RNA-primed PCR.

H Shibata, T Tahira and K Hayashi

Genome Res. 1995 5: 400-403
Access the most recent version at doi:10.1101/gr.5.4.400

PCR METHODS AND APPLICATIONS

RNA-primed PCR

Hiroki Shibata, Tomoko Tahira, and Kenshi Hayashi[1]

Division of Genome Analysis, Institute of Genetic Information, Kyushu University, Maidashi 3-1-1,
Higashi-ku, Fukuoka 812-82, Japan

We show that RNA can serve as a primer in PCR. Use of r*Tth* DNA polymerase is essential because it has strong reverse transcriptase activity. RNA primers can be obtained by in vitro transcription and are less costly than DNA primers, which are chemically synthesized. RNA-primed PCR also opens the possibility that a specific amplification reaction can be achieved in the absence of knowledge of the target nucleotide sequence.

PCR is a widely used technique to amplify DNA regions that are bracketed by two oligonucleotide primers of known sequences.(1) Usually, those primers are DNA, which are chemically synthesized. With the technological progress of automated DNA synthesizers, the cost of the primer synthesis remains a major part of the expense of the projects that involve extensive use of PCR. Also, previous knowledge of nucleotide sequences of primer regions is a prerequisite for the synthesis of specific primers.

We show here that in vitro-transcribed oligoribonucleotides can serve as primers in PCR. In principle, any unknown nucleotide sequences on DNA can be copied in quantity as single-stranded RNAs by attaching phage promoters and transcribing them in vitro.(2,3) Thus, RNA-primed PCR opens the possibility of amplifying target sequences inexpensively without previous knowledge of primer sequences.

MATERIALS AND METHODS

Templates and Chemically Synthesized Primers

Two cloning vectors, pGEM2 and pBluescript SK(+), served as templates for primer extension and RNA-primed PCR experiments (Fig. 1). Four DNA primers, SP6-30, G410, T7-30, and BS430, and one RNA primer, RS28, were chemically synthesized (Table 1). All primers were gel-purified by the standard protocols.(4)

RNA Primers Synthesized by In Vitro Transcription

An RNA primer, RGB32, was enzymatically synthesized from *Bam*HI-digested pGEM2 as a template (Fig. 1A; Table 1) using an in vitro transcription kit (RiboMAX large-scale RNA production system-SP6, Promega). RGB32 included the entire sequence of the chemically synthesized RNA primer RS28 and an additional four nucleotides at the 3′ end (Table 1). Another RNA primer, RSX30, was also obtained by in vitro transcription using a kit (RiboMAX large-scale RNA production system-T7, Promega) with *Xho*I-digested pBluescript SK(+) serving as a template (Fig. 1B; Table 1). Both in vitro transcription reactions were performed using 2.5 μg of linearlized plasmid DNA serving as templates in a reaction mixture of 50 μl that contained a trace amount of [α-^{32}P]UTP (3000 Ci/mmole; Amersham). After phenol extraction and ethanol precipitation, the transcripts were separated by electrophoresis in 10% polyacrylamide gel containing 7 M urea. Products of expected lengths (detected by autoradiography) were eluted from the gel and recovered by the standard procedures.(4) Primary transcripts showed considerable heterogeneity,(2,3) and 100–500 pmoles of purified RNA primers could be recovered from each 50-μl in vitro transcription reaction. However, this amount was sufficient for >20 reactions of PCR.

