A Lock-docking Oligo(dT) Primer for 5' and 3' RACE PCR

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We describe a method that can be used to obtain and sequence 3' and 5' ends of cDNA transcripts directly from PCR products. The method employs a modified oligo(dT) primer that enables it to "lock-dock" at the junction of gene-specific cDNA sequence and a natural (3') or appended (5') poly(A) tail. As a result, discrete, first-round PCR products are obtained that are easily isolated and sequenced directly.

The rapid amplification of cDNA ends (RACE) procedures of Frohman⁽¹⁾ have been used successfully to obtain and sequence 3' and 5' cDNA ends when some sequence from the internal portion of a gene is known.^(2,3) Various modifications and improvements of the RACE procedure have also been successfully employed.⁽⁴⁻⁷⁾ The original RACE techniques involve the use of three primers, one for cDNA synthesis and two for use in the PCR. In the 3' RACE procedure, the cDNA synthesis primer incorporates multiple cloning sites (mcs) on the 5' side of an oligo(dT) sequence [(mcs)oligo(dT)]. For the PCR, the downstream primer consists of the mcs region, and the upstream primer is specific for a portion of the internal region of the mRNA. Because the (mcs)oligo(dT) primer used in cDNA synthesis binds at any point along the poly(A) tail of an mRNA transcript, a population of products of varying length is usually obtained.⁽⁷⁾ Therefore, subsequent to the PCR, the amplification products are often characterized by Southern blotting before cloning and screening. We describe here a modification of the RACE (mcs)oligo(dT) primer that has enabled us to amplify the 3' ends of the canine brain Glut1 and "Glut3-like" members of the glucose transporter family and directly sequence their discrete PCR products. (Henceforth, we will refer to Glut3-like simply as Glut3.) This circumvents the need for Southern blotting, cloning, and screening. Glut3 has recently been shown to be present in neurons in brain,⁽⁸⁻¹⁰⁾ and Glut1 has been shown to be an abundant glucose transporter at the blood-brain barrier.^(11,12) The 2190-base-length sequence of the Glut3 3' untranslated region (UTR) has been determined in human fetal skeletal muscle,⁽¹³⁾ and 212 bases of the mouse brain

Glut3 3'-UTR have been reported.⁽⁹⁾ Although the sequence of the Glut1 3'-UTR has been determined from several species,⁽¹⁴⁻¹⁸⁾ it has not yet been reported for the canine species.

METHODS AND MATERIALS

mRNA Isolation

Approximately 1 gram of fresh canine brain cortex tissue was obtained and immediately frozen in liquid nitrogen. Poly(A)⁺ RNA was extracted from the tissue using a commercially available kit (Micro Fast Track Kit, Invitrogen, San Diego, California).

Primer Design for cDNA Synthesis

Ordinarily, the synthesis of cDNA is performed with an (mcs)oligo(dT) primer, as outlined in the 3'-RACE procedures of Frohman et al.⁽²⁾ However, in the method reported here, a modification was made to the 3' end of the (mcs)oligo(dT) primer to enable it to lock in at the beginning of polyadenylation rather than at random points along a potentially lengthy poly(A) tail. Because the base adjacent to the 5' side of RNA polyadenylation is a U, C, or G, and the next upstream base is an A, U, C, or G, a "lock-docking" oligo(dT)₁₆ primer was designed as follows:

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(MCS)TTTTTTTTTTTTTTTAA
                             CC
                             GG
                             T
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Ten of the 12 permutations in this primer will contain at least one G or C (83.3%). If a third set of bases (TACG) is added at the 3' end of the primer, there is an increased chance (91.7%) that base-pairing will involve a G or a C, but the number of permutations increases from

12 to 48. These calculations are made with the assumption that there is a random distribution of the last two nucleotides prior to the poly(A) tail in the target cDNA.

A similar primer has been used to sequence the 3' ends of cloned cDNAs containing a poly(A) tail.⁽¹⁹⁾ For this purpose, a single non-T (A, C, or G) nucleotide at the 3' end of the primer was sufficient.

