[Short Communication]

Study to find the reaction condition of a small amount of single-stranded template DNA in Sanger sequencing reactions

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SUMMARY

Single-stranded template DNA adsorption was found on a plastic container's wall. The adsorption decreased the reaction efficiency of Sanger sequencing reactions. The amount of adsorbed DNA per unit contact area of a plastic container's wall was 2 fmol/mm². Adding polyoxyethylene (20) sorbitan monolaurate effectively reduced the amount of DNA adsorbed. A detectable amount of DNA fragments was produced from only 0.05 pmol of single-stranded template DNA by reducing the amount of adsorbed DNA.

Key words: Sanger sequencing reactions, adsorption, polyoxyethylene sorbitan monolaurate.

INTRODUCTION

DNA sequencing techniques¹⁾ enable us to access the genetic information encoded in the base sequence of DNA and provide fundamental data to molecular biology and biotechnology. The standard amount of single-stranded template DNA (ssDNA) provided in Sanger sequencing reactions is conventionally about 0.5 pmol. However, the sequencing reactions should often be redone using the same template DNA for the following reasons:

(1) the optimum dideoxynucleotide to deoxynucleotide molar ratios are changeable over various chain length sequences.²⁾

(2) the optimum enzyme for the sequencing reactions is also variable over different target sequences.³⁻⁵⁾

(3) a G/C rich area in a sequence sometimes causes "band compression" with the bands being

closer than would be expected. In that case, the use of dITP³⁾ or deoxy-7-deaza-guanosine triphosphate^{3,6)} in place of dGTP is recommended.

(4) M13 phage's growth is affected by the length of inserted DNA. If the length is too long, the concentration of the phage becomes smaller than expected and detectable amounts of DNA fragments are not produced in the sequencing reactions.

Preparing a large amount of ssDNA could be suggested for retrying the sequencing reactions. However, preparing a large amount of ssDNA by prolonging the incubation hours for M13 phage or by incubating the phage using a large volume of liquid and condensing it is not a good idea. These methods yield impurities affecting the reliability of the sequencing reactions. Consequently, the optimum reaction condition of a small amount of ssDNA should be found. However, problems that

シーケンシング反応における鋳型 DNA 微量時の反応条件の検討

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occur when using a small amount of ssDNA are not known, because the reaction efficiencies of various amounts of single-stranded template DNA (ssDNA) have not been examined. This report discloses the problems caused by using a small amount of ssDNA, and then finds the optimum reaction condition for that case.

MATERIALS AND METHODS

MATERIALS

M 13 mp 8 ssDNA was from Amersham (Tokyo, Japan). Fluorescent-labelled primer was from Yuki-gosei (Tokyo, Japan). Sequenase was from United States Biochemical (Cleveland, Ohio). Klenow fragment was from Takara (Kyoto, Japan). Plastic containers were mainly from Bioplastics (Osaka, Japan).

METHODS

The change of reaction efficiency, which is defined as the ratio of the amount of produced DNA fragments to that of provided ssDNA, was examined for various amounts of ssDNA. Since it is difficult to evaluate the reaction efficiency directly from the DNA fragment spectra,⁷⁾ the ratio of the reaction efficiency of the measured sample to that of a control sample was obtained by comparing the peak intensities of each group of A fragments (reaction products made by A termination solution).

FITC (Fluorescein isothiocyanate) labelled primers were annealed to ssDNA by being heated at 60°C for 15 min, and then passively cooled at room temperature for 10 min. The amount of the primer was equivalent to the amount of ssDNA. After adding DNA polymerase (Klenow fragment), the annealing reactions were split immediately into four aliquots, and the appropriate elongation/termination mixes were added. Following the elongation/termination reaction, chase solution was added. The final steps were adding formamide stop solution, and then storing the reaction products at -20°C.

The reaction products were heated at 95°C for 1 min prior to loading the samples onto a 4%, 40 cm long, 0.2 mm thick polyacrylamide gel which included 7 M urea. The electrophoresis was car-

ried out at 1,700 volts for 4 h. DNA fragments were detected by the apparatus that we developed.⁷⁾

RESULTS AND DISCUSSION

Single-stranded DNA adsorption

The ratio of reaction efficiency for various amounts of ssDNA is shown in Fig. 1. The reaction efficiency rapidly decreased when the amount of ssDNA was smaller than 0.4 pmol. The reason for the decrease in reaction efficiency must be disclosed to find the optimum condition when using a small amount of ssDNA in Sanger sequencing reactions.

