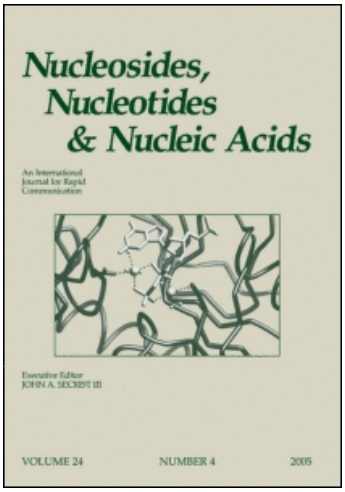

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APPROACHES TO SOLID PHASE DNA SEQUENCING

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ABSTRACT

A novel approach to enable two-directional solid-phase DNA sequencing, involving immobilization of double stranded plasmid to avidin agarose, is described. A plasmid vector, pRIT28, has been designed to allow enzymatic incorporation of biotinylated dUTP into two alternative sites.

INTRODUCTION

The proposal to sequence the human genome has focused the attention to the need to develop automated methods for various operations in sequencing (1). Attempts to develop automated sequencing reactions by a centrifugal reagent handling device has been described (2), as well as systems for high-speed sequencing using a laboratory robot (3).

We have recently described a novel method for solid-phase DNA sequencing using the avidin-biotin system (4). This approach is based on the immobilization of double stranded plasmid DNA to a solid support and subsequent use of the immobilized DNA as template for sequencing reactions. The affinity gel containing the immobilized template is consecutively used for the four different dideoxy-nucleotide reactions. This solid-phase procedure might facilitate automated handling of liquids in µl quantities and is therefore well adapted for automatization.

Here, we describe an improved procedure to use this approach for solid-phase DNA sequencing, involving the use of a new sequencing vector, pRIT28. Using this vector both

strands may be separately immobilized, and the insert sequenced from both directions.

MATERIALS AND METHODS

DNA techniques

Restriction enzymes were obtained from Pharmacia, Sweden. DNA manipulations and purifications were performed according to standard procedures (5). 11-bio-dUTP was obtained from BRL and used to terminally label double stranded DNA according to the suppliers recommendations. Avidin agarose was obtained from Sigma chemicals.

Construction of the plasmid pRIT28

The plasmid pTZ18R (Pharmacia, Sweden) was partially digested with BglI and a synthetic oligonucleotide linker 5'-CCATGACAATGGAGTGCTGGTTACCGATATCGAA-3' (and its complementary sequence) was inserted near the 3'-end of the lacZ' gene. This synthetic fragment contains BstXI, EcoB, BstEII and EcoRV recognition sequences. The BglI site, used for the insertion, was destroyed in the process. The reading frame was changed in the last part of the lacZ' gene, but the color of the colonies remained light blue when introduced into E.coli strain RRI ΔM15 with IPTG/Xgal selection (5). This construction was partially digested with PvuII and a synthetic oligonucleotide linker 5'-GGCCAGGGAGGCCAGATCTGAGCGGCCGCTGCTG-3' (and its complementary sequence) was inserted upstream of the lacZ' gene. This fragment contains SfiI, BglII, EcoB, and NotI recognition sequences. The PvuII site used for this insertion was destroyed simultaneously. The resulting plasmid, denoted pRIT28, is suitable for two-directional solid-phase sequencing.

Sequencing reactions using immobilized template DNA

The immobilized biotinylated double stranded DNA (4) was converted into single stranded form by incubation with 0.15 M NaOH for 15 minutes at 37°C. The avidin agarose gel, with immobilized template DNA, was subsequently washed

with 0.15 M NaOH and H_2O. Sequencing reactions were performed using both ^{35}S labelled dATP and ^{32}P end labelled primer. In both cases 1 µg of the plasmid, immobilized on 1 µl avidin agarose gel, were mixed with equimolar amounts of the respective primer, 1u Klenow polymerase, 5 µl of the appropriate nucleotide mix (4) and the volume was adjusted to 10 µl with a buffer containing 10 mM Tris HCl (pH 7.5), 10 mM $MgCl_2$, 200 µg/ml Bovine serum albumin and 100 mM NaCl. For the ^{35}S protocol 0.5 µl ^{35}S-dATP (12.5 µCi/µl) was also added. In both cases the reaction mixtures, at a total volume of 10 µl, were incubated 20 minutes at 37°C. Thereafter 4 µl of a chase solution (0.5 mM dNTPs) was added followed by 15 minutes incubation at 37°C. After completed reactions the supernatant was removed after centrifugation and the newly synthesized oligonucleotides were eluted by incubation with 5 µl formamide/sequencing dye mix at 37°C for 10 minutes. After centrifugation the supernatant was removed and diluted with 5 µl H_2O. 3 µl was loaded on a polyacrylamide sequencing gel. The avidin agarose gel, with immobilized template DNA, was regenerated by washing with 0.15 M NaOH and TE (10 mM Tris (pH 7.5), 1mM EDTA).

