RNA (2001), *7*:774–781. Cambridge University Press. Printed in the USA.

METHOD

Primer-independent RNA sequencing with bacteriophage ϕ6 RNA polymerase and chain terminators

EUGENE V. MAKEYEV and DENNIS H. BAMFORD

Department of Biosciences and Institute of Biotechnology, FIN-00014, University of Helsinki, Finland

ABSTRACT

Here we propose a new general method for directly determining RNA sequence based on the use of the RNA-dependent RNA polymerase from bacteriophage ϕ6 and the chain terminators (RdRP sequencing). The following properties of the polymerase render it appropriate for this application: (1) the ϕ6 polymerase can replicate a number of single-stranded RNA templates in vitro. (2) In contrast to the primer-dependent DNA polymerases utilized in the sequencing procedure by Sanger et al. (*Proc Natl Acad Sci USA*, 1977, *74*:5463–5467), it initiates nascent strand synthesis without a primer, starting the polymerization on the very 3′-terminus of the template. (3) The polymerase can incorporate chain-terminating nucleotide analogs into the nascent RNA chain to produce a set of base-specific termination products. Consequently, 3′ proximal or even complete sequence of many target RNA molecules can be rapidly deduced without prior sequence information. The new technique proved useful for sequencing several synthetic ssRNA templates. Furthermore, using genomic segments of the bluetongue virus we show that RdRP sequencing can also be applied to naturally occurring dsRNA templates. This suggests possible uses of the method in the RNA virus research and diagnostics.

Keywords: bacteriophage ϕ6; bluetongue virus; chain terminators; dsRNA virus; RNA-dependent RNA polymerase; RNA sequencing

INTRODUCTION

The determination of nucleic acid sequences is undoubtedly one of the most broadly used techniques in modern life sciences. In 1977, two principally different strategies were developed, which have provided the basis for the remarkable progress now witnessed in gene and genome sequencing. The strategy proposed by Sanger et al. (1977) utilizes a DNA polymerase to extend the primer annealed to the nucleic acid template of interest. Polymerization is terminated via incorporation of base-specific chain-terminating nucleotides. The reaction products are then separated according to their size and visualized by a radioactive or nonradioactive detection method so that the template sequence can be deduced from the resultant separation pattern (Fig. 1A). The second approach (Maxam & Gilbert, 1977) uses the base-specific cleavage of the terminally labeled nucleic acid target, the reaction products being analyzed exactly as in the first approach (Fig. 1B).

Both strategies were initially reported for DNA, but later, and this is in the scope of the present report, they were adapted to determine RNA sequences. Although useful for some applications, these RNA sequencing methods, however, have not become as popular as their DNA counterparts partly due to the following reasons. The strategy of the RNA base-specific cleavage is technically challenging and laborious. This applies to both its chemical (Peattie, 1979) and enzymatic (Donis-Keller et al., 1977) variants. On the other hand, a more convenient technique of RNA sequencing with the use of reverse transcriptase and chain-terminating agents (Hamlyn et al., 1978; Zimmern & Kaesberg, 1978) requires that part of the template sequence has to be known a priori to design the complementary primer. In DNA sequencing, this problem is easily overcome by cloning the unknown fragment into a vector and using a primer complementary to the vector DNA. The solution for RNA is less straightforward, unless the RNA is

Reprint requests to: Dennis H. Bamford, Viikki Biocenter, P.O. Box 56, Viikinkaari 5, FIN-00014, University of Helsinki, Finland; e-mail: dennis.bamford@helsinki.fi.

