Determination of transcription starting site of *Mus musculus* Melanocortin 3 Receptor gene using "New 5'RACE"

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Method Article

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Abstract

Introduction

TSS \(Transcription starting site) determination is key to the study of transcriptional regulation, and "New 5´ RACE" \(rapid amplification of cDNA ends) is a challenge method for determining multiple transcription starting sites . Since Melanocortin 3 receptor \(MC3R) plays an important role in the regulation of body obesity, protocol for obtaining 5´UTR Transcription starting site of mouse Melanocortin 3 Receptor is presented. **Introduction** The 5´ends of transcripts give important information about transcription initiation sites ; RACE \(rapid amplification of cDNA ends) PCR is useful for quickly obtaining full length cDNA from mRNA for which only part of the sequence is known. And "new 5´RACE" is changed by adding an anchor ligation to the 5´end of the mRNA before reverse transcription, thus, the anchor sequence becomes incorporated into the first-strand cDNA if and only if the reverse transcription proceeds through the entire length of the mRNA of interested . On the other hand, promoter of Melanocortin receptors are among those promoters of G-protein coupled receptors, which usually characterized as TATA less, GC rich. Melanocortin 3 receptors and Melanocortin 4 receptors are involved in the regulation of energy homeostasis . Therefore, it should be significant to determine their transcription starting site. Since the intact mRNA has a 7-methyl guanosine CAP \(figure1) on its 5´end. The 5´RACE could target its intact mRNA with improved specificity, through dephosphorylation with Calf Intestinal Alkaline Phosphatase \(CIAP) to dephosphorylate degraded uncapped mRNA \(figure1), then Tobacco Acid Pyrophophatase \(TAP) treatment to remove cap from full length mRNA \(Figure 2), and 5´RACE adaptor ligation to decapped mRNA. Due to the advantage of the adaptor, the "new 5´RACE" could produced the first strand cDNA by reverse transcription from the full length mRNA of targeted. With the nested PCR, it is like that the length of the primer become longer \(the length of outer Plus that of inner primer), so that the contamination could be reduced and a higher specificity to the target sequence could be ensured. With TOPO Clone \(Ligation) and Transformation, they are sent for sequencing. The transcription starting site could be determined from the statistics of the sequencing results. There are 5'RACE for TSS determination for promoter of estrogen receptor , of human PPARg gene , etc. But there is no detail report of 5'RACE determination of TSS on Melanocortin 3 Receptor. Here a detail protocol based on our first hand experiments is disclosed. \(Figure 2) **Discussion** **Primer Design** Alter the stringency while designing primers so that all of the resulted three factors \(hairpin, dimmers and false priming) meet the standards for primer design guidelines. \(As it shown in tableA2-primer analysis result by primer premier, the experience should be -8.0kcals/mol, though we have -10.9kcal/mol for A-O-318 in the false primer, and -16.5kcal/mol for S-In-28, it still works). In the primer design for the gene specific inner control primer 3, we designed two inner control primers \(3a and 3b) instead of one, this way, we could get better primer match and easy way for primer design for the case of mMC3r. As a result, it shows \(Table A2) good in analysis result as well as experiment. In addition, since the commercial available RACE kit has its limitations3, commercial systems are often geared toward the construction of universal pools of full-length cDNAs. So the use of GSP-RT primer, gives more potency for the reverse transcription steps starting more specifically. \(Figure E, I and A1'c is from GSP-RT 496) **Reverse Transcription** Other than GSP-RT-601\(figure E, A1'd), the GSP-RT-496 \(figure E, A1'c) starts closer to the 5'end of transcripts and result a higher specific cDNA. Besides, the 5'UTR of mMC3R gene is characterized as a TATA less and GC rich. \(as high as 58.5% near the CpG island, detail data not shown here), Therefore, in order to get a fully open, instead of using M-MLV Reverse transcriptase in the kit, we used the SuperScript III Reverse transcriptase, \(it is an engineered version of M-MLVR, reduced RNAse H activity and increased thermal stability, can be used to synthesize first-strand cDNA at temperature up to 55°C, providing increased specificity, higher yield of cDNA and more full length product than other reverse transcriptase.) **Outer and Nested PCR** In each PCR, the Touchdown PCR \(Table Db, Table Eb) is used. It could avoid low Tm priming during the earlier cycles. Touchdown PCR increase specificity and reduced background amplification. By starting at a high annealing temperature, only gene-specific cDNA is amplified, allowing the target product to accumulate. Decreasing the annealing temperature through the remaining PCR cycles permits efficient amplification of tagged, gene-specific template. Since the empirical screening by determination of annealing temperature is time consuming, especially for the case of small amount presenting target template. As a result, Touchdown PCR is not only a method for determining the optimum cycling conditions for a specific PCR but also as a potential one-step method for approaching optimal amplification, specially, when the degree of identity between the primer and template is unclear. **Sequence and statistics** The consensus transcription start site -368 upstream of ATG should be identified.

