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Exoquence DNA sequencing

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ABSTRACT

We have developed a strategy for DNA sequencing based on exonuclease III digestion followed by double strand specific endonuclease digestion and direct dideoxynucleotide sequencing reaction. This strategy eliminates the need for subcloning, oligonucleotide primers, and prior knowledge of the DNA to be sequenced. All template and primer duplexes needed for sequencing a complete insert can be prepared in one day from uncharacterized starting DNA. Sequence information can be obtained from different regions of the DNA simultaneously. The method uses double-stranded DNA to generate single-stranded template and primer, and thus produces high quality sequence results. Commercially available dideoxy-sequencing kits are well suited for this method. The strategy should be applicable for both automatic and routine laboratory DNA sequencing.

INTRODUCTION

The DNA sequencing technique has been under scientific investigation since the discovery of the structure of DNA. At present, DNA sequencing requires the preparation of appropriate templates and primers, sequencing reaction (1,2), separation of the reaction products, and reading of the sequence. Efforts to improve and automate these processes have been made (3–8). However, the preparation of appropriate templates and primers currently requires several steps and a great deal of time. This procedure is probably the most difficult one to be automated.

Sequencing strategies based on *E.coli* exonuclease III (Exo III) have been employed since 1982 (9). Although improvements have been made (10), Exo III sequencing methods were not popular until the publication of an Exo III recession strategy (11). However, conventional Exo III recession requires subcloning, growing bacterial cultures and purifying nucleic acids. The recently published ExoMeth strategy successfully eliminates the need for primer generation and subcloning (12). However, this method relies on modified nucleotides, which are expensive and eliminate the use of most commercially available sequencing kits. The use of all four modified nucleotides is inhibitory to the polymerase reaction. This method also limits the number of enzymes which can be used to reduce the primer size.

Here we report a new Exo III-based DNA sequencing method, Exoquence, with which sequences from totally uncharacterized DNA can be determined with no need for subcloning, generating primers, or incorporating modified nucleotides. This technique is extremely rapid and suited for automated or routine laboratory sequencing.

MATERIALS AND METHODS

Primary enzyme digestions

'A and B' or 'A and C' digestions in Fig. 1 refer to the primary enzyme digestions. Low DNA concentration and large reaction volume were used in the restriction digestion at appropriate buffer and incubation temperature. After primary digestions, the DNA was extracted with phenol-chloroform, precipitated by ethanol, washed with 70% ethanol, lyophilized, and resuspended in water at a concentration of 1 $\mu g/\mu l$. The phenol-chloroform extraction may be omitted, because the presence of these enzymes in the further steps is inconsequential. Ethanol precipitation is needed to concentrate the DNA and to remove sodium ions which inhibit Exo III deletion.

Exo III deletions

The digested DNA was digested with Exo III (NEB, Stratagene, BRL) in Exo III Buffer (50 mM Tris·HCl pH8.0, 5 mM MgCl, 10 mM β-mercaptoethanol) at 37°C at a DNA concentration of 0.2 $\mu g/\mu l$. Two procedures were used to mix the reaction contents: 1). The DNA and the buffer were mixed and incubated at 37°C for 5 minutes to allow the temperature to equilibrate; the Exo III was then mixed with other reaction contents as quickly as possible. 2). The DNA, the buffer, and Exo III were mixed and incubated for 5 minutes at 0°C to allow the enzyme to bind to the DNA. The reaction mixture was then incubated at 37°C. 25 μl aliquots were removed at 30-second intervals, and reaction were terminated with 75 μl of 40 mM pre-cooled EDTA. Under these conditions, Exo III will remove about 440 b/min. To allow the sequence obtained from one recession to overlap with the sequence obtained from the previous recession, 30 seconds or less is recommended for each time interval. The aliquots were left on ice until all reactions were completed. Twenty units of Exo III per microgram of DNA were normally used, but other Exo III:DNA ratios gave comparable results (see Discussion).

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The Exo III in each aliquot was inactivated at 70°C for 10 minutes. The DNA was then extracted with phenol-chloroform, precipitated by ethanol, and resuspended in water at a concentration of 0.05 μg/μl. The heat inactivation may not be needed, since the phenol-chloroform extraction will get rid of the Exo III. Low DNA concentration and large reaction volume are preferred for the secondary restriction digestion. 1−2 μg of the DNA can be taken out for the analysis of the resection.

