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A One-Tube Method of Reverse Transcription-PCR To Efficiently Amplify a 3-Kilobase Region from the RNA Polymerase Gene to the Poly(A) Tail of Small Round-Structured Viruses (Norwalk-Like Viruses)

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Amplification of a 3-kb genome region from the RNA polymerase gene to the 3′ poly(A) tail of small round-structured virus (SRSV) by reverse transcription-PCR (RT-PCR) has been difficult to achieve because of a stable secondary structure in a region between the RNA polymerase gene and the 5′ end of the second open reading frame. We have developed a one-tube RT-PCR method to efficiently amplify this region. The method comprises three procedures: purification of poly(A)$^+$ RNA from a starting RNA solution by oligo(dT)$_{30}$ covalently linked to latex particles, buffer exchange, and continuous RT and PCR in a single tube containing all reaction components. The key elements of this method are (i) first-strand cDNA synthesis with the Superscript II version of RNase H$^-$ Moloney murine leukemia virus reverse transcriptase at 50°C for 10 min by using the RNA-oligo(dT)$_{30}$ hybrid on the latex particles as the template and primer, and (ii) PCR by *Taq* and *Pwo* DNA polymerases mixed together with a mixture of 12 phased oligo(dT)$_{25}$ antisense primers. The detection threshold of the one-tube RT-PCR method was as little as 0.2 ng of the crude RNA used as the source of the template. Using this method, we obtained 3-kb products from 24 SRSV strains previously characterized into four genetic groups. These included 5 P1-A, 4 P1-B, 5 P2-A, and 10 P2-B strains. Because SRSVs have not yet been cultivated in vitro, this novel method should facilitate molecular characterization of SRSVs to provide a firm scientific foundation for improvements and refinements of SRSV diagnostics.

Small round-structured viruses (SRSVs), also called Norwalk-like viruses, are members of the family *Caliciviridae* which have a single-stranded RNA genome with positive polarity, are 7.6 to 7.7 kb in length, and have a poly(A) tail at the 3′ end (13, 17, 22, 24, 39). The genome contains three open reading frames (ORFs) and two small untranslated regions at the 5′ and 3′ termini. The largest ORF, ORF1, encodes a polyprotein precursor for nonstructural proteins that includes a putative RNA polymerase gene located toward the carboxyl terminus (17, 24, 29). The second ORF, ORF2, encodes the viral capsid protein, and the smallest ORF, ORF3, encodes a protein of undefined function (8, 15, 18, 21, 25–27). The viruses of this group have not yet been cultivated in vitro, no practical animal model has been developed to study them, and virus concentration in stool samples is usually very low.

SRSVs are the major cause of outbreaks of nonbacterial acute gastroenteritis and appear to be a common cause of sporadic episodes of gastroenteritis in children and adults (23, 35, 42). The genetic or pathogenetic relationships between the strains that cause outbreaks and those that simultaneously circulate in the same communities have not been studied, in part due to the lack of suitable methods to screen for SRSVs in large field studies. The reverse transcription-PCR (RT-PCR), currently used in many laboratories to detect and characterize SRSVs, is based on the nucleotide sequence of the RNA polymerase region that has the highest degree of nucleotide sequence conservation. While the RNA polymerase region may be suited to broadly detect SRSV strains, the ORF2 encoding the capsid protein is of particular interest because its sequence and expression provide clues to genetic and antigenic relationships among SRSVs that would be important for the development of improved diagnostic methods. As more information on the sequence from the RNA polymerase region has become available, many researchers have directed their efforts toward amplification of a region extending from the RNA polymerase gene to the poly(A) tail. The RT-PCR products obtained from the RNA polymerase region are small (100 to 500 bp) (3, 11, 12, 14, 20, 31, 33, 44), whereas the distance from this region to the poly(A) tail is about 3 kb and includes two stable secondary structures predicted in locations upstream from ORFs 2 and 3 (22). The RT-PCR protocols routinely used to amplify the RNA polymerase region have usually been inefficient for amplification of this region. To date, RT-PCR amplification of the 3-kb region of SRSVs has been reported for only three strains. Of these, Southampton virus was amplified by a protocol that required 0.5 g of a stool sample (24), an amount corresponding to 70 to 1,000 times more than that used for short products from the RNA polymerase region (3, 12, 14, 20). For the remaining two strains, Lordsdale and Mexico viruses, details of the amplification protocols have not been reported (13, 19).

We have systematically examined the RT-PCR conditions required to overcome the previous difficulties and have developed a one-tube RT-PCR method that permits routine amplification of the 3-kb region of genetically distinct SRSV strains present in low concentrations in stool samples. The availability of this novel RT-PCR method should facilitate molecular characterization of SRSVs. It may also be applicable with other single-stranded RNA viruses in which amplification of a long

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TABLE 1. SRSV strains used in this study

Strain code	Strain identification[a]	Sense primer(s) used
S1	P1-A/11523/93/La[b]	SR48
S2	P1-A/2036/92/US[c]	SR48 + SR50 + SR52
S3	P1-A/11873/94/Md	CL6 or SR48
S4	P1-A/8438/94/Ar	CL10
S5	P1-A/8548/94/Fl	CL14 or SR48
S6	P1-B/11551/93/Fl[d]	CL18
S7	P1-B/11821/93/Md[d]	CL18
S8	P1-B/11860/94/Pa	CL18
S9	P1-B/11641/94/Hi	CL18
S10	P2-A/2555/93/Pa	CL22
S11	P2-A/11885/94/La	CL22
S12	P2-A/8377/94/Al	CL22
S13	P2-A/8387/94/Md	CL22
S14	P2-A/11756/95/Vt	CL22
S15	P2-B/4880/86/US[c]	SR46
S16	P2-B/12700/92/UK[c]	SR46
S17	P2-B/12678/94/Md	CL26
S18	P2-B/12044/94/Va	CL26
S19	P2-B/8410/94/Hi	CL26
S20	P2-B/12630/94/US	CL26
S21	P2-B/12732/94/La	CL28
S22	P2-B/12275/94/Vt	CL28
S23	P2-B/11941/95/Fl	CL26
S24	P2-B/12096/95/Ak	CL26

[a] Probe group/strain designation/year/state or country of detection.
[b] The strain is identical to LA-Oyst-G1 described in the multistate oyster-associated outbreak (2).
[c] From Ando et al. (3).
[d] From Ando et al. (2).

region of the genome has been difficult because of its stable secondary structure or the small amount of the template RNA available.

