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银鲫转录调控因子*lbh-b* cDNA克隆与表达分析

李文华[1,2] 汪 洋[1] 李 志[1] 桂建芳[1] 周 莉[1]

(1. 中国科学院水生生物研究所, 淡水生态与生物技术国家重点实验室, 武汉 430072;
2. 华侨大学, 福建省分子医学重点实验室, 厦门 361021)

摘要: *Lbh* (Limb-bud and heart)基因是脊椎动物中高度保守的转录调控因子, 在早期胚胎发育及某些人类疾病的发病过程中发挥着重要作用。我们前期在银鲫(*Carassius gibelio*)垂体转录组中筛选到一个在垂体中大量表达的基因*lbh-b*。为了进一步研究*lbh*基因在银鲫的表达特征, 首先采用RACE方法克隆了银鲫*lbh*基因家族的成员*lbh-b*基因(*Cglbh-b*)。*Cglbh-b*的cDNA全长1526 bp, 开放阅读框549 bp, 共编码182个氨基酸。生物信息学分析表明*CgLbh-b*蛋白与其他脊椎动物的Lbh蛋白同源性在68%以上, 可能也是无序蛋白质家族的成员之一。成体组织RT-PCR分析表明*Cglbh-b*仅在银鲫的垂体、端脑、卵巢及眼睛中表达。不同胚胎发育时期的表达分析表明, 在受精卵至原肠胚中*Cglbh-b*转录产物是以母源形式存在的mRNA, 其合子转录起始于尾芽期。胚胎整体原位杂交结果显示从受精后2d到受精后3d, *Cglbh-b*大量表达于脑和眼睛。此外, 随着卵子成熟*Cglbh-b*在银鲫垂体中的表达上调。这些结果暗示, *Cglbh-b*可能在调控银鲫脑和眼睛的发育以及卵子成熟过程中发挥着重要作用。

关键词: 银鲫; *lbh-b*; 胚胎发育; 卵子成熟

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脊椎动物*Lbh*基因是一个高度保守的编码无序蛋白(Intrinsic disordered protein, IDP)的转录调控因子[1,2], 在早期胚胎发育[3]及某些人类疾病的发病过程中起着重要作用[4]。Lbh最早克隆于小鼠(*Mus musculus*)中, 与其他已知的蛋白家族没有同源性, 并根据它表达于胚胎肢芽和心脏的这种独特的表达模式而命名[3]。从结构上来讲, Lbh没有明显的二级或三级结构, 它是构象可根据转录活性变化而变化的高度无序的蛋白质[1]。人*LBH* cDNA全长为2927 bp, 编码105个氨基酸, 大量表达于胚胎及成体心脏。在哺乳动物细胞中, 过表达人类*LBH*能够激活活化剂蛋白-1(Activator protein-1, AP-1)和血清应答元件(Serum response element, SRE)的表达, 暗示*LBH*在有丝分裂过程中发挥着重要功能[2]。人类*LBH*基因缺陷会导致先天性心脏病, *Lbh*基因的下调表达会直接影响转录因子*Nkx2.5*和*Tbx5*的功能, 从而影响心脏的正常发育[4]。在类风湿性关节炎的滑膜病变过程中, *LBH*也是其中一个候选的致病基因[5]。此外, *LBH*基因在恶性基底细胞样乳腺癌中过量表达[6]。在软骨内成骨发生过程中, *Lbh*可以在某种程度上通过干扰*Runx2*或*VEGF*的表达来调节早期骨化中心的形成[7]。同时, *LBH*还可作为经典Wnt/β-catenin信号通路中的一个直接靶基因来调控正常上皮细胞和肿瘤上皮细胞的发育过程[6]。目前, 研究人员对低等脊椎动物*lbh*基因的功能知之甚少。在斑马鱼和爪蟾中, *lbh*具有介导颅神经嵴细胞迁移的功能[8]。我们在斑马鱼中克隆了表达于脑和眼睛的*lbh*基因家族的新成员*lbh-like*, 敲降*lbh-like*会抑制多种光受体特异基因, 如*opsins*、*gnat*1、*gnat*2和*irbp*的表达, 同时发现它作用于*otx*2基因上

