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A Method for Accurate Determination of Terminal Sequences of Viral Genomic RNA

Ziming Weng and Zhongguo Xiong[1]

Department of Plant Pathology, University of Arizona, Tucson, Arizona 85721

A combination of ligation-anchored PCR and anchored cDNA cloning techniques were used to clone the termini of the saguaro cactus virus (SCV) RNA genome. The terminal sequences of the viral genome were subsequently determined from the clones. The 5′ terminus was cloned by ligation-anchored PCR, whereas the 3′ terminus was obtained by a technique we term anchored cDNA cloning. In anchored cDNA cloning, an anchor oligonucleotide was prepared by phosphorylation at the 5′ end, followed by addition of a dideoxynucleotide at the 3′ end to block the free hydroxyl group. The 5′ end of the anchor was subsequently ligated to the 3′ end of SCV RNA. The anchor-ligated, chimerical viral RNA was then reverse-transcribed into cDNA using a primer complementary to the anchor. The cDNA containing the complete 3′-terminal sequence was converted into ds-cDNA, cloned, and sequenced. Two restriction sites, one within the viral sequence and one within the primer sequence, were used to facilitate cloning. The combination of these techniques proved to be an easy and accurate way to determine the terminal sequences of SCV RNA genome and should be applicable to any other RNA molecules with unknown terminal sequences.

Many animal viruses and >90% of all plant viruses have single-stranded RNA genomes.[1] However, accurate determination of the viral RNA terminal sequences is tedious and difficult. Traditional cDNA cloning techniques often generate clones missing the most terminal nucleotides. Several techniques have recently been developed to resolve the 5′- and the 3′-terminal nucleotides of viral RNA genomes. One such method determines the viral RNA 5′ terminus by direct runoff sequencing with reverse transcriptase.[2,3] A major problem with this method is that the sequencing product corresponding to the ultimate nucleotide at the 5′ terminus appears as a heavy band in an autoradiograph. This band masks a significant area of the sequencing gel, making it difficult to resolve the last few nucleotides.[4–7] Several modifications have improved the direct RNA sequencing technique,[8] but their results are inconsistent.

Another commonly used method to determine both the 5′- and 3′-terminal sequences is the addition of a homopolymer tail to viral RNA termini by poly(A) polymerase or to the end of the first strand cDNA using terminal deoxyribonucleotidyl transferase (TdT).[9] The terminal regions of the viral genome are then either cloned directly into vectors or amplified by PCR with appropriate primers.[10] However, subsequent sequencing of the dsDNA may not differentiate the beginning of the viral genome from the end of the homopolymer sequence.

A more difficult and complicated technique to identify the 3′-terminal sequences is the direct chemical sequencing of the viral RNA.[4,5,11–13] In this technique, a radioactively labeled pCp nucleotide is ligated to the 3′ terminus of the viral RNA using RNA ligase. The 3′-terminal nucleotide is identified by complete alkaline hydrolysis of the end-labeled RNA followed by two dimensional electro-chromatography.

Although these techniques can sometimes successfully determine the terminal sequences of the viral RNA genome, they are often unable to resolve the ultimate terminal residues. In this paper, we describe a combination of two techniques that can accurately identify the 5′ and the 3′ termini of an RNA viral genome. One technique is adopted from ligation-anchored PCR[14] to resolve 5′-terminal sequences. Another, termed anchored cDNA cloning, was developed to resolve 3′-terminal sequences. In the anchored cDNA cloning, an oligonucleotide anchor of defined sequence is ligated to the 3′ terminus of the viral RNA. Synthesis of the first strand cDNA is then primed with an oligonucleotide complementary to the anchor sequence. The dsDNA generated in subsequent steps is cloned and sequenced. We have used these techniques to accurately identify the terminal sequences of the saguaro cactus virus (SCV) RNA genome.

SCV is a member of the *Carmovirus* genus. It causes an asymptomatic systemic infection in the giant saguaro cactus (*Carnegiea gigantea*) in the southwestern United States and northern Mexico.[15] Virions of SCV are ~32 nm in diameter, consisting of a positive-sense single-stranded RNA genome ~4 kD in length and capsid protein subunits of ~38 kd in size.[15–17]

MATERIALS AND METHODS

Virus and RNA Purification

SCV was isolated from naturally occurring saguaro cacti in Saguaro National

[1]Corresponding author.
E-MAIL zxiong@ag.arizona.edu; FAX (520) 621-9290.

