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Nonspecific, Nested Suppression PCR Method for Isolation of Unknown Flanking DNA

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ABSTRACT

We report the development of a simple, sensitive and robust two-step PCR method for the isolation of unknown sequences flanking characterized regions of genomic DNA or cDNA. The method requires 100 bp

or less of a known sequence upstream of an oligonucleotide primer binding site. A first round of suppression PCR is conducted at low stringency with a polymerase lacking exonuclease activity to generate a mixture of products including fragments of the desired flanking sequence that are often greater than 1 kb in length. The desired fragments are then amplified from the mixture in a second round of suppression PCR using an extended oligonucleotide in combination with a polymerase exhibiting exonuclease activity. These fragments are subsequently identified by hybridization with the 100 bp of known sequence or simply by cloning and sequencing. The method is widely applicable and allows isolation of novel cDNA from very low abundance transcripts.

INTRODUCTION

Numerous methods exist for the PCR-based amplification of unknown sequences flanking regions of characterized DNA (5). Most of these attempt to circumvent the absence of an oligonucleotide binding site in the unknown region either by ligation of a known sequence, using, for example, the "vectorette" (1) and ligation-mediated suppression PCR (11) approaches, or by circularization (through ligation) of the molecule to be amplified by inverse PCR (13). Successful attempts to amplify flanking DNA by PCR have been made using nonspecific binding of oligonucleotide primers in the uncharacterized regions (4,7-9,14) but such strategies have been complicated by problems such as short PCR product length, difficulty in isolating desired fragments from among many undesired products, methodological complexity and insensitivity. Therefore, a simpler, more reliable method to isolate flanking DNA from low copy number sources is desirable.

Suppression PCR, where a single oligonucleotide primer is used to amplify a DNA fragment that possesses terminal inverted repeats, discourages the formation of short PCR fragments. When fragment lengths are short or the primer concentration is relatively low, the inverse repeats have a tendency to anneal to one another, thus blocking oligonucleotide primer binding and DNA synthesis (6,12).

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Under PCR conditions in which a single primer can bind to incompletely complementary DNA, those DNA strands that possess a completely complementary binding site will be replicated preferentially because they only require one nonspecific binding event to allow exponential amplification (by suppression PCR). Where a completely complementary binding site does not exist, nonspecific binding will usually give only low-frequency linear amplification, and these single-stranded fragments will not be observable among the PCR products. Here, we use this phenomenon, together with a novel nested suppression PCR method, to isolate uncharacterized DNA sequences flanking known regions of genomic DNA and cDNA.

MATERIALS AND METHODS

Refer to Figure 1 for a diagram of the strategy. For details of oligonucleotides including melting temperature calculations, see Table 1.

Step 1: Nonspecifically Primed Suppression PCR (NSPS-PCR)

Ideally, this step is performed using an oligonucleotide binding site that will give inclusion of 100 or more bases of known sequence in the desired PCR product to allow detection by probe hybridization. However, it is possible to proceed to step 2 without having tested the products of the step 1 reaction. Indeed, we have seen amplification of products in step 2 reactions from step 1 reactions in which no product was detectable with Southern analyses.

An oligonucleotide (typically 20 nucleotides length) that primes towards a region of flanking DNA is tested in 25 μ L reactions with *Taq* DNA polymerase (Stratagene, La Jolla, CA, USA) and a range of annealing temperatures, primer concentrations and Mg^{2+} concentrations. Reaction constituents are 20 ng genomic DNA, 0.1–1.6 μ M oligonucleotide primer, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 0.5–4 mM $MgCl_2$, 2 U *Taq* DNA polymerase. PCR cycling was as follows: 35 cycles of 30 s at 94°C, then 1 min at the oligonucleotide's calculated annealing temperature minus 5°C–30°C, then a ramp of

+0.5°C/s to 72°C and 3–4 min at 72°C.

As a guide, when multiple reaction products are visible after agarose gel electrophoresis, then nonspecific priming by the oligonucleotide is occurring and these reactions can then be analyzed by filter transfer and probe hybridization to detect the presence of desired products. Note that extension times in the reactions should be long enough to allow amplification of fragments up to 2 kb or more in length.

