DNA Sequencing by Capillary Electrophoresis Using Short Oligonucleotide Primer Libraries

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ABSTRACT

Two strategies for DNA sequencing by primer walking using short oligonucleotide primer libraries have been successfully employed along with capillary electrophoresis using replaceable polymer solutions of linear polyacrylamide and fluorescence detection. A 3.5-kb stretch of the single-stranded M13mp18 template was sequenced with T7 PRISM dye-terminator/Sequenase[®] chemistry. An in-house base-calling program offered read lengths of roughly 450 bases with an average of 97.8% accuracy.

INTRODUCTION

Fast separation methodologies are being developed for large-scale DNA sequencing, e.g., capillary electrophoresis (CE) (16) and ultrathin slab gels (4,33). The inherent properties of CE—short analysis times and ease of automation—are well-suited to sequencing (3,22,30). Furthermore, the throughput using single capillary instruments can be potentially increased through multiple capillary array instruments (15,24,28). Cross-linked, gel-filled capillaries have been used to obtain size-dependent separation of DNA sequencing fragments (3,7,11). Although extended sequences have been obtained with such matrices (1), these materials can be disadvantageous in CE because of potentially poor gel stability.

Our laboratory introduced the use of low-viscosity, replaceable linear polyacrylamide (LPAA) solutions for the separation of sequencing reaction products (30). Recently, other polymer solutions have been used for DNA sequencing as well (8,9). The relatively low viscosity of these solutions permits replacement of the sieving matrix after each run, thus providing extended column lifetime, speed of separation and ease of automation. Previously, we reported the use of LPAA solutions to obtain sequencing information up to 350 bases in 30 min (30). These polymer solutions were optimized to provide robust separation of short stretches of sequences in a minimum analysis time,

using a simple two-peak height–two-color base coding.

The strategy frequently used for large-scale operations is shotgun sequencing, mainly because this approach is well-suited to automated instruments (13,37). However, there are drawbacks to this approach: its tedious and laborious subcloning steps, high redundancy (5–8 per base pair) and time-consuming manual sequence editing.

Directed sequencing strategies provide potential advantages over shotgun procedures, i.e., higher efficiency (a larger number of bases read per unit of time), less redundancy, fewer cloning steps and straightforward data assembly (36). The sequencing information obtained from the initial sequencing analysis, starting from the known sequence (e.g., a cloning vector), is used to determine the priming site for a second sequencing reaction. Primer-walking methods have been applied to large-scale sequencing projects with some success (32).

The lack of automated procedures and the necessity for synthesis of new primers for each reaction have limited the routine application of primer walking, in spite of its higher sequencing efficiency (23). In comparison to typical slab-gel operation, the increased separation speed and ease of automation offered by CE provide significant potential for the development of a closed loop instrument based on primer walking. However, custom synthesis of oligonucleotides 18–20 bases in length is generally expensive and slow.

Table 1. Purification of Sequencing Products from SSB-Hexamer Primed Reactions

Digest *ts*SSB-1 protein by adding 2 μ L of 2 μ g/ μ L pronase and 1.3 μ L of 10% (wt/vol) SDS to the sequencing reaction. The digestion reaction is performed at 65°C for 20 min.

Prepare a Sephadex® (Pharmacia Biotech) microcolumn (CentriSep™; Princeton Separations, Adelphia, NJ, USA) according to the manufacturer's specifications.

After digestion, transfer the sequencing reaction mixture onto the top of the Sephadex column.

Centrifuge the microcolumn for 4 min at 3000 rpm, collect the sample and vacuum dry under centrifugation.

Resuspend the sample in 40 μ L of dimethyl sulfoxide (DMSO) for 30 min.

Wash an ultrafiltration microcolumn (300 000 molecular weight cutoff) (Ultrafree-MC; Millipore, Bedford, MA, USA) extensively with water, centrifuging at 5000 rpm for 5 min. Pipet 40 μ L of DMSO onto the microcolumn, then centrifuge for 5 min at 5000 rpm in a fixed-angle microcentrifuge (Model 5415C; Eppendorf North America, Madison, WI, USA). Discard the DMSO spun through.