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Park, Tucson, Arizona, in the spring of 1993. The virus was maintained and propagated in *Chenopodium capitatum*. SCV was purified according to Nelson and Tremaine[17] and Xiong and Lommel.[15] *C. capitatum* tissue 10–14 days after inoculation was used for purification. The final purified virus was resuspended in a small volume of 0.1 M sodium acetate buffer, pH 5.0.

RNA was extracted from fresh virus preparations.[5] Proteinase K and SDS were added to give final concentrations of 1 mg/ml and 0.1%, respectively. After 10 min incubation at 37°C, virions were dissociated by 2 min incubation at 60°C in the presence of 1% SDS. Viral RNA was then purified by extraction with organic solvents and ethanol precipitation. Purified viral RNA was resuspended in a small volume of distilled, sterile water for the subsequent studies.

RNA Sequencing

Direct RNA sequencing of the 5′ terminus of SCV genome was carried out as described by Geliebter.[3] An oligonucleotide primer, SCV160 (5′-CCCGCACCAGGTCAACGG-3′), complementary to nucleotides 149–166 of SCV genome (Z. Weng and Z. Xiong, in prep.) was synthesized. A small amount of the primer (0.1 μg) was labeled in a 20 μl reaction containing 50 mM Tris-HCl (pH 7.4), 10 mM $MgCl_2$, 5 mM DTT, 0.1 mM spermidine, 100 μCi [γ-^{32}P]ATP, and 10 units of T4 DNA kinase. The reaction mix was incubated at 37°C for 30 min and then heated to 65°C for 5 min. Variable amounts of the viral RNA were mixed with 5 ng of the ^{32}P-labeled SCV160 primer. The mixture was then heated to 80°C for 3 min and allowed to anneal at 57°C for 45 min, a temperature of 5°C below the denaturation temperature (Td)[3] of the primer. RNA was sequenced using either avian myeloblastosis virus (AMV) reverse transcriptase (GIBCO-BRL, Bestheda, MD) or Moloney-murine leukemia virus (M-MLV) reverse transcriptase (GIBCO-BRL) as described by Geliebter et al.[3] TdT (GIBCO-BRL) was used to help eliminate pauses by reverse transcriptase.[8]

5′-Terminal Cloning

Clones representing the 5′ terminus of SCV genome were obtained by ligation-anchored PCR.[14] Three micrograms of SCV RNA was first reverse-transcribed into cDNA with 500 units of Superscript II RNase H$^-$ reverse transcriptase (GIBCO-BRL) using 1 μg of primer SCV750 (5′-CGAGGTAAGATAGGCCCC-3′) that is complementary to viral RNA nucleotides 755–772 (Z. Weng and Z. Xiong, in prep.). SCV RNA and primer SCV750 were mixed, denatured by heating to 70°C for 6 min, and then quickly chilled in ice water. Subsequent reverse transcription was carried out at 42°C for 1 hr in a 20-μl reaction containing 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 10 mM DTT, and 0.5 mM each of dATP, dCTP, dGTP, and dTTP. Residual RNA was removed by the addition of NaOH to a final concentration of 150 mM and boiling for 5 min. The denatured cDNA was neutralized with an equal molar amount of HCl and precipitated with the addition of 2 volumes of ethanol.

