Promoter for Sindbis Virus RNA-Dependent Subgenomic RNA Transcription

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Sindbis virus is a positive-strand RNA enveloped virus, a member of the *Alphavirus* genus of the *Togaviridae* family. Two species of mRNA are synthesized in cells infected with Sindbis virus; one, the 49S RNA, is the genomic RNA; the other, the 26S RNA, is a subgenomic RNA that is identical in sequence to the 3' one-third of the genomic RNA. Ou et al. (J.-H. Ou, C. M. Rice, L. Dalgarno, E. G. Strauss, and J. H. Strauss, *Proc. Natl. Acad. Sci. USA* 79:5235-5239, 1982) identified a highly conserved region 19 nucleotides upstream and 2 nucleotides downstream from the start of the 26S RNA and proposed that in the negative-strand template, these nucleotides compose the promoter for directing the synthesis of the subgenomic RNA. Defective interfering (DI) RNAs of Sindbis virus were used to test this proposal. A 227-nucleotide sequence encompassing 98 nucleotides upstream and 117 nucleotides downstream from the start site of the Sindbis virus subgenomic RNA was inserted into a DI genome. The DI RNA containing the insert was replicated and packaged in the presence of helper virus, and cells infected with these DI particles produced a subgenomic RNA of the size and sequence expected if the promoter was functional. The initiating nucleotide was identical to that used for Sindbis virus subgenomic mRNA synthesis. Deletion analysis showed that the minimal region required to detect transcription of a subgenomic RNA from the negative-strand template of a DI RNA was 18 or 19 nucleotides upstream and 5 nucleotides downstream from the start of the subgenomic RNA.

Positive-strand RNA viruses are defined by the ability of the genomic RNA to function as an mRNA. For some viruses, such as picornaviruses and flaviviruses, the genomic RNA is the only viral mRNA synthesized in infected cells. There are many other viruses in which one or more subgenomic mRNAs are also synthesized. In these cases, some of the viral proteins are translated from the subgenomic species, thus creating the potential for independently regulating the levels of viral proteins in infected cells, e.g., replication proteins required in catalytic amounts versus structural proteins required in stoichiometric amounts. Two distinct mechanisms for generating subgenomic RNAs have been defined. One is the initiation of transcription at an internal promoter site on the minus strand of the genomic RNA. This mechanism has been most clearly established for the alphaviruses (19, 23, 25, 27) and for the plant virus brome mosaic virus (BMV) (5, 15). In contrast, subgenomic RNA synthesis by coronaviruses involves discontinuous RNA synthesis (1). A leader RNA, which dissociates from its template and then rebinds at various sites along the minus strand of the genome, serves as a primer for synthesis of a nested set of subgenomic RNAs.

Sindbis virus is the type species of the alphaviruses. The single-stranded RNA genome (49S RNA) is capped at the 5' terminus, polyadenylated at the 3' terminus, and, excluding the poly(A) sequence, is 11,703 nucleotides long (reviewed in reference 28). In infected cells, the 49S RNA is a template for full-length complementary minus strands which then serve as templates for the synthesis of both genomic RNA and the subgenomic 26S RNA. The genomic RNA is the mRNA for the nonstructural proteins required for replication and transcription of viral RNAs. The subgenomic RNA is

4,106 nucleotides long and is colinear with the 3'-terminal one third of the 49S genome. It is capped and polyadenylated, but unlike the 49S RNA, the 26S RNA does not serve as a template for minus-strand synthesis and is not packaged into mature virions. It is translated into a polyprotein that undergoes cotranslational and posttranslational cleavages to produce the virion structural proteins. The subgenomic RNA is transcribed and translated more efficiently than the genomic RNA, and the structural proteins are produced in greater abundance in infected cells than are the nonstructural proteins.

A comparison of the sequences of genomic RNAs of a number of alphaviruses in the region spanning the start of the subgenomic RNA, the junction region, identified a conserved 21-nucleotide domain encompassing 19 nucleotides upstream and 2 nucleotides downstream (-19 to +2) of the initiation site. These nucleotides were proposed to be the promoter, on the minus-strand template, for RNA-dependent subgenomic mRNA synthesis (19). Further evidence supports this hypothesis. They share some similarity to the sequence of the promoter for the synthesis of BMV subgenomic RNA (5, 15). Also, the conserved sequence has been shown to be at least part of the promoter, since a three-base insertion in it dramatically reduces the levels of subgenomic RNA produced by the mutant virus (6).

