Jpn. J. Vet. Res. 53(3－4)：159-168, 2006

FULL PAPER

A rapid and simple Transcriptional sequencing method for GC-rich DNA regions

Masaki Izawa[1),5)], Nobuo Kitamura[1)], Nanae Odake[1)], Fuminori Maki[1)], Kaoru Kanehira[1)], Hideyuki Nemoto[1)], Mitsuyo Yamaguchi[1)], Atsushi Yamashita[3)], Nobuya Sasaki[4)], Masahira Hattori[2),3)], Shinji Kanayama[1)] and Yuko Yoneda[1)]

(Accepted for publication : February 15, 2006)

Abstract

In genome sequencing project, we encounter the DNA regions that often contain stable secondary structure with high GC content. These regions are difficult to not only amplify by PCR for template preparations, but also determine the DNA sequences using standard Cycle sequencing (CS) method. Transcriptional sequencing (TS) is a unique DNA sequencing method using RNA polymerase, and is based on the principles of the chain-termination method, which is a powerful method to analyze GC-rich sequences. In this study, we examined the multiple displacement amplification (MDA) to overcome low efficiency of PCR amplification in GC-rich regions and subjected to TS reaction. Combination of MDA and TS (MDA-TS) was extremely successful with GC content ranging from 65% to 85%, which are difficult to analyze with PCR and CS. We also report plasmid vector, pTS1, which has the stronger T7 and T3 promoters than those of conventional vectors, and the sequence that decreases transcriptional efficiency was removed from its multiple cloning sites. pTS1 resulted in the improved sequencing accuracy and reduced reaction time up to 5 min. These results showed that MDA-TS is a rapid and accurate method for the analysis of GC-rich templates.

[1)]Nippon Genetech Co., Ltd., 1-1-24, Arakawa, Toyama, 930-0982, Japan.
[2)]RIKEN Genomic Sciences Center, 1-7-22, Suehiro Tsurumi, Yokohama, Kanagawa 230-0045, Japan
[3)]Laboratory of Genome Information, Kitasato Institute for Life Sciences, Kitasato University, 1-15-1 Kitasato, Sagamihara, Kanagawa 228-8555, Japan
[4)]Laboratory of Experimental Animal Science, Graduate School of Veterinary Medicine, Hokkaido University, Kita-ku, Sapporo, 060-0818, Japan
[5)]Corresponding author : Masaki Izawa
Nippon Genetech Co., Ltd., 1-1-24, Arakawa, Toyama, 930-0982, Japan.
Tel：+81-76-443-9561 Fax：+81-76-443-9120
E-mail : m-izawa@nippongenetech.com

Keywords : DNA sequencing ; Transcriptional sequencing ; Multiple displacement amplification

Introduction

A number of genome sequencing projects are currently in progress. However, in the case of human genome project, the latest genome sequence is not yet complete and still contains sequencing gaps. Closing of sequence gaps in humans and other organisms where having a complete set of genetic sequence information is critical to project success[9]. One reason for existence of the gaps is often regions that are GC-rich or contain highly repetitive elements. These gaps are recalcitrant to sequencing by available techniques. In order to fully understand the genome structures, effective method for obtaining accurate sequences in these "difficult" regions should be needed. Owing to their high GC sequences exhibit strong base-base interactions, which lead to superstructures and consequently to regions with higher melting temperatures. Therefore, PCR enzymes fall off their templates, causing premature termination of in the standard cycle sequencing (CS) method.

In contrast, Transcriptional sequencing (TS) has been developed based on the T7 RNA polymerase (RNAP) chain termination reaction with a fluorescent dye attached to 3'-deoxynucleoside triphosphate (3'-dNTP). TS shows excellent features compared to CS. It achieves a simple sequencing reaction using an isothermal reaction at 37℃. This method enables us to reduce the amount of template required, and to carry out PCR direct sequencing without elimination of PCR primers and dNTPs[11, 16]. In the direct sequencing of PCR products by using CS, un-incorporated dNTPs and primers must be eliminated to avoid inhibiting the sequencing reaction. In addition to these properties, RNAP can unwind dsDNA without the need of a denaturing step. This property had a great advantage for analyzing GC-rich regions of mouse genomic DNA sequences and palindromic sequences[10, 17].

Traditional methods to prepare DNA templates for sequencing require overnight cultures followed by multiple steps aimed at isolating and purifying plasmid DNA. These protocols are often laborious and time-consuming. Preparation of templates from clinical samples is a bottleneck in genotyping and DNA sequencing analysis and is frequently limited by the amount of specimen available. In these cases, PCR amplification is very useful. However, lack of amplification of certain target DNA as well as the occurrence of non-specific bands often results in time-consuming PCR setup procedures, especially for templates with high GC content. In contrast, the multiple displacement amplification (MDA) is recently developed, which uses phi29 DNA polymerase to exponentially amplify circular DNA templates for sequencing as an alternative to general plasmid preparations[1]. The protocol is simple, requiring less than 20 minutes of hands-on time from bacterial colonies. Moreover, MDA is reported to be efficient amplification method for high GC regions[19].

