Reverse Transcriptase Adds Nontemplated Nucleotides to cDNAs During 5'-RACE and Primer Extension

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Dayue Chen and John T. Patton

National Institutes of Allergy and Infectious Diseases,
National Institutes of Health,
Bethesda, MD, USA

ABSTRACT

In determining the terminal sequences of the genomic dsRNAs of rotavirus by 5'-rapid amplification of cDNA ends (5'-RACE), it was found that most of the viral cDNAs contained extra nucleotides at their 5' termini that had not been reported before on any rotavirus sequence. Although the extra nucleotides could be dA, dC, dG, or dT residues, the extra nucleotides on the cDNAs usually consisted of a single dT residue. Experiments performed with DNA/RNA duplexes indicated that reverse transcriptase has an associated terminal nucleotidyl transferase (TdT)-like activity, which can add nontemplated nucleotides to the 3' ends of DNA, and that reverse transcriptase was responsible for the presence of the extra nucleotides detected on the 5'-RACE cDNAs. The TdT-like activity of reverse transcription was specific for double-stranded substrates (i.e., DNA/DNA or DNA/RNA duplexes) and was active over a wide range of temperatures and enzyme concentrations. Both commercially available Moloney murine leukemia virus and avian myeloblastosis virus reverse transcriptases contained the TdT-like activity. This work implies that 5'-RACE and primer extension assays must be used carefully in determining the terminal sequences of nucleic acids because, under standard reaction conditions, reverse transcriptase can add nontemplated nucleotides to the 3' ends of cDNAs following template-directed synthesis.

INTRODUCTION

Both rapid amplification of cDNA ends (RACE) and primer extension assays use reverse transcriptase to catalyze the template-directed synthesis of a cDNA. The RACE procedure is frequently used to generate full-length cDNA clones and to determine the precise terminal sequences of cellular and viral RNAs (5–7). In the initial step of RACE, a cDNA is produced by incubating an RNA with reverse transcriptase and one of two types of primers, either oligo-dT (3'-RACE) or a specific deoxyoligonucleotide that anneals closely to the 5' end of the RNA (5'-RACE). In the case of 5'-RACE, a homopolymer tail is then added to the end of the cDNA product with terminal deoxynucleotidyl transferase (TdT). Afterwards, the cDNAs are amplified by PCR using a gene-specific primer and an oligonucleotide complementary to the homopolymer tail. The amplified cDNAs can be sequenced directly or cloned into a plasmid for further characterization. In a typical primer extension assay, a primer anneals to the RNA of interest through specific base pairing and is extended by reverse transcriptase until the growing chain of cDNA reaches the 5' end of the RNA template. The technique has long been used to measure the level of gene expression and to map the 5' end of RNAs (8,9).

In this study, we report that commercially purchased Moloney murine leukemia virus (MMLV) and avian myeloblastosis virus (AMV) reverse transcriptases contain a TdT-like activity that can add one or more nontemplated nucleotides to the 3' end of cDNAs upon the completion of template-directed synthesis. Under typical

reaction conditions, the termini of 70%–80% of 5'-RACE cDNAs prepared with MMLV reverse transcriptase contained one or more nontemplated nucleotides. The TdT-like activity preferentially adds A residues to cDNAs and uses DNA/RNA or DNA/DNA duplexes as substrates. Because of this activity, strict reliance on 5'-RACE and primer extension to define the 5'-terminal sequence of RNA can yield misleading results.

MATERIALS AND METHODS

Cloning and Sequencing of 5'-RACE Products

dsRNA was purified from rotavirus SA11-4F (2), heated to 100°C for 2 min, and used as a template for making gene 8 cDNAs. The primer for cDNA synthesis, 5'-ATTAGAGACGTTTGCTTGCGTCAC-3', was complementary to nucleotides 322–345 of the gene 8 (+)RNA. Reaction mixtures (50 µL) contained 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 2.5 mM MgCl₂, 10 mM dithiothreitol, 400 µM each of the dNTPs, 0.5 µg dsRNA, 100 nM primer, and 200 U MMLV reverse transcriptase (SUPERSCRIPT II™; Life Technologies, Rockville, MD, USA) and were incubated at 45°C for 50 min. The cDNA products were purified with GlassMax® spin cartridges (Life Technologies) and tailed by incubating them with 10 U TdT (Life Technologies) and 200 µM dGTP, dATP, or dCTP in a volume of 25 µL for 10 min at 37°C. The tailed cDNAs were amplified by 30 cycles (94°C for 60 s, 50°C for 45 s, 72°C for 90 s) of PCR using an anchor primer and the gene 8 primer, 5'-aatggatccAGAGCTTCATA

