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 DNA sequences 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.Genomatix.de/en/index.html) 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 PCRs was performed to identify the optimal amplification conditions using betaine (Sigma), DMSO (Merck), glycerol (Merck), formamide (Scharlau), DTT (Sigma) (1.9). 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-HCl (pH=8.8), 100 mM of KCl, 100 mM of (NH₄)₂SO₄, 1% Triton X-100 and 1 mg/ml of nuclease-free BSA] (Fermentas, Lithuania) instead of 10 x PCR buffer 1% Triton X-100 and 1 mM (Fermentas, Lithuania) instead of 10 x PCR buffer 1% Triton X-100 and 1 mM. 

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

Table 1. Primers used for amplification of nucleotides

<table>
<thead>
<tr>
<th>Amplified DNA (species)</th>
<th>Primers sequence</th>
<th>Length of product</th>
<th>Product GC%</th>
<th>Accession No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>PeP Promoter (Mouse)</td>
<td>F: 5’ GCT ATT AAT GAA AGG ACT GCT TCG AGG TCT CAG 3’&lt;br&gt;R: 5’ GCT AGC CTA GGG TCA GGT TAG GAG AGT AGG 3’</td>
<td>875 bp</td>
<td>70.01%</td>
<td>NC_000070</td>
</tr>
<tr>
<td>Androgen receptor (Exon 1) (Human)</td>
<td>F: 5’ ACC CTC AGC CGC GGC TCC TTC ATC 3’&lt;br&gt;R: 5’ CGT GGA TAG GGC ACT CGT CTC ACC 3’</td>
<td>270 bp</td>
<td>71.80%</td>
<td>NC_000023</td>
</tr>
<tr>
<td>Eukaryotic releasing factor 3a (Exon 1) (Human)</td>
<td>F: 5’ CAT TTC TCG TTC TCT GTC CAC 3’&lt;br&gt;R: 5’ CTG GTC CGC GCA GTC AGG 3’</td>
<td>142 bp</td>
<td>75.94%</td>
<td>NC_000016</td>
</tr>
<tr>
<td>Elongation factor 1a promoter (Human)</td>
<td>F: 5’ GCT ATT AAT CGG GAG GCT CGG GTCT 3’&lt;br&gt;R: 5’ GGC AGC TCA CGA CAC CGT AA 3’</td>
<td>1186 bp</td>
<td>60.09%</td>
<td>NC_000020</td>
</tr>
</tbody>
</table>

F and R, are referred as forward and reverse primers respectively. Ase1 (ATTAAAT) and Nhel (GCTAGC) restriction sites are underlined.
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(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.

Figure 1. DNA sequence, GC and CpG nucleotide composition graph of the putative promoter of PeP gene. A) Sequence of DNA fragment consisting of PeP gene (Accession no: NC_000070) putative promoter. The initiation codon is underlined. B) Genetyx software was used to plot the GC-graph and CpG plot programs (http://www.ncbi.nlm.nih.gov/). Translation start site (TSS) is located at position +21 relative to the transcription start site. Primer sequences are shown below the graph. C) Two different secondary structures predicted graphs of target DNA based on the Mfold web server (http://mfold.bioinfo.rpi.edu/) for nucleic acid folding and hybridization prediction as noted in the Materials and Methods section. The internal energy required for formation of these constructs is indicated as kcal/mole.

Figure 2. Effects of additives and PCR buffers on amplification of the putative promoter of PeP gene. Betaine (0-1 M) and DMSO (0-10% v/v) (lanes 2-8) were added to the different PCR reaction buffers to obtain amplification of the putative promoter region of the PeP gene. The desired PCR products (875 bp) are indicated by arrow heads while the star represents the nonspecific band. Lane one represents the DNA ladder (100 bp).

Figure 3. Optimization of amplification of the PeP gene putative promoter region by increasing MgCl2. The same PCR conditions of figures 2B (lanes 5-7) and 2C (lanes 2 and 5) were repeated using 3 and 4 mM MgCl2, respectively (A, B).

Figure 4. Optimized condition for amplification of the GC-rich putative promoter of the mouse PeP gene by different PCRs. The amplification of the 875 bp fragment was carried out by conventional (A) and touch-down PCR (B) using the mentioned annealing temperatures (Ta) and PCR conditions as described for figure 3a (lane 4).

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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 satisfaction 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 concentrations of MgCl₂, betaine and DMSO would yield the highest amount of several amplicons.

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References