Here, we demonstrate that the combination of MDA and TS allows successfully rapid template preparations and sequencing reactions for difficult templates.

Materials and Methods

Enzymes and Nucleotides

Ribonucleotides were purchased from

Yamasa Co. Inc. and Sigma/Aldrich Inc. The N-hydroxysuccinimidyl esters of BODIPY dyes were purchased from Molecular Probes. Yeast pyrophosphatase was purified according to a previously described method[15]. T7 RNAP mutant contained 172-173 deleted residues[12,13] and F644Y[11]. The mutated polymerase gene was constructed by PCR-mediated site directed mutagenesis. Methods for mutant protein expression and purification have been described elsewhere[11,16]. BODIPY-labeled 3' dNTPs were synthesized based on the report[16].

Plasmid constructions and preparations

pBluescript SK (pBS) and pGEM3Zf (pGEM) were purchased from Stratagene and Promega, respectively. pTS1 and its recombinant plasmids were purified with alkaline-SDS treatment and CsCl gradient-ultracentrifugation. PCR amplification of the epidermal growth factor receptor (*EGFR*) gene, reaction was carried out in a 20-μl volume. It contained 200 ng human genomic DNA (Promega), 6pmol T7 or T3 promoter-tagged primer, 250 μmol of each dNTPs, and 0.5 U GeneTaqNT (Nippon Gene). PCR reactions were carried out using the MJ Research PTC-200 DNA Engine according to the following protocol: initial denaturing step at 94℃ for 3 min, followed by 30 cycles of 94℃ for 45 sec, 55℃ for 45 sec, and 72℃ for 3 min.

For the construction of the pTS1 vector, we synthesized 8 ssDNAs with the following sequences: 5'-AgCTgTgCgCgCAAATTAACC CTCACTAAAgggAgAgAgCT-3';5'-CTCTCC CTTTAgTgAgggTTAATTTgCgCgCAC-3';5'-CCTgCAggCTAgCTTgCgCAAggATCCTAggC CTgAAgCTT-3';5'-gTCgACAAgCTTCAggC CTAggATCCTTgCgCAAgCTAgCCTgCAggAg CT-3';5'-gTCgACgAATTCACCCgggAAgAT CTTgCTTACgTACgCgTggTACCATgCA-3'; 5'-TggTACCACgCgTACgTAAgCAAgATCTT CCCgggTgAATTC-3';5'-TTCTCCCTATAgTgAgTCgTATTATgCgCgC-3';5'-AATTgCgCg CATAATACgACTCACTATAgggAgAATgCA-3'. These ssDNAs were phosphorylated using T4 polynucleotide kinase with ATP, annealed, and ligated. The ligated oligonucleotides showed the cohesive sequences of *Eco* RI and *Hind* III sites at their terminals, and were introduced into pUC19 vector cut by *Eco* RI and *Hind* III.

For rapid DNA sequencing, the sequencing products were purified using Gel filtration columns. TS or CS analysis was done using an ABI 310 Genetic analyzer and about 200 ng of plasmid DNA. The LC1 clone represented the pTS1 plasmid clone containing a fragment of the lambda phage genomic DNA, and Human GC-rich DNAs were cloned into pTS1 plasmid. Experiments were done in triplicates to verify the accuracy of the results.

CS

CS was performed with the Dynamic ET terminator cycle sequencing kit (Amersham Bioscience, Fig. 4) and the Big dye terminator cycle sequencing kit (Applied Biosystems, Figs. 3 and 6) according to the manufacturer's protocol. For Dynamic ET terminator cycle sequencing kit (Fig. 4), excess dye-labeled terminators were removed by EtOH precipitation, according to the instruction manual. For rapid DNA sequencing (Figs. 3 and 6), sequencing products were purified with Centri-Sep column (Princeton separations). Following sequencing, the percentage of correct base calls up to 400-bases from the first signal, was calculated. PCR and MDA products were treated with the shrimp alkaline phosphatase and exonuclease I (Amersham Biosciences) to inactivate primers and excess dNTPs. The sequencing forward primer M13 (-21) was used, it was located in the vicinity of the T7 promoter.

