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Note

Cloning of Full-Length cDNA of Teleost Corticotropin-Releasing Hormone Precursor by Improved Inverse PCR

Hisato KUNIYOSHI,† Yukio FUKUI, and Yoichi SAKAI

Graduate School of Biosphere Science, Hiroshima University, 1-4-4 Kagamiyama, Higashi-hiroshima, Hiroshima 739-8528, Japan

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We designed a new inverse PCR protocol combined with switching mechanism at 5′ end of RNA transcript (SMART) technology, and applied it to the cloning of teleost corticotropin-releasing hormone precursor cDNA. Due to the advantages of both techniques, this method can efficiently amplify the complete 5′- and 3′-ends of cDNA in a single reaction, and might prove to be an alternative to the conventional rapid amplification of cDNA ends (RACE) approaches.

Key words: inverse PCR; corticotropin-releasing hormone precursor; teleost; *Halichoeres tenuispinnis*

Rapid amplification of cDNA ends (RACE)[1,2] is an efficient strategy to obtain sequence information on either 3′- or 5′-regions flanking a known sequence of cDNA, but the conventional RACE method often amplifies non-specific products, which can result from the use of only one gene-specific primer.[2,3] In such cases, nested PCR is necessary to increase specificity.[2]

An alternative strategy is inverse PCR.[4] In this method, double-strand cDNA (ds-cDNA) is circularized by self-ligation and used as a PCR template. With a pair of gene-specific primers arranged in a back-to-back orientation, the cDNA fragment containing both 3′- and 5′-ends can be amplified using a standard PCR protocol (Fig. 1). Inverse PCR has two advantages: (1) non-specific PCR products are less likely to be amplified because of the use of two gene-specific primers,[3,5] and (2) both 3′- and 5′-regions are amplified in a single reaction. In spite of these advantages, inverse PCR has not been as popular as RACE.

To improve inverse PCR, we combined it with SMART (switching mechanism at 5′ end of RNA transcript) technology,[6] commercialized by Clontech in their SMART™ PCR cDNA Synthesis Kit (Fig. 1). SMART technology utilizes the template-switching activity of MMLV-reverse transcriptase to add an anchor sequence to the 3′-end of newly synthesized first-strand cDNA. In combination with an oligo-dT primer with a specific 5′ heel sequence, first-strand cDNA with anchor sequences at both ends is synthesized. The cDNA population is amplified by long-distance PCR (LD-PCR) with a primer, 5′ PCR primer

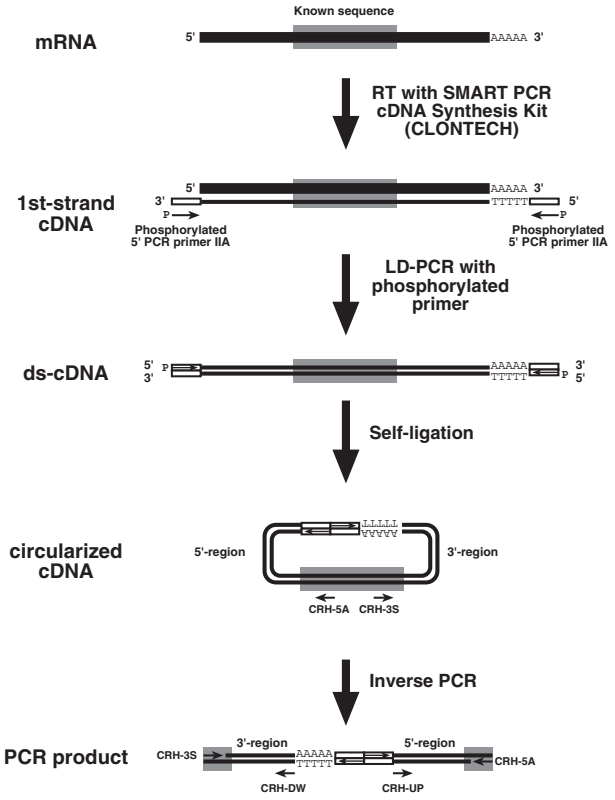

Fig. 1. Schematic Outline of Improved Inverse PCR.

Thick and thin lines represent mRNA and cDNA respectively. Arrows indicate primers. White boxes at the ends of cDNAs represent the anchor sequences introduced with the SMART™ PCR cDNA Synthesis Kit. The known sequence obtained by degenerate PCR is shaded.

† To whom correspondence should be addressed. Fax: +81-82-424-2459; E-mail: hkuni@hiroshima-u.ac.jp

Abbreviations: CRH, corticotropin-releasing hormone; ds-cDNA, double-strand cDNA; LD-PCR, long-distance polymerase chain reaction; RACE, rapid amplification of cDNA ends; SMART, switching mechanism at 5′ end of RNA transcript

IIA (kit supplied), which corresponds to the anchor sequences. To make possible the subsequent self-ligation, we used the 5′ PCR primer IIA that had been phosphorylated with T4 polynucleotide kinase (Takara, Kyoto, Japan). The resulting ds-cDNA was circularized by self-ligation and then used as the template for inverse PCR.