Primer Extension

*Pvu*II-digested pGEM2 served as a template for chain elongation reactions using RNA and DNA primers. The reactions were performed in 7.5 μl (Sequenase reaction) or 10 μl (all other reactions), which contained 0.6 μM of the primer and 0.8 μg of the template, using the following enzymes in various conditions: (1) Sequenase version 2.0 DNA polymerase (Amersham) in the sequencing conditions [37°C for 5 min in 26.7 mM Tris-HCl (pH 7.5), 33.3 mM NaCl, 13.3 mM $MgCl_2$, 6.7 mM DTT, and Sequenase version 2.0 DNA polymerase at 0.2 U/μl]; (2) *AmpliTaq* DNA polymerase (Perkin Elmer Cetus) under PCR conditions [74°C for 5 min in 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2.5 mM $MgCl_2$, and *AmpliTaq* DNA polymerase at 0.1 U/μl]; (3) r*Tth* DNA polymerase (Perkin-Elmer Cetus) in the condition of reverse transcription [70°C for 5 min in 10 mM Tris-HCl (pH 8.3), 90 mM KCl, 1 mM $MnCl_2$, and r*Tth* DNA polymerase at 0.25 U/μl]; and (4) r*Tth* DNA polymerase in the PCR condition [74°C for 5 min in 10 mM Tris-HCl (pH 8.3), 98 mM KCl, 4% glycerol, 0.04% Tween 20, 0.6 mM EGTA, 0.2 mM $MnCl_2$, 2 mM $MgCl_2$, and r*Tth* DNA polymerase at 0.05 U/μl]. All reactions also contained 40 μM dGTP, 40 μM dATP, 40 μM TTP, 4 μM dCTP, and 3 μCi of [α-^{32}P]dCTP (3000 Ci/mmole; Amersham). Denaturing and annealing conditions were at 100°C for 2 min followed by chilling on ice. The internally labeled products were analyzed by urea–polyacrylamide gel electrophoresis and autoradiography following the standard procedures.(4)

RNA-primed PCR

RNA-primed PCR was performed in 10

---

[1] Corresponding author.
E-MAIL khayashi@gen.kyushu-u.ac.jp; FAX 81-92-632-2375.

5:400–403 ©1995 by Cold Spring Harbor Laboratory Press ISSN 1054-9803/95 $5.00

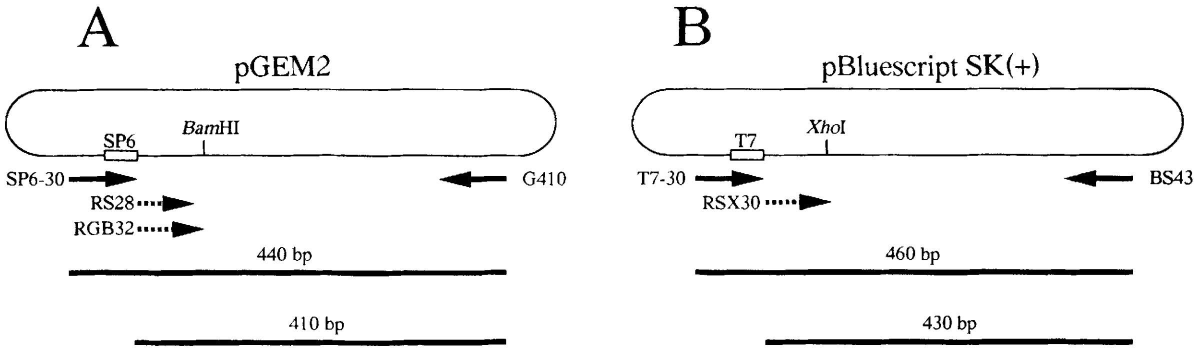

FIGURE 1 Locations of primers on template plasmids. (*A*) pGEM2 and its four primers are shown. RGB32 was obtained by an in vitro runoff transcription of *Bam*HI-digested pGEM2. (*B*) pBluescript SK(+) and its three primers are shown. RSX30 was obtained by in vitro runoff transcription of *Xho*I-digested pBluescript SK(+). DNA primers are shown by arrows. RNA primers are shown by broken lines with arrowheads. Transcription promoters are represented by open boxes. Expected PCR products and their lengths are indicated below.

mM Tris-HCl, 90 mM KCl, 1 mM $MnCl_2$, 200 μM of each of the four dNTPs, and 0.25 U/μl of r*Tth* DNA polymerase in a 5-μl reaction mixture using 10 pg to 1 ng of pGEM2 or pBluescript SK(+) serving as templates and 0.6 μM of each of the oligoribonucleotides serving as primers. The mixture was subjected to 20 cycles of 94°C for 30 sec and 70°C for 3 min in GeneAmp PCR System 9600 (Perkin-Elmer Cetus). The PCR products were resolved by electrophoresis in 1.5% agarose gel and stained by ethidium bromide.(4)

RESULTS AND DISCUSSION

Amplification using RNA as primers in PCR requires two reactions that are not involved in conventional DNA-primed PCR: (1) DNA chain elongation must start from RNA primers at the beginning of the reaction in each thermal cycle; and (2) the chain elongation must be completed by reverse transcribing RNA template, again in each thermal cycle.