Heat the sample dissolved in DMSO for 5 min at 95°C.

Place the heated solution on the microcolumn and heat for an additional 5 min at 100°C.

Spin the column for 5 min at 5000 rpm. The recovered purified sample is injected electrokinetically into the capillary column for 10 s at a constant electric field of 200 V/cm.

Several reports (for example, Reference 26) have presented faster and smaller scale synthesis of oligonucleotides compared to traditional methods. However, these approaches are not fully sufficient for large-scale sequencing.

In 1989, the strategy of primer walking using a finite size oligonucleotide library was introduced (34). It was later shown that strings of contiguous hexamers, in combination with single-stranded binding protein (SSB)-saturated templates, can prime sequencing reactions efficiently (i.e., sufficient sequencing products for detection) and specifically, using both radioactivity and fluorescence detection with either single- or double-stranded templates (17,25). A related strategy for directed sequencing has also been reported based on the use of modified modular primers (heptamers and pentamers) without SSB (18,19). This general approach (i.e., the use of short oligonucleotides as primer for DNA sequencing reactions) is seen as a possible cost-effective, closed-end system for

large-scale sequencing.

This paper presents the results on the first successful application of sequencing chemistries using short oligonucleotide libraries and CE analysis in a single capillary prototype instrument using replaceable polymer solutions. DNA sequencing reactions were performed on M13mp18 template with fluorescence-labeled dideoxynucleotides (Applied Biosystems PRISM™ T-7 dye-labeled dideoxy terminators [Perkin-Elmer/Applied Biosystems Division (PE/ABI), Foster City, CA, USA]) for Sequenase® Version 2.0 [United States Biochemical (USB), Cleveland, OH, USA] and analyzed by CE using replaceable linear polyacrylamide matrices (LPAA). The fast analysis of the sequencing fragments in CE provides an overall increase in sequencing speed. A CE-based automated sequencer, utilizing modular primer walking, should provide complementary technology to automated shotgun sequencing strategies for large-scale sequencing efforts.

MATERIALS AND METHODS

Instrument Description

The basic design of a CE instrument using a single laser line excitation (argon ion laser 488 nm) and two photomultipliers (PMT) for on-column fluorescence detection of sequencing reaction products has been described previously (30). The instrument described in this paper differs only in the detector and the capillary column heating device. The detector is an intensified photodiode array instrument (Model 1420; EG&G Princeton Applied Research, Princeton, NJ, USA) for full spectral detection of labeled dideoxynucleotides (5). The thermostat on the heating device regulates the temperature of the separation column, providing constant temperature (e.g., 30° $\pm$ 0.5°C) for 15 cm of the effective length of the capillary. A 15-cm piece of copper tubing is filled with heat-conductive epoxy (Norlabs, Greenwood, CT, USA) and wrapped with a flexible heating element equipped with a thermistor connected directly to a temperature controller (Omega Engineering, Stamford, CT, USA). The remaining segment of the capillary is left unheated due to physical limitations.

The data are recorded in two formats: acquisition of the full spectra and real-time monitoring of the signal at two pre-selected diode groups. The schematic of the data-acquisition system has been published previously (5). Stored data are analyzed with the aid of the laboratory-developed software, which allows viewing of the four-color dye channels, spectral information, base calling, etc.

