JBCR Journal of Biomedical & Clinical Research
Journal of Medical University of Pleven

Journal of Biomedical and Clinical Research 17(1): 95–105 (2024)
DOI: 10.3897/jbcr.e121803

Research Article

Design of primers and optimization of PCR conditions for the detection of alternatively spliced isoforms of mouse ChAT mRNA

Desislava Marinova[1], Stefan Trifonov[1]

1 *Department of Anatomy, Histology, Cytology and Biology, Medical University - Pleven, Pleven, Bulgaria*

Corresponding author: Stefan Trifonov (stefantrifonov@outlook.com)

Summary

Acetylcholine plays an important role as a neurotransmitter in the central and peripheral nervous systems. Its biosynthesis is catalyzed by choline acetyltransferase (ChAT). In mice, the ChAT gene has three 5'-noncoding (R, N, and M) and 14 coding exons, from which seven mRNA isoforms (M, N1, N2, R1, R2, R3, and R4) are transcribed. They differ in the 5'-noncoding ends and encode the same protein (common ChAT). An additional isoform that lacks coding exons from 5 to 8 encodes a smaller protein (peripheral ChAT). This research aimed to design and check the specificity of primers targeting R3, N1, N2, M, peripheral ChAT, and common ChAT isoforms. The optimal PCR conditions and specificity of the primers were tested in a series of PCR reactions. Specially designed DNA fragments or plasmids containing isoform-specific nucleotide sequences were used as templates. The appropriate annealing temperature, which yields sufficient specificity for each tested primer pair, was as follows: R3 – 60 °C; N1 – 63 °C; N2 – 65 °C; M – 65 °C; peripheral ChAT – 65 °C and common ChAT – 63 °C.

Key words: Choline acetyltransferase, primer, polymerase chain reaction, alternatively spliced isoforms

OPEN ACCESS

Academic editor: Milena Atanasova
Received: 27 February 2024
Accepted: 13 March 2024
Published: 10 June 2024

Citation: Marinova D, Trifonov S (2024) Design of primers and optimization of PCR conditions for the detection of alternatively spliced isoforms of mouse ChAT mRNA. Journal of Biomedical and Clinical Research 17(1): 95–105. https://doi.org/10.3897/jbcr.e121803

Introduction

Most protein isoforms arise from alternative splicing of the primary RNA transcripts. Splicing provides an extensive collection of protein isoforms that display different properties and serve various functions (Graveley 2001; Liu et al. 2022). This way, alternative splicing is one of the main mechanisms to expand biological diversity. As a neurotransmitter, acetylcholine (Ach) has important functions both in the central (Armstrong et al. 1983; Li et al. 2018) and in the peripheral nervous system (Tooyama and Kimura 2000; Bellier and Kimura 2007). Choline acetyltransferase (acetyl-CoA, choline O-acetyltransferase, ChAT), a cytoplasmic enzyme in cholinergic neurons, catalyzes the biosynthesis of Ach from acetyl-coenzyme A and choline. Then, the vesicular acetylcholine transporter (VAChT) translocates Ach into the synaptic vesicles (Brown 2006, 2019). In the brain, ACh is involved in fundamental processes like arousal, sleep, learning, memorizing, cognition, and movement (Obermayer et al. 2017; Solari and Hangya 2018), whereas in the spinal cholinergic motor neurons it is the neu-

rotransmitter at the neuromuscular junction (Mille et al. 2021). In the peripheral nervous system, cholinergic neurons control the functions of visceral organs, and, specifically in the enteric nervous system, they play an essential role in controlling and regulating the functions of the gastrointestinal tract (Furness 2016; Johnson et al. 2018). ChAT serves as a reliable marker of cholinergic neurons, both in the central and in the peripheral nervous system. Morphologic studies and molecular and genetic analysis of ChAT will give us insight into cholinergic neurons' physiological and pathological states.

A single gene encodes the enzyme ChAT in all species studied so far (Wu and Hersh 1994). Seven mRNA isoforms, named M, N1, N2, R1, R2, R3 and R4, are transcribed from the ChAT gene in mice (Misawa et al. 1992) (Fig. 1A). There are five variants in rats (R1, R2, N1, N2, and M) (Kengaku et al. 1993) and six variants in humans (R, N0, N1, N2, M and H) (Misawa et al. 1997; Oda et al. 2004). The gene encoding ChAT in mice has 17 exons. In the 5'-end, there are three noncoding exons (R, N, and M), followed by 14 successive coding exons (Ishii et al. 1990; Misawa et al. 1992) (Fig. 1A, B). The entire gene for VAChT is located in the first intron of the ChAT gene locus between R and N noncoding exons. Exon R consists of two parts (Ra and Rb) that are alternatively spliced in R1, R2, R3, and R4 ChAT mRNA splicing isoforms (Fig. 1A). The third M noncoding exon is located between the N exon and the first *N*-terminal coding ChAT exon (Fig. 1A). It contains an initial alternatively spliced portion (Ma) of about 30 nucleotides, which is unique only for isoform M. All these alternative splicing isoforms differ from each other in their 5'-noncoding ends and share the same coding regions, so they produce the same protein with molecular weight of 67 kDa, known as common ChAT (cChAT). The function of the noncoding exons is unclear. They probably provide different stability, translational efficiency, compartmentalization, and three-dimensional structures for the corresponding ChAT mRNA isoforms. The existence of several different promoters increases the possibility that different ChAT mRNA isoforms might be expressed in different types of cholinergic neurons and non-neuronal cells. A variant of ChAT mRNA with spliced coding exons from 5 to 8 was also described in rats (Tooyama and Kimura 2000; Bellier and Kimura 2007) (Fig. 1B). Splicing preserves the reading frame because the number of deleted nucleotides is an exact multiple of 3. This alternatively spliced mRNA encodes a protein with a molecular weight of 49 kDa. It is expressed predominantly in peripheral nerve cells and fibers and was named ChAT of a peripheral type (pChAT) (Nakajima et al. 2000; Chiocchetti et al. 2003).