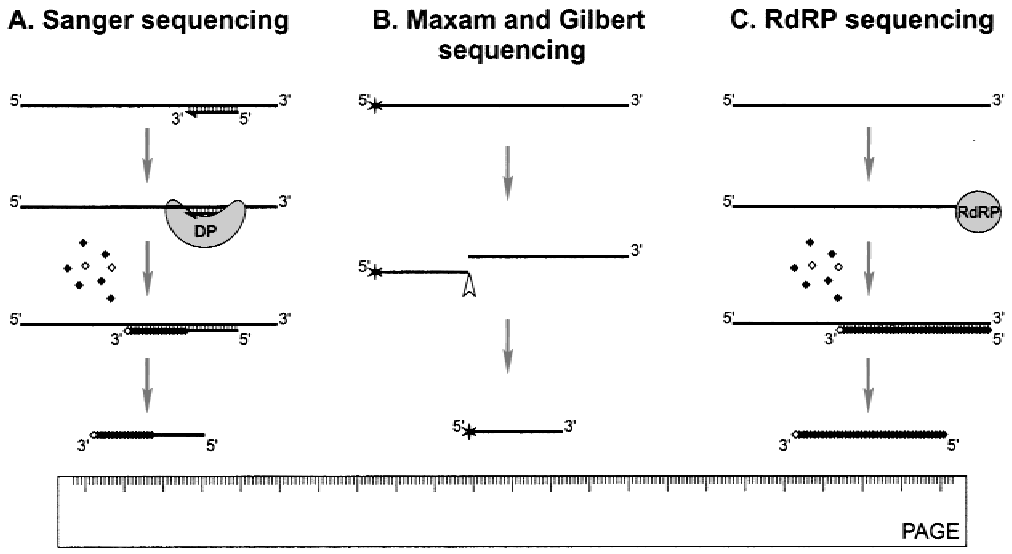

FIGURE 1. Principle of the RdRP sequencing (**C**) in comparison with the Sanger (**A**) and Maxam and Gilbert (**B**) sequencing procedures. Black diamonds in **A** and **C** show normal nucleotide substrates; open diamonds correspond to base-specific chain terminating agents. DP: DNA polymerase elongating a template-complementary primer. RdRP: RNA-dependent RNA polymerase initiating complementary strand synthesis *de novo*. Stars in **B** indicate terminal label; the arrowhead represents base-specific cleavage. The ruler shows the reaction product separation step usually being high-resolution denaturing polyacrylamide gel electrophoresis (PAGE). See text for further details.

first converted into cDNA and then processed by the standard methods of genetic engineering.

At the moment, the cDNA-mediated approach is the strategy of choice for mRNA sequencing because it is possible to utilize a standard oligo(dT) primer complementary to the poly(A) tail. However, for the RNA species lacking poly(A), for example, genomic RNAs of some viruses (Fields et al., 1996) or noncoding RNAs (Eddy, 1999), cDNA synthesis becomes more problematic, generally being achieved through either RNA tailing (see, e.g., Attoui et al., 2000, and references therein) or random priming (Sambrook et al., 1989). In addition to being time consuming, the cDNA strategy may alter the sequence of the original RNA molecule due to the low fidelity of reverse transcriptase (Patel & Preston, 1994, and references therein).

To this end, we devised a novel strategy called RdRP sequencing, in which a nucleic acid molecule is directly sequenced with a nonspecific RNA-dependent RNA polymerase (RdRP) and chain-terminating nucleotide analogs bypassing the cDNA step (Fig. 1C). The method does not require primer, and thus can be used to analyze completely unknown RNA species. The feasibility of such an approach has been demonstrated earlier using the polymerase from bacteriophage Qβ to sequence a Qβ-specific RNA template (Kramer & Mills, 1978), as well as the brome mosaic virus (BMV) replicase to analyze BMV (−)sense RNA synthesis (Miller et al., 1986). However, both polymerases show pronounced specificity to their authentic RNA templates (Blumenthal, 1980; Klovins & van Duin, 1999; Kim et al., 2000, and references therein), which compromises their general sequencing application. In contrast, the RdRP from dsRNA bacteriophage ϕ6 (also referred to as P2 polymerase) has been reported to efficiently replicate a broad range of both phage-specific and heterologous ssRNAs in vitro, initiating the synthesis at the very 3′ terminal template nucleotide (Makeyev & Bamford, 2000a). The experiments presented here extend this information and directly demonstrate that ϕ6 RdRP, together with the 3′-deoxyribonucleotide 5′-triphosphates (3′-dNTPs) terminators, can indeed be used to sequence synthetic and natural RNA templates with homogeneous 3′ termini.

RESULTS

Effect of the template 3′-terminal nucleotide on the replication efficiency

The P2 polymerase of bacteriophage ϕ6 has been previously shown to accept a number of different ssRNA templates (Makeyev & Bamford, 2000a). This encourages the use of the P2 polymerase in the RdRP sequencing procedure outlined in Figure 1C. It has been noticed, however, that the replication efficiency depends on the nature of 3′ end of the template. To characterize this phenomenon in more detail, we compared the replication efficiency of five variants of sΔ^+ ssRNA (ϕ6 s$^+$ segment with an internal deletion; Makeyev & Bamford, 2000b). One of the variants had the natural ϕ6 terminus (… CUCUCUCUC**U3′**) and was designated as sΔ^+(∅); the others contained four different 1-nt additions: sΔ^+(A3′), sΔ^+(C3′), sΔ^+(G3′), and sΔ^+(U3′). C3′

and, to a lesser extent, G3′ stimulated the replication by several-fold, whereas A3′ and U3′ were neutral or somewhat inhibitory (Fig. 2A,C). As evident from Figure 2B,D, the same effect of the single-nucleotide extensions (C3′ > G3′ > A3′ > U3′) was also observed for five different firefly luciferase mRNAs: luc(∅), luc(A3′), luc(C3′), luc(G3′), and luc(U3′). These experiments show that while being generally unspecific, the P2 polymerase prefers templates with 3′ terminal C. This is likely to reflect more efficient initiation when the first base pair is being stabilized by three hydrogen bonds rather than two because G3′ is also a good initiation signal. Furthermore, some other reasons for the bias to C3′ have recently been inferred from the crystal structure of the P2 polymerase (Butcher et al., 2000, 2001). In addition to the terminal nucleotide, the nature of the 3′ penultimate base has also been shown to influence P2-directed replication, for example, CC3′ being a more efficient initiation signal than AC3′ (Makeyev & Bamford, 2000b). Despite these preferences, substantial amounts of dsRNA can be produced using less favorable ssRNA templates by simply increasing the P2 concentration in the reaction mixture (Makeyev & Bamford, 2000b).