Secondary enzyme digestion

The R digestion in Fig. 1 is referred to as the secondary enzyme digestion. R is usually an enzyme which recognizes a four base pair DNA sequence (ie, R is a frequent cutter). 10 units of R enzyme and appropriate buffer were directly added into the purified resection aliquots, and were then incubated at 37°C for 1 hour. An enzyme which needs 65°C or 55°C (eg, Taq I) to digest DNA may not be used, since high temperatures may denature the template/primer duplexes. After R digestion, the DNA was extracted with phenol-chloroform, precipitated by ethanol, washed with 70% ethanol, lyophilized, and resuspended in 8 μl of water. At this point, the DNA was ready for the sequencing reaction.

Sequencing

Sequenase Version 1.0 DNA sequencing kits (USB) were used to do all the sequencing in multi-well plates. [α-^{35}S] dATP (Amersham) was used to label the DNA. The procedure was the same as the protocol recommended except that the denaturation and the primer annealing steps were eliminated. After the sequencing reaction, the samples were denatured at 85°C for 2 minutes, chilled on ice, and loaded onto a conventional sequencing gel. Gel running, exposing, and film developing were the same as in standard dideoxy sequencing methods.

Analysis of Exo III recession

1−2 μg of Exo III treated DNA was digested by mung bean nuclease (NEB, Stratagene) according to recommended conditions. Then the DNA was extracted with phenol-chloroform, precipitated by ethanol, washed with 70% ethanol, lyophilized, resuspended in 10 μl water, and analyzed on a 1% agarose TBE mini-gel.

RESULTS

Exoquence strategy and general comments

The cloned DNA to be sequenced is first linearized to make the insert ends susceptible to and the vector ends resistant to Exo III digestion (Figure 1). Exo III removes nucleotides progressively and synchronously from susceptible 3′ ends of double-stranded DNA and leaves extensive single stranded 5′ protrusions (13). The recessed strand will serve as primer, while the intact strand will serve as template in the dideoxy sequencing reaction. A series of time points of Exo III digestion create successive sets of primer/template heteroduplexes for sequencing a complete insert. The primers generated by Exo III in each time point are reduced in length by secondary enzyme digestion (R digestion in Figure 1). Therefore, all the appropriate primer/template duplexes needed to sequence a complete insert are ready for dideoxy sequencing at this point. The procedures described above can be finished in a single day regardless of the length of the insert.

Blunt ends, 5′ overhangs, and 3′ end overhangs with less than 4 bases are susceptible to Exo III, whereas 3′ overhangs with 4 or more bases or DNA ends filled with a-thio-deoxynucleotides are resistant (9, 11, 14, and 15). The 3′ overhangs with 4 or more bases can be generated by restriction digestion, terminal deoxynucleotide transferase polymerization, or λ exonuclease digestion. The restriction enzyme digestion is by far the easiest method and was used exclusively in this study.

Enzyme A in Fig. 1A is the one which produces blunt ends or 5′ overhangs, and its site must be located near the end of the insert to allow Exo III to recess toward the insert. Enzyme B must be the one which produces 4-base 3′ overhangs.

The strategy in Fig. 1A assumes that enzymes A and B have only one restriction site each on the plasmid DNA. To assure a single cut on the plasmid, Not I and other rare cutters (which recognize more than six base DNA sequences) can be used as enzyme A. However, so far no enzyme which recognizes more than a six base sequence and produces 4-base 3′ end overhangs is commercially available for enzyme B. When the insert is long (>3kb), it is reasonably probable that enzyme B will have more than one site on the plasmid. Such a situation is illustrated in Figure 1B. The enzyme with multiple restriction sites on the plasmid and producing 4-base 3′ overhangs is defined as enzyme C. Regardless of the number of enzyme C sites, none of the DNA fragment ends produced by enzyme C digestion can be digested by Exo III. Therefore, in an aliquot collected at any one time point, only one set of template/primer duplexes generated is available for sequencing.

Any plasmid DNA suitable for double-stranded DNA sequencing and containing appropriate polylinker sites flanking the insert can be used for the Exoquence method. The larger the insert, the more DNA is needed. The length of the insert DNA determines the number of time points taken. The starting DNA can be circular or linear double-stranded DNA as long as unidirectional Exo III digestion can be achieved.