Although the structure of the mouse ChAT gene and its regulatory elements have attracted growing scientific interest during the past decades, knowledge about the specific expression patterns of the splicing isoforms of ChAT mRNA and the activity of the different promoters in mouse brain regions and non-neuronal tissue has remained insufficient. Until now, only some ChAT mRNA splicing isoforms have been discriminated by *in situ* hybridization, semiquantitative reverse transcription PCR (Trifonov et al. 2009) and immunohistochemistry (Muroishi et al. 2000). These methods are only qualitative or semi-quantitative, and a truly quantitative and reproducible evaluation of the expression of ChAT mRNA splicing isoforms is still needed.

Due to extreme sequence similarities between different ChAT mRNA isoforms, designing a reliable and robust method for measuring their expression

is challenging. The standard PCR assay and real-time quantitative PCR have many advantages and are attractive techniques for studying the expression of different alternatively spliced transcripts (Too 2003; Camacho Londoño and Philipp 2016). This study aimed to design isoform-specific primers targeting the unique exon-exon junctions of alternatively spliced ChAT mRNA isoforms, test their specificity, and optimize the PCR conditions for detecting them.

Materials and methods

In silico primer design, dilution, and preparation of primer aliquots

Primer design is a crucial initial step in any experiment utilizing PCR to target and amplify a known nucleotide sequence of interest (Chuang et al. 2013). When designing primers, it is important to consider the primer melting temperature, GC content, number of GC clamps and the amplicon length. Properly designed primers will increase PCR amplification efficiency (Bustin and Huggett 2017). We designed forward and reverse primers targeting R3, N1, N2, M, pChAT, and cChAT mRNA splicing isoforms using the Primer-BLAST program (Ye et al. 2012). All primers met the requirements of the default settings, which were modified as follows: melting temperature was calculated to be between 60 °C and 65 °C; GC content of 40% to 60%. The primer pairs used in the PCR analysis are listed in Table 1. Among all R isoforms, the R3 type is the only one containing exons Rb and Mb, so the primers' location was chosen to be

Table 1. Sequence of primers used for PCR.

mRNA for ChAT splicing isoforms (primer name)	Sequence 5` → 3`	Location of primers	Product length (bp)	Annealing temp. (t °C)
R3				
Forward (qRb_Fwd)	TGAGCCTTCCTAAGCCTCTAC	Exon Rb	149	60
Reverse (qMb_Rev1)	CCGCCACCTAGGTTCTGTAG	Exon Mb		
N1				
Forward (qN_Fwd)	ATCCAGGCTCTATCATCTGAGG	Exon N	170	63
Reverse (qMb_Rev1)	CCGCCACCTAGGTTCTGTAG	Exon Mb		
N2				
Forward (qN_Fwd)	ATCCAGGCTCTATCATCTGAGG	Exon N	112	65
Reverse (qN2_Rev)	CAGAGCTGCCGAC**ATCAA**	Exon P1_N		
M				
Forward (qMa_Fwd)	GAGAGGTGTGGCTGGTTTG	Exon Ma	119	65
Reverse (qMb_Rev2)	GCAGCAGTACCAGAGGTTG	Exon Mb		
Peripheral ChAT				
Forward (qPC_Fwd)	TAGCCCTGCTGTGATCTTTG	Exon P3	125	65
Reverse (qPC_Rev)	TGTTGCCTGTCATC**CTGTC**	Exon P9_P4		
Common ChAT				
Forward (qCC_Fwd)	CCAATCCATTCCCACTGACTG	Exon P5	95	63
Reverse (qCC_Rev)	GAAGCCGGTATGATGAGAAGAG	Exon P5		

The five base pairs, crossing the unique exon-exon junction in the reverse primers for N2 and pChAT isoforms are denoted in bold font.

