Analysis of Sindbis Virus Promoter Recognition In Vivo, Using Novel Vectors with Two Subgenomic mRNA Promoters

RAMASWAMY RAJU AND HENRY V. HUANG*

*Department of Molecular Microbiology, Washington University School of Medicine, Box 8230,
660 South Euclid Avenue, St. Louis, Missouri 63110-1093*

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Four types of Sindbis virus vectors, each carrying two promoters for subgenomic mRNA synthesis, were designed to measure relative promoter strengths and to survey potential contextual effects on promoter strengths. One of the promoters in each vector was used as the reference promoter, while the other was the one being tested. We used these vectors to measure the relative strengths of four promoters: the minimal promoter, an extended sequence believed to have full promoter activity, and two mutant promoters, one with an inactivating 3-nucleotide insertion called CR4.1 and the other with a 4-nucleotide deletion called $\Delta 4$. The strengths of the promoters were measured by quantitating the RNA transcribed from each promoter in vivo and also by assaying for chloramphenicol acetyltransferase activity encoded by one of the two transcripts. We found that the relative strengths of the promoters were similar in different contexts. The complete promoter was 6-fold more active, the $\Delta 4$ promoter was (surprisingly) about twice as active, and the CR4.1 promoter was 100-fold less active than the minimal promoter. At least two contextual effects were identified that can alter the activity of one or both promoters in the vectors. One effect is that given identical promoters, the 3'-proximal promoter on the minus-strand template can be more active than the 5'-proximal promoter. This may be due to preferential association of one or more components of the transcription complex for the 3' end of the minus-strand template. A second effect is promoter competition, particularly when the promoters are closely spaced.

Sindbis virus is the type species of the *Alphavirus* genus of the *Togaviridae* family. It is an arbovirus transmitted primarily by mosquitoes to a variety of vertebrate hosts (20). The genome of Sindbis virus consists of a single-stranded RNA molecule of positive polarity and is 11,703 nucleotides (nt) long (19). The genomic 49S RNA is capped at the 5' end and polyadenylated at the 3' end. The polymerase components are translated from the 5' two-thirds of the genome. Early during infection, the polymerase complex uses the plus-sense genomic RNA to synthesize a full-length minus-strand complement. The minus strand in turn is used as template for the synthesis of genome-size plus strands. The most abundant intracellular viral RNA species is a 26S subgenomic mRNA transcribed from an internal initiation site on the minus-strand template (17). The 26S RNA is 4,106 nt long, capped, polyadenylated, and colinear with the 3' one-third of genomic plus-strand RNA. The 26S subgenomic RNA encodes the three virion structural proteins, and this RNA is not packaged into virion particles.

The functional organization of the polymerase components and the *cis*-acting recognition motifs on the viral RNAs of Sindbis virus have been under intense investigations. The availability of cDNA clones of Sindbis virus that can be transcribed in vitro to produce infectious transcripts has greatly facilitated such analysis. Nucleotide sequence comparison of members of the alphavirus genus revealed at least four conserved RNA motifs (20). One of these is a 21-nt conserved sequence spanning the initiation site for the 26S subgenomic RNA. This conserved region, called the junction region, was proposed to be the RNA promoter on the minus strand for the 26S mRNA synthesis (12). A defective interfering (DI) genome of Sindbis virus lacks this conserved

sequence, and it does not express any subgenomic RNA (10). A DI-derived subgenomic RNA can be obtained upon insertion of the conserved sequence along with flanking sequences into the DI genome (9). Deletion analyses demonstrated that the minimal promoter capable of directing the synthesis of detectable amounts of DI-derived subgenomic RNA consists of 19 nt 3' and 5 nt 5' of the initiation site on the minus-strand template. (For simplicity, we will adopt a convention whereby this promoter is denoted as 19/5.) Interestingly, the minimal 19/5 promoter seems not to be as active as, e.g., a 98/117 promoter (9). We also demonstrated that a three-base insertion, called the CR4.1 mutation, at the promoter region drastically reduces synthesis of the subgenomic RNA (6). In these studies, the various promoter segments were inserted into DI genomes and the promoter activity was analyzed through expression of an mRNA coding for chloramphenicol acetyltransferase (CAT). However, the absolute strength of a promoter might depend not only on its precise sequence, but also on contextual factors. For example, local RNA structures and proximity to the 5' or 3' terminus may influence the promoter activity. Although never found with naturally occurring alphaviruses, the presence of other promoters might also influence the promoter activity. We therefore developed four novel Sindbis virus vectors designed to estimate the strength of a given promoter in different contexts and relative to different reference promoters. With these vectors, we were able to measure the relative strengths of different promoters despite the different contexts in which they were placed. We confirm that the CR4.1 mutation described earlier (6) essentially inactivates the promoter. We also show that a minimal promoter carrying a 4-base deletion is more active than the minimal promoter itself. The behavior of the different promoters in different contexts also provides clues to the mechanism of transcription initiation at internal RNA promoters.

* Corresponding author.

MATERIALS AND METHODS

General recombinant DNA procedures. Restriction endonucleases, T4 and T7 DNA polymerase, Moloney murine leukemia virus reverse transcriptase, T4 DNA ligase, SP6 DNA-dependent RNA polymerase, RNasin, and DNase I were obtained from various commercial sources and used essentially as recommended by the manufacturers, except as noted below. Plasmids were constructed, characterized, and purified by standard procedures with minor modifications (11). Radioactive materials were obtained from Amersham Corp., ICN Pharmaceuticals, Inc., or Du Pont Co.

Plasmid constructions. (i) **JUNCAT.** The plasmid JUNCAT (8) contains the promoter for the SP6 DNA-dependent RNA polymerase followed by a polylinker sequence (including, in order, *Apal*, *KpnI*, *ClaI*, *HindIII*, and *XbaI* sites) and then the bacterial *cat* gene. Beyond the *cat* gene were the 3' terminal sequences [position 11087 through the poly(A) sequence, nucleotide positions according to reference 19] of Sindbis virus and an *SspI* site for linearization for run-off transcription.