MATERIALS AND METHODS

Virus samples. The 24 strains of SRSVs from outbreaks of acute gastroenteritis used in this study had previously been characterized into four genetic groups: P1-A, P1-B, P2-A, and P2-B (3). These strains are identified by probe group, specimen designation, year, and state or country of detection and were designated strains S1 to S24 for convenience in this study (Table 1). Strain S1 was used to optimize the one-tube RT-PCR conditions described below.

RNA. RNA was extracted from 100 μl of a 10% stool suspension as described previously (3), concentrated by ethanol precipitation, and suspended in 20 μl of H_2O. For strain S1, a 10% stool suspension was prepared from 1.7 g of a single stool sample and was kept at 4°C, and RNA was extracted several times in separate groups of 10 to 30 tubes during the development of the method. After each extraction, the RNA was pooled from the tubes, the RNA concentration was determined spectrophotometrically, and aliquots (2 to 10 μl/tube) were made for use in a single-tube per experiment. In the typical experiments performed to optimize the reaction conditions and determine the detection threshold of the method as described below (see Fig. 1 to 3), the RNA concentration was broadly estimated to be 10 ng per μl by spectrophotometry (optical densities at 260, 280, and 320 nm, 0.406, 0.326, and 0.177, respectively), but the concentration of the poly(A)$^+$ RNA was not determined. The RNA extracts from the 24 SRSV strains were kept at −70°C and used in the RT-PCR within 3 months.

RT-PCR primers. Nineteen primers were used in this study (Table 2). The oligo$(dT)_{30}$-Latex primer (Oligo-dT30 Super; Takahara Co. Ltd., Otsu, Japan) is an oligo$(dT)_{30}$ covalently linked at or near its 5′ end to the carboxyl residues on the surface of the latex particles. The VN primer is a mixture of 12 combinations of 40-mer oligonucleotides [linker-$(dT)_{25}$-VN, where V may be dG, dA, or dC and N may be any one of the four deoxynucleotides] in which the 12 components were synthesized and purified separately and then mixed to make a solution with an equal concentration of each component. The TT primer is a single 40-mer primer with the same sequence as the VN primer, except that two 3′ nucleotides, V and N, are substituted by two dTs. All but oligo$(dT)_{30}$-Latex primers were synthesized in the Biotechnology Core Facility of the Centers for Disease Control and Prevention (Atlanta, Ga.).

RT-PCR. Five microliters of the RNA solution was mixed with an equal volume of 10% (wt/vol) oligo$(dT)_{30}$-Latex suspension in a 0.5-ml microtube, heated at 90°C for 1 min, and rapidly cooled in ice-water. After 3 min in ice-water and the subsequent addition of 1.1 μl of 5 M NaCl (final 0.5 M), the tube was incubated at 37°C for 20 min and was then centrifuged at about 16,000 × *g* for 3 min at room temperature. The latex particles containing the RNA-oligo$(dT)_{30}$ hybrid were suspended in a total of 1 ml of washing buffer (10 mM Tris-HCl [pH 7.6 at room temperature], 0.5 M NaCl, and 1.0% [vol/vol] Triton X-100), transferred to a 1.5-ml microtube at room temperature, and centrifuged as described above. After being placed on ice, the latex particles were suspended in 1 ml of ice-cooled RT-PCR buffer (50 mM Tris-HCl [pH 8.7 at room temperature], 40 mM KCl, 2.5 mM $MgCl_2$, and 0.5% [vol/vol] Triton X-100), transferred to a fresh 1.5-ml tube, mildly pipetted on ice, and centrifuged at room temperature as described above. The latex particles were then suspended (on ice) in 400 μl of the ice-cooled RT-PCR reaction mixture (50 mM Tris-HCl [pH 8.7 at room temperature], 40 mM KCl, 2.5 mM $MgCl_2$, 0.5 mM [each] dATP, dGTP, dCTP, and dTTP [deoxynucleoside triphosphates; dNTPs], 2 mM [without accounting for carryover from the enzyme storage buffers] dithiothreitol, 0.5% [vol/vol] Triton X-100, 2% [vol/vol] [without accounting for the carryover] glycerol, 0.4 units of human placental RNase inhibitor [Boehringer Mannheim Corp.] per μl, 0.25 μM

TABLE 2. RT-PCR primers

No.	Primer	Length	Sequence (5′ to 3′)[a]	Polarity[b]	Location[c]
1	Oligo$(dT)_{30}$-Latex	30	Latex particle-$(t)_{30}$	−	
2	VN primer	40	gct gga gtc tag a $(t)_{25}$ (a/g/c)(a/g/c/t)	−	
3	TT primer	40	gct gga gtc tag a $(t)_{25}$ tt	−	
4	Mon189	25	gca cca att atg gct tgg gcc att a	−	6972
5	SR33	21	tgt cac gat ctc atc atc acc	−	4888
6	SR46	21	tgg aat tcc atc gcc cac tgg	+	4766
7	SR48	21	gtg aac agc ata aat cac tgg	+	4766
8	SR50	21	gtg aac agt ata aac cac tgg	+	4766
9	SR52	21	gtg aac agt ata aac cat tgg	+	4766
10	CL6	23	aac agc ata aat cac tgg **CTG AT**	+	4769
11	CL10	24	**ATC CTA ACT CTA TGT GCA TTG TCA**	+	4787
12	CL14	24	aac agc ata aat cac tgg **CTA AAT**	+	4769
13	CL18	24	gcc cac tgg **ATT CTA ACT TTG AGT**	+	4778
14	CL22	20	atc gcc cac tgg **TTG CTT AC**	+	4775
15	CL26	20	atc gcc cac tgg **CTC CTC AC**	+	4775
16	CL28	21	atc gcc cac tgg **CTT CTA ACT**	+	4775
17	SR34	20	tcg taa atg atg atg gcg tc	+	5352
18	SR62	23	tat cag $(^{t}_{c})$t$(^{a}_{c})$ aag cct gtg gga ac	+	6888
19	SR60	20	tga g$(^{c}_{t})$t c$(^{c}_{t})$g gtg gct tca tg	+	7293

[a] The sequences specific for individual strains are highlighted by the boldface capital letters. VN primer, SR60, and SR62 are degenerate primers, with nucleotide variations indicated in parentheses.
[b] −, negative; +, positive.
[c] Equivalent location of the 5′ end of the primer within the unique genomic sequence (7,654 bases) of Norwalk virus (GenBank accession number, M87661).