We previously reported the construction of cDNA clones of defective interfering (DI) RNAs of Sindbis virus (12) and the full-length Sindbis virus genome (22) that can be transcribed *in vitro* to produce biologically active transcripts. These clones have been used to map *cis*-acting sequences required for DI RNA replication and packaging (12) and the casual sequence changes for a variety of Sindbis virus mutant phenotypes (8, 9, 16, 21, 22). Two factors make it difficult to analyze the sequence requirements for subgenomic RNA synthesis by using the full-length cDNA clone of the Sindbis virus genome. First, the clone is somewhat

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FIG. 1. Diagram of a cDNA clone of a DI genome with a junction region insert. The promoter for the SP6 DNA-dependent RNA polymerase is immediately upstream of the KDI25.3 cDNA; the unique *EcoRI* site was used for runoff transcription in vitro. The 2,443-nucleotide (nt) cDNA is also shown in a linear form, and the unique *NaeI* site was used to insert the junction region sequences to give clone JNSH. An enlarged view of the junction region insert in JNSH is shown. The junction region insert extends from -98 to +117 relative to the start of the subgenomic RNA. The junction sequence from -19 to +5 is shown, with the start of the subgenomic RNA indicated by the arrow.

cumbersome to manipulate because of its large size and the lack of unique restriction sites around the junction region. The second, more serious impediment is that the open reading frame for the nonstructural protein nsP4 overlaps the conserved sequence and the initiation site for subgenomic RNA synthesis. Thus, alterations in this region are constrained by the need to preserve the nsP4 protein sequence. In this study, we used Sindbis virus DI cDNA clones to define the promoter element for subgenomic RNA synthesis. Cells infected by a DI clone containing the junction region from -98 to +117 synthesized both DI genomes and a subgenomic RNA of the size and sequence expected if the promoter on the minus strand of the DI genome was functional. This made possible the deletion analysis of the junction region within the DI RNA, to define the minimum region required for transcription of subgenomic RNA.

MATERIALS AND METHODS

DI cDNA clones. KDI25, a plasmid containing the cDNA of a Sindbis virus DI RNA, has been described in detail previously (12). All junction region-containing DI cDNA clones were constructed by inserting different lengths of upstream and downstream junction sequences into KDI25.3 or KDI25.3NP, both derivatives of KDI25. These derivatives have 157 bases (starting at the *MstI* site at 11546; numbering according to reference 27) from the 3' terminus of the Sindbis virus genome instead of the 67 bases in KDI25. This change in the 3' terminus was engineered such that run-off transcripts would not contain the 21-base C tail (introduced during the original cDNA cloning) that was present in KDI25. KDI25.3NP is identical to KDI25.3 with the addition of a short polylinker region (CATCGATAAGCTTCTAGAGATCTGCAGGTC) inserted at the *NaeI* site at base 1340 (Fig. 1). The *NaeI* site is unique in KDI25.3 and KDI25.3NP. (The other *NaeI* site at 2245 in the KDI25 cDNA was eliminated in the construction of these derivatives.) This restriction site was used for the insertion of junction fragments. For all the clones, the region of the

insert was sequenced by the dideoxy-chain termination method (24) to verify that the inserted sequences were those expected.

Clones JNSH and JNHS. JNSH was constructed by inserting the 227-base-pair *SspI* (7500)-to-*HincII* (site in polylinker) fragment from TSCAT (30) into KDI25.3 which had been linearized at the unique *NaeI* site (Fig. 1). The insert contains 215 bases of Sindbis virus sequence (from 7500 to 7714, or -98 to +117 relative to the subgenomic start site at 7598) and 12 bases of the polylinker. The insertion in JNSH was in the same sense as that in Sindbis virus. A clone, JNHS, with the insert in an inverted orientation was also made.

Clones JNSR, JNBH, and JNBR. JNSR is identical to JNSH except that the downstream junction sequences from +15 to +117 have been deleted. JNBH and JNBR contain the junction sequences from -49 to +63 and -49 to +14, respectively. These clones were constructed by digesting a subclone of the junction region (P27.JBP) with the appropriate restriction enzymes, blunt ending the recessed ends, and ligating the fragments with KDI25.3 linearized at *NaeI*. The subclone, P27.JBP, was generated by inserting a 388-base fragment from *BamHI* (7334) to *PstI* (site in polylinker) spanning the junction region from TSCAT into proteus 27. Proteus 27 is a vector which contains the SP6 DNA-dependent RNA polymerase promoter upstream from a polylinker (H. V. Huang, unpublished data).