(ii) **JUNCAT-C.** Plasmid JUNCAT-C contains the minimal 19/5 promoter inserted between the *KpnI* and *XbaI* sites of JUNCAT. The minimal promoter insert was obtained through a polymerase chain reaction (PCR)-based saturation mutagenesis procedure (13).

(iii) **JUNCAT-8.** Plasmid JUNCAT-8 contains an altered minimal promoter called $\Delta 4$ (see Fig. 2) that has a deletion of positions -19 through -16, plus a 20-nt insertion due to a PCR artifact, inserted between the *KpnI* and *XbaI* sites of JUNCAT. This promoter sequence was a byproduct during a PCR-based saturation mutagenesis procedure (13).

(iv) **JC-TR and JC-TR.A.** The JUNCAT plasmid was cut with *ClaI* (polylinker), and the ends were rendered blunt with T4 DNA polymerase and further cut with *NcoI* (in *cat*). The plasmids TRCAT (21) and TRCAT.A (6) were cut with *SspI* (7499) and *NcoI* (in *cat*). The large *ClaI-NcoI* fragment from the JUNCAT plasmid was ligated with the *SspI-NcoI* fragment of TRCAT to get JC-TR. JC-TR contains the 98/14 promoter. The large *ClaI-NcoI* fragment of JUNCAT was also ligated to the *SspI-NcoI* fragment of TRCAT.A to get JC-TR.A. JC-TR.A contains exactly the same promoter as JC-TR but with the CR4.1 three-base insertion mutation (6).

(v) **TAPA.** Plasmid TAPA (8) was derived from Toto1000 (16). It contains the complete Sindbis virus genome positioned downstream of an SP6 DNA-dependent RNA polymerase promoter. In addition, this plasmid contains an *Apal* to *XbaI* polylinker just 3' of the termination codon of the E1 glycoprotein gene.

(vi) **TT20 series plasmids.** The TAPA plasmid was cut with *Apal* and *PvuI*, and the larger fragment was ligated to the larger *Apal-PvuI* (promoter and *cat* containing) fragment of JUNCAT-C to get TT20a, JUNCAT-8 to get TT20b, JC-TR to get TT20c, JC-TR.A to get TT20d, and JUNCAT to get TT20e (Fig. 1A).

(vii) **XNA-1.** The TAPA plasmid was linearized with *XbaI*, and the ends were rendered blunt with T4 DNA polymerase and ligated to destroy the *XbaI* site. The resulting plasmid (TAPA-1) was digested with *NarI* (11311) and *PvuI* (in the vector). The plasmid Toto1003 was digested with *NarI* (7869) and *PvuI*, and the large fragment was isolated and ligated to the smaller fragment from TAPA-1. The resulting plasmid (XNA-1) contains the Sindbis virus nonstructural region and the junction region (with a 6-nt *XbaI* site inserted at position 7612 [16] up to position *NarI* (7869) of Toto 1003, fused to *NarI* (11311) through the poly(A) sequences from

TAPA. The out-of-frame fusion of the *NarI* sites leads to an open reading frame of 74 amino acids of the capsid protein amino terminus plus two extra residues. Toto1003 is identical to Toto1002 (16) except that a deletion (11450 to 11641, replaced with 5'-CGAGG-3') was present in the 3' untranslated region. The segment of Toto1003 used to make XNA-1 does not include the deletion.

(viii) **TR20 series plasmids.** The plasmid XNA-1 was cut with *Apal* and *PvuI*, and the larger fragment was ligated to the same promoter-*cat* fragments used to make the TT20 clones to obtain TR20a, -b, -c, -d, and -e clones (Fig. 1B).

(ix) **JNTS-TR.** The plasmid JNTSCAT (kindly provided by B. Weiss and R. Levis) was cut with *StyI* (the Sindbis virus DI KDI-25, position 1325 [10]), and the ends were rendered blunt with T4 DNA polymerase and further cut with *NarI* (11311). The plasmid TRCAT was cut with *HpaI* (6919) and *NarI* (11311). The larger JNTSCAT fragment was ligated to the smaller TRCAT fragment to generate JNTS-TR. JNTS-TR contains the KDI-25 5' sequences (10), followed by TRCAT sequences (*HpaI* [6919] to *NarI* [11311]), including a 6-nt *XbaI* site inserted at 7612 and the *cat* sequences), and Toto1000-derived sequences from 11311 through the 3' end. JNTSCAT consists of the 5' region of KDI-25 up to the *NaeI* site (KDI-25 position 1340) plus the 3' region from the *SspI* (7499) site through the poly(A) sequences of TSCAT (21).

(x) **DIF20 series plasmids.** The plasmid TR20e was digested with *PvuI* and partially digested with *XbaI*, and the 2.8-kb fragment was ligated to the larger fragment of *XbaI*- and *PvuI*-digested JNTS-TR to get DIF20e. DIF20e was further digested with *Apal* and *PvuI*, and the larger fragment was ligated to the same promoter-*cat* fragments used to make the TT20 clones to obtain DIF20a, -b, -c, and -d clones (Fig. 1C).

(xi) **JNTS-CA.** The plasmid JNTSCAT was cut with *StyI* (KDI-25 position 1325), and the ends were rendered blunt with T4 DNA polymerase and further cut with *NarI* (11311). The plasmid JUNCAT-C was cut with *NheI* (site upstream of the SP6 DNA-dependent RNA polymerase promoter), and the ends were rendered blunt with T4 DNA polymerase and further cut with *NarI* (11311). The larger fragment of JNTSCAT and the smaller fragment of JUNCAT-C were ligated to get JNTS-CA. This plasmid contains KDI-25 sequences, a duplicated SP6 DNA-dependent RNA polymerase promoter, and polylinker sequences starting with an *Apal* site, followed by the minimal 19/5 promoter, the *cat* gene, and 3' untranslated sequences from Sindbis virus position 11311 through the poly(A) sequences.