Two convenient, nearly complementary oligonucleotide primers F500 and R500 were chosen for the ligation-anchored PCR (Fig. 1A). These two primers were previously synthesized to clone cotton geminiviruses and contained degenerated nucleotides (A. Nadeem, M. Nelson, and Z. Xiong, in prep.). One nmole of the anchor oligonucleotide F500 (5′-AGCCTCTAGAACATTCCCATGTAYAGRAAGCC-3′) was first phosphorylated with 10 units of polynucleotide kinase (GIBCO-BRL) in the presence of 10 nmoles of ATP. The 3′ end of F500 was subsequently blocked by 10 units of TdT in the presence of 14 nmoles ddATP. Approximately 14 nmoles of the 5′-phosphorylated and the 3′-blocked anchor F500 was ligated to 1.2 μg of the first strand cDNA at 22°C for 18 hrs in a 10 μl reaction containing 50 mM Tris-HCl (pH 8.0), 10 mM $MgCl_2$, 10 μg/ml of BSA, 25% (wt/vol) of PEG 8000, 1 mM hexamine cobalt chloride, 20 μM ATP, and 5 units of T4 RNA ligase (GIBCO-BRL). The ligation reaction was terminated by adding 32 μl of ligase termination solution containing 0.5 M NaCl, 10 mM Tris-HCl (pH 8.0), and 1 mM EDTA. Two primers, SCV160 and R500 (5′-CTGTGAGCTCGGCTTYCTRTACATGGGCCTGT-3′), nearly complementary to the anchor F500 (Fig. 1A), were used to amplify the viral 5′-terminal fragment with 40 cycles of a touchdown PCR program. A schematic of this procedure is shown in Figure 2.

The following touchdown PCR condition was used to amplify the viral 5′ terminus. PCR was carried out in 25 μl of 10 mM Tris-HCl (pH 9.0), containing 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% gelatin, 0.1% Triton X-100, 0.25 mM dNTP, 1.25 units of a mixture of *Taq* DNA polymerase (Promega):*Pfu* DNA polymerase (Stratagene, La Jolla, CA) [20:1, (vol/vol)], 0.4 μM each of the two primers, and variable amount of anchored cDNA (2.5–25 ng). The mixture was overlaid with 25 μl of light mineral oil and incubated at 94°C for 3 min in a Temp-tronic thermocycler (Thermolyne, IL) prior to the touchdown cycles. The initial PCR cycle consisted of denaturation at 94°C for 45 sec, annealing at 60°C for 60 sec,

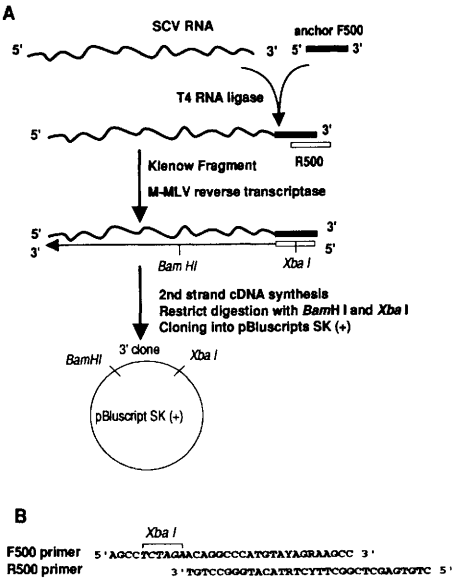

FIGURE 1 Schematic of anchored cDNA cloning strategy. (*A*) Alignment of the nearly complementary sequences of anchor F500 and primer R500. A restriction site *Xba*I (TCTAGA) engineered within the 5′ terminus of the primer R500 is italicized. Symbols representing degenerated nucleotides in the primers are Y = C, T; and R = G, A. Prior to reverse transcription reaction, Klenow fragment was used to fill the gap at the 3′ termini of the primers. (*B*) Anchored cloning strategy for cloning the 3′ terminus of SCV RNA. Anchor oligonucleotide F500 (solid bar), 5′ phosphorylated and 3′ blocked with ddATP, was ligated to the 3′ terminus of SCV RNA (wavy line) by T4 RNA ligase. The anchor-ligated RNA was reverse transcribed into cDNA (straight line) using primer R500 (hollow bar) complementary to the anchor. The cDNA was converted into ds-cDNA, which was subsequently digested with *Bam*HI in the viral sequence and *Xba*I in the primer sequence and cloned into pBluescript SK(+).