Step 2: Reamplification of Desired Products by Nested Suppression PCR (Reamplification PCR)

To amplify specifically desired products from the first round of suppression PCR, the initial reaction is diluted 10^{-3} in water and then used in a second suppression PCR as described below. Note that the primer used for this PCR is identical to that used in step 1 except that it is extended by six nucleotides (corresponding to the known sequence) at the 3' end. The 3'-extended oligonucleotide may be shortened by up to four nucleotides at the 5' end if desired.

Reaction constituents for a 25 μ L reamplification suppression PCR are 1 μ L 10^{-3} diluted nonspecific reaction product, 0.13–0.4 μ M oligonucleotide primer, 2 mM $MgSO_4$, 20 mM Tris-HCl (pH 8.8), 10 mM KCl, 10 mM ammonium sulfate, 0.1% Triton® X-100 (Union Carbide Chemicals and Plastics, Danbury, CT, USA), 0.1 mg/mL nuclease-free BSA, 0.5 U *PfuTurbo*™ (Stratagene).

PCR cycling is as follows: 35 cycles of 30 s at 95°C, then 30 s at the oligonucleotide's calculated melting temperature minus 5°C–10°C, then a ramp of +0.5°C/s to 72°C, and finally 4 min at 72°C. (Note: Lower annealing temperatures can be used and may assist suppression of small fragment synthesis. If the calculated melting temperature is above 77°C, then the annealing and ramp steps can be deleted.)

Modification of the Procedure for Amplification of cDNA from Low-Abundance Transcripts

To amplify 5' unknown sequence from low-abundance transcripts, we performed first-strand cDNA synthesis

on whole RNA using a gene-specific primer biotinylated at its 5' end and ThermoScript™ thermostable reverse transcriptase (Life Technologies, Rockville, MD, USA) at 65°C to selectively copy the desired transcript. A typical reaction used 3 pmol biotinylated primer with 130–150 ng whole cell RNA in a total volume of 20 μ L and was other-

Figure 1. Principle of the NSPS-PCR/reamplification PCR method. Step 1: A primer complementary to known sequences is used in NSPS-PCR with *Taq* DNA polymerase under conditions promoting nonspecific oligonucleotide binding to amplify DNA fragments that include novel flanking sequences. When nonspecific priming creates a high-affinity (oligonucleotide complementary) binding site within a synthesized strand, dsDNA with inverse, terminal, high-affinity sites can form that is then amplified exponentially. Step 2: An overlapping primer extended by six nucleotides at its 3' end allows specific reamplification of desired products using *Pfu* DNA polymerase. During the first cycles of the reamplification PCR (A), DNA is initially synthesized only from the completely complementary site in the known sequence. During later cycles (B), the DNA polymerase has truncated the primer sufficiently at its 3' end so that it is completely complementary to the sequence at the opposite end of the fragment and thus exponential amplification can occur.

wise used according to the manufacturer's instructions. The reaction was terminated by heating to 85°C for 10 min, and then the RNA of the RNA/cDNA hybrids was degraded by addition of 2 U RNase H (Roche Molecular Biochemicals, Mannheim, Germany) followed by a 2-h incubation at 37°C.

The desired cDNA was purified using streptavidin-coated magnetic particles with a calculated biotin binding capacity fourfold greater than the molar amount of the biotinylated primer present in the original reverse transcriptase reaction. Buffers promoting binding of long DNA molecules were used (KilobaseBINDER™ kit; Dynal AS, Oslo, Norway). After DNA binding and washing, the magnetic beads were resuspended in 30 µL water. To amplify unknown flanking cDNA from the desired transcript, up to 15 µL of the beads were used in a 50-µL NSPS-PCR with a primer binding 3' to the site of

the biotinylated primer and having a calculated melting temperature of greater than 72°C. PCR cycling was as follows: 5 cycles of (2 min at 25°C, then a ramp of +0.5°C/s up to 50°C, then 3 min at 50°C), 3 cycles of (2 min at 25°C, then a ramp of +0.5°C/s up to 70°C then 3 min at 70°C), 35 cycles of (40 s at 94°C then 4 min at 72°C), followed by 10 min at 72°C. Note that during the ramped cycles and the 72°C step of the first 10 of the 35 non-ramped cycles, settled beads were resuspended in the reaction by pipetting rapidly and briefly in the elongation step. The PCR products were subjected to reamplification PCR as described above.