TCAACATAGCC-3', which contained a sequence complementary to nucleotides 285–308 of the gene 8 (+)RNA (uppercase) and a *Bam*HI site (underlined). The oligonucleotides 5'-ccggaattccccccccccccccc-3' (*Eco*RI site underlined), 5'-ggccacgcgtcgactagtacgggiugggiugggiig-3' (*Sal*I site underlined, I = inosine), and 5'-cggctcgagttttttttttttttt-3' (*Xho*I site underlined) were the anchor primers for dG-, dC-, and dA-tailed cDNAs, respectively. After purification, the PCR products were digested with *Bam*HI and *Eco*RI (dG-tailed cDNA), *Bam*HI and *Xho*I (dA-tailed cDNA), or *Bam*HI and *Sal*I (dC-tailed cDNA), and were ligated into the *Bam*HI/*Eco*RI sites (dG-tailed cDNA) or *Bam*HI/*Sal*I sites (dA- and dC-tailed cDNAs) of pUC19. Clones containing cDNA inserts were sequenced with a T7 Sequenase® version 2.0 DNA Sequencing kit (Amersham Pharmacia Biotech, Piscataway, NJ, USA) using a gene 8 primer that annealed to a region 60 nucleotides downstream of the 5' end of the gene 8 (+)RNA.

Preparation of T7 Run-Off Transcripts

Gene 8 (+)RNAs were made by run-off transcription of SP65 g8R with T7 RNA polymerase (13). Unincorporated nucleotides were removed by passing RNA samples through Micro-Select®-D, G-50 spin columns (Eppendorf-5 Prime, Westbury, NY, USA). The quality of the RNA transcripts was evaluated by electrophoresis on 1% formaldehyde agarose gels (14).

Preparation of DNA/RNA Duplexes

Gel-purified oligonucleotides were used to prepare DNA/RNA duplexes. Oligonucleotide I, 5'-GGCGACTGAGACGCTTTA-3', is complementary to residues 7–24 of the gene 8 (+)RNA and forms a DNA/RNA duplex that leaves a 5'-RNA overhang of six nucleotides (Figure 1B, duplex I). Oligonucleotide II, 5'-GGCGACTGAGACGCTTTAAAGCC-3', is complementary to the first 24 residues of the

gene 8 RNA and forms a DNA/RNA duplex in which the 5' end of the RNA is blunt-ended with the 3' end of the oligonucleotide (Figure 1B, duplex II). Oligonucleotide III, 5'-GGCGACTGAGACGCTTTAAAGCCg-3' (lowercase is nonviral), is also complementary to the first 24 nucleotides of the gene 8 RNA but forms a DNA/RNA duplex with a 3'-DNA overhang of a single nucleotide (Figure 1B, duplex III). The oligonucleotides were 5'-end-labeled in a reaction mixture (20 µL) containing 60 mM Tris-HCl (pH 7.8), 10 mM MgCl₂, 200 mM KCl, 50 µCi [γ -³²P] ATP (3000 Ci/mmol; NEN® Life Science Products, Boston, MA, USA), 50 pmol oligonucleotide, and 4 U T4 polynucleotide kinase (Life Technologies). After incubation for 30 min at 37°C, ³²P-labeled oligonucleotides were recovered by phenol-chloroform-isoamyl alcohol extraction and ethanol precipitation and passed twice through Sephadex G-25. To prepare the DNA/RNA duplexes, 5 pmol ³²P-labeled or unlabeled oligonucleotides and 10 pmol gene 8 (+)RNA in 50 mM Tris-HCl (pH 8.3), 3 mM MgCl₂, and 55 mM KCl were heated to 100°C for 2 min and annealed by incubating overnight at 42°C.