TS

TS was carried out at 37℃ for 5 min (Figs. 4 and 6). In Figs. 1 and 3, duration is shown in the figure legends. A 20-μl reaction mixture contained 40mM Tris-HCl (pH 8.0), 8 mM $MgCl_2$, 5 mM DTT, 25 mM NaCl, 0.4 mM $MnCl_2$, 2mM spermidine, 0.05% Tween 20, 500 μM ITP, 250 μM 5-Br-UTP, 250 μM ATP, 250 μM CTP, 1 mM GMP, BODIPY FL-3'-dGTP, BODIPY R6G-3'-dATP, BODIPY564/570-3'-dUTP, BODIPY 581/591-3'-dCTP, 0.005 U of yeast PPase, and 50U of T7 RNAP. The labeled fragments were purified using CentriSep column and analyzed with the ABI 310 Genetic analyzer. Automatic base calling was performed using the base-call software of each sequencer. To assemble sequencing data, sequencing chromatograms were analyzed with Sequencher software (Gene Codes). PCR and MDA products were used as templates without any purification.

MDA

MDA was carried out using the Templiphi amplification kit (Amersham Bioscience) according to the instruction manual. Samples of human DNA that could not be analyzed by CS were used in RIKEN human genome sequencing project. They were introduced into pTS1 vector. For agarose gel electrophoresis, Each 5 μl of MDA and PCR products were loaded onto a gel. Following electrophoresis, the gel was stained with EtBr, and pictured using a CCD detecting system. TS was carried out with 1 μl MDA-amplified DNA in 20 μl of TS reaction.

Results

The accuracy and quickness of TS method

DNA sequencing is widely used not only in biological research, but also in gene diagnostic analysis. The ideal sequencing reaction should be accurate, quick, and easy to perform, enabling automation of a large number of reactions. Currently, CS chemistry is widely used. However, CS has the inconvenience of a long reaction time (1- to 3-hours) due to its requirement of temperature cycling. In TS reaction, the original protocol needs 1hr, which has been optimized for the conventional slab gel DNA sequencers. In this study, we examined the reduction of reaction time because the current capillary instruments are much more sensitive than the slab gel systems. Single nucleotide polymorphisms (SNPs) and deletion in *EGFR* genes which have been shown to be associated with Iressa (gefitinib) -sensitivity to non-small-cell-lung cancer (NSCLC)[14] and which are routinely investigated for diagnostic purposes were chosen to test if the TS can be used as a time-saving method.

We amplified a human *EGFR* with minimum T7 and T3 promoter sequences, which were designed to be located respectively at both terminals of the PCR product. PCR product was directly added to TS reaction mix and incubated at 37℃ for 5, 10, 15 and 30 min. TS products were purified using sephadex gel filtration columns, then analyzed using a capillary sequencer. Every result in 5 to 30 min reaction revealed 100% sequencing accuracy for deletion regions (underlines in Fig. 1) and over 99.5% in entire PCR products. Thus, we have found that 5 min reaction produces more than sufficient signal for detailed analysis at the required level of accuracy using capillary instruments as shown in Fig. 1. The ability of accuracy and quickness of TS indicates the usefulness of this method as a diagnostic tool.

Highly efficient transcription using the pTS 1 vector

At present, two independent methods for preparing DNA templates are available. One

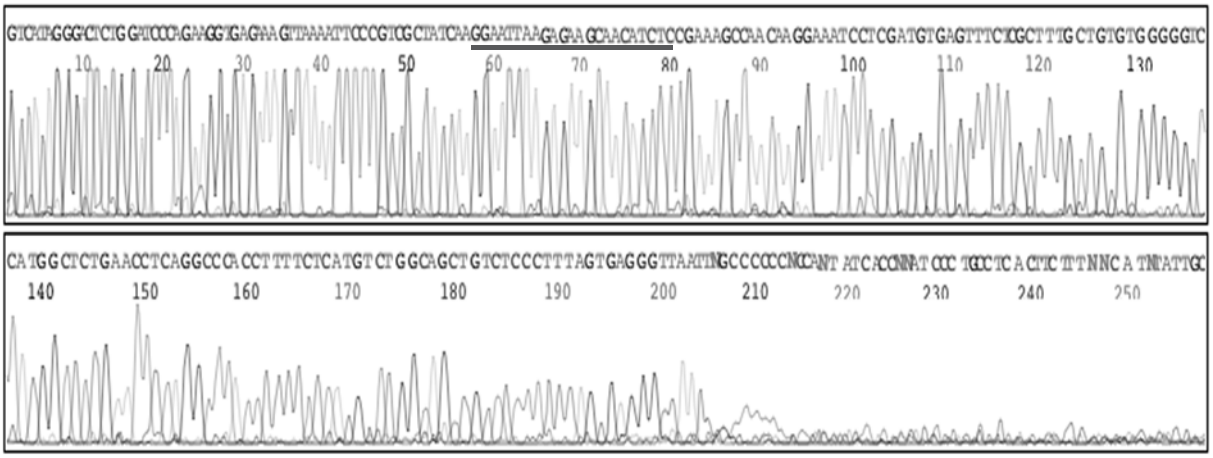

Fig. 1. Rapid TS sequencing for PCR product.
Detection of deletion by direct Transcriptional sequencing. The T7 and T3 phage promoter sequences can be appended to both of the PCR primers and incorporated into the *EGFR* PCR product (281bp). Using T7 and T3 RNAPs, the *EGFR* gene could be transcribed from both ends for 5 minutes. The electropherogram shows only T7 side-sequence of the *EGFR* exon 19. The lines show the locations of deletion in NSCLCs.