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作者简介: 李文华(1987—), 女, 吉林白城人; 讲师; 主要从事水生生物发育遗传研究。E-mail: liwenhua11211@163.com

通信作者: 周莉, E-mail: zhouli@ihb.ac.cn

游参与调节光受体的分化过程[9]。

银鲫(*Carassius gibelio*)是我国重要的淡水养殖鱼类之一[10], 它是一类有着超过156条染色体的多倍体鱼类[11—13], 在其天然种群中存在着丰富的、遗传异质的克隆系[14, 15]。因其特殊的多重生殖方式[16—19]和性别决定机制[20], 已成为研究发育遗传的独特生物学模型[14]。为了鉴定在鱼类垂体发生及生殖调控过程中起重要作用的新基因, 我们首先进行了银鲫垂体转录组测序, 从转录组测序结果中筛选得到在垂体中大量表达的cDNA片段(Contig_16003), 该基因与斑马鱼*lbh-like*基因同源, 推测该基因可能在银鲫早期胚胎发育及内分泌调控过程中发挥着重要作用。本文根据该cDNA片段, 克隆了其全长cDNA, 并分析了它的进化、结构无序性和表达特征。

1 材料与方法

1.1 实验材料

银鲫饲养于中国科学院水生生物研究所关桥实验基地。2龄银鲫雌性个体的垂体、下丘脑、端脑、中脑、小脑、延髓、脊髓、肝脏、肾脏、脾脏、心脏、肠、皮肤、鳃、卵巢、眼睛以及2龄银鲫雄性个体的精巢组织用于成体组织RT-PCR分析。同时取2龄银鲫雌性个体和雄性个体各4尾的垂体, 用于RT-PCR分析。

在繁殖季节, 对性成熟的银鲫雌性个体腹腔注射促性腺激素释放激素和多巴胺的混合物进行人工催产[19]。每间隔2h, 随机取3条银鲫的垂体, 直至自然排卵。所取垂体用于分析在卵子成熟过程中目的基因的表达变化。

取银鲫受精后发育至8 胞期、64胞期、256胞期、囊胚期、50%外包期、尾芽期、4体节期、15体节期、2d、3d和4d的胚胎用于早期发育时序RT-PCR分析。用于RNA提取的每种组织和每组胚胎所取的样品量分别为30 mg和 30颗胚胎。取4 胞期、64胞期、50%外包期、2d、3d和4d的50—100颗胚胎用于全胚原位杂交实验[21]。

1.2 *Cglbh-b*基因cDNA序列扩增及序列分析

取2龄银鲫雌性个体的垂体提取总RNA构建SMART cDNA文库。总RNA提取、SMART cDNA合成和RACE-PCR参照本实验室方法及SMART cDNA合成试剂盒(Clontech)操作手册[22]。根据转录组测序得到的Contig_16003部分序列设计引物(表 1)。PCR扩增条件为: 95℃预变性1min, 95℃变性15s, 58℃退火30s, 68℃延伸2min, 36个循环。

使用PONDR®VL-XT软件对*CgLbh-b*基因的氨基酸序列进行蛋白质无序化预测分析[23]。从GenBank (http://www.ncbi.nlm.nih.gov/)数据库下载*Lbh*基因家族蛋白序列(表 2), 使用MAFFT软件对序列进行对位排列[24], 并用BioEdit软件进行人工校正[25]。选择Jones-Taylor-Thornton (JTT) 模型, 使用MEGA 6.1软件构建邻接法(Neighbor-Joining, NJ)系统发育树[26], 并进行1000次自展重复。