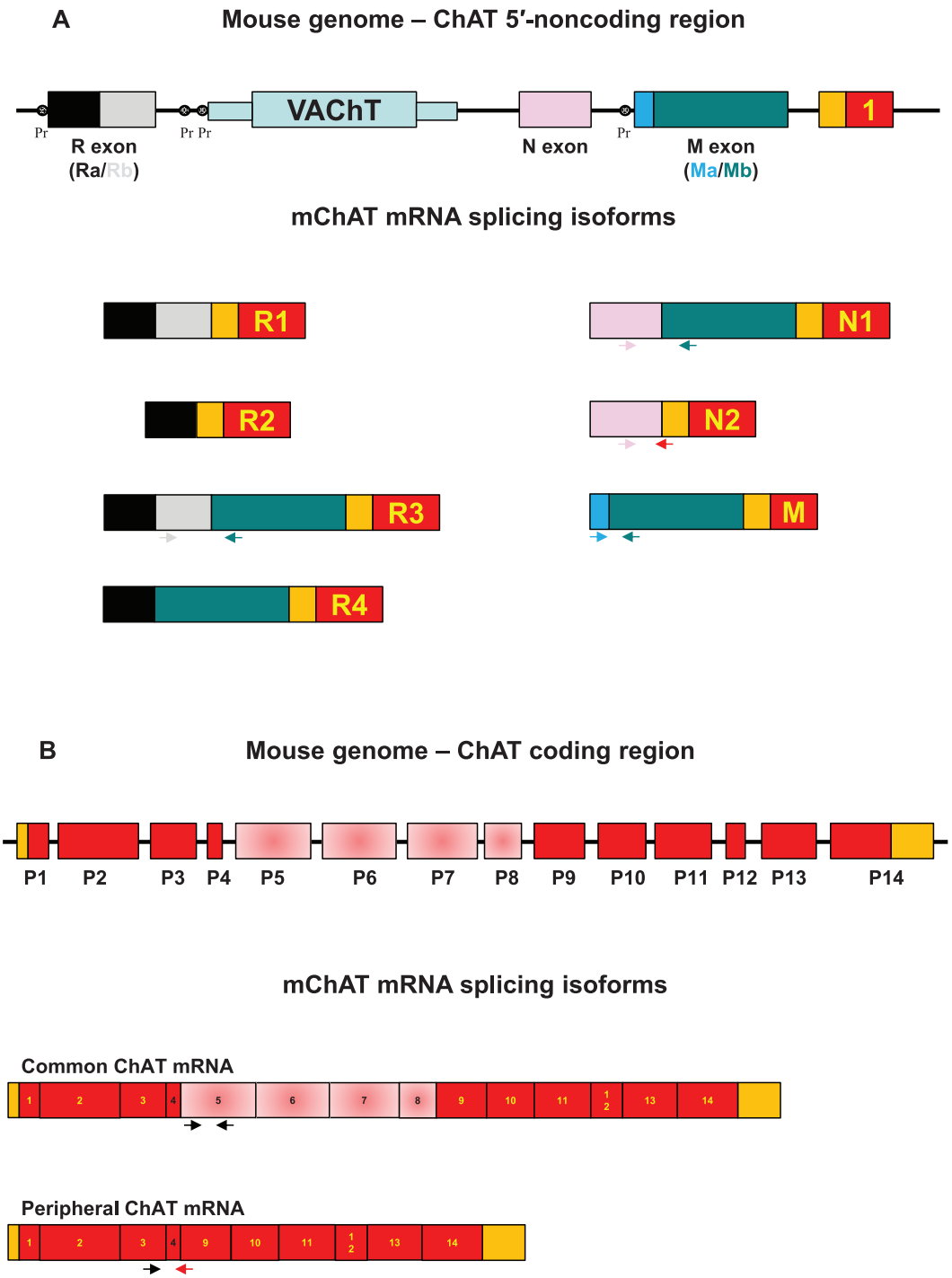

Figure 1. Schematic diagram showing the structure of cholinergic gene locus and the splicing pattern of multiple mRNA isoforms for ChAT. **A.** 5'-noncoding region and isoforms produced by alternative splicing of R, N and M noncoding exons. **B.** Coding region and the two alternatively spliced variants – cChAT and pChAT. Arrows indicate the locations of isoform-specific forward and reverse primers. Pr, promotor region; cChAT, common choline acetyltransferase; pChAT, peripheral choline acetyltransferase.

in these unique exons (Fig. 1A). N1 and N2 ChAT mRNA splicing isoforms are closely related, and the only difference is the presence of exon Mb in the N1 isoform. The forward primer used to detect the N1 and N2 isoforms was common and located in exon N. The reverse primer for isoform N1 was chosen to be in exon Mb, while the reverse primer for N2 isoform encompassed the exon

splice junction of exon P1 and exon N (Vandenbroucke et al. 2001). There were five base pairs at the 3' end of the primer overlapping with the adjacent exon (Exon P1_N, Fig. 1A). Similarly, for the pChAT isoform that lacks a protein-coding exons from P5 to P8, the forward primer was located in exon P3, while the reverse primer encompassed with 5 base pairs the exon splice junction (Exon P9_P4, Fig. 1B). Lyophilized primers (Sigma-Aldrich) were initially resuspended in TE buffer (10 mM Tris pH 8.0, 1 mM EDTA) to a concentration of 100 pmol/µl. The stocks were further diluted in TE buffer to a final concentration of 10 pmol/µl and stored at -20 °C.