PCR Fragment Purification, Cloning and Sequencing

PCR products were electrophoresed on 1% agarose gels in TAE buffer (40 mM Tris base, 40 mM acetic acid, 1

mM EDTA) including 0.5 mg/mL ethidium bromide (Sigma, St. Louis, MO, USA), excised under long-wave UV illumination and purified using a Wizard PCR Preps kit (Promega, Madison, WI, USA) before cloning into the pCR-Blunt II-TOPO vector of the Zero Blunt™ TOPO™ PCR cloning kit (Invitrogen, Carlsbad, CA, USA). Automated sequencing was performed using the ABI PRISM™ dye primer cycle sequencing ready reaction kit (PE Biosystems, Foster City, CA, USA) and M13 sequencing primers. Sequence comparisons were made using the FAS-TA program (3).

Alkaline Blotting and Radioactive Probe Hybridizations

Alkaline blotting and radioactive probe hybridizations were conducted with Hybond®-N+ nylon filter membrane (Amersham Pharmacia Biotech,

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Table 1. Details of Oligonucleotides

Oligonucleotide Name	Sequence	Target Gene/ cDNA	Annealing Temperature	Concentration in Reaction (μM)	Purpose
Mila364	5'-CACTCCTGAGTGAGGATACTGCGGC-3'	<i>D. rerio</i> tyrosinase	50°C	0.1–0.8	NSPS-PCR
Mila364ext3	5'-CACTCCTGAGTGAGGATACTGCGGCCCG-3'	<i>D. rerio</i> tyrosinase	65°C	0.13	reamp. PCR
Mila364ext6	5'-CCTGAGTGAGGATACTGCGGCCCGTTGG-3'	<i>D. rerio</i> tyrosinase	65°C	0.13	reamp. PCR
Mila283B	Biotin-5'-GGCCGAGGCGCAGTCATCG-3'	<i>B. floridae</i> Notch	~65°C	0.15	rev. transcr.
Mila339	5'-TTCCGCAGTTCAAGCAGATGTTTGGGG-3'	<i>B. floridae</i> Notch	25–75°C	1.6	NSPS-PCR
Mila339ext6	5'-CGCAGTTCAAGCAGATGTTTGGGGACTGCA-3'	<i>B. floridae</i> Notch	72°C	0.13 and 0.4	reamp. PCR

^aPrimer annealing/synthesis temperature for reverse transcription.
Melting temperatures (T_m) of oligonucleotides were estimated using the formula; $T_m = [(\text{No. of A and T bases}) \times 2 + (\text{No. of G and C bases}) \times 4]^\circ\text{C}$.

Little Chalfont, England, UK) according to the manufacturer's instructions. DNA probes were labeled by random priming with a ^{32}P -dATP as described in Reference 10. Autoradiography was conducted with Fuji RX Medical X-ray film (Fuji Photo Film, Tokyo, Japan).

RESULTS AND DISCUSSION

Cloning of DNA Flanking a Region of Known Zebrafish Tyrosinase Gene Sequence

To facilitate cloning of the promoter of the tyrosinase gene of the zebrafish, *Danio rerio*, we initially used degenerately primed PCR to clone a fragment of cDNA predicted to encode amino acids 41–236 of the protein (Camp, Kortschak, Tamme and Lardelli, unpublished results). Isolation of a genomic clone containing this gene from a lambda bacteriophage library was hindered by nonspecific hybridization signals when this cDNA fragment was used as a probe. Thus, we decided to attempt NSPS-PCR to clone sequences upstream of the known region (Figure 1).

