Amplification of GC-rich Putative Mouse PeP Promoter using Betaine and DMSO in Ammonium Sulfate Polymerase Chain Reaction Buffer

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Abstract

**Background:** Recently, we have shown that peroxisomal protein expression was induced upon retinoic acid treatment in mouse embryonic stem cells during the process of neurogenesis. Thus, characterization of the respective promoter could elucidate the molecular aspects of transcriptional regulation of this gene.

**Methods:** Using the conventional software programs for promoter prediction, a putative promoter region was identified approximately 561 bp upstream of the peroxisomal protein coding sequence. In order to clone this region with a GC-content of 71.01%, a cocktail of ammonium sulfate buffer supplied with two additive components, betaine and dimethyl sulfoxide, and a high concentration of MgCl2 was used.

**Results:** The modulated polymerase chain reaction composition significantly improved the amplification of GC-rich DNA target sequences. Improved amplification of this region was due to reduction in the formation of secondary structures by the GC-rich region.

**Conclusion:** Therefore, this polymerase chain reaction composition could be generally used to facilitate the amplification of other GC-rich regions as verified by amplification of different GC rich regions.

**Keywords:** Amplification, GC-rich region, Peroxisomal protein, Promoter

Introduction

Polymerase Chain Reaction (PCR) is a conventional method for amplification of DNA regions while templates with high percentages of GC bases including eukaryotic promoter regions are not easily amplified (1-4). In this state, PCR results in production of undesired truncated fragments instead of bona fide product (1,5).

Several approaches have been proposed to overcome this problem including additives usage in different PCR procedures (2,6). Different additives were utilized to amplify GC-rich regions including betaine (N,N,N-trimethylglycin), dimethyl sulfoxide (DMSO), formamide, glycerin, glycerol, dithiothreitol (DTT), Bovine Serum Albumin (BSA), non-ionic detergents such as Triton X-100, nucleotide analogs 7-deaza dGTP, ethylene glycol and 1, 2-propanediol, and reducing agents such as β-mercapto-ethanol (1-9). These additives not only prevent secondary structure formation of both templates and primers, but also increase the annealing chance of primers to plate strands at temperatures lower than the melting temperature of primers (1,3,5,7,9).

In the present study, we have utilized a combination of betaine and DMSO to amplify the putative promoter region of mouse peroxisomal protein (PeP). Mouse PeP which has been identified as a fibronectin type III domain containing protein (FNDC5; NCBI accession no: NC_000070) is supposed to undergo proteolytic cleavage and releasing to the extracellular space (10,11). Our data have elucidated that its expression increased upon Retinoic Acid (RA) treatment during the process of neurogenesis (12).

Very recently Bostrom et al have indicated that the cleaved form of PeP named irisin is a secreting myokine which its production could be induced after exercise, may improve the glucose homeostasis by regulating the obesity (11). To find out molecular mechanisms involved in the regulation of PeP expression, a putative promoter region at position -561/+101 relative to the Transcription Start Site (TSS), upstream of the PeP gene was chosen for amplification and further cloning.
Materials and Methods

To predict a potential promoter region of mouse PeP (Accession No: NC_000070), approximately 2x10^6 bp upstream of the PeP gene was submitted to the Genomatix (http://www.Genom-axt.de/en/index.html) and Proscan (http://www.proscan.co.za/) software programs. DNA extraction was performed on i) one Caucasian individual after signing a consent form and ii) a heart tissue sample of NMRI mouse. The ethics approval to use specimen was obtained from Royan Institute Bioethics Committee (Approval ID. No: EC-90-1077). Then 857 bp upstream of the PeP gene including a part of the transcriptional region was chosen for amplification using specific primers in conventional PCR (Table 1). Then, a set of PCR was performed to identify the optimal amplification conditions using betaine (Sigma), DMSO (Merck), glycerol (Merck), formamide (Scharlau), DTT (Sigma) (1,2). Moreover, different approaches of PCR including touch-down PCR (2,6), slow-down PCR (2) and hot-start PCR were applied. Finally, the successful amplification condition for amplicon (875 bp), included 10x PCR buffer AMS (composed of 750 mM of Tris-HCl (pH=8.8), 200 mM of (NH₄)₂SO₄, 0.1% Tween 20) (Cinna-Gen, Iran) and 4 mM Mg²⁺  (Cinnagen, Iran). To improve PCR efficiency, different concentrations of betaine (0.5 to 1 M) and DMSO (5-10% v/v) were applied. Simultaneously, similar amplification condition was repeated in Pfu buffer [200 mM of Tris-HCI (pH=8.8), 100 mM of KCl, 100 mM of (NH₄)₂SO₄, 1% Triton X-100 and 1 mg/ml of nuclelease-free BSA] (Fermentas, Lithuania) instead of 10 x PCR buffer AMS. Touch-down PCR was carried out in an Eppendorf thermocycler (Eppendorf, Germany) with one step of initial denaturation at 95°C for 5 min followed by twenty repetitive cycles, including 94°C for 10 s, a variable annealing step and an elongation step at 72°C for 4 min. The variable annealing step was started at a temperature of 66°C which decreased by 0.5°C/ cycle to reach a temperature of 56 °C for 30 s. Subsequently, additional amplification was continued for 20 cycles with a constant annealing temperature of 56°C for 30 s. PCR was terminated after final elongation step at 72°C for 10 min.