RESULTS

The basic concept

The general principle of the method is outlined in Fig. 1. The target DNA is cloned into the multilinker region of the sequencing vector pRIT28 and may be sequenced from both directions using the Sanger method (6). The plasmid is linearised with a restriction enzyme which yields 5'-protruding ends (BstEII or BglII) followed by digestion with an enzyme which yields blunt-ends or 3'-protruding ends (EcoRV or SfiI). The 5'-protrusions are filled in using Klenow polymerase and 11-bio-dUTP, dATP, dCTP and dGTP. After desalting, the mixture is passed over an avidin agarose column. This leads to directed immobilization of

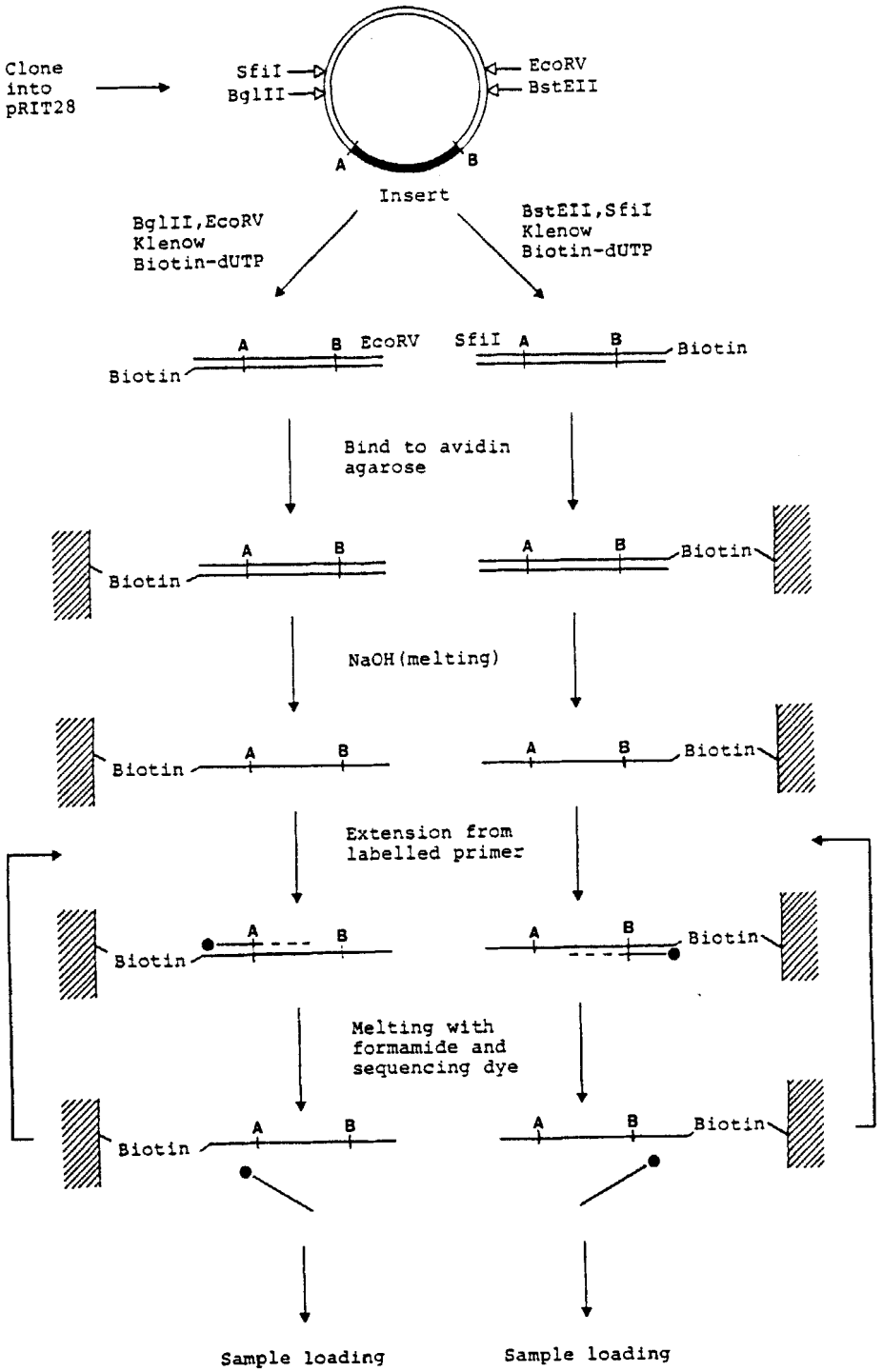

FIG.1. A schematic drawing of the basic concept of the solid-phase sequencing using the biotin-avidin system. See text for details.