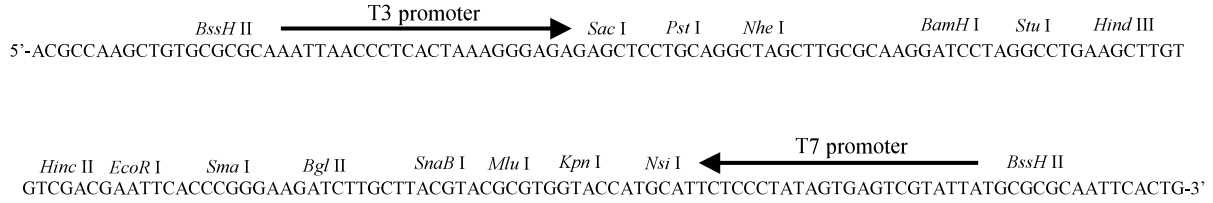

Fig. 2. Structure of pTS1 multiple-cloning sites.
Multiple-cloning site flanked by minimum T3 and T7 promoters in pTS1 vector. Directions of each arrow show directions of transcription. Detail of pTS1 construction is described under Materials and Methods.

is PCR and the other is DNA cloning using a plasmid vector. In order to further improve sequencing ability of TS, we developed a new cloning vector, pTS1 (Fig. 2). This vector was derived from pUC 19, and had multiple-cloning site and phage promoters (T7 and T 3) for initiation of transcription. Each promoter was located at end of the multiple-cloning sites in order to sequence both strands, respectively. The region from -17 to +6 promoter sequences were introduced into pTS1, which is the minimal length essential for strong activity. In most commercially vectors such as pBS and pGEM, the region from +4 to+6 is different from the consensus promoter. Variants of the+1 to+6 regions were reported to decrease transcription efficiency[8]. Further, GC-rich restriction sites such as *Not* I were removed, because these sites decrease efficiency of the transcription in pBS and pGEM (data not shown). In TS analysis with pBS, pGEM and pTS1 vectors, sequencing accuracies versus time were compared. Percentages of correct base calls up to 400-bases from the first signal in TS were calculated (Fig. 3). With pTS1, even after one min sequencing time, over 99% sequencing accuracy was obtained while with pBS and pGEM accuracies were 89.5% and 91.0% respectively. After a 5 min sequencing time, sequencing accuracy

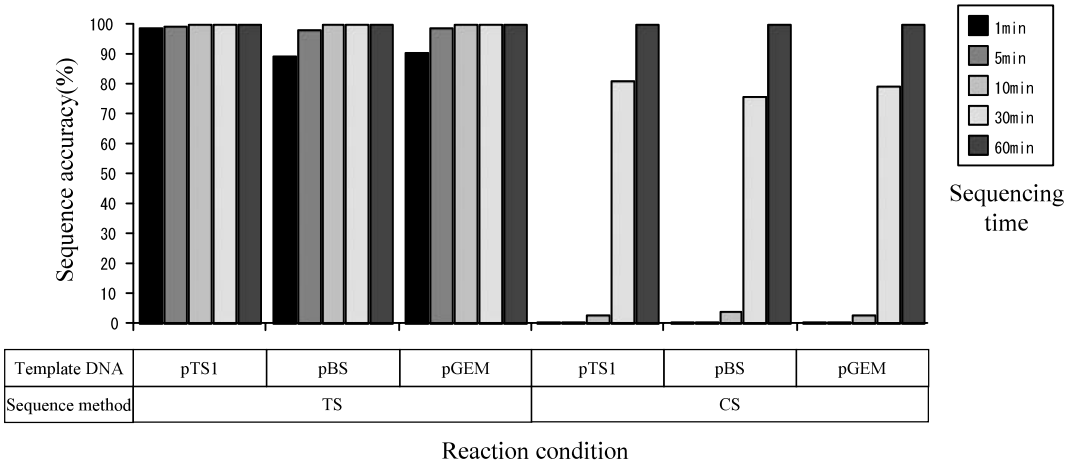

Fig. 3. Comparison of sequence accuracy with pTS1 and commercial vectors.
Comparison of sequence accuracy within 400-base-long regions of TS and CS by various plasmids, pTS1, pGEM and pBS. 200 ng of each plasmid were incubated for 1, 5, 10, 15 and 30 min using both methods. The individual quality of the sequences was assessed by calculating the percentage of correct basecalls within 400-base-long sections from the first signal.