表 1 引物序列

Tab. 1 Primers sequences

引物 Primer	引物序列(5′—3′, 下划线为T7启动子) Sequence (5′—3′, T7 sequence are underlined)	用途 Usage
5′-RACE-R1	GATGACAAACTGACACTTTCTGTAGGCT	5′RACE-PCR
5′-RACE-R2	CACCCACTGAACTCCTCACGGTCCTTC	5′RACE-PCR
3′-RACE-F1	TTTCCCCTTGCCTTACCGTCTTTATCCT	3′RACE-PCR
3′-RACE-F2	GTGCAGAGCTCCACGCTGTCCATGCT	3′RACE-PCR
5′UPM	AAGCAGTGGTATCAACGCAGAGTAC	5′RACE通用引物
3′UPM	GTGGTATCAACGCAGAGTACTTTTTT	3′RACE通用引物
β-actin F	AGCACGGTATTGTGACTAACTG	内参引物
β-actin R	TCGAACATGATCTGTGTCATC	内参引物
lbh-b F1	AGCCTACAGAAAGTGTCAGTTTGTC	RT-PCR
lbh-b R1	AGGATAAAGACGGTAAGGCAAGG	RT-PCR
lbh-b F2	TTTGTCCCTTTTCTTTGTATTTCTG	荧光定量PCR
lbh-b R2	TATTGCAAAGATGAAATCTGAATGA	荧光定量PCR
Anti *lbh-b* F	AGCATGGACAGCGTGGAGCTC	原位杂交
Anti *lbh-b* R	TAATACGACTCACTATAGGGATGGTAGTGGTCCTGATGCAT	原位杂交
Sense *lbh-b* F	TAATACGACTCACTATAGGGAGCATGGACAGCGTGGAGCTC	原位杂交
Sense *lbh-b*R	ATGGTAGTGGTCCTGATGCAT	原位杂交

表 2　*LBH*基因同源DNA序列

Tab. 2　The homologous DNA sequences of *LBH*

物种 Species	序列号 GenBank accession No.
罗非鱼Tilapia Lbh	XP_003442986
慈鲷科鱼Cichlids Lbh	XP_005934338
河豚Fugu rubripes Lbh	XP_011616972
青鳉Medaka Lbh-like	XP_011488553
古比鱼Guppy Lbh	XP_007576459
鸡Chick Lbh	NP_001026209
非洲爪蟾African clawed toad Lbh	NP_001081507
人Human LBH	NP_112177
小鼠Mouse Lbh	NP_084275
斑马鱼Zebrafish Lbh-a	NP_956814
银鲫Gibel carp Lbh-b	KT_696607
斑马鱼Zebrafish Lbh-like	XP_001336471
古比鱼Guppy Lbh-like	XP_007575999
青鳉Medaka Lbh-like isoform X3	XP_011481185
罗非鱼Tilapia Lbh-like	XP_003441738
慈鲷科鱼Cichlids Lbh-like	XP_005927379
斑马拟丽鱼Zebra mbuna Lbh-like	XP_014265505
鸡Chick Lbh-like	XP_421381
热带爪蟾Western clawed frog Lbh-like isoform X2	XP_002938037
古比鱼Guppy Lbh-like	XP_007574801
比目鱼Southern platfish Lbh-like	XP_014327879
斑马鱼Zebrafish Lbh-like	XP_009305375
慈鲷科鱼Cichlids Lbh-like	XP_005951713
斑马拟丽鱼Zebra mbuna Lbh-like	XP_004554795
罗非鱼Tilapia Lbh-like	XP_005453463
青鳉Medaka Lbh-like isoform X1	XP_004082740
河豚Fugu rubripes Lbh-like	XP_011618576
古比鱼Guppy Lbh-likeisoform X1	XP_007540130
比目鱼Southern platfish Lbh-like	XP_005811327

