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A Rapid and Efficient PCR-Based Method for Synthesizing High-Molecular-Weight Multimers of Oligonucleotides

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We have developed a simple PCR based approach for synthesizing high-molecular-weight DNA multimers of short oligonucleotide elements in yields sufficient for preparing DNA affinity columns or for using as probes to detect sequence-specific DNA binding proteins. The technique is rapid, efficient and produces multimers consisting of 30 - 100 repeating units.

Transcription factors and other sequence-specific DNA binding proteins are routinely purified on columns of high molecular weight DNA containing multiple repeats of the relevant binding site coupled to sepharose or adsorbed to cellulose. Published values for protein enrichment by this technique range from 30 - 1000 fold using single or multiple rounds of DNA affinity chromatography (1-4).

One of the main difficulties with this technique is the preparation of adequate amounts of DNA multimers containing repeats of the relevant DNA binding sequence, which typically ranges between 5 and 15 nucleotides in length. Two basic approaches have been described in the literature for constructing polymeric repeats of these binding elements. The first is a DNA ligase-driven reaction (5) which requires high concentration of the oligonucleotides and frequently produces multimers with a relative small number of binding site repeats (3-20). In our laboratory, this approach has been highly variable with respect to size and yield of multimers. Alternatively, a cloning approach has also been developed in which multiple repeats of a DNA binding sequence are inserted into a high copy plasmid such as PUC19 and propagated in

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E. coli (6). Although this approach provides a more homogenous product (in terms of the multimer size distribution), yields can be low, deletions within the insert can arise during replication and plasmid sequences may be present on the affinity column.

In this report we describe an alternative approach for preparing DNA multimers which makes use of PCR. The procedure is described using oligonucleotides containing the heat shock transcription factor binding element (HSE) but we have also used this method to prepare large amounts of multimers containing other DNA recognition elements. By starting with short oligonucleotides containing two directly repeated binding sequences, we have prepared multimers ranging in length from 2 kb - 4 kb following 35 cycles of PCR. Typically between 20 - 50 µg of high molecular weight DNA can be prepared in a typical (100µl) reaction.

Materials and Methods

Synthesis of oligonucleotides. All oligonucleotides in this study were prepared using an Applied Biosystems model 391 DNA Synthesizer.

Conditions for PCR

Conditions were chosen for optimal annealing, extension and denaturation of the oligonucleotide shown in Figure 1. In some experiments the conditions of annealing/extension differed slightly and these changes are indicated in the figure legend. Denaturation was performed at 95°C for 1 minute followed by a 2 minute period of renaturation and extension at 65°C. These steps were followed by a 1 minute incubation at 37°C. Vent DNA polymerase (New England Biolabs) was used for all of the experiments reported. Taq polymerase (Perkin Elmer/Cetus) was also found to work well in these experiments and produced high molecular weight DNA products (data not shown). All experiments were performed using an Ericomp Programmable Cyclic Reactor. Typical reactions (100µl) contained 10 ng each primer, 12 mM Mg SO_4, 3 mM each dNTP, BSA (100 µg/ml), Vent polymerase (12 units) and 1x polymerase buffer provided by the manufacturer.

5' CATGAATTCCAGAACGTTCGCATGAATTCCAGAACGTTCG 3'

Figure 1. Sequence of the HSE-containing oligonucleotide used for PCR.

The DNA sequence of one strand of the 40 bp oligonucleotide FS3 is shown. The heat shock transcription factor binding site is singly underlined. The *EcoRI* restriction site is marked with a double line.

Characterization of product DNA

DNA was fractionated by electrophoresis in 1% agarose gels (Tris-acetate buffer) and visualized by staining with ethidium bromide. DNA concentrations were either estimated from the intensity of ethidium bromide staining and comparing to a DNA standard or the concentration was determined spectrophotometrically after phenol:chloroform extraction, precipitation in ethanol (-70^{o}C, 67%) and extensive dialysis against TE (10mM Tris.HCl pH 7.5, 0.1mM EDTA). Molecular weights were estimated from the migration position of 1 kb ladder markers (BRL) and from ϕX174 fragments (New England Biolabs).