The lock-docking primer was synthesized by Bio-Synthesis, Inc. (Denton, Texas) at no extra charge for the degeneracy at the 3' end. Alternatively, one can purchase bulk CPG-linked nucleotides and empty columns from various suppliers (Applied Biosystems, Foster City, California or Cruachem, Sterling, Virginia). From these components, one can mix any combination of the four CPG-linked nucleotides to start a synthesis.

cDNA Synthesis

First-strand cDNA synthesis was performed using 1 μ g of canine cortex poly(A)⁺ RNA as template and Moloney murine leukemia virus (Mo-MLV) reverse transcriptase (GIBCO BRL, Grand Island, New York) as follows: To a 1.5-ml microfuge tube, additions were made, in order, of 4 μ l of 5 $\times$ Mo-MLV reaction buffer (GIBCO BRL), 8 μ l of a mixture that is 1.25 mM in each dNTP (Perkin Elmer Cetus, Norwalk, Connecticut), and 16 ng of the modified (mcs)oligo(dT)₁₆ primer dissolved in 1 μ l of dH₂O. The mRNA template was diluted in dH₂O to yield a final reaction volume of 20 μ l, and then was denatured at 75°C for 3 min, and quenched on ice before addition to the above mixture. Two hundred units of Mo-MLV reverse transcriptase were added to the mixture, and the mixture was allowed to incubate 40 min at 37°C and 30 min at 42°C to encourage longer transcripts. The resultant cDNA-RNA hybrid was treated with 2 units of RNase H (Amersham, Arlington Heights, Illinois) and incubated 20 min at 37°C, followed by heating at 95°C for 5 min to inactivate the enzymes. The cDNA was diluted to 1:10 and 1:100 for use in the PCR, without synthesis of the second strand of cDNA.

PCR for Canine Glut3

Two separate PCRs were performed on

the cDNA template using two different, canine-specific upstream 20-mer primers. Primer A (CTTCCTCATCGTCTTCTTGG) was designed to prime synthesis at a site located 172 bases upstream of the predicted location of the termination codon of Glut3, and primer B (TTTGAGGAAATCACCCGAGC) at a site 40 bases downstream of primer A. Each of these primers was used in conjunction with a 19-mer truncated version of the mcs sequence that is part of the (mcs)oligo(dT)₁₆ primer. The sequence of the 19-mer truncated primer is CCGCATGATCTAGATATCGA; the entire mcs sequence is CCGCATGCGGCCGAGATCTAGATATCGA, which contains the restriction enzyme sites *Sph*I/*Not*I/*Bgl*II/*Eco*RV/*Cl*aI. The PCR conditions were based on the "touchdown" PCR protocol.⁽²⁰⁾ The annealing temperature was decreased 1°C every second cycle from 60°C to a touchdown temperature at 50°C; this final annealing temperature was maintained for 10 cycles. Denaturation was done for 5 min at 94°C in the first cycle, and 1 min in each cycle thereafter; touchdown annealing was performed as described above. Extension times were gradually increased as follows: 10 cycles at 2 min each, followed by 10 cycles at 3 min each, then 10 cycles at 4 min each. A final cycle was allowed to extend for 15 min. The increasing of extension times enabled completion of a longer (more than 2000-bp) transcript as nucleotide concentration was being lowered. Reactions were performed in final volumes of 100 μ l, using AmpliWax PCR Gems (Perkin-Elmer Cetus, Norwalk, Connecticut) to facilitate final mixing of primer and template at a temperature high enough to prevent possible undesirable primer dimerization from occurring prior to the first denaturation cycle. A lower layer consisted of 2.5 μ l of 10 $\times$ PCR buffer (100 mM Tris-HCl, pH 8.3, 500 mM KCl), 10 μ l of 25 mM MgCl₂, 16 μ l of a dNTP solution that is 1.25 mM in each dNTP (dATP, dCTP, dGTP, dTTP), and 20 pmoles of each primer. After addition of one AmpliWax PCR Gem, heating at 80°C for 5 min and cooling to room temperature, the upper layer was added. The upper layer consisted of 60 μ l of deionized H₂O, 10 μ l of 10 $\times$ PCR buffer (see above), 2.5 units of *Taq* DNA polymerase (AmpliTaq, Perkin-Elmer Cetus), and 1 μ l of a 1:10 dilution of the canine brain cortex cDNA as described above. Final concentrations in the PCR

reaction were 200 μ M in each dNTP, 62.5 mM KCl, 2.5 mM MgCl₂, and 12.5 mM Tris-HCl (pH 8.3). Cycling was done in a DNA Thermal Cycler (Perkin-Elmer Cetus).