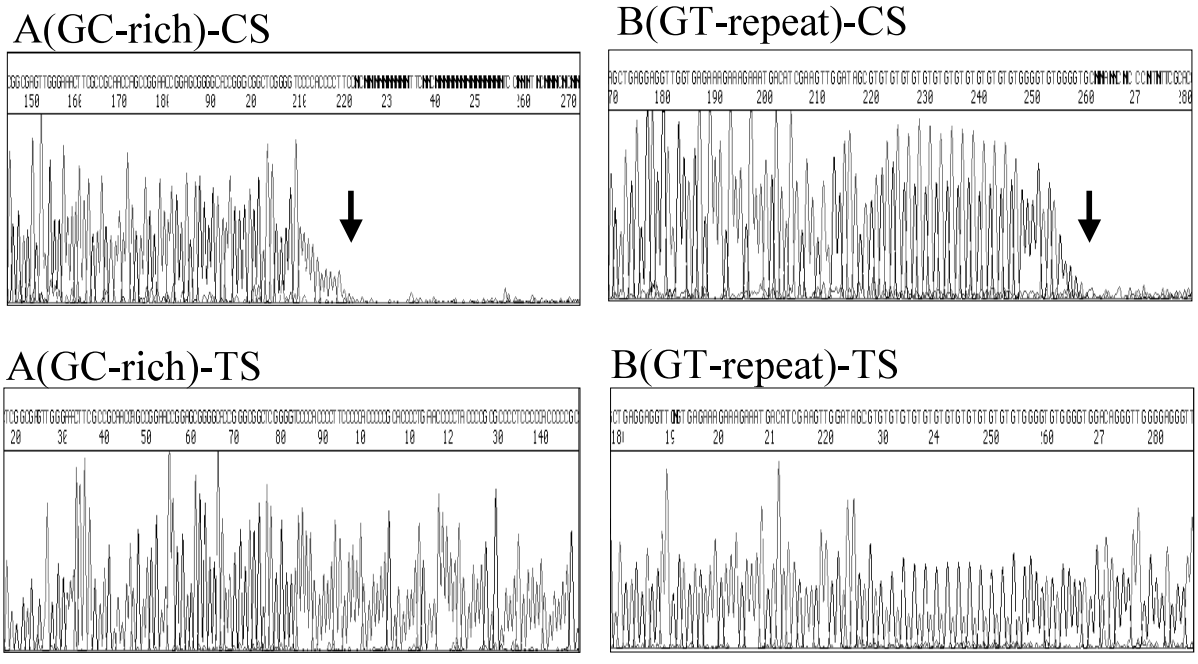

Fig. 4. Comparison of TS with CS results for DNA sequence of difficult templates.
The GC-rich (A) or GT repetitive (B) regions of human genomic clones were sequenced. The upper and lower panels show chromatographs with CS and TS, respectively. Peaks suddenly disappear at the points indicated by arrows in CS. On the other hand, peaks continue beyond the termination points in TS.

with pTS1 was over 99.5%. Thus, pTS1 will be able to improve sequencing accuracy and reduction of reaction.

The combined use of MDA and TS enables efficient sequence analysis of GC-rich DNAs.

TS is an effective method to analyze GC rich DNA sequences and repetitive regions[10,17]. RNAP can dissolve dsDNA due to its own helicase-like activity. TS does not require any denaturing step, and its high rate of specificity makes it greatly advantageous to analyze GC-rich DNAs. Although TS is an excellent method to analyze GC-rich DNAs, it is limited by an insufficient output of PCR products, therefore, an efficient amplification method is necessary to utilize the advantage of TS. Though various methods for GC-rich DNA amplification were developed, they are not enough to analyze GC-rich DNA[4]. On the contrary to them, MDA should be a successful DNA amplification method for DNA regions with 80% GC content[19]. First, to evaluate the reliability of TS, we prepared pTS1 plasmids containing GC-rich region (A) and GT repetitive region (B), respectively, and analyzed them with CS and TS. The sequence signal from CS fell very low and was unclear patterns impossible for base-calling, while TS results showed a sharp sequence pattern without a decrease in signal at a particular region (Fig. 4, lower panel). Secondly, we tested whether various types of GC-rich DNAs could be amplified by PCR or MDA from *E. coli* colonies (Fig. 5). Several human GC-rich DNAs were amplified by PCR. However, non-specific bands are visible in all amplified-samples, which are not good for next sequencing reactions (samples 2, 3, 5 in the upper panel of Fig. 5). In other lanes 4, 6 and 7, only faint bands are detectable. Even when PCR cycles were extended to more than 45 cycles and addition of several GC-destabilizing agent such as dimethyl sulfoxide could not lead satisfactory results. On the other hand, all clones were successfully amplified with MDA (Fig. 5, lower panel). Fig. 6 shows that the DNA sequences of MDA product were analyzed using CS and TS. In the chromatogram of Fig. 6, GC content was 82.4%. The sequence signal of CS fell very low, and was too weak to perform base-calling (Fig. 6, upper panel). Consis-