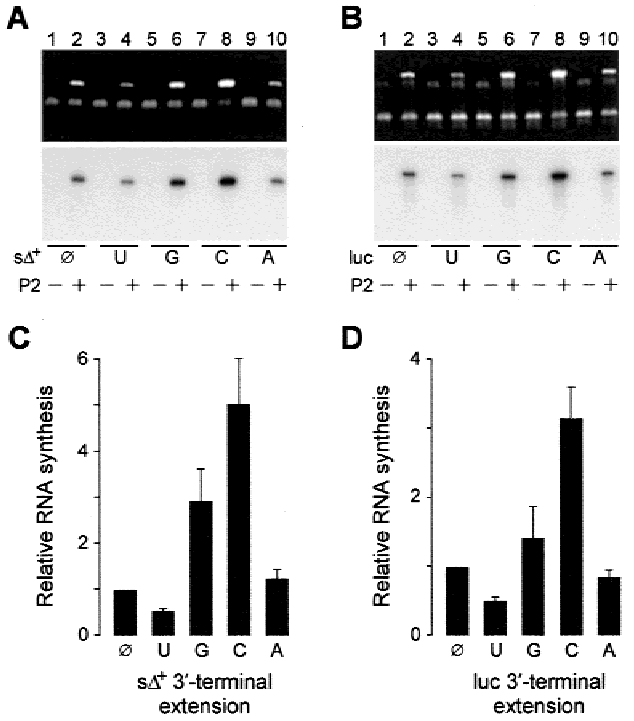

FIGURE 2. Effect of the 3′-terminal nucleotide of the template on the replication efficiency. **A:** Agarose gel analysis of the replication mixtures programmed with 70 μg/mL (0.32 μM) of different ssRNA templates: lanes 1 and 2: sΔ+(∅); lanes 3 and 4: sΔ+(U3′); lanes 5 and 6: sΔ+(G3′); lanes 7 and 8: sΔ+(C3′); and lanes 9 and 10: sΔ+(A3′). The templates were prepared by T7 transcription of PCR fragments amplified from pEM15 with the upstream primer ON1 and the appropriate downstream primer ON2, ON3, ON4, ON5, or ON6. Even lane numbers correspond to the reaction mixtures containing 0.5 μM of P2 polymerase, odd numerals are for the controls without polymerase. Both the ethidium bromide (EtdBr) staining (upper panel) and the autoradiogram (lower panel) are shown. **B:** A similar experiment carried out with five variants of the firefly luciferase mRNA (70 μg/mL, or 0.12 μM): lanes 1 and 2: luc(∅); lanes 3 and 4: luc(U3′); lanes 5 and 6: luc(G3′); lanes 7 and 8: luc(C3′); and lanes 9 and 10: luc(A3′). The templates were produced by T7 transcription of the PCR fragments amplified from pT7luc with ON1 and one of the ON7–ON11 primers. **C** and **D:** Phosphorimager analysis of the reactions presented in **A** and **B**, respectively. Bars show the template efficiency of different ssRNA variants normalized to that of the unmodified templates: sΔ+(∅) in **C** and luc(∅) in **D**. Results are the mean of three independent experiments ± SEM.

3′-dNTPs inhibit P2-directed replication

To serve as a sequencing enzyme, the polymerase must accept chain-terminating agents. For several RNA polymerases including those from *Escherichia coli* and bacteriophages T7 and Qβ (Kramer & Mills, 1978; Axelrod & Kramer, 1985), nucleotide-specific chain termination has been achieved using 3′-deoxyribonucleoside 5′-triphosphates (3′-dNTPs; Fig. 3A). We studied the