Templates used in PCR reactions to test the specificity of isoform-specific primers

De novo designed double-stranded gBlock Gene Fragments (Integrated DNA Technologies) and a plasmid pGEM-T Easy (Promega, Madison, WI) containing isoform-specific nucleotide sequences were used as templates. The gBlock-1 fragment contained the 5'-noncoding region of isoform R3 (exons Rb, Mb, P1, and part of P2) and a portion of exon P7, the whole P8 exon and part of exon P9 (Fig. 2). It was used as a template to amplify R3 isoform using primers qRb_Fwd

Figure 2. The sequence of gBlock-1 fragment contained the 5'-noncoding region of isoform R3 (exons Rb, Mb, P1 and part of P2) and part of exon P7, the whole P8 exon and part of exon P9. The exact location of forward and reverse primers is marked with an arrow. The 5 bases and the 3' end of the primers qN2-Rev and qPC-Rev encompassing the exon splice junction are also indicated.

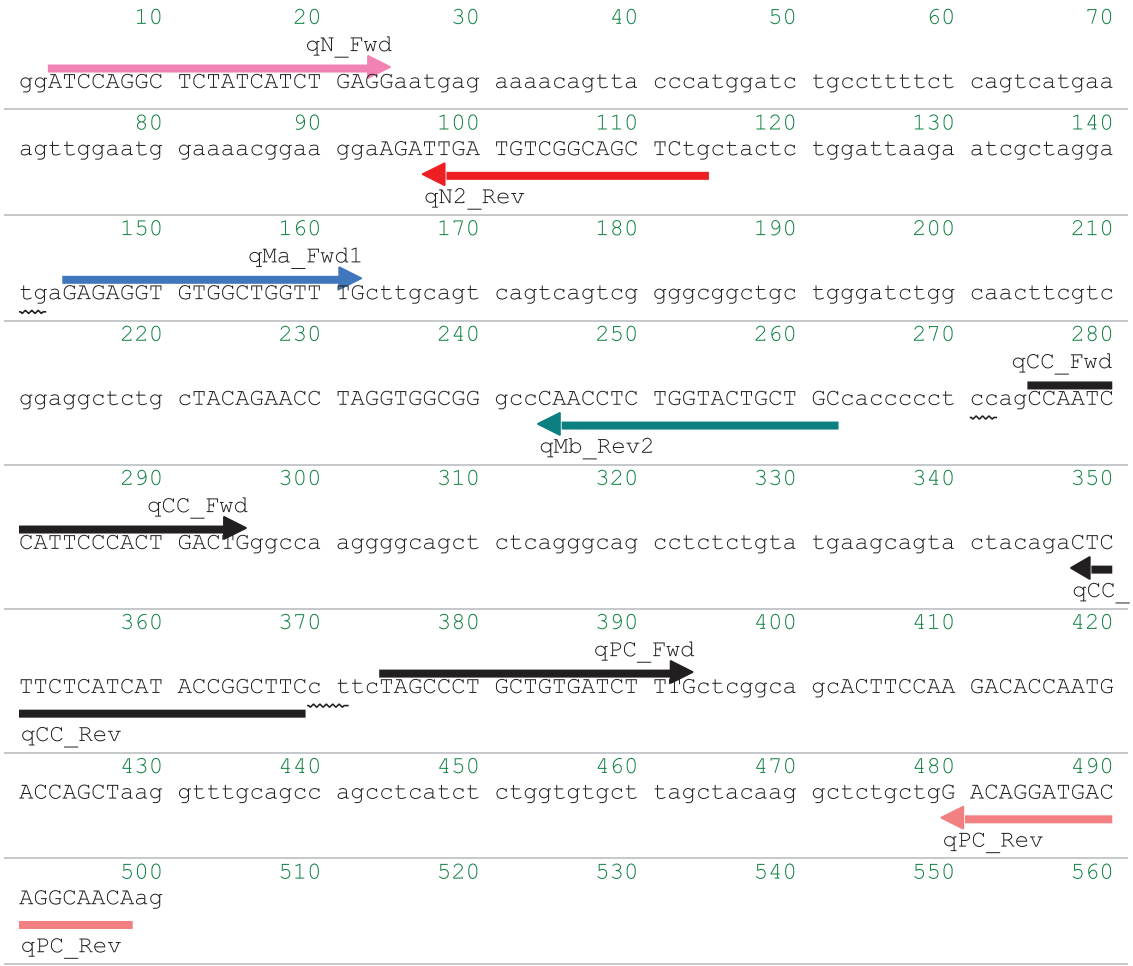

Figure 3. The sequence of gBlock-2 fragment contained specific regions of N2, M, pChAT and cChAT isoforms. The exact location of forward and reverse primers is marked with an arrow.

and qMb_Rev1 (Fig. 2). It was also used to check the specificity of the reverse primer (qN2_Rev) encompassing the exon splice junction between exon P1 and N for isoform N2 with primer qRb_Fwd (expected product length of 280 bp (Fig. 2). Similarly, the gBlock-1 fragment was used to check the specificity of the qPC_Rev primer which encompassed the exon splice junction between exon P9 and exon P4 for isoform pChAT by combining it with qRb_Fwd (expected product length of 618 bp Fig. 2). The gBlock-2 fragment contained sequences specific for N2, M, pChAT and cChAT isoforms (Fig. 3) and was used to test the corresponding primer pairs (Table 1). Plasmid pGEM-N1 containing part of the 5'-non-coding region of isoform N1 was generated in a previous study (Trifonov et al. 2009) and was used to test the specificity of primers amplifying the N1 isoform.