1.3　RT-PCR和实时荧光定量PCR

用总RNA提取试剂盒(Promega)提取银鲫各组织及各发育时期胚胎的总RNA。用M-MLV逆转录酶(Promega)和随机引物进行逆转录, 具体操作步骤见说明书。RT-PCR和实时荧光定量PCR的操作流程按照Li等[9]描述的方法进行, 所用引物序列见表 1。

1.4　胚胎原位杂交

根据*Cglbh-b*基因的cDNA序列, 设计原位杂交引物(表 1)。在引物的5′端加入T7启动子序列, 然后PCR扩增获得带有T7 启动子的长度为1267 bp的DNA片段。用T7聚合酶(Roche)进行体外转录, 获得地高辛(Digoxigenin)标记的反义和正义RNA探针。胚胎原位杂交的具体操作步骤参见按照Li等[9]和Xiao等[27]描述的方法进行。

2　结果

2.1　*Cglbh-b*基因克隆及序列特征分析

通过RACE PCR扩增得到*Cglbh-b*全长cDNA序列。*Cglbh-b*全长1526 bp (GenBank: KT696607), 其中包含一个174 bp的5′-UTR, 一个803 bp的3′-UTR, 一个549 bp的开放阅读框, 共编码182个氨基酸。同时, 在poly (A) 尾上游20 bp处含有一个多腺苷酸化加尾信号AATAAA。将*Cg*Lbh-b蛋白与已报道的脊椎动物Lbh蛋白进行多重序列比对, 比对结果显示*Cg*Lbh-b与斑马鱼Lbh-like相似性最高, 高达96%。同时, *Cg*Lbh-b蛋白与斑马鱼Lbh-a蛋白(NP_956814)、人LBH蛋白(NP_112177)、小鼠Lbh蛋白(NP_084275)及鸡Lbh蛋白(NP_ 001026209)的相似性分别为73%、68%、68%及71%。

小鼠Lbh是有着无序结构和可变构象蛋白质家族的一员[1]。在本研究中, 我们采用生物信息学技术分析了*Cg*Lbh-b的结构特征。与小鼠Lbh类似, *Cg*Lbh-b含有很多无序残基, 其中包含29% 的带电残基(Arg/Lys/Glu/Asp)、14%极性残基(Ser/Thr)和17%的螺旋断裂残基(Pro/Gly)。此外, Lbh-b蛋白的第78到第155个氨基酸被PONDR® VL-XT软件预测为氨基酸折叠的不稳定区域。上述的结果显示*Cg*Lbh-b可能与小鼠的Lbh类似, 同为无序蛋白质家族的成员。

2.2　硬骨鱼*Lbh*基因家族的进化关系和基因组加倍

为了理清硬骨鱼类*Lbh*基因家族的进化关系, 我们检索获得了一些脊椎动物*Lbh*相关基因, 并构建了系统进化树。进化树的拓扑结构与已知的脊椎动物亲缘关系一致, 脊椎动物*Lbh*基因进化树含有3个分支(图 1)。由于硬骨鱼类在进化过程中存在基因组加倍事件[28, 29], 所以, 硬骨鱼类*Lbh*相关基因比四足动物多, 形成了3个分支。分支A同时包含四足动物和硬骨鱼*Lbh*基因的直系同源基因, 我们将其命名为*Lbh-a*。分支B和分支C分别被命名为*Lbh-b*和*Lbh*-like, 这些序列在硬骨鱼类中是Lbh-a的旁系同源基因。根据该系统树, 我们克隆的斑马鱼Lbh-like[9]应更名为*Lbh-b*。

硬骨鱼类Lbh氨基酸序列多重比对和蛋白结构域分析显示*Lbh*基因具有进化保守性。Lbh-a、Lbh-b和Lbh-like直系同源基因内部的氨基酸平均一致性分别为80.22%、79.24%和59.82%。3个分支两两之间的序列一致性分别为: 53.67% (Lbh-a和Lbh-b)、44.37% (Lbh-a和Lbh-like)和45.52% (Lbh-b和Lbh-like)。与人类和小鼠的*Lbh*基因类似, 鱼类*Lbh-a*和*Lbh-b*基因含有N端疏水区、核定位信号