Utilizing SMART technology, our inverse PCR protocol has additional advantages. Since the template-switching phenomenon occurs when reverse transcriptase has reached the end of the mRNA template, full-length first-strand cDNAs but not prematurely terminated ones are tagged with the anchor sequence, and therefore enriched during LD-PCR.[6] This property is favorable for the cloning of full-length cDNA. Furthermore, only 0.05–1 μg of the starting total RNA is required. We applied this protocol to the cloning of corticotropin-releasing hormone (CRH) precursor cDNA of a marine wrasse, *Halichoeres tenuispinnis*.

First we obtained a partial sequence of the wrasse CRH precursor cDNA by degenerate PCR. First-strand cDNA was synthesized from wrasse brain total RNA using an RNA PCR Kit (Takara). Based on the amino acid sequences conserved among teleost CRH precursors,[7] we designed two degenerate primers, sense primer CRH-1 (5′-GGNGARGARTAYTTYATHMG-3′) and antisense primer CRH-2 (5′-TGYTCNGCNC-GNGACATYTC-3′) (Y; C/T, R; A/G, H; A/C/T, M; A/C, N; A/C/G/T) (Fig. 3). PCR cycling conditions were 30 cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s. A 280-bp product encoding the CRH precursor was amplified. On the basis of its nucleotide sequence, a pair of oppositely directed primers (CRH-5A and CRH-3S) was designed for inverse PCR (Fig. 1 and Fig. 3).

Next we performed improved inverse PCR to obtain the sequences of the 5′- and 3′-regions. First-strand cDNA was synthesized from 1 μg of wrasse brain total RNA using the SMART™ PCR cDNA Synthesis Kit (Clontech, Palo Alto, CA, USA) according to the manufacturer's instructions. Double-strand cDNA was prepared by LD-PCR using PrimeSTAR™ HS DNA polymerase (Takara), which can produce blunt-ended ds-cDNA. A PCR reaction was set up in a total volume of 50 μl containing 1 μl of the first-strand cDNA (equivalent to 100 ng of total RNA), 0.8 μM phosphorylated 5′ PCR primer IIA, 0.2 mM each dNTP, 1.25 U PrimeSTAR™ HS DNA polymerase, and 1 × PrimeSTAR™ Buffer (Takara). After an initial 1-min denaturing step at 95 °C, 15 cycles of amplification were performed using a cycle profile of 95 °C for 5 s, 65 °C for 5 s, and 68 °C for 6 min. After reagents and primers were removed with MicroSpin S-400 HR Columns (GE Healthcare, Piscataway, NJ, USA), ds-cDNA was circularized using a DNA Ligation Kit Ver. 2.1 (Takara). Inverse PCR was carried out in a 50-μl reaction mixture containing 1 μl of circularized ds-cDNA, 0.5 μM primers (CRH-5A and CRH-3S), 0.2 mM each dNTP, 1.25 U *Ex Taq* Hot Start Version (Takara), and 1 × *Ex Taq* Buffer (Takara). PCR parameters were 95 °C for 1 min and 35 cycles of 95 °C for 30 s, 67 °C for 30 s, and 72 °C for 2 min, followed by 72 °C for 7 min. As shown in Fig. 2, an approximately 1.0-kbp fragment was amplified from the circularized ds-cDNA but not from the linear ds-cDNA (*i.e.*, before self-ligation). Sequence analysis revealed that the 1.0-kbp amplicon contained both 3′- and 5′-regions of the wrasse CRH precursor cDNA. We found heterogeneity in the length of the 3′-untranslated region, which resulted from a difference in the polyadenylation site (Fig. 3, arrowheads). A 250-bp fragment is likely to have been a non-specific product because it was amplified from linear ds-cDNA as well as circularized ds-cDNA (Fig. 2).

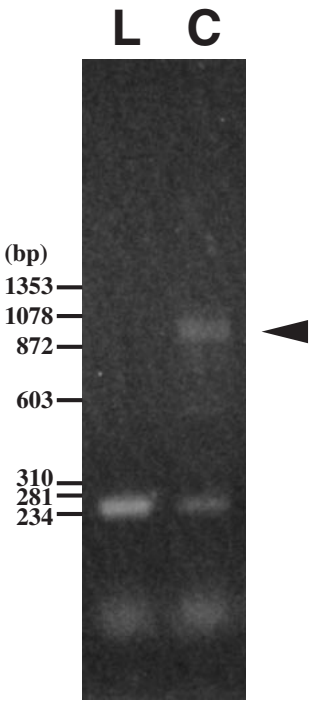

Fig. 2. Inverse PCR for *H. tenuispinnis* CRH Precursor cDNA.
PCR products were electrophoresed on a 2% agarose gel. A 1.0-kbp product (indicated by an arrowhead) was amplified from the circularized ds-cDNA (lane C) but not from the linear ds-cDNA (lane L). The sizes of markers are indicated on the left.

