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Novel alternative splice variants of chicken *NPAS3* are expressed in the developing central nervous system

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ABSTRACT

We report isolation of novel splice variants of chicken *Neuronal Per-Arnt–Sim domain protein 3* (*cNPAS3*) gene distinct from the previously predicted *cNPAS3* at the 5′ end. Newly identified *cNPAS3* splice variants feature N-terminus coding sequences with high degrees of homology to human *NPAS3* (*hNAPS3*). We also show that the alternative splicing pattern of *NPAS3* is conserved between chicken and human. RNA *in situ* hybridization indicated that the expression of *cNPAS3* in the developing central nervous system (CNS) is limited to the ventricular zone and only partially overlaps with that of chicken *Reelin* (*cReelin*), the only known regulatory target gene of *NPAS3* in the adult brain. Overexpression of *cNPAS3* by *in ovo* electroporation had little effect on the expression of *Sox2*, a marker for neural precursors, or of Isl1/2, a marker for early differentiating motor neurons. Taken together with the little effect of *cNPAS3* overexpression on *cReelin*, it is noted that the function of *NPAS3* in the developing CNS remains to be determined. Still, identification of proper cDNA sequences for *cNPAS3* should represent a solid beginning of the understanding process.

1. Introduction

Neuronal PAS domain protein 3 (*NPAS3*) gene encodes a basic helix–loop–helix (bHLH) transcription factor (Brunskill et al., 1999). The bHLH subfamily to which *NPAS3* belongs is defined by the presence of PAS (Per–Arnt–Sim) domain, which functions in diverse physiological contexts including environmental adaptation to hypoxia and circadian regulation (McIntosh et al., 2010). *NPAS3* appears to be orthologous to *Trachealess* of *Drosophila melanogaster* and accordingly functions in lung development in vertebrates but is also expressed highly in the brain (Brunskill et al., 1999; Shin et al., 2010; Zhou et al., 2009). Much attention has been brought to *NPAS3* after the discovery of a translocation between chromosomes 9 and 14 in a familiar schizophrenia case that resulted in disruption of *NPAS3* (Kamnasaran et al., 2003). Several genome wide association analyses have since supported the association of *NPAS3* with pathogenesis of several neurological disorders (Huang et al., 2010; Kempermann et al., 2008; Pickard et al., 2006). Behavioral and anatomical analyses of gene-targeted mouse models also provided evidences consistent with such hypotheses. Specifically, Erbel-Sieler and coworkers showed that mice having compound mutations of *NPAS1* and *NPAS3* display behavioral and neuroanatomical abnormalities seen in schizophrenia (Erbel-Sieler et al., 2004). *NPAS3* deficient mice show reduced *Reelin* expression and decreased adult neurogenesis in hippocampal dentate gyrus which are also seen in schizophrenia brains (Brunskill et al., 2005; Grayson et al., 2005; Pieper et al., 2005).

Although *NPAS3* is expressed as early as embryonic day 9 in the CNS of mouse embryo, functional analysis during early embryogenesis has been limited (Brunskill et al., 1999). This may be in large part due to that anatomical alterations are subtle and that *NPAS3* deficient mice are essentially viable (Brunskill et al., 1999; Erbel-Sieler et al., 2004). Here, we report isolation of three distinct cDNAs representing splice variants of *NPAS3* in chicken (*cNPAS3*), a model system used extensively for gene function analysis during early CNS development. Interestingly, the deduced amino acid sequences are distinct from that of a previously predicted *cNPAS3* (XM_421232.3) at the N-terminus but are highly homologous to amino acid sequences from human splice variants of *NPAS3*. *cNPAS3* is expressed in the ventricular zone of developing CNS in a similar manner to the mouse *NPAS3* (Brunskill et al., 1999). Although ectopic expression of *cNPAS3* showed no detectable effect in the early development of neural tube, isolation of definitive *cNPAS3* should represent the first step for functional analysis using the chicken model.

Abbreviations: NPAS3, Neuronal Per–Arnt–Sim domain protein 3; CNS, central nervous system; *Sox2*, SRY (sex determining region Y)-box 2; Isl1/2, Insulin gene enhancer protein 1/2 (Islet1/2); cDNA, complementary DNA; bHLH, basic helix–loop–helix; RACE, rapid amplification of cDNA ends; HH stage, Hamburger–Hamilton stage; PCR, polymerase chain reaction; S, sense; AS, antisense; RT-PCR, reverse transcription-polymerase chain reaction; IgG, immunoglobulin G; ORF, open reading frame; UTR, untranslated region; PAS, Per–Arnt–Sim.