(xii) **JNTS-C.** The *Apal* site of JNTS-CA was destroyed by cutting, treatment with T4 DNA polymerase, and ligation. This manipulation resulted in the inactivation of the duplicated SP6 DNA-dependent RNA polymerase promoter located adjacent to the *Apal* site. Hence, JNTS-C has only one functional SP6 DNA-dependent RNA polymerase promoter, immediately upstream of the DI 5' sequences.

(xiii) **DIC20 series plasmids.** The 2.8-kb fragment obtained by partial *XbaI* and complete *PvuI* digestion of TR20e was ligated to the corresponding larger fragment obtained from JNTS-C to get DIC20e. DIC20e was further digested with *Apal* and *PvuI*, and the larger fragment of this digestion was ligated to the same promoter-*cat* fragments used to make the TT20 clones to obtain DIC20a, -b, -c, and -d clones (Fig. 1D).

(xiv) **GEM-3CAT.** The pGEM-3 plasmid (Promega Biotec; kindly provided by Chang Hahn) was digested with *EcoRI* and *XbaI* and ligated to a corresponding *cat* gene fragment obtained from TRCAT to obtain GEM-3CAT.

FIG. 1. Structures of plasmids. The names of the plasmids and the relevant restriction sites used in cloning are shown. The SP6 DNA-dependent RNA polymerase promoter and the start site for in vitro transcripts are indicated by the right-angle arrow. The *Sst*I linearization site for run-off transcription indicates the end of the in vitro transcripts. The open rectangles marked P1 and P2 denote the reference promoter and the test promoter positions, respectively. Each group of plasmids (TT20, TR20, DIF20, and DIC20) has five members, all of which possess the same reference promoter but each of which carries a unique test promoter at P2. Shown below each plasmid are the sizes of the expected in vivo viral RNAs (in kilobases). The mRNA-2 (1.45 kb) is identical for all the plasmids. NS, Nonstructural region; S, structural region.

Cells and viruses. Primary and secondary cultures of chicken embryo fibroblasts were prepared and used to determine virus stock titers as described before (18). BHK-21 cells, grown in minimal essential medium (MEM) supplemented with 10% tryptose phosphate and 10% bovine calf serum, were used for all transfections, virus growth, and RNA and CAT analyses (6).

In vitro transcription. Plasmids were linearized with *Sst*I, phenol-chloroform extracted twice, and ethanol precipitated with glycogen (20 µg/ml) and 0.1 M sodium chloride, and the DNA pellets were dissolved in water. A 2-µg amount of linearized plasmids was transcribed at 37°C with 15 U of SP6 DNA-dependent RNA polymerase in a 30-µl final volume. The reaction conditions were as described before (16), but including 10 mM NaCl, 250 µM cap analog, and trace amounts of [³H]UTP. The amount of RNA synthesized was estimated by adsorption of a small sample to DE-81 paper. The DNA template was then digested with 1 µg of RNase-free DNase I, phenol-chloroform extracted twice, and ethanol precipitated. The pellets were dissolved in 25 µl of water. Ten percent of the sample was glyoxylated and analyzed on a 1% agarose gel (see Fig. 3). RNAs with significant degradation were not used for transfection. Under these conditions, DNase I-treated RNA samples gave higher transfection efficiencies than untreated samples (results not shown).

cat-specific plus-sense riboprobes were prepared by transcribing the pGEM-3CAT plasmid, linearized with *Eco*RI, with SP6 DNA-dependent RNA polymerase.

Transfection of vector RNA and harvesting virus. Sindbis virus-infected or uninfected BHK-21 cells (35- or 60-mm dishes) were washed twice with 3 ml of Ca²⁺- and Mg²⁺-free phosphate-buffered saline (PBS), overlaid with a mixture containing 1 to 2 µg of in vitro transcripts, 12 µg of Lipofectin (Life Technologies, Inc.), and 200 µl of PBS, and constantly rotated for 10 min at room temperature. The cells were washed two times with 3 ml of PBS, overlaid with 1 ml of MEM containing 10% fetal bovine serum, and incubated at 37°C for 20 h. The medium was harvested, virus titers were determined (TT20 series only), and the medium was divided into portions and stored at -80°C. In the case of transfections requiring prior Sindbis virus infection, the BHK-21 cells were infected with 5 PFU of Sindbis virus (S. Schlesinger's laboratory strain) per cell for 1 h at 30°C.

Infection with recombinant virus, RNA labeling, and harvest. BHK-21 cells were infected with 10 PFU of TT20 series viruses per cell or with 5 to 7% of the first-passage supernatant from the other series. Inocula were diluted to 200 µl with PBS, adsorbed onto BHK-21 cells at 37°C for 1 h, and washed twice with 3 ml of PBS, 0.5 ml (35-mm dishes) or 1 ml (60-mm dishes) of medium was added, and the infection

FIG. 2. Sequences of the test promoters located at the P2 site. All a clones carry the 19/5 promoter sequence at the P2 site. Similarly, all b, c, and d clones carry the Δ4, 98/14, and CR4.1 promoters, respectively. The CR4.1 promoter is identical to sequence c except that it has the additional 3-base insertion. The sequence in the e clones is a vector polylinker sequence upstream of the *cat* gene. The size of mRNA-1 made by all the a, b, and e clones is 80 nt shorter than the mRNA-1 made by the c and d clones because of the longer promoter sequence of c and d variants.

was continued for 3 h. Dactinomycin was then added to 1 μg/ml, followed 5 to 10 min later with 50 to 200 μCi of [³H]uridine (depending on the level of uridine incorporation into viral RNAs). At the end of 4 h, the cells were washed with PBS, scraped into 1 ml of PBS, and suspended with a Pasteur pipette. A 100-μl aliquot was pelleted at low speed and processed for CAT assays. The remaining 900-μl sample was pelleted and extracted with RNazol (Cinna/Biotech Laboratories International, Inc.), and the RNA was isolated and ethanol precipitated. This RNA sample was used for estimation of the amount of minus strands and for the quantitation of mRNA levels.