Conventional polymerase chain reaction

PCR was carried out on qTower^3 G Thermal Cycler (Analytik Jena, Germany). For the PCR, 0.25 µM forward and reverse primers, 10 µl 2× Luna Universal qPCR Master Mix (New England BioLabs, USA), and 2 µl of template DNA (10 000 copies for gBlock-1 and gBlock-2 and 20 pg/µl for pGEM-N1) were supplemented with dH_2O to a final volume of 20 µl. The qTower^3 G Thermal Cycler (Analytik Jena) and Luna Universal qPCR Master Mix (New England BioLabs) allowed for

real-time detection to be carried out at the elongation step and the generation of the melt curve after amplification. When using the reverse primers encompassing the exon splice junction (qN2_Rev and qPC_Rev), PCR conditions had to be optimized by adding 3% dimethyl sulfoxide (DMSO) to achieve complete specificity. DMSO stabilizes secondary DNA structures and prevents nonspecific primer annealing. The following PCR protocol was applied: 95 °C for 60 sec, followed by 40 cycles of 95 °C for 15 sec; 60, 63, or 65 °C (primer pair specific; see Table 1) for 10 sec; 60 °C for 30 sec. All PCR products were visualized on a 2% agarose gel stained with ethidium bromide.

Results and discussion

We managed to amplify the fragments with expected size for R3 (149 bp), N1 (170 bp), N2 (112 bp), M (119 bp), pChAT (125 bp) and cChAT (95 bp) isoforms of ChAT mRNA using the isoform-specific primers and the corresponding templates (Table 1 and Fig. 4A). It was possible to design unique primer pairs for R3, N1, M and cChAT mRNA isoforms because they have at least one unique exon. N2 and pChAT isoforms lacked such exons, so another previously used design strategy was implemented (Trifonov et al. 2014a). An isoform-specific reverse primer with 5 base pairs crossing the unique exon junction of each isoform was used (Table 1 and Fig. 4A).

Due to the extreme sequence similarities between different ChAT mRNA isoforms, designing a reliable and robust method for measuring their expression is challenging. The PCR has many advantages and is an attractive technique for studying the expression of different splicing isoforms (Too 2003; Ohkawara et al. 2010). In conventional and quantitative PCR, three approaches can be implemented to quantify the expression levels of different ChAT splicing isoforms: 1) specific primer or primer pair for variants having unique exon or nucleotide sequence; 2) boundary-spanning primer hybridizing to the sequence encompassing the exon-exon junction with the opposing primer in a common exon (Vandenbroucke et al. 2001; Wong et al. 2002); 3) boundary spanning probe encompassing the exon splice junction and common primers (Vandenbroucke et al. 2001; Wong et al. 2002). The use of common primers and unique boundary-spanning probes has been repeatedly reported as unreliable because co-amplification of other variants impairs correct quantification at a very low level of the alternative transcript (Vandenbroucke et al. 2001; Too 2003). When a specific primer or a primer pair for a dedicated splicing isoform cannot be designed, the use of boundary-spanning primer is recommended as a method of choice, especially if the difference in isoform abundance is substantial (Vandenbroucke et al. 2001; Trifonov et al. 2014).

The appropriate annealing temperature, which yields sufficient specificity for each tested primer pair, was as follows: R3 – 60 °C; N1 – 63 °C; N2 – 65 °C; M – 65 °C; common ChAT – 63 °C and peripheral ChAT – 65 °C. The optimal annealing temperature is the range of temperatures where the efficiency of PCR amplification is maximal without nonspecific products. Optimization of the primer annealing involves lowering the reaction temperature to make it possible for the primers to bind to their complementary DNA. The optimal annealing temperature depends on the PCR results. In the case of lack of amplification or low quantity of the amplified product, the annealing temperature should be