PCR for Canine Glut1

The PCR conditions used to amplify a Glut1 3' end fragment were nearly identical to those used for Glut3. The template used was 1 μ l of the 1:100 dilution of the canine brain cortex cDNA. T4 gp32 protein (60 pmoles) was also added to each reaction mixture⁽²¹⁾ (United States Biochemical, Cleveland, Ohio). Two canine-specific upstream primers, primer C (AAGTTCCTGAGACCAAAGGC) and primer D (CGGACCTTTGATGAGATGTC), were used in two separate PCRs, each with the downstream truncated mcs primer. Canine-specific primer C was designed to prime synthesis at a site located 124 bases upstream of the predicted termination codon, and canine-specific primer D at a site located 20 bases downstream from primer C.

Sequencing Methods

PCR products were electrophoresed in 1% low-melting-point agarose gels (Sea-Plaque GTG Agarose, FMC Bio Products, Rockland, Maine), the appropriate bands (see Results) were cut out, and the agarose was removed using a Gene-Clean Kit (Bio 101, La Jolla, California). The PCR-amplified DNA was sequenced with Sequenase Version 2.0 (United States Biochemical, Cleveland, Ohio) using a protocol modified for the sequencing of PCR products.⁽²²⁾ The two primers used to sequence the Glut3 band were primers A and B (described above). The primer used to sequence the Glut1 band was primer D (described above).

To a 1.5-ml microfuge tube were added 300 ng of the DNA (dissolved in 8 μ l of TE pH 8.0), 1 pmole of primer (dissolved in 1 μ l of dH₂O), and 1 μ l 1 M NaOH; the mixture was incubated at 68°C for 10 min. Four microliters of TDMN buffer [14 μ l of 1 M TES (free acid, Sigma, St. Louis, Missouri), 6 μ l of 1 M HCl, 4 μ l of 1 M Mg Cl₂, 2 μ l of 5 M NaCl, 21.5 μ l of dH₂O, and 2.5 μ l of 1 M DTT that is added shortly before use] were added, and the mixture was incubated at room temperature for 10 min. Then added, in order, were 2 μ l of Labelling Mix dilution (diluted 1:5 with dH₂O, Se-

quenase Kit, United States Biochemical), 1 μ l of manganese buffer (0.15 M sodium isocitrate, 0.1 M MnCl_2), 1 μ l of [α - ^{35}S]-dATP (370 Mbq/ml, Amersham, Arlington Heights, Illinois), and 2 μ l of Sequenase enzyme dilution (diluted 1:8 in Sequenase Enzyme Dilution Buffer, United States Biochemical). Following incubation for 5 min at 20°C, 3.5 μ l of the reaction mix was added to 2.5 μ l of each of the termination mixes (G,A,T,C) at 37°C, and incubation was continued at 37°C for 10 min. Finally, 4 μ l of sequencing Stop Solution (United States Biochemical) was added to each reaction. The reactions were denatured at 95°C for 3 min and quenched on ice for 5 min prior to loading on a 6% polyacrylamide sequencing gel.

RESULTS

The PCR amplification of canine brain cDNA using the truncated mcs/A primer pair yielded one discrete electrophoretic band of 2400-bp (Fig. 1, lane 2). The PCR with the truncated mcs/B primer pair yielded the expected 40-bp smaller band as determined by gel electrophoresis and ethidium bromide staining (Fig. 1, lane 3). Sequencing of the 2400-bp product with both primers A and B verified that it is derived from canine Glut3 (Fig. 2). The sequence obtained with primer B (Fig. 2, left) includes a region upstream of the termination codon, TAA, and a portion of the 3'-UTR for Glut3. The sequence obtained with primer A overlaps that obtained with primer B (Fig. 2, right).