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2. Materials and methods

2.1. Rapid amplification of cDNA ends (RACE)

5′ RACE was performed using RNA extracted from neural tubes of Hamburger–Hamilton (HH) stage 29 chicken embryos and E11 mouse embryos with GeneRacer™ Kit (Invitrogen) following the manufacturer's protocol. The PCR reactions were performed with PfuUltraII Fusion HS DNA polymerase (Agilent) using GeneRacer™ 5′ Primer and a *cNPAS3*-specific oligonucleotide primer, *cNPAS3*-AS1 and the following first round cycling condition: 94 °C for 5 min, 35 cycles of 94 °C for 30 s, 65 °C for 30 s, 72 °C for 40 s and a final extension step at 72 °C for 7 min. The second and third (when necessary) rounds using GeneRacer™ 5′ Nested Primer and *cNPAS3*-AS2 were carried out for 30 and 25 cycles respectively. Both of the anti-sense *cNPAS3*-specific primers were designed based on the predicted *cNPAS3* sequence (XM_421232.3) and are listed in Table 1. To clone amplified cDNAs in pGEM-T Easy vector (Promega), final 5 PCR cycles were typically performed using GoTaq® DNA Polymerase (Promega). For mouse *NPAS3* (*mNPAS3*), 5′ RACE was performed using *mNPAS3*-specific primers, *mNPAS3*-AS1 and -AS2 (Table 1).

For the 3′ RACE reaction, total RNA extracted from a chicken embryo was reverse-transcribed using GeneRacer™ Oligo dT primer. PCR reactions were carried out for two rounds with PfuUltraII polymerase using two gene-specific sense primers (*cNPAS3*-S1 and -S2; Table 1), two kit-provided anti-sense primers (GeneRacer™ 3′ Primer and GeneRacer™ 3′ Nested Primer) and the following cycling condition: 94 °C for 5 min, 35 cycles of 94 °C for 30 s, 65 °C for 30 s, 72 °C for 2 min and a final extension step at 72 °C for 7 min.

2.2. Isolation of full-length chicken NPAS3 splice variants

Total RNA preparations were obtained from neural tubes of HH stage 29 chicken embryos with TRIzol (Invitrogen). Random hexamer-primed reverse transcription was performed using SuperScript™ First-Strand Synthesis System for RT-PCR (Invitrogen) following the manufacturer's protocol. First-strand cDNA served as templates for PCR amplification, and cloning was performed in two parts: to amplify the 5′-half fragment, the sense primer *cNPAS3*-S3 and the antisense primer *cNPAS3*-AS3 were used, and the PCR product of 5′-half fragment served as templates for the subsequent round of PCR with primers, *cNPAS3*-S4 (containing XhoI site and Kozak-HA sequence) and *cNPAS3*-AS3. To clone the 3′ half, cNPAS3-S5 and cNPAS3-AS4 (containing BglII site) were used as primers. All PCR products were cloned in pGEM-T Easy vector for sequence confirmation and subsequent cloning. To obtain full-length *cNPAS3* cDNAs, HA-tagged 5′-half fragment was isolated by NcoI digestion and inserted into 3′-fragment containing plasmid, likewise NcoI-digested. Expression plasmids were subsequently constructed by isolating XhoI/BglII fragments and inserting them into the pCAGGS vector.

Table 1
Sequences of oligonucleotide primers.