We first examined the priming activity of an RNA primer using various DNA polymerases in various conditions (Fig. 2B). Chain elongation starting from RNA primer was observed in two combinations, by Sequenase (Amersham) in the sequencing condition (lane 2) and by r*Tth* DNA polymerase (Perkin-Elmer Cetus) in the RT condition (lane 6), although elongation from RNA primer was always less efficient than that from DNA primer (lanes 1,3,5,7).

Successful chain elongation from RNA primer and the fact that r*Tth* DNA polymerase possesses strong reverse transcriptase activity(5) encouraged us to perform PCR using r*Tth* DNA polymerase in the reverse transcription condition with RNA as one of the primers. As shown in Figure 3, amplification from pGEM2 using RNA/DNA primer pairs RS28/G410 (lane 2) and RGB32/G410 (lane 3) yielded bands of the same expected length (410 bp), which was slightly shorter than the band of the product of a DNA/DNA primer pair, SP6-30/G410 (440 bp, lane 1). Similar results were obtained using pBluescript SK(+) as a template, that is, 430-bp product from an RNA/DNA primer pair RSX30/BS430 (lane 5) and 460-bp product from a DNA/DNA primer pair T7-30/BS430 (lane 4). None of the products were observed when the reactions were performed without template DNA. We confirmed that the products were resistant to exonuclease I (data not shown), and therefore, to double-stranded DNA. Direct sequencing of the PCR products after purification (Prep-A-Gene, Bio-Rad) also revealed that the fragments had the expected nucleotide sequences (data not shown).

We sought factors critical for RNA-primed PCR by running the reaction under various conditions. The presence of appropriate concentration of manganese ion seems to be critical, as no product was observed above 2 mM or below 0.5 mM $MnCl_2$ (data not shown). This may reflect requirement of rather narrow range of Mn^{2+} concentration in steps of either initiation of chain elongation from RNA primer or reverse transcription of RNA template, both of which are essential in RNA-primed PCR. Use of Mn^{2+} instead of Mg^{2+} is known to reduce the fidelity of some thermoresistant DNA polymerase.(6) We did not examine this point in detail, but the quality of the ladder of direct sequencing of RNA-primed PCR product suggested that at least the majority of the product carried faithful sequence. Extension steps shorter than 3 min resulted in dramatic reduction of the product, perhaps suggesting slow reactions during initial chain elongation or reverse transcription.

Efficiency of amplification in RNA-primed PCR seems to be low, as judged from more cycles needed than in the conventional PCR. Still, RNA-primed PCR was successful starting from as little as 10 pg of template plasmid DNA simply by increasing number of thermal cycles to 40. Thus, concentration of template DNA is not critical to obtain faithful amplification products in RNA-primed PCR (Fig. 4). In those experi-

Table 1. DNA/RNA primers

Template	Name	Sequence (5'-3')	Chemistry
pGEM2	SP6-30	CATACACATACGATTTAGGTGACACTATA	D
	RS28	GAAUACACGGAAUUCGAGCUCGCCCGGG	R
	RGB32	GAAUACACGGAAUUCGAGCUCGCCCGGGGAUC	R
	G410	GTCGGGTTTCGCCACCTCTGACTTGAGCTG	D
pBluescript SK(+)	T7-30	GGCCAGTGAATTGTAATACGACTC	D
	RSX30	GGGCGAAUUGGGUACCGGGCCCCCCCUCGA	R
	BS430	ACGACCGAGCGCAGCGAGTCAGTGAGCGAG	D

ments, similar levels of products were observed using either linear or circular plasmid DNA as a template. The amplification was also successful in the presence of excess human DNA (10 pg of pGEM2 and 1 ng of human genomic DNA), although smeared background was sometimes observed (data not shown). There seems to be no clear-cut upper limit in the length of target in RNA-primed PCR. A target fragment of 2 kb could be amplified by increasing elongation steps to 5 min in another primer–template combination (data not shown).