Assay for the Addition of Non-templated Nucleotides by Reverse Transcriptase to DNA/RNA Duplexes

Reaction mixtures (25 µL) contained 0.5 pmol ³²P-labeled DNA/RNA duplex, either 500 µM each dNTP or 500 µM one dNTP, 200 U MMLV reverse transcriptase or 5 U AMV reverse transcriptase, and the buffer provided by the supplier of the reverse transcriptase (Life Technologies) and were incubated for 60 min at 45°C, unless otherwise indicated. After extraction with phenol-chloroform-isoamyl alcohol and precipitation with ethanol, the sizes of ³²P-labeled oligonucleotides in the mixtures were analyzed by electrophoresis on 20% polyacrylamide sequencing gels containing 7 M urea and autoradiography.

RESULTS

Sequence Analysis of Gene 8 cDNA Clones Generated by 5'-RACE

To determine the 5'-terminal se-

Figure 1. DNA/RNA duplexes I, II, and III. Terminal structure of DNA/RNA duplexes formed by annealing oligonucleotides I, II, and III to gene 8 (+)RNA made by T7 transcription. Sequences of the oligonucleotides are given as well as the relevant sequences of the RNA. The nonviral nucleotide of oligonucleotide III is shown in lowercase.

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quence of the gene 8 (+)RNA of rotavirus SA11-4F by 5'-RACE (4), cDNAs were prepared from virion-derived gene 8 dsRNA using MMLV reverse transcriptase. The cDNAs were tailed with dATP, dCTP, or dGTP, amplified by PCR, and cloned into pUC19. Of 29 dATP-tailed clones, sequence analysis showed that 22 (75.9%) had the 5'-terminal sequence, 5'-GGCTTTT-3' (Figure 2A). Based on earlier work (1,12), this is the expected sequence for the 5' end of the gene 8 RNA. However, seven clones (24.1%) contained either one (G or C) or two (GT, GC, or CT) extra nucleotides upstream of the expected 5'-terminal sequence (Figure 2A). Of 34 dCTP-tailed clones, the 5'-terminal sequence of only 10 (24.9%) agreed with expected gene 8 sequence (Figure 2B). For these 10 clones, the origin of the first two nucleotides GG in the 5'-terminal sequence (5'-GGCTTTT-3') is masked because of dCTP-tailing of the first-strand cDNA. Of the remaining 24 dCTP-tailed clones, 18 (52.9%) contained an extra T, and 6 (17.6%) contained an extra C upstream of the expected 5'-terminal sequence (Figure 2B). Similar to the dCTP-tailed clones, the majority (19 of 22 clones, 84.6%) of the dGTP-tailed clones contained one or two extra nucleotides at their 5' ends (Figure 2C). Of the 19 clones, 17 (77.3%) contained an extra T, one contained an extra GT, and one contained an extra TT. These results showed that the 5'-terminal sequences of the gene 8 cDNA clones produced by 5'-RACE were heterogeneous and that, in total, the majority of the clones contained one or more additional nucleotides upstream of the expected 5'-terminal sequence. The extra nucleotides were detected more often with cDNAs tailed with dCTP or dGTP than those tailed with dATP. For cDNAs tailed with dCTP or dGTP, the extra nucleotides upstream of the expected 5'-terminal gene 8 sequence were usually T residues.

When gene 8 (+)RNA made by T7 transcription was used as the template for 5'-RACE, instead of virion-derived RNA, the 5' ends of the gene 8 cDNA clones contained extra nucleotides in frequencies similar to those observed above (data not shown). Thus, even when the RNA used for 5'-RACE were made by transcribing a gene 8 cDNA

beginning with the sequence 5'-GGCTTTT-3', analysis of the clones indicated that the 5' end of the RNA was heterogeneous in sequence and contained extra nucleotides upstream of the expected 5'-terminal sequence.

Addition of Nontemplated Nucleotides to DNA/RNA Duplexes by Reverse Transcriptase

Previous reports have indicated that the reverse transcriptases of AMV, MMLV, and HIV have an associated TdT-like activity (3,10,11,15). To test

whether or not this activity was responsible for the presence of the extra nucleotides, oligonucleotides I (18 nucleotides), II (24 nucleotides), and III (25 nucleotides) were 5'-end-labeled with ^{32}P and then annealed to gene 8 (+)RNAs, yielding DNA/RNA duplexes that contained a 5'-RNA overhang (I), a blunt-end (II), and a single-base 3'-DNA overhang (III), respectively (Figure 1). The terminal structure of duplex II mimics the blunt-end generated by reverse transcriptase during first-strand synthesis on an RNA template. The three DNA/RNA duplexes were