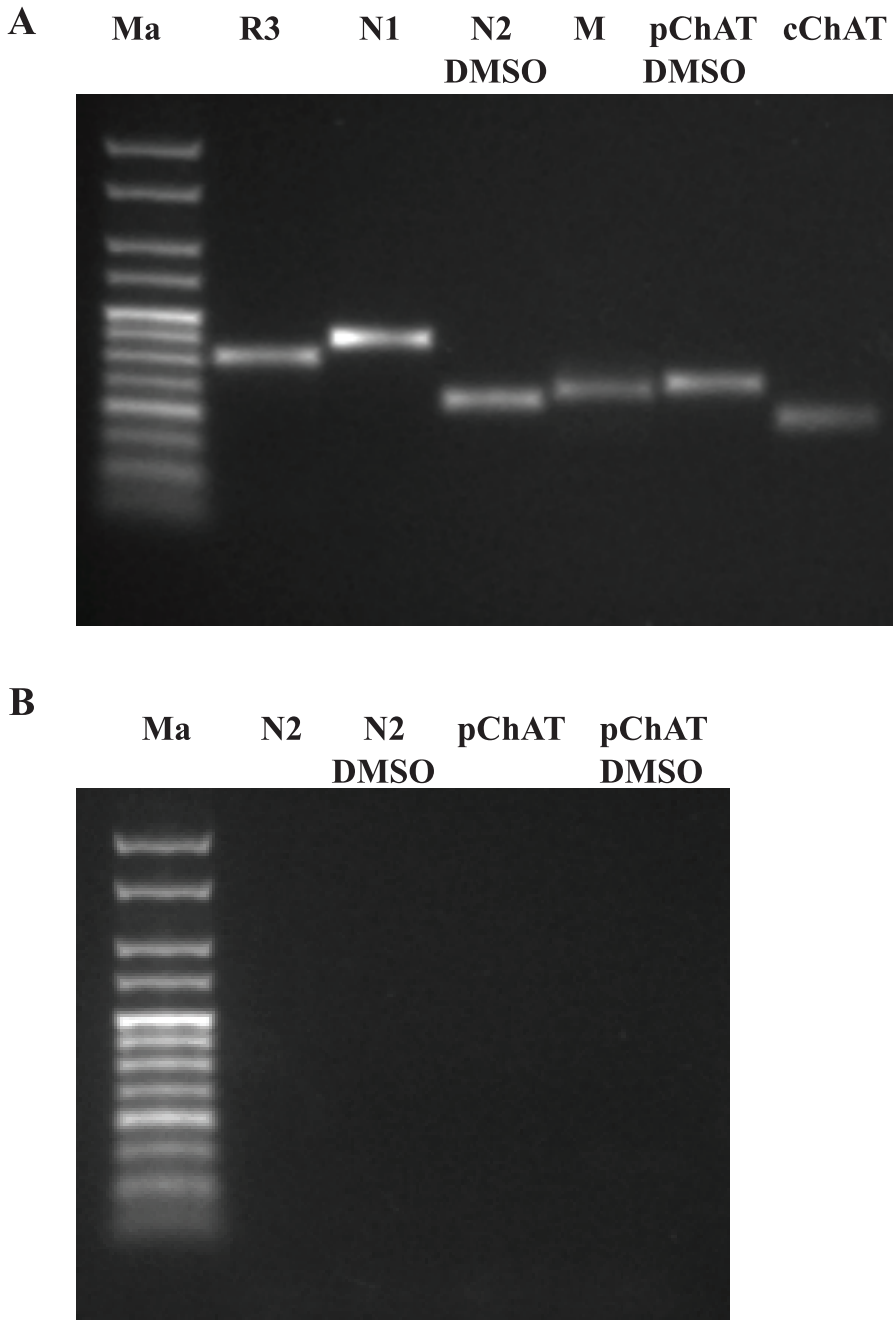

Figure 4. A. Gel electrophoresis of amplified fragments with expected size for R3 (149 bp), N1 (170 bp), N2 (112 bp), M (119 bp), pChAT (125 bp) and cChAT (95 bp) isoforms of ChAT mRNA using the isoform-specific primers and the corresponding templates (gBlock-1 for R3 isoform; gBlock-2 for N2, M, pChAT and cChAT isoforms; pGEM-N1 for N1 isoform). **B.** Gel electrophoresis of control reaction of amplification with primer qRb-Fwd, isoform-specific reverse primers for N2 and pChAT and gBlock-1 as a template, with or without DMSO. Ma, DNA ladder marker HyperLadder 25 bp (Bioline); DMSO, dimethyl sulfoxide.

lowered in increments of 2–3 °C. When nonspecific amplification products exist, the annealing temperature should be raised in increments of 2–3 °C (Rychlik et al. 1990). Due to the homology at the 3' end of exon N and Mb for isoform N2 and exon P4 and P8 for isoform pChAT, the annealing temperature had to be increased to 65 °C. This temperature resulted in a lack of nonspecific amplification in the control reaction with primer qRb-Fwd and gBlock-1 as a template, as detected on 2% agarose gel (Fig. 4B). When observing the amplification in re-

al-time, in the control reactions, there was an increase in the fluorescence signal in the last several cycles. Also, a small second peak, along with the desired one, was noticeable in the melt curve analysis, which suggested some nonspecific binding of the primer. DMSO (3%) was added to these reactions, which yielded complete specificity without interfering with the amplification (Fig. 4A, B).