[each] positive-sense primer(s) and VN primer, 0.05 U of *Taq* DNA polymerase [Boeringer Mannheim Corp.] per μl, 6.25×10^{-3} U of *Pwo* DNA polymerase [Boehringer Mannheim Corp.] per μl, and 3 U of the Superscript II version of RNase H^- Moloney murine leukemia virus [MoMLV] reverse transcriptase [Life Technologies, Inc., Gaithersburg, Md.] per μl). The suspension was divided into five aliquots of 75 μl each in thin-walled tubes (MicroAmp Reaction Tube; Perkin-Elmer) and was immediately transferred to a thermal cycler (Gene Amp PCR System 9600; Perkin-Elmer) that was preheated to 50°C for a hot start (10). The thermocycling format used for RT-PCR was as follows: one cycle of RT at 50°C for 10 min, followed by denaturation at 94°C for 2 min; 40 amplification cycles with denaturation at 94°C for 15 s, annealing at 50°C for 1 min, and extension at 72°C for 3 min; and a final incubation at 72°C for 7 min, followed by cooling at 4°C. The amplification products were separated from the latex particles by centrifugation at about $16,000 \times g$ for 3 min, and 10 μl was analyzed by electrophoresis on a 0.8% (wt/vol) agarose gel, followed by staining with 0.5 μg of ethidium bromide (EtBr) per ml for 1 h. Water was used as the negative control that was treated by the same procedures described above.

In typical experiments for optimization of the reaction conditions, the RNA-oligo$(dT)_{30}$ hybrid derived from 10 μl of the starting RNA solution was suspended in 1 ml of RT-PCR buffer, dispensed into 20 to 40 tubes on ice, and then centrifuged and suspended in 75 μl of the RT-PCR mixtures which differed in their components. In the course of the optimization, when the concentrations of *Taq* and *Pwo* DNA polymerases had not yet been optimized, 0.075 U of the enzyme mixture (*Taq-Pwo*; at an unpublished ratio) from the Expand Long Template PCR system (Boehringer Mannheim Corp.) per μl was used in the experiments described below (Fig. 1A and Fig. 2A through D).

Southern hybridization. The 3-kb products of the RT-PCR were confirmed by Southern hybridization by using the six digoxigenin-labeled oligonucleotides comprising the four probe sets (P1-A, P1-B, P2-A, and P2-B) as described previously (3). For the P1-A strains, three probes (probes SR63d, SR65d, and SR69d) comprising the P1-A probe set were used together for strains S2 to S5, while the single probe SR65d was used for strain S1.

Analysis of single-stranded cDNA. The RT-PCR products showing aberrant migration patterns were analyzed for susceptibility to digestion by using mung bean (MB) nuclease (Life Technologies, Inc.) and for the possibility of making a transition to the 3-kb duplex form by reassociation treatments after denaturation. The amount of the RT-PCR product, pooled from 20 tubes and ethanol precipitated, was adjusted to approximately 3 μg per 128 μl of a test solution (34 mM KCl and 2 mM $MgCl_2$) and was subjected to two continuous cycles of denaturation-quenching treatments: the first cycle, heating at 95°C for 10 min, followed by immediate quenching in ice-water (rapid cooling); and the second cycle, heating at 95°C for 10 min, followed by gradual quenching from 80 through 40°C over a period of 13 h (slow cooling). Before and after each step of denaturation, five aliquots of 8 μl of product were collected from the test solution. Reaction with 0, 0.01, 0.1, 1, or 10 units of MB nuclease was conducted in 10 μl of solution containing 100 mM sodium acetate (pH 5.0), 1 mM zinc acetate, 500 mM NaCl, 10 mM L-cysteine, and 50% (vol/vol) glycerol at 37°C for 30 min. The reaction products were analyzed by agarose gel electrophoresis and Southern hybridization as described above.

Detection threshold. The RNA-oligo$(dT)_{30}$ hybrid, derived from 10 μl of the starting RNA and suspended in 1 ml of the RT-PCR buffer, was subjected to a 10-fold serial dilution (500 μl/dilution) by using the RT-PCR buffer as the diluent. After centrifugation, the precipitate from each dilution was suspended in 185 μl of the reaction mixture, dispensed into duplicate thin-walled tubes (75 μl/tube), and subjected to the RT-PCR, followed by agarose gel electrophoresis and Southern hybridization as described above. The reaction mixture without the RNA-oligo$(dT)_{30}$ hybrid was used as the negative control for RT-PCR and Southern hybridization.

RESULTS

Difficulties in initial attempts. Our initial attempts to develop a method for the amplification of a 3-kb region from the RNA polymerase gene to the 3′ poly(A) tail were based on reaction conditions similar to those used to amplify a small region in the RNA polymerase gene (3, 4). In these procedures, avian myeloblastosis virus (AMV) reverse transcriptase was used for the first-strand cDNA synthesis and the temperature for the extension step in PCR was 60°C. We sometimes could obtain the 3-kb product by using these reaction conditions. However, the amount of the product was very small (Fig. 1A, lanes 1 and 8), and the results were often hardly reproducible. The difficulties in amplification of the 3-kb region were not ascribed to an inability of AMV reverse transcriptase to transcribe a long region, nor were they due to a significant reduction in activity of *Taq* and *Pwo* DNA polymerases when they were used at 60°C instead of the commonly recommended 72°C (34), because RT-PCR yielded a large amount of internal products, including a 2.4-kb product spanning a region from the poly(A) tail to the 5′ end of ORF2 (Fig. 1A, lanes 2, 3, 9, and 10). We found that our difficulties were consistently linked to the production of cDNA fragments with an unexpectedly small size (Fig. 1A, left, lanes 1 and 4 to 8). These small products were not a result of primer oligomerization or contamination of nontarget nucleic acid because they specifically hybridized with the probe derived from the RNA polymerase region (Fig. 1A, right, lanes 1 and 4 to 8). The truncated RT-PCR products could result from either a mispriming of the VN primer due to its hybridization with an adenosine-rich site(s) on the template or a deletion of sequence due to incorrect polymerization across the bases of an intrastrand hairpin-like secondary structure (7, 38).