The PCR amplification of canine brain cDNA using the truncated mcs/C primer pair yielded one discrete electrophoretic band of 1025-bp (Fig. 3, lane 2). The PCR with the truncated mcs/D primer pair yielded the expected 20-bp smaller band (Fig. 3, lane 3). Sequencing of the 1025-bp product with primer D verified that it is derived from the canine Glut1 target (Fig. 4). This sequence includes a region upstream of the termination codon, TGA, and a portion of the 3'-UTR.

The lengths of the PCR products for both Glut1 and Glut3 are consistent with the sizes of these two glucose transporter mRNA transcripts as determined by Northern blot analysis.⁽⁸⁾ Each product also correlates well with the predicted length based on human sequence data.

All Glut3 and Glut1 products were ob-

FIGURE 1 PCR products of the canine cortex Glut3 3' end, run on a 1% agarose gel. (Lane 1) DNA standard (λ DNA-HindIII + EcoRI; Boehringer Mannheim, Indianapolis, Indiana). The templates used in lanes 2 and 3 are 1 μ l of a 1:10 dilution of canine cortex cDNA. (Lane 2) The 2400-bp product obtained using the Glut3 primer A in conjunction with the truncated mcs primer; (lane 3) the 2360-bp product obtained using the Glut3 primer B in conjunction with the truncated mcs primer; (lane 4) negative control (no template). Primers are described in Methods and Materials.

tained with one round (31 cycles) of the PCR.

DISCUSSION

To isolate and sequence the 3' ends of cDNA, the RACE procedure, which employs PCR technology, was developed.⁽²⁾ The advantage that the PCR gives to the cloning step of this procedure is to increase greatly the probability that a clone of interest will be detected in the screening process. Our approach circumvents the several steps involved in cloning and screening. By using the lock-docking oligo(dT) primer modification, single, discrete PCR products for the 3' ends of the canine brain cortex Glut3 and Glut1 poly(A)⁺ RNAs were obtained. Thus, this primer served the purpose for which it was designed; namely, to lock in at the 3' end of gene-specific sequences and their poly(A) junctions.

Subsequently, the PCR yields discrete products that can be sequenced directly.

Although the sequencing of PCR-amplified DNA inserted in plasmids is straightforward, a problem of sequence fidelity arises because replication by *Taq* polymerase introduces random errors.⁽²³⁾ When a single PCR-generated product is cloned and sequenced, any error in the sequence of the cloned PCR insert will be retained. Such errors are undetected unless several clones are sequenced, and the correct sequence is determined by the rule of majority. In contrast, direct sequencing of a PCR product gives the correct sequence because the random *Taq* polymerase errors are outnumbered by correct copies.⁽²⁴⁾

The sequencing protocol given in this report has provided a consistently reliable method for sequencing PCR products. One helpful feature of this protocol

FIGURE 2 Sequence of a portion of the 3' end of canine cortex Glut3 obtained from the 2400-bp Glut3 PCR product. (Left) Sequence obtained with the Glut3 primer B. Reading from bottom to top (5' to 3'): 52 bases at the 3' end of the internal portion of canine Glut3, a TAA termination signal, and the beginning of the 3'-UTR of the gene. (Right) Sequence obtained using the Glut3 primer A. This sequence overlaps that obtained with the Glut3 primer B. Primers are described in Methods and Materials.

FIGURE 3 PCR products of the canine cortex Glut1 3' end, run on a 1% agarose gel. (Lane 1) DNA standard, the same as in Fig. 1. The templates used in lanes 2 and 3 are 1 μ l of a 1:100 dilution of canine cortex cDNA. (Lane 2) The 1025-bp product obtained using the Glut1 primer C in conjunction with the truncated mcs primer; (lane 3) the 1005-bp product obtained using the Glut1 primer D in conjunction with the truncated mcs primer; (lane 4) negative control (no template). Primers are described in Methods and Materials.

is that DNA precipitation is not required. Once sequence data has been gathered for a portion of these two cDNA ends, new primers can be designed to "walk" to the ends of these two transcripts. The truncated mcs primer has been used more successfully than the oligo(dT)mcs primer as a sequencing primer. Sequence obtained for the Glut1 smaller product with the truncated mcs primer was of comparable quality to that obtained in Figure 4 (data not shown), but sequence obtained for the other PCR products at the 3'-distal ends was of lesser quality. Because it is difficult to read sequence within 40-bp of the sequencing primer with the low copy number of template used here, readable sequence most likely begins beyond the AAUAAA polyadenylation signal, as this signal has not been observed. The average polyadenylation site starts 16 nucleotide units downstream of the AAUAAA signal. Also, the labeling isotope used was [α - 35 S]dATP and would not have been incorporated

over the initial 16-base-length stretch of Ts, thereby decreasing the intensity of signal close to the primer.

