Reverse transcription, amplification and sequencing of poliovirus RNA by Taq DNA polymerase

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Abstract A model for virion RNA of the poliomyelitis virus, which does not pass the stage of DNA copies during biogenesis, demonstrates that Taq DNA polymerase is capable of synthesizing 960-bp cDNA with the specific primer. When comparing the nucleotide sequence of the starting virion RNA and recombinant DNAs, isolated from several independent clones, copying and amplification of virion RNA appear accurate (one substitution per 960 bp). A comparison of Taq and Tth DNA polymerases in RT/PCR indicates that the sensitivity of Taq polymerase seems to be two orders of magnitude higher than that of Tth polymerase. The RNA detection level under the chosen conditions approached 10^4 RNA copies per test. The present investigation indicates the great versatility of Taq polymerase, which promoted the reverse transcription reaction of RNA, cDNA amplification, screening of recombinant clones as well as sequencing of recombinant DNA. Thus application of Taq polymerase is rather promising not only to detect nucleic acids in biological samples, but also for isolating and cloning individual genes, encoded on DNA and/or on RNA templates.

Key words: RNA; cDNA; Taq polymerase; Reverse transcription; Sequence; Amplification; Gene cloning

1. Introduction

The thermostable DNA polymerase discovered by Chien [1] in 1976 became widespread in the polymerase chain reaction (PCR) [2,3]. The highly sensitive PCR method is currently applied to analyze not only DNA molecules, but also RNA preparations isolated from a great variety of sources. The universal approach used to establish gene expression [4], subcloning [5] and to diagnose RNA viruses [6] through reverse transcription and amplification (RT/PCR) includes two stages: (a) cDNA synthesis on the RNA template by mesophilic reverse transcriptase (AMV, M-Mlu), (b) amplification of the cDNA fragment limited with specific primers induced by Taq DNA polymerase [7]. The approach was further improved by running two enzymatic reactions, one after the other, with a common buffer run in one tube [8]. The researchers had to overcome certain difficulties to obtain appropriate results: fast RNA degradation, need for use of RNAs inhibitors, which decreased PCR efficacy, high lability of viral reverse transcriptase as well as a complex secondary structure present in RNA molecules considerably impeding the synthesis of full-size cDNA molecules [7].

All these factors promoted the search for thermophilic enzymes capable of synthesizing DNA copies on RNA templates. First attempts to study RT activity of native Taq DNA polymerase revealed that the enzyme displayed the polymerase activity in poly(rA)-oligo$(dT)_{12}$ as a template, but it did not detect cDNA synthesis with the mRNA-oligo$(dT)_{12}$ primer or with poly(rC)-oligo$(dG)_{12-18}$ as a template [9]. In 1989, Jones and Foulkes [10], who were the first to utilize coupled reverse transcription and amplification reaction by Taq polymerase, synthesized an amplified 358 bp long mRNA fragment of glucose-6-phosphate dehydrogenase (G6PD). Although the authors used high concentrations of cellular RNA (up to 5 μg per 20 μg of incubation mixture), the yield was low. Besides, it was not specific enough due to a weak signal at Southern blot hybridization. Tse and Forget [11] also investigated RT activity of Taq polymerase in the coupled RT/PC reaction to detect expression of the spectrin gene in human peripheral blood cells. They proved the enzyme to be capable of catalyzing specific synthesis in RT/PCR using 40 ng of the starting RNA template. But in this case hybridization should be performed with a labelled probe to detect specific reaction products.

Myers and Gelfand [12] tried to analyze RT activity as well. They were the first to propose the use of Mn^{2+} ion in the RT reaction, and of Mg^{2+} ion in PCR. The maximum length of fragments obtained by the investigators did not exceed 500 bp. According to comparative analysis of RT activities of two thermostable DNA polymerases, recombinant Tth polymerase (cellular RNA as template and artificial RNA templates obtained by T7 RNA polymerase) was 100-fold more efficient than Taq polymerase. However, other researchers [13] did not reveal significant differences, when comparing reverse transcriptase activities of thermophilic polymerases.