the biotinylated DNA fragments (Fig. 1). Note that depending on the choice of enzymes the target DNA is immobilized in either orientation. Single stranded DNA is obtained by melting the strands by alkaline treatment, and simultaneous elution of the non-biotinylated strand. The sequencing primer, nucleotides and Klenow polymerase are added to the immobilized single stranded template and the sequencing reaction is performed. The extended oligonucleotides can be labelled using different strategies, either by a labelled primer or during the extension. The newly synthesized labelled oligonucleotides are eluted by a formamide dye mix leaving the template available for the next sequencing reaction. The annealing/extension is repeated to obtain specific fragments for all four nucleotides and the four samples are loaded on a sequencing gel.

The sequencing vector pRIT28

A new multi-purpose plasmid vector, pRIT28, was constructed to enable sequencing using the concept outlined in Fig.1. A schematic drawing of the plasmid is shown in Fig.2. The plasmid is a high-copy number vector with a multilinker site in the lacZ' region which enables the blue/white selection common for many cloning vehicles. For solid-phase sequencing, linkers were inserted approximately 200 base pairs upstream and downstream from the multi-cloning site. Cleavage with BglII or BstEII yields protruding 5'-ends, which can be used to incorporate biotinylated uracil using Klenow polymerase. Cleavage with EcoRV or SfiI yields ends which are not biotinylated using the same procedure.

When the linker containing the BstEII site was inserted into the BglI site in the 3'-end of the lacZ' gene, this affected the ability of the gene product to complement the ß-galactosidase and resulted in light blue colonies. Despite this there was still a distinct difference between white and light blue colonies (4), facilitating selection on IPTG/Xgal plates.