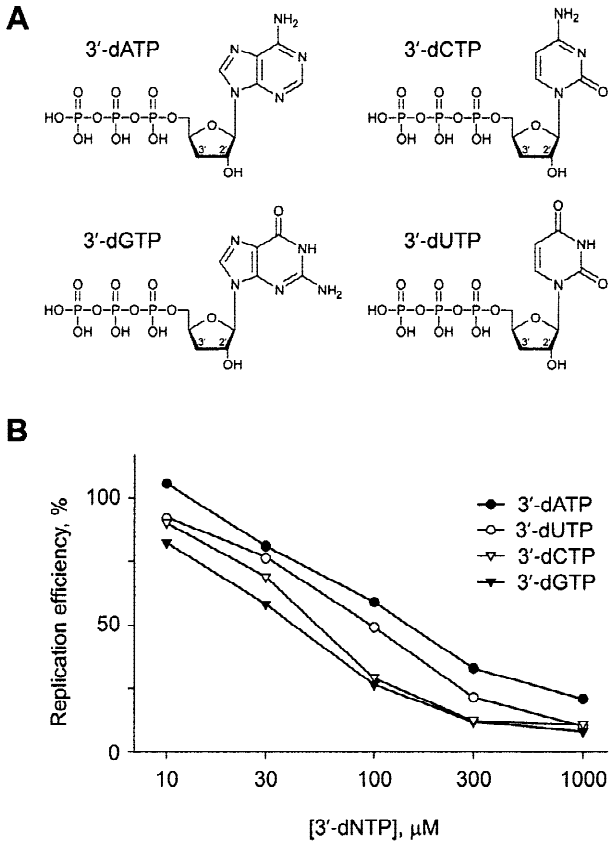

FIGURE 3. Inhibition of RNA replication by 3′-dNTPs. **A:** Chemical structures of the four 3′-dNTPs used in this study as chain terminators. **B:** P2 replication mixtures containing 100 μg/mL (0.42 μM) of sΔ− ssRNA template (T7 transcript of pEM16 cut with *Bpu*AI), 0.5 μM of P2, and 1 mM each of the four NTPs were incubated for 1 h at 30 °C in the presence of 10 μM to 1 mM of one of the four 3′-dNTPs. Replication efficiency was determined by TCA precipitation and plotted semilogarithmically as a function of 3′-dNTP concentration. 100% corresponds to the control replication without 3′-dNTPs.

TABLE 1. 3′-dNTP concentrations (μM) causing 50% inhibition of the ssRNA replication.[a]

ssRNA	3′-dATP	3′-dCTP	3′-dGTP	3′-dUTP
sΔ^-	150	50	40	100
luc(∅)	90	50	45	60

[a]Final concentration of each NTP in the reaction mixture was 1 mM.

effect of the four 3′-dNTPs on P2-catalyzed replication. All of them inhibited RNA synthesis in a concentration-dependent manner as measured by the decrease in the amount of the TCA-precipitable labeled products (Fig. 3B). Different 3′-dNTPs caused 50% inhibition at 40–150 μM concentrations under the conditions employed (Table 1). The inhibition was specific, as the addition of any of the four 2′,3′-ddNTPs up to 150 μM did not affect RNA synthesis (not shown). Regardless of the template 3′-terminal sequence, the inhibitory effect of 3′-dGTP or 3′-dCTP was reproducibly stronger than that of 3′-dUTP or 3′-dATP (Table 1). This might indicate that 3′-dNTPs act predominantly by terminating chain elongation rather than inhibiting the initiation step. The following experiments directly demonstrate the chain termination effect of 3′-dNTPs.

Sequencing of synthetic ssRNA templates

To assess the utility of the P2 polymerase for the RdRP sequencing, four reactions were set up, each containing one of the four 3′-dNTPs, and the reaction products were analyzed on a high-resolution PAGE under denaturing conditions. Figure 4 shows the resultant sequencing patterns obtained for three different RNA templates: sΔ^- containing native ϕ6 s$^+$ strand initiation signal (…UUUUUUCC3′); s$^+$/*Eco*47III, ϕ6 s$^+$ segment lacking the natural 158-nt-long 3′ end (the terminal sequence is …UGCCAUAAGC3′); and sΔ^+(G3′) containing ϕ6 (−)strand initiation signal extended with the 3′ G (…UCUCUCUCUG3′). The patterns are unambig-

