Since January 2020 Elsevier has created a COVID-19 resource centre with free information in English and Mandarin on the novel coronavirus COVID-19. The COVID-19 resource centre is hosted on Elsevier Connect, the company's public news and information website.

ANALYTICAL BIOCHEMISTRY **252,** 62–70 (1997)
ARTICLE NO. AB972307

Effective Amplification of 20-kb DNA by Reverse Transcription PCR

Volker Thiel,*,[1] Ayoub Rashtchian,† Jens Herold,* David M. Schuster,† Nin Guan,† and Stuart G. Siddell*

**Institute of Virology, University of Würzburg, 97078 Würzburg, Germany; and †Life Technologies Inc., 9800 Medical Center Drive, Rockville, Maryland 20850*

Received May 12, 1997

Polymerase chain reaction has been applied to the amplification of long DNA fragments from a variety of sources, including genomic, mitochondrial, and viral DNAs. However, polymerase chain reaction amplification from cDNA templates produced by reverse transcription has generally been restricted to products of less than 10 kilobases. In this paper, we report a system to effectively amplify fragments up to 20 kilobases from human coronavirus 229E genomic RNA. We demonstrate that the integrity of the RNA template and the prevention of false priming events during reverse transcription are the critical parameters to achieve the synthesis of long cDNAs. The optimization of the polymerase chain reaction conditions enabled us to improve the specificity and yield of product but they were not definitive. Finally, we have shown that the same reverse transcription polymerase chain reaction technology can be used for the amplification of extended regions of the dystrophin mRNA, a cellular RNA of relatively low abundance.

Polymerase chain reaction (PCR)[2] and related techniques have become an almost indispensable tool in biological research and medicine. Consequently, there is a constant effort to improve the sensitivity and specificity of the procedure and to extend the application of PCR and related techniques to an increasing range of problems. For example, PCR technology has been adapted to the amplification of long DNA templates and to DNA templates of a more complex nature, e.g., human genomic DNA and mitochondrial DNA (1–5). PCR, combined with reverse transcription (RT), is also a suitable method for the amplification of DNA from RNA templates. However the application of RT-PCR to the generation of long DNA products remains problematic. Thus, only a few reports describe RT-PCR protocols that enable the amplification of DNAs longer than about 7 kilobases (kb) (6–10). Most probably the integrity and purity of RNA templates and the reverse transcription reaction are the critical parameters prior to the amplification of the cDNA.

In this paper we report experiments that describe the parameters critical in producing RT-PCR products of up to 20 kb. We focused on the requirements of the RNA template, the reverse transcription reaction, and the amplification of the cDNA by PCR. To carry out these studies, we used the genomic RNA of the human coronavirus HCV 229E as template (11). The HCV 229E genomic RNA has an approximate length of 27,000 nucleotides (nt) and the homogeneity of the RNA can be readily visualized by hybridization analysis (12). Finally, we have shown that in addition to viral RNAs, which are relatively abundant in the infected cell, cellular mRNAs, such as the dystrophin mRNA, also represent suitable templates for the generation of long DNA products by RT-PCR.

MATERIALS AND METHODS

Preparation of Polyadenylated RNA

Viral RNA. MRC-5 cells were infected with HCV 229E (12) at an m.o.i. of 10 and incubated at 33°C for 18 h. Polyadenylated RNA (poly(A)-RNA) was then isolated using two different methods. In the first method, the total cytoplasmic RNA was prepared by NP-40 lysis, phenol extraction, and ethanol precipitation and poly(A)-RNA was isolated by chromatography on

[1] To whom correspondence should be addressed at Institute of Virology, University of Würzburg, Versbacher Strasse 7, 97078 Würzburg, Germany. Fax: +49 931 201 3934. E-mail: v.thiel@rzbox.uni-wuerzburg.de.

[2] Abbreviations used: PCR, polymerase chain reaction; RT, reverse transcription; PBS, phosphate-buffered saline; DTT, dithiothreitol; m.o.i., multiplicity of infection.

0003-2697/97 $25.00

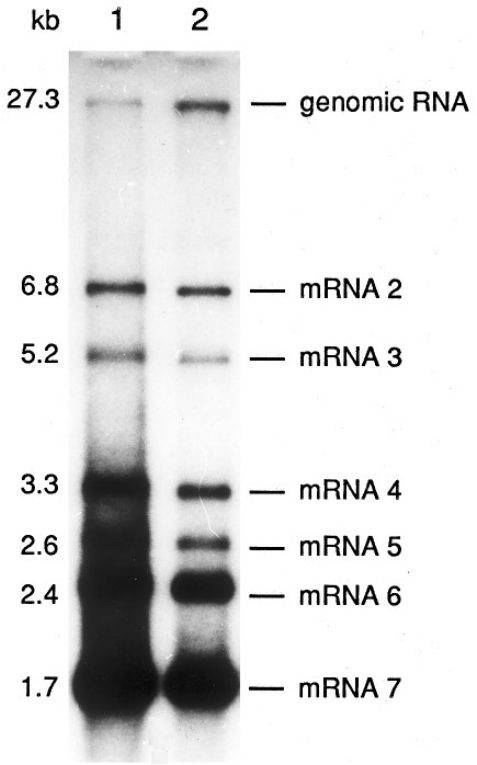

FIG. 1. Hybridization analysis of HCV 229E specific RNAs. Poly(A)-RNA was isolated from HCV 229E-infected cells by affinity chromatography using poly(U)-Sepharose (lane 1) or oligo(dT)$_{25}$ magnetic beads (lane 2) and 0.5 μg RNA was separated by electrophoresis in a formaldehyde 1% agarose gel. The virus RNAs were hybridized with the HCV 229E-specific, 5′-end-labeled oligonucleotide 55 and visualized by autoradiography.