Reagents

Materials - Mouse Brain, hypothalamus Total RNA is from Clontech - RLM-RACE kit from Ambion - Transcription super III from Invitrogen - Platinum PCR SuperMix High Fidelity is from Invitrogen - SuperScriptIII RT is from Invitrogen - TOPO Cloning kit is from Invitrogen - One shot Efficiency DH5a-T1 Competent Cells for transformation is from Invitrogen - EcoRI is from New England Biolabs - DNA extractions kits are from Qiagen

Procedure

1| Check the quality of the RNA and design primers as decribed in "Protocol step A":http://www.nature.com/protocolexchange/system/uploads/2436/original/nprot.2010.28_-_protocol_step_A.pdf?1358506599 2| Perform RNA processing \(B), Reverse Transcription \(C) and Outer 5'RLM-RACE PCR \(D) as described in "Protocol step B":http://www.nature.com/protocolexchange/system/uploads/2437/original/nprot.2010.28_-_protocol_step_B.pdf?1358506624, "step C":http://www.nature.com/protocolexchange/system/uploads/2438/original/nprot.2010.28_-_protocol_step_C.pdf?1358506643 and "step D":http://www.nature.com/protocolexchange/system/uploads/2439/original/nprot.2010.28_-_protocol_step_D.pdf?1358506663. 3| Perform Nested PCR\(2+4) as described in "Protocol step E":http://www.nature.com/protocolexchange/system/uploads/2440/original/nprot.2010.28_-_protocol_step_E.pdf?1358506687 4| Perfom TOPO TA Cloning as described in "Protocol step F":http://www.nature.com/protocolexchange/system/uploads/2441/original/nprot.2010.28_-_protocol_step_F.pdf?1358506712

Timing

This way, an ideal result could be offered by this protocol within a week \(if every step is smooth).

Anticipated Results

Multiple transcriptional start sites may be identified in addition to the major site at position -368 from the initiating methionine that corresponds to the predicted transcription start site according to GenBank. In a word, on the base of general steps of new 5'RACE, we use more RNA than what is suggested in the TAP treatment, use GS-RT Primer, SuperScriptIII and a higher temperature in the reverse transcription reaction. Two gene specific inner control sense primers are designed and used instead of one, with Platinum PCR Supermix, the reagent taking and mixing time for either outer PCR and nested PCR was reduced. Further more, with the touchdown PCR, for each of the outer and nested PCR processing, the time for screening and optimum the PCR reaction condition was also saved.