RNA analysis. Equivalent amounts of RNA samples were glyoxylated and electrophoresed through 1 or 1.5% agarose gels in 10 to 15 mM phosphate buffer, pH 7 (3). The gels were then washed extensively with industrial-grade methanol, impregnated with diphenyloxazole (3% in methanol) for 3 to 6 h, soaked in water for 1 h, dried under vacuum at 60°C, and exposed to preflashed X-ray film (Kodak X-Omat AR). The subgenomic mRNA bands were cut out, using the autoradiograms as guides, and the radioactivity in each band was determined by liquid scintillation. The background was determined from counting the adjoining region below the bands of interest. The relative intensities of mRNA bands were also estimated by densitometric scanning.

Dot-blot assay for quantitating minus strands. To compare the intracellular level of *cat*-specific minus-strand templates, 10% of the RNA samples were glyoxylated and spotted onto Hybond (Amersham Corp.) nylon membranes soaked in 1.5 M sodium chloride. (Filtration of the samples through commercial blotting apparatuses did not give consistent results.) The membranes were dried at 80°C for 30 min and soaked in 50 mM Tris (pH 8.5) at 65°C. The membranes were prehybridized and then hybridized (14) with a *cat*-specific plus-sense riboprobe transcribed from pGEM3-CAT.

Primer extension analysis. A minus-sense oligonucleotide corresponding to nucleotide positions 17 to 34 downstream of the initiation codon of the *cat* gene was 5' end labeled, annealed to intracellular RNAs, and extended with Moloney murine leukemia virus reverse transcriptase essentially as described before (15). The primer-extended samples were ethanol precipitated and analyzed on 6% acrylamide sequencing gels along with DNA sequence ladders generated

with the same primer annealed to the respective DNA clones to identify the mRNA start site.

Quantitation of CAT expression. Cell extracts were prepared by two freeze-thaws, the cytoplasmic supernatant was obtained by centrifugation, and an aliquot of the extract was assayed for CAT (5, 21). Based on this preliminary screening, a more accurate assay was performed by choosing volumes of the extracts for the assay so that the chloramphenicol conversion fell in the linear range of 25 to 40%. In addition, the assay mixtures were adjusted with Sindbis virus-infected cell extracts to equalize the protein content in each. Finally, the *cat* expression levels from various samples were normalized with respect to the amount of intracellular *cat*-specific minus-strand RNA in each.

DNA sequencing. Dideoxynucleotide chain-termination reactions with T7 DNA polymerase (Sequenase; United States Biochemical) were carried out according to the manufacturer's instructions.

RESULTS

The strength of a promoter might depend both on its sequence and on contextual influences. We therefore designed novel Sindbis virus derivatives (TT20, TR20, DIF20, and DIC20; Fig. 1) to identify possible contextual effects and to test whether the relative strength of different promoters may be measured accurately, independent of contextual effects. Each derivative carries two promoters; one is the reference promoter (labeled P1 in Fig. 1), and the other is the test promoter (labeled P2 in Fig. 1; sequences shown in Fig. 2). The four test promoters were the 19/5 promoter, which is the minimal sequence that directs detectable subgenomic mRNA synthesis (9); a 98/14 promoter, which probably has full promoter function (9, 21); a mutant promoter called Δ4 that has a 4-nt deletion and a 20-nt insertion relative to the minimal promoter; and a mutant promoter with the CR4.1 3-nt insertion mutation that drastically reduces transcription (6). The reference promoters were the fully active promoter (9, 21) and the minimal 19/5 promoter (9).

The promoter strengths were measured by quantitating the *in vivo* incorporation of label into each subgenomic RNA species, as well as by CAT expression, which is predominantly due to transcription from the P2 promoter (see below). In general, *in vitro* transcripts (Fig. 3) of the TT20 series clones were transfected into cultured BHK-21 cells (see Materials and Methods for detailed protocols). Virus was harvested and used to infect cells in a second passage, in which subgenomic RNA synthesis and CAT expression were measured. In all the experiments, the amount of intracellular templates (negative-sense full-length genomic RNA) was used to normalize the CAT protein expressed in different infections. Similarly, *in vitro* transcripts of clones from the TR20, DIF20, and DIC20 series were transfected into BHK-21 cells, except that the cells were preinfected with Sindbis virus as helper. The virus produced by this first passage was then used to infect cells in a second passage to measure the promoter strengths. Some of the clones were expected to and did produce two distinguishable subgenomic RNAs: a larger mRNA-1 transcribed from the reference promoter and a smaller mRNA-2 transcribed from the test promoter (Fig. 3). The results are summarized in Table 1 and represent averages of results from three separate experiments.

RNA and CAT expression by TT20 clones. The TT20 clones consist of the complete Sindbis virus genome with a second test promoter (labeled P2 in Fig. 1) inserted immediately 3'

FIG. 3. Comparison of in vitro transcripts of the clones and their RNA products in vivo. Ten percent of the in vitro transcripts of a 30- μ l reaction labeled with trace quantities of [3 H]UTP (lanes SP6), and 10 to 15 μ g of [3 H]uridine-labeled intracellular RNA (lanes VIVO) extracted from cells infected with virus derived from the in vitro transcripts were glyoxylated and electrophoresed on a 1% agarose gel. Except for the TT20a clone, the other three clones have Sindbis virus present as helper. Hence, Sindbis virus 49S genomic and 26S subgenomic mRNAs are found in addition to the genomic RNA of each clone. The mRNA-1 and the mRNA-2 (faintly visible) expressed from the reference promoter (P1) and the test promoter (P2; in this case the 19/5 promoter), respectively, are also indicated.

of the termination codon of the E1 gene. In addition, the bacterial *cat* gene was inserted 3' of the test promoter. The reference promoter at the P1 position is the wild-type Sindbis virus promoter, in its normal position, with the normal flanking sequences extending at least 3.5 kb on either side. In vitro transcripts were transfected into cells, and the resulting virus was used to infect cells in a second passage. Viral RNAs were labeled in vivo, isolated, and electrophoresed on agarose gels. Figure 4A shows a short exposure of a typical gel. The genomic RNA and the mRNA-1 expressed from all the clones are clearly visible. Figure 4B shows the overexposed autoradiogram. Although mRNA-2 becomes visible for clones TT20a, -b and -c, we could not detect any mRNA-2 for the TT20d and -e samples even when the gel was exposed to film for much longer times.