Oligonucleotide primer Mila364 binds 81–105 bases inside the boundary between known zebrafish tyrosinase gene exonic sequences and flanking se-

quences that must include the gene's promoter. The oligonucleotide primes DNA synthesis towards this flanking region and is predicted to have a melting temperature of 80°C. NSPS-PCR was performed on 40 ng zebrafish genomic DNA with oligonucleotide primer Mila364 and *Taq* DNA polymerase at a range of temperatures and Mg^{2+} concentrations. An annealing temperature of 50°C together with 2 mM Mg^{2+} gave nonspecifically primed DNA fragments in sufficient quantities to be observable on an agarose gel stained with ethidium bromide (data not shown). Since low primer concentrations favor large fragment amplification in suppression PCR, we also performed a series of reactions at a range of primer concentrations. After electrophoresis of the products through an agarose gel, they were transferred to a filter and hybridized with a radioactive tyrosinase cDNA probe to detect tyrosinase gene products. Weak and indistinct signals were observed. These results suggested that tyrosinase gene sequences had been amplified (data not shown).

During NSPS-PCR, amplification of undesired sequences also occurs if a second nonspecific priming event occurs on the single-stranded product of an initial nonspecific event. For complex substrates such as genomic DNA,

the chances of this occurring are quite high. Thus, a method of specifically reamplifying the desired sequence from this background of undesired products is required.

One commonly used strategy for selective PCR reamplification is to use a novel primer nested within the known sequence together with one of the original primers. However, for NSPS-PCR, the original primer binds at both ends of all the PCR products. Thus, in such a nested primer reaction, all DNA fragments would be reamplified.

If the original oligonucleotide is extended at its 3' end by a number (n) of nucleotides, then it primes DNA synthesis at the known end of the desired fragments. However, the probability that it primes at the novel end is only $1/4^n$, that is, the probability of no 3' end mismatch, assuming random distribution and equal amounts of A, C, G and T nucleotide residues in the template. We reasoned that we might get selective amplification of fragments of the tyrosinase gene DNA by using a mixture of the original primer (Mila364) and a 3' extended primer because the latter primer would compete with the former for binding to all fragment termini and thus would inhibit DNA synthesis on undesired templates. Tests of this strategy using various mixture

ratios of the original primer and a primer extended by three nucleotides (Mila364ext3), and with either *Taq* or *Pfu* DNA polymerase, showed that selective amplification did occur. Interestingly, by far the greatest selective amplification occurred when *Pfu* DNA polymerase was used with the extended oligonucleotide but no original primer. This can be explained by the 3' → 5' exonuclease activity of proofreading polymerases such as *Pfu* (Figure 1). Also, proofreading thermostable polymerases have little tendency to favor synthesis of short fragments over longer ones (2) and thus the *Pfu* reactions amplified longer fragments than in the *Taq* reactions (data not shown).

We then tested the effects on the efficiency of the tyrosinase gene DNA amplification of different lengths of 3' primer extension and of multiple rounds of PCR with progressively extended primers. Primers extended by three or six nucleotides at their 3' ends (Mila364ext3 and Mila364ext6, respectively) were used in single rounds of reamplification PCR with 10^{-3} or 10^{-5} dilutions of the initial NSPS-PCR product. The products of the Mila364ext3 reamplification reactions were then diluted before being subjected to a second round of reamplification PCR using Mila364ext6. By far the greatest and most specific amplification of tyrosinase sequences was achieved using a single round of reamplification PCR with the six nucleotide extended primer Mila364ext6 on the 10^{-3} dilution of the NSPS-PCR (Figure 2). (Note that some undesired products can still be amplified.) This is probably due to the greater number of cycles of linear amplification of the desired sequence by the six-nucleotide extended primer that must occur before it has been truncated enough to amplify all fragments exponentially. In subsequent experiments with other genes, we have noted that high-efficiency reamplification by using *Pfu* DNA polymerase and a six-nucleotide-extended primer is a general phenomenon.

