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On the cover:

A confocal image of pachytene staged maize meiotic chromosomes hybridized with a probe for knob heterochromatin. The knob probe is detected by fluorescein-conjugated antibodies, and the chromosomes are counterstained with propidium iodide as described in the accompanying article on pages 256-263 by E.R. Makowski and S.E. Ruzin of the University of California at Berkeley.

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Benchmarks

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Direct Dideoxy Sequencing of Genomic DNA by Ligation-Mediated PCR

The ability to amplify single copy sequences from genomes of high complexity by polymerase chain reaction (PCR) allows the direct nucleotide sequence determination of genomic DNA without prior cloning (5). Useful applications of genomic sequencing are, for example, the determination of mutations in specific alleles of genes that specify a heritable genetic disorder (Reference 5, and references therein). Obtaining sequences directly from the genome, however, requires that at least some sequence information is already known to be able to select appropriate primers for PCR amplification.

The ligation-mediated PCR (LM-PCR) technology (3) enables the determination of a completely unknown genomic sequence next to a known region. This may be desirable if, for example, a cDNA has been cloned and the promoter sequence of the corresponding gene needs to be determined without further library screening and cloning (1). During LM-PCR, a double-stranded linker oligonucleotide is ligated to genomic DNA breaks, providing a generic sequence that can be used for amplification in combination with a specific primer residing in a known sequence. LM-PCR has been applied successfully for *in vivo* footprinting and genomic sequencing using the chemical method introduced by Maxam and Gilbert (Reference 3, and references therein). Chemical sequencing, however, is time-consuming and prone to errors due to the inherent problems of the Maxam and Gilbert chemistry.

We describe a modification of the LM-PCR methodology that enables the use of commercial kits for standard dideoxy sequencing (6) to directly determine original genomic sequences. We have used this technique to sequence the promoter region of the *Drosophila* hsp27 gene directly from our fly strain and recorded a number of deviations from the published sequence, which had complicated our initial attempts to analyze the promoter by *in vivo* footprinting.

The method that relies on a previously published variation of the original LM-PCR protocol called "Linker Tag Selection (LTS)-LM-PCR" (4) is outlined in Figure 1, A-G. (A detailed step-by-step protocol is available from the authors upon request.) Genomic DNA is cut with a restriction enzyme at a suitable distance from the known sequence used as starting point (A). If the existence of such a restriction site is not known from Southern blot experiments, a screen with a small selection of frequently cutting enzymes will likely reveal a suitable site. The resulting fragments are heat-denatured, and specific primer 1 is annealed to the known sequence. Primer 1 is extended by Vent® (exo⁻) DNA polymerase (New England Biolabs, Beverly, MA, USA) until the end of the restriction fragment is reached (B). To this blunt end, a short double-stranded linker (made of two complementary oligonucleotides; the longer

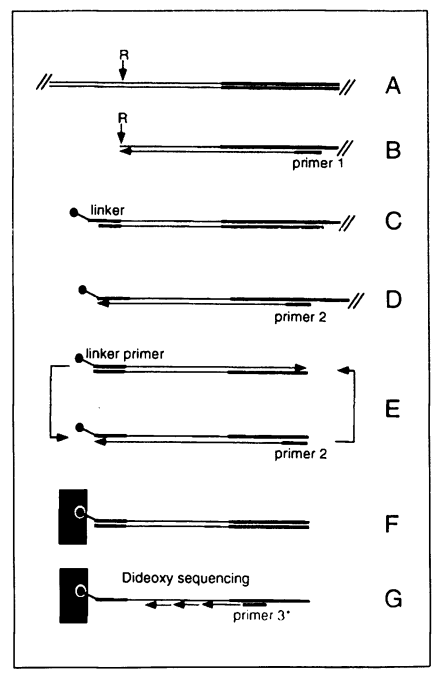

Figure 1. Schematic representation of steps involved in LTS-LM-PCR genomic sequencing. For details refer to the text. Bold lines: known genomic sequence. Thin lines: unknown sequences to be determined. Arrows: primer extension reactions. R: Restriction site. The linker primer is biotinylated (filled circle) at its 5′ end. The surface of the paramagnetic beads used for immobilization is shown as a shaded box.

