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Evaluation of hypothalamic murine and human melanocortin 3 receptor transcript structure

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ABSTRACT

The melanocortin 3 receptor (MC3R) is involved in regulation of energy homeostasis. However, its transcript structure is not well understood. We therefore studied initiation and termination sites for hypothalamic murine *Mc3r* and human *MC3R* transcripts. Rapid Amplification of cDNA Ends (RACE) was performed for the 5′ and 3′ ends of murine and human hypothalamic RNA. 5′ RACE experiments using hypothalamic murine RNA indicated mouse hypothalamus expresses two major *Mc3r* transcription start sites: one with a 5′ UTR approximately 368 bases in length and another previously unknown transcript with a 5′ UTR approximately 440 bases in length. 5′ RACE experiments using human hypothalamic RNA identified a 5′ UTR beginning 533 bases upstream of the start codon with a 248 base splice. 3′ RACE experiments using hypothalamic murine RNA indicated the 3′ UTR terminates approximately 1286 bases after the translational stop codon, with a previously unknown 787 base splice between consensus splice donor and acceptor sites. 3′ RACE experiments using human *MC3R* transcript indicated the 3′ UTR terminates approximately 115–160 bases after the translational stop codon. These data provide insight into melanocortin 3 receptor transcript structure.

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1. Introduction

The melanocortin-3 receptor (MC3R) is a G-protein-coupled receptor derived from a single-exon gene that is mainly expressed in the hypothalamus, liver and adipose tissue [1–3] and plays an important role in energy homeostasis. Inactivation of both alleles of *Mc3r* in mice is associated with positive energy balance through several mechanisms, including increased energy intake, changes in the balance of substrate oxidation, and increased metabolic efficiency [4–7]. Human linkage and polymorphism association studies also suggest MC3R is important for human energy homeostasis [8–10]. To our knowledge, however, no studies have evaluated the transcript structure of murine *Mc3r* although a recent paper has reported the transcript structure for the human *MC3R* [11]. Untranslated regions (UTRs) play important roles for gene expression, including providing sites for RNA splicing as well as potentially regulating mRNA stability, localization, and translational efficiency [12,13]. We therefore studied initiation and termination sites for hypothalamic murine and human *MC3R* transcripts. In addition, we evaluated the sequence of the 5′ and 3′ UTRs of murine Mc3r and human *MC3R*.

2. Materials and methods

2.1. Tissue samples

Murine Hypothalamic Total RNA and Human Hypothalamic Poly-A RNA were purchased from Clontech (Mountain View, CA). The total murine RNA was treated with DNase I (Ambion/Roche, Grand Island, NY) according to the manufacturer's instructions to minimize potential genomic contamination.

2.2. 5′- and 3′-rapid amplification of cDNA ends (RACE)

We amplified the 5′-ends of murine *Mc3r* and human *MC3R* RNA by RNA ligase mediated amplification of cDNA ends [14] (RLM-

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RACE) using the strategy outline in the First Choice® RLM-RACE RNA Ligase Mediated RACE Kit (Ambion, Grand Island, NY) (1). 50 μg of DNase-treated total murine hypothalamic RNA was dephosphorylated with calf intestine phosphatase (CIP), and then a phenol: chloroform extraction was performed to retrieve the CIP-treated RNA. The RNA was digested by tobacco acid pyrophosphate (TAP) to remove the 5′ cap structure, and ligated to a 5′ RACE adapter (5′-GCTGATGGCGATGAATGAACACTG) at 5′-ends using T4 RNA ligase. The ligated RNA was transcribed into cDNA from 5′ adapter ligated mRNA primed with oligo(dT)$_{20}$ using Superscript® III reverse transcriptase (Invitrogen, Grand Island, NY) in a total reaction volume of 20 μL, and then used as a template for subsequent PCR. To amplify the 5′ ends of human *MC3R*, 1.25 μg of human hypothalamic poly-A RNA was digested by TAP to remove the 5′ cap structure, and ligated to the same 5′ RACE adapter at 5′-ends using T4 RNA ligase. The 5′ adapter-ligated murine and human RNA was transcribed into cDNA using oligo(dT)$_{20}$ and Superscript® III reverse transcriptase (Invitrogen) in a total reaction volume of 10 μL, and then used as a template for subsequent nested PCR. Primers specific to the 5′ RACE adapter (Ambion, First Choice® RNA Ligase Mediated (RLM RACE Kit) were used (Table 1). All other gene specific primers for both murine and human melanocortin-3-receptor DNA sequences (Table 1) were synthesized by Invitrogen/Life Technologies Corporation (Carlsbad, CA, USA). The primer combinations for each of the nested PCR reactions can be found in Fig. 1.