incubated with MMLV or AMV reverse transcriptase, at concentrations suggested by the supplier, in the presence of all four dNTPs or one individual dNTP. The sizes of the ^{32}P -labeled oligonucleotides of the DNA/RNA duplexes were evaluated by electrophoresis (Figure 3A). When DNA/RNA duplex II was incubated with all four dNTPs and MMLV or AMV reverse transcriptase, 90% and 30%, respectively, of the oligonucleotides recovered from the reaction mixtures had increased in size by one or more nucleotides (lanes 3 and 11). When the four dNTPs were incubated individually with MMLV and AMV reverse transcriptase, in all cases the products included oligonucleotides that had increased in size by at least one nucleotide (lanes 4–7 and 12–15). However, the efficiency with which the non-templated nucleotides were added to the oligonucleotides varied depending

on which dNTP was present (lanes 4–7 and 12–15) and was calculated to follow the order $\text{A} > \text{G} \geq \text{C} > \text{T}$. The addition of two or more nontemplated nucleotides to oligonucleotides occurred much less frequently than did the addition of a single nucleotide, and the addition of two or more nontemplated nucleotides was most efficient in reaction mixtures that contained both dATP and MMLV reverse transcriptase. Because oligonucleotides that contained a single nontemplated nucleotide (i.e., the 25-nucleotide products) exhibited slight differences in electrophoretic mobility depending on which nucleotide was added (lanes 4–7 and 12–15), it was possible to conclude that in the presence of all four dNTPs, dATP was added to oligonucleotides more efficiently than the other three dNTPs (lanes 3 vs. 5 and lanes 11 vs. 13).

Due to the six nucleotide 5'-RNA overhang, incubation of DNA/RNA du-

plex I with reverse transcriptase is expected to extend the oligonucleotide from 18 to 24 nucleotides via template-dependent cDNA synthesis (Figure 1). If reverse transcriptase transfers nucleotides to the cDNA in a non-template-dependent manner upon the completion of template-dependent synthesis, then the length of the oligonucleotide will exceed 24 nucleotides. Indeed, such experiments revealed that greater than 90% of the total extended oligonucleotides recovered from reaction mixtures containing MMLV reverse transcriptase were 25 nucleotides or more in size (Figure 3A, lanes 8 and 9). In contrast, when AMV reverse transcriptase was used, approximately 25% of the total extended oligonucleotides were 25 nucleotides in length (lanes 16 and 17). The results obtained with DNA/RNA duplexes I and II indicate that both these commercially purchased reverse transcriptases have TdT-like

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activity, which adds nontemplated nucleotides to the 3'-end of cDNAs. Unlike duplexes I and II, MMLV and AMV reverse transcriptase did not add nontemplated nucleotides to oligonucleotides I and II (Figure 3B). Thus, the substrate for the TdT-like activity of reverse transcriptase does not include ss-DNA. In related experiments, it was shown that the TdT-like activity of reverse transcriptase could add nontemplated nucleotides to blunt-ended DNA/DNA duplexes but could not add nucleotides to ssRNA (data not shown).

The addition of the first nontemplated nucleotide to the oligonucleotides of duplexes I (5'-RNA overhang) and II (blunt-end) occurred more frequently in reaction mixtures containing reverse transcriptase and all four dNTPs than did the addition of the second or third nontemplated nucleotide (Figure 3A). This suggested that the transfer of one nontemplated nucleotide produced 3' overhangs on these duplexes, which then made them poorer substrates for the TdT-like activity of reverse transcriptase. To test this, we compared the ability of MMLV and AMV reverse transcriptase to transfer nontemplated

nucleotides to DNA/RNA duplexes that contained a 5'-RNA overhang (I), a blunt-end (II), and a single-base 3'-DNA overhang (III). The results showed that MMLV and AMV reverse transcriptase added nontemplated nucleotides to 91% and 25%, respectively, of the extended oligonucleotides of duplex I (Figure 4, lanes 5 and 8), to 87% and 26% of the oligonucleotides of duplex II (lanes 6 and 9), but to only 27% and 0% of the oligonucleotides of duplex III (lanes 7–10). The decreased frequency of the transfer of nontemplated nucleotides to the oligonucleotides of DNA/RNA duplex III relative to those of duplexes I and II showed that the TdT-like activity of reverse transcriptase adds nucleotides to blunt-ended substrates more efficiently than to substrates with 3' overhangs.