To demonstrate whether optimized PCR condition was applicable for other GC enriched regions, similar procedure was repeated for amplification of different loci on human genome (Table 1). Secondary structures predictions of the putative promoter of PeP and other amplified loci were assessed by Mfold web server (http://mfold.Bio-info.rpi.edu) (14).

Results

Putative promoter prediction and amplification

Computational analyses predicted a putative promoter region in location of -561 to +101 relative to the TSS of PeP gene (Figure 1A) with high GC content (approximately 71.01%) (Figure 1B). Secondary structure prediction of this region indicated formation of nine independent structures owing to its high internal energy in the range of -199.73 to -209.77 kcal/mol (data not shown). Two extreme structures with the highest and the lowest internal energy are shown in figure 1C. Cloning of this region was carried out using different PCRs to obtain proper amplification. However, there were no product yields regardless of different additives (Figure 2A). Data indicated utilization of a mixture containing 10x PCR buffer AMS with different amounts of betaine (final: 0.5, 0.75 and 1 M), and 5-10% (v/v) of DMSO improved PCR yield (Figure 2B, lanes 5-7). Meanwhile, substitution of Pfu buffer with 5% (v/v) DMSO in the PCR reaction resulted in amplification of desired fragment (Figure 2C, lane 2).

Improvement of the PCR yield

Interestingly increased amounts of MgCl₂ (3 and 4 mM) improved PCR yields. Particularly, PCR buffer AMS supplied with DMSO (10% v/v), betaine (final concentration: 1 M) and 4 mM of MgCl₂ improved PCR efficiency (Figure 3). While, utilization of touch down PCR did not enhance the PCR product amounts compared to the conventional PCR approach (Figure 4).

To verify the efficacy of our approach, Androgen Receptor (Exon 1) and Eukaryotic Releasing Factor 3a (Exon 1) genes from a human source and a portion of human Elongation Factor 1a Promoter encompassing 71.8, 75.94 and 60.09% GC bases respectively were chosen for amplification (Table 1). The expected 270 bp PCR product band for exon 1 of human Androgen receptor was observed significantly when 5-10% DMSO, 0.5-0.75 M betaine was used in PCR buffer AMS or Pfu buffer (Figure 5A, left panel). Similarly, the amplified region of Eukaryotic Releasing Factor 3a was predicted a potential promoter region of mouse PeP (Accession No: NC_000070), approximately 2x10^6 bp upstream of the PeP gene was submitted to the Genomatix (http://www.Genom-axt.de/en/index.html) (13) and Proscan (http://www.proscan.co.za/) (Version 1.7) software programs. DNA extraction was performed on i) one Caucasian individual after signing a consent form and ii) a heart tissue sample of NMRI mouse, which was obtained from Royan Institute (Tehran, Iran) using DNeasy Blood and Tissue Kit (Qiagen, Germany). The ethics approval to use specimen was obtained from Royan Institute Bioethics Committee (Approval ID. No: EC-90-1077). Then 857 bp upstream of the PeP gene including a part of the transcriptional region was chosen for amplification using specific primers in conventional PCR (Table 1). Then, a set of PCR was performed to identify the optimal amplification conditions using betaine (Sigma), DMSO (Merck), glycerol (Merck), formamide (Scharlau), DTT (Sigma) (1,2). Moreover, different approaches of PCR including touch-down PCR (2,6), slow-down PCR (2) and hot-start PCR were applied. Finally, the successful amplification condition for amplicon (875 bp), included 10x PCR buffer AMS (composed of 750 mM of Tris-HCl (pH=8.8), 200 mM of (NH₄)₂SO₄, 0.1% Tween 20) (Cinna-Gen, Iran) and 4 mM Mg²⁺  (Cinnagen, Iran). To improve PCR efficiency, different concentrations of betaine (0.5 to 1 M) and DMSO (5-10% v/v) were applied. Simultaneously, similar amplification condition was repeated in Pfu buffer [200 mM of Tris-HCI (pH=8.8), 100 mM of KCl, 100 mM of (NH₄)₂SO₄, 1% Triton X-100 and 1 mg/ml of nuclelease-free BSA] (Fermentas, Lithuania) instead of 10 x PCR buffer AMS. Touch-down PCR was carried out in an Eppendorf thermocycler (Eppendorf, Germany) with one step of initial denaturation at 95°C for 5 min followed by twenty repetitive cycles, including 94°C for 10 s, a variable annealing step and an elongation step at 72°C for 4 min. The variable annealing step was started at a temperature of 66°C which decreased by 0.5°C/ cycle to reach a temperature of 56 °C for 30 s. Subsequently, additional amplification was continued for 20 cycles with a constant annealing temperature of 56°C for 30 s. PCR was terminated after final elongation step at 72°C for 10 min.