The 3′-ends of the cDNA were also amplified by RACE (First Choice® RLM-RACE RNA Ligase Mediated RACE Kits, Ambion). 50 μg of DNase-treated total murine hypothalamic RNA and 1.5 μg of human hypothalamic poly-A RNA were transcribed into cDNA with a 3′ RACE adapter (5′-GCGAGCACAGAATTAATACGA CTTTTTTTTTTTTT) and used as a template for PCR reactions using nested PCR (see Table 1 for gene-specific primers).

PCR products from the 5′- and 3′-RACE reactions were cloned into a PCR2.1-Topo Vector (Invitrogen) by TA cloning. Vectors were then transformed into One Shot MAX Efficiency DH5α-T1R Competent Cells (Invitrogen) and the bacteria were grown overnight on

Table 1
Primers used in this study.

Primer	Primer sequence (5′–3′)
Adapter specific primers	
5′ RACE (Adapter) Outer Primer (KO)	GCTGATGGCGATGAATGAACACTG
5′ RACE (Adapter) Inner Primer (KI)	CGCGGATCCGAACACTGCGTTTGCTGGCTTTGAT
3′ RACE (Adapter) Outer Primer (K3O)	GCGAGCACAGAATTAATACGACT
3′ RACE (Adapter) Inner Primer (K3I)	CGCGGATCCGAATTAATACGACTCACTATAGG
5′ RACE Inner Control Primer (For Thymus Control)	GAAGTAGATGGTGGGCAGGAAGAT
5′ PCR Control Primer (For Thymus Control)	GCAGCAGGTAGCAGTGAC
3′ RACE Control Primer	AGCAGTTGGTTGGAGCAAACATC
Gene specific primers	
Primer_C_5′_Mouse_Mc3r	TCCTGCTGCCTGTCTTCTGTTTCT
Primer_D_5′_Mouse_Mc3r	GCTCACCAGCATGTCGGCT
Primer_E_5′_Mouse_Mc3r	GCCACCAGGGAGATGCAAATCATAGAG
Primer_M_5′_Human_MC3R	TGTTCAGCCAACACTGCCTAATGG
Primer_N_5′_Human_MC3R	TTTCTTCAGCAACCAGAGCAGCAG
Primer_O_5′_Human_MC3R	AGCATCATGGCGAAGAACATGGTG
Primer_P_5′_Human_MC3R	AGAAGATGAACACGCCCAGGAGAA
Primer_Q_3′_Human_MC3R	TGTTGAAGTGGGCAGTGTAGCAGA
Primer_R2_3′_Human_MC3R	GAAGGGGGCCCAGCAGAAGA
Primer_S2_3′_Human_MC3R	GCGTCTGTGGCGTGGTGTTC
Primer_T_3′_Human_MC3R	TCGAGGACCAGTTTATCCAGCA
Primer_I_3′_Mouse_Mc3r	TAGCCCAAGTTCATGCTGTTGCAG
Primer_J2_3′_Mouse_Mc3r	TGGTGGGGCAGGTGATGATGA
Primer_K2_3′_Mouse_Mc3r	TCGCCATGGTGCTCCTCATG
Adapter specific primers	
5′ RACE (Adapter) Outer Primer (KO)	GCTGATGGCGATGAATGAACACTG
5′ RACE (Adapter) Inner Primer (KI)	CGCGGATCCGAACACTGCGTTTGCTGGCTTTGAT
3′ RACE (Adapter) Outer Primer (K3O)	GCGAGCACAGAATTAATACGACT
3′ RACE (Adapter) Inner Primer (K3I)	CGCGGATCCGAATTAATACGACTCACTATAGG
5′ RACE Inner Control Primer (For Thymus Control)	GAAGTAGATGGTGGGCAGGAAGAT
5′ PCR Control Primer (For Thymus Control)	GCAGCAGGTAGCAGTGAC
3′ RACE Control Primer	AGCAGTTGGTTGGAGCAAACATC
Gene specific primers	
Primer_C_5′_Mouse_Mc3r	TCCTGCTGCCTGTCTTCTGTTTCT
Primer_D_5′_Mouse_Mc3r	GCTCACCAGCATGTCGGCT
Primer_E_5′_Mouse_Mc3r	GCCACCAGGGAGATGCAAATCATAGAG
Primer_M_5′_Human_MC3R	TGTTCAGCCAACACTGCCTAATGG
Primer_N_5′_Human_MC3R	TTTCTTCAGCAACCAGAGCAGCAG
Primer_O_5′_Human_MC3R	AGCATCATGGCGAAGAACATGGTG
Primer_P_5′_Human_MC3R	AGAAGATGAACACGCCCAGGAGAA
Primer_Q_3′_Human_MC3R	TGTTGAAGTGGGCAGTGTAGCAGA
Primer_R2_3′_Human_MC3R	GAAGGGGGCCCAGCAGAAGA
Primer_S2_3′_Human_MC3R	GCGTCTGTGGCGTGGTGTTC
Primer_T_3′_Human_MC3R	TCGAGGACCAGTTTATCCAGCA
Primer_I_3′_Mouse_Mc3r	TAGCCCAAGTTCATGCTGTTGCAG
Primer_J2_3′_Mouse_Mc3r	TGGTGGGGCAGGTGATGATGA
Primer_K2_3′_Mouse_Mc3r	TCGCCATGGTGCTCCTCATG