Effect of Reverse Transcriptase Concentration and Temperature on the Addition of Nontemplated Nucleotides

To evaluate the effect of reverse transcriptase concentration on the addition of nontemplated nucleotides,

DNA/RNA duplex I (5'-RNA overhang) was incubated with serial dilutions of MMLV and AMV reverse transcriptase. Analysis of the extended oligonucleotides (i.e., those ≥ 24 nucleotides) from the assays showed that the TdT-like activity decreased by eightfold as the amount of MMLV reverse transcriptase was reduced from 100 to 12.5 U (Figure 5A, lanes 6–9). Further reduction in the amount of MMLV reverse transcriptase had little effect on TdT-like activity (lanes 10–12). In contrast, dilution of AMV reverse transcriptase had no impact on its TdT-like activity (lanes 13–15). Higher concentrations of AMV reverse transcriptase were not available for testing. These results indicate that reverse

Figure 3. Addition of nontemplated nucleotides to DNA/RNA duplexes. (A) ^{32}P -labeled DNA/RNA duplexes I (5'-RNA overhang; lanes 8, 9, 16, and 17) and II (blunt-end; lanes 2–7 and 1–15) and (B) ^{32}P -labeled oligonucleotides I (lanes 8, 9, 16, and 17) and II (lanes 2–7 and 10–15) were incubated with MMLV or AMV reverse transcriptase and all four dNTPs (dN) or one of the four dNTPs (dG, dA, dT, and dC), or in the absence of dNTPs (w/o). The sizes of oligonucleotides recovered from reaction mixtures were determined by electrophoresis and autoradiography. End-labeled oligonucleotides were used as size markers (lane 1). The positions of end-labeled oligonucleotides used in the assays are indicated with arrows: Oligo I (18 nucleotides) and Oligo II (24 nucleotides).

Figure 4. Effect of terminal structure on addition of nontemplated nucleotides. DNA/RNA duplexes I (5'-RNA overhang), II (blunt-end), and III (3'-DNA overhang) were incubated with all four dNTPs in the absence of reverse transcriptase (lanes 2–4), or in the presence of 200 U MMLV reverse transcriptase (lanes 4–7) or 5 U AMV reverse transcriptase (lanes 8–10). Oligonucleotides from the mixtures were analyzed by electrophoresis and autoradiography and quantified by phosphorimaging. Values were calculated by dividing the level of products containing greater than or equal to one nontemplated nucleotide by the total level of products that were greater than or equal to 24 nucleotides (duplex I and II) or greater than 25 nucleotides (duplex III) and multiplying by 100. Asterisks indicate that the total level of products exclude 18-mer unextended products.

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transcriptase concentration affects the frequency of the addition of nontemplated nucleotides and that the level of TdT-like activity is lower at lower reverse transcriptase concentrations.

Reverse transcription is routinely performed at elevated temperature

(45°C–50°C) to reduce the impact of RNA secondary structure on cDNA synthesis. To examine the effect of temperature on the TdT-like activity of reverse transcriptase, DNA/RNA duplex I (5'-RNA overhang) was incubated with MMLV and AMV reverse tran-

scriptases at 25°C–50°C. The results showed that temperature was not a significant factor in the template-dependent elongation (i.e., cDNA synthesis) of the 18-nucleotide oligonucleotide primer of the duplex (Figure 5B, lanes 5–14). However, the addition of nontemplated nucleotides was affected by temperature. At 25°C, approximately 36% and 48% of the extended oligonucleotides generated by MMLV and AMV reverse transcriptase, respectively, contained nontemplated nucleotides (lanes 5 and 10). At 32°C–45°C, the level of extended oligonucleotides with nontemplated nucleotides increased to 90% in assays containing MMLV reverse transcriptase (lanes 6–8). Unlike the results with MMLV reverse transcriptase, the level of extended oligonucleotides with nontemplated nucleotides decreased to 20% in assays containing AMV reverse transcriptase at 32°C–45°C (lanes 11–13). At the highest temperature (50°C), nontemplated nucleotides were present on only approximately 27% and 19% of the extended products recovered from assays containing MMLV and AMV reverse transcriptase, respectively (lanes 9 and 14). Therefore, although functioning over a wide range of temperatures, the TdT-like activity of reverse transcriptase is influenced by temperature and is reduced at 50°C.