one being biotinylated at its 5′ end) is ligated in the presence of polyethylene glycol (PEG) (C). The biotinylated linker primer is now used for PCR amplification with Vent DNA polymerase (New England Biolabs) in combination with specific primer 2, which is based more than 3′ from primer 1 in the known sequence (Figure 1) in order to increase the specificity of the amplification (D,E). The biotinylated amplification products are immobilized on streptavidin-coated paramagnetic beads (Dynabeads® M-280 streptavidin, Dynal, Oslo, Norway) and subjected to direct solid-phase sequencing (G). The immobilized fragments are alkali denatured, and the complementary strand removed by washes. Radiolabeled specific primer 3, again located 3′ to primer 2, is annealed and extended in the presence of an adequate deoxy/dideoxynucleotide mixture which finally generates the sequencing ladder (6).

The genomic sequences shown in Figure 2 were obtained using the LTS-LM-PCR conditions as described (4) with the following modifications. Genomic DNA was digested overnight with the indicated restriction enzymes, phenol/chloroform-extracted, ethanol-precipitated and resuspended in water at 1 mg/mL. One microgram of this DNA was processed through the first primer extension, linker ligation and PCRs as described (4). To the PCR (50 µL), an equal volume of 5 M NaCl, 10 mM Tris-HCl pH 8.5, 1 mM EDTA containing 1 mg of Dynabeads M-280 streptavidin, washed according to the manufacturer's specifications, were added, and the reaction was incubated for 30 min at room temperature on a rotating wheel. The beads were washed once with 100 µL BW solution (Dynal, supplier's information), resuspended in 100 µL freshly diluted 150 mM NaOH and incubated for 5 min at room temperature and 2 min at 50°C. The beads were washed once with 150 mM NaOH, twice with 10 mM Tris-HCl, pH 8.5, 1 mM EDTA, pH 8.0 and once with Vent buffer [10 mM KCl, 10 mM $(NH_4)_2SO_4$, 20 mM Tris-HCl pH 8.8, 4 mM $MgSO_4$, 0.01% Triton® X-100]. They were then resuspended in 15 µL of labeling mixture containing Vent buffer, 200 µM deoxyribonucleoside triphosphates (dNTPs) and 0.4 pmol of kinased primer 3 (2×10^6 cpm). This mixture (3.5 µL) was then added to each of four tubes containing 3 µL of the base-specific dNTP/ddNTP mixtures (A-mix: 900 µM ddATP, 30 µM dATP, 100 µM dCTP, 100 µM dGTP, 100 µM dTTP; C-mix: 480 µM ddCTP, 30 µM dATP, 37 µM dCTP, 100 µM dGTP, 100 µM dTTP; G-mix: 400 µM ddGTP, 30 µM dATP, 100 µM dCTP, 37 µM dGTP, 100 µM dTTP; T-mix: 720 µM ddTTP, 30 µM dATP, 100 µM dCTP, 100 µM dGTP, 33 µM dTTP) in Vent buffer. The reactions were heated at 95°C for 1 min, immediately transferred to a 50°C water bath and incubated for 30 min. One microliter (2 U) of Vent (exo⁻) DNA polymerase was added, and the tubes were transferred to 76°C for 10 min. After chilling on ice, the beads were washed with water, the synthesized strands were eluted with 2 µL of loading buffer (67% formamide, 0.0035% xylene cyanol, 0.0035% bromophenol blue, 6.7 mM EDTA, 50 mM NaOH) as previously described (4) and separated on a sequencing gel. Exposure times varied from 2 h to 2 days depending on the efficiency of the overall process.