PCR reaction conditions are available from the authors. *Note:* Primer K2 was used in the outer and inner PCR primer pair in the amplification of 3′ murine MC3R RNA.

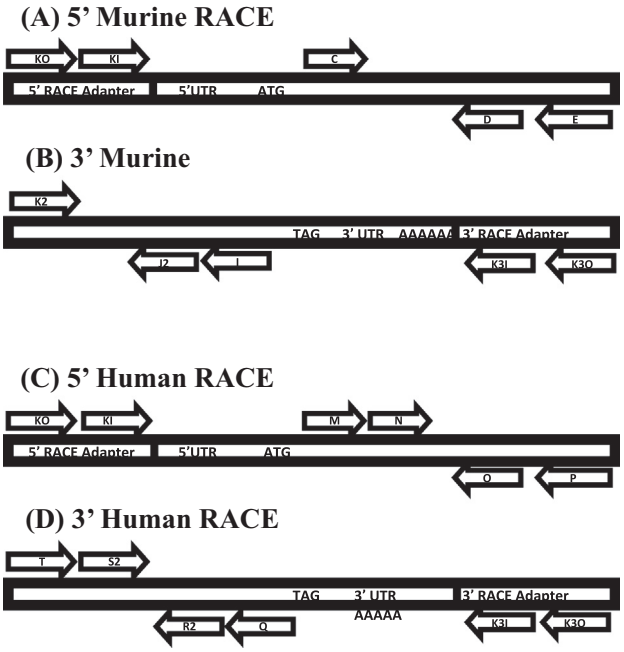

Fig. 1. Nested PCR Design for RACE experiments. (A) Amplification of 5′ Murine MC3R RNA sequences after ligation of adaptor sequences. Outer PCR primer pairs are KO + E (for 5′ RACE) and C + E (for gene-specific control reaction). Inner PCR primer pairs are KI + D (for 5′ RACE) and C + D (for gene-specific control reaction). (B) Amplification of 3′ Murine MC3R RNA sequences after cDNA synthesis with poly(T) adaptor sequence. Outer PCR primer pairs are K3O + K2 (for 3′ RACE) and I + K2 (for gene-specific control reaction). Inner PCR primer pairs are K3I + K2 (for 3′ RACE) and J + K (for gene-specific control reaction). (C) Amplification of 5′ Human MC3R RNA sequences after ligation of adaptor sequences. Outer PCR primer pairs are KO + P (for 5′ RACE) and M + P (for gene-specific control reaction). Inner PCR primer pairs KI + O (for 5′ RACE) and N + O (for gene-specific control reaction). (D) Amplification of 3′ Human MC3R RNA sequences after cDNA synthesis with poly(T) adaptor sequence. Outer PCR primer pairs are K30 + T (for 3′ RACE) and Q + T (for gene-specific control reaction). Inner PCR primer pairs are K3I and S2 (for 3′ RACE) and R2 and S2 (for gene-specific control reaction).