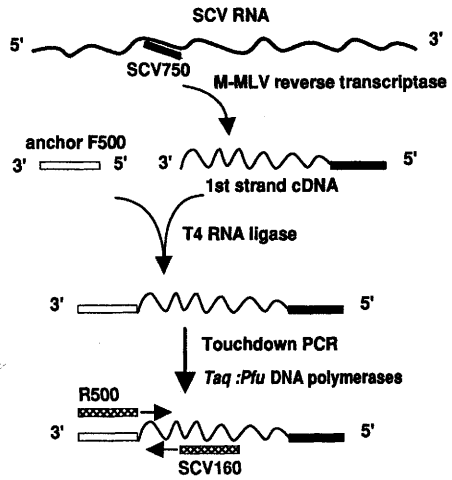

FIGURE 2 Schematic of ligation-anchored PCR strategy for cloning the 5′ terminus of SCV RNA. SCV RNA (heavy line) was reverse transcribed into cDNA (thin line) using primer SCV750 (solid bar) complementary to viral nucleotides 755–772. Anchor oligonucleotide F500 (hollow bar), 5′ phosphorylated and blocked with ddATP at the 3′ terminus, was ligated to the 3′ terminus of the cDNA. The 5′ end of the SCV genome was amplified by touchdown PCR using the anchor-ligated first-strand cDNA as template and two primers (crosshatched bars), R500 complementary to anchor F500 and SCV160 complementary to viral RNA at 149–166. The amplified ds-cDNA of the 5′ terminus was blunt-ended and then cloned into pBS(+) plasmid digested with *Sma*I.

and polymerization at 72°C for 30 sec. The annealing temperature was then lowered by 0.4°C per every cycle in the first 20 cycles and maintained at 52°C in the last 20 cycles. The PCR products were blunt-ended with the Klenow polymerase and deoxyribonucleotides, phosphorylated with kinase, and ligated into *Sma*I-digested and dephosphorylated pBS(+) plasmid (Stratagene, CA).

3′-Terminal Cloning

A new technique, designated as anchored cDNA cloning, was developed to clone the 3′ terminus of the SCV genome (Fig. 1A). The anchor primer, F500, was phosphorylated and blocked as described above. Approximately 14 pmoles of the pretreated anchor F500 was ligated directly to 3 μg of SCV RNA with T4 RNA ligase under the same reaction conditions described above. The anchor-ligated viral RNA was reverse transcribed into cDNA using primer R500, which contains a *Xba*I restriction site. Primers F500 and R500 (Fig. 1B) are not completely complementary. They were initially designed to clone geminivirus DNA A components (A. Nadeem, M. Nelson, and Z. Xiong, in prep.) and were used for convenience. Annealing of R500 to F500 created a gap between the 3′ termini of R500 and SCV RNA genome (Fig 1A). This gap was first filled-in with Klenow fragment. cDNA was then generated by reverse transcription with Superscript II RNase H^- reverse transcriptase. The second strand cDNA was synthesized using a combination of *Escherichia coli* RNase H, *E. coli* DNA polymerase I, *E. coli* DNA ligase, and T4 DNA polymerase.[5]

A convenient *Bam*HI restriction site ~1000 nucleotides upstream of the 3′ terminus of the SCV genome (Z. Weng and Z. Xiong, in prep.) and the *Xba*I site within primer R500 were used to clone the DNA fragment representing the viral 3′ terminus into pBluescript SK(+) (Stratagene, CA).

Nucleotide Sequencing

Clones representing both the 3′ and 5′ termini of the SCV genome were sequenced by the dideoxynucleotide chain termination method[18] using Sequenase version 2.0 (U.S. Biochemical, Cleveland, OH). The complete sequence of the 5′-terminal clones was resolved using T7 and T3 primers complementary to sequences adjacent to the multiple cloning sites in pBS(+). Nested clones for the 3′ terminal clones were generated using Exonuclease III digestion,[5] and subsequently sequenced with T3 and T7 primers.

RESULTS AND DISCUSSION

Cloning and Determination of the 5′-terminal Sequence

To determine the 5′-terminal sequence of the SCV RNA genome, we initially used direct RNA sequencing with reverse transcriptase (M-MLV-RT or AMV-RT) and primer SCV160.[3] When the sequencing products were electrophoresed on a 6% denaturing polyacrylamide gel and autoradiographed, a heavy band of radioactivity resulting from the termination of sequencing products at the ultimate nucleotide at the viral RNA 5′ terminus appeared. This band blocked resolution of the last few nucleotides in the autoradiograph (Fig. 3). Further attempts using TdT extension[8] and variable amounts of SCV RNA template (5, 1, and 0.5 μg) in the sequencing reaction failed to completely resolve the 5′ terminus by RNA sequencing (data not shown).