The mRNA-1 and mRNA-2 positions were cut from the dried gel and the radioactivity was counted. Table 1 shows the relative molar ratios between the two mRNAs expressed by each clone. If we assume that the P1 promoters (identical in all the clones) are equally active, the relative strengths of the test promoters at the P2 position may be deduced (Table 1). By this estimation, the 98/14 promoter is 5.7-fold more active than the minimal 19/5 promoter; the Δ 4 promoter is 1.7-fold stronger, while the CR4.1 promoter is essentially inactive in this system.

The first suggestion of a contextual effect, called the 3'-proximal effect, can also be seen. The 98/14 promoter at the P2 position of TT20c is believed to be fully functional (9, 21), yet it was utilized only 20% as well as the presumed equally active promoter at the P1 position (Table 1). This suggests that the 3'-proximal promoter is utilized more efficiently than the 5'-proximal promoter on the minus-strand template. Since mRNA-1 is 3.7 times longer than mRNA-2, it is expected to be more sensitive to degradation, and the actual molar ratio of the mRNAs is a minimal estimate, which reinforces our conclusion.

The P1 reference promoter present at the junction of the nonstructural and structural regions drives the synthesis of a

bicistronic mRNA-1 encoding both the Sindbis virus structural polyprotein and CAT. This bicistronic mRNA is an extremely poor message for CAT synthesis, since in the absence of a P2 promoter very little CAT was made (TT20e, Table 1). CAT is expressed predominantly from mRNA-2 produced from the P2 promoter. The relative CAT expression from the P2 promoters agrees well with the relative promoter strengths estimated from RNA analysis. The Δ 4 promoter, which has a 4-nt deletion at the minimal promoter, gave 2.5-fold more CAT protein than the minimal 19/5 promoter. The 98/14 promoter gave 7.3-fold-higher amounts of CAT. The CR4.1 promoter expressed 57-fold less CAT, as expected from our previous results (6). As we could not detect any mRNA-2 made by the CR4.1 clone, the trace quantities of CAT detected could have been the result of, e.g., a low level of RNA nicking in vivo or a very low level of internal *cat* AUG recognition on the bicistronic mRNA-1.

RNA and CAT expression by TR20 clones. The P1 and P2 promoters of the TT20 vectors are separated by 3.7 kb. To determine whether interpromoter distance affects promoter strengths, we constructed the TR20 series (Fig. 1B) of clones. The RNA genomes derived from these plasmids have the nonstructural region of Sindbis virus, encompassing the junction region up to a position 14 nt 5' of the initiation site on the minus-strand template. There is a 320-nt distance between the P1 and P2 promoters. The *cat* gene is immediately after the P2 promoter position. The test promoters, the same as those in the TT20 clones, were placed at the P2 position of the TR20 vector. As this series of vectors do not have the structural protein genes, Sindbis helper virus was used during the first passage in order to package the amplified vector RNA. The intracellular viral RNA isolated from infected cells therefore includes the Sindbis virus genomic and subgenomic RNAs (Fig. 3 and 5). In addition, the TR20 series full-length genomic RNA, mRNA-1 derived from P1, and mRNA-2 derived from P2 were found (Fig. 3 and 5). mRNA-1 and mRNA-2 differ by only 24% in size.

An overexposed gel of the intracellular viral RNA (Fig. 5) of TR20 series-infected cells demonstrated that the mRNA-1 and mRNA-2 bands were well resolved, with relatively low levels of background, allowing accurate determination of the amount of both mRNAs. Clones TR20d and TR20e, with the CR4.1 mutant promoter or with no promoter at the P2 position, respectively, gave no detectable mRNA-2 even when the infected cells were labeled for 6 h (Fig. 5B). We previously found synthesis of a very low level of mRNA from a virus containing only the CR4.1 promoter (6). In the absence of any other functional promoter that might compete with it, the CR4.1 promoter may be recognized by the transcription complex for transcription initiation, albeit inefficiently.

Table 1 shows that the molar ratios of mRNA-2 to mRNA-1 for TR20a, -b, and -c were uniformly six- to sevenfold higher than that for TT20a, -b and -c, while the relative promoter strengths deduced from the mRNA-2/mRNA-1 molar ratios are exactly that obtained from the TT20 clones. Comparison of the amounts of the two mRNAs made by these clones suggests that the increases in the molar ratios are mostly due to decreases in the amounts of mRNA-1 made by the clones. This is confirmed by the observation that CAT expression by the TR20 clones did not increase compared with that by the TT20 clones (Table 1), as would be expected if mRNA-2 had increased by six- or sevenfold. The TR20c clone consistently produced more mRNA-2 than mRNA-1. The reason for this is yet unclear, but the results are consistent with a decrease in the 3'-

TABLE 1. Promoter strengths measured by quantitation of RNA and CAT

Clone	P1 promoter ^a	P2 promoter ^b	Molar ratio, ^c mRNA-2/mRNA-1	Relative promoter strength ^d	CAT trimers produced ^e (10 ¹⁰)	Relative CAT expression ^f
TT20 series						
a	ns/s	19/5	0.04	1.0	3.9	1.0
b		Δ4	0.06	1.7	9.9	2.5
c		98/14	0.21	5.7	29	7.3
d		CR4.1	— ^g	0.0	0.07	0.02
e		None	—	0.0	0.03	0.01
TR20 series						
a	ns/14	19/5	0.25	1.0	2.8	1.0
b		Δ4	0.42	1.7	5.6	2.0
c		98/14	1.55	6.2	17.7	6.3
d		CR4.1	—	0.0	0.04	0.01
e		None	—	0.0	0.02	0.01
DIF20 series						
a	679/14	19/5	0.13	1.0	0.8	1.0
b		Δ4	0.23	1.8	1.4	1.8
c		98/14	0.81	6.4	3.2	4.0
d		CR4.1	—	0.0	0.02	0.03
e		None	—	0.0	0.005	0.01
DIC20 series						
a	19/5	19/5	0.64	1.0	0.4	1.0
b		Δ4	2.75	4.3	1.4	3.5
c		98/14	7.43	11.7	3.5	8.8
d		CR4.1	—	0.0	0.01	0.03
e		None	—	0.0	0.003	0.01

^a The reference promoter (P1) in each vector. For each promoter, the sequences 3' and 5' of the RNA initiation site (indicated as 3'/5') on the minus-strand template are shown. ns/s, Entire nonstructural region and the entire structural region 3' and 5', respectively; ns/14, entire nonstructural region and 14 nt 3' and 5', respectively; 679/14, 679 nt and 14 nt 3' and 5', respectively; 19/5, 19 nt and 5 nt 3' and 5', respectively.