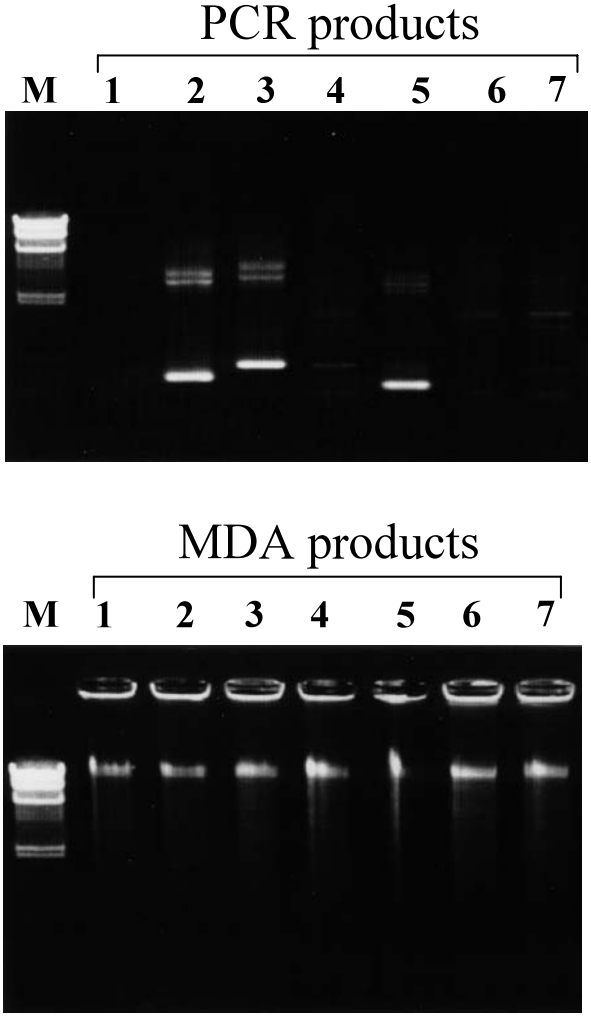

Fig. 5. Comparison of MDA with PCR for DNA amplification from bacterial colonies
Agarose gel electrophoresis of PCR products (upper panel) and MDA products (lower panel) amplified from seven human genomic GC-rich clones. M represents the molecular weight marker.
The results show that all fragments can be obtained with a high yield in MDA.

tently, sequencing of these PCR amplified-products were not possible due to very low efficiency of the CS reaction (data not shown). On the other hand, TS results revealed a sequence pattern without any decrease in signals (Fig. 6, lower panel). By using other templates with GC contents ranging from 60 to 85%, TS also showed discrete patterns without any sudden signal declines and resulted in high sequencing accuracy from both ends (more than 99.5%). Our aim was not only to obtain specific templates but also to obtain satisfactory sequencing results. Thus, these results showed that MDA-TS was a superior method for the analysis of GC-rich templates.

Discussions

The international human genome sequencing consortium reported that the final human genome sequence contained 341 gaps, and these could not be analyzed with current cloning and/or sequencing methods using standard CS. There is a possibility that these gaps contain important biological functions[2]. In the DNA sequence analysis using CS, the difficult DNA templates usually are found to have regions containing a high degree of secondary structure or a GC content of 60% or greater. They usually contain gene-expression regulatory elements including promoters and enhancers, and they play important roles in various cellular events[3]. Promoter regions of genes are in the main focus for detecting novel SNPs. SNPs in these regions frequently affect transcription and may be relevant for pathogenesis of diseases and pharmacogenetics purposes and are therefore termed regulatory SNPs (rSNPs)[6].