Clones JNB6, JN34R, and JN19R. JNB6 is identical to JNBR except that bases +7 to +14 downstream from the initiation site for subgenomic RNA synthesis are deleted. This was done by annealing and ligating complementary oligonucleotides corresponding to -6 to +5 of the junction region which have an *AvaII*-compatible end at the 5' end of the plus strand and an *XbaI*-compatible end at the 5' end of the minus strand into π AN7-JSX (see below) which was linearized with *AvaII* and *XbaI*. The resulting subclone, π AN7-JS6, contains the junction region from -98 to +6 (owing to a T[U in the corresponding RNA] in the *XbaI* site which corresponds to +6 in the junction region). A *BsmI*-to-*XbaI* fragment from the subclone corresponding to nucleotides -49 to +6 of the junction region was inserted into the polylinker region of KDI25.3NP to give JNB6. Clones JN34R and JN19R have junction sequences from -34 to +14 and -19 to +14, respectively, inserted into the polylinker region of KDI25.3NP. To construct these clones, complementary oligonucleotides, from -34 to -7 for JN34R and from -19 to -7 for JN19R, of the junction region and flanked by a *BamHI*-compatible end at the 5' end of the plus strand and an *AvaII*-compatible end at the 5' end of the minus strand were inserted into π AN7-JSX linearized with *BamHI* and *AvaII*. The resulting subclones, π AN7-J34R and π AN7-J19R, contained, respectively, junction sequences from -34 to +14 and -19 to +14 within the π AN7 polylinker region. JN34R and JN19R were generated by isolating *SmaI*-to-*XbaI* fragments containing the respective junction regions and ligating them into the polylinker region of KDI25.3NP.

The subclone π AN7-JSX was constructed by inserting the *SspI* (7499)-to-*XbaI* (in polylinker) junction region fragment (from -98 to +14) from TRCAT (30) into π AN7 cut with *HincII* and *XbaI*. π AN7 (13) has no *AvaII* sites. By inserting the junction region, which has a single *AvaII* site at position -6 from the initiation site for subgenomic RNA transcription, manipulations of the junction region were greatly facilitated.

Clone JN22. JN22, also designated JN20/2, has junction

sequences from -20 to +2. The clone was constructed by annealing complementary oligonucleotides specific to junction sequences -19 to +2 and ligating them into KDI25.3 linearized at *NaeI*. Owing to a C nucleotide at the 3'-terminal position of the vector which corresponds to nucleotide -20 of the junction region, the resulting clone contained junction sequences from -20 to +2. For this insert, clones with the junction insert in the normal and inverted orientations were isolated.

Clone JNB2. JNB2 is identical to JNBR except that the sequences from +3 to +14 downstream of the initiation site for subgenomic RNA synthesis were deleted. This was accomplished by inserting an *AvaII* fragment from JN22 (junction sequences -20 to +2) from position 1353 (corresponding to -6 from the subgenomic RNA start site) to position 1641 into a plasmid, containing a large region of Sindbis virus junction sequences, at the *AvaII* site at -6 from the initiation site for subgenomic RNA synthesis. The resulting clone contained Sindbis virus sequences from 6267 to 7599 (corresponding to base -1331 to base +2 at the subgenomic start site) fused to KDI25.3 sequences from 1341 to 1641. A fragment containing the junction region from -49 to +2 and the downstream DI sequences was then cloned back into KDI25.3 at the appropriate sites.

Clone JN19/6. JN19/6 has junction sequences from -19 to +6. JN19/6 was constructed by making a subclone, π AN7-JN25, and then isolating the junction region fragment and inserting it into the polylinker region of KDI25.3NP. π AN7-JN25 was made by ligating an *ApaLI*-to-*AvaII* fragment from π AN7-J19X which contains Sindbis virus junction sequences from -19 to -7 to an *AvaII*-to-*ApaLI* fragment from π AN7-JS6 which contains Sindbis virus junction sequences from -6 to +6. The resulting clone, π AN7-JN25, contained the junction region from -19 to +6. A *SmaI*-to-*XbaI* fragment containing this region was isolated and ligated into the *ClaI* (filled-in)-*XbaI* sites of KDI25.3NP.

Clone JN20/5. JN20/5 has junction sequences from -20 to +5. Complementary oligonucleotides spanning the junction region from -19 to +5 were annealed and ligated into KDI25.3NP at a *PstI* site which was blunt ended with T4 DNA polymerase. Treatment of the *PstI*-linearized DNA with T4 DNA polymerase resulted in a 3'-terminal C in the KDI25.3NP vector. C is the nucleotide at position -20 of the junction region, and therefore the resulting clone has a junction region from base -20 to +5.

Clones JN19/4, JN19/3, and JN17/6. JN19/4, JN19/3, and JN17/6 have junction sequences from -19 to +4, -19 to +3, and -17 to +6, respectively. These clones were constructed by annealing complementary oligonucleotides which have a *ClaI*-compatible end at the 5' end of the plus strand and an *XbaI* compatible end at the 5' end of the minus strand and ligating them into KDI25.3NP linearized with *ClaI* and *XbaI*.