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Figures

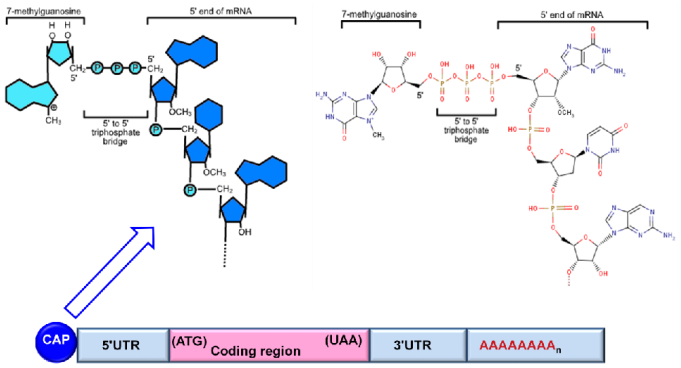

Figure 1: 5'CAP of mRNA

Figure 1

Figure 1

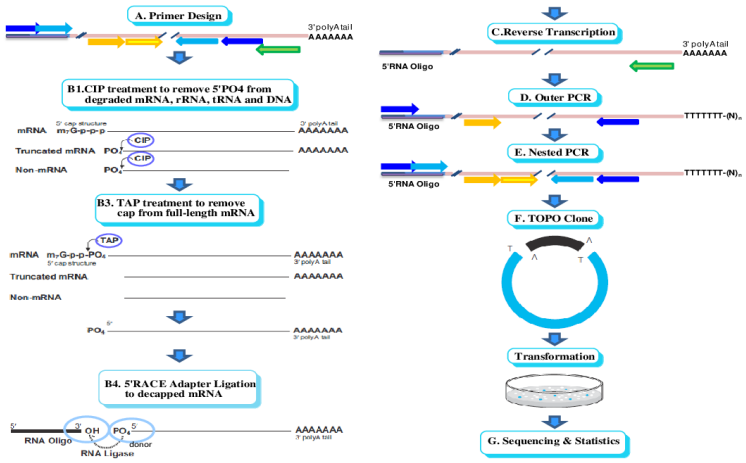

Figure 2: Steps Diagram of mMC3r 5'RACE Protocol

Figure 2

Figure 2

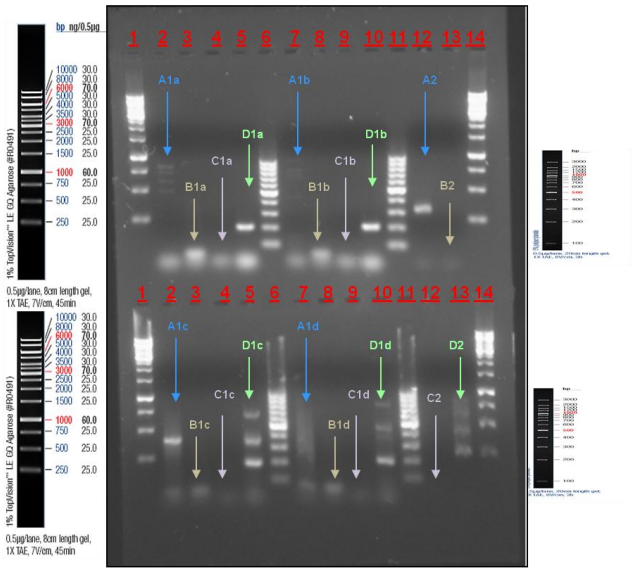

Figure D: Gel of (mMC3r 5'RACE) Outer PCR

a,b,c,d are Out PCR groups using different RT template from previous RT reaction;

a: ambion's random decamer

b: invitrogen's random primer

c: GSP-RT-496

d: GSP-RT-601

A1: (1+5) outer PCR;

B1: TAP negative control;

C1: Template negative control;

D1: (3a+5) inner control;

A2(1'+5'), B2[(TAP(-)], C2[Template(-)], D2(3'+5') : kit control using mouse thymus as template;

The other lanes are markers.

Figure 3

Figure D Figure for Step D

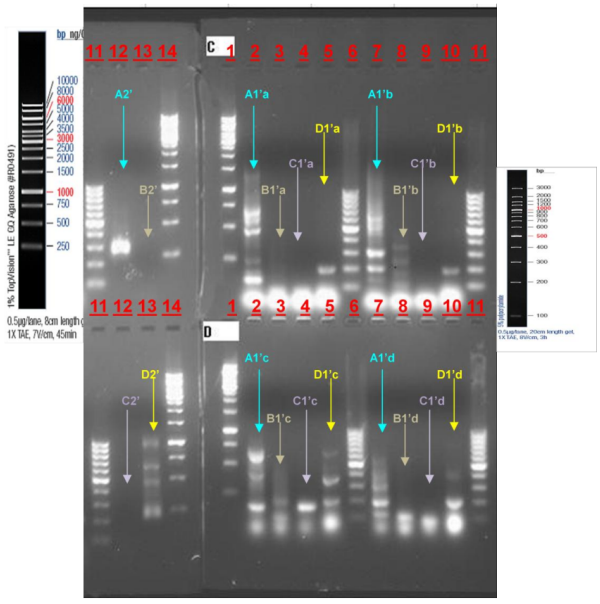

Figure E: Gel of (mMC3r 5'RACE) Nested PCR
a,b,c,d are Nested PCR groups using group a,b,c,d of out PCR as template;
A1': (2+4) nestedPCR;
B1': TAP negative control of nested PCR;
C1': Template negative control of nested PCR;
D1': (3b+4) inner control of nested PCR;
A2'(2'+4'), B2'[(TAP(-)], C2'[Template(-)], D2'(3'+5') : kit control using mouse thymus as template;
The other lanes are markers.

Figure 4

FIgure E Figure for step E

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