Results

The basic approach for preparing high molecular weight multimeric DNA is outlined in Figure 2. Complementary single-stranded oligonucleotides were synthesized containing two direct repeats of the DNA binding site. When these oligonucleotides were annealed under suitable conditions, three possible structures were formed - a fully duplex dimeric molecule, a partial duplex with 3' overhangs and a partial duplex with 5' overhangs. Polymerization by Vent DNA polymerase converted this last structure to a molecule containing three binding site repeats. Upon denaturing and annealing these trimeric DNA's, longer multimers were produced. Significantly, all the DNA in the reaction has the potential to be converted to multimeric molecules in subsequent cycles of denaturation, annealing and polymerization.

The FS3 oligonucleotide shown in Figure 1 (together with its complement) was one of the substrates used for optimizing the PCR reaction conditions. This oligonucleotide contained a repeat of the well-characterized heat shock element (HSE) consisting of the module nGAAn in alternating orientations (8). The oligonucleotide also contained *EcoRI* restriction sites within each of the two direct repeats which were used to characterize the reaction products.

Preliminary experiments were performed to increase the yield of multimeric DNAs. The amount of high molecular weight product was optimized by a) decreasing the initial oligonucleotide concentration; b) increasing the concentration of nucleoside triphosphates; c) increasing the Mg^{++} concentration; d) reducing the annealing temperature and 3) increasing the amount of Vent DNA polymerase in the reaction. A typical reaction time course is shown in Figure 3. Under these conditions, the amount of high molecular weight product appears to be maximal following 35 cycles of PCR. The sizes of the product molecules range from 1 kb to greater than 5 kb as judged by