^b The test promoter (P2) in each vector. The 19/5, Δ4, 98/14, and CR4.1 promoter sequences are shown in Fig. 2. "None" indicates the absence of a second promoter.

^c The amount of radioactivity recovered from each mRNA-2 band was divided by the amount of radioactivity (divided by 3.7 for the TT20 series or by 1.24 for the other vectors to correct for size differences) recovered from the mRNA-1 band. The ratios represent the average of results from three separate experiments. The average variation in the mRNA ratios (three independent experiments) was 5%.

^d The mRNA-2/mRNA-1 molar ratio of each P2 promoter relative to that of the 19/5 P2 promoter in the a clone is shown.

^e The total number of CAT protein molecules from 2×10^5 cells infected with each vector was normalized to the amount of minus-strand templates produced in each infection. The CAT expression shown represents the average from three separate experiments, the same ones used to measure the mRNA-2/mRNA-1 molar ratios. The average variation in CAT expression (three independent experiments) was 15%, after normalization to the minus-strand levels.

^f CAT expression relative to that from the 19/5 P2 promoter in the same vector series.

^g —, mRNA-2 was undetectable.

proximal effect when the promoters are brought close together. As with the TT20 clones, the relative CAT expression levels given by the different test promoters agree well with the relative promoter strengths estimated from RNA analysis.

RNA and CAT expression by DIF20 clones. The P1 promoter of the TT20 and TR20 clones overlap the nsP4 gene, which most likely encodes the polymerase (1, 2, 7), and thus the P1 promoter cannot be altered at will. The DIF20 clones are similar to the TR20 clones except for the substitution of a DI 5' terminus (10) and the deletion of the nonstructural region upstream of the P1 promoter (Fig. 1). The P1 promoter extends 679 nt 3' and 14 nt 5' of the initiation site on the minus strand.

Figure 6 shows the RNA isolated from cells infected with the DIF20 clones. The Sindbis helper virus genomic and subgenomic RNA were present in all the samples, as was the DI genomic RNA. (The Sindbis virus 26S mRNA and the DIF20 genomic RNA comigrated in this 1.5% gel. In a 1% gel, these two RNAs were clearly separable [Fig. 1].) The DIF20a, -b, and -c clones gave both mRNA-1 and mRNA-2 bands, but even extended labeling periods of DIF20d- and

DIF20e-infected cells again failed to detect any mRNA-2 (Fig. 6B).

The DIF20c P2 promoter seems to be less active than the P1 promoter. Since available evidence (9) (the results with TR20c) suggests that their strengths should be comparable, the results suggest that the 3'-proximal promoter on the minus-strand template is recognized better than the 5'-proximal promoter, as seems to be the case with the TT20 clones.

Table 1 shows that the DIF20 clones gave mRNA-2/mRNA-1 molar ratios that were uniformly three- to fourfold higher than those of the corresponding TT20 clones. The increased ratios are again likely to be due to decreased mRNA-1 levels, as reflected by the amount of mRNA-1 and mRNA-2 made, and by the lack of increase in CAT expression. The increases in molar ratios are less than that seen with the TR20 clones (Table 1). The reason for this is unclear, but one possibility is that since the DIF20 promoters are much closer to the 3' end of the minus strand than the TR20 promoters, the 3'-proximal effect is stronger in the DIF20 clones. Despite the increased molar ratios, the relative promoter strengths deduced from the molar ratios are

FIG. 4. Intracellular RNA of the TT20 clones. BHK-21 cells (35-mm dishes) were infected with 10 PFU of TT20 viruses per ml and labeled with 50 μ Ci of [3 H]uridine per ml between 3 and 4 h postinfection and harvested, and intracellular RNA was extracted. From 6 to 8 μ g (30%) of the intracellular RNA was glyoxylated and analyzed on a 1.5% agarose gel. (A) Underexposed gel; (B) overexposure of the gel in panel A to see mRNA-2. The reference promoter (P1) is the wild-type promoter, and a, b, c, d, and e refer to the test sequence at the P2 location (Fig. 1 and 2). Mock-infected cells (lanes m) treated with dactinomycin did not incorporate any significant radioactivity. Note that the exposures for these gels are much shorter than those shown in the subsequent figures.

identical to those for the TT20 and TR20 clones. Thus, relative promoter strengths may be consistently measured even though the contexts are quite different.

RNA expression by clones of the DIC20 series. The TT20, TR20, and DIF20 vectors possess extended, presumably fully functional promoters as the reference promoter at the P1 position. We also tested the effect of putting a weaker promoter at the P1 position. The DIC20 clones (Fig. 1D) are identical to the DIF20 clones except that the P1 promoter is a 19/5 promoter. Virus stocks were prepared as above and used to infect BHK-21 cells, and RNA and CAT expression were then analyzed. The expected Sindbis virus genomic and subgenomic RNAs and the DIC20 genomic RNA were

FIG. 5. Intracellular RNA of the TR20 clones. BHK-21 cells (35-mm dishes) were infected with 100 μ l of TR20 viruses diluted to 200 μ l with PBS and labeled between 3 and 4 h postinfection with 50 μ Ci of [3 H]uridine per ml (A); the cells were harvested and the intracellular RNA (7 to 10 μ g) was analyzed on gels. Panel B represents intracellular RNA labeled between 3 and 9 h postinfection with 20 μ Ci of [3 H]uridine per ml. The samples are labeled as in Fig. 4. S indicates RNA from Sindbis virus-infected cells. Note that the mRNA-1's of c and d are slightly longer than the mRNA-1 of a, b, and e because the c and d clones possess longer P2 promoters.