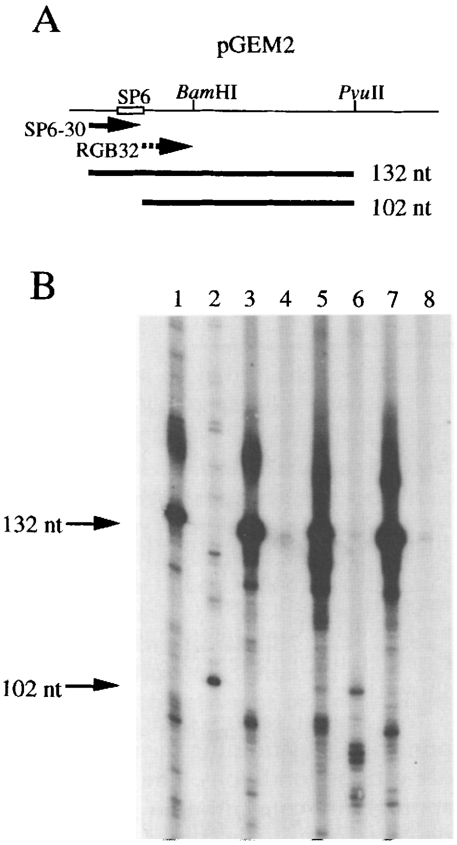

FIGURE 2 Primer extension using various DNA polymerases. (*A*) Location of primers. pGEM2 was digested with *Pvu*II and used as a template. Products from each primer are indicated by thick lines, with their expected lengths in nucleotides. (*B*) Chain elongation from DNA primer SP6-30 (lanes *1,3,5,7*) and RNA primer RGB32 (lanes *2,4,6,8*) by DNA polymerases. Employed enzymes are Sequenase v. 2.0 DNA polymerase (Amersham) under the sequencing conditions (lanes *1,2*), *AmpliTaq* DNA polymerase (Perkin-Elmer Cetus) under PCR conditions (lanes *3,4*), r*Tth* DNA polymerase (Perkin-Elmer Cetus) under reverse transcription conditions (lanes *5,6*), and r*Tth* DNA polymerase under PCR conditions (lanes *7,8*).

In aqueous solution, the RNA–DNA duplex is more stable than DNA–DNA duplex.(7) Therefore, higher annealing temperature must be required in the RNA-primed PCR than in the conventional PCR to perform amplification reaction of similar specificity, if primers of the same lengths are used. In PCR where one primer is RNA and the other is DNA, such as that described here, priming from RNA may be less specific than that from DNA. For RNA-primed PCR from template of higher complexity, it may be necessary to use two RNA primers, or, one RNA primer and the other longer DNA primer, and to adopt high annealing temperature to achieve maximal selectivity of the amplification.

Several techniques using hemispecific amplification have been developed to amplify unknown region adjacent to known sequence. These include inversed PCR,(8) ligation-mediated PCR,(9) and vectorette PCR.(10,11) However, in all of these techniques, specificity of amplification relies on at least one primer, which must be located in the region of known sequence. Thus, further extension of amplification units to the next unknown region inevitably requires sequence determinations and chemical primer synthesis based on this information. Combination of RNA-primed PCR with one of the hemispecific amplification can, in principle, negate requirement of sequence determination in the PCR-based genome walking, and drastically shorten its turnaround time.

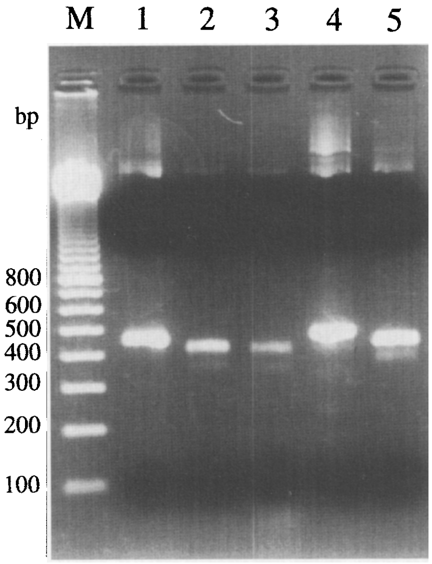

FIGURE 3 RNA-primed PCR. pGEM2 served as a template for amplification between G410 as a reverse primer and SP6-30 (lane *1*), RS28 (lane *2*), or RGB32 (lane *3*) as forward primers. pBluescript SK(+) served as a template for amplification between BS430 as a reverse primer and either T7-30 (lane *4*) or RSX30 (lane *5*) as forward primers. A 100-bp ladder was loaded in lane M.