Using NSPS-PCR and Reamplification PCR to Isolate cDNA from Low-Abundance Gene Transcripts

Having developed a method to gen-

erate and isolate unknown flanking sequences from regions of known DNA, we then attempted to clone 5' sequences flanking a known region of cDNA derived from the *Notch* gene transcript of the chordate, *Branchiostoma floridae* (Holland, Tamme, Burgtorf, Holland, Lehrach, Pontarotti and Lardelli, unpublished observations) for which no bacteriophage library of randomly primed cDNA currently exists. *Notch* genes in chordates are expressed at low abundances during embryogenesis, typically less than 1 in 3×10^5 of all mRNA transcripts in an embryo (M. Lardelli, unpublished observations).

We synthesized the first strand of *B. floridae Notch* cDNA from whole RNA (extracted from embryos 24 hours after fertilization) using a *Notch*-specific biotinylated oligonucleotide. After purification of this cDNA on streptavidin-coated magnetic beads, we performed

Figure 2. Reamplification PCR of NSPS-PCR-derived tyrosinase sequences using three- or six-nucleotide extended primers. Identical sample volumes of reactions were electrophoresed through an agarose gel next to size marker DNA fragments (M, sizes in kb are indicated). "3n extn" and "6n extn" indicate single rounds of reamplification PCR using primers extended at their 3' end by either three or six nucleotides, respectively. "-3" and "-5" indicate whether 10^{-3} or 10^{-5} dilutions of the initial NSPS-PCR were used. "6n extn 2nd reamp" indicates the products of a second round of reamplification PCR using the six-nucleotide extended primer on the 10^{-3} diluted products of the three-nucleotide extended PCR. "*" indicates the largest fragment observed to possess tyrosinase gene sequence. This image is an edited composite of a larger image, but the relative brightness of the components has been preserved.

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Figure 3. Isolation of novel cDNA from a low-abundance transcript. Agarose gel electrophoresis of PCR products next to size marker DNA fragments (M, sizes in kb are indicated). Left panel shows NSPS-PCR on *B. floridae* cDNA captured on magnetic beads. A range of PCR products is observed as a smear. Right panel shows two reamplification reactions of the previous PCR with differing concentrations of a primer extended by six nucleotides at its 3' end. PCR sample volumes are equal. Two distinct bands were observed. "*" indicates the approximately 700 bp fragment containing *B. floridae* *Notch* cDNA. This image is an edited composite of a larger image, but the relative brightness of the components has been preserved.

NSPS-PCR using *Taq* DNA polymerase and a primer (Mila339) binding 28 bases downstream of the most upstream known sequence with an annealing temperature 28°C below its calculated melting temperature (see Materials and Methods). Gel electrophoresis of the reaction products showed a smear that indicated the presence of a large variety of DNA fragments. We then reamplified this PCR using a primer extended at the 3' end by six nucleotides (Mila339ext6). Two strong bands of approximately 400 and 700 bp were observed after gel electrophoresis of the reaction products (Figure 3). Cloning and sequencing of these products demonstrated that the 700 bp fragment contained novel *B. floridae* *Notch* flanking cDNA, while the 400 bp fragment did not. Our success with amplification of cDNA from the *B. floridae* *Notch* transcript and with the NSPS-PCR/reamplification strategy overall suggests that this procedure will be a robust, powerful and widely applicable method for isolation of cDNA from low-abundance transcripts.

CONCLUSION

The initial tests to find optimal conditions for NSPS-PCR may require numerous reactions, but these are rapidly performed using microtiter PCR plates and temperature cyclers that allow differential annealing temperatures across the heating block. Commonly, the number of different fragments produced by the reamplification PCR is small enough to allow identification of desired products by cloning and sequencing. If this is not so, Southern hybridization can be used to identify the desired products.

When amplification of flanking sequences from less complex DNA sources such as YACs or bacterial genomes is desired, other methods such as ligation-mediated suppression PCR may give more predictable results. However, the much larger, repetitive and often highly methylated genomes of vertebrates and some plant species may lack the restriction sites required for these methods. The method described here is both sensitive and reliable, yet it minimizes the molecular biological handling of the DNA substrate to PCR and dilution only.

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