Effect of primer composition. The difficulties that we experienced in our initial attempts were overcome by the procedures described in Materials and Methods (Fig. 1B, lane 1). We found the use of the RNA-oligo$(dT)_{30}$ hybrid on the latex particles in the RT step to be extremely important, as demonstrated by complete elimination of the 3- and 2.4-kb products when the RNA-oligo$(dT)_{30}$ hybrid was denatured immediately before RT (Fig. 1B, lanes 6 to 9). Similarly, the importance of using the VN primer in the PCR step was clearly demonstrated by the absence of the 3-kb product when the VN primer was substituted by the TT primer (Fig. 1B, lane 2). However, substitution of the VN primer by the TT primer did not result in a substantial reduction in the yield of the 2.4-kb product (Fig. 1B, lanes 3 and 4). The differences between the results obtained with the VN primer and the TT primer suggest a strong secondary structure in a region between the 2.4- and 3-kb nucleotides from the poly(A) tail, a region between the RNA polymerase gene and the 5′ end of ORF2.

In addition, the 3-kb product was consistently accompanied by a faintly staining product, corresponding to 2.5 kb, that migrated relatively faster (Fig. 1B, left, lane 1). Unexpectedly, the hybridization signal of the faint product was comparable in intensity to that of the 3-kb product (Fig. 1B, right, lane 1). We also found that the substitution of the VN primer by the TT primer resulted in production of a cDNA fragment with the same migration pattern that could be detected only by Southern hybridization (Fig. 1B, right, lane 2). The product at the 2.5-kb position was digested by the MB nuclease (0.1, 1, and 10 U/μl) and was converted to a MB nuclease-resistant 3-kb duplex form by slow cooling after denaturation (data not shown). The results from this analysis suggest that the product at the 2.5-kb position represents the two complementary 3-kb single-stranded DNAs (ssDNAs), the accumulation of which is a result of self-annealing of each strand of the 3-kb cDNA by rapid cooling between denaturation and primer annealing in the PCR step. Of note, optimum RT-PCR conditions for the 3-kb product were quite different from those for the short products of the RNA polymerase region (3), since our method did not yield a 123-bp product visible by EtBr staining (Fig. 1B, lane 10). This fact explains why our new RT-PCR method resulted in the accumulation of the 3-kb ssDNA fragments rather than the truncated double-stranded DNA (dsDNA) that was a source of difficulties in our initial attempts. Besides the product at the 2.5-kb position, we sometimes noticed by Southern hybridization another product detectable at about the 5-kb position (e.g., Fig. 1B, right, lane 1). This product was also susceptible to MB nuclease digestion and shifted from the 5- to 3-kb position by slow cooling after denaturation (data not shown), suggesting that this product represents two dimers, each of which comprises two 3-kb fragments of ssDNA, with polarities that are the same within a dimer but complementary