poly(U)-Sepharose (13). In the second method, the cells were washed twice with ice-cold PBS and then scraped and pelleted in 10 ml ice-cold PBS. The cell pellet was resuspended in 1.5 ml lysis buffer (10 mM Tris–HCl, pH 7.5, 140 mM NaCl, 5 mM KCl, 1% NP-40) and incubated for 30 s on ice. The cell lysate was centrifuged at 1500*g* for 1 min and the supernatant was incubated for 5 min at 23°C with 5 mg oligo(dT)$_{25}$ Dynabeads (Dynal) resuspended in 1.5 ml of 2× binding buffer (20 mM Tris–HCl, pH 7.5, 1 M LiCl, 2 mM EDTA, 1% SDS). The oligo(dT)$_{25}$ magnetic beads were washed twice with wash buffer (10 mM Tris–HCl, pH 7.5, 150 mM LiCl, 1 mM EDTA) and the bound poly(A)-RNA was eluted in 2 mM EDTA, pH 8.0, for 2 min at 65°C.

Cellular RNA. Poly(A)-RNA from human heart was a gift from Dr. Chris Gruber and had been isolated using TRIzol reagent (Life Technologies) and oligo(dT)-cellulose chromatography according to the manufacturer's instruction.

Analysis of Viral RNAs

Poly(A)-RNA from HCV 229E-infected MRC-5 cells was electrophoresed on 2.2 M formaldehyde–1% agarose gels. The gels were dried and hybridized with ^{32}P-(5′-end)-labeled oligonucleotide 55, (Table 1, Fig. 2) as described (14).

Reverse Transcription

Fifty to 500 ng of poly(A)-RNA from HCV 229E-infected MRC-5 cells were used for reverse transcription in a volume of 20 μl with 1 μl (200 U) SuperScript II reverse transcriptase (Life Technologies), 4 μl of 5× first-strand buffer (Life Technologies), 2 μl of 10 mM dNTPs, 2 μl of 0.1 M DTT, 0.5 μl of RNasin (50 U/μl) (Pharmacia), and 30–100 ng of oligonucleotide primer (Table 1, Fig. 2). The reactions were incubated for 90 min at 42°C and then heated for 2 min at 94°C and chilled on ice.

First-strand cDNA synthesis with poly(A)-RNA from human heart was done using the SuperScript Preamplification System (Life Technologies), oligo(dT) primer, and 1 μg human heart poly(A)-RNA essentially according to the manufacturer's instructions. The cDNA synthesis reaction was incubated at 45°C for 45 min followed by heat inactivation of the reverse transcriptase at 70°C for 10 min and removal of the mRNA template by incubation with 2U RNase H for 20 min at 37°C (10).

Polymerase Chain Reaction

PCR amplifications were done in thin-wall PCR tubes (Perkin–Elmer) using a Perkin–Elmer GeneAmp PCR System 9600. Aliquots of 0.2–3 μl reverse transcription reaction were used as PCR templates. PCRs were done in a total volume of 50 μl with 1 unit Elongase Enzyme Mix (Life Technologies) and final concentrations of 60 mM Tris–SO_4 (pH 9.1), 18 mM $(NH_4)_2SO_4$, 2 mM $MgSO_4$, 0.2 mM dNTPs, and 0.2–0.4 μM oligonucleotide primer (Table 1, Fig. 2 and 7A). Unless indicated otherwise, the PCR cycles were 1 min, 94°C, followed by 30 cycles of 20 s denaturation at 94°C, 30 s annealing at 50°C, and elongation for 1 min/kb expected product length at 72°C. During the last 18 cycles, the elongation time was increased by 30 s in each successive cycle. The reaction was terminated by a 10-min elongation at 72°C.

RESULTS

Previous studies have shown that the RNAse H-deficient reverse transcriptase, SuperScript II, is capable of copying RNAs of at least 7.5 kb (15). However, there has been no systematic study of the maximum length of cDNA synthesis that can be achieved with this enzyme. The availability of HCV 229E genomic RNA, combined with long PCR technology, now makes it possible to study in more detail the capabili-

TABLE 1

Description of HCV 229E and Dystrophin-Specific Oligonucleotides

Oligonucleotide	Sequence (5′ to 3′)[a]	Position[b]	Orientation[c]	Application
159	cgatcgcggccgctggccgaataggccATGGCCTGCAACCGTGTGACACT	293–315	+	PCR
147	AAACCAGTCTGCTCATCA	3860–3877	+	PCR
127	cgatcgcggccgctggccgaataggccatgGCTGATTACCGTTGCGCTTGT	9071–9091	+	PCR
16	CTGTCGCTGGCATTCCATC	8040–8058	+	PCR
89	TCATGGTGTATTTAGTAAGAT	12830–12850	−	PCR
85	ACACACGGTGTATGTCCTCATT	12979–13000	−	RT
11	gagaggatccGCAAAACAAACATTTTATTTAGTTGAGAC	20554–20582	−	PCR
32	TATAGGCATTGCGCAACCACCGG	21747–21769	−	RT
55	AGAAACTTCATCACGCACTGG	26802–26822	−	HYB[d]
421U	TCATGCCCTGAACAATGTCAACAA	421–444	+	PCR
2784U	CCCCATCAGAGCCAACAGCAAT	2784–2805	+	PCR
10358L	GGGGATGCTTCGCAAAATACCT	10358–10379	−	PCR
13207L	TCAGGAACACCCCAAAACCAAAGT	13207–13230	−	PCR

[a] The nucleotides corresponding to HCV 229E or dystrophin sequences are shown in capitals. The nucleotides shown in small case were added for cloning purposes.
[b] The position refers to the nucleotide sequence of HCV 229E genomic RNA or dystrophin mRNA.
[c] Oligonucleotides with mRNA orientation are designated as +.
[d] HYB, hybridization.

ties of SuperScript II and the parameters that are critical for its effective use.