Base-Calling Software

The raw spectra were first smoothed using principal component analysis, which also reduced the data to a compact form for manipulation. Color separation was then achieved by solving a matrix equation by ordinary linear regression, $\tilde{Y} = \tilde{R}\tilde{C}$, where $\tilde{Y}$ is the matrix of raw data, $\tilde{R}$ a matrix containing the four dye-terminator spectra and $\tilde{C}$ the solution matrix, a four-channel electropherogram in which each channel is the fluorescence signal of one of

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Table 2. Hexamer Primers Used for Sequencing on M13mp18

Reaction No.	Sequence Hexamer #3 (5' End of the Primers)	Sequence Hexamer #2 (Middle Hexamer)	Sequence Hexamers #1 (3' End of the Primers)	G+C	3' End Position on M13mp18
1	AACTCC	AACAGG	TCAGGA	9	291
2	GGCATA	GTAAGA	GCAACA	8	639
3	TTTTAA	GAACTG	GCTCAT	6	806
4	GCAACG	GCTACA	GAGGCT	11	1324
5	CACCAG	TACAAA	CTACAA	7	1760
6	GTTTTA	ACGGGG	TCAGTG	9	2106
7	ATTCAT	ATGGTT	TACCAG	6	2714
8	CCACAA	GAATTG	AGTTAA	6	3004
9	GAATCA	TTACCG	CGCCCA	10	3405
10	GCTCAA	CAGTAG	GGCTTA	9	3752

the four dyes. The matrix $\tilde{R}$ was obtained by searching the most intense spectra in the raw data for the best matches to the reference spectra of the four dye terminators. After color separation, noise was removed by Wiener filtering (27). Baseline correction was then performed by subtracting from each point the smallest value in a short interval on adjacent sides.

After this initial processing, peak detection and base calling were performed concurrently with a moving time window containing about ten bases at any moment. Peaks were determined as maxima in the data that matched a model of peak height, shape and spacing. Since these parameters changed with migration time, each called peak was used to update the model. Calls were made by a graph-theoretic method, which determined the most evenly spaced set of peaks (after dye mobility correction), with due consideration given to peak height and width (2). Sequencing was terminated when irregularity in peak spacing increased above an arbitrary threshold, indicating unreliable calls.

Capillary Coating and Sieving Matrix Preparation

The capillaries were coated to suppress the electroosmotic flow and to provide high separation efficiency, as described (12). Absence of electroosmotic flow also improves both reproducibility and ruggedness of the

electrophoretic separation. The sieving matrix (4%T/1× TBE/30% [wt/vol] formamide/3.5 M urea) was developed to extend the sequence readout per run in a reasonable analysis time over that previously published (30). A separate paper will present the optimization of LPAA solutions for the separation of single-stranded DNA fragments. The synthesis of LPAA solutions was performed using the conventional ammonium persulfate/*N,N,N',N'*-tetramethylethylenediamine (APS-TEMED) redox system. The polymer was elongated by using small amounts of both radical initiator and catalyst and a low polymerization temperature (0°C) to provide high molecular weight LPAA polymer fibers. Since oxygen is a radical quencher, an inert atmosphere must be provided for the polymerization reaction.

In brief, 1 mL of acrylamide solution (4% acrylamide [wt/vol] in TBE-buffer [1× Tris/boric acid/ethylenediaminetetraacetic acid, pH 8.3]; 30% wt/vol formamide; 3.5 M urea) was degassed for 2 h by purging with helium. With a microsyringe, 1 μ L each of 10% TEMED and 10% APS was added to the sealed vial. After briefly vortex mixing, the polymerization was carried out in an ice bath (0°C) for 24–48 h. After polymerization, the sieving matrix was kept in the freezer (–20°C) to avoid decomposition of the buffer denaturants. Care must be exercised to avoid exposure of the polymer solutions to room temperature for pro-

longed time. (Note: Formamide can rapidly decompose in basic solution at room temperature.)

The separation performance and reproducibility of the capillary system were determined using a FAM-labeled (–21) primer (PE/ABI) and M13mp18 ssDNA template (New England Biolabs, Beverly, MA, USA), and the sequencing reaction was terminated with dideoxythymidine triphosphate (dd-TTP) (USB). These reactions were prepared according to the USB Sequenase protocol, with the exception that the labeling step was omitted and the dNTP/ddNTP ratio in the extension/termination was changed to 400 from typically 10–50.