Most commercially available enzymes can be used in the Exoquence DNA sequencing method. Enzymes B or C must digest completely (>90%). Kpn I, Pst I, Sac I, BstX I were used in this study for enzyme B, and each gave satisfactory results. Complete digestion by enzyme A is preferable but not obligatory, because the reduced amount of template resulting from incomplete digestion can be offset by using larger amounts of DNA. All enzymes producing blunt ends, 5′ overhangs and 3′ overhangs with less than 4 bases can be used for enzyme A as long as they have a single site on the plasmid. To assure complete digestions by enzymes A and B or enzymes A and C, we found the following precautions advisable. Check the enzyme quality before actual digestion. Digest the DNA with enzyme B or C first. Relative distance between enzymes A and B or A and C in the polylinker region is important. If the distance is long enough (>20 bp), both enzymes will digest completely regardless of whether the enzymes digest together or separately. This distance is generally less than 100 base pairs, so the short DNA fragments produced (the right-most fragments in Figs. 1A and B) will be completely digested to single-stranded DNA by Exo III and thus have no effects on sequencing. The enzyme R is used to reduce the primer length, so it should be a frequent cutter which recognizes a four base sequence. Since the DNA fragment to be sequenced is uncharacterized, enzyme R is chosen randomly.

Generally more than one enzyme is needed as enzyme R to sequence a DNA fragment. Four frequent cutting enzymes should