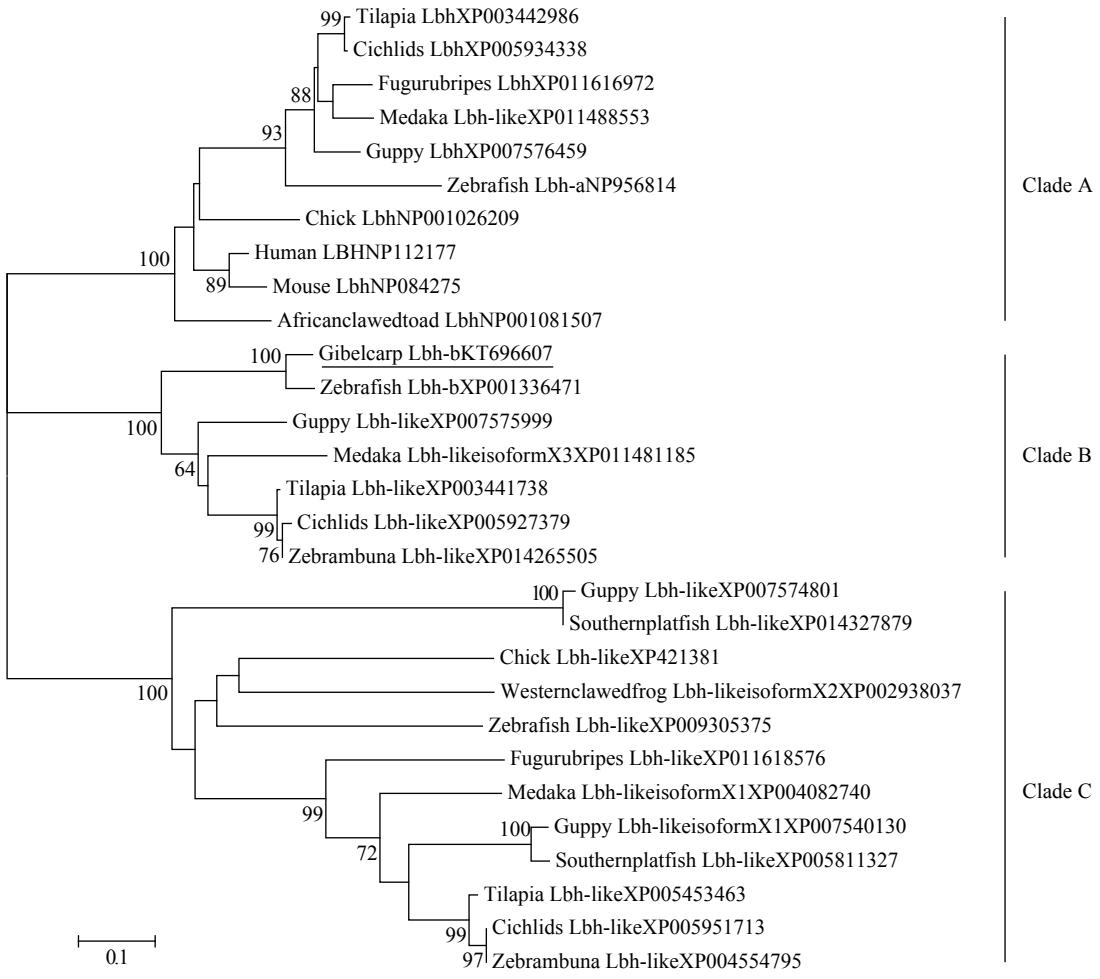

图 1 基于NJ法构建的不同脊椎动物Lbh的系统发育树

Fig. 1 Phylogenetic tree of Lbh in vertebrates based on NJ method

下划线所示为*Cg*Lbh-b, Bootstraps 1000检验各分支置信度, 只显示置信度大于50的分支

*Cg*Lbh-b is underlined. The percentages of replicate trees in the bootstrap test (1000 replicates) are calculated, and values large than 50 are shown next to the branches