Alternative mRNA splicing is widely used to produce structurally and functionally diverse proteins from one gene (Lopez 1998). According to Croft et al. (Croft et al. 2000), more than 35% of human genes express variably spliced products. Expression levels of these transcripts in different cells, tissues, and organs are often unknown. Hence, the primers for R3, N1, N2, M, pChAT, and cChAT mRNA isoforms developed in this study can be used to quantify these isoforms in different mouse tissues and organs.

Conclusion

PCR is fundamental to molecular biology and a key practical molecular technique for clinical laboratory investigations. However, the method's efficiency depends on identifying unique primer sequences and designing PCR-efficient primers. In our study, primers specific for R3, N1, N2, M, pChAT, and cChAT mRNA isoforms were successfully designed and tested. Determining the expression levels of ChAT mRNA splicing isoforms in adult mouse brains and various mouse non-neuronal tissues might help elucidate their role in the pathogenesis of various diseases.

References

Armstrong DM, Saper CB, Levey AI, Wainer BH, Terry RD (1983) Distribution of cholinergic neurons in rat brain: demonstrated by the immunocytochemical localization of choline acetyltransferase. The Journal of Comparative Neurology 216: 53–68. https://doi.org/10.1002/cne.902160106

Bellier J-P, Kimura H (2007) Acetylcholine synthesis by choline acetyltransferase of a peripheral type as demonstrated in adult rat dorsal root ganglion. Journal of Neurochemistry 101: 1607–1618. https://doi.org/10.1111/j.1471-4159.2007.04458.x

Brown DA (2006) Acetylcholine. British Journal of Pharmacology 147(S1): S120–S126. https://doi.org/10.1038/sj.bjp.0706474

Brown DA (2019) Acetylcholine and cholinergic receptors. Brain and Neuroscience Advances 3: 239821281882050. https://doi.org/10.1177/2398212818820506

Bustin S, Huggett J (2017) qPCR primer design revisited. Biomolecular Detection and Quantification 14: 19–28. https://doi.org/10.1016/j.bdq.2017.11.001

Camacho Londoño J, Philipp SE (2016) A reliable method for quantification of splice variants using RT-qPCR. BMC Molecular Biology 17: 8. https://doi.org/10.1186/s12867-016-0060-1

Chiocchetti R, Poole D, Kimura H, Aimi Y, Robbins H, Castelucci P, Furness J (2003) Evidence that two forms of choline acetyltransferase are differentially expressed in subclasses of enteric neurons. Cell and Tissue Research 311: 11–22. https://doi.org/10.1007/s00441-002-0652-6

Chuang L-Y, Cheng Y-H, Yang C-H (2013) Specific primer design for the polymerase chain reaction. Biotechnology Letters 35: 1541–1549. https://doi.org/10.1007/s10529-013-1249-8

Croft L, Schandorff S, Clark F, Burrage K, Arctander P, Mattick JS (2000) ISIS, the intron information system, reveals the high frequency of alternative splicing in the human genome. Nature Genetics 24: 340–341. https://doi.org/10.1038/74153

Furness JB (2016) Integrated neural and endocrine control of gastrointestinal function. In: Brierley S, Costa M (Eds) The enteric nervous system. Springer International Publishing, Cham, 159–173. https://doi.org/10.1007/978-3-319-27592-5_16

Graveley BR (2001) Alternative splicing: increasing diversity in the proteomic world. Trends in Genetics 17: 100–107. https://doi.org/10.1016/S0168-9525(00)02176-4

Ishii K, Oda Y, Ichikawa T, Deguchi T (1990) Complementary DNAs for choline acetyltransferase from spinal cords of rat and mouse: nucleotide sequences, expression in mammalian cells, and in situ hybridization. Brain Research. Molecular Brain Research 7: 151–159. https://doi.org/10.1016/0169-328X(90)90092-R

Johnson CD, Barlow-Anacker AJ, Pierre JF, Touw K, Erickson CS, Furness JB, Epstein ML, Gosain A (2018) Deletion of choline acetyltransferase in enteric neurons results in postnatal intestinal dysmotility and dysbiosis. The FASEB Journal 32: 4744–4752. https://doi.org/10.1096/fj.201701474RR

Kengaku M, Misawa H, Deguchi T (1993) Multiple mRNA species of choline acetyltransferase from rat spinal cord. Brain Research. Molecular Brain Research 18: 71–76. https://doi.org/10.1016/0169-328X(93)90174-N

Li X, Yu B, Sun Q, Zhang Y, Ren M, Zhang X, Li A, Yuan J, Madisen L, Luo Q, Zeng H, Gong H, Qiu Z (2018) Generation of a whole-brain atlas for the cholinergic system and mesoscopic projectome analysis of basal forebrain cholinergic neurons. Proceedings of the National Academy of Sciences 115: 415–420. https://doi.org/10.1073/pnas.1703601115

Liu Q, Fang L, Wu C (2022) Alternative splicing and isoforms: from mechanisms to diseases. Genes 13: 401. https://doi.org/10.3390/genes13030401

Lopez AJ (1998) Alternative splicing of pre-mRNA: developmental consequences and mechanisms of regulation. Annual Review of Genetics 32: 279–305. https://doi.org/10.1146/annurev.genet.32.1.279