FIG. 6. Intracellular RNA of the DIF20 clones. BHK-21 cells (60-mm dishes) were infected with 100 μ l of DIF20 viruses diluted to 200 μ l with PBS and labeled with 100 μ Ci of [3 H]uridine per ml between 3 and 4 h postinfection (A). Another set of plates were infected and labeled between 3 and 9 h postinfection and harvested (B). For each set, 14 to 16 μ g of RNA was glyoxylated and analyzed on a 1.5% agarose gel. The samples are labeled as in Fig. 4. S indicates RNA from Sindbis virus-infected cells.

clearly detectable in infected cells. In addition, the DIC20a, -b, and -c clones gave the expected mRNA-1 and mRNA-2 bands. The Δ 4 and 98/14 test promoters in the DIC20b and -c clones were clearly stronger than the 19/5 reference promoter (Fig. 7, Table 1). As was found with the corresponding TT20, TR20, and DIF20 clones, the DIC20d and -e clones gave no evidence of an mRNA-2 band. The DIC20 clones gave relative promoter strengths and relative CAT expression levels that were significantly different from those of the TT20, TR20, and DIF20 clones. At least part of the difference can probably be attributed to the use as reference promoter of the relatively weak 19/5 promoter, which might compete less well against the stronger promoters. Indeed, the presence of stronger P2 promoters resulted in increased amounts of mRNA-2 and decreased amounts of mRNA-1 (Fig. 7).

Although the promoters present at the P1 and P2 positions of the DIC20a clone are expected to be identical in strength, the P2 promoter was recognized 0.64-fold as well as the P1 promoter (Table 1). This result suggests that the 3'-proximal promoter is recognized better.

Transcription initiation sites of mRNA-2. The RNA samples obtained from all the infections were analyzed by primer extension to map the initiation sites. The primer used was a

FIG. 7. Intracellular RNA of the DIC20 clones. BHK-21 cells (60-mm dishes) were infected with 100 μ l of DIC20 viruses diluted to 200 μ l with PBS and labeled between 3 and 4 h postinfection with 200 μ Ci of [3 H]uridine per ml. From 12 to 14 μ g of the RNA was glyoxylated and analyzed on a 1.5% agarose gel. The samples are labeled as in Fig. 4.

FIG. 8. 5' ends of mRNA-2 derived from all the vectors. Cytoplasmic RNAs (10 μ g) extracted from infected and uninfected cells were annealed to 1 pmol of 5'-end $^{32}\text{PO}_4$ -labeled negative-sense primer and extended by reverse transcriptase. One third of the reaction products was electrophoresed on a 6% sequencing gel along with a corresponding sequence ladder (lanes labeled ACGT). The sequence ladders in panels a, b, c, d, and e are from plasmids carrying the respective (a, b, c, d, and e) promoters at the P2 position. (Note that the a, b, and c promoters have identical sequences around the initiation site.) The primer extension products are indicated by the name of the vector from which the mRNA is derived. Thus, the sequence of a clones is followed by the primer extension products of mRNA-2 made from promoter a from the different clones indicated above the lane. Lane m contains primer extension products from intracellular RNA from mock-infected cells.

cat gene-specific negative-sense oligonucleotide. Figure 8 shows that all the clones that gave detectable amounts of mRNA-2 also gave patterns of extension products identical to that of the Sindbis virus subgenomic mRNA (17). The patterns are consistent with mRNA-2 initiating at the +1 position. (The multiple bands found are also observed with the Sindbis virus subgenomic mRNA [17] and might be due to the 5' cap or might reflect heterogeneity in the transcription initiation site.) The RNA samples obtained from cells infected with any of the vectors containing d and e at the P2 position did not show any specific extension products.

CAT expression. The relative CAT expression by all the clones agrees well with the relative promoter strengths (Table 1), and thus CAT expression is quite reliable for promoter strength analysis. Since CAT is produced predominantly from mRNA-2, this suggests that the differences at the 5' ends of the different mRNA-2's do not significantly affect the translation efficiencies. CAT expression by the DIF20 and DIC20 clones was generally lower than that by the TT20 and TR20 clones. The DIF20 and DIC20 clones do not encode the nonstructural proteins, and their CAT expression depends on replication and transcription factors supplied *in trans* by helper virus. Thus, their CAT expres-

sion might be less efficient than that of the TT20 and TR20 clones, which do encode the nonstructural proteins.

DISCUSSION

We measured the strengths of four different test promoters relative to a reference promoter *in vivo*. The measurements were made with four different vectors and two types of reference promoters to provide different contexts and to investigate contextual effects on promoter recognition. The results show that the relative promoter strengths are consistent from vector to vector when measured relative to a given reference promoter. This is an important result, since quantitative measures of promoter strengths are necessary for addressing a number of questions. For example, we have used these vectors to measure the strength of a large number of promoters derived from saturation mutagenesis, to identify the importance of individual nucleotides in the promoter (13).

Previous observations suggest that the minimal 19/5 promoter is less active than the wild-type promoter (9). The sequences that are responsible for enhancing the activity of the minimal 19/5 promoter have not been identified. The