(NLS)和富含谷氨酸的C端酸性结构域[9]。但是, Lbh-like基因仅含有富含谷氨酸的C端酸性结构域。

2.3 *Cglbh-b*主要表达于垂体、脑和眼睛中

我们采用RT-PCR方法分析了*Cglbh-b*在银鲫成体组织中的表达情况。与斑马鱼*lbh-b*的表达情况类似[9], *Cglbh-b*也主要表达于垂体、端脑、卵巢及眼睛, 而在所检测的其他组织包括下丘脑、中脑、小脑、延脑、脊髓、肝脏、肾脏、脾脏、心脏、肠、皮肤、鳃和精巢中没有表达(图 2A)。

接着, 为了研究*Cglbh-b*在银鲫早期胚胎发育过程中的表达图式, 我们分别进行了RT-PCR和胚胎原位杂交实验。结果表明, 在4胞期到50%外包期胚胎中, *Cglbh-b*转录产物是以母源形式存在的mRNA(图 2B); 广泛分布于动物极的每个细胞中(图 3A–C)。*Cglbh-b*的合子转录起始于尾芽期 (图 2B)。从受精后2d到受精后3d, *Cglbh-b*大量表达于脑和眼睛中(图 3D、E)。在受精后4d, 杂交信号则主要集中于眼部(图 3F)。

2.4 卵子成熟过程中*Cglbh-b*在垂体中的表达上调

我们采用实时定量PCR方法检测了*Cglbh-b*在银鲫雌雄个体垂体中的表达差异。如图 4A所示, *Cglbh-b*在雌性个体垂体中的表达量显著高于雄性个体(约为4.66倍, *t*-test, P=0.0021)。接下来, 我们分析了在卵子成熟过程中*Cglbh-b*在垂体中表达量的变化。结果如图 4B所示, 在注射催产激素2h后, *Cglbh-b*在垂体中的表达开始上调; 在卵母细胞进入减数分裂中期 I 时(注射催产激素4h后), *Cglbh-b*的表达水平达到最高(上调约3倍), 并一直维持着较高水平的表达; 直到注射催产激素10h后, 这时卵母细胞发育成熟进入第二次减数分裂的中期 II 并开始产卵。*Cglbh-b*的表达水平下调并逐步恢复到未注射催产激素时的表达水平。上述的结果表明

*Cglbh-b*可能在卵子发生和成熟的生殖调控过程中发挥着重要作用。

3　讨论

卵母细胞的减数分裂成熟在卵子发生和产生有育性的卵子等过程中发挥着十分重要的作用[30—32]。高通量转录组测序是发掘调控卵巢成熟过程新基因的有力工具[33, 34]。本研究从银鲫垂体转录组中获得*lbh*基因家族新成员*Cglbh-b*, 通过生物信息学分析揭示了*Cglbh-b*基因的结构无序性及它与其他