The RNA Template

HCV 229E genomic RNA has two major advantages for the studies reported here. First, as a viral RNA, it is relatively abundant in the infected cell. Second, coronaviruses are positive-strand RNA viruses, and the genomic RNA has a 3′ polyadenylate tract that can be used for affinity chromatography (13). Human epithelium MRC-5 cells were infected with HCV 229E, and the poly(A)-RNA was isolated by poly(U)-Sepharose chromatography or chromatography with homopolymeric oligonucleotide $(dT)_{25}$ coupled to magnetic beads. The poly(A)-RNAs were separated by gel electrophoresis and the viral mRNAs were visualized by hybridization analysis using the HCV 229E-specific oligonucleotide 55 (Fig. 1). In both RNA preparations it is possible to identify the genomic RNA (27.3 kb) and the six subgenomic mRNAs (1.7–6.8 kb) that are characteristic of coronavirus infection. The hybridization analysis indicates that the material isolated with oligo$(dT)_{25}$ magnetic beads (lane 2) is less degraded than material isolated by poly(U)-Sepharose (lane 1).

In order to generate HCV 229E-specific cDNAs, we did reverse transcriptions with HCV 229E-specific oligonucleotides and the RNA templates described above. To amplify DNA products from the HCV 229E cDNAs, we inserted aliquots of the RT reaction, without further purification, into the PCR. In a first series of experiments, we carried out the RT reaction using the HCV 229E-specific oligonucleotide 85 (Table 1, Fig. 2) as the RT primer. Then we tried to amplify, by PCR, DNA fragments that extended 4.8 kb (PCR primers 16 and 89) or 9 kb (PCR primers 147 and 89) upstream of the RT primer binding site. Up to a distance of 4.8 kb from the RT priming site, we were able to obtain the expected PCR products, regardless of the poly(A)-RNA preparation that we used as template for the RT reaction (Fig. 3, lanes 1 and 3). However, when we tried to synthesize the 9.0-kb PCR product, we only succeeded when we did the RT reaction with the poly(A)-RNA template prepared by the Dynabeads method (Fig. 3, lanes 2 and 4). Increasing the amount of poly(U)-Sepharose-purified poly(A)-RNA up to 1 μg in the RT reaction did not lead to the synthesis of the 9-kb PCR product. This result indicates that the quality of the RNA preparation, i.e., the integrity of the template rather than its abundance, is a critical factor when longer RT-PCR products are desired.

The RT Reaction

Adjusting the amount of RT-primer. First, we established an RT-PCR protocol that enabled us to generate a DNA product with a size of 12.6 kb. As RNA template we used the poly(A)-RNA that was prepared from HCV 229E-infected cells using oligo$(dT)_{25}$ magnetic beads. We primed the reverse transcription with the oligonucleotide 85 and 3 μl of the RT reaction then served as template for a subsequent PCR reaction with the primers 159 and 89. As is shown in Fig. 4A (lane 3), this protocol resulted in a PCR product with the expected size of 12.6 kb and an additional smaller product of 3.9 kb. Restriction enzyme analysis revealed that

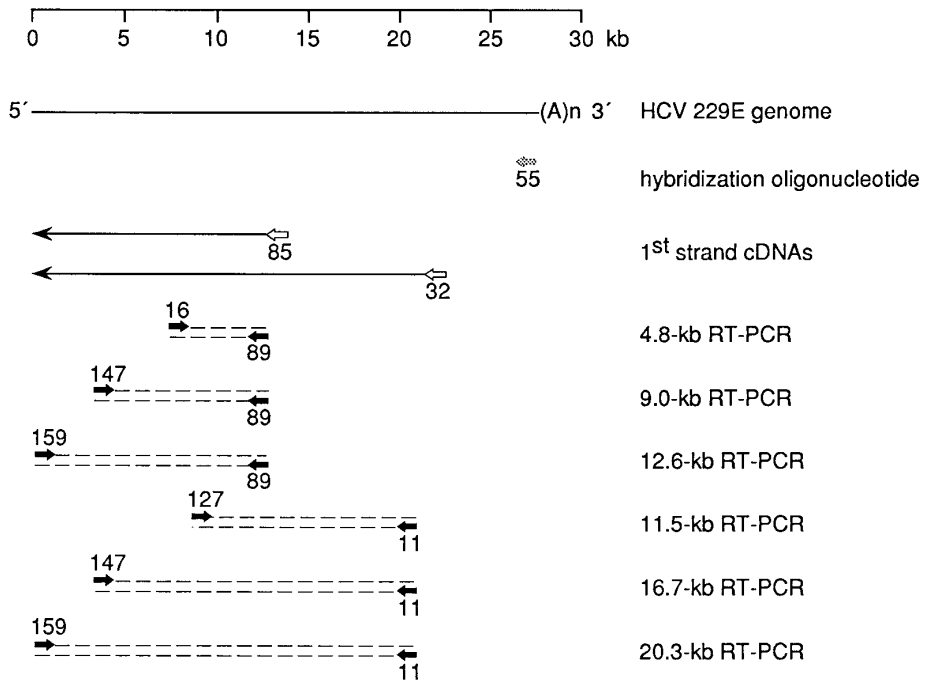

FIG. 2. Schematic representation of HCV 229E-specific oligonucleotides and RT-PCR products. The oligonucleotides are indicated as arrows according to their orientation and position relative to the HCV 229E genomic RNA. The hybridization oligonucleotide 55 is drawn in grey, the RT primers in white, and the PCR primers in black. The expected sizes of the RT-PCR products are indicated.