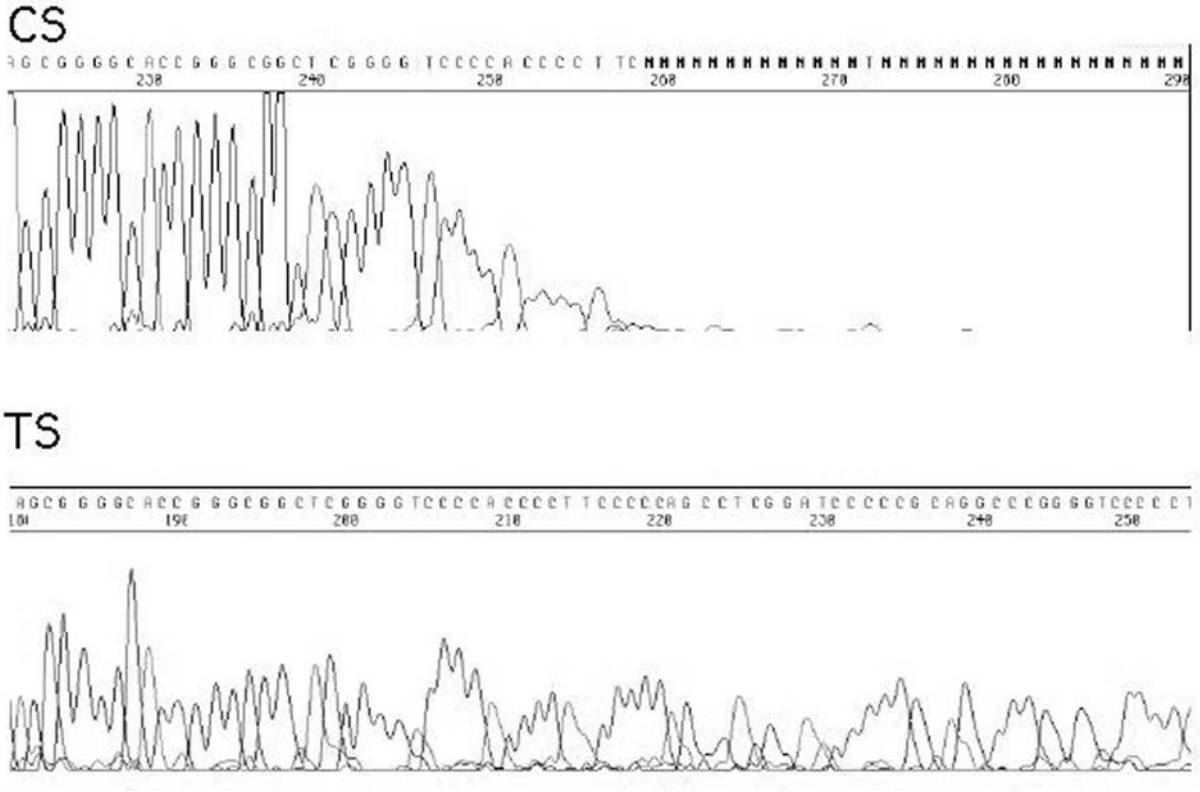

Fig. 6. DNA sequencing electrochromatogram of a highly GC-rich MDA product using CS and TS.
Sample No. 2 of MDA reaction in Fig. 5 was subjected to direct-sequencing using CS (upper panel) and TS (lower panel). CS yielded a greatly reduced signal and finally became unreadable. In contrast to the results, all MDA clones could be analyzed completely by TS (data not shown).

PCR has become a routine technique for template preparation. However, due to very high GC content PCR setup procedures can be very time consuming and the establishment of specific PCR conditions for GC-rich templates can often be difficult or unsuccessful. The use of various additives such as formamide, DMSO, betaine or 7-deaza-dGTP[4,14], special procedures like the use of hotstart PCR or protocols like the touchdown PCR have been reported to improve the amplification of the DNA template[5]. In our difficult templates, the obtaining specific PCR products remain impossible using these techniques (data not shown). On the other hand, we successfully amplified all GC-rich DNA templates using MDA. Furthermore, amplification of templates with GC-contents of up to 85% was successful.

When standard CS techniques have failed on GC-rich or highly repeat regions, varieties of approaches have been developed to overcome these problems. The addition of certain reagents, such as ThermoFidelase (Fidelity Systems Inc., USA), relaxes the secondary structures of templates, and sometimes can yield clearer results. Replacement of dGTP with 7-deaza-dGTP or deoxy inosine triphosphate (dITP) prevents reaction products from forming secondary structures[14,18]. However, we could not overcome these problems using them in the all of difficult templates. We reported that the TS could overcome the difficulty in sequencing the problematic regions in previous and this papers[17]. Therefore, we examined the combination of MDA and TS which resulted in the successful amplification and sequencing of extremely GC-rich targets. Further, new designed vector, pTS1 effectively reduced the reaction time. By reducing the time (MDA and TS for 20 and 5 min, respectively), hands-on time in template preparation and sequencing, the MDS-TS delivers a cost-effective method for producing sequence data of consistent quality and quantity. For sequencing GC-rich region, MDA-TS is superior method unlike the general combination use of PCR and CS. More work is necessary to assess the performance of MDA-TS as templates with 85% < GC content may be particularly difficult to amplify. Thus, we suggest that MDA-TS can contribute the filling the gap of GC-rich region and discover new information on gene deletion and rSNPs.

Finally, the utility of MDA-TS is not limited to genomic applications. MDA-TS is isothermal and rapid reaction without any purification steps will facilitate time-saving and easy handling. This makes possible a high-throughput, high quality application to clinical diagnosis in veterinary and medical sciences.

Acknowledgements

We thank all our collaborators and colleagues for technical assistance and helpful discussions. This work was partially supported by the Akiyama foundation (to N.S.).