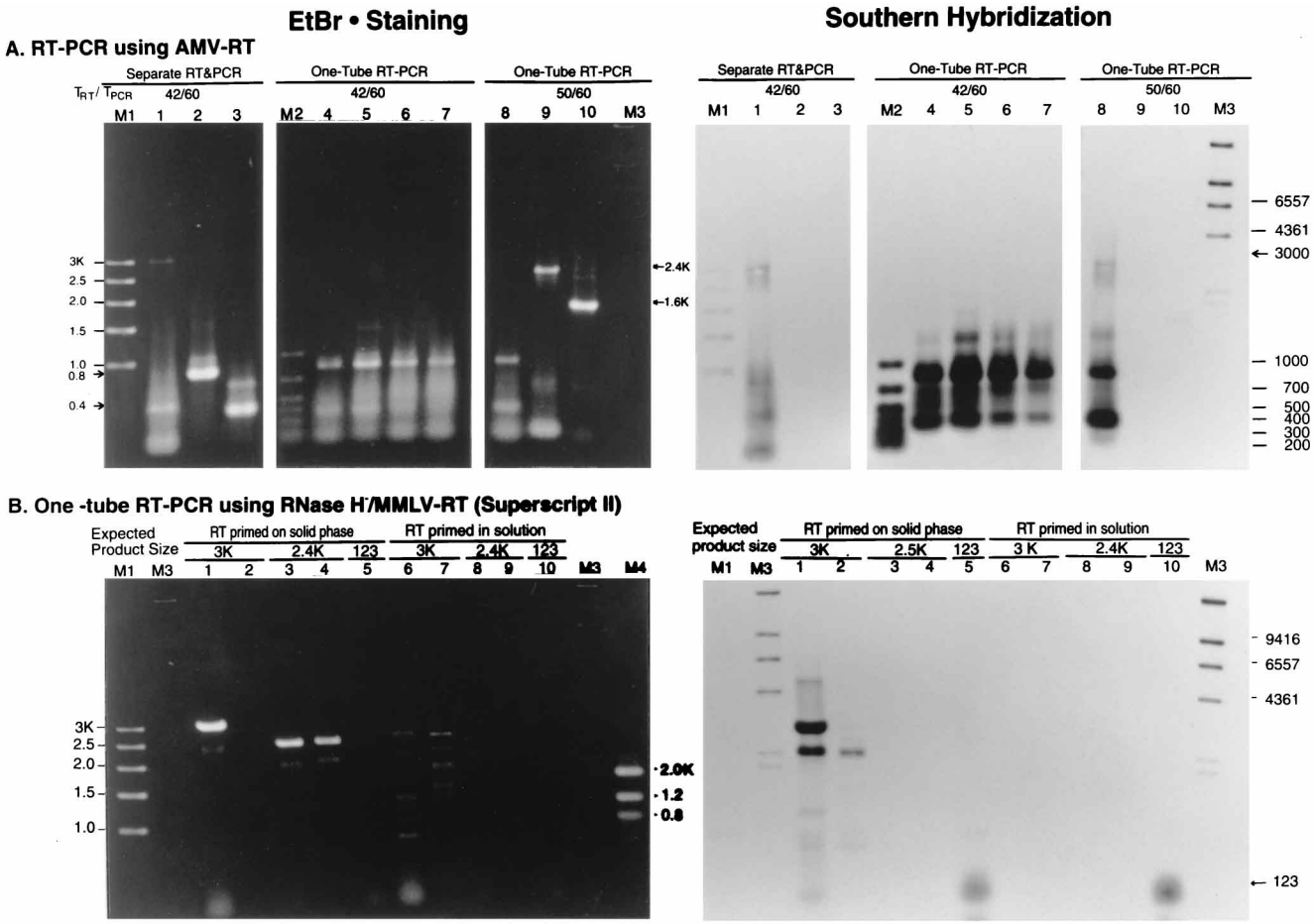

FIG. 1. Effect of primer composition. Poly(A)$^+$ RNA, purified with oligo(dT)$_{30}$-Latex, was used as the template. (A) Amplification products from RT-PCR in which AMV reverse transcriptase (Molecular Genetic Resources, Tampa, Fla) was used in the RT step. Lanes 1, 2, and 3, RT and PCR performed separately under the reaction conditions similar to those described previously (4), with the following modifications: for the primers used in the RT and PCR steps, 300 nM VN primer in combination with 300 nM (each) SR48, SR50, and SR52 (lane 1), SR62 (lane 2), or SR60 (lane 3); for the RT step, at 42°C for 1 h in 20 μl; and for the PCR step, 2 mM $MgCl_2$ and 0.2 mM (each) dNTP and 40 amplification cycles with denaturation at 94°C for 15 s, annealing at 42°C for 1 min 30 sec, and extension at 60°C for 5 min. Before and after the RT step, RNA was denatured at 90°C for 1 min in the presence of 5% (vol/vol) dimethyl sulfoxide and digested with 0.2 U of RNase H per μl at 37°C for 30 min, respectively. Lanes 4 to 7, one-tube RT-PCR performed under the reaction conditions similar to those reported recently (3), with the following modifications: for the primers, 310 nM (each) VN primer and SR48; for the concentration of $MgCl_2$, 1.75 mM (lane 4), 2.00 mM (lane 5), 2.25 mM (lane 6), or 2.50 mM (lane 7); for the DNA polymerase, 0.035 U of the *Taq-Pwo* enzyme mixture (Boehringer Mannheim) per μl; for the thermocyle format, one cycle of RT at 42°C for 45 min, followed by denaturation at 94°C for 2 min; 40 amplification cycles with denaturation at 94°C for 15 s, annealing at 42°C for 1 min 30 s, and extension at 60°C for 10 min; and a final incubation at 68°C for 7 min. Lanes 8 to 10, the conditions were similar to those for the one-tube RT-PCR, with the following modifications: for the primer combination, 310 nM (each) VN primer and SR48 (lane 8), VN primer and SR34 (lane 9), or Mon189 and SR34 (lane 10); for the RT step, at 50°C for 15 min and annealing in the PCR step at 50°C for 1 min and 30 s. The expected product sizes for lanes 1 and 4 to 8 were 3 kb, and those for lanes 2, 3, 9, and 10 were 0.8, 0.4, 2.4, and 1.6 kb, respectively. T_{RT}/T_{PCR}, temperatures of primer extension in the RT and PCR steps, respectively; 3K, 3-kb DNA. (B) One-tube RT-PCR with the Superscript II version of MoMLV reverse transcriptase in the RT step. Lanes 1 to 5, the components of the reaction mixture and the thermocycle conditions for RT-PCR are described in Materials and Methods, except for the primers used in lanes 2 to 5; lanes 6 to 10, products from a similar method with a modification in which the RNA-oligo(dT)$_{30}$ hybrid, formed on the surfaces of the latex particles, was denatured at 94°C for 3 min immediately before RT-PCR and the enzymes were added to the reaction mixture after denaturation. The primer pairs used were as follows: VN primer and SR48 (lanes 1 and 6), TT primer and SR48 (lanes 2 and 7), VN primer and SR34 (lanes 3 and 8), TT primer and SR34 (lanes 4 and 9), and SR33 and SR48 (lanes 5 and 10). (A and B) Lanes M1 to M4, DNA molecular markers, as follows: lane M1, DNA ladder XII (Boehringer Mannheim); lane M2, digoxigenin-labeled marker XI (Boehringer Mannheim); lane M3, digoxigenin-labeled marker II (Boehringer Mannheim); and lane M4, low-molecular-mass DNA ladder (Life Technologies). The amount of 3-kb dsDNA in lane M1 corresponds to 100 ng.

between the dimers. These two dimers might hybridize with each other at the region of the strong secondary structure, as predicted above.

Effects of other parameters. In addition to the primer components, we systematically examined the following parameters to optimize the reactions: pH of the RT-PCR mixture, concentrations of chemical and enzyme components (KCl, $MgCl_2$, dNTPs, primers, cosolvents [Triton X-100, Tween 20, Nonidet P-40, dimethyl sulfoxide, glycerol], MoMLV-reverse transcriptase, and DNA polymerases [*Taq*, *Pwo*]), and the thermocycling formats. Representative results from these experiments indicated that the optimum combination of $MgCl_2$ and dNTP concentrations depended on the primer extension temperature in the PCR step (Fig. 2A, left), a visible product was not obtained in the absence of nonionic detergents (Fig. 2B, left), and the optimum pH (based on 50 mM Tris-HCl at room temperature) and temperature in the RT step were 8.7 and 50°C, respectively (Fig. 2C, left). Similarly, MoMLV reverse transcriptase at a range of from 3.0 to 4.0 U/μl and the mixture of *Taq* and *Pwo* polymerases (unpublished ratio; from Expand