Sequencing Reaction Protocol

Two main strategies were used for DNA sequencing using short oligonucleotide primers and labeled dideoxynucleotides: (i) hexamers with single-stranded binding protein temperature-sensitive mutant (*ts*SSB-1, made available to Brookhaven National Laboratories by courtesy of C.W. Fuller, Amersham Life Sciences Inc.)-saturated templates and (ii) modified modular primers. For these studies, the DNA sequencing samples using modified modular primers were kindly provided by L. Kotler and L. Ulanovsky of the Weizmann Institute in Israel. A protocol previously reported was followed (18,19).

The protocols for DNA sequencing using hexamers and SSB-saturated templates with both radioactivity (0.7 μ g of M13mp18) and fluorescence detection (2 μ g of M13mp18) have also been reported previously (17,25). The protocol used in this work was slightly modified by using *ts*SSB-1 and including 1% (wt/vol) sodium dodecyl sulfate (SDS) in the digestion reaction. The use of SDS with pronase improved the proteolytic digestion of *ts*SSB-1 (31).

In addition, the concentration of NAN-ddG in the PRISM T-7 mixture was increased from 8.0 μ M to 13.0 μ M by the addition of NAN-ddG to compensate for the poor excitation of the NAN dye (maximum absorption at 555 nm) by the 488-nm line of the argon ion laser. The amount of template DNA used per reaction was the same as previously described (25). In future work, a tunable dye laser will be used to compensate for the poor excitation of NAN and al-

Figure 1. Separation of FAM-labeled primer (-21) sequencing reaction products from M13mp18 terminated with dideoxythymidine triphosphate. The electrophoretic conditions were the following: capillary effective length = 30 cm (total length = 40 cm); column temperature 25°C; sieving matrix 4% T LPAA/30% (wt/vol) formamide/3.5 M urea; constant electric field = 200 V/cm.

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Table 3. Sequencing Results on M13mp18 Using SSB-Hexamer Primed Reactions and Capillary Electrophoresis Analysis

Primer 3' End Site on M13mp18	Site On M13mp18 for the First Base Sequenced	Site On M13mp18 for the Last Base Sequenced	Total Number of Bases Sequenced	Miscalls	% Accuracy
291	253	7165	337 ^a	8	97.6
639	603	144	459	11	97.6
806	775	202	573 ^b	6	99.0
1324	1281	868	413	4	99.0
1760	1703	1251	452	24 ^c	94.0
2106	1998	1598	400	11	97.3
2714	2676	2181	495	8	98.4
3004	2972	2540	432	3	99.3
3752	3704	3188	516	13	97.5
			av. number of bases sequenced	av. number of miscalls	av. accuracy
			456	10	97.8%

^aThe short read length was due to weak signal from this sample.
^bThe highest priming efficiency of the sites tested.
^cThe number of errors was higher than the average because of mispriming.

low for the use of the regular PRISM T7 mixture. Increasing the NAN-ddG concentration in the deoxynucleotide-dideoxynucleotide mixture increased the probability for reaction termination with the ddG terminator. Since the intensity of the fluorescence signal was dependent on both excitation efficiency and concentration, the signal increased.

Purification of Sequencing Reaction Products

Electrokinetic injection was required to introduce the sequencing fragments into the capillary column. To maximize the signal, small ions, non-incorporated deoxynucleotides and dideoxynucleotides, hexamer primers, template DNA and *ts*SSB-1 digestion products were removed from the sample. Maximum signal was required to accommodate hexamers from weak priming sites. The presence of SSB protein molecules in the sequencing sample interfered with both the injection and separation of sequencing fragments in CE. The use of a temperature-sensitive mutant *ts*SSB-1 was necessary to purify the sequencing sample for CE analysis. The DNA-protein complex between *ts*SSB-1 and the template DNA is disrupted above 47°C

(21). Therefore, at the temperature of 65°C, pronase could effectively digest the protein into short peptides or amino acid components. The detailed purification method for DNA sequencing of samples using hexamer-SSB chemistry and CE analysis is outlined in Table 1. Investigations are ongoing on automated procedures for sample purification.