current results show that the 98/14 promoter is consistently six- to sevenfold more active than the minimal 19/5 promoter. They suggest that the responsible enhancing sequences reside within 98 nt 3' and 14 nt 5' of the minimal promoter on the minus-strand template. The mutant $\Delta 4$ promoter was found to be about 1.7-fold more active than the minimal promoter. The deletion in the $\Delta 4$ promoter resulted in the juxtaposition of the remnant promoter sequences with flanking vector sequences, which gives a fortuitous partial match with the wild-type sequence (Fig. 2). The net effect is that an A at position -16 relative to the initiation site was changed to a C, and the A at -18 was changed to a G. The junction region consensus sequence of alphaviruses actually has G at -18 (9). Sindbis virus and Venezuelan equine encephalitis virus are the only two of eight alphaviruses that have been characterized that have an A at this position. Furthermore, in the course of testing the ability of Sindbis virus to recognize the junction region sequences of other alphaviruses and use them as promoters, we found that the A to G change at -18 is probably a neutral mutation (8). It is possible therefore that the -16 A to C change by itself may be an upmutation that gives better promoter recognition. However, the $\Delta 4$ promoter also includes a 20-nt insertion after position +13, whose effect on promoter activity is unknown. We cannot exclude the possibility that the deletion altered the flanking sequence in such a way that some inhibitory effect was eliminated. The fourth promoter that we tested had the CR4.1 mutation, previously shown to give very low but detectable levels of transcription (6). Transcription from this promoter was not detectable with any of the clones (Table 1). It is possible that detectable levels of transcription from the CR4.1 promoter occur only if no other stronger promoter is present to compete with it. The previous positive results were obtained when the CR4.1 promoter was the only promoter available to the transcription proteins, while all of the current experiments included one or more additional promoters.

The results also show that the context is important for promoter action. At least two types of contextual effects were found. One kind of contextual effect is promoter competition. The DIC20a, DIC20b, and DIC20c clones showed particularly strong promoter competition (Fig. 7). The utilization of the 19/5 promoter at the P1 position decreased dramatically as progressively stronger promoters were placed at the P2 position. A simple explanation of promoter competition is that the transcription initiation complex assembles more efficiently and/or is more stable at stronger promoters. Thus, for any less than saturating concentration of transcription factors, the occupancy of the promoters will depend on their relative strengths.

Another kind of contextual effect is that given two promoters with the same strength on a single genome, the promoter that is closer to the 3' terminus of the minus-strand template is more active. In the case of TT20a, mRNA-1 was expressed from the normal Sindbis virus promoter while mRNA-2 was driven by the 19/5 promoter. The relative strengths of these is 6:1. Thus, one might expect that the TT20a clone should give one-sixth or 0.17 as much mRNA-2 as mRNA-1. Instead, mRNA-2 was only 0.04-fold as abundant as mRNA-1 (Table 1). Similarly, TT20b gave an mRNA-2/mRNA-1 ratio of 0.06 (Table 1), when the relative promoter strengths would predict a ratio of $1.7/5.7 = 0.3$. The TT20c clone has promoters of equal strengths at both P1 and P2 positions, yet it expressed 0.21-fold as much mRNA-2 as mRNA-1. The P2 promoter is 3.8 kb downstream of the P1 promoter in the TT20 clones. In the case of

the DIF20 clones, in which the interpromoter distance is decreased to 320 nt, the 3'-proximal effect is less pronounced. However, the DIF20c clone, with promoters of equal strengths at both P1 and P2 positions, still gave higher mRNA-1 than mRNA-2 levels (Table 1).

The 3'-proximal effect can also be seen in the DIC20a, DIC20b, and DIC20c clones, in which the P2 promoters are as active as or more active than the 19/5 P1 promoter. The DIC20a clone has the 19/5 promoter in both the P1 and the P2 positions. Though the molar amounts of mRNA-1 and mRNA-2 are expected to be equal, there was 0.64-fold as much mRNA-2 as mRNA-1 (Table 1). Similarly, since the $\Delta 4$ promoter was found to be 4.3-fold stronger than the 19/5 promoter (compare DIC20b and DIC20a), the DIC20b clone would be expected to make 4.3-fold less mRNA-1 than mRNA-2, but it was only 2.8-fold less (Table 1). Similarly the DIC20c clone was expected to give 11.7-fold less mRNA-1 than mRNA-2, yet gave only a 7.4-fold decrease. None of the TR20 clones showed any preference for the 3'-proximal promoter. In fact, the TR20c clone had promoters of equal strengths yet consistently produced more mRNA-2 than mRNA-1. The reason for this is not clear. It is possible that other sequence elements present in the nonstructural region of TR20 might modulate promoter activity, opposing the 3'-proximal effect. Since the DIF20 clones do not have the nonstructural region, this inhibition may be absent. Though the TT20 clones have the nonstructural region, the two promoters are spaced far apart (3.8 kb) and the inhibition might not extend to the P2 position. If this is true, then lengthening the distance between the promoters in the TR20c construct should result in clones that exhibit the 3'-proximal effect. We favor an alternate possibility, that the distance between the promoters relative to the distance from the 3' end of the minus-strand template determines the strength of the 3'-proximal effect. If the promoters are far apart and both are far from the 3' end (e.g., the TT20 clones), then the 3'-proximal promoter is heavily favored. If the distance between the promoters and the 3' end is short (e.g., the DIF20 and DIC20 clones), then the 3'-proximal effect is smaller. Finally, if the promoters are close together relative to their distance from the 3' end, the 3'-proximal effect might disappear altogether.

The results show that the 3'-proximal promoter on the minus-strand template is preferentially utilized in most of the clones. This appears to be opposite to the preferential utilization of the 5'-proximal promoter by brome mosaic virus (4). The mechanism by which the Sindbis virus transcription complex gains access to the internal promoter is not known. However, the 3'-proximal effect can be explained if one or more components of the transcription complex for initiation of transcription show a preference for the 3' terminus of the minus strand. The polymerase is certainly such a component. The minus strand is also the substrate for full-length plus-strand synthesis, and there is necessarily a 3' to 5' movement of the polymerase along the minus-strand template during plus-strand synthesis. The polymerase might bind at the 3' terminus and scan 3' to 5' along the minus strand; the 3' promoter would be encountered first, and therefore it might be used preferentially. The observation that the TT20 clones, which have a large distance between the promoters, show the greatest 3'-proximal effect strongly supports this model. At this time we cannot exclude other models, e.g., that the 3'-proximal effect is due to different RNA secondary structures. The molecular mechanisms underlying the contextual effects are yet to be determined, nor is it known whether the effects are in fact

independent or interrelated. It is clear, however, that promoter utilization is not dependent simply on the combination of a particular promoter with particular transcription factors, as it also occurs in a rich context. It is likely that further characterization of the role of the context will provide important clues for understanding the regulation and mechanism of promoter recognition *in vivo*.

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