Unfortunately, the above experiments lacked any high grade analysis of RT/PCR products, such as comparative analysis of nucleotide sequences of amplified cDNAs and parent RNAs. In addition, there was no negative control (RNase treatment of RNA preparations), which makes the results less reliable.

To avoid these shortcomings, in this study we used virion RNA as a model RNA template. As is well known, its RNA is never present with DNA copies in nature [14], and this in turn totally excludes pseudo-positive results in RT/PCR. Besides, the nucleotide sequence of poliomyelitis virus [15] enabled comparison of nucleotide sequences of cloned cDNA fragments after RT/PCR and the starting poliovirus RNA. With highly purified virion RNA, the sensitivity of the above method can be established very accurately. We utilized this enzyme not only to synthesize cDNA copies of virion RNA in the RT reaction, and for their amplification, but also to screen recombinant clones and to sequence recombinant DNA.

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Abbreviations: RT, reverse transcription; PCR, polymerase chain reaction

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As was shown earlier [16,17], a functionally active gene for glycoprotein of rabies virus might be isolated using virion RNA of the given virus as a template, and/or by AMV reverse transcriptase, Taq polymerase and PCR amplification. Taq polymerase in RT/PCR can copy the virion RNA fragment containing about 1000 nucleotide bases thus promoting isolation of active genes at minimum amounts of virion RNA (up to 10^4 copies) or total preparations of cellular RNA. The activity of Taq and Tth polymerases was compared to characterize Taq polymerase RT activity in detail.

2. Materials and methods

2.1. Viral and bacterial strains and plasmids

Poliomyelitis virus (Mahoney strain) was reproduced in a cell culture from green monkey kidney (CGMK). After total culture degeneration, the virus-containing suspension was purified to obtain the virus, and virus RNA was extracted as described [18]. Virion RNA was centrifuged before RT/PCR, then vacuum-dried and dissolved in sterile DEP-H_2O at a concentration of 0.2 μg/μl. *E. coli* DH5 served as a bacterial strain grown in LB medium. Plasmid pBluescript II SK(+) was utilized as a bacterial vector (Stratagene, La Jolla, CA, USA).

2.2. Enzymes, oligodeoxynucleotides, nucleotides

Recombinant Taq and Tth DNA polymerases, DNA ligase of phage T4, T4-polynucleotide kinase, and restriction endonucleases *Kpn*I and *Bam*HI were obtained in the Biotechnology laboratory of the Shemyakin and Ovchinnikov Institute of Bioorganic Chemistry, Russia. Deoxynucleoside triphosphates and dideoxynucleoside triphosphates (Boehringer-Mannheim GmbH, Germany), [γ-^{32}P]ATP (GNC, FSI, Obninsk, Russia), oligodeoxynucleotides: *Kpn*I primer (5′-GCTGGTACCAGTCCCATATGC-3′), *Bam*HI primer (5′-GTATCCGTCGAAGTGTGATGGATCCG-3′), F primer (5′-TGTAAAACGACGGCCAGT-3′), R primer (5′-CACAGGAAACAGCTATGAC-3′), and *Seq*I primer (5′-GGTGACCAGTACCATCACTG-3′) were synthesized on a model 308002 DNA synthesizer (Applied Biosystems, Beckman, USA) and purified by PAG electrophoresis.