Two clones, JNSH.5' and JNSH.554, were constructed that contained a junction region identical to that in JNSH (-98 to +117) but differed in the 5'-terminal sequences. JNSH.5' contains the Sindbis virus 5' end from position 1 to 125 instead of the tRNA^{Asp}, and JNSH.554 has a 75-nucleotide deletion from nucleotides 167 to 241 in the DI region (130 to 204 in the Sindbis virus genome) which removes the 51-base highly conserved region present at the 5' end of alphaviruses (20). The 5' terminus of JNSH was replaced with the 5' sequences from DI-549, which contains the Sindbis virus 5' end (29), and DI-554, which contains the 75-nucleotide deletion (12), to give JNSH.5' and JNSH.554, respectively.

Transcription, transfection, and passaging of DI cDNA

clones. Runoff transcripts were synthesized in vitro by SP6 DNA-dependent RNA polymerase utilizing as templates plasmids linearized at the unique *EcoRI* site immediately 3' of the DI poly(A) sequences. The reaction conditions for transcription, transfections, and passaging were as described by Levis et al. (12). Briefly, cells were infected with Sindbis virus and transfected with transcribed RNA after treatment with 200 μ g of DEAE-dextran per ml (molecular weight 500,000)-50 mM Tris hydrochloride (pH 7.5) in Eagle minimal essential medium with Earle salts. After 12 to 15 h at 37°C, the medium was harvested. For passage 2 and passage 3, one-fourth of the medium from the previous passage was used to infect new monolayers.

RNA isolation and gel electrophoresis. Typically, RNA was isolated from cells infected with passage 2 virus. Infected chicken embryo fibroblasts were incubated with 10 μ Ci of [³H]uridine per ml in the presence of 1 μ g of actinomycin D per ml for 7 h at 37°C or 12 to 15 h at 30°C. Cells were rinsed three times with 50 mM Tris hydrochloride (pH 7.4)-0.1 M NaCl at 4°C and then lysed with 0.5% Triton X-100-0.5% 1,5-naphthalenedisulfonic acid-50 mM Tris hydrochloride (pH 7.4)-0.1 M NaCl-1 mM EDTA. Samples were extracted and precipitated with ethanol (14). Incorporation of [³H]uridine was determined by precipitation with 10% trichloroacetic acid. The RNAs were denatured in glyoxal and dimethyl sulfoxide and analyzed by electrophoresis on 1% agarose gels (2).

S1 nuclease analysis and Northern (RNA) hybridization analysis. The procedure for S1 nuclease analysis of in vivo-labeled RNAs was as previously described (11). For Northern blot analysis, RNAs were denatured and separated on a 1% agarose gel and transferred to Biotrans nylon membrane (ICN Biomedicals, Inc., Costa Mesa, Calif.) in 20 $\times$ SSC (3 M NaCl and 0.3 M sodium citrate, pH 7.0). Conditions for probe preparation and blotting were according to the supplier.

Primer extension analysis. The primer (corresponding to nucleotides 1664 to 1681 of the JNSH DI cDNA clone) and RNA were coprecipitated and then annealed by suspending the samples in 5 μ l of 100 mM Tris hydrochloride (pH 7.5)-1 mM EDTA, heating the mixture to 70°C for 10 min, and slowly cooling it to room temperature. Extensions were done at room temperature for 30 min in a final volume of 10 μ l in 75 mM KCl-50 mM Tris hydrochloride (pH 7.5)-10 mM dithiothreitol-3 mM MgCl₂-250 μ M deoxynucleoside triphosphates-100 μ g of bovine serum albumin per ml-10 μ Ci of [α -³⁵S]dATP per ml-200 U of Moloney murine leukemia virus reverse transcriptase. The reactions were stopped by the addition of 80% formamide loading buffer. The extension products were analyzed on a 6% sequencing gel. A sequence ladder of JNSH or JN20/5 with the same primer was included as a size standard.

RESULTS

Junction region sequences are functional in a DI RNA context. We first determined whether a subgenomic RNA could be produced from a DI genome. A DI clone called JNSH was constructed that had 215 nucleotides of the junction region inserted in the same polarity as in Sindbis virus (Fig. 1). The insert consists of nucleotides 7500 to 7714 of the Sindbis virus genome, nucleotide 7598 being the initiating nucleotide of the subgenomic RNA. Thus, JNSH has 98 nucleotides upstream and 117 nucleotides downstream from the start site. A DI cDNA clone called JNHS that contained the insert in an inverted orientation was also constructed.