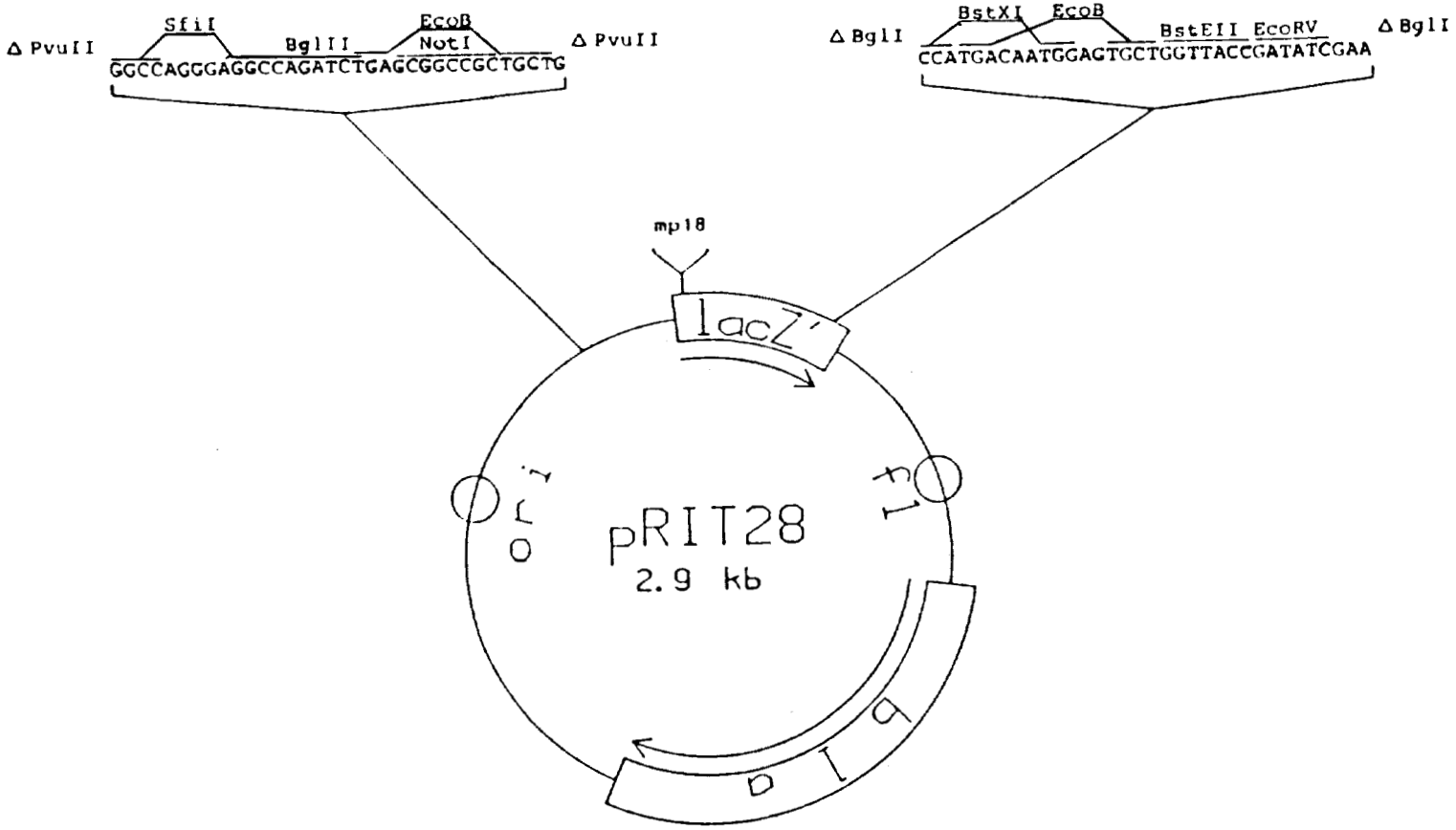

FIG.2. The sequencing vector, pRIT28, with the nucleotide sequence of the synthetic linkers inserted in the flanking regions. Abbreviations: bla, ß-lactamase gene; ori, origin of replication; f1, origin of replication of phage f1; lacZ', part of the ß-galactosidase gene.

Solid-phase sequencing

To evaluate the solid-phase approach, plasmid pRIT28 with a synthetic DNA insert, was digested with BstEII and EcoRV and the 5'-protrusions were filled in using 11-bio-dUTP and the appropriate dNTPs. After desalting on a Sephadex G-50 column (Pharmacia, Sweden), approximately 1 µg plasmid DNA was used for the immobilization to 1 µl avidin agarose.

The subsequent strand specific elution was performed as described by Ståhl et al.(4). The sequencing reactions were initiated from the RIT primer (7), complementary to a region downstream from the multilinker region. Dual protocols with both ^{32}P labelled primer, and incorporation of ^{35}S-dATP during extension, were run in parallel.

A simplified procedure was used in which the annealing and Klenow reaction were performed simultaneously. The primer was included in the extension mixture and the annealing step prior to the extension reaction could be omitted. After extension and chase elongation (4), the supernatant was removed and the gel extensively washed. The newly synthesized oligonucleotides were eluted with 5 µl formamide/dye mix and ready for loading on a sequencing gel. The affinity gel containing the single stranded template was thereafter used for another round of sequencing reactions using a new nucleotide mix.

The protocol was followed for all four dideoxy-nucleotides and the eluted samples were loaded on a sequencing gel. Autoradiograms of DNA fragments separated by electrophoresis using both protocols are presented in Fig.3. Clearly readable sequences are obtained, which correlates well with the expected sequence of the insert.

DISCUSSION

A procedure for solid phase sequencing using the enzymatic method to generate base specific fragments has been described recently (4). The immobilization procedure involves enzymatic incorporation of 11-bio-dUTP and binding