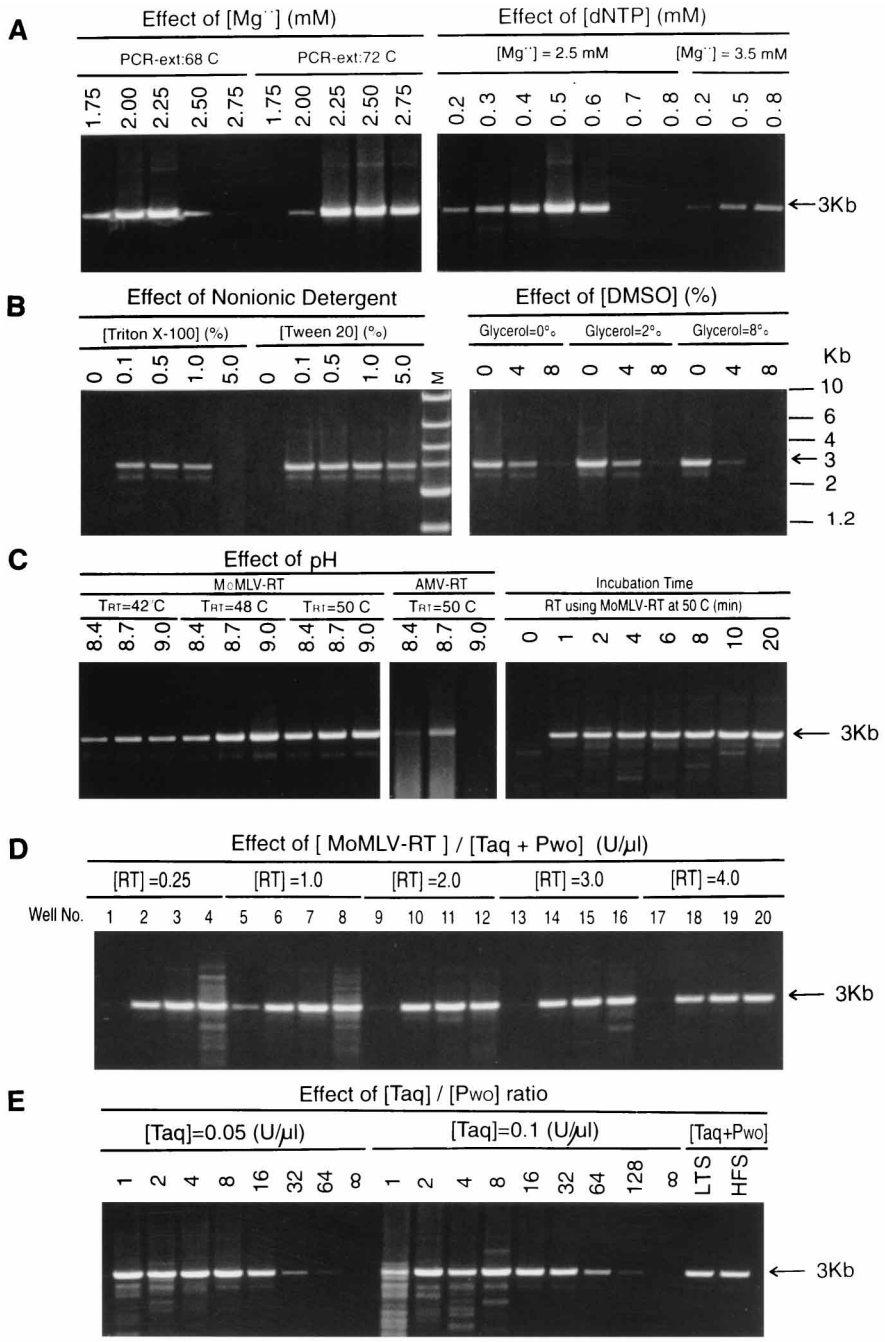

FIG. 2. Results of optimization of RT-PCR conditions. The reaction was run under the conditions described in Materials and Methods except for the parameters indicated. (A) Effect of concentrations of $MgCl_2$ and dNTPs. In the left gel, the $MgCl_2$ concentration was changed under the conditions in which each dNTP was present at 0.5 mM. Extension in the PCR step was performed at 68°C for 5 min or 72°C for 3 min. In the right gel, the dNTP concentration was changed under the conditions in which the $MgCl_2$ concentration was 2.5 or 3.5 mM and extension in the PCR step was performed at 72°C for 3 min. (B) Effect of nonionic detergents, dimethyl sulfoxide (DMSO), and glycerol. The effect of nonionic detergent was examined in the absence of dimethyl sulfoxide and glycerol (left gel), while the effects of dimethyl sulfoxide and glycerol were examined in the presence of 0.5% (vol/vol) Triton X-100 (right gel). M, a mixture of a high-molecular-mass DNA ladder and a low-molecular-mass DNA ladder (Life Technologies). The amount of 3-kb dsDNA marker corresponds to 60 ng. (C) Effect of pH of the reaction mixture and incubation temperature and time in the RT step. RT was carried out by using 3 U of the Superscript II version of MoMLV reverse transcriptase (left and right gels) per μl or 0.2 U of AMV reverse transcriptase (Molecular Genetic Resources, Tampa, Fla.) per μl (middle gel). (D) Effect of MoMLV reverse transcriptase/*Taq* plus *Pwo* ratio. [Taq+Pwo], concentration of an enzyme mixture (*Taq* and *Pwo* DNA polymerase, at an unpublished ratio) included in a commercially available PCR kit (Expand Long Template PCR System; Boehringer Mannheim): 0.025 U/μl, wells 1, 5, 9, 13, and 17; 0.05 U/μl, wells 2, 6, 10, 14, and 18; 0.075 U/μl, wells 3, 7, 11, 15, and 19; and 0.1 U/μl, wells 4, 8, 12, 16, and 20. (E) Effect of *Taq*/*Pwo* ratio. The *Taq*/*Pwo* ratio was changed from 1 to 64 or 128 by changing the concentration of the *Pwo* DNA polymerase under conditions in which the concentration of *Taq* DNA polymerase was 0.05 U/μl (left eight lanes) or 0.1 U/μl (middle nine lanes). ∞, absence of the *Pwo* DNA polymerase. LTS and HFS, 0.075-U/μl enzyme mixtures included in the Expand Long Template PCR System and Expand High Fidelity PCR System (Boehringer Mannheim), respectively.