the smaller product was amplified from a region of the HCV 229E genome, ranging from approximately nucleotide (nt) 300 to nt 4200. Since the reverse transcription reaction was performed at 42°C, we reasoned that this product could result from the amplification of a cDNA generated during the RT reaction by a "less stringent" priming event. Indeed, analysis of the HCV 229E genomic RNA sequence revealed a stretch of 6 nucleotides (nt 4205–4210) that match exactly to the 3′ end of primer 85 (Fig. 4B). Thus, a priming event at this position, during the RT reaction, would result in the synthesis of a cDNA with the primer sequence at its 5′ end. Since we inserted aliquots of the reverse transcription reaction directly into the PCR, we also introduce significant amounts of the primer 85, and it is likely that during the PCR, the 3′ truncated cDNA was amplified with the upstream primer 159 and the reverse transcription primer 85.

To test this hypothesis, and to try to circumvent such problems, we performed two further experiments. First we changed the temperature of the reverse transcription reaction from 42 to 45°C in order to minimize the occurrence of "less stringent" priming events. However, when we used this approach, we only obtained small amounts of the PCR product with the expected size of 12.6 kb without diminishing the amount of the 3.9-kb PCR product (data not shown). The second strategy was to reduce the primer concentration in the reverse transcription reaction, in order to reduce the amount of RT primer carryover into the subsequent PCR. The result of this experiment is shown in Fig. 4A (lanes 1 and 2). If we reduced the RT primer concentration from 0.75 to 0.5 μM, the amount of the 3.9-kb PCR product decreases significantly without affecting the yield of the 12.6-kb product (lane 2). If we reduced the RT primer concentration to 0.25 μM, only small amounts of the 3.9-kb product were synthesized, again without affecting the yield of the 12.6-kb product (lane 1). We also tried to reduce the RT primer concentration to 0.15 μM but, in this case, the yield of the 12.6-kb product decreased significantly (data not shown).

The PCR Reaction

Adjusting the amount of PCR template. Our next goal was to establish a RT-PCR protocol that enabled us to amplify cDNAs longer than 12.6 kb. On the basis of the results described above, we looked for an RT primer that would fulfill two criteria. First, in order to synthesize cDNAs over 12.6 kb in length, the priming site should be located at least 20 kb downstream from the 5′ end of the HCV 229E genomic RNA. Second, this primer has to be highly specific, in order to minimize the occurrence of "less stringent" priming events during the reverse transcription reaction. Therefore, we chose RT-primer 32 at a concentration of 0.25 μM. The

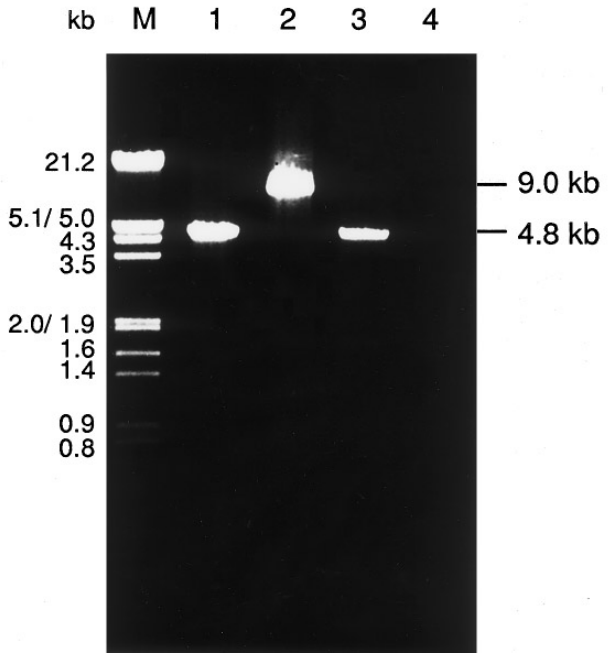

FIG. 3. RT-PCR products using different preparations of HCV 229E poly(A)-RNA as RT template. Poly(A)-RNA prepared with oligo$(dT)_{25}$ magnetic beads (lanes 1 and 2) or poly(U)-Sepharose chromatography (lanes 3 and 4) were used as template for reverse transcription with oligonucleotide 85. 3-μl aliquots of the RT reaction were then used for PCR reactions with the oligonucleotides 16 and 89 (lane 1 and 3) or oligonucleotides 147 and 89 (lanes 2 and 4). 5-μl aliquots of the PCR reactions were separated on a 0.6% agarose gel. Lane M shows 400 ng of *Hin*dIII–*Eco*RI-digested λDNA.

subsequent PCR was done with 2 μl of the RT reaction as template and oligonucleotides 127 and 11 as upstream and downstream PCR primers, respectively (Table 1, Fig. 2). As is shown in Fig. 5, it was possible to amplify a DNA fragment with the expected size of 11.5 kb (lane 1) using this procedure. However, when we did the PCR with primers 147 and 11 (Table 1, Fig. 2), we obtained not only the desired product with the expected size of 16.7 kb but also a second product with a size of approximately 4.9 kb (lane 2). Again, by restriction enzyme analysis, we identified this 4.9-kb fragment as an amplification product of the HCV 229E genomic RNA. Our interpretation of this result was as described above, so again we tried to lower the RT primer concentration in the reverse transcription reaction. However, in this case, we decreased the total yield of both RT-PCR products (data not shown). Since we could not reduce the RT primer concentration, we decided to reduce the amount of RT reaction used as a template in the PCR. When we decreased the volume of the RT reaction from 2 μl to 1.5 or 0.75 μl per 50-μl PCR reaction, there was a clear decrease in the amount of 4.9-kb product without a decrease in the yield of full-length RT-PCR product (Fig. 5, lanes 2–4).

