Preparation and Application of Digoxigenin-Labeled Ucp2 Gene RNA Probe: Postprint

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Date: 2018-07-11T00:00:00+00:00

Abstract

Objective: To prepare digoxigenin-labeled specific RNA probes for detecting Ucp2 gene expression in early mouse embryos. Methods: Total RNA was extracted from mouse embryonic brain tissue, primers were designed, and the Ucp2 gene fragment was obtained by RT-PCR and cloned into the pGEM-T vector. Using Sp6, T7, and Ucp2-specific primers, transcription templates were obtained by PCR amplification, and digoxigenin-labeled sense and antisense Ucp2 RNA in situ hybridization probes were generated via Sp6 and T7 RNA polymerases. After detecting the titer of the labeled probes, the specificity and hybridization efficiency of the prepared probes were analyzed by whole embryo in situ hybridization. Results: Sense and antisense probes for the Ucp2 gene were successfully obtained. The antisense probe could efficiently and sensitively detect high expression of the Ucp2 gene in the nervous system of mouse embryos at E9.5 and E10.5, while the sense probe failed to detect any expression signals. Conclusion: Specific and efficient digoxigenin-labeled Ucp2 RNA in situ hybridization probes were successfully prepared, laying a foundation for further investigation of Ucp2 gene expression in mouse embryonic tissues, particularly its localization in neural tissues.

Full Text

Preamble

Title: The Preparation and Application of the Ucp2 Gene RNA Probe Labeled by Digoxin

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Abstract

Objective: To prepare a digoxigenin-labeled specific RNA probe for detecting Ucp2 gene expression in early mouse embryos. **Methods**: Total RNA was extracted from mouse embryonic brain tissue, and primers were designed to obtain the Ucp2 gene fragment via RT-PCR. The fragment was cloned into the pGEM-T vector. Transcription templates were obtained through PCR amplification using Sp6, T7, and Ucp2-specific primers. Sense and antisense Ucp2 RNA probes labeled with digoxigenin were then synthesized using Sp6 and T7 RNA polymerases. After testing the probe titer, whole embryo in situ hybridization was performed to evaluate probe specificity and hybridization efficiency. **Results**: Sense and antisense Ucp2 probes were successfully obtained. The antisense probe efficiently and sensitively detected high Ucp2 expression in the nervous system of mouse embryos at E9.5 and E10.5, while the sense probe detected no expression signal. **Conclusion**: Specific and efficient digoxigenin-labeled Ucp2 RNA in situ hybridization probes were successfully prepared, laying the foundation for further studies on Ucp2 gene expression in mouse embryonic tissues, particularly in neural tissue localization.

Keywords: Ucp2; RNA probe; whole embryo in situ hybridization

Introduction

Neural tube defects (NTDs) are severe congenital malformations of the central nervous system. In some Chinese provinces, the incidence reaches as high as 19.9‰, representing a major cause of congenital deformities and disabilities in newborns. Neural tube closure is a precisely regulated dynamic process that can be disrupted by environmental or genetic factors, leading to NTDs. Recent studies have demonstrated that diabetes, obesity, and reactive oxygen species are potential risk factors for NTDs, all of which are associated with uncoupling protein 2 (Ucp2), making Ucp2 a candidate gene for NTDs. Both Lupo PJ et al. and our research group have shown that the insertion/deletion polymorphism in the Ucp2 3' -untranslated region may be a risk factor for neural tube defects. However, the specific relationship between Ucp2 and NTDs remains unclear.

To further investigate the relationship between Ucp2 and NTDs and explore the underlying mechanisms, it is essential first to characterize its expression and localization during neural tube development. Nucleic acid probes are widely used for gene detection, and digoxigenin (DIG) has become a common label for nucleic acid probes due to its high sensitivity, specificity, and lack of radioactive hazards. To date, no reports have described DIG-labeled Ucp2 RNA probes. This study utilized RNA polymerase to incorporate DIG-11-dUTP into RNA products during in vitro transcription to prepare DIG-labeled Ucp2 RNA probes. Whole embryo in situ hybridization was employed to detect Ucp2 mRNA expression during neural tube development in mouse embryonic tissues, thereby

validating probe efficacy. The successful preparation of this probe will provide an efficient tool for subsequent Ucp2 gene detection studies.

Materials and Methods

1.1.2 Major Reagents

T7 RNA polymerase and SP6 RNA polymerase were purchased from Promega. DNA/RNA labeling mix (DIG-11-dUTP) was obtained from Roche. PCR Master Mix was purchased from TIANGEN.