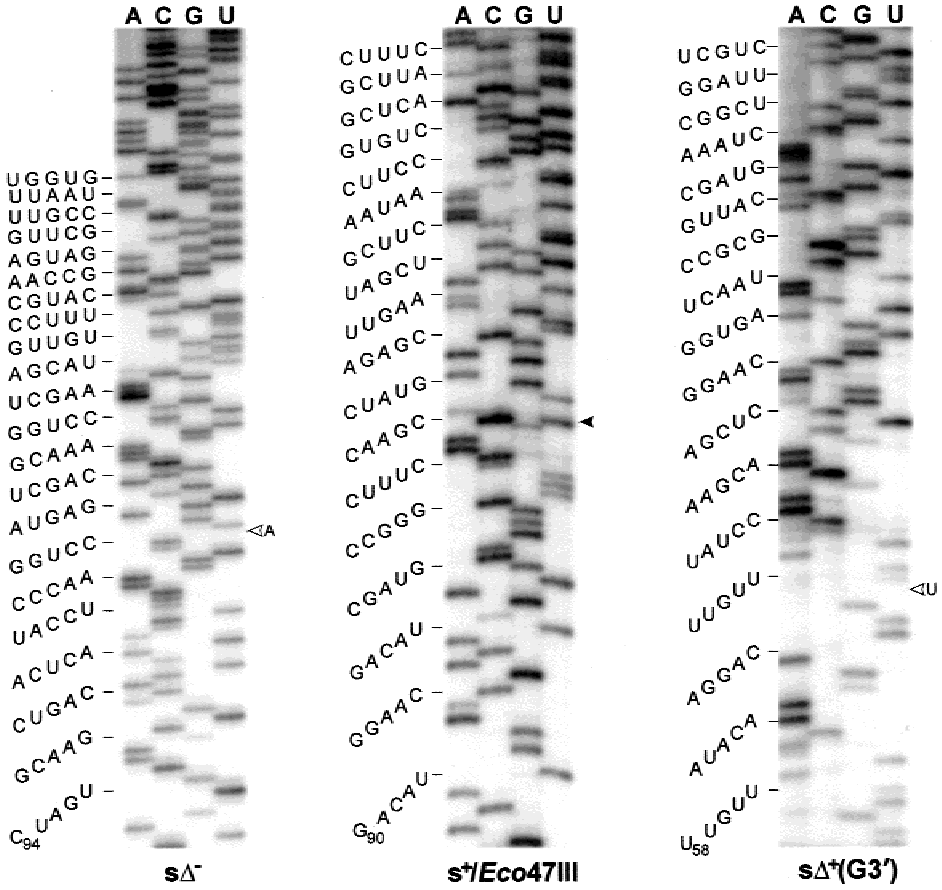

FIGURE 4. Sequencing of ϕ6-related RNAs using P2 RdRP. Aliquots of the P2 reaction mixtures containing base-specific 3′-dNTP chain terminators were analyzed by high-resolution denaturing PAGE to obtain RNA sequencing patterns. Three ssRNA templates were employed in this experiment: sΔ^- (25 μg/mL); s$^+$/*Eco*47III (100 μg/mL; produced by T7 transcription of pLM659 cut with *Eco*47III); and sΔ^+(G3′) (100 μg/mL). The final concentration of P2 was 0.5 μM. Sequence interpretation is indicated on the left. Numeral indices show the distance of nucleotide residues from the replication initiation site.

uously readable apart from occasional "compressed" regions (one of which is shown with the black arrowhead) and several cases of weak termination (indicated with the open arrowheads). Up to 200–300 nt could be determined in one sequencing experiment. Deduced sequences match the known sequence of the ϕ6 small RNA segment (GenBank accession number M12921). The only difference detected was a single-nucleotide insertion (UUGAAUAGCU in s^+/Eco47III instead of the expected UUGAUAGCU). However, this extra A was also found in pLM659 plasmid, the source of s^+/Eco47III, using the standard DNA sequencing with modified T7 DNA polymerase (Sequenase 2.0; USB). In addition to the shown results, satisfactory sequencing patterns were also produced using $s\Delta^+$, $s\Delta^+$(C3′), and $s\Delta^+$(A3′). $s\Delta^+$(U3′) gave only a poorly readable pattern due to the low signal-to-noise ratio.

The sequencing procedure was also applied to ϕ6-unspecific RNAs. Two different 3′-terminally modified luciferase RNAs were used for this purpose (Fig. 5): luc(C3′) extended with a single 3′ cytosine and luc3′end(s^-) containing nine additional nucleotides from the 3′ end of ϕ6 s^- segment (…UUUUUUUCC3′). Readable patterns were obtained in both cases allowing one to correctly determine the luciferase mRNA 3′ proximal sequence (GenBank #M15077). As expected, luc3′end(s^-) termination products were 8 nt longer than the corresponding luc(C3′) products (compare, e.g., the mobility of the bands marked with the asterisks). A legible pattern was also produced for the unmodified luciferase template luc(∅), although this required longer exposure to the X-ray film (not shown).

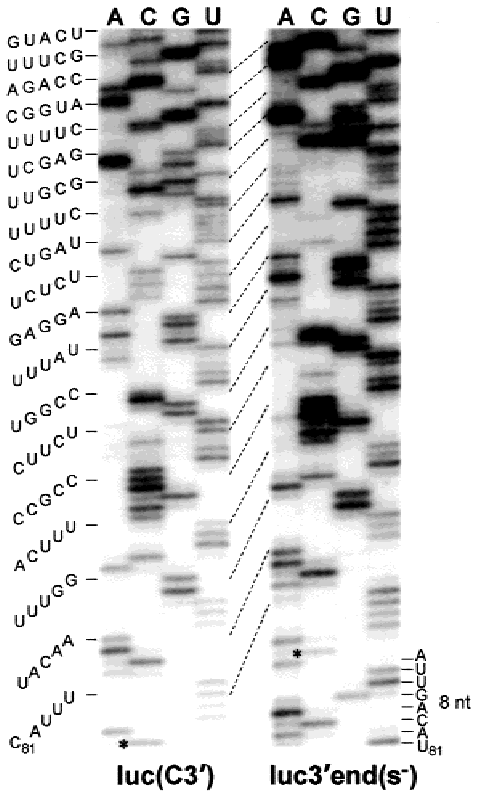

FIGURE 5. Sequencing of luciferase mRNA. Two variants of the firefly luciferase mRNA, luc(C3′) and luc3′end(s^-), were prepared by T7 transcription of the pT7luc PCR fragments amplified with the upstream primer ON1 and either of the downstream primers ON10 or ON12, respectively. The RNA (100 μg/mL) were sequenced with P2 polymerase (0.5 μM) and analyzed with PAGE. The luc3′end(s^-) RNA is 8 nt longer than luc(C3′), which accounts for the 8-nt shift in the sequencing patterns as shown with the dashed lines. Sequence reading is shown for the luc(C3′) (on the left), and for the 8-nt fragment of luc3′end(s^-) (on the right). Numeral indices show the distance of nucleotide residues from the replication initiation site.