DISCUSSION

This study was prompted by our initial observation that the majority of clones generated by the 5'-RACE procedure contained extra nucleotides upstream of the 5' end of the rotavirus gene 8-specific sequence. By analyzing DNA/RNA duplexes with defined terminal sequences, we have demonstrated that reverse transcriptase can add one, and less commonly two or three, nucleotides in a template-independent manner to the 3' end of cDNAs upon the completion of template-dependent synthesis. The TdT-like activity of reverse transcriptase is substrate specific, as it catalyzed the addition of nontemplated nucleotides to DNA/RNA and DNA/DNA duplexes, but not to ssDNA or ssRNA. In comparison to DNA/RNA duplexes with blunt-ends, the TdT-like

Figure 5. Impact of reverse transcriptase concentration and reaction temperature on the addition of nontemplated nucleotides. (A) DNA/RNA duplex I (5'-RNA overhang) was incubated with the indicated units of MMLV or AMV reverse transcriptase and all four dNTPs for 1 h at 45°C (A) or with 20 U MMLV or 5 U AMV reverse transcriptase and all four dNTPs for 1 h at the indicated temperatures (B). The oligonucleotides from the mixtures were analyzed by electrophoresis and autoradiography and quantified by phosphorimaging. Values were obtained by dividing the level of the primer extension products containing nontemplated nucleotides (> 24 nucleotides) by the total extension products (≥ 24 nucleotides) and multiplying by 100. End-labeled oligonucleotide size marker (lane 1), oligonucleotide I (18 nucleotides, panel B, lane 4), II (24 nucleotides, lane 3), and III (25 nucleotides, lane 2).

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activity added nucleotides to duplexes with 3'-DNA overhangs much less efficiently. While any of the four dNTPs were used as substrates, dATP was the preferred substrate for TdT-like activity. Reverse transcriptase added nontemplated nucleotides to cDNAs at all enzyme concentrations examined, but only at high concentrations of reverse transcriptase (≥ 100 U) was the addition of more than one nontemplated nucleotide to cDNAs readily detected. The TdT-like activity of reverse transcriptase functioned over a wide range of temperatures (25°C–50°C) but exhibited peak activity at temperatures that are typically used in generating cDNAs by RACE and primer extension (32°C–45°C).

5'-RACE and primer extension have been widely used to determine the precise terminal sequences of cellular and viral RNAs. Under typical reaction conditions, MMLV reverse transcriptase can add nontemplated nucleotides to 70%–80% of 5'-RACE clones (Figure 1) and to greater than 90% of primer extension products (Figure 4),

while AMV reverse transcriptase adds nontemplated nucleotides to 40% of 5'-RACE clones (data not shown) and to 25% of primer extension products (Figure 4). These findings raise concerns about the strict reliance of using these methods to determine the terminal sequences of RNAs. For cellular RNA, the presence of nontemplated nucleotides may be readily identified by comparing the terminal sequence of the cDNA to the genomic DNA sequence from which the RNA was derived. However, this approach is not applicable for cDNAs of RNA viruses because of the lack of a genomic DNA sequence to make a sequence comparison. Indeed, when using 5'-RACE or primer extension to determine the terminal sequences of RNAs of RNA viruses, it may be necessary to confirm its accuracy by an alternative method such as direct RNA sequencing.

Despite significant effort, we could not find assay conditions under which the TdT-like activity of reverse transcriptase was abolished. However, our analysis indicates that it is possible to reduce the incidence of the addition of nontemplated nucleotides in 5'-RACE and primer extension by using less reverse transcriptase, by raising the reaction temperature, or by a combination of both. Since the addition of nontemplated nucleotides cannot be completely eliminated, 5'-RACE clones can be expected to exhibit some degree of sequence heterogeneity at the junction of cDNAs and homopolymer tail. To avoid misinterpreting the sequence information gained from 5'-RACE clones, we recommend that at least two different dNTPs should be used in homopolymer tailing and that several cDNA clones should be independently prepared and their sequences comparatively analyzed.

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Address correspondence to:

Dr. John T. Patton
Laboratory of Infectious Diseases
National Institutes of Allergy and Infectious Diseases
National Institutes of Health
7 Center Drive, MSC 0720, Room 117
Bethesda, MD 20892, USA.
e-mail: jpatton@niaid.nih.gov