Finally, full-length cDNA was amplified by end-to-end PCR with primers, CRH-UP and CRH-DW, corresponding to the 5′- and 3′-ends of cDNA respectively (Fig. 1 and Fig. 3). First-strand cDNA prepared using the RNA PCR Kit (Takara) was used as a template. To suppress errors in the polymerase reaction, end-to-end PCR was carried out using the Expand High Fidelity^{PLUS} PCR System (Roche, Basel, Switzerland). PCR parameters were 35 cycles of 94 °C for 10 s, 55 °C for 30 s, and 72 °C for 1 min. A 1.0-kbp product was amplified. Since Northern blot analysis probed with wrasse CRH precursor cDNA detected an approximately 1.0-kb tran-

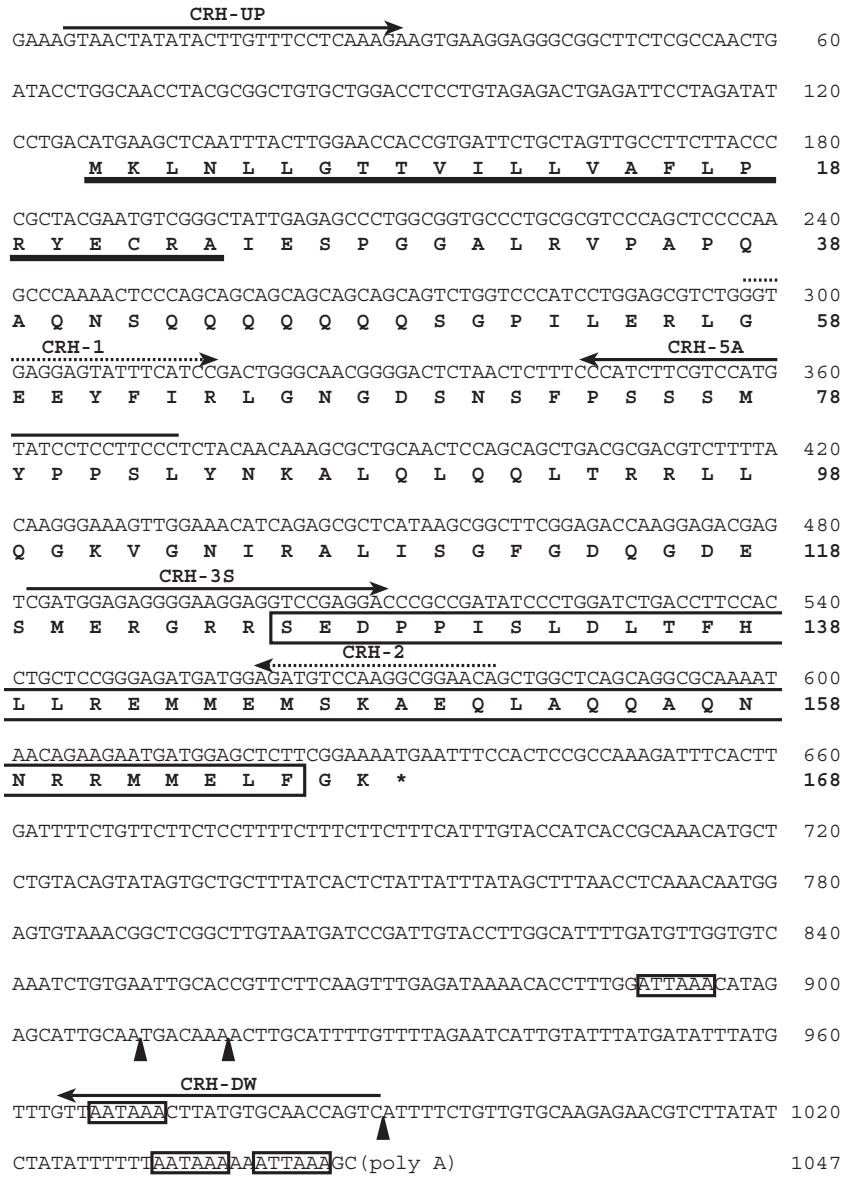

Fig. 3. Nucleotide and Deduced Amino Acid Sequences of the Longest *H. tenuispinnis* CRH Precursor cDNA.
The putative signal peptide is underlined. The amino acid sequence for CRH mature peptide is boxed. Broken and solid arrows indicate the degenerate and gene-specific primers respectively. Putative polyadenylation signal sequences in the 3′-untranslated region are boxed. Arrowheads indicate the polyadenylation sites of shorter transcripts.

script (data not shown), it is probable that the amplicon included the full-length sequence of CRH precursor mRNA.

As shown in Fig. 3, the wrasse CRH precursor cDNA (accession no. DQ073097) encoded a 168-amino-acid protein. Similarly to CRH precursors from other species, the wrasse CRH precursor is composed of a putative signal peptide, a cryptic region, and the CRH mature peptide. The CRH mature peptide is located between a potential proteolytic cleavage signal (Arg-Arg) and a C-terminal amidation signal (Gly-Lys).

In this report, we indicate that inverse PCR combined with the SMART system is a useful method, comparable to RACE. So far, we have succeeded in cloning the cDNAs of several neuropeptides from various teleost species (unpublished results). We believe that our inverse PCR protocol should prove to be a powerful tool for cloning of full-length cDNAs.

Acknowledgments

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