Sequencing of viral dsRNAs

RdRP sequencing can only be used for templates with homogeneous 3′ ends. Therefore, one of the applications for this method might be determining the sequence of RNA viral genomes lacking the poly(A) tail. Among others, dsRNA viruses represent perhaps the most obvious target for the new sequencing method because they both have defined 3′ termini and, on the other hand, are relatively difficult to analyze using conventional sequencing strategies. Genomes of many dsRNA viruses are segmented (Fields et al., 1996), but individual segments can be purified by agarose or polyacrylamide gel electrophoresis and then used separately for the sequencing reactions. To test the utility of this approach, dsRNA segments of the bluetongue virus (BTV; serotype 1), an economically important member of genus *Orbivirus*, family *Reoviridae* (van Regenmortel et al., 2000) were separated on a 1% low melting agarose gel and purified using a standard phenol extraction procedure (Sambrook et al., 1989). BTV dsRNA segments encoding structural proteins VP5 and VP6 (Fig. 6A) were denatured at 100 °C for 1 min, chilled on ice, and added to the sequencing reaction. Since the (+) and (−) strands of orbiviruses differ in their 3′-terminal sequences (Mertens & Sangar, 1985), we rationalized that the P2 polymerase might more efficiently initiate at one of the two strands to produce a legible chain-termination pattern. Indeed, P2 initiated preferentially at the 3′ end of the (+) strands (ends in …ACUUAC3′) rather than the (−) strands (ends in …UUUAAC3′), allowing one to read the (−) strand sequences of the two BTV segments (Fig. 6B,C). Both patterns match corresponding GenBank sequences (M36713 and D10905). The experiment was also repeated with the BTV segment encoding structural protein VP7; and again a readable pattern for the (−) strand was obtained (not shown) that corresponded to the known sequence (accession number X53740).

DISCUSSION

The data presented here show that sequence of RNA molecules can be determined using a method called