The largest cDNA clone mapped to the 5′-terminal region of SCV RNA genome was also sequenced using the same primer. This cDNA clone was obtained using a conventional cDNA cloning technique (Z. Weng and Z. Xiong, in prep.). Sequences derived from the cDNA clone and directly from the viral RNA were identical. The only difference was that the 5′ terminus of the cDNA clone was shorter than the 5′ terminus of the genomic RNA by several nucleotides (data not shown). It was obvious that the conventionally generated clone did not contain the last few nucleotides at the 5′ terminus of SCV RNA.

Ligation-anchored PCR[14] was then used to resolve the 5′-terminal sequence.

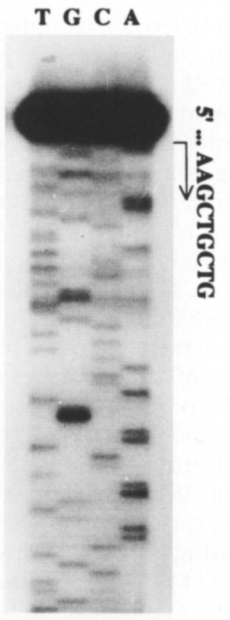

FIGURE 3 Reverse transcription runoff sequencing of the 5′ terminus of SCV RNA. Primer SCV160, complementary to viral nucleotides 149–166, was end-labeled with [γ-^{32}P]ATP and used for sequencing. The 5′ terminus was sequenced by the dideoxynucleotide chain termination reaction using M-MLV reverse transcriptase. Sequencing products were resolved on a 6% polyacrylamide gel containing 6 M urea. Notice that the heavy radioactive band at the top of the gel overshadows other bands.

In the initial attempt, the first-strand cDNA containing the SCV RNA 5′ terminus was synthesized using reverse transcriptase primed with SCV160. This product was subsequently ligated to the anchor oligonucleotide F500. The ligated cDNA was amplified by 40 cycles of touchdown PCR using SCV160 and R500 primers. When the PCR products were resolved on a 2% agarose gel, a smear of DNA rather than a discrete DNA band was observed (data not shown). Altering the amount of first-strand cDNA template in the PCR reaction did not improve the results (data not shown). Nonspecific binding of SCV160 primer to the viral RNA during reverse transcription and the subsequent use of the same primer in the PCR might have contributed to the DNA smear.

To circumvent this problem, primer SCV750, complementary to SCV nucleotides 755–772, was used to prime first strand cDNA synthesis. This cDNA was then ligated to anchor primer F500 and amplified with SCV160 and R500 primers. A discrete PCR product of ~200 bp was observed as expected (Fig. 4). The amount of cDNA template had a significant effect on the quality and quantity of the PCR product. The best result was obtained by using the least amount (2.5 ng) of the first-strand cDNA template.

The PCR amplified DNA fragment was purified from agarose gels with GeneClean (ISC, BioExpress, UT) and cloned into pBS (+) plasmid at the *Sma*I site. Twelve independent recombinant plasmids containing the PCR product in both orientations were sequenced. The sequence of a representative clone at the junction between SCV 5′ terminus and anchor oligonucleotide is presented in Figure 5. Because a primer with a defined sequence was used in the PCR cloning, the beginning of the viral sequence can be easily identified. The first nucleotide adjacent to the known sequence of the anchor primer was assigned as the first nucleotide at the 5′ terminus of the SCV RNA genome. The first 10 nucleotides of SCV RNA 5′ terminus thus determined, 5′-GGGTAAGCTG...-3′, are identical to the first 10 nucleotides in the genome of carnation mottle virus, the type member of the *Carmovirus* genus. The terminal nucleotide sequence obtained from ligation-anchored PCR clones was identical to that determined by direct RNA sequencing and included the additional 4 nucleotides that were not resolved by direct RNA sequencing (Figs. 3 and 5).