FIG. 2. Analysis of RNAs produced in cells infected with DI particles and helper Sindbis virus. (A) Appearance of a subgenomic-sized RNA in chicken embryo fibroblasts infected with JNSH in the presence of Sindbis virus. Cells were infected with Sindbis virus and transfected with in vitro transcripts of KDI25.3 (2,443 nucleotides), JNSH (2,670 nucleotides), or JN22 (2,465 nucleotides) (passage 1). RNAs were labeled with [3 H]uridine during the formation of passages 2 and 3 in the presence of 1 μ g of actinomycin D per ml. SIN refers to RNA from cells infected only with Sindbis virus. The other samples are designated by the names of the respective DI clones. T refers to the respective RNA transcripts used for transfection; 2 and 3 refer to the passage during which the RNA was isolated. * denotes the subgenomic-sized [1,220 nucleotides, plus poly(A)] RNA. (B) S1 nuclease analysis of [3 H]uridine-labeled DI RNAs. In vitro transcripts or RNA isolated from cells during the formation of passage 3 were hybridized to JNSH cDNA and then digested with S1 nuclease. Samples were denatured and analyzed on a 1% agarose gel. Lanes 1 and 2 contain the in vitro RNA transcript of JNSH (2,670 nucleotides). Lanes 3 and 4 contain RNA isolated during passage 3 of JNSH DI RNA (2,670-nucleotide genomic and 1,220-nucleotide subgenomic RNA). Lane 5 contains RNA from cells infected only with Sindbis virus. Lanes 1 and 2 contain RNAs that were not hybridized or digested with S1 nuclease. Lanes 3 to 5 contain RNAs that were hybridized and digested. (C) Primer extension products from Sindbis virus RNAs isolated from cells synthesizing JNSH (lanes labeled DIJNSH) genomic and subgenomic RNAs during the formation of passage 3. A primer (corresponding to nucleotides 1664 to 1681 of the JNSH DI cDNA clone) was annealed to in vitro transcripts or to in vivo-labeled RNAs. Extensions were done in the presence of [α - 32 S]dATP with Moloney murine leukemia virus reverse transcriptase. Products were analyzed on a 6% sequencing gel and compared with a sequence ladder of JNSH DNA (lanes labeled KDIJNSH) generated with the same primer. The arrow indicates the sequence ATAGTC...corresponding to the 5' end of the subgenomic mRNA. P3 refers to the JNSH RNA labeled in vivo during passage 3, T refers to the JNSH in vitro transcript RNA, and V refers to RNA isolated from JNSH virus particles.

RNA transcribed in vitro from these clones was transfected into chicken embryo fibroblasts in the presence of helper Sindbis virus. At 12 h later, the medium was harvested and samples were used to infect new cells (passage 2). Virus samples harvested after 12 h (passage 2 virus) were used to infect cells (passage 3) in the experiments described below. Passaging allowed those DI RNAs that were competent for replication and packaging to be amplified and to be more easily detected.

The DI genomic RNAs detected in cells during the formation of passage 3 were identical in size to the input transcripts (Fig. 2A). By passage 3 of JNSH, a new RNA species was clearly detected. The initial criteria that it was a subgenomic RNA were as follows: (i) it had the size [1220 nucleotides, plus poly(A)] expected for a subgenomic RNA if the junction region inserted into JNSH was functional; and (ii) Northern blot hybridizations showed that it contained sequences identical to those in the 3' region of the DI genome (data not shown). Only the DI-specific genomic and the helper-specific RNAs were detected in cells infected with either the parental KDI25.3 (Fig. 2A) or JNSH, in which the junction sequences were inverted (data not shown). The novel JNSH RNA species was characterized by S1 nuclease analysis. The JNSH DI RNAs were labeled in vivo with [3 H]uridine during passage 3, hybridized to the JNSH cDNA clone, treated with S1 nuclease, and separated by gel electrophoresis. The DI RNA from passage 3 cells infected with Sindbis virus and JNSH gave two protected fragments (Fig. 2B). One corresponded to the genomic RNA, and the other had a size that corresponded to that expected for the DI

subgenomic RNA. The input in vitro transcripts showed protection of a single RNA the size of the JNSH genome. Thus, the novel RNA species produced by cells coinfecting with JNSH and Sindbis virus was smaller than and colinear with the 3' region of the JNSH genome.

The 5' end of the RNA was mapped by primer extension. The primer used was complementary to a region in the JNSH genome that would be 243 bases downstream from the expected 5' terminus of a subgenomic RNA if it initiated at the correct site on the negative strand. It was annealed to RNA isolated from cells infected with Sindbis virus plus JNSH, to in vitro transcripts of JNSH, or to RNA isolated from extracellular virus particles from cells coinfecting with Sindbis virus and JNSH. Extension products were analyzed on a sequencing gel and compared with a sequence ladder of JNSH generated via primer extension of the DNA in the presence of dideoxynucleotides by using the same primer (Fig. 2C). The sequence in the DNA that corresponds to the region in the genomic RNA surrounding the junction is CCTAAATAGTC (19), with the underlined base being the start site for 26S subgenomic RNA. All virus-specific RNAs contained the primer recognition sequence, but only the JNSH RNA obtained from passage 3 produced an extension product that corresponded to the size expected for the JNSH subgenomic RNA if it initiated at the same nucleotide position as the Sindbis virus subgenomic RNA. Like the 26S subgenomic RNA of Sindbis virus, the DI subgenomic RNA was not packaged into extracellular virus particles (Fig. 2C).