LB Agar plates containing 50 μg/mL ampicillin and coated with 40 μL of 40 mg/mL XGAL (US Biological, Salem, Massachusetts). Randomly selected 10–24 clones per plate were selected for sequencing from each experiment.

Plasmid DNA was extracted with QIAprep Spin Miniprep Kit (Qiagen) according to the manufacturer's instructions. Purified plasmids were sequenced by Macrogen (Rockville, MD, USA).

2.3. Sequence analysis

The sequencing results were analyzed using Sequencher (Gene Codes Corp, Ann Arbor, MI, USA). For 5′ RACE analysis, sequences were aligned with the consensus murine *Mc3r* or human *MC3R* genomic sequence from GenBank (murine version: NM_008561.3 GI:142371951, human version: NG_012200.1 GI:238018074) along with 1000 bases upstream bases upstream of the consensus 5′ start sites for gene translation. Considering the adenosine base pair of the start codon as position 0, the relative sizes of the observed 5′ UTRs were determined. For 3′ RACE analysis sequences were aligned with the consensus human *MC3R* or murine *Mc3r* genomic sequences from GenBank along with 2000 bases downstream of the consensus stop codon (TAG). Considering the thymine base pair of the stop codon as position 0, the relative sizes of the observed 3′ UTRs were determined.

2.3.1. SplicePort

The consensus genomic sequences from GenBank for both the human and murine melanocortin 3 receptors were entered into SplicePort (http://spliceport.cbcb.umd.edu) to identify potential splice donor and splice acceptor sites. A score threshold measurement was assigned to evaluate the likelihood that each site would serve as a splice donor/acceptor. The donor and acceptor sites of any splices predicted by SplicePort along with their corresponding score threshold measurements, were evaluated against splices observed in the RACE experiments.

2.3.2. Transcription factor binding site analysis

The consensus sequences from GenBank for both the human and murine melanocortin 3 receptors were entered into TFBind (http://tfbind.hgc.jp). TFBind identifies potential transcription factor binding sites. The location of any transcriptional start sites observed in the RACE experiments were compared with the presence of any transcription initiation sequences identified by TFBind in the consensus sequences.

2.3.3. Poly(A) Signal Miner

The consensus sequences from GenBank for the human melanocortin 3 receptor were entered into Poly(A) Signal Miner (http://dnafsminer.bic.nus.edu.sg/PolyA.html). Poly(A) Signal Miner predicts polyadenylation signals in human DNA sequences using both upstream and downstream sequence elements [15].

3. Results

3.1. Murine 5′ Mc3r RLM-RACE

1107 PCR products sequenced from five independent murine *Mc3r* 5′ RLM RACE experiments contained *Mc3r* sequence. A total of 296 sequences had a transcriptional start site (TSS) between 325 and 385 bases upstream of the start of gene translation, with 210 sequences starting 368 bases upstream (Fig. 2A). A total of 809 sequences had a transcriptional start site (TSS) between 414 and 465 bases upstream of the start of gene translation, among which 741 sequences had a TSS 440 bases upstream (Fig. 2A).

3.1.1. Murine Mc3r TFBind analysis

Associated putative initiator sequences were found for both the murine *Mc3r* -440 TSS and the murine *Mc3r* -368 TSS. For the -440 TSS, a putative initiator sequence began at -441 bases and had a score of 0.939 which was the second highest for potential initiator sequences in the 5′ UTR region. For the -368 TSS, a putative initiator sequence began at -370 bases with a score of 0.916, which was among the top 6 potential initiator sequences (Fig. 3A).

3.1.2. Murine 5′ Mc3r ATCT repeats

The GenBank consensus sequence for murine *Mc3r* contains 8 repeats of the sequence ATCT from 164 to 132 bases upstream of the start codon. None of the clones from the murine 5′ RACE experiments had 8 ATCT repetitions. Three clones had 6 ATCT repeats, while the remaining 111 sequences had 7 ATCT repeats.