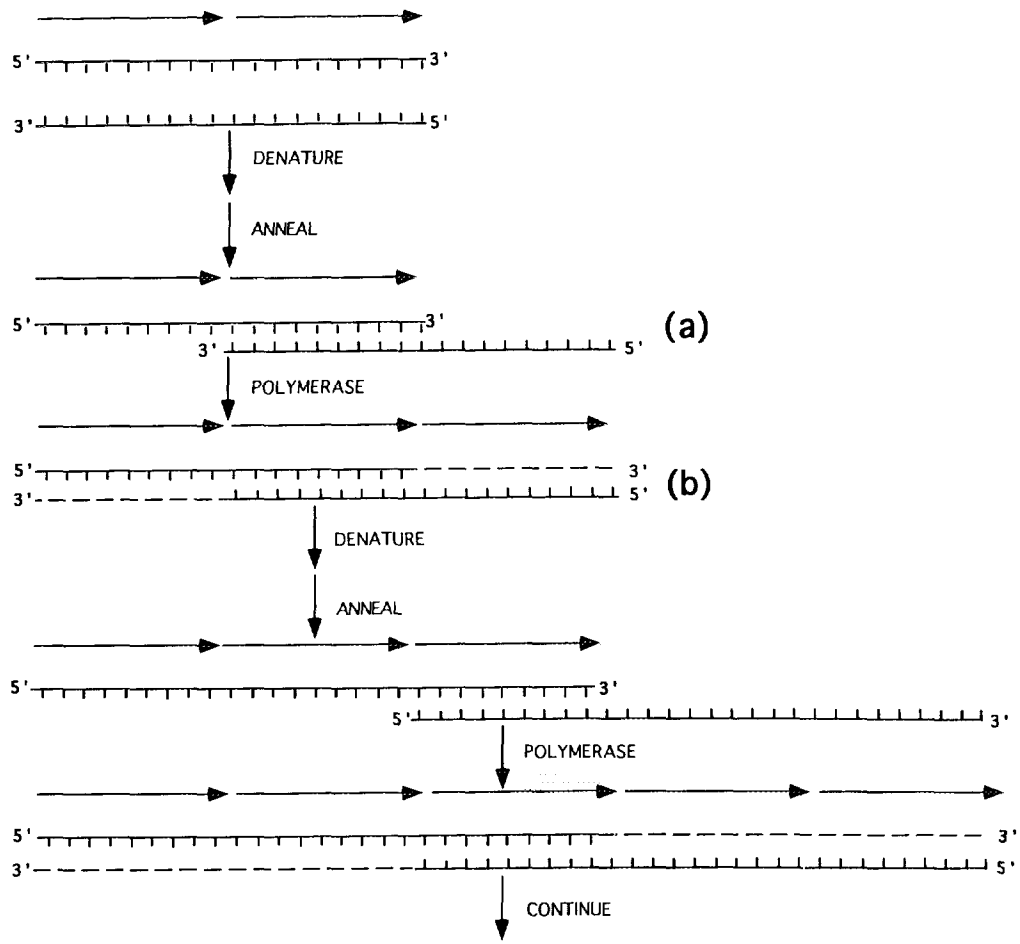

Figure 2. Synthesis of multimers using PCR and partially duplex oligonucleotides. The scheme for preparing high-molecular-weight DNA's containing direct repeats of a DNA binding element (identified by arrow) is shown. Annealing the dimeric oligonucleotide yields a partially duplex molecular containing 5' overhangs (structure a). The 3' ends of these intermediates can be extended by DNA polymerase to yield a product containing 3 copies of the binding site (structure b). By repeating the denaturation, annealing and polymerization steps, large multimeric DNA's are produced.

migration of the DNA in 1% agarose. Both the amount of product and the average chain length increased over the first 21 cycles. In several experiments the average length of product DNA decreased after 25 cycles of PCR.

Multimer synthesis required dNTP's, Vent DNA polymerase and complementary oligonucleotides (data not shown). Moreover, following digestion with *EcoRI*, the

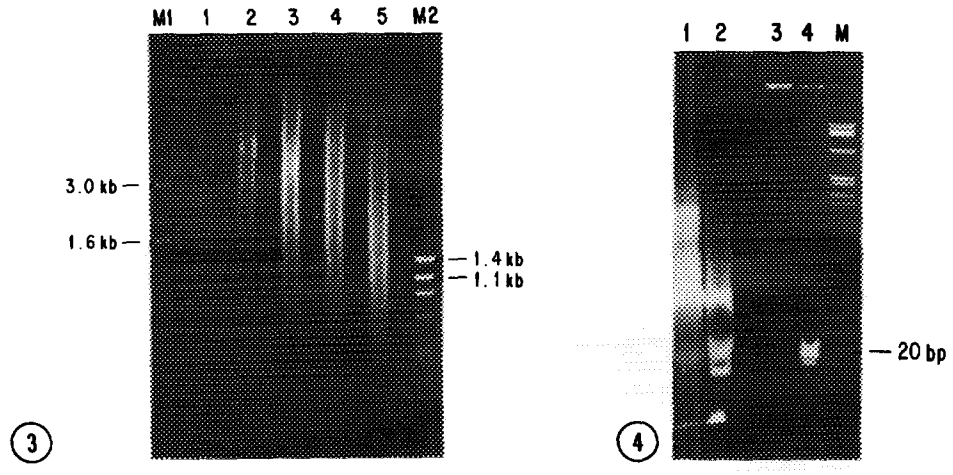

Figure 3. Time course of multimer synthesis.

Reaction conditions were as described in Materials and Methods. After each 7 cycle interval, an aliquot (18µl) was removed and the DNA was fractionated in a 1% agarose gel and stained with ethidium bromide. Lane 1, 7 cycles; lane 2, 14 cycles; lane 3, 21 cycles; lane 4, 28 cycles; lane 5, 35 cycles. Lane M1, 1-kb ladder markers lane M2, ΦX174 HaeIII fragment markers.