Mille T, Quilgars C, Cazalets J, Bertrand SS (2021) Acetylcholine and spinal locomotor networks: The insider. Physiological Reports 9(3): e14736. https://doi.org/10.14814/phy2.14736

Misawa H, Ishii K, Deguchi T (1992) Gene expression of mouse choline acetyltransferase. Alternative splicing and identification of a highly active promoter region. Journal of Biological Chemistry 267: 20392–20399. https://doi.org/10.1016/S0021-9258(19)88714-8

Misawa H, Matsuura J, Oda Y, Takahashi R, Deguchi T (1997) Human choline acetyltransferase mRNAs with different 5'-region produce a 69-kDa major translation product. Brain Research. Molecular Brain Research 44: 323–333. https://doi.org/10.1016/S0169-328X(96)00231-8

Muroishi Y, Kasashima S, Nakanishi I, Oda Y (2000) Immunohistochemical and in situ hybridization studies of choline acetyltransferase in large motor neurons of the human spinal cord. Histology and Histopathology 15: 689–696. https://doi.org/10.14670/HH-15.689

Nakajima K, Tooyama I, Yasuhara O, Aimi Y, Kimura H (2000) Immunohistochemical demonstration of choline acetyltransferase of a peripheral type (pChAT) in the enteric nervous system of rats. Journal of Chemical Neuroanatomy 18: 31–40. https://doi.org/10.1016/S0891-0618(99)00058-7

Obermayer J, Verhoog MB, Luchicchi A, Mansvelder HD (2017) Cholinergic modulation of cortical microcircuits is layer-specific: evidence from rodent, monkey and human brain. Frontiers in Neural Circuits 11: 100. https://doi.org/10.3389/fncir.2017.00100

Oda Y, Muroishi Y, Misawa H, Suzuki S (2004) Comparative study of gene expression of cholinergic system-related molecules in the human spinal cord and term placenta. Neuroscience 128: 39–49. https://doi.org/10.1016/j.neuroscience.2004.06.002

Ohkawara S, Tanaka-Kagawa T, Furukawa Y, Nishimura T, Jinno H (2010) Development of a SYBR Green real-time polymerase chain reaction assay for quantitative detection of human N-methyl-D-aspartate receptors subtype 1 splice variants. Journal of Health Science 56: 527–533. https://doi.org/10.1248/jhs.56.527

Rychlik W, Spencer WJ, Rhoads RE (1990) Optimization of the annealing temperature for DNA amplification in vitro. Nucleic Acids Research 18(21): 6409–6412. https://doi.org/10.1093/nar/18.21.6409

Solari N, Hangya B (2018) Cholinergic modulation of spatial learning, memory and navigation. European Journal of Neuroscience 48: 2199–2230. https://doi.org/10.1111/ejn.14089

Too H-P (2003) Real time PCR quantification of GFRalpha-2 alternatively spliced isoforms in murine brain and peripheral tissues. Brain Research. Molecular Brain Research 114: 146–153. https://doi.org/10.1016/S0169-328X(03)00169-4

Tooyama I, Kimura H (2000) A protein encoded by an alternative splice variant of choline acetyltransferase mRNA is localized preferentially in peripheral nerve cells and fibers. Journal of Chemical Neuroanatomy 17: 217–226. https://doi.org/10.1016/S0891-0618(99)00043-5

Trifonov S, Houtani T, Hamada S, Kase M, Maruyama M, Sugimoto T (2009) In situ hybridization study of the distribution of choline acetyltransferase mRNA and its splice variants in the mouse brain and spinal cord. Neuroscience 159: 344–357. https://doi.org/10.1016/j.neuroscience.2008.12.054

Trifonov S, Yamashita Y, Kase M, Maruyama M, Sugimoto T (2014) Glutamic acid decarboxylase 1 alternative splicing isoforms: characterization, expression and quantification in the mouse brain. BMC Neuroscience 15: 114. https://doi.org/10.1186/1471-2202-15-114

Vandenbroucke II, Vandesompele J, Paepe AD, Messiaen L (2001) Quantification of splice variants using real-time PCR. Nucleic Acids Research 29: e68. https://doi.org/10.1093/nar/29.13.e68

Wong Y, Sia G, Too H (2002) Quantification of mouse glial cell-line derived neurotrophic factor family receptor alpha 2 alternatively spliced isoforms by real time detection PCR using SYBR Green I. Neuroscience Letters 320: 141–145. https://doi.org/10.1016/S0304-3940(01)02282-0

Wu D, Hersh LB (1994) Choline acetyltransferase: celebrating its fiftieth year. Journal of Neurochemistry 62: 1653–1663. https://doi.org/10.1046/j.1471-4159.1994.62051653.x

Ye J, Coulouris G, Zaretskaya I, Cutcutache I, Rozen S, Madden TL (2012) Primer-BLAST: a tool to design target-specific primers for polymerase chain reaction. BMC Bioinformatics 13: 134. https://doi.org/10.1186/1471-2105-13-134