3.2. Human 5′ MC3R RACE

1932 PCR products sequenced from six independent human *MC3R* 5′ RLM RACE experiments contained *MC3R* sequence. All 1932 sequences had a TSS from 528 to 567 bases upstream of the start of gene translation, with 1326 sequences starting 533 bases upstream. In all PCR products sequenced, a 248 base splice was observed from 140 to 388 bases upstream of the start codon (Fig. 2C).

3.2.1. Human MC3R TFBind analysis

An associated putative initiator sequence was found for the human *MC3R* -533 region TSS. The initiator sequence began 533 bases upstream of the start codon and had a score of 0.948 which was the second highest for potential initiator sequences in the human *MC3R* 5′ UTR region. This initiator sequence could conceivably account for the multiple TSSs seen from 517 to 544 bases upstream of the second ATG (Fig. 3C).

3.2.2. Human MC3R SplicePort analysis

The donor and acceptor sites for the 248 base pair splice that was apparent in all human *MC3R* 5′ sequences were evaluated using SplicePort. Analysis of the GenBank consensus human *MC3R* sequence by SplicePort identified a potential splice donor site 388 base pairs upstream of the second translational start site (ATG2). SplicePort also identified a potential splice acceptor site 140 base pairs upstream of ATG2. The score for this splice donor site was -0.0451, which corresponds to approximately 99% specificity. [16] This donor site had the highest score among potential donor sites within the Human *MC3R* 5′ UTR. The score for the splice acceptor site was -0.0834, which corresponds to approximately 99% specificity. This acceptor site had the second highest score among potential acceptor sites within the Human *MC3R* 5′ UTR.

3.3. Murine Mc3r 3′ RACE

1612 PCR products sequenced from seven independent murine *Mc3r* 3′ RACE experiments contained *Mc3r* sequence. 1613 sequences terminated 1280–1291 bases after the translational stop codon, of which 1485 sequences terminated 1286 bases downstream. Of these 1485 sequences, 1479 had a 787 base pair splice from 171 bases to 958 bases downstream of the stop codon (Figs. 2B and 3B).

3.3.1. Murine Mc3r SplicePort Analysis

The donor and acceptor sites for the 787 base pair splice that was observed for murine *Mc3r* were evaluated using SplicePort. Analysis of the GenBank consensus murine *Mc3r* sequence identified a potential splice donor site 171 base pairs downstream of the stop codon. A potential splice acceptor site 958 base pairs