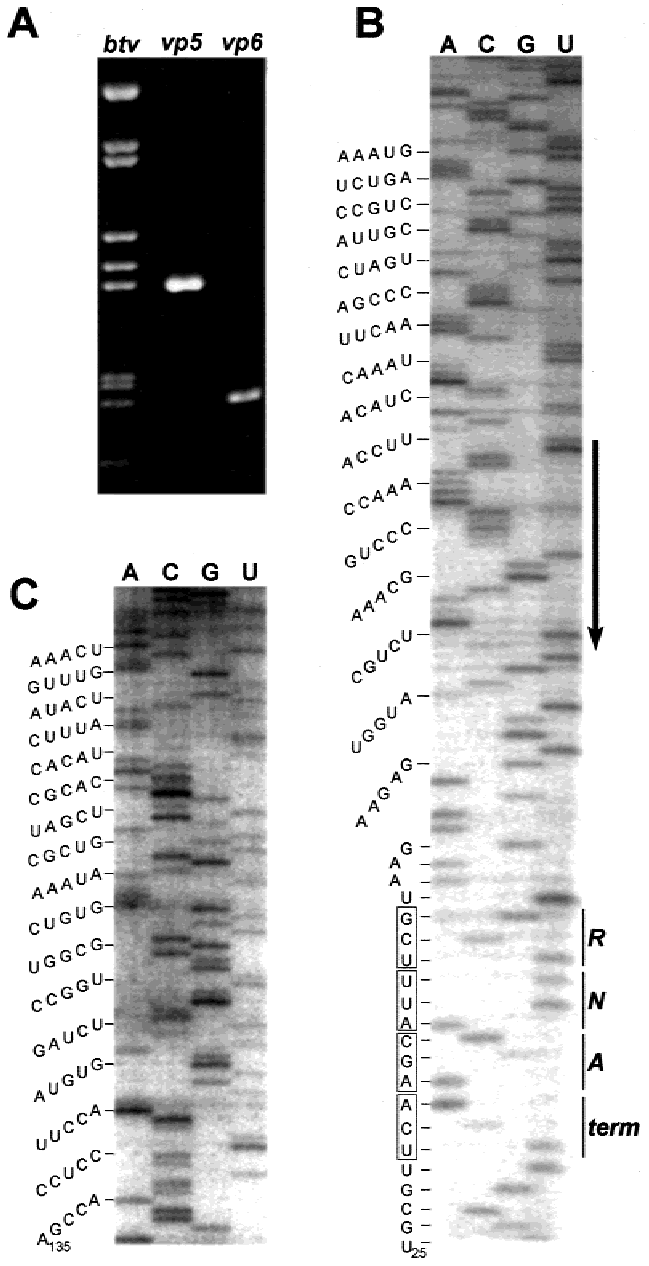

FIGURE 6. Sequencing of genomic RNA from bluetongue virus. **A:** Agarose gel electrophoresis of the total genomic dsRNA (200 ng) extracted from BTV virions (lane *btv*) along with the purified segments encoding viral proteins VP5 and VP6 (lanes *vp5* and *vp6*, ~100 ng and ~25 ng of the individual segments, respectively). The two segments were heat denatured and sequenced with P2 polymerase (0.25 μM). **B:** *vp5* sequencing pattern. The arrow on the right shows the direction of the VP5 open reading frame (ORF). Positions of the last three coding triplets are indicated with the one-letter code; *term* stands for the UGA stop codon. Each of the four sequencing lanes was produced using ~100 ng of the *vp5* template. **C:** *vp6* pattern. Each lane corresponds to ~25 ng of the *vp6* template. Sequence readings (shown on the left of the panels) correspond to the (−) sense strands. Numeral indices show the distance of nucleotide residues from the replication initiation site.

RdRP sequencing. This technique provides a fast and easy way to directly analyze unknown nucleic acid samples, bypassing the steps of primer design and synthesis, reverse transcription, and cloning. RdRP sequencing requires homogenous RNA templates with a uniform 3′ end. This suggests possible applications of the method in analyzing viral and perhaps nonpolyadenylated cellular RNAs.

Notably, we directly demonstrate the utility of the method for determining 3′-proximal sequences of the BTV genomic segments. Although it still remains to be tested whether or not RdRP sequencing can also be utilized for other types of RNA, the method is immediately suitable for the research and diagnostics of pathogenic orbivirus species. In this respect, we envision the RdRP sequencing approach to complement traditional techniques of molecular virology (Arens, 1999; Attoui et al., 2000). Figure 7 illustrates how the RdRP sequencing information can facilitate virus subtyping. The new method could be especially useful for fast characterization of unknown isolates when the lack of prior information hampers the primer-dependent techniques such as RT-PCR or Sanger sequencing.

In our hands, 100–400 ng of individual BTV segments were sufficient to produce the four sequencing lanes (Fig. 6). This amount of RNA can be readily obtained for BTV and other cultivable dsRNA viruses via amplification in the embryonating eggs or/and cell cultures (Clavijo et al., 2000). The sensitivity of RdRP sequencing might be further improved by changing the autoradiographic detection to fluorescent or chemiluminescent ones (McCormick et al., 1992; Sasaki et al., 1998). Our preliminary data show that P2 can incorporate some fluorescent and biotinylated nucleotide analogs into the RNA product. Other modifications may include utilization of 7-deaza-GTP to overcome the "compression" problem (Fig. 4 and not shown), as it has been described for the transcriptional sequencing technique (Sasaki et al., 1998). In addition, we have recently crystallized the ϕ6 P2 polymerase (Butcher et al., 2000), and obtained a high-resolution X-ray structure of the protein alone as well as complexed with the template and NTP substrates (Butcher et al., 2001). This information can be used to design a sequencing enzyme with superior properties.

MATERIALS AND METHODS

Plasmids

Plasmid pEM15 (Makeyev & Bamford, 2000b) was used to prepare sΔ^+ RNA and its 3′-terminally modified variants. RNAs sΔ^- and s$^+$/*Eco*47III were transcribed from pEM16 (Makeyev & Bamford, 2000b) and pLM659 (Gottlieb et al., 1992), respectively. Firefly (*Photinus pyralis*) luciferase mRNAs were produced from pT7luc (Kolb et al., 2000).

Preparation of ssRNA substrates

RNAs were prepared by in vitro transcription with T7 RNA polymerase as described (Makeyev & Bamford, 2000a). Tem-

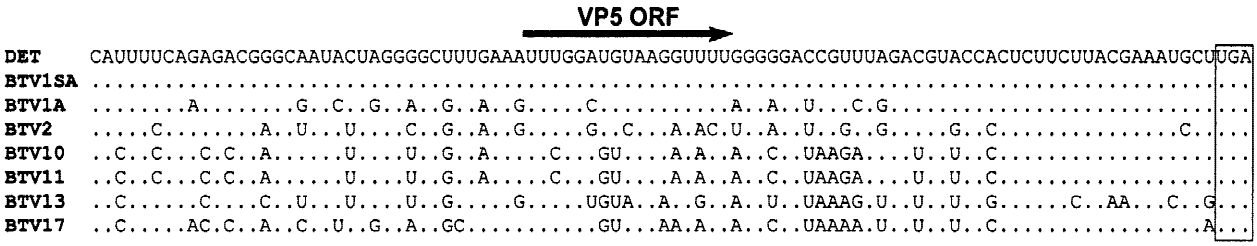

FIGURE 7. Alignment of the *vp5* gene fragment determined in Figure 6B (DET) with the sequences of *vp5* from the following BTV strains: serotype 1, South Africa (BTV1SA, GeneBank accession number M36713); serotype 1, Australia (BTV1A, M21845); serotype 2 (BTV2, X62283); serotype 10 (BTV10, D12532); serotype 11 (BTV11, U03285); serotype 13 (BTV13; X54308); serotype 17 (BTV17, X55359). The arrow shows the direction of the VP5 ORF. The stop codons are boxed. Note that the determined sequence is identical to the South African, but not Australian, strain of BTV1, thus indicating the origin of the virus used in the sequencing experiment.