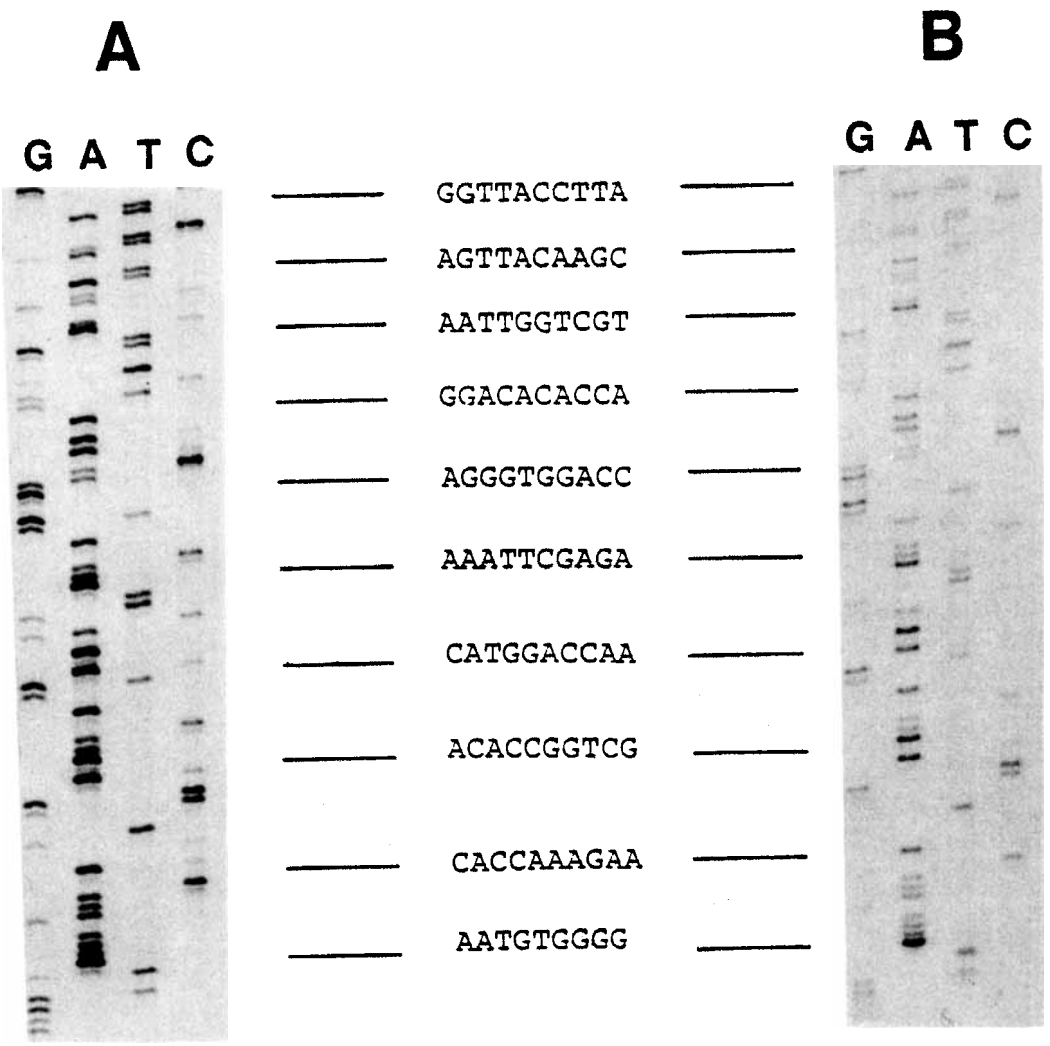

FIG.3. Autoradiograms of sequencing gels with samples obtained by solid-phase sequencing using A: ^{35}S-dATP incorporation and B: ^{32}P end labelled primer.

of the biotinylated fragment to avidin agarose followed by strand specific elution. In this procedure we used either ^{32}P labelled primer or incorporation of ^{35}S-dATP to specificly label the newly synthesized oligonucleotides with similar results.

The procedure presented here is based on the same concept, but has several advantages compared to the original

strategy. Using alternative restriction sites, and two different primers, the insert can be sequenced from both directions, which doubles the amount of information collected from each clone. The new sequencing vector pRIT28 is more versatile than pRIT27 (4) due to additional sites recognized by "rare cutters" such as NotI and SfiI. We here show that the extended material can be eluted by a formamide/dye mix suitable for the electrophoresis which is a great advantage compared with the alkaline elution followed by neutralization and ethanol precipitation that was used in the original procedure.

Furthermore the results presented in Fig.3 also demonstrate that no primer annealing step is needed. This further simplifies automatization of the solid phase sequencing procedure.

Although we have only shown that the solid-phase strategy outlined in Fig.1 can be used for DNA sequencing, a number of other applications requiring immobilized single or double stranded DNA may also be considered. Recently, a suitable scheme for solid phase in vitro mutagenesis based on the same concept has been designed (unpublished).

It is important to evaluate if the solid-phase strategy, outlined in Fig.1, can be incorporated into a general sequencing strategy. The solid-phase strategy, involving a fluorescence labelled primer, may provide the missing link in a completely automated and consecutive scheme for all operations in sequencing, involving an automated electrophoresis station (1) with on line detection and a station for automated preparative plasmid purification (T. Moks and M. Uhlén, unpublished). This would enable sequencing in considerably larger scale than is possible at present.

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