.
16. **Lehtovaara, P., H. Söderlund, S. Keränen, R. F. Pettersson, and L. Kääriäinen.** 1982. Extreme ends of the genome are conserved and rearranged in the defective interfering RNAs of Semliki Forest virus. J. Mol. Biol. **156**:731–748.
17. **Maxam, A. M., and W. Gilbert.** 1980. Sequencing end-labeled DNA with base-specific chemical cleavages. Methods Enzymol. **65**:499–560.
18. **Monroe, S. S., J.-H. Ou, C. M. Rice, S. Schlesinger, E. G. Strauss, and J. H. Strauss.** 1982. Sequence analysis of cDNA's derived from the RNA of Sindbis virions and of defective-interfering particles. J. Virol. **41**:153–162.
19. **Monroe, S. S., and S. Schlesinger.** 1983. RNAs from two independently isolated defective interfering particles of Sindbis virus contain a cellular tRNA sequence at their 5′ ends. Proc. Natl. Acad. Sci. U.S.A. **80**:3279–3283.
20. **Nelson, T., and D. Brutlag.** 1979. Addition of homopolymers to the 3′-ends of duplex DNA with terminal transferase. Methods Enzymol. **68**:41–50.
21. **Ou, J.-H., C. M. Rice, L. Dalgarno, E. G. Strauss, and J. H. Strauss.** 1982. Sequence studies of several alphavirus genomic RNAs in the region containing the start of the subgenomic RNA. Proc. Natl. Acad. Sci. U.S.A. **79**:5235–5239.
22. **Ou, J.-H., E. G. Strauss, and J. H. Strauss.** 1981. Comparative studies of the 3′-terminal sequences of several alphavirus RNAs. Virology **109**:281–289.
23. **Ou, J.-H., E. G. Strauss, and J. H. Strauss.** 1983. The 5′-terminal sequences of the genomic RNAs of several alphaviruses. J. Mol. Biol. **168**:1–15.
24. **Ou, J.-H., D. W. Trent, and J. H. Strauss.** 1982. The 3′-non-coding regions of alphavirus RNAs contain repeating sequences. J. Mol. Biol. **156**:719–730.
25. **Perrault, J.** 1981. Origin and replication of defective interfering particles. Curr. Top. Microbiol. Immunol. **93**:151–207.
26. **Pettersson, R. F.** 1981. 5′-Terminal nucleotide sequence of Semliki Forest virus 18S defective interfering RNA is heterogenous and different from the genomic 42S RNA. Proc. Natl. Acad. Sci. U.S.A. **78**:115–119.
27. **Rice, C. M., and J. H. Strauss.** 1981. Nucleotide sequence of the 26S mRNA of Sindbis virus and deduced sequence of the encoded virus structural proteins. Proc. Natl. Acad. Sci. U.S.A. **78**:2062–2066.
28. **Schlesinger, M. J., and L. Kääriäinen.** 1980. Translation and processing of alphavirus proteins, p. 371–392. *In* R. W. Schlesinger (ed.), The togaviruses, biology, structure, replication. Academic Press, Inc., New York.

29. **Seed, B.** 1982. Diazotizable arylamine cellulose papers for the coupling and hybridization of nucleic acids. Nucleic Acids Res. **10**:1799–1810.
30. **Sekiya, T., Y. Kuchino, and S. Nishimura.** 1981. Mammalian tRNA genes: nucleotide sequence of rat genes for $tRNA^{Asp}$, $tRNA^{Gly}$, and $tRNA^{Glu}$. Nucleic Acids Res. **9**:2239–2248.
31. **Smith, A. J. R.** 1979. The use of exonuclease III for preparing single stranded DNA for use as a template in the chain terminator sequencing method. Nucleic Acids Res. **6**:831–848.
32. **Smith, D. R., and J. M. Calvo.** 1980. Nucleotide sequence for the *E. coli* gene coding for dihydrofolate reductase. Nucleic Acids Res. **8**:2255–2274.
33. **Söderlund, H., S. Keränen, P. Lehtovaara, I. Palva, R. F. Pettersson, and L. Kääriäinen.** 1981. Structural complexity of defective-interfering RNAs of Semliki Forest virus as revealed by analysis of complementary DNA. Nucleic Acids Res. **9**: 3403–3417.
34. **Stark, C., and S. I. T. Kennedy.** 1978. The generation and propagation of defective-interfering particles of Semliki Forest virus in different cell types. Virology. **89**:285–299.
35. **Steinmetz, M., J. G. Frelinger, D. Fisher, T. Hunkapiller, D. Pereira, S. M. Weissman, H. Urehara, S. Nathenson, and L. Hood.** 1981. Three cDNA clones encoding mouse transplantation antigens: homology to immunoglobulin genes. Cell **24**:125–134.
36. **Stellwag, E. J., and A. E. Dahlberg.** 1980. Electrophoretic transfer of DNA, RNA and protein onto diazobenzyloxymethyl (DBM)-paper. Nucleic Acids Res. **8**:299–317.
37. **Stollar, V.** 1979. Defective interfering particles of togaviruses. Curr. Top. Microbiol. Immunol. **86**:36–66.
38. **Stollar, V.** 1980. Defective interfering alphaviruses, p. 427–457. *In* R. W. Schlesinger (ed.), The togaviruses, biology, structure, replication. Academic Press, Inc., New York.
38a. **Strauss, E. G., and J. H. Strauss.** 1983. Replication strategies of the single-stranded RNA viruses of eukaryotes. Curr. Top. Microbiol. Immunol. **105**:1–98.
39. **Vieira, J., and J. Messing.** 1982. The pUC plasmids, an M13mp7-derived system for insertion mutagenesis and sequencing with synthetic universal primers. Gene **19**:259–268.
40. **Volckaert, G., J. Tavernier, R. Derynck, R. Devos, and W. Fiers.** 1981. Molecular mechanisms of nucleotide-sequence rearrangements in cDNA clones of human fibroblast interferon mRNA. Gene **15**:215–223.
41. **Walseth, T. F., and R. A. Johnson.** 1979. The enzymatic preparation of [α-^{32}P]nucleoside triphosphates, cyclic [^{32}P]AMP, and cyclic [^{32}P]GMP. Biochim. Biophys. Acta **526**:11–31.
42. **Weiss, B., and S. Schlesinger.** 1981. Defective-interfering particles of Sindbis virus do not interfere with the homologous virus obtained from persistently infected BHK cells but do interfere with Semliki Forest virus. J. Virol. **37**:840–844.