RESULTS AND DISCUSSION

Capillary Electrophoretic Separation of Sequencing Reaction Products with 4% T LPAA Sieving Matrix

In our previous work, a 6% T LPAA matrix was used for the separation of sequencing fragments of at least 350 bases in 30 min (30). The synthesis and electrophoretic conditions were optimized to obtain fast sequencing analysis of short stretches of DNA using simple instrumentation. In order to extend the read length to 450–500 bases, a lower concentration of LPAA was selected (4% T). To achieve sufficient separation efficiency, the polymerization parameters were optimized to synthesize LPAA of high molecular weight. Such LPAA can be synthesized by means of the conventional redox ini-

tiation process (APS and TEMED) by decreasing the polymerization temperature and by decreasing the amount of radical initiator used (14). The average molecular weight for the laboratory-synthesized LPAA (4% T) was determined by size-exclusion chromatography to be approximately 10⁶ Da with polydispersity of 2.2 (American Polymer Standards, Mentor, OH, USA).

The use of 4% T/1× TBE/30% (wt/vol) formamide/3.5 M urea LPAA with coated capillaries allowed separation to base 500 in roughly 60 min. As an example, Figure 1 presents the electropherogram for the separation of a M13mp18–FAM-labeled primer (-21) sequencing reaction terminated with ddTTP. Using this LPAA solution, sequencing fragments ranging in size from 27 bases to 498 bases could be resolved by one base difference in 53 minutes.

Optimization of Sample Purification and Injection

The amount of DNA sequencing reaction products injected into the column and the degree of separation affect both the read length and the accuracy of the base calls. Sequencing chemistries, based on the use of short oligonucleotides as primers in combination

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Figure 2. Separation of SSB-hexamer primed sequencing reaction products. The electrophoretic conditions were the same as for Figure 1 except that the temperature was 30°C. The reaction was primed at position 3' 806 in M13mp18 with three hexamers and 6.5 µg of *ts*SSB-1.

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Figure 3. Separation of modified modular primed sequencing reactions products. The electrophoretic conditions were the same as for Figure 2. The primers used for this reaction were three degenerate, phosphorylated heptamers and one pentamer at position 3' 2892 on M13mp18.

with dye-labeled dideoxynucleotides, in general yielded lower amounts of products than other sequencing chemistries, e.g., labeled primers or cycle-sequencing. Furthermore, the signal using short primers could differ as much as 50-fold, depending on the priming site or the template. The use of these novel sequencing chemistries thus required maximization of the amount of purified material introduced in the capillary column. The electrokinetic injection of sequencing fragments was optimized and will be described in a separate report.

The use of labeled dideoxynucleotides, large quantities of hexanucleotides (μM) and SSB protein (μg) imposed unusual conditions for the purification of sequencing fragments. A two-step purification process was designed to eliminate impurities. The salts and SSB digestion products were removed with a MicroSpin Sephadex[®] column (Pharmacia Biotech, Piscataway, NJ, USA) and the template DNA was removed by ultrafiltration in a denaturant solvent. As a result of these purification steps, long injection times (20 s) could be used without significant loss in current or separation. Loss of current or column failure is frequently found to be caused by template present in the sample (29,35).

Sequencing Results on M13mp18 ssDNA

Sequencing using single-stranded binding protein-saturated templates and hexamer primers

Templates saturated with *E. coli* SSB protein have been used for sequencing to minimize the secondary structure of the DNA template and increase the processivity of DNA polymerase (Sequenase Version 2.0) (20). SSB protein has also been utilized in hexamer string sequencing to improve the specificity of short oligonucleotides annealing to the template (17). The presence of the SSB protein in the sequencing sample, however, interfered with both the separation and injection of sequencing products. The use of the *ts*SSB-1 mutant offers great advantage for the application of this chemistry to

CE. In order to remove the *ts*SSB-1 from the sequencing sample, pronase was used to digest the protein at 65°C. *ts*SSB-1 proteins allowed for the proteolytic digestion upon dissociation of the DNA-protein complex in the sequencing sample (6,25). It is noteworthy that no sequencing ladders could be detected when the wild-type SSB was utilized for the sequencing reactions (results not shown).