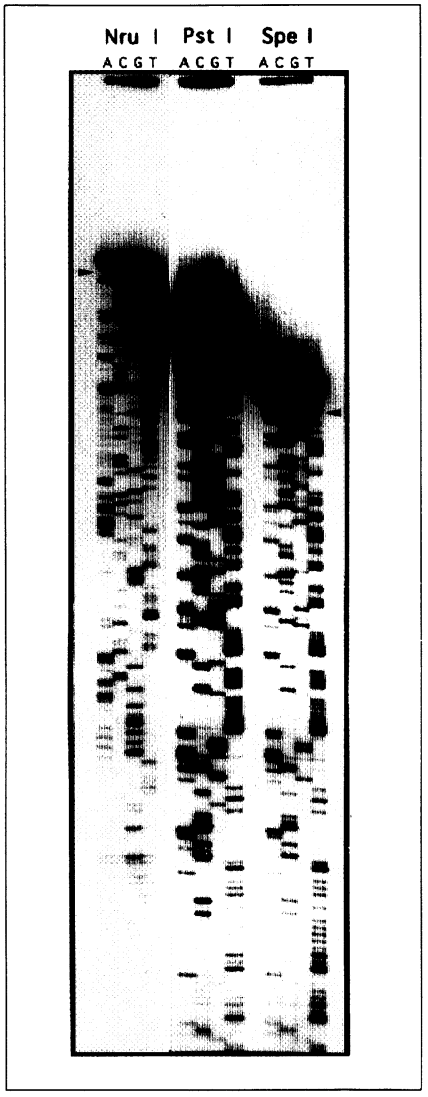

Figure 2. Genomic sequences obtained by LTS-LM-PCR after digestion of genomic DNA with *Nru*I, *Pst*I and *Spe*I. The arrowhead points to the complete restriction fragment. The three gene-specific primers used for reaction with *Nru*I digestion were Primer 1: 5′AAGCAATTATTAGCCTGTC3′, primer 2: 5′TAAGCGACATTTAAAAGCCCCTTTG3′ and primer 3: 5′TTAAAAGCCCCTTTGAATACGCCCCGTAAT3′, and for reactions with *Pst*I and *Spe*I digestion: Primer 1: 5′GTCATCCTCCAGCAAATGC3′, primer 2: 5′GGTGCGGTAGTCATGATCCA3′ and primer 3: 5′TCATGATCCAACTCCCGGGCCAAGTGC3′. Biotinylated linker consisted of the short oligonucleotide 5′GAATTCAGATC 3′ and the long 5′ biotinylated oligonucleotide 5′CACCCGGGAGATCTGAATTC 3′ as described (4).

Figure 2 shows genomic sequences of the *Drosophila* hsp27 gene promoter obtained with 2 different sets of primers and 3 different restriction enzymes (*Nru*I, *Pst*I and *Spe*I). These enzymes create blunt-ended, 3′-protruding and 5′-protruding fragments, respectively, which demonstrates the wide applicability of our approach. Sequences 400 bp from the first specific primer were readily obtained, and longer sequences may be sampled provided that the restriction site is not too close to the known sequence. We have, however, observed poor results when using a rare cutting restriction enzyme such as *Xmn*I, which could reflect the low efficiency of primer annealing after thermal denaturation of larger genomic fragments. Following the newly determined sequence, the linker oligonucleotide sequence can be identified demonstrating the ability of the polymerase to synthesize DNA even very close to the bead (not shown). The use of the Vent DNA polymerase ensures a low error rate during the PCR amplification because of the 3′–5′ exonuclease proofreading activity of the enzyme. We previously noted that the use of the Vent (exo⁻) DNA polymerase is crucial for the first and last primer extension reactions (4). We have, however, not evaluated other heat stable polymerases that have recently become

available, such as *Pfu* DNA polymerase (Stratagene, La Jolla, CA, USA) or *UlTma*™ (Perkin-Elmer, Norwalk, CT, USA) for their performance in these reactions.

We have tested the feasibility of direct genomic sequencing with chain termination reactions using an LM-PCR strategy on a promoter of known sequence. The method should find wide application for the determination of unknown genomic sequences adjacent to a patch of limited sequence information. Using a small selection of restriction enzymes that cut the genome relatively frequently, the chances are good that new genomic sequence is obtained. The new sequence information can then be used to design further primers as part of a "walking sequencing" strategy.

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