Long Template PCR system) at a range of from 0.05 to 0.075 U/μl gave the best results among the combinations examined (Fig. 2D). Although MoMLV reverse transcriptase showed an inhibitory effect on *Taq* and *Pwo* DNA polymerases (e.g., Fig. 2D, lanes 6 and 7 versus lanes 18 and 19), it was much less prominent than that reported for AMV reverse transcriptase (36). Under the conditions in which MoMLV reverse transcriptase was 3 U/μl, neither *Taq* polymerase nor *Pwo* polymerase alone could produce a visible amount of the 3-kb cDNA fragment (Fig. 2E, lane ∞, for *Taq* polymerase; data not shown for *Pwo* polymerase). The optimum *Taq*/*Pwo* ratio determined in this study was 8 to 16, which may be comparable to those used in the enzyme mixtures (*Taq* and *Pwo*; at an unpublished ratio) included in the commercially available kits (Fig. 2E, right).

One striking finding of the optimized reaction conditions was the extremely fast production of the first-strand cDNA during the RT step: the 3-kb product became clearly visible by EtBr staining after a 1-min incubation at 50°C, reached steady state by 8 to 10 min, and did not increase with an incubation time of ≥20 min (Fig. 2C, right; data not shown for 30 min). The rapid production of the 3-kb cDNA fragments was solely ascribed to the activity of MoMLV reverse transcriptase (data not shown), not to the cooperative function of MoMLV reverse transcriptase and *Taq* polymerase, which might exhibit RT activity (37, 41).

Of note, the better results were obtained from the use of freshly prepared RNA (data not shown). In addition, the following procedures were of particular importance among the procedures described in Materials and Methods (data not shown): (i) prepare the RT-PCR mixture by adding *Taq* and *Pwo* DNA polymerases and MoMLV reverse transcriptase immediately before it was mixed with the RNA-oligo$(dT)_{30}$ hybrid, (ii) keep the reaction mixture at 0°C before and after mixing with the hybrid, and (iii) hot start the RT within 30 min after the addition of MoMLV reverse transcriptase to the reaction mixture.

Detection threshold. The detection threshold of this method was estimated to be 0.2 ng of the starting RNA, which resulted in a 3-kb product barely visible by EtBr staining but clearly detectable by Southern hybridization (Fig. 3). The 3-kb ssDNA, migrating faster than the double-stranded product, was consistently detected by Southern hybridization (Fig. 3B). The background ladder detected by Southern hybridization with 20 ng of the starting RNA may be an artifact associated with an excess amount of latex particles (250 μg/reaction) to which the template-primer-enzyme complex may stick (16).

Application of the new method. Amplification of the remaining 23 SRSV strains by using the optimized conditions resulted in 3-kb products clearly visible by EtBr staining (Fig. 4). This collection included 4 P1-A, 4 P1-B, 5 P2-A, and 10 P2-B strains. The specificity of the products was confirmed by Southern hybridization, which also detected the ssDNA product migrating at about 2.5 kb (data not shown). The negative control consisting of water did not show the specific product (data not shown).

A success rate of amplification of the 3-kb genome region of SRSVs was about 75% upon further application of this method to RNAs prepared from stool specimens from individuals involved in 43 outbreaks, including 24 outbreak specimens used in this study (data not shown). The unsuccessful amplification resulted from the RNAs which were kept at −70°C for 6 months to 3 years, suggesting degradation of RNA during the storage.

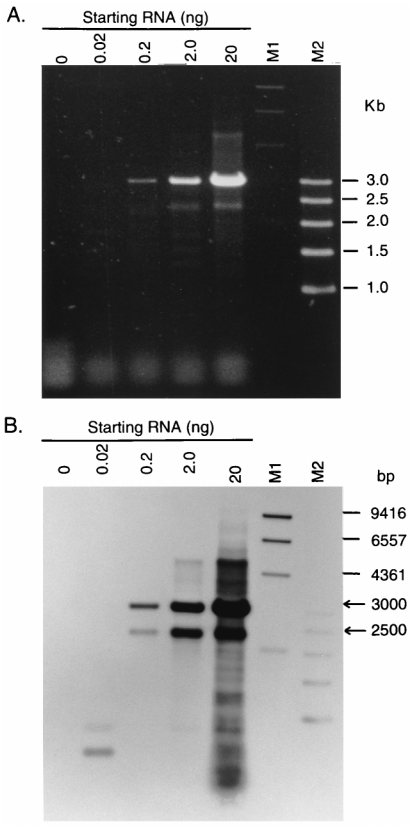

FIG. 3. Detection threshold of the one-tube RT-PCR method. The procedures are described in Materials and Methods. Lanes M1 and M2, digoxigenin-labeled DNA molecular marker II and DNA marker XII (Boehringer Mannheim), respectively. The amount of the 3-kb marker in lane M2 corresponds to 100 ng.

DISCUSSION

In the past 3 years, portions of the RNA polymerase gene from many strains of SRSV have been sequenced on the basis of the RT-PCR products derived from this region (3, 4, 11, 15, 19, 33, 40, 44). However, efforts to use these sequences for positive-sense primers to amplify a 3-kb region from the RNA polymerase gene to the poly(A) tail have largely been unsuccessful, delaying progress in the genetic and antigenic characterization of SRSVs. The difficulties might have been associated with the secondary structure within the target sequence, the use of oligo(dT) primers with low priming efficiency (6, 28), the relatively large size of the amplification target requiring highly processive polymerization, and low concentration of the highly purified template RNA available from stool samples. The results of the present study consistently suggested that the secondary structure is the most problematic among the factors making amplification of the 3-kb RT-PCR product difficult. We have developed a method which overcomes these difficulties and which allows for the efficient and reproducible amplification of the 3-kb region from stool samples containing a diverse set of SRSV strains.