The optimized purification scheme was then applied to SSB-hexamer primed reactions, and the reaction products were analyzed by CE. Figure 2 shows an example of the separation of sequencing fragments starting at site 3' 806 on M13mp18. The separation was achieved for fragments ranging in size from 31 to 520 bases in 65 minutes of analysis. A comparison with the GenBank sequence of M13mp18 revealed that the sequence information for 460 bases was obtained with an accuracy of 97.8%.

Sequencing using modified modular primers

A reaction performed with a modular primer of one pentamer and three degenerate phosphorylated heptamers to prime the reaction at the 3' position 2892 of M13mp18 was analyzed by CE. The pentamer was the front (3' primer end) module. The two middle and back oligonucleotides (5' end of the primer) were 5'-phosphorylated and degenerate at the last two 3' bases (19).

Figure 3 presents the separation of DNA sequencing fragments using this modified modular primer sequencing chemistry. Fragments ranging in size from 43 to 520 bases in length could be resolved with one base difference. The accuracy for 467 bases sequenced was 99.6% vs. the M13mp18 sequence (10). Thus, in Figures 2 and 3, the two chemistries yielded sequencing information for more than 460 bases in about one hour using CE with 4%T LPAA solutions. These promising results led us to determine the sequence of a portion of M13mp18.

Sequencing on M13mp18 single-stranded template

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Because of the availability of primers, SSB-hexamer primed chemistry was utilized to sequence a section of ssM13mp18 vector. Table 2 presents the sequence of the selected hexamer primers used for the sequencing reactions. Included in this table are the GC contents of the hexamers and the position on M13mp18 where the 3' end of the front hexamer annealed. In separate studies, these hexamers were shown to yield moderately to highly successful sequencing reactions on slab gels with both radioactivity and fluorescence detection. With further experience it is anticipated that rules regarding the efficiency of hexamer priming will be established.

The sequencing results obtained using M13mp18 are summarized in Table 3. The raw sequence data were analyzed using laboratory-developed sequencing software, followed by manual editing (see Base-Calling Software section). The average number of bases sequenced for these reactions was roughly 450, and the average number of errors was 10 compared with the GenBank sequence (10), representing an average accuracy of 97.8%. There are two main sources of error: the poor excitation of NAN dye label (ddG) by the 488-nm argon line laser and the deconvolution of the dye spectra by the sequencing software. Given that the maximum absorption of the NAN dye is 555 nm, a second laser line in conjunction with an argon ion laser would have to be used to efficiently excite all four labeled dideoxynucleotides. Upon improved excitation of the NAN dye, the number of errors in the sequence call should decrease, thus improving accuracy further.

Nevertheless, the data represent initial promising results in the application of CE to primer walking DNA-sequencing strategies for genomic scale projects. Further developments in robotics, instrumentation and data analysis are required in order to apply CE routinely and effectively to DNA sequencing analysis. At present our laboratory is developing a multiple capillary array and appropriate robotics with a multiplex purification system in order to transform CE into a tool for large-scale sequencing in combination with primer walking and other sequencing

strategies.

CONCLUSION

M13mp18 was used as a model to assess the reliability of DNA sequencing using short oligonucleotide primer libraries and CE. The use of hexamers with SSB, together with modified modular primer sequencing chemistries, were successfully applied to CE with replaceable sieving matrices. These initial results provide promising evidence that these sequencing chemistries in combination with capillary electrophoresis have the potential to provide a fast, fully automated, closed-end system for DNA sequencing.

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