Optimization of the PCR cycle conditions. In parallel to the experiments described above, we set out to optimize the PCR cycle conditions, in order to improve the yield of long RT-PCR products. In most DNA template PCR protocols that aim to produce longer products, the elongation step is performed at 68°C. Therefore, we repeated the RT-PCR reactions described above (Fig. 5, lanes 2–4), but changed the elongation temperature from 72 to 68°C. With this change, the amount of full-length (16.7 kb) PCR product synthesized is significantly increased (Fig. 5, lanes 5–7). This result encouraged us to believe that it should be possible to effectively synthesize RT-PCR products longer than 16.7 kb.

In an attempt to effectively amplify DNA of more than 20 kb by RT-PCR, we used the protocol described above with RT primer 32 and PCR primers 159 and 11 (Table 1, Fig. 2). In contrast to our standard protocol, the PCR cycle conditions for this exper-

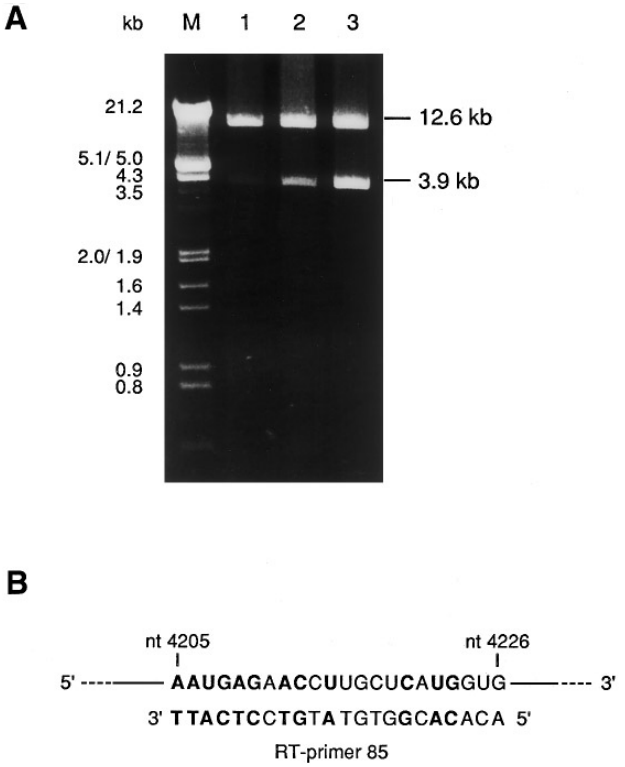

FIG. 4. Effect of RT primer concentration on the generation of "fortuitous" PCR products. (A) Reverse transcriptions were performed with HCV 229E poly(A)-RNA and 0.75 μM (lane 3), 0.5 μM (lane 2), and 0.25 μM (lane 1) concentrations of RT primer 85. 3-μl aliquots of the RT reaction were then used for PCR reactions with the oligonucleotides 159 and 89. 10-μl aliquots of the PCR reactions were separated on a 0.8% agarose gel. Lane M shows 400 ng of *Hin*dIII–*Eco*RI-digested λDNA. (B) Comparison of the putative false priming site in the HCV 229E sequence (nt 4205–4226) and the sequence of the RT primer, oligonucleotide 85. Complementary nucleotides are shown in bold.

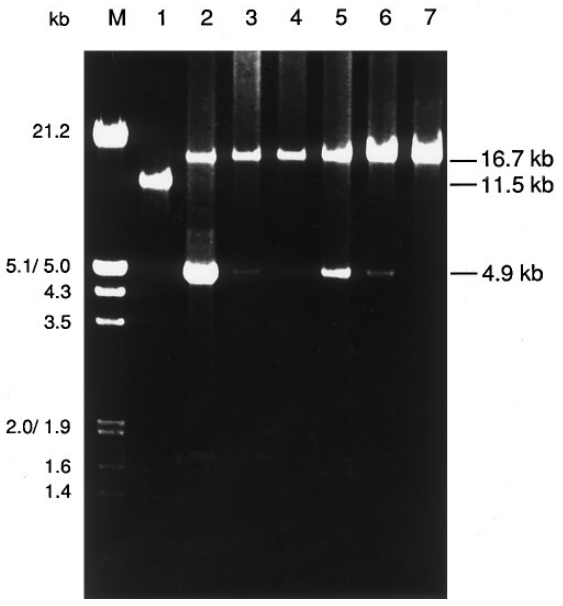

FIG. 5. Effect of RT reaction carryover on the generation of "fortuitous" PCR products. Reverse transcriptions were performed with HCV 229E poly(A)-RNA and 0.25 μM concentrations of RT primer 32. 2-μl aliquots (lanes 1, 2, and 5), 1.5-μl aliquots (lanes 3 and 6), or 0.75-μl aliquots (lanes 4 and 7) of the RT reaction were then used for PCR reactions with the oligonucleotides 127 and 11 (lane 1) or oligonucleotides 147 and 11 (lanes 2 to 7). Elongation temperatures were 72°C (lanes 1 to 4) or 68°C (lanes 5 to 7). 5-μl aliquots of the PCR reactions were separated on a 0.6% agarose gel. Lane M shows 400 ng of *Hin*dIII–*Eco*RI-digested λDNA.