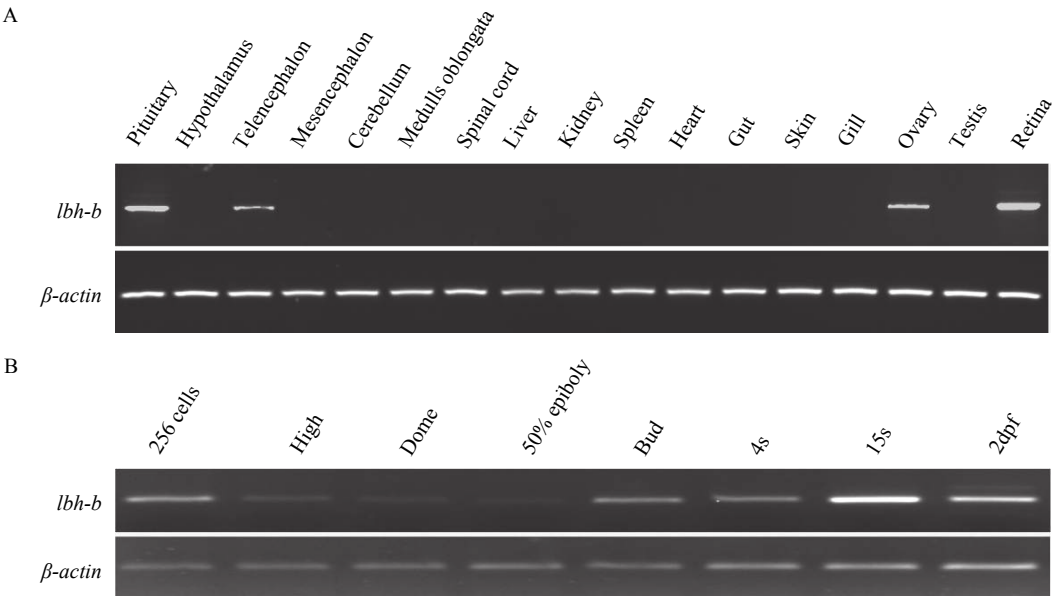

图 2　*Cglbh-b*在银鲫成体组织(A)和胚胎发育过程中(B)的表达分析

Fig. 2　Semi-quantitative RT-PCR detects the *Cglbh-b* expression in adult tissues (A) and in embryos during embryogenesis (B)

*β-actin*作为阳性对照The *β-actin* was used as internal control; pituitary: 垂体, hypothalamus: 下丘脑, telencephalon: 端脑, mesencephalon: 中脑, cerebellum: 小脑, medulla oblongata: 延髓, spinal cord: 脊髓, liver: 肝脏, kidney: 肾脏, spleen: 脾脏, heart: 心脏, gut: 肠道, skin: 皮肤, gill: 鳃, ovary: 卵巢, testis: 精巢, retina: 视网膜, 256 cells: 256胞期, high: 高期, dome: 椭球期, 50% epiboly: 50%外胞期, bud: 尾芽期, 4s: 4体节期, 15s: 15体节期, 2 dpf: 受精后2d