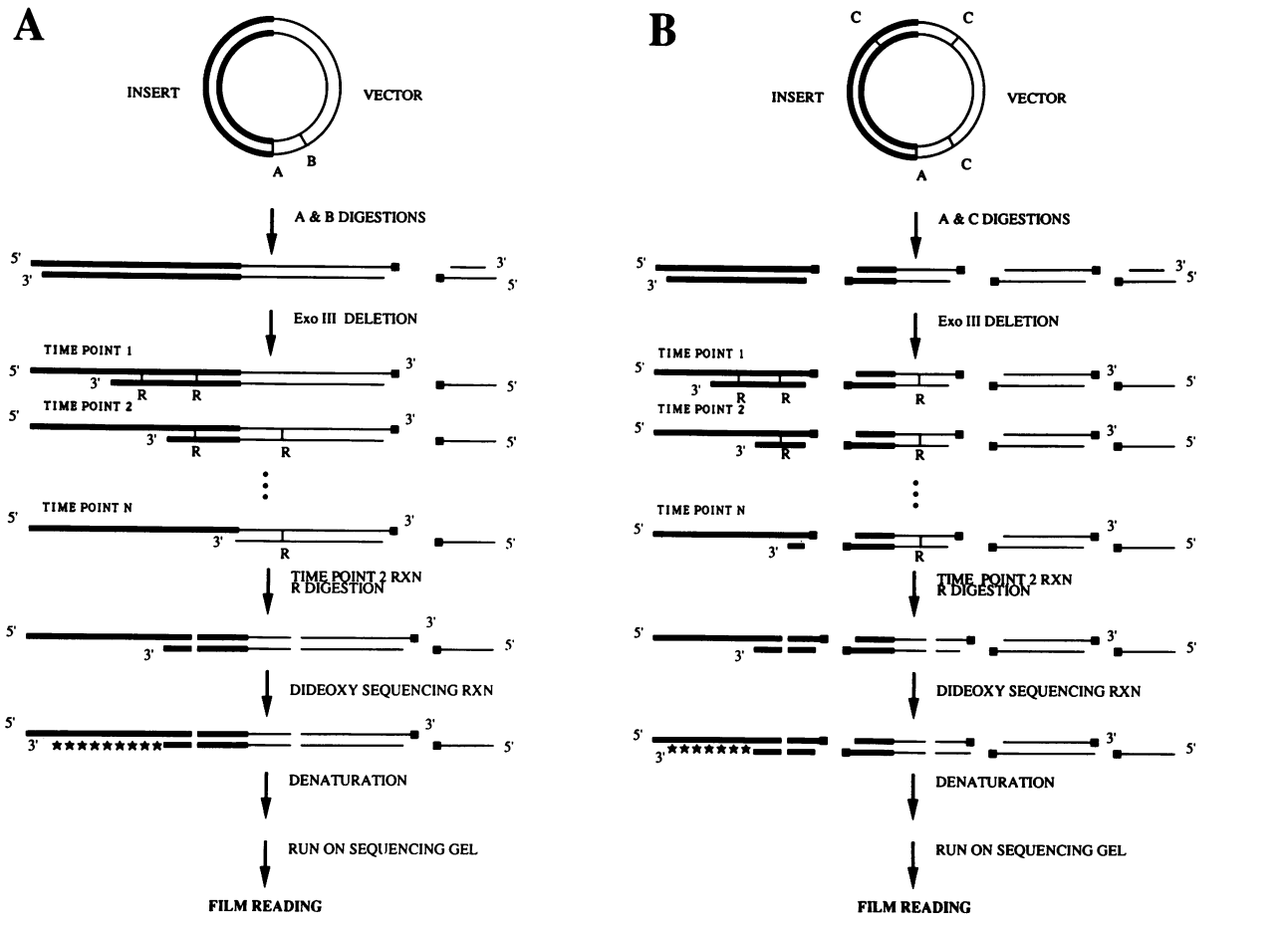

Figure 1. Schematic representation of Exoquence DNA sequencing strategies. A, B, C, and R are restriction enzymes. The thick and thin lines represent the insert and vector sequences, respectively. The solid squares are the 3′ ends produced by B or C digestion which block the Exo III recession. The stars represent the labeled DNA strand. Strategy A represents the situation for a single 3′ overhang site (enzyme B) and strategy B assumes multiple sites (enzyme C). Enzyme R is a randomly chosen, frequent cutter. For clarity, only a few of the R sites are illustrated. The R sites are unknown since the insert is uncharacterized.

be sufficient to sequence any unknown DNA, since the combined frequency of their restriction sites is one in every 64 base pairs. Enzymes requiring six or more base recognition sequences are impractical to be used as enzyme R unless the position of their sites is known; for example, when the resection end points are near the other polylinker site. The location of the sequence obtained is determined only by the order of the time points and the length of Exo III recession. If these numbers are chosen properly, the distal sequence obtained from one time interval will overlap with the proximal sequence obtained from the immediately previous time interval.

Theoretically enzyme A in Fig. 1 can also have multiple recognition sites on the starting DNA. As long as the region to be sequenced is longer than any DNA fragments produced by A digestion, the short DNA fragments (like the right-most fragments in Figs. 1A and B) will be digested to single stranded DNA. Consequently they will have no effect on sequencing.

In conventional dideoxy sequencing, the primers used in one sequencing reaction are homogenous, which means they have the same 5′ ends, 3′ ends, and base composition. In Exoquence DNA sequencing, the primer base composition and 5′ ends, which are produced by R digestion, are homogenous. The 3′ ends

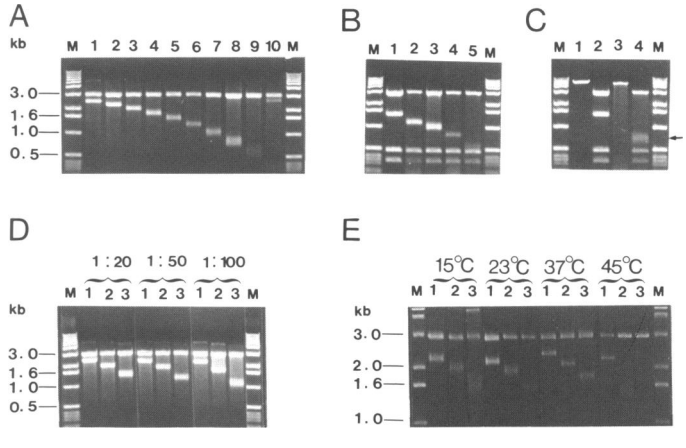

Figure 2. Analysis of Exo III recessions. **(A)** and **(B)** are the resections produced by Exoquence strategies A and B, respectively. The range of the recession, reflected by the width of the digested bands, is not proportional to the length recessed: compare the arrow-indicated band in lane 4 of **(C)** with that in lane 4 of (B), or with that in lane 8 of (A). **(D)** Effect of different DNA to Exo III molar ratios on range. **(E)** Effect of varying temperature. Numeric numbers indicate different aliquots. M is the 1 kb DNA ladder marker (BRL).