Both the 3' end PCR products of Glut3 and Glut1 can be obtained without the use of the AmpliWax PCR Gems (Perkin-Elmer Cetus). However, the yield and specificity of product improves when using this wax overlay as compared to the use of a mineral oil overlay. Also, the Glut1 3' end PCR products, when produced using a mineral oil overlay rather than a wax overlay, include an additional 1425-bp product (data not shown). This band is fainter than the 1025-bp band and has not been identified.

The use of the T4 gp32 protein enhances the specificity and yield of the Glut1 1025-bp product considerably (data not shown). The T4 gp32 protein was not used to obtain the Glut3 products shown here but has subsequently

FIGURE 4 Sequence of a portion of the canine cortex Glut1 3' end. Sequence was obtained from the 1025-bp Glut1 PCR product with Glut1 primer D. Reading from *bottom* to *top* (5' to 3'): 12 bases at the 3' end of the internal portion of canine Glut1, a TGA termination signal, and the beginning of the 3'-UTR of Glut1. Primers are described in Methods and Materials.

been used to advantage. Also, with the large Glut3 products, we have subsequently found it advantageous to increase the concentration of dNTPs. A final concentration in the PCR of 250 μ M in each dNTP and 3 mM $MgCl_2$ significantly increases the yield of the Glut3 products. The PCR reactions work equally well in 50- μ l or 100- μ l final volumes.

Preliminary evidence that Glut3 and Glut1 transcripts were being targeted in the PCR was achieved by the use of two primers for each. The predicted smaller-sized PCR product was observed for each Glut3 and Glut1 when the internal primers, B and D, respectively, were employed (40-bp smaller for Glut3, and 20-bp smaller for Glut1; Figs. 1 and 3). Should this approach not succeed due to primer failure, new primers (displaced slightly from the first) may be synthesized, or one may choose to do a Southern blot.

Another expected advantage of the lock-docking primer approach is the ability to detect directly the differential use of multiple polyadenylation sites by the cell. Under the PCR conditions used for the Glut3 and Glut1 3' ends as described here, only one discrete PCR product was observed for each upon gel electrophoresis of the products. However, it is also possible that this primer could initiate cDNA synthesis at an internal stretch of As, yielding a discrete PCR product.

We are planning to use the lock-docking primer in the 5'-RACE protocol⁽¹⁾ to obtain the 5' ends of canine Glut3 and Glut 1. This procedure involves the production of cDNA using a gene-specific first-strand primer located near the 5' end of the mRNA. After excess gene-specific primer is removed (to avoid tailing the primer), the cDNA will be tailed at the 3' end of the newly synthesized first strand using dATP and terminal deoxynucleotidyl transferase. The lock-docking oligo(dT) primer will be used to produce the second strand of cDNA. The PCR will then be performed with the truncated mcs primer and a second, gene-specific downstream primer located internally to the one used to produce the first strand of cDNA. The products will be characterized as outlined above for the 3' end.

In summary, the advantages of the lock-docking procedure are speed, direct sequencing of PCR products, and multi-

ple use of primers. In addition, when searching for members of multigene families with a degenerate/lock-docking primer pair, the resultant PCR products should all be distinguishable owing primarily to the variable lengths of 3'-UTRs. Discrete PCR products of differing sizes should also be observed for alternately terminated transcripts or, in some cases, alternately spliced transcripts. Overall, the methods employed here have the potential to speed up vastly the searches for, and sequencing of, the 5' and 3' ends of any mRNAs of interest for which some sequence is known for the internal portion of the mRNA.

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