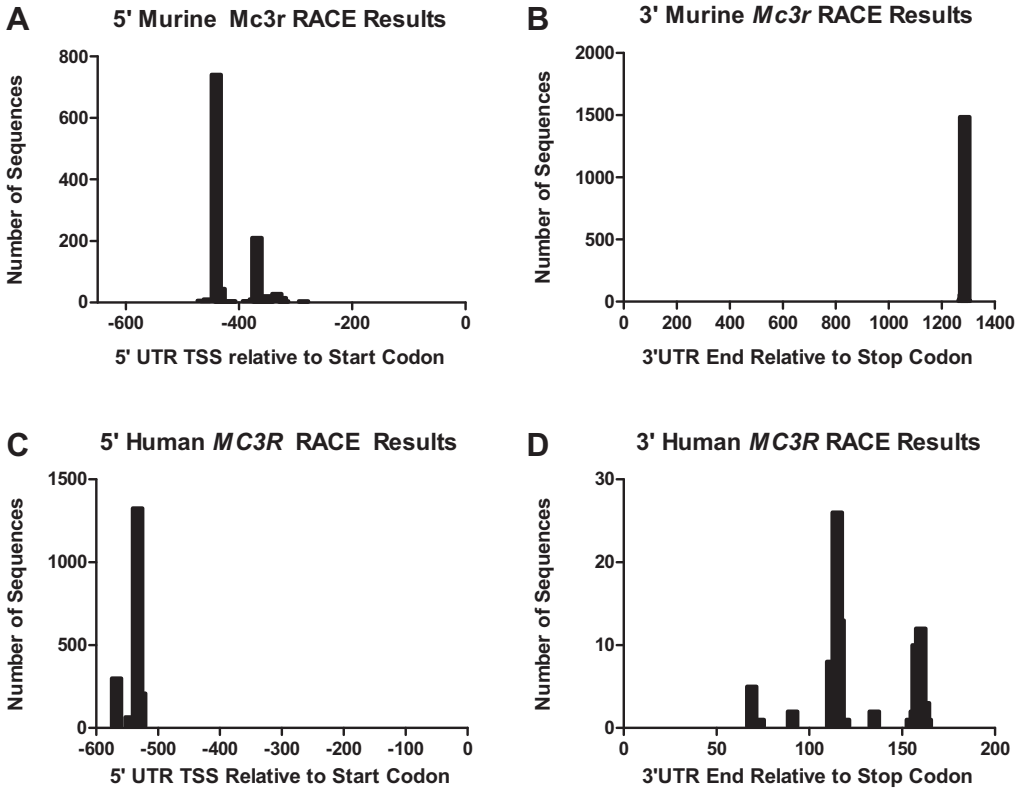

Fig. 2. The number of PCR product sequences for each TSS observed in RACE experiments. (A) 5′ RLM-RACE using murine *Mc3r* mRNA and *Mc3r* specific primers. The two predominant 5′ UTR lengths that were observed started 440 and 368 base pairs upstream of the start codon. (B) 3′ RACE using murine *Mc3r* mRNA and *Mc3r* specific primers. The predominant 3′ UTR observed was 1286 bases downstream of the translational stop codon. A 787 base pair splice from 171 bases to 958 bases downstream of the stop codon was observed. (C) 5′ RLM-RACE using human poly-A *MC3R* mRNA and *MC3R* specific primers. The predominant 5′ UTR length observed started 533 base pairs upstream of the start codon. A 248 base splice was observed in all the clones from 140 to 388 bases upstream of the start codon. (D) 3′ RACE using human poly-A *MC3R* mRNA and *MC3R-specific* primers. The predominant 3′ UTR observed was at 116 and 160 bases downstream of the translational stop codon.

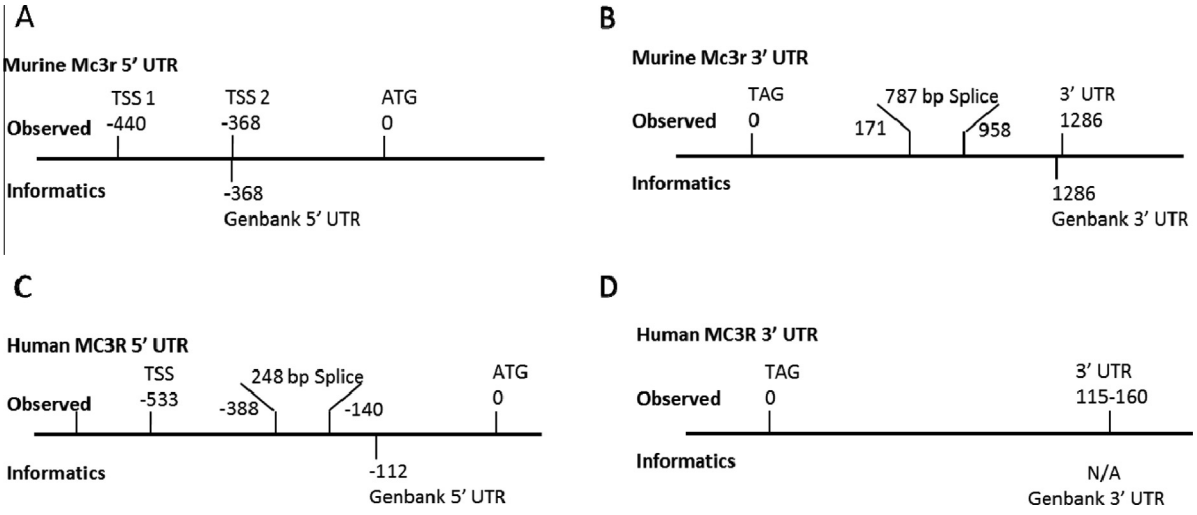

Fig. 3. Transcript structure for murine *Mc3r* and human *MC3R*. Current findings in comparison to available bioinformatics results. (A) Murine *Mc3r* 5′ UTR transcript structure. Bioinformatics suggested a single TSS 368 bases upstream of the start of gene translation; TSS's were observed at 368 and 440 bases upstream of the start of gene translation. (B) Murine *Mc3r* 3′ UTR. Bioinformatics suggested the 3′ UTR terminates 1286 bases after the translational stop codon. The 3′ UTR terminated 1286 bases after the translational stop codon, with a previously unknown 787 base pair splice from 171 bases to 958 bases downstream of the murine *Mc3r* stop codon. (C) Human *MC3R* 5′ UTR. Bioinformatics indicated a single TSS 112 bases upstream of the start of gene translation. The TSS was found 527–544 bases upstream of the start of *MC3R* gene translation and the 5′UTR contains a 248 base splice from 140 to 388 bases upstream of the human *MC3R* start codon. (D) Human *MC3R* 3′ *UTR*. There was no predicted 3′ *UTR* by bioinformatics. The 3′ UTR terminated 115–160 bases after the translational stop codon. N/A: no available bioinformatic information.