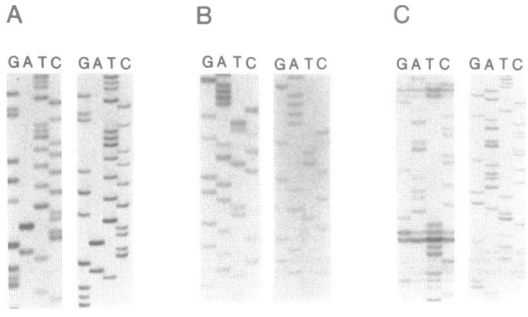

Figure 3. Comparison of Exoquence DNA sequencing (right panels) with conventional double-stranded DNA sequencing (left panels). **(A)** Sequences obtained by strategy A in figure 1. **(B)** Sequences obtained by strategy B in figure 1. **(C)** A typical artifact region produced with conventional double-stranded sequencing compared with the same region sequenced by strategy B of figure 1.

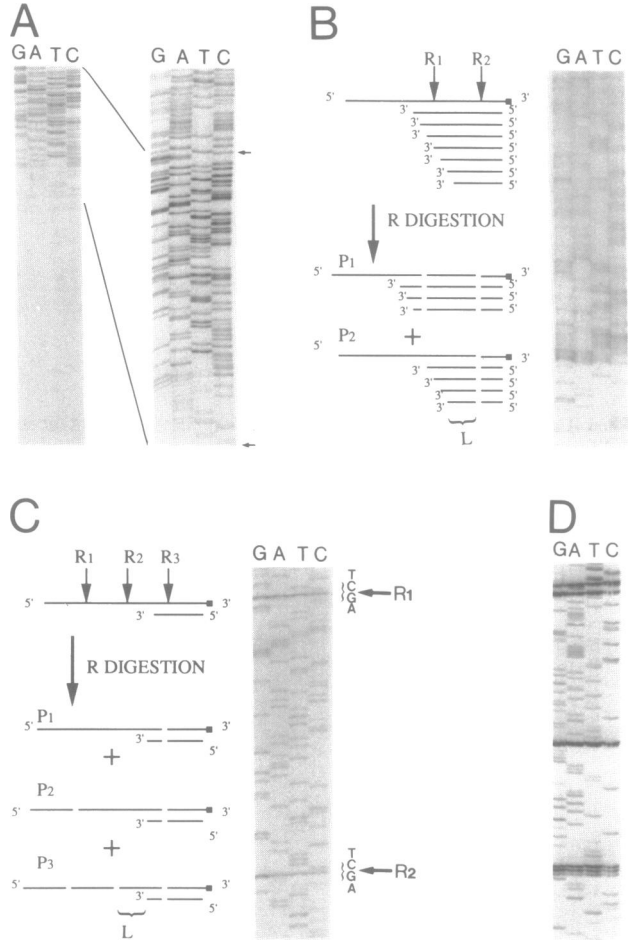

Figure 4. Troubleshooting Exoquence DNA sequencing. **(A)** The secondary enzyme digestion can produce long primers(>50 bases). In this case, the gel on the left has to be run longer (right) to resolve the identical sequences, indicated between arrows. **(B)** If there is a restriction site (R_1) inside the recession range, partially overlapped and partially non-overlapped sequences can be produced as shown in the schematic on the left. **(C)** If an enzyme R cuts single strand DNA at lower efficiency (sites on single strand DNA indicated by R_1 and R_2), sequence results typical of 'hard stops' are produced. The enzyme in this case was Alu I (AGCT). **(D)** Artifact that has not yet been solved.

Table 1. Summary of secondary enzymes (27) used in this study

Res \ Enz	Acc I	Afl III	Alu I	Ava I	Ava II	BamH I	Dde I	Dpn I	EcoR I	Hae III	Hinf I	Hpa II	Kpn I
+	3	1	4		2	1	2	5	1				2
+?				1				2					
?			2					3		1			
-?										1			
-			4	1			2	1			5	1	

Res \ Enz	Mse I	Msp I	Nco I	Nde I	Pst I	Pvu I	Pvu II	Rsa I	Sau3A I	Sph I	Stu I	Sty I	Xba I	Xho I
+			1	3			2	11	2	2	2	1	1	3
+?						1								
?		4						1	2					3
-?														
-	2				1									1

Hinf I never worked. Mse I, Msp I, Hpa II, and Hae III never gave good results. Rsa I, Alu I, Dpn I, and enzymes recognizing 6-base sequences always gave good results. Of 91 sequencing reactions, 51 worked (+), 19 did not work (−), and 21 had various problems (+?, ?, and −?). About 56% of reactions were successful.

of primers, which are the end points of the Exo III-digested strands, are heterogenous. During the dideoxy sequencing reaction, the 3′ ends are extended, and the extension stops when a dideoxynucleotide (ddNTP) is incorporated. Therefore the heterogenous 3′ ends of primers produced by Exo III resection are inconsequential to the sequencing reaction.