Primers	Sequences (5′ → 3′)
cNPAS3-AS1	CTTCTCCTGCGTTGGGCACCTTATAC
cNPAS3-AS2	CAACAACTTTGCCAACTCTATGAATTC
cNPAS3-S1	CTGGCACTATTCGGTATGCTCCAG
cNPAS3-S2	GGAATGTGTTCACAACTGCCGAAG
cNPAS3-S3	AGCCCGTCTGGCTAAGCAG
cNPAS3-AS3	TGTCAGATTCAGGGTCTTCACTG
cNPAS3-S4	CTCGAGACCATGTACCCATACGATGTTC CAGATTACGCTATGGCGCCCACCAAGCC
cNPAS3-S5	CAGGCATAGTCACTTGGACTTG
cNPAS3-AS4	AGATCTTCAATCCTCTTTCCGTTCCAG
cNPAS3-S6	AGTTGTTGCCACTGCCTGC
cNPAS3-S7	GATCCTTCCAGGCGAGAACG
cNPAS3-AS5	CATGTGGCAGTCAATCCTGAC
cNPAS3-AS6	GCTTGTAGATTCCACTGGCTCAGG
mNPAS3-AS1	GCTGGTCGTCTCCACTGGCTCAG
mNPAS3-AS2	GGATGATGGATGCCTTGTCGAG

2.3. RNA in situ hybridization

RNA *in situ* hybridization on frozen embryo sections was performed using the methods previously described with minor modifications (Ma et al., 1998). Detailed protocols are available upon request. The probe template for *cNPAS3* was generated by PCR amplification using a pair of primers, *cNPAS3*-S6 and *cNPAS3*-AS5 and cloning the product into pGEM-T Easy vector. To generate probe templates for the splice variants, PCR products amplified with c*NPAS3*-S7 and *cNPAS3*-AS1 (Table 1) were separated by agarose gel electrophoresis and cloned into pGEM-T Easy vector. The probe for *Sox2* was derived from a full length *cSox2* cDNA clone (Bylund et al., 2003). The probe for *cReelin* was designed based on a previous report (Yip et al., 2011).

2.4. In ovo electroporation

Eggs were incubated at 38 °C in an atmosphere of 70% humidity. Approximately 5 μl of expression vectors (0.5 μg/μl) were mixed with trypan blue and electroporated into the neural tube of HH stage 15–16 chicken embryos with five 50 ms pulses at 22 V using a BTX electroporator (Harvard Apparatus). After 24 hr or 48 hr incubation, embryos were isolated and processed for *in situ* hybridization and immunohistochemistry.

2.5. Immunohistochemistry

Primary antibodies and titers used were: rat anti-HA (Roche, 1:500) and mouse anti-Isl1/2 (Developmental Studies Hybridoma Bank, 1:20). Secondary antibodies were goat anti-rat IgG Alexa 488 and goat anti-mouse IgG Alexa 594 (Invitrogen, 1:300). Detailed protocols are available upon request.

3. Results

3.1. Identification of novel alternative splice variants of chicken NPAS3

We noted that the N-terminal amino acid sequence of the full-length *cNPAS3* open reading frame (ORF) predicted from a sequence (XM_421232.3) obtained by automated computational analysis was not homologous with that of *hNPAS3* or *mNPAS3*. After multiple attempts to amplify cDNA based on the predicted *cNAPS3* failed, we performed 5′ RACE using cDNA derived from the neural tube of HH stage 29 chicken embryos. Thus isolated three cDNA clones had 5′-UTR nucleotide and deduced N-terminal amino acid sequences that were identical amongst them but were distinct from those specified by XM_421232.3 (Fig. 1A, B). Importantly, the sequence of the first 17 amino acids of the novel cNPAS3 protein from the first exon ORF was identical to that of hNPAS3 protein. On the basis of this result, we isolated three full-length cDNAs representing alternative splice variants (KC294571, KC294572 and KC294573; Fig. 1C). The 3′ end of *cNAPS3* was determined by 3′RACE. The three variants are deduced to encode three distinct proteins each comprised of 878, 895 and 908 amino acids. NPAS3 proteins have bHLH domain and PAS domain, and all three chicken isoforms retain these domains (Fig. 1B). Each of the c*NPAS3* splice variants has a matching human counterpart: *cNPAS3*-1 (KC294571) to *hNPAS3*-4 (NM_001165893.1), *cNPAS3*-2 (KC294572) to *hNPAS3*-3 (NM_173159.2) and *cNPAS3*-3 (KC294573) to *hNPAS3*-1 (NM_001164749.1) (Fig. 1C). We also noted that the N-terminal amino acid sequence of mouse NPAS3 (NP_038808.2 deduced from NM_013780.2) does not match that of human or novel chicken NPAS3 proteins. We thus carried out 5′ RACE and defined a novel m*NPAS3* sequence (KC294574). The predicted first 17 amino acids now matches perfectly with those of human and chicken