Extending the reaction time did not increase the reaction efficiency (data not shown). This supposes that the reactions were saturated, and that the adsorption of ssDNA onto a plastic container's wall caused the decrease of reaction efficiency. The tendency of the reaction efficiency in various amounts of ssDNA can be explained by assuming 0.16 pmol of ssDNA adsorption in Fig. 1. The amount of ssDNA adsorbed per unit contact area was estimated to be 2 fmol/mm². To verify the presence of the ssDNA adsorption, the ratio of the reaction efficiency for various volumes of the reaction liquid obtained experimentally was compared with that obtained by assuming 2 fmol/mm² of adsorption. The experimental value agreed with the expected value (data not shown). Thus the adsorption as-

Fig. 1. Ratio of reaction efficiency for various amounts of ssDNA.

The amount of ssDNA of the control sample is 0.4 pmol. ● indicates the ratio of the reaction efficiency obtained experimentally. The line indicates the ratio of the reaction efficiency when assuming 0.16 pmol of ssDNA adsorption.

sumption was verified. Therefore, decreasing the amount of adsorbed ssDNA is crucial for successfully generating reactions with a small amount of ssDNA. The amount of adsorbed ssDNA was almost the same when a Sarstedt tube or an Eppendorf tube was used as a container (data not shown).

Method to test reaction reliably with a small amount of ssDNA

The detergent polyoxyethylene (20) sorbitan monolaurate is commonly used to prevent protein adsorption, because the detergent can be adsorbed into a container's wall more than in other materials. Sanger sequencing reactions were performed with the detergent, and the effect of adding the detergent was examined. Figure 2 shows that a detectable amount of DNA fragments, for analysing the DNA sequence, could not be obtained from 0.25 pmol of ssDNA when there is no method to reduce the DNA adsorption. Addition of the detergent enabled us to obtain a detectable amount of DNA fragments when using the same amount of ssDNA. The amount of ssDNA adsorbed became negligible when adding the detergent.

A detectable amount of DNA fragments were

produced from only 0.05 pmol of ssDNA, as shown in Fig. 3. Consequently, the amount of ssDNA in Sanger sequencing reactions can be reduced to one tenth of the conventional reaction system.

Addition of surplus FITC labelled primers or plasmid DNA was also effective (data not shown). However, this method will not always do, because these surplus DNA hybridize to the template DNA when the sequence of sample DNA is similar to the added DNA. Other methods, such as siliconizing a plastic container's wall and addition of transfer RNA, were not effective. Therefore, addition of the detergent polyoxyethylene (20) sorbitan monolaurate is recommended as the most effective method.

We also tried to increase the reaction efficiency when double-stranded DNA were used as the template. However, the reaction efficiency could not be increased by adding polyoxyethylene (20) sorbitan monolaurate. We guess that the adsorption of template DNA was negligible since the adsorption of denatured template DNA was prevented by the adsorption of surplus recombined double-stranded DNA.

Fig. 2. Effect of added detergent polyoxyethylene (20) sorbitan monolaurate on DNA fragment spectra.

The upper indicates the DNA fragment spectra obtained with the detergent. The lower indicates the DNA fragment spectra without the detergent. The amount of ssDNA was 0.25 pmol. The ordinate represents fluorescence signal intensities and the abscissa represents migration time. The numbers indicate the length of the DNA fragments. The detergent was added to the annealing reaction liquid with a 0.1% of concentration.

Fig. 3. DNA fragment spectra obtained from 0.05 pmol of ssDNA by the addition of polyoxyethylene (20) sorbitan monolaurate.

The ordinate represents fluorescence signal intensity and the abscissa represents migration time. The numbers indicate the length of DNA fragments. The volume of the annealing reaction was 2 μ l. All of the reaction product was loaded onto a polyacrylamide gel.

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REFERENCES

- 1) Messing, J.: *Methods Enzymol.*, **101**: 20, 1983.
- 2) Johnston-Dow, L. et al.: *Biotechniques*, **5**: 754, 1987.
- 3) Tabor, S. and Richardson, C.C.: *Proc. Natl. Acad. Sci. USA*, **84**: 4767, 1987.
- 4) Toneguzzo, F. et al.: *Biotechniques*, **6**: 460, 1988.
- 5) Innis, M. A. et al.: *Proc. Natl. Acad. Sci. USA*, **85**: 9436, 1988.
- 6) Mizusawa, S. et al.: *Nucl. Acids Res.*, **14**:

1319, 1986.

- 7) Kambara, H. et al.: *Bio/Technology*, **6**: 816, 1988.

要 旨

プラスチック容器壁に一本鎖鋳型 DNA が吸着する現象を見いだした。サンガー法によるシーケンシング反応では、鋳型 DNA 量が微量のときにはこの吸着の影響が顕著に現れる。プラスチック容器壁への DNA 吸着量は単位接触面積当り 2 fmol/mm² であった。ポリオキシエチレン (20) ソルビタンモノラウレートを反応液に添加することにより、DNA 吸着量を低減できた。この方法によりわずか 0.05 pmol の鋳型 DNA から解析可能な DNA 断片群を調製できた。