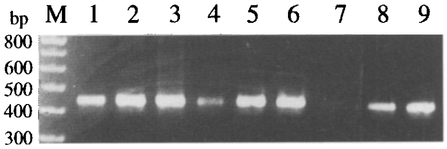

FIGURE 4 The effect of template concentration and numbers of thermal cycles in RNA-primed PCR. The amplification reactions were performed between RSX30 and BS430 using pBluescript SK(+) linearized by *Sca*I as a template at various concentrations: 1 ng (lanes *1–3*), 100 pg (lanes *4–6*) and 10 pg (lanes *7–9*) per 5 μl. Numbers of thermal cycles were 21 (lanes *1,4,7*), 32 (lanes *2,5,8*), and 40 (lanes *3,6,9*).

Several strategies are conceivable to obtain RNA primers of desired sequence by in vitro transcription. For instance, a common oligonucleotide adaptor carrying a promoter sequence can be attached by ligation to the desired region. Truncation of template for in vitro transcription can be achieved by digesting the ligation product with appropriate choice of restriction enzyme that cuts at the site downstream of transcription initiation. In vitro transcription should then give RNA primer of the desired sequence.

This is the first report that RNA can serve as a primer in PCR, providing the possibility of obtaining primers inexpensively by in vitro transcription. The procedures presented here, especially purification of RNA primers after in vitro transcription, still requires many manipulations. With further optimization and simplification of each procedures, RNA-primed PCR should find wider applications such as in entirely PCR-based chromosome walking without chemical DNA synthesis.

ACKNOWLEDGMENTS

This work was supported by Grants in Aid for Scientific Research on Priority Areas (Cancer), and for Creative Basic Research (Human Genome Analysis) from the Ministry of Education, Science, and Culture of Japan. H.S. is a recipient of fellowship from the Japan Society for the Promotion of Science for Young Scientists.

The publication costs of this article were defrayed in part by payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 USC section 1734 solely to indicate this fact.

REFERENCES

1. Saiki, R.K., D.H. Gelfand, S. Stoffel, S.J. Scharf, R. Higuchi, G.T. Horn, K.B. Mullis, and H.A. Erlich. 1988. Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. *Science* **239:** 487–491.
2. Melton, D.A., P.A. Krieg, M.R. Rebagliati, T. Maniatis, K. Zinn, and M.R. Green. 1984. Efficient *in vitro* synthesis of biologically active RNA and RNA hybridization probes from plasmids containing a bacteriophage SP6 promoter. *Nucleic Acids Res.* **12:** 7035–7056.
3. Milligan, J.F., D.R. Groebe, G.W. Witherell, and O.C. Uhlenbeck. 1987. Oligoribonucleotide synthesis using T7 RNA polymerase and synthetic DNA templates. *Nucleic Acids Res.* **15:** 8783–8798.
4. Sambrook, J., E.F. Fritch, and T. Maniatis. 1989. *Molecular cloning: A laboratory manual*, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.
5. Myers, T.W. and D.H. Gelfand. 1991. Reverse transcription and DNA amplification by a *Thermus thermophilus* DNA polymerase. *Biochemistry* **30:** 7661–7666.
6. Leung, D.W., E. Chen, and D.V. Goeddel. 1989. A method for random mutagenesis of a defined DNA segment using a modified polymerase chain reaction. *Technique* **1:** 11–15.
7. Casey, J. and N. Davidson. 1977. Rates of formation and thermal stabilities of RNA:DNA and DNA:DNA duplexes at high concentrations of formamide. *Nucleic Acids Res.* **4:** 1539–1552.
8. Triglia, T., M.G. Peterson, and D.J. Kemp. 1988. A procedure for *in vitro* amplification of DNA segments that lie outside the boundaries of known sequences. *Nucleic Acids Res.* **16:** 8186.
9. Mueller, P.R. and B. Wold. 1989. In vivo footprinting of a muscle specific enhancer by ligation mediated PCR. *Science* **246:** 780–786
10. Riley, J., R. Butler, D. Oglivie, R. Finniear, D. Jenner, S. Powell, R. Anand, J.C. Smith, and A.F. Markham. 1990. A novel, rapid method for the isolation of terminal sequences from yeast artificial chromosome (YAC) clones. *Nucleic Acids Res.* **18:** 2887–2890.
11. Arnold, C. and I.J. Hodgson. 1991. Vectorette PCR: A novel approach to genomic walking. *PCR Methods Applic.* **1:** 39–42.

Received August 2, 1995; accepted in revised form October 18, 1995.