downstream of the stop codon was also identified. The score for this splice donor site was 0.989, which corresponds to greater than 99% specificity. This donor site had the highest score among potential donor sites within the 3′ UTR. The score for the splice acceptor site was 0.678, which corresponds to greater than 99% specificity. This acceptor site also had the highest score among potential acceptor sites within the 3′ UTR.

3.4. Human MC3R 3′ RACE

There was greater difficulty obtaining PCR products containing the 3′ *MC3R* UTR sequence compared to 3′ *Mc3r*, 5′ *MC3R*, and 5′ *Mc3r* sequences. After multiple redesigns of primers, varying the amount of RNA in reactions, and studying several sets of PCR conditions, we were able to obtain a total of 97 PCR products from four independent 3′ RACE experiments that contained *MC3R* sequence. 33 sequences terminated 135–160 bases after the translational start codon, of which 30 sequences terminated 158–160 bases downstream. Another 49 sequences terminated 112 to 116 bases after the translational stop codon, and 8 sequences terminated between 69 and 91 bases after the stop codon (Figs. 2D and 3D). However, we attributed the 69–91 base sizes to nonspecific polyT primer binding because of the presence of several consecutive adenine bases within the *MC3R* sequence at these locations.

3.4.1. Human MC3R Poly(A) Signal Analysis

Sequence analysis of 3′ RACE clones containing *MC3R* sequence revealed no consensus downstream GT box. Upstream poly-adenylation signals TATAA, AAGAA, and AATATA were found for the sequences that terminated 112–116, and 158–160 bases after the translational stop codon.

4. Discussion

In the present study, we characterized the transcript structure of human *MC3R* and murine *Mc3r*, identifying novel aspects of both 5′ and 3′ transcripts. For the murine *Mc3r* 5′ UTR, bioinformatics analysis suggested a single TSS 368 bases upstream of the start of gene translation. Our results confirmed a TSS 368 bases upstream of the start of gene translation, but also identified a functional TSS 440 bases upstream. For the murine *Mc3r* 3′ UTR, bioinformatics analysis suggested the 3′ UTR terminates 1286 bases after the translational stop codon. Our results confirmed that the 3′ UTR terminates 1286 bases after the translational stop codon, but identified a novel 787 base pair splice from 171 bases to 958 bases downstream of the murine *Mc3r* stop codon. For the human *MC3R* 5′ UTR, bioinformatics indicated a single TSS 112 bases upstream of the start of gene translation. Our results demonstrated that the TSS is found 527–544 bases upstream of the start of *MC3R* gene translation and also that the 5′UTR contains a 248 base splice from 140 to 388 bases upstream of the human *MC3R* start codon. These results were very recently also demonstrated by Park et al. who similarly identified the 3′UTR for the human *MC3R* as terminating 116 bp downstream of the stop codon [11]. Our results confirm that the 3′ UTR is quite short and likely terminates only 115–160 bases after the translational stop codon. This observation is not unique; other genes have also demonstrated short 3′ UTRs [17–19]. Additional studies may be needed to delineate the human *MC3R* 3′ UTR, as we obtained fewer 3′ MC3R sequences than for the other UTRs studied, which limited our ability to define dominant UTR sizes.

These results represent a detailed analysis of the transcript structure of the human *MC3R* and murine *Mc3r*. Our data suggest the possibility of transcript heterogeneity for both human *MC3R* and murine *Mc3r* that could conceivably enable tissue-specific gene regulation [12]. Further studies are needed to evaluate which of the two murine *Mc3r* transcripts is preferentially expressed in the hypothalamus and elsewhere and determine if there is tissue-specific regulation of mouse *Mc3r* transcript distribution.

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