The results demonstrated that a DI genome containing a 215-nucleotide junction region insert was replicated and

TABLE 1. Minimal junction region sequences that allow subgenomic RNA synthesis

Junction region ^a	DI clone	Junction region sequences ^{a,b}	Subgenomic RNA synthesis ^{b,c}
Positive orientation	JNSH	-98 to +117	+
	JNSR	-98 to +14	+
	JNBH	-49 to +63	+
	JNBR	-49 to +14	+
	JNB6	-49 to +6	+
	JNB2	-49 to +2	-
	JN34R	-34 to +14	+
	JN20/5	-20 to +5	+
	JN22	-20 to +2	-
	JN19R	-19 to +14	+
	JN19/6	-19 to +6	+
	JN19/4	-19 to +4	-
Negative orientation	JN19/3	-19 to +3	-
	JN17/6	-17 to +6	-
	JNHS	-98 to +117	-
	JN12	-19 to +2	-

^a Positive and negative orientations indicate that the junction sequences were inserted in the same or inverted polarity, respectively, as that in Sindbis virus.

^b Numbers indicate the extent of junction sequence relative to the subgenomic RNA start site.

^c Detection of a subgenomic RNA by passage 3.

packaged. The junction region insert was functional; a DI-derived subgenomic RNA was synthesized if the junction region was inserted in the same polarity as that in Sindbis virus. The subgenomic RNA initiated at the same nucleotide used to initiate viral subgenomic RNA synthesis, was colin-

ear with 3' downstream sequences of the DI genome, and was not packaged into viral particles.

Deletion analysis of junction region. We also tested a clone called JN22 that had a 22-nucleotide junction region insert, from -20 to +2 relative to the initiating nucleotide. The insert is one nucleotide longer than the -19 to +2 conserved sequence identified by Ou et al. (19) and proposed to be at least part of the *cis*-acting sequences required for subgenomic RNA transcription. JN22 was amplified during passaging in the presence of Sindbis helper virus but gave no evidence of any subgenomic RNA synthesis (Fig. 2A). Since the 21-base conserved region seemed not to be sufficient for subgenomic RNA synthesis, we constructed derivatives of JNSH that have smaller junction region inserts to determine the minimum sequence required for detectable promoter activity (Table 1). Subgenomic RNA was detected if the junction region insert in the DI genome had at least 19 nucleotides upstream and at least 5 nucleotides downstream of the initiation site. Figure 3A shows the results for some of the clones with the smallest junction region inserts. Clones JN19/6 and JN20/5 were two with the smallest junction region inserts, from -19 to +6 and from -20 to +5, respectively, that still directed subgenomic RNA synthesis from the minus strand of the DI genome. We demonstrated that the smaller RNA in the JN20/5-infected cells was actually a subgenomic RNA by doing S1 nuclease protection experiments and primer extensions as described above. The S1 nuclease analysis showed that the smaller RNA detected in JN20/5-infected cells was protected by the JN20/5 cDNA clone to give a fragment of the expected size (Fig. 3B). The primer extensions employed as templates the JN20/5 in vitro transcript, JN20/5 passage 3 RNAs, and passage 3 RNAs