2.3. RT/PC reaction

The RT mixture (20 μl) contained 67 mM Tris-HCl (pH 8.8), 16.6 mM $(NH_4)_2SO_4$, 0.25 mM each of deoxynucleoside triphosphates (dATP, dCTP, dGTP, dTTP), 2 mM $MnCl_2$ or 1.5 mM $MgCl_2$, 20 pM *Bam*HI primer (reverted), 5 units of Taq or Tth DNA polymerase and variable amounts of virion RNA of poliovirus (0.2 μg to 2 pg). RNA template was added to the RT mixture heated to 60°C, 40 μl of mineral oil was then overlaid and incubated for 3 min at 60°C, the process continued for 15 min at 70°C. Following the RT reaction, 80 μl of PCR mixture was added, containing 67 mM Tris-HCl (pH 8.8), 16.6 mM $(NH_4)_2SO_4$, 0.01% Tween-20, 0.75 mM EGTA, 0.25 mM each of the four deoxynucleoside triphosphates, 2 mM $MgCl_2$ and finally 20 pM *Kpn*I of the primer (direct). The general incubation mixture (100 μl) was then amplified in a DNA Thermal Cycler (Perkin-Elmer Cetus Instruments) as follows: 1 min at 94°C, 1 min at 56°C, and 1.5 min at 72°C for 35 cycles. Aliquots (5 μl) were analyzed by electrophoresis on 1% agarose gel.

2.4. General cloning methods

RT/PCR incubation mixture containing the cDNA amplified fragment was extracted with chloroform and precipitated with 3 volumes of ethanol. The amplified fragment and plasmid pBluescript II SK(+) were hydrolyzed with restriction enzymes *Bam*HI and *Kpn*I, and ligated with T4 DNA ligase. *E.coli* DH5 cells were transformed according to Maniatis et al. [19]. The recombinant clones were screened as described in [20] by amplification of the inserted fragment by PCR and subsequent electrophoresis in 1% agarose gel. The recombinant clones were further utilized to determine the nucleotide sequence.

2.5. Sequencing

DNA recombinant sequencing was carried out by Taq polymerase according to [21], with some modifications. (a) Kinase mixture. Primer labelling performed in the kinase mixture (10 μl) containing 100 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 10 mM DTT, 30 μCi [γ-^{32}P]ATP (5000 Ci/mM) 5–10 pM primers and 2.5 units of T4 polynucleotide kinase. The mixture was incubated for 30–60 min at 37°C. It was heated in a boiling bath for 2 min to achieve inhibition. The incubation mixture contained kinase mixture (3–5 μl), recombinant DNA (1–5 μg), 1 μl 10×Taq buffer (50 mM Tris-HCl (pH 8.8), 50 mM $MgCl_2$), 2–5 units Taq DNA polymerase and 2.0 μl terminating mixture. Four tubes (A, C, G, T) were filled with PCR mixture. The terminating mixture was overlaid with mineral oil (15 μl), and amplified as follows: 97°C for 3 min, 56°C for 1 min, 72°C for 1 min, 94°C for 45 s; 20 cycles. Sequences of the ply2 and ply12 inserts in both the 3′ and 5′ directions were obtained.

3. Results and discussion

Earlier studies of the reverse transcriptase activity of thermophilic DNA polymerases testified to the significance of the ion composition and the ionic strength for effective cDNA synthesis with these enzymes [22]. We utilized two different ion compositions of the incubation mixture to run a combined RT/PCR on the poliovirus RNA: (a) using Mn^{2+} at the reverse transcription stage and Mg^{2+} in PCR, and (b) using standard buffer for amplification with 1.5 mM $MgCl_2$. The 960 bp specific DNA fragment was observed only in Mn/Mg-containing ion systems. Such a selectivity at the RT stage was typical of Taq and Tth DNA polymerases. With Taq polymerase, the specific product was obtained in higher amounts, but shorter cDNA molecules were not synthesized. The experiments on detection of the RNA of the tick-borne encephalitis virus and mRNA of avian adenovirus CELO provided similar results (data not shown). In the following we compare the reverse transcriptase activity of Taq and Tth polymerases using different amounts of virion RNA template (5–200 ng) (Fig. 1). The amount of the product in RT depended primarily on the concentration of the starting RNA for Tth DNA polymerase (Fig. 1, lanes 1–4), but addition of the template (5 ng) to the sample revealed that the 960 bp fragment was hardly detectable on agarose gel. By contrast,