iment were 12 cycles of 20 s at 94°C, 30 s at 50°C, and 27 min at 68°C, followed by 18 cycles where the elongation time was increased by 30 s in each successive cycle. The result is shown in Fig. 6. Using this protocol, it was possible to synthesize a DNA product with the expected size of 20.3 kb (lane 1). The identity of this product was confirmed by restriction analysis, however, the yield of the product was relatively poor. Also, we observed an unacceptable amount of heterogeneous DNA products that were clearly visible in agarose gel electrophoresis. Therefore, we did a series of experiments to optimize the PCR conditions by varying the cycle profile. As is shown in Fig. 6 (lane 2), a profile of five cycles with 5 s at 94°C, 20 s at 50°C, 23 min at 68°C, followed by 25 cycles where the elongation time was increased by 30 s in each successive cycle and the annealing step was left out increased the specificity of the PCR and greatly enhanced the synthesis of the 20.3-kb RT-PCR product. To demonstrate the reproducibility of this optimized RT-PCR protocol we amplified DNAs of 19.2, 19.5, and 20.2 kb in length using different PCR primers (data not shown). The specificity and product yields of these RT-PCRs were equivalent to those for the 20.3-kb RT-PCR shown in Fig. 6 (lane 2).

Amplification of Long Cellular mRNAs

The experiments described above were all done with poly(A)-RNA from HCV 229E-infected cells as RNA template in the RT reaction. In this material, the viral RNAs constitute 10–20% of the total RNA and the 27 kb viral genomic RNA represents approximately 4% of the viral RNAs or 0.4–0.8% of the total RNA (S. Siddell, unpublished). Obviously, we were interested to know if the RT-PCR protocols described above could also be used for less abundant RNAs, for example, cellular mRNAs.

The human dystrophin gene was used to study the long RT-PCR amplification of cellular mRNAs. The exon structure of this gene has been characterized and it has been shown to transcribe a 14-kb mRNA with a

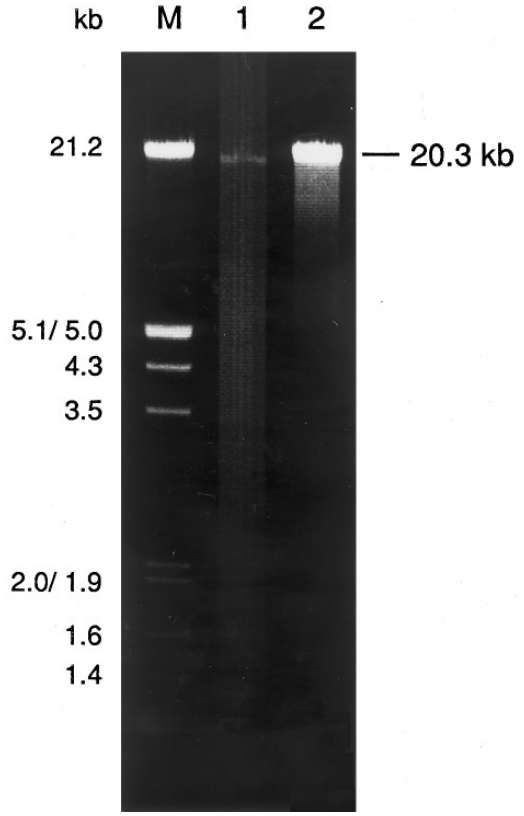

FIG. 6. PCR cycle optimization of a 20-kb RT-PCR. Reverse transcriptions were performed with HCV 229E poly(A)-RNA and RT primer 32 at a concentration of 0.25 μM. 0.75-μl aliquots of the RT reaction were then used for PCR reactions with the oligonucleotides 159 and 11. A PCR cycle profile of 12 cycles of 20 s at 94°C, 30 s at 50°C, and 27 min at 68°C, followed by 18 cycles where the elongation time was increased by 30s in each successive cycle, is shown in lane 1 (10 μl product). A PCR cycle profile of 5 cycles of 5 s at 94°C, 20 s at 50°C, and 23 min at 68°C, followed by 25 cycles where the elongation time was increased by 30 s in each successive cycle and the annealing step was left out is shown in lane 2 (5-μl product). The PCR reactions were separated on a 0.6% agarose gel. Lane M shows 400 ng of *Hin*dIII–*Eco*RI digested λDNA.