Optimizing Exo III conditions to reduce range

Exo III recession is processive unless there are significant nicks in the starting DNA (recession can start at the nicks). Even though Exo III recession is fairly synchronized under standard conditions, a range of endpoints leaving heterogeneous 3′ ends still exists. We define the distance between the shortest and longest 3′ end as the range. A limited range is crucial for success with our method.

Exo III recessions were analyzed by removing the single-stranded portion of the heteroduplexes with mung bean nuclease, digesting the DNA with an enzyme to liberate the insert, and running the blunt-ended products on an agarose gel. Recessions generated by using strategies represented in Figs. 1A and 1B are shown in Figs. 2A and 2B, respectively.

It is reported that the Exo III range is about 4 to 10% of the recessed DNA length. We observed that the range was not always proportional to the length of recession; ie, the range associated with a short recession was the same as that associated with a long recession: Compare the width of the arrow-indicated band in Lane 4 of Fig. 2C with that in Lane 8 of Fig. 2A; the recessions are 1.6 and 0.6 kbp, respectively. Often recessions of approximately equivalent length produce significantly different ranges: Compare the width of the arrow-indicated band in Lane 4 of Fig. 2C with that in Lane 4 of Fig. 2B; both recessions are about 0.6 kbp. More important factors in determining the range are the quality of the enzyme, which unfortunately is difficult to control, and the digestion conditions. However, the DNA to enzyme molar ratios of 1:2 and 1:10 (data not shown), 1:20, 1:50, and 1:100 (Fig. 2D) did not significantly influence the range. A number of digestion temperatures (15°C, 23°C, 37°C, and 45°C) can be used, but the smallest range was achieved at 37°C (Fig. 2E).

Comparison of exoquence and conventional sequencing results

Sequences obtained by strategy A in Fig. 1 (Fig. 3A, right panel) and strategy B in Fig. 1 (Fig. 3B, right panel) compare favorably to sequences obtained by a conventional double-stranded DNA sequencing method (Fig. 3A and 3B, left panels). Thus, it does not matter how many restriction sites enzyme C has on the plasmid, as long as it does not cut in the region where the sequence needs to be determined.

Since Exoquence uses double-stranded DNA to generate single-stranded template and primer, the results are often better than conventional double strand sequencing. For example, in the region of DNA shown in Fig. 3C, the compression artifact produced in the standard method was resolved in the Exoquence method (Fig. 1, strategy B).

Troubleshooting the exoquence method

Since selection of the secondary enzyme digestion is random, in practice there is no need to measure the precise recession length within each Exo III time point. By knowing the approximate Exo III digestion rate, one can sufficiently estimate where the end points are located. So Exo III analysis can be omitted when actually doing the sequencing. Even after the secondary enzyme digestion, the primers may still be too long for the sequences to be resolved by standard gel running conditions (Fig. 4A, left panel). In most instances, this problem can be solved simply by running the gel longer (Fig. 4A, right panel).

If the range is wide, enzyme R may have a site within the range (Fig. 4B). L represents the length of the shortest primer produced by R_2 digestion. If L is longer than 20 bases but shorter than 200 bases, the sequences generated from two sets of primers are partially overlapped and partially non-overlapped (Fig. 4B, top and bottom). If L is shorter than 20 bases, the sequences will be completely overlapped and if L is longer than 200 bases, the sequences will be totally non-overlapped (data not shown). If enzyme R has more than one site within the range, more than two sets of primers will be generated. These sequences will be totally overlapped and uninterpretable.