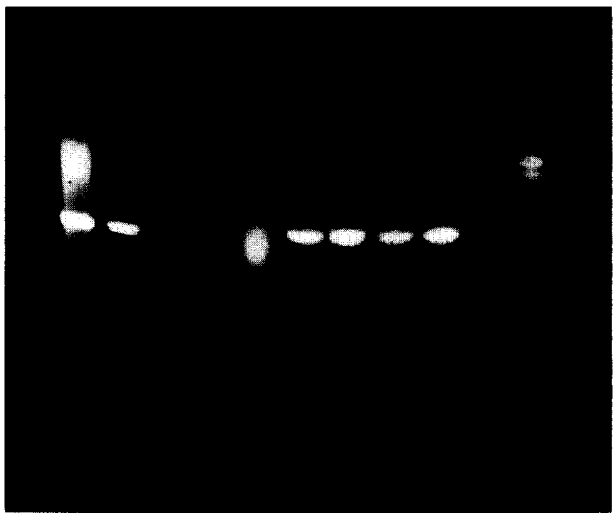

Fig. 1. Reverse transcription and PCR amplification of the poliomyelitis virus RNA fragment (960 bp) by Tth (1–5) and Taq (6–10) DNA polymerases at different amounts of virion RNA in the reaction mixture: 200 ng (1, 6), 50 ng (2, 7), 20 ng (3, 8), 5 ng (4, 9). Lanes 5, 10: control of the reaction specificity with direct primer at the RT stage. Lane II: marker of molecular mass (the *Taq*I digest of pBR322 DNA).

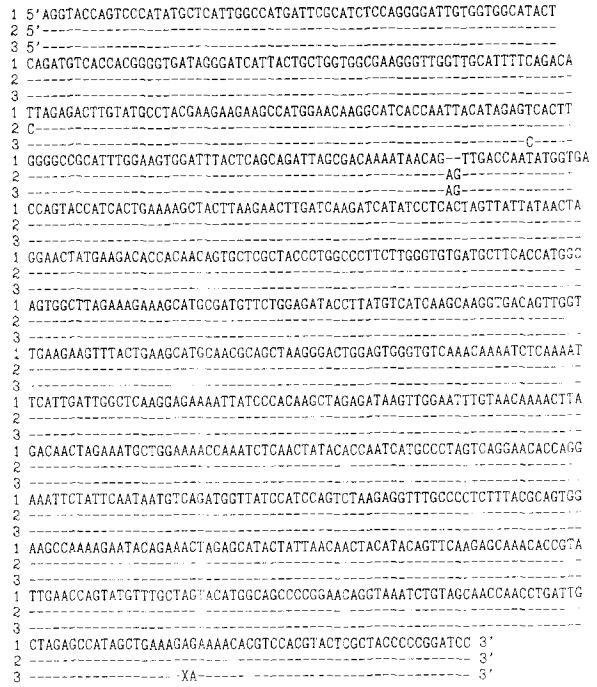

Fig. 2. Comparative analysis of nucleotide sequences. 1: Nucleotide sequence of the genome fragment of poliovirus from bp 3630 to bp 4576 from the 5′ terminus [16]. 2: Nucleotide sequence of the recombinant DNA isolated from clone ply2. 3: Nucleotide sequence of the recombinant DNA isolated from clone ply12. X indicates that the given nucleotide base (A) is absent.

under the same conditions Taq polymerase displayed much higher activity (100-fold as much) (Fig. 1, lanes 6–9), as a result up to 20 μg of the cDNA fragment was obtained from 5 ng of poliovirus RNA.