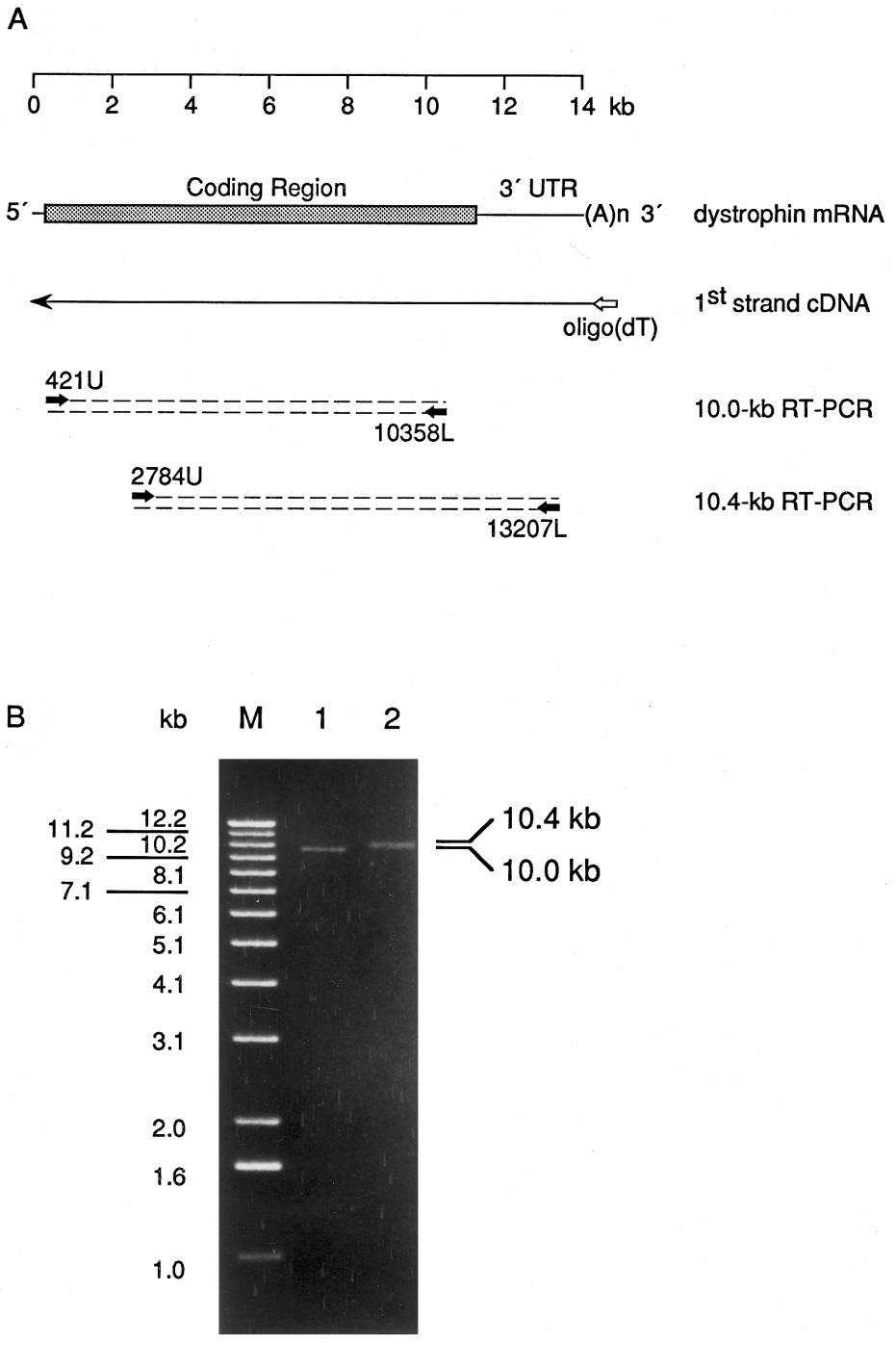

FIG. 7. RT-PCR from human dystrophin mRNA. (A) Schematic representation of the human dystrophin mRNA-specific oligonucleotides and RT-PCR products. The oligonucleotides are indicated as arrows according to their orientation and position relative to the dystrophin mRNA. The RT primer, oligo(dT), is drawn in white and the PCR primers in black. The expected sizes of the RT-PCR products are indicated. (B) Reverse transcriptions were performed with human heart poly(A)-RNA and oligo(dT) as RT primer. 0.2-μl aliquots of the RT reaction were then used for PCR reactions with the PCR primers 421U and 10358L (lane 1) or 2784U and 13207L (lane 2). 20-μl aliquots of the PCR reactions were separated on a 0.8% agarose gel. Lane M shows 100 ng of a 1-kb DNA ladder (Life Technologies).

2.7-kb region of 3′ untranslated sequence (16; Fig. 7A). The dystrophin mRNA is expressed in low amounts, approximately 0.01–0.001% of the mRNA in muscle (17, 18), and, thus, it serves as a good test system for the RT-PCR amplification of low abundance cellular mRNAs.

Poly(A)-RNA from human heart (1 μg) was used as template for an oligo(dT)-primed reverse transcription reaction as described under Materials and Methods. Subsequently, two different sets of PCR primers were tested for the amplification of cDNA segments using the following cycle profile: 94°C, 15 s; 62°C, 15 s; 68°C, 12 min for 35 cycles with an initial 1-min denaturation at 94°C. The PCR-primers 421U and 10358L produced a 10.0-kb DNA fragment from the dystrophin cDNA, while the PCR primers 2784U and 13207L amplified a 10.4-kb segment of the cDNA (Fig. 7B, lanes 1 and 2). These results show that cDNAs of at least 13.5 kb (including the 3′ untranslated region) can be effectively synthesized by reverse transcription of the dystrophin mRNA and that this cDNA can be readily amplified by PCR.

DISCUSSION

In this study we have adapted the concept of long PCR technology to reverse transcription PCR. The nature of RT-PCR requires the synthesis of a cDNA by reverse transcription prior to its amplification in the PCR reaction. We have demonstrated that there is no limitation concerning the ability of reverse transcriptase to synthesize cDNAs of up to 20 kb in sufficient amounts. However, to achieve this goal, a number of critical parameters have to be kept in mind. First, the integrity of the RNA template is important. Depending on the source of the RNA template, a method of preparation should be chosen that minimizes degradation of the RNA. In our hands, cell or tissue lysis can be achieved by detergent (NP-40) or TRIzol (Life Technologies) reagent, as appropriate, and oligo(dT)-based affinity chromatography with magnetic beads, or cellulose matrix, has proven to be reliable for the isolation of poly(A)-RNA. In our opinion, excessive phenol extraction and ethanol precipitation should be avoided and, where possible, the RNA should be protected by inhibitors such as RNasin.