1.2.1 Preparation of cDNA Template

Mouse embryonic brain tissue was collected, and total RNA was extracted using Trizol reagent. RNA integrity was assessed by 1% agarose gel electrophoresis. The RNA was reverse-transcribed into cDNA using a reverse transcription kit from TAKARA and stored at -20°C.

1.2.2 Ucp2 Primer Design and RT-PCR

Primers for the Ucp2 probe were designed based on the mouse Ucp2 gene cDNA sequence (http://www.ncbi.nlm.nih.gov/gene/22228): F-UCP2-1: TTGGTTTCAAGGCCACAGAT, R-UCP2-1: GAGATTGGTAGGCAGCCATT; F-UCP2-2: CCAACAGCCACTGTGAAGTT, R-UCP2-2: GCTGCTCATAGGTGACAAACA. The expected length of the probe template amplified by UCP2-F2/R2 was 850 bp.

Nested PCR was performed with two reaction systems. Reaction system one contained 10 l of 2×MasterMix (Transgene), 7 l ultrapure water, 1 l each of forward and reverse primers (F1 and R1, 5 M), and 1 l cDNA. Reaction system two contained 10 l of 2×MasterMix, 7 l ultrapure water, 1 l each of forward and reverse primers (F2 and R2, 5 M), and 1 l of the first-round PCR product. PCR conditions were: 95°C for 3 min, 35 cycles of (95°C for 30 s, 56°C for 30 s, 72°C for 1 min), followed by 72°C for 10 min, and storage at 4°C.

1.2.3 Cloning of Target Fragment into pGEM-T Easy Vector and Transformation

PCR products were ligated into the pGEM-T Easy vector. The ligation reaction consisted of 2.5 l 2×Rapid Ligation Buffer, 0.5 l T4 ligase (TAKARA), 0.5 l pGEM-T Easy vector (Promega), and 1.5 l amplified product, incubated at room temperature for 1 h. The ligation product (5 l) was added to 100 l Trans5α competent *E. coli*, placed on ice for 30 min, heat-shocked at 42°C for 1 min, then placed on ice for 2 min. After adding 125 l LB liquid medium, the cells were shaken for 45 min (37°C, 210 rpm) and plated, then incubated inverted at 37°C for 12-14 h.

1.2.4 Selection and Sequencing of Positive Transformants

Sixteen transformants were selected from each plate for PCR identification. The PCR reaction contained 10 l 2×MasterMix (Transgene), 7 l ultrapure water, 1 l each of UCP2-F2 and UCP2-R2 primers (5 M), and 1 L template. Reaction conditions were: 95°C for 2 min, 35 cycles of (94°C for 30 s, 58°C for 30 s, 72°C for 1 min), followed by 72°C for 10 min, and storage at 4°C. PCR products (4 l) were analyzed by 1% agarose gel electrophoresis. Positive transformants were selected for sequencing verification.

1.2.5 In Vitro Transcription of DIG-Ucp2 RNA Probe

Probe template preparation: Plasmid was extracted from bacterial culture containing the correctly sequenced target fragment using a plasmid mini-prep kit (GENERAY). The plasmid was designated pGEMT-Ucp2, with the insert oriented in the SP6 direction. pGEMT-Ucp2 was used as a template for PCR using 10 l 2×MasterMix (Transgene), 7 l ultrapure water, 1 l each of UCP2-F2 (or UCP2-R2) and T7 (or SP6) primers (5 M), and 1 L template. Reaction conditions were: 95°C for 2 min, 35 cycles of (94°C for 30 s, 58°C for 30 s, 72°C for 1 min), followed by 72°C for 10 min, and storage at 4°C. PCR products were purified using a PCR clean-up kit (Axygen) to collect linearized templates. The elution solvent was nuclease-free ultrapure water, with a final volume of 15 l.

In vitro transcription: Purified templates were used for in vitro synthesis with T7 RNA polymerase using the MAXIscript SP6/T7 kit (Ambion, USA). Following the manufacturer' s instructions, the reaction contained 2 l 10×transcription buffer, 1 l each of 10 mM ATP, CTP, and GTP, 0.6 l 10 mM UTP, 1 l 10 mM DIG-11-UTP, 2 l RNA polymerase mix (SP6/T7), and 11.4 l template DNA. After gentle mixing and centrifugation, the reaction was incubated at 37°C for 1 h. DNase I (1 l, Ambion, USA) was added and incubated at 37°C for 15 min to remove the template. The volume was increased to 50 l with 30 l DEPC-treated water, followed by addition of 5 l nuclease-free 3 M sodium acetate (pH 5.2) and 3 volumes of absolute ethanol. Precipitation was performed overnight at -80°C. After centrifugation at 12,000 g for 20 min at 4°C, the supernatant was removed, the pellet was air-dried in a fume hood, and resuspended in 20 l nuclease-free ultrapure water.