Unlike Myer et al. [12], we used a more alkaline RT solution (Tris-HCl, pH 8.8) and higher concentrations of Mn^{2+} (2 mM) and dNTPs (250 μM), $(NH_4)_2SO_4$ was used instead of KCl. The PCR buffer composition was slightly changed by using 0.01% Tween-20 instead of glycerol. The higher activity of Taq polymerase in RT/PCR as compared with Tth polymerase is induced by the peculiarities of the RNA template and the greater sensitivity of Tth polymerase to impurities in RT/PCR and also by much faster cDNA synthesis by Taq DNA polymerase. The RT/PCR sensitivity displayed by Tth polymerase was greater by one order of magnitude than that reported by other authors [10–12]. It can be compared with the sensitivity displayed by reverse transcriptase of viruses and of Taq polymerase in the coupled RT/PCR [6] as well as with the detection level of the radioactive hybridization signal [23]. The Taq polymerase gave a detectable signal with 10^4 copies of target RNA of poliomyelitis virus, when analyzing the specific cDNA fragment by electrophoresis on agarose gel. To synthesize cDNA, we chose the virion RNA fragment located between the restriction sites *Kpn*I and *Bam*HI, it was used for cloning the amplified fragment. At the same time, the 960 bp fragment is of much interest as a model for synthesis and isolation of individual viral, bacterial and cellular genes by Taq DNA polymerase.

The specificity of the product obtained in RT/PCR was later confirmed by sequencing. To analyze recombinant clones, we applied the PCR technique proposed by Simon et al. [20]. The procedure makes it possible to use bacterial cells from fresh-cultivated clones. As a result, the technique accelerates the search for necessary clones and simplifies their assay as well. Since 1988, Taq polymerase has been in wide use for DNA sequencing [21,24–26], whereas double-stranded DNA can be directly sequenced without a prior denaturation step. Furthermore, the primer specificity increases, and the problem of GC-rich DNA region sequencing is solved. Taq polymerase, unlike Sequenase, is characterized by high stability and can be compared with this enzyme by the number of errors upon sequencing [25].

Depending on the enrichment of the DNA template with AT pairs or GC bases during this process Taq polymerase might initiate discrimination against the insertion of certain dideoxynucleoside triphosphates [25]. We also tried to optimize terminating mixtures to sequence cloned cDNA copies of the virion RNA.

Using the F and R primers complementary to the region of pBluescript II DNA which flanks the insertion site as well as the *Seq*I primer complementary to the recombinant DNA sequence we succeeded in establishing the nucleotide sequence of the DNA fragment inserted into the vector.

Additionally, comparative analysis was performed with the nucleotide sequence of polioviral genome [15] (Fig. 2). Comparative analysis of the nucleotide sequence of recombinant DNA isolated from clone ply2 showed that the sequence differed in one C for T substitution and insertion of two AG nucleotides. The nucleotide sequence of recombinant DNA isolated from clone ply12 differed in two nucleotide substitutions (C for T in one case and A for G in the other), one deletion of nucleotide base A, and, as in case with clone ply2, the insertion of two nucleotide bases AG. According to other researches [27] the independent property of the above substitutions occurs due to amplification. The insertion of two nucleotide bases AG revealed upon sequencing of several independent clones confirmed its presence in the poliovirus genome of the given strain. Copying of the virion RNA by Taq polymerase is characterized with high accuracy and can be compared with commonly used reverse transcriptase of avian myeloblastosis virus.

Thus the RT/PCR method can be applied to diagnose genetic disorders and viral diseases, as well as for isolation and cloning of individual genes from any RNAs obtained from bacteria and viruses.

We regard Taq polymerase as unique, reliable, and highly efficient. Besides, it is capable of replacing reverse transcriptase in RT, and Sequenase in sequencing.

Diagnosis of viral diseases, in particular those causing death and epidemics, should be fast and reliable. Taq DNA polymerase successfully demonstrated all these properties, providing its application for express detection of viruses of RNA and DNA origin.

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