Second, the conditions of the reverse transcription reaction strongly influence the outcome of the subsequent PCR. In our experience, the major problem that arises is "less stringent" priming during the RT reaction. The fortuitous cDNAs that are synthesized and the small amounts of RT-primer that are carried over into the PCR are responsible for most of the background amplification products observed. Also, it should be noted that previous studies for amplification of long mRNAs has required digestion of the RNA with RNase H after cDNA synthesis (10). This step did not seem to be necessary for amplification with HCV 229E genomic RNA. This may be due to the fact that in these amplification experiments HCV 229E RNA was relatively abundant, since the experiments with dystrophin mRNA required treatment with RNase H prior to amplification.

Finally, as is the case for all PCRs, the cycle conditions have to be optimized according to the amount of template, the PCR primers, and the cycle profile.

Having shown that it is possible to amplify 20-kb DNAs by RT-PCR, it is interesting to briefly consider the possible applications of this technique. Our own major interest is the RT-PCR amplification of viral RNA sequences and the generation of infectious cDNA clones. Previous studies with picornaviruses, alphaviruses, and flaviviruses have demonstrated how infectious cDNA clones can greatly facilitate studies on the molecular biology and pathogenesis of important human viruses (19–22). It would be highly desirable to extend this approach to RNA viruses with genomes of more than 10 kb, for example, coronaviruses and arteriviruses. The feasibility of the RT-PCR approach in the generation of an infectious cDNA has already been demonstrated for the potato virus Y (9) and for hepatitis A virus (6).

Finally, another important application of long RT-PCR technology will be the amplification of cellular mRNAs, particularly those of low abundance. We have shown that RT-PCR can be used for the amplification of 10-kb products from dystrophin mRNA and we see no reason why the same technology could not be applied, perhaps in combination with nested-set PCR, to other, perhaps even less abundant, cellular mRNAs. This type of application could provide important information in relation to diagnostic procedures and will also find a wide variety of uses in the areas of molecular and cellular biology.

ACKNOWLEDGMENT

This work was supported by the Deutsche Forschungsgemeinschaft (SFB 165/B1).

REFERENCES

1. Barnes, W. M. (1994) *Proc. Natl. Acad. Sci. USA* **91,** 2216–2220.
2. Cheng, S., Fockler, C., Barnes, W. M., and Higuchi, R. (1994) *Proc. Natl. Acad. Sci. USA* **91,** 5695–5699.
3. Cheng, S., Chang, S. Y., Gravitt, P., and Respess, R. (1994) *Nature* **369,** 684–685.
4. Cheng, S., Higuchi, R., and Stoneking, M. (1994) *Nature Genet.* **7,** 350–351.
5. Cheng, S., Chen, Y., Monforte, J. A., Higuchi, R., and Van Houten, B. (1995) *PCR Methods Appl.* **4,** 294–298.
6. Tellier, R., Bukh, J., Emerson, S. U., and Purcell, R. H. (1996) *Proc. Natl. Acad. Sci. USA* **93,** 4370–4373.
7. Martinez, J. M., Breidenbach, H. H., and Cawthon, R. (1996) *Genome Res.* **6,** 58–66.
8. Chumakov, K. M. (1996) *J. Virol.* **70,** 7331–7334.
9. Fakhfakh, H., Vilaine, F., Makni, M., and Robaglia, C. (1996) *J. Gen. Virol.* **77,** 519–523.
10. Nathan, M., Mertz, L. M., and Fox, D. K. (1995) *Focus* **17,** 78–80.

11. Herold, J., Raabe, T., and Siddell, S. (1993) *Arch. Virol.* [*Suppl.*] **7,** 63–74.
12. Raabe, T., Schelle-Prinz, B., and Siddell, S. G. (1990) *J. Gen. Virol.* **71,** 1065–1073.
13. Siddell, S. (1983) *J. Gen. Virol.* **64,** 113–125.
14. Meinkoth, J., and Wahl, G. (1984) *Anal. Biochem.* **138,** 267–284.
15. Gerard, G. F., Schmidt, B. J., Kotewicz, M. L., and Campbell, J. H. (1992) *Focus* **14,** 91–93.
16. Koenig, M., Hoffmann, E. P., Bertelson, C. J., Monaco, A. P., Feener, C., and Kunkel, L. M. (1987) *Cell* **50,** 509–517.
17. Hoffman, E. P., Monaco, A. P., Feener, C. C., and Kunkel, L. M. (1987) *Science* **238,** 347–350.
18. Tennyson, C. N., Shi, Q., and Worton, R. G. (1996) *Nucleic Acids Res.* **24,** 3059–3064.
19. Racaniello, V. R., and Baltimore, D. (1981) *Science* **214,** 916–919.
20. Rice, C. M., Levis, R., Strauss, J. H., and Huang, H. V. (1987) *J. Virol.* **61,** 3809–3819.
21. Rice, C. M., Grakoui, A., Galler, R., and Chambers, T. J. (1989) *New Biol.* **1,** 285–296.
22. Sumiyoshi, H., Hoke, C. H., and Trent, D. W. (1992) *J. Virol.* **66,** 5425–5431.