FIG. 3. Production of subgenomic RNAs from DI templates containing deletions in the junction region. (A) Pattern of virus-specific intracellular RNAs from cells infected during passage 3 with DI particles that contain various lengths of the junction region in the RNA genome. SIN refers to cells infected with Sindbis virus only. Numbers under the other lanes refer to the number of nucleotides of upstream and downstream junction sequence in each of the DI RNAs: 19/6, JN19/6; 20/5, JN20/5; 19/4, JN19/4; 19/3, JN19/3; and 20/2, JN22. Excluding the poly(A) sequences, the sizes of the genomic RNAs (and the expected subgenomic RNAs, when detected) are, respectively, 2,462 (1,091), 2,459 (1,078), 2,454, 2,453, and 2,424 nucleotides. (B) Protection of [³H]uridine-labeled DI JN20/5 RNAs from S1 nuclease digestion. The samples are JN20/5 DI RNA isolated from cells during passage 3 (genomic RNA = 2,459 nucleotides, subgenomic RNA = 1,078 nucleotides; lanes 2 and 3), JN20/5 in vitro transcripts (2,459 nucleotides, lanes 4 and 5), and RNA from cells infected only with Sindbis virus during passage 3 (lanes 6 and 7). Samples of the RNAs were hybridized to the JN20/5 plasmid clone and then digested with S1 nuclease (lanes 2, 4, and 6). Parallel samples (lanes 3, 5, and 7) were not hybridized to the template cDNA before treatment with S1 nuclease. Samples were denatured and analyzed on a 1% agarose gel after digestion. Lane 1 is JN20/5 passage 3 RNA without hybridization and without S1 nuclease digestion. (C) Analysis of the extension products obtained from Sindbis virus RNAs isolated from cells during the formation of passage 3. The procedures and the primer were identical to those described in the legend to Fig. 2C. Passage 3 (P3) RNAs were either from cells synthesizing DI [labeled as DI(20/5)] genomic and subgenomic RNA or from cells synthesizing only Sindbis virus (SIN) 49S and 26S RNA. T refers to JN20/5 in vitro transcript. The products were compared with a sequence ladder of JN20/5 DNA [labeled as KDI(20/5)] generated with the same primer (lanes labeled G, A, T, and C). The arrow indicates the sequence ATAGTG...corresponding to the 5' end of the subgenomic mRNA.

		-20		-10		+1		+10	
SIN	GAAAUAAAGC		AUCUCUACGGUGGUCCUAAAUAGU					CAGCAUAGUA	
SFV	CCUG-U-UA-		-C-----C-----U-G					UGCGU--A--	
MBV	CCCG-U-UUG		-C-----C-----					UGCGUG-A--	
RRV	CCC--CGUA-		-C-----C-----A					UGCAGAGAC-	
ONN	CCCG-CGUAA		CCU-G-----C--A-----G					U-CGCACUAC	
WEE	A-CCC--UCA		CC-----CU-A-----G					UGA-G-----	
EEE	C-CCCC-UAA		CC-----CU-A-----G					UU-UGC-U--	
VEE	-CCCCU-UAA		C-----CUAA-----G-A					-UA-GACA--	

FIG. 4. Sequences at the alphavirus initiation site for subgenomic RNA synthesis. Sequences from -29 to +15, in the plus sense, are shown for eight alphaviruses. The minimal Sindbis virus promoter is shown in bold type. Dashes indicate sequence identity with Sindbis virus. SIN, Sindbis virus (19); SFV, Semliki Forest virus (19); MBV, Middleburg virus (19); RRV, Ross River virus (4); ONN, O'Nyong Nyong virus (J. Strauss, unpublished data); EEE, eastern equine encephalitis virus (3); WEE, western equine encephalitis virus (7); and VEE, Venezuelan equine encephalitis virus (10).

from cells infected with Sindbis virus alone. Only JN20/5 passage 3 RNA gave an extension product that corresponded to the expected size for the DI-derived subgenomic RNA (Fig. 3C). The initiation site is the same as that used for synthesis of the Sindbis virus 26S subgenomic RNA.

Cells infected with JN19/6 and JN20/5 synthesized subgenomic RNAs, but those infected with JN19/4 (Fig. 3A) and JN17/6 (data not shown), with junction region inserts of -19 to +4 and -17 to +6, respectively, did not produce any detectable subgenomic RNA. Therefore, under the conditions described, transcription of a subgenomic RNA required that the template contain at least 18 or 19 nucleotides upstream and 5 nucleotides downstream of the initiating nucleotide.

Diverse 5' termini are consistent with subgenomic RNA synthesis. Tsiang et al. (29) showed that a replication-competent DI genome can have a variety of 5' termini: the Sindbis virus 5' end, a deleted form of the 26S subgenomic RNA 5' terminus, or tRNA^{Asp}. All the DI cDNA clones described above have the tRNA^{Asp} sequence replacing the first 30 nucleotides of the 5' end of the Sindbis virus plus strand (17). We altered the 5' end of the DI genome to determine whether this region in the minus-strand template has any role in subgenomic RNA synthesis. We tested two derivatives of JNSH. One has the wild-type 5'-terminal sequences restored. The other has a deletion covering the 51-nucleotide highly conserved sequence from base 155 to 204 in the Sindbis virus genome (20) of unknown function, while retaining the tRNA^{Asp} at the 5' terminus. Both DI RNAs were replicated and in both cases the infected cells analyzed at passage 3 produced subgenomic RNAs of the expected size (data not shown). Thus, if sequences at the 3' terminus of the negative strand are important for subgenomic RNA synthesis, both the normal genomic sequences and the tRNA sequences can function in this capacity.