plates for the T7 transcription were prepared by either cutting plasmid DNA with restriction endonucleases or by PCR amplification. For PCR, oligonucleotide ON1 (Table 2) containing the T7 promoter sequence was always used as the upstream primer. ON2 to ON6 served as downstream primers for the amplification of the Δs^+ fragment from pEM15. ON7 to ON12 were downstream primers to amplify the luciferase gene from plasmid pT7luc (Kolb et al., 2000). RNA preparations were dissolved in sterile water, and the RNA concentration was measured (A_{260}). The quality of each preparation was checked by electrophoresis in either a 5% polyacrylamide gel containing 7 M urea or a 1% agarose gel.

Replication assay

P2 polymerase was obtained from RNA-Line, Finland. RNA replication was assayed in 10 μL mixtures containing 50 mM Tris HCl, pH 8.9, 80 mM NH_4OAc, 6% (w/v) PEG4000, 5 mM $MgCl_2$, 1 mM $MnCl_2$, 0.1 mM EDTA, 0.1% Triton X-100, 1 mM each of ATP, CTP, GTP, and UTP (Pharmacia), 0.25 mCi/mL of [α-^{32}P]UTP (Amersham, 3,000 Ci/mmol) and 0.8 U/μL RNasin. Reactions were supplemented with 25–100 μg/mL of ssRNA templates. The P2 polymerase concentration was 0.5 μM. In the control experiments, the P2 preparation was replaced with an equal volume of buffer containing 50% glycerol, 50 mM Tris HCl, pH 8.0, 100 mM NaCl, 0.1 mM EDTA, 0.1% Triton X-100, and 0.4 mg/mL BSA. The mixtures were incubated at 30 °C for 1 h and analyzed by standard agarose gel electrophoresis (Makeyev & Bamford, 2000a). Replication efficiency was determined by phosphorimager analysis of the product bands. In some experiments, RNA synthesis was quantified by TCA precipitation followed by scintillation counting (Axelrod & Kramer, 1985).

TABLE 2. Oligonucleotides used in this study.

Name	Sequence
ON1	5′CGCGTAATACGACTCACTATAG
ON2	5′AGAGAGAGAGCCCCCGA
ON3	5′AAGAGAGAGAGCCCCCGA
ON4	5′CAGAGAGAGAGCCCCCGA
ON5	5′GAGAGAGAGAGCCCCCGA
ON6	5′TAGAGAGAGAGCCCCCGA
ON7	5′TAAGCTTGGGCTGCAGGT
ON8	5′ATAAGCTTGGGCTGCAGGT
ON9	5′CTAAGCTTGGGCTGCAGGT
ON10	5′GTAAGCTTGGGCTGCAGGT
ON11	5′TTAAGCTTGGGCTGCAGGT
ON12	5′GGAAAAAAATAAGCTTGGGCTGCAGGT

RdRP sequencing

The sequencing reactions (5–10 μL) were carried out in principle as described above for the replication assay with some modifications. The mixtures contained 1 mM ATP, 0.2 mM CTP, 1 mM GTP, and 0.02 mM of unlabeled UTP as well as 0.5–1 mCi/mL of [α-^{32}P]UTP. To obtain the nucleotide-specific termination products, the mixtures were supplemented with 300 μM 3′-dATP (Sigma), 20 μM 3′-dCTP, 80 μM 3′-dGTP, or 4–8 μM 3′-dUTP (Biolog, Germany). After incubation for 1 h at 30 °C, the reactions were stopped by adding 9 vol of 2.5 M NH_4OAc, 100 mM EDTA, and precipitated with ethanol. Alternatively, sequencing reactions were passed through AutoSeq G-50 spin columns (Pharmacia) preequilibrated with water and vacuum dried. The RNA pellets were dissolved in sample buffer (95% formamide, 10 mM EDTA, 0.05% bromophenol blue, and 0.05% xylene cyanol FF), incubated at 100 °C for 2 min and then analyzed on a 6% polyacrylamide gel containing 7.5 M urea.

ACKNOWLEDGMENTS

We thank Sarah Butcher for the critical reading of the manuscript and Peter Mertens for providing genomic RNA of BTV. Expert technical assistance by Riitta Tarkiainen and Marja-Leena Perälä is greatly appreciated. This work was supported by the Academy of Finland “Finnish Centre of Excellence Program 2000-2005” (162993, 164298), TEKES (4509024), and the European Union (BIO4-CT97-2364).

Received October 12, 2000; returned for revision November 17, 2000; revised manuscript received March 1, 2001

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