Some restriction enzymes have single strand endonuclease activity (Fig. 4C). Since the single strand activity is usually lower than double strand activity, P_1 will be the major product. The sequences generated will look like those of Fig. 4C. In that example the enzyme was Alu I. If single strand activity is high, P_3 will be the major product. The portion of the sequence ladder that can be resolved is indicated by L in Fig. 4C (L is the distance between the primer and the nearest R site). This problem can be avoided by not using enzymes with high single strand activity, such as Hha I, HinP I, and Mnl I.

DISCUSSION

We present here a new rapid DNA sequencing technique applicable to a variety of DNA types and base compositions. We elected not to test Exoquence using starting DNA of known sequence. Instead this method was used to sequence both strands of a 2.5 kbp, completely uncharacterized DNA insert in a Bluescript vector. The same DNA was also sequenced by a conventional double-stranded DNA sequencing technique. The sequences deduced by both methods were identical. Since both strands of 2.5 kbp were sequenced, this method can sequence at least one strand of 5 kbp. The theoretical length of DNA that can be sequenced by this method is twice the length that Exo III can recess synchronously, assuming both polylinker regions contain appropriate sites to allow unidirectional recessions. The fact that Exo III recession remains synchronous for 14 kbp (11) suggests that much larger inserts could be sequenced by our method. Modifications of standard protocols to accommodate large inserts, such as increasing the amount of input DNA, also should be applicable to our method.

We provide evidence that sequences obtained by Exoquence are as good as those obtained by the conventional method. As anticipated in any method, problems in this method are also inevitable. Most are solved by logical troubleshooting as shown in figure 4. However not all problems have logical solutions. Some sequence results, such as those in figure 4D, arise for reasons we have not yet determined. Even so, the sequence information generated in this case can still be used to synthesize primers. Primers generated by this method could significantly accelerate conventional DNA sequencing. For the rare long stretches of DNA that have no appropriate four base secondary restriction sites, sequence information cannot be determined by Exoquence. For the data reported here, about half of the reactions gave readable sequence. Among the reactions which did not work, half were caused by primers produced by secondary enzyme digestions that were too long to allow gel resolution and another half resulted from poor secondary enzymes (enzyme R in Fig. 1). So far, 27 enzymes have been used as secondary enzymes (Table 1). Some consistently gave good results (eg, RsaI), whereas some never gave good results (eg, Hinf I). We reasoned that the enzymes of the later category were contaminated by exonuclease or endonuclease activity. By avoiding these enzymes, a higher rate of productive sequencing reactions is assured.

The most obvious and reasonable way to further improve the Exoquence DNA sequencing method is to further reduce the Exo III recession range. Efforts were made to reduce the range in this study. Two procedures were used to mix the Exo III with other reaction contents (see Materials and Methods). In the first procedure, no matter how quickly we mixed Exo III with the other reaction contents, some Exo III molecules still bound DNA faster than others, giving them a 'head start' and subsequently generating a certain range. It has been shown that Exo III can bind DNA at low temperature but will not further digest it, and once the enzyme binds to DNA, it will not dissociate (16, 17). Based on this property, the second procedure was employed. Occasionally, we observed very short ranges, but the overall ranges were similar to those generated by quickly mixing the reaction contents at 37°C (data not shown). We assume that the range produced in this case results from the non-uniform temperature increase within the reaction tube. Perhaps synchronous heating of the reaction contents can be achieved by microwave or other sophisticated techniques. If the range is reduced to zero or close to zero, more frequent cutters or a combination of frequent cutters can be used in secondary enzyme digestions. Most primer/template duplexes generated will be appropriate, eliminating the problems of Figs. 4A and 4B.

Recent reports (19–21) show that polymerase chain reaction (PCR) products can be directly sequenced. However a subcloning or a walking strategy is still needed to sequence a PCR product greater than 1 kbp. By designing restriction sites resistant to Exo III resection within the PCR primers, several kbp of PCR products should be sequencable by the Exoquence method without subcloning or further synthesis of squencing primers. Combining

Exoquence with PCR theoretically allows one to bypass the entire process of cloning, subcloning, and isolation of templates.

The primer/template duplexes generated by the Exoquence method should be good substrates for fluorescence-tagged chain-terminating reactions (5). Therefore, they can be used for automatic sequencing based on conventional strategy. The tremendous simplification of preparing appropriate templates and primers by the Exoquence method makes the automation of the whole sequencing process, from starting DNA clone to obtaining final sequencing results, possible.

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