To demonstrate whether optimized PCR condition

Table 1. Primers used for amplification of nucleotides

| Amplified DNA (species) | Primers sequence | Length of product | Product GC% | Accession No. |
|-------------------------|------------------|------------------|-------------|---------------|
| PeP Promoter (Mouse)    | F: 5' GCT ATT AAT GAA AGG ACT GGT CTG AGG | 875 bp          | 70.01%      | NC_000070     |
|                         | R: 5' GCT AGC CTA GGG TCA GTG TAG GAG   |                 |             |               |
| Androgen receptor (Exon I) (Human) | F: 5' ACC CTC AGC CGC TTC TCT ATC 3' | 270 bp          | 71.80%      | NC_000023     |
|                         | R: 5' CGT AGA TAG GGC ACT CTG CTC ACC 3' |                 |             |               |
| Eukaryotic releasing factor 3a (Exon I) (Human) | F: 5' CAT TCG TTC TCT GTC CAC 3' | 142 bp          | 75.94%      | NC_000016     |
|                         | R: 5' GTC GCA CCA GCA GTG AGG 3'       |                 |             |               |
| Elongation factor 1a promoter (Human) | F: 5' GCT ATT AAT CGT GAG GCT CCG GT3' | 1186 bp         | 60.09%      | NC_000020     |
|                         | R: 5' GCC AGG ACT TCA CGA CAC CTG AA3' |                 |             |               |

F and R, are referred as forward and reverse primers respectively. AseI (ATTAAAT) and Nhel (GCTAGC) restriction sites are underlined.

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PeP Promoter Amplification

(142 bp) was visualized when 5-7.5% DMSO, 0.5-0.75 M betaine in PCR buffer AMS reaction was used (Figure 5A, right panel). Moreover, amplification for promoter region of Elongation Factor 1a was achieved when 4 mM of MgCl₂ was added to the Pfu buffer (Figure 5A, middle panel and Figure 5B). Secondary structure prediction of aforementioned amplicons indicated formation of secondary structures due to presence of high internal energy (Figure 5C).

Discussion

Amplification of GC-rich genomic regions requires time-consuming work to be optimized. Additives such as betaine (1,3,5,9), DMSO (1,3,5,6,8), form-amide (1,3), glycerol (1,6), glycerin (7), non-ionic detergents such as Triton X-100 (1), nucleotide analogs 7-deaza dGTP (5-7), DTT (3), ethylene glycol (9), 1,2-propanediol (9), reducing compounds such as β-mercapto-ethanol (3) and their combination have been utilized to optimize amplification. However, effects of these additives on amplification of different fragments are not similar (9). For instance, betaine not only acts to reduce the DNA thermal melting transition dependent on base pair composition, but also does not allow DNA polymerase to pause near hairpin-loop structures (7). Thus, a combination of betaine and DMSO make GC-rich fragments of DNA accessible for DNA polymerase (7). However, in this research no efficient amplification was seen using both betaine and DMSO. Previous reports have indicated that amplification of 71% GC-rich DNA fragments was achieved by the addition of 16.6 mM (NH₄)₂SO₄, 3.1 mM MgCl₂ and 0.01% Tween 20 (3). In the present study, in order to amplify mouse PeP promoter with 71.01% GC, two different reaction buffers (10x PCR reaction buffer AMS and Pfu buffer) containing (NH₄)₂SO₄ were utilized. The application of these reaction buffers resulted in efficient amplification.
due to reduction in the formation of secondary structure due to GC-rich regions. Computational analysis confirmed that secondary structure formation of a putative region of mouse PeP promoter was highly possible. Previous studies have shown that proper concentration of Mg²⁺ ion increased the productivity and fidelity of DNA polymerases due to the increased DNA/DNA interactions and formation of complexes with dNTPs. However, higher amounts of MgCl₂ may inhibit polymerase activity (15-17). In the present study, increased MgCl₂ concentration (4 mM) was efficient. In some instances, modifications of the PCR procedure including a touch-down PCR is needed to gain satisfactory PCR products. However, utilization of this procedure did not yield more product than the conventional PCR. Our data demonstrated that addition of (NH₄)₂SO₄ in PCR reaction with suitable concentration of MgCl₂, betaine and DMSO would yield the highest amount of several amplicons.

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