1.2.6 Whole Embryo In Situ Hybridization of Ucp2 Expression at E9.5 and E10.5

Pregnant mice were euthanized to obtain E9.5 and E10.5 embryos, which were fixed overnight in 4% paraformaldehyde (PFA) in PBS at 4°C. Standard procedures were followed as previously described. The control group was hybridized with the sense Ucp2 probe simultaneously with the experimental antisense probe group.

Results

2.1 Identification of Recombinant Plasmid pEGMT-Ucp2

The recombinant plasmid pEGMT-Ucp2 was constructed as a template for synthesizing DIG-Ucp2 RNA probes. Plasmids extracted from transformed bacteria were identified by PCR using UCP2-F2 and UCP2-R2 primers. As shown in [Figure 1: see original paper], lanes 2 and 4 showed positive clones with a single band near 850 bp, consistent with the expected size. The plasmid from the positive clone in lane 2 was selected for sequencing analysis, and BLAST alignment confirmed the correct sequence (see NM_{011671}).

2.2 Preparation of DIG-Ucp2 RNA Probe

Single-stranded antisense and sense Ucp2 RNA probes incorporating DIG-11-dUTP were synthesized in vitro using SP6 and T7 RNA polymerases. After purification, agarose gel electrophoresis demonstrated good probe integrity [Figure 2: see original paper]. The concentration of the DIG-Ucp2 RNA probe was determined to be 1488.03 ng/ l by UV spectrophotometry (OD260/280 = 1.98).

2.3 Whole Embryo In Situ Hybridization Results

Whole embryo in situ hybridization revealed that the antisense probe efficiently detected Ucp2 expression primarily in the forebrain, hindbrain, midbrain, and neural tube development sites of E9.5 and E10.5 embryos. The sense probe detected no Ucp2 mRNA expression signals [Figure 3: see original paper].

Discussion

NTDs are severe congenital malformations of the central nervous system. Elucidating their molecular mechanisms requires first understanding the expression patterns of relevant genes during neural tube closure, making in situ hybridization technology particularly important for NTD research. Whole embryo in situ hybridization differs from conventional hybridization performed on slides with cells or tissue sections by examining intact embryos, allowing observation of probe binding sites in the whole organism and providing more comprehensive spatial information.

Nucleic acid probes were initially labeled with radioactive isotopes, which, despite high sensitivity, specificity, and resolution, suffer from high production costs, short half-lives, and radioactive hazards requiring specialized laboratories and protective measures, limiting their application. Biotin-labeled probes offer high sensitivity but are restricted in biological samples due to endogenous biotin and biotin-binding proteins that cause non-specific binding. DIG-labeled probe detection has become widely used in recent years due to its excellent specificity, high sensitivity, lack of radioactive hazards, simple operation, and stable results.

UCP2 is a member of the mitochondrial uncoupling protein family, a carrier protein located on the inner mitochondrial membrane involved in energy metabolism, reactive oxygen species production, insulin secretion regulation, and fatty acid metabolism. Our group and international colleagues have studied the relationship between the Ucp2 3' -UTR insertion/deletion polymorphism and NTDs in human populations, but the molecular mechanisms of Ucp2 in neural tube defects remain unclear. This study used mouse embryos to design and prepare DIG-labeled Ucp2 RNA probes. Based on the full-length Ucp2 mRNA nucleotide sequence, primers were designed to obtain a Ucp2 cDNA fragment by RT-PCR. The recombinant plasmid pEGMT-Ucp2 was successfully constructed as a template for in vitro transcription of Ucp2 RNA probes, with correct sequence confirmed by PCR and sequencing. Using the in vitro transcription system, sense and antisense DIG-Ucp2 RNA probes incorporating DIG-11-dUTP were generated via SP6 and T7 RNA polymerases. The antisense probe demonstrated high specificity and strong signals, efficiently detecting high Ucp2 expression in the forebrain, hindbrain, midbrain, and neural tube during the critical neural tube development stages of E9.5 and E10.5. The sense probe showed no binding and served as an effective negative control, consistent with published literature.

This study successfully prepared DIG-Ucp2 RNA probes using digoxigenin-labeled nucleic acid probe technology, effectively detecting high Ucp2 expression in the forebrain, hindbrain, midbrain, and neural tube during critical embryonic neural tube development stages. This provides a powerful tool for further investigation of Ucp2' s role in the mechanisms underlying NTDs.

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Note: Figure translations are in progress. See original paper for figures.