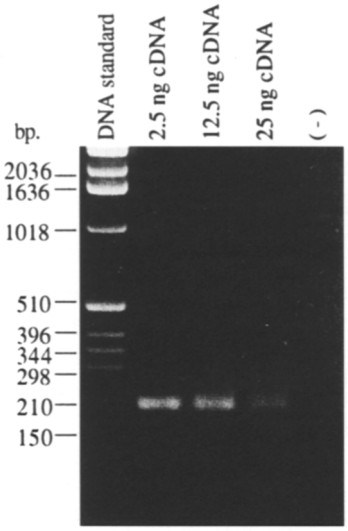

FIGURE 4 DNA fragments representing the 5′ terminus of SCV RNA amplified by ligation-anchored PCR. Anchor-ligated cDNA template was amplified by 40 cycles of touchdown PCR with primer R500 complementary to the anchor primer F500, and primer SCV160 complementary to SCV nucleotides 149–166. A mixture of *Taq* DNA polymerase and *Pfu* DNA polymerase [20:1, (vol/vol)] was used in the PCR reaction. 2.5, 12.5, and 25 ng of anchor-ligated cDNA template were used in a 25-μl PCR reaction. The negative control (–) contained no added cDNA template. Ten microliters of PCR-amplified products were electrophoresed in a 2% agarose gel. The molecular weight of 1 kb DNA standard (BRL, Bethesda, MD) is labeled to the *left*.

Of the 12 PCR clones from which the 5′-terminal sequence of SCV was determined, eight clones were identical. In two of the remaining clones, the 5′-terminal residue was an A instead of a G residue. In another clone, the second G residue was changed to an A. The first four nucleotides in the other clone were absent, possibly owing to premature termination of the reverse transcription reaction. The discrepancy in the sequences is unlikely to be PCR-generated errors, as transitions between A and G residues are also common in SCV genome (Z. Weng and Z. Xiong, in prep.).

3′-Terminal Sequence Determination

To clone the 3′ terminus, SCV RNA was directly ligated to the oligonucleotide anchor F500. The anchor-ligated RNA was reverse transcribed into cDNA using primer R500 which is nearly complementary to anchor F500. When R500 was annealed to F500, a gap of ten nucleotides between the 3′ terminus of the R500 primer and the 3′ terminus of the SCV genomic RNA is formed (Fig. 1B). This gap was first filled with Klenow polymerase and deoxyribonucleotides. cDNA was subsequently synthesized by reverse transcription, followed by the second strand synthesis.[5] Restriction digestion with *Xba*I in the primer and *Bam*HI in the viral sequence (Z. Weng and Z. Xiong, in prep.) (Fig. 1A) generated a DNA fragment of ~1000 bp. This DNA fragment containing the SCV 3′ terminus and the anchor was cloned into pBluescript SK(+). Three independent clones were sequenced from both directions to determine the 3′ sequence of SCV RNA genome. A typical sequencing gel showing the junction between

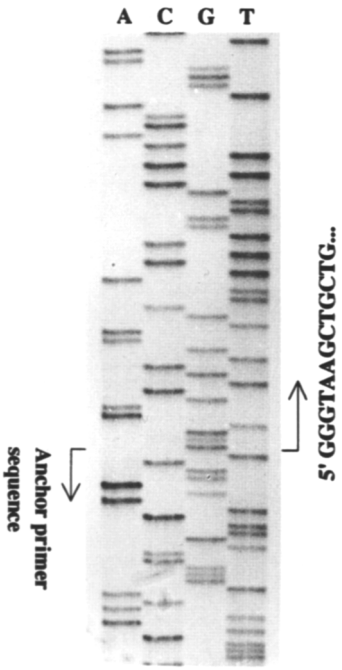

FIGURE 5 The 5′-terminal sequence of SCV RNA and the adjacent anchor primer sequence. The 200-bp inserts generated by ligation-anchored PCR were cloned and sequenced from both directions using T7 and T3 primers. A representative sequencing gel containing the junction between the viral RNA sequence and the primer sequence is shown. The 5′-terminal sequence of SCV RNA genome and the 3′ end of the anchor primer F500 are labeled. The first nucleotide above the anchor sequence was determined as the first nucleotide of the 5′ terminus of SCV genome.

the 3′ viral RNA sequence and the primer sequence is shown in Figure 6. As with the 5′-terminal sequence, the first nucleotide adjacent to the known primer sequence is defined as the last nucleotide at the 3′ terminus of the SCV RNA genome. Thus, the SCV RNA 3′-terminal sequence is 5′- ...TCTCCCGCCC-3′.