References

1) Dean, F. B., Nelson, J. R., Giesler, T. L. and Lasken, R. S. 2001. Rapid amplification of plasmid and phage DNA polymerase and multiply-primed rolling circle amplification. *Genome Res.,* 11 : 1095-1099.

2) Eichler, E. E., Clark, R. A. and She, X. 2004. An assessment of the sequence gaps : unfinished business in a finished human genome. *Nature Reviews,* 5 : 345-354.

3) Egger, G., Liang, G., Aparicio, A. and Jones, P. A. 2004. Epigenetics in human disease and prospects for epigenetic therapy. *Nature,* 429 : 457-463.

4) Henke, W., Herdel, K., Jung, K., Schnorr, D. and Loening, S. A. 1997. Betaine im-

proves the PCR amplification of GC-rich DNA sequences. *Nucleic Acids Res.,* 25 : 3957-3958.

5) Hecker, K. H. and Roux, K. H. 1996. High and low annealing temperatures increase both specificity and yield in touchdown and stepdown PCR. *Biotechniques,* 20 : 478-485.

6) Hudson, T. J. 2003. Wanted : regulatory SNPs. *Nature Genetics,* 33 : 439-440.

7) Ikeda, H., Ishikawa, J., Hanamoto, A., Shinose, M., Kikuchi, H., Shiba, T., Sakaki, Y., Hattori, M. and Omura, S. 2003. Complete genome sequence and comparative analysis of the industrial microorganism Streptomyces avermitilis. *Nature Biotech.,* 21 : 526-531.

8) Imburgio, D., Rong, M., Ma, K. and McAllister, W. T. 2000. Studies of promoter recognition and start site delection by T7 RNA polymerase using a comprehensive collection of promoter variants. *Biochemistry,* 39 : 10419-10430.

9) International Human Genome Sequencing Consortium, Finishing the euchromatic sequence of the human genome 2004. *Nature,* 431 : 931-945.

10) Ishikawa, T., Hayashida, Y., Hirayasu, K., Ozawa, K., Yamamoto, N., Tanaka, T. and Matsuura, S. 2003. Use of transcriptional sequencing in difficult to read areas of the genome. *Anal. Biochem.,* 316 : 202-207.

11) Izawa, M., Sasaki, N., Watahiki, M., Ohara, E., Yoneda, Y., Muramatsu, M., Okazaki, Y. and Hayashizaki, Y. 1998. Recognition sites of 3'-OH group by T7 RNA polymerase and its application to Transcritpional sequencing. *J. Biol. Chem.,* 273 : 14242-14246.

12) Lyaknov, D. L., He, B., Zhang, X., Studier, F. W., Dunn, J. J. and McAllister, W. T. 1998. Pausing and termination by bacteriophage T7 RNA polymerase. *J. Mol. Biol.,* 280 : 201-213.

13) Lyaknov, D. L., He, B., Zhang, X., Studier, F. W., Dunn, J. J. and McAllister, W. T. 1997. Mutant Bacteriophage T7 RNA polymerases with altered termination properties. *J. Mol. Biol.,* 269 : 28-40.

14) Mutter, G. L. and Boynton, K. A. 1995. PCR bias amplification of androgen receptor alleles, a trinucleotide repeat marker used in clonality studies. *Nucleic Acids Res.,* 23 : 1411-1418.

15) Paez, J. G., Janne, P. A., Lee, J. C., Tracy, S., Greulich, H., Gabriel, S., Herman, P., Kaye, F. J., Lindeman, N., Boggon, T. J., Naoki. K., Sasaki, H., Tujii, Y., Eck, M. J., Sellers, W. R., Johnson, B. E. and Meyerson, M. 2004. EGFR mutations in lung cancer : correlation with clinical response to Gefitinib therapy. *Science,* 304 : 1497-1500.

16) Sasaki, N., Izawa, M., Watahiki, M., Ozawa, K., Tanaka, T., Yoneda, Y., Matsuura, S., Carninci, P., Muramatsu, M., Okazaki, Y. and Hayashizaki, Y. 1998. Transcriptional sequencing : A method for DNA sequencing using RNA polymerase. *Proc. Natl. Acad. Sci. USA,* 95 : 3455-3460.

17) Shibata, K., Izawa, M., Hayashizaki, Y. and Watahiki, M. 2003. Practical application of transcriptional sequencing for GC-rich templates. *J. Stru. Func. Genom.,* 4 : 35-39.

18) Turner, S. L. and Jenkins, F. J. 1995. Use of deoxyinosine in PCR to improve amplification of GC-rich DNA. *Biotechniques,* 19 : 48-52.

19) Yan, J., Feng, J., Hosono, S. and Sommer, S. S. 2004. Assessment of multiple displacement amplification in molecular epidemiology. *Biotechniques,* 37 : 136-143.