The precise mechanisms underlying the high efficiencies of RT and PCR have not been analyzed in this study. In effect, the efficiency of RT in this method could follow from an increased efficiency of RT initiation, a high rate of strand extension, and, probably, a high processivity (number of bases incorporated during a single round of primer extension) of RNase H^- MoMLV reverse transcriptase. Our particularly important

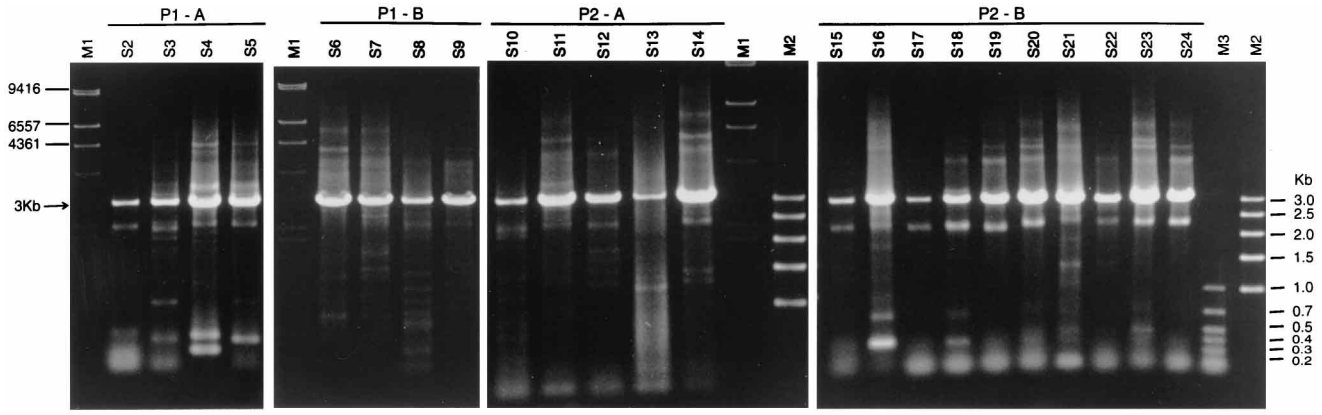

FIG. 4. Results of application of the one-tube RT-PCR method to SRSV strains representing four probe groups. The positive-sense primers used for the individual strains are indicated in Table 1. Lanes M1 to M3, DNA molecular markers, as follows: lane M1, digoxigenin-labeled marker II (Boehringer Mannheim); lane M2, DNA ladder XII (Boehringer Mannheim); and lane M3, digoxigenin-labeled marker X1 (Boehringer Mannheim). The amount of the 3-kb dsDNA marker in M2 corresponds to 100 ng.

finding demonstrated the need for conducting RT on the solid surfaces of latex particles rather than in solution, and this step most likely resulted in the formation of a stable enzyme-template-primer complex on the surfaces of the latex particles. The mechanism of this phenomenon may be comparable to that reported for the initiation of avian retrovirus reverse transcriptase, for which an enzyme-template-tRNA primer complex is required to be maintained in a specific orientation, albeit in a solution and not on the solid phase (1). Our additional finding that a relatively large amount of the first-strand cDNA needed for the 3-kb amplicon was produced with 1 min of incubation at 50°C indicates that the chain elongation rate of RT by this method is at least 50 nucleotides per second, a rate about 10-fold higher than that reported for MoMLV reverse transcriptase (43). This extremely high transcription rate may be associated with the increased temperature of 50°C that would result in the stimulation of transcription activity of the enzyme and partial disruption of the secondary structure. It is also possible that this high transcription rate is an intrinsic characteristic of the Superscript II version of RNase H^- MoMLV reverse transcriptase (43). The temperature and the incubation time established for the RT step in this study are comparable to those used in a one-tube RT-PCR with AMV reverse transcriptase reported for human immunodeficiency virus type 1 mRNA (30). However, the reaction conditions of pH 8.7, a temperature of 50°C, and an incubation time of 10 min optimized for MoMLV reverse transcriptase in our method are quite different from those recommended by the company supplying this enzyme (32). In our study, MoMLV reverse transcriptase molecules were not sequestered away from the transcript immediately upon their first dissociation. Therefore, we could not determine the processivity of this enzyme. Nevertheless, the finding of the extremely fast production of a relatively long product described above suggests that the processivity of this enzyme must be very high under these conditions for RT. Taken together, the amount of product in the RT step of this method might reach a level that could not have been achieved by the RT procedures of the RT-PCR methods routinely used to amplify the RNA polymerase region of SRSVs.

In the PCR step of this method, the high efficiency of amplification for the 3-kb region may be mainly associated with the negative-sense primers and the two DNA polymerases. Our results indicated the importance of using a mixture of 12 combinations of phased oligo$(dT)_{25}$ primers (VN primer) as compared with the commonly used oligo$(dT)_{27}$ primer (TT primer). These results confirm the importance of the two 3′ bases, VN, in VN primers for the efficient initiation of primer extension from the 3′ poly(A) tail, as reported by other researchers (6, 28). Similarly, our results indicated that the combined use of *Taq* and *Pwo* DNA polymerases is crucial for amplification of the 3-kb region. These results suggest that a reciprocal action of *Taq* polymerase with high processivity and *Pwo* polymerase with proofreading activity (3′ to 5′ exonuclease activity) is required to effectively transcribe this target region with a stable secondary structure, as has been reported for amplification of long targets from bacteriophage and human genomic DNAs (5, 9). Of note, our finding that the optimum combination of $MgCl_2$ and dNTP concentrations depends on the primer extension temperature is an additional element that allowed us to optimize the thermocycle conditions for amplification of the 3-kb product.

Using this new method, we could amplify the 3-kb region from the 24 SRSV strains representing four probe groups (3). The amount of the RT-PCR products was high for all 24 strains examined and sufficient for future studies of cloning, sequencing, and expression of the capsid protein and the protein of unknown function encoded by ORF3. While SRSVs have not yet been cultivated in vitro, this novel method has a great advantage over other RT-PCR methods in that its use precludes the possibility of mistaking the sequences derived from different strains for a single origin. Such mistakes may happen in a dual or mixed infection of SRSVs (2, 40), when two different genome regions (e.g., the RNA polymerase and the viral capsid regions) are amplified by separate RT-PCR assays. In addition, the amount and purity of the starting RNA required in this method are comparable to, or less than, those required in the RT-PCR methods currently used for the diagnosis of SRSV infections (3, 12, 14, 20). Moreover, the use of the universal VN primer precludes the necessity to newly design the negative-sense primers from the sequences in ORF3 or the 3′ untranslated region that may be more diverse than those in the RNA polymerase region (8, 13, 19, 24). In summary, the availability of this novel method should facilitate the molecular characterization of SRSVs, especially in genetic and antigenic relationships, to provide a firm scientific foundation for improvements and refinements of SRSV diagnostics. It is possible that this method may be useful in the study of not only other single-stranded RNA viruses but also eukaryotic mRNAs

(28), in which amplification of a long genome region has been difficult because of the secondary structure or the small amount of the template RNA available.

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