Of the three clones sequenced, two had identical terminal sequences. The remaining clone had a one nucleotide variation. Nucleotide 11 from the 3′ terminus of this clone was a T residue instead of a C residue in the other two clones. C → T transition in other parts of SCV genome is also common (Z. Weng and Z. Xiong, unpubl.).

Because the purpose of using Klenow DNA polymerase prior to reverse transcription is to fill the gap between R500 primer and the viral sequence, using a primer that is completely complementary to the anchor would eliminate the need for a DNA polymerase reaction before reverse transcription. The two primers, R500 and F500, were used in this study only because they were already available in the laboratory.

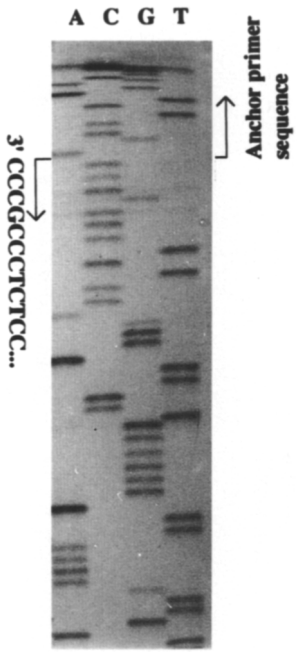

FIGURE 6 The 3′-terminal sequence of SCV RNA and the adjacent anchor primer sequence. Clones containing the 3′ terminus of SCV RNA genome were obtained by the anchored cDNA cloning technique. Three independent clones representing the 3′ terminus of SCV genome were sequenced. A typical sequencing gel containing the junction between the 3′ terminus sequence of SCV RNA and the 5′ terminus of the anchor primer F500 is shown here. The viral 3′-terminal sequence and the anchor sequence are labeled. The last nucleotide below the anchor sequence was determined as the last nucleotide of the 3′ terminus of SCV genome.

Two Techniques are Interchangeable for Determining the Sequences of Both Termini

Both the ligation-anchored PCR and anchored cDNA cloning strategies are similar and interchangeable for determining the 5′- and 3′-terminal sequences of RNA viral genomes as well as other RNA molecules with undetermined termini. Ligation-anchored PCR could be used to determine the 3′ terminus sequence if the anchor is first ligated directly to the 3′ terminus of the RNA followed by cDNA synthesis using a primer complementary to the anchor. The cDNA can then be amplified by PCR using two primers, one primer corresponding to sequences near the 3′ terminus of the RNA molecule and the other complementary to the anchor. The PCR products can then be cloned and sequenced. Similarly, anchored cDNA cloning can be used to determine the nucleotides at the 5′ terminus. An anchor can be ligated to the 3′ terminus of the first-strand cDNA. Double-stranded (ds)-cDNA can then be synthesized using normal cloning methods with an oligonucleotide complementary to the anchor primer for second strand synthesis. The ds-cDNA can be cloned with two restriction enzymes, one enzyme cleaving in the anchor primer and the other in the RNA sequence near the 5′ terminus.

The anchored cDNA cloning technique avoids PCR amplification, which is believed to have a higher misincorporation rate. Thus, one may not need to sequence many clones to ensure the correct terminal sequences. However, a convenient restriction site in the sequence near the terminus is required for cloning. On the other hand, ligation-anchored PCR can generates numerous clones for easy screening and sequencing. Sequence variations among PCR-generated clones were observed in this study, but the amount of variations is comparable to that among independent clones of SCV RNA genome generated by traditional cDNA cloning (Z. Weng and Z. Xiong, in prep.).

Like most plant RNA viruses, SCV consists of a single-stranded RNA genome. These two techniques, therefore, can be widely applied to determine the terminal sequences of these RNA viruses. These techniques should also be applicable to cellular RNAs. Although most mRNAs have 3′-terminal poly(A) tails, pre-mRNA is heterogeneous and does not contain a universal 3′-terminal sequence. These techniques could be easily adapted to determine the terminal sequences of the pre-mRNA.

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