DISCUSSION

A survey of the sequence relatedness among several alphaviruses indicated that 19 nucleotides upstream and 2 nucleotides downstream from the start of the subgenomic domain of the genome were highly conserved (19). This finding suggested that the conserved region constituted a promoter region for the initiation of transcription from the negative-strand template (19). Naturally occurring DI genomes of Sindbis virus lack junction region sequences and do not produce subgenomic RNAs (18, 26). We found that a subgenomic RNA could be transcribed from a DI template, using a DI RNA that contains an insertion of 215 nucleotides encompassing 98 nucleotides upstream and 117 nucleotides

downstream from the start of 26S RNA. The identification of subgenomic RNA was based not only on the size of the RNA but also on nuclease S1 analysis and on the correct site of initiation of the RNA. Deletion analysis showed that cells infected with DI genomes containing nucleotides -20 to +5 or -19 to +6 of the junction region produced subgenomic RNAs, but those with nucleotides -19 to +4 or -17 to +6 did not. Thus, a minimum of 18 to 19 nucleotides upstream and 5 downstream of the initiation site are required for transcription. With the availability of more alphavirus junction region sequences, it is now clear that the sequence conservation first defined by Ou et al. (19) extends over a region essentially identical to the Sindbis virus promoter sequence identified here (Fig. 4). The sequence differences within this region among the alphaviruses may represent positions of lesser importance for recognition, or they may be sites that are characteristic of the different viruses.

No subgenomic RNA was detected in cells synthesizing DI RNAs in which the 215-base junction region fragment had been inserted in an inverted orientation. In this case, the subgenomic RNA would have been transcribed from the positive-strand template. It is possible that the product was not detected because it was unstable. Alternatively, for example, the polymerase complex synthesizing minus strands may lack the polypeptides required for the internal initiation of transcription. Another possibility is that the recognition of the promoter is context dependent and additional sequences or structural elements of the minus-strand template are required to coordinate initiation of subgenomic RNA synthesis.

Cells synthesizing DI RNAs derived from clones JNSH, JNBH, and JN34R, which have junction region inserts of -98 to +117, -49 to +63, and -34 to +14, respectively, produced more subgenomic than genomic RNA (see, e.g., Fig. 2A), a characteristic also observed for cells infected with Sindbis virus. In contrast, cells infected with DI RNA JN19/6 or JN20/5 produced substantially decreased amounts of subgenomic RNA compared with the DI genomic RNA (Fig. 3A). Several factors may contribute to the different levels of transcription of subgenomic RNA. Flanking DI RNA sequences (or secondary structures) brought into proximity with the promoter may affect transcription initiation. Alternatively, the flanking, junction region sequences may have a role in transcription, perhaps by serving as additional *cis*-acting elements that modulate the level of transcription directed by the core promoter. This latter possibility is similar to the model proposed for the transcription of BMV subgenomic RNA. Initiation of BMV subgenomic RNA occurs at an internal polymerase-binding site on the minus

strand of RNA3. Marsh et al. (15) using an in vitro transcription-replicase system showed that efficient subgenomic RNA synthesis from a BMV RNA3 minus-strand template requires a region of 62 nucleotides spanning the subgenomic initiation site. French and Ahlquist (5) showed that a smaller region, 36 nucleotides long, is sufficient for low-level synthesis of BMV subgenomic RNA in vivo but that the efficiency is enhanced by additional upstream and downstream sequences.

The low level of genomic RNA produced in cells infected with JNSH suggests that the minus strands of DI RNAs with strong subgenomic RNA promoters serve as templates for high levels of subgenomic RNA transcription and are not as available as templates for replication. This may be one reason that naturally occurring DI genomes do not contain junction region sequences. DI RNAs which allow subgenomic RNA synthesis will produce less of the genomic RNA for packaging into particles and would not be amplified as efficiently as DI genomes without a functional promoter.

Since DI RNAs do not encode any functional replication, transcription, and packaging proteins, the proteins responsible for subgenomic RNA initiation and elongation must be supplied in *trans* by the coinfecting helper virus. The viral protein or proteins required for subgenomic RNA transcription have not been identified. Of the four viral nonstructural proteins, mapping of temperature-sensitive mutations of Sindbis virus suggests that nsP4 has RNA polymerase elongation activity (8), while mutations in nsP2 can alter the relative amounts of genomic and subgenomic RNA produced in infected cells (9). The subgenomic RNA is capped and methylated, and a methyltransferase activity has been mapped to nsP1 (16). We describe elsewhere the introduction of a *cis*-acting mutation in the promoter of Sindbis virus that results in drastic reductions in viral subgenomic RNA synthesis (6). Pseudorevertants of the mutant virus have been isolated that have gained the ability to recognize, in *trans*, the mutant junction region. It is likely that mapping of the second-site mutations in these pseudorevertants will help identify the viral proteins involved in promoter recognition.

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