Figure 4. *EcoRI* digestion of the HSE - containing oligonucleotide multimers.

High-molecular-weight DNA was prepared by PCR as described in the legend to Figure 3 for 35 cycles. Following the synthetic reaction, the DNA was digested with *EcoRI* restriction endonuclease and fractioned through a 8% acrylamide gel. Lane 1, annealed oligo FS3 and its complement, untreated; lane 2, annealed oligo FS3 and its complement, EcoRI treated; lane 3, PCR multimers, untreated; lane 4, PCR multimers, EcoRI treated; lane M, ΦX174 HaeIII fragments. The 20-bp EcoRI fragment is indicated.

expected 20 bp oligonucleotide was released in high yield (Figure 4). This result is consistent with that the product molecules being linear repeated multimers.

The yield of high molecular weight product was determined for reactions containing 3 mM of each dNTP following 35 cycles of PCR. The DNA was removed, dialyzed exhaustively against 4 liters of buffer (TE) and a sample was taken for spectrophotometric measurement. We have prepared several hundred micrograms of high molecular weight multimeric DNA in a few hours using the Ericomp multisample thermal cycler. In several experiments, the yield of DNA ranged from 20 - 50 µg/reaction. We have used this DNA to prepare DNA affinity columns for purifying transcription factors from *Saccharomyces cerevisiae* (Tony Schmitt, unpublished).

This yield of DNA is greater than expected based upon a simple assumption that the concentration of 3' ends in the PCR reaction is determined by the initial oligonucleotide primer concentration. Based upon the amount of primer used in the experiments described above, we would expect a yield of only 2 - 3µg of DNA with an average chain length of 5000 bp. It is likely, however, that 'new' primer termini are produced in the reaction due to strand breakage. These DNA fragments could provide additional primer termini for subsequent rounds of polymerization that would further amplify the final yield of high molecular weight DNA.

Discussion

We have described a PCR-based method for preparing high molecular weight DNAs starting from oligonucleotides containing short, directly repeated sequence elements. Unlike conventional PCR, which uses primers to anneal to and direct synthesis from separate DNA templates, in this approach the primers are complementary single-stranded oligonucleotides that can form partial duplex molecules which serve as substrates for DNA polymerase. Multiple cycles of denaturing, annealing and polymerization produce increasingly longer multimeric DNAs. Because of the relative ease of preparation, its speed and the size and yield of the product molecules, we believe that this procedure has several advantages over current methods for preparing oligonucleotide multimers for such applications as DNA sequence affinity chromatography and southwestern detection of sequence-specific DNA binding proteins. Unlike the DNA ligation method, the procedure described in this report uses small amounts of oligonucleotides. The average products of the reaction are multimers ranging from 30 - 100 repeats of the starting oligonucleotide. This size range rivals or exceeds those of both plasmid and ligation methods. Moreover, scaling up the reaction is easily accomplished without a corresponding increase in the time required for preparation.

While the mechanism shown in Figure 2 adequately explains the increase in the size of the DNA, it does not account for the greater than expected yield of products we routinely observed. We propose that this amplification is due to fragmentation of the high molecular weight DNAs during PCR and their subsequent extension. The decrease in the size distribution of product DNA between cycles 27 and 35 (Figure 3) is consistent with the idea that considerable strand breakage occurs during PCR. Finally, should also be noted that the inclusion of a final round of DNA synthesis in the presence of labeled dNTP's should provide a simple method for preparing DNA probes of relatively high specific activity that can be used for hybridization or Southwestern

analysis. Alternatively, we have carried out the final polymerization step in the presence of low amounts of ^{3}H-labeled dNTP's. This step provides an accurate means for quantifying recovery of the DNA and determining its coupling efficiency to sepharose during affinity column preparation.

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