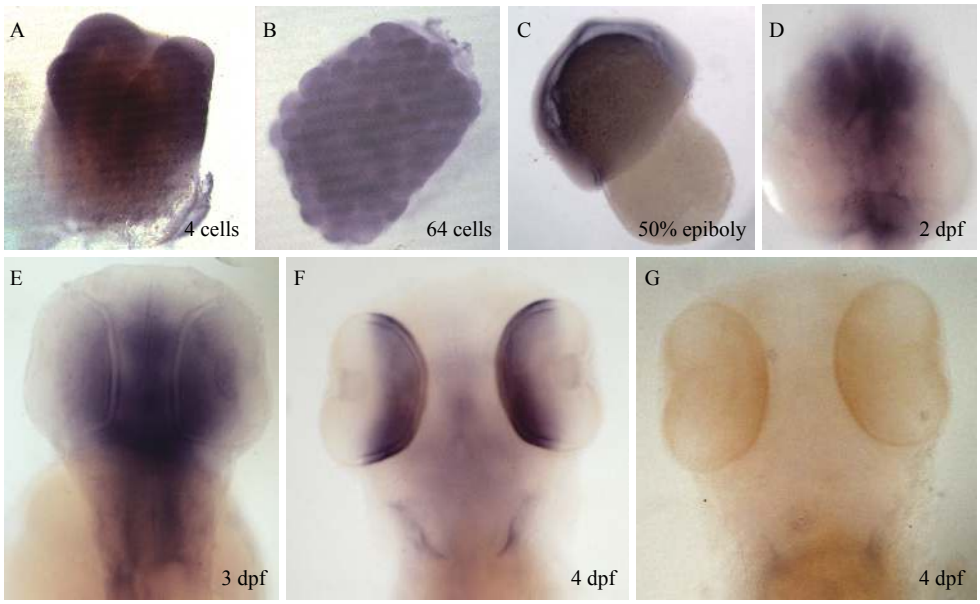

图 3　整体原位杂交检测*Cglbh-b*在银鲫胚胎的时空表达特征

Fig. 3　Whole-mount *in situ* hybridization with *Cglbh-b* antisense probe (A–F) and sense probe (G)

(A–F)反义探针检测信号; (G)正义探针检测信号; 4 cells: 4胞期, 64 cells: 64胞期, 50% epiboly: 50%外胞期, 2 dpf: 受精后2d, 3 dpf: 受精后3d, 4 dpf: 受精后4d

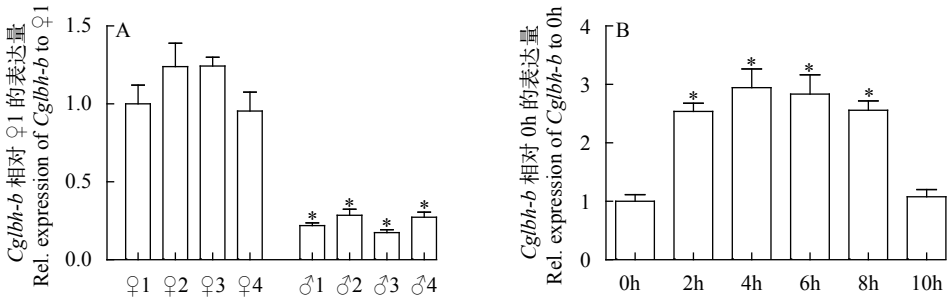

图 4 *Cglbh-b*在银鲫雌雄个体垂体(A)和卵子成熟过程中垂体(B)中的表达分析

Fig. 4 The expression of *Cglbh-b* in pituitaries between female and male individuals (A) or during oocyte maturation (B) by real-time PCR

♀代表雌性个体; ♂代表雄性个体。*表示与♀1或0h相比具有显著性差异(P<0.05)

"♀" represents pituitary of female individual. "♂" represents pituitary of male individual. Asterisks (*) indicate significant differences (P<0.05) between other pituitaries of ♀1 or 0h

*lbh*基因家族成员的进化关系。此外, 还揭示了该基因主要表达于垂体、脑和眼睛中, 以及在银鲫雌雄个体垂体中的表达差异和卵子成熟过程中表达变化。

在生理条件下, 无序蛋白质具有高度的灵活性, 同时缺少稳定的二级或三级结构[35, 36]。具有无序结构域的蛋白质或无序蛋白质参与很多必要的生物学过程及人类疾病的发生[37—39]。小鼠Lbh蛋白质不含有可识别的二级或三级结构但具有结构无序性特征[1]。我们的分析结果表明鱼类Lbh-b与小鼠Lbh一样, 含有大量的无序残基, 是无序蛋白质家族的一员。这暗示Lbh-b与Lbh或其他含有无序结构域的蛋白质类似, 可以在不同的生理状态下获得不同的功能。

*Cglbh-b*和斑马鱼*lbh-b*在早期胚胎发育中表达图式极其相似[9]。从胚胎原位杂交结果来看, *Cglbh-b*与斑马鱼*lbh-b*都大量表达于脑和眼睛(图 3D, E)。且在受精后4d, 杂交信号均主要集中于眼部(图 3F)。尽管*Cglbh-b*与斑马鱼*lbh-b*的表达位置类似, 但两者的表达时间却有先后之别。*Cglbh-b*呈母源性表达, 其合子转录起始于尾芽期(图 2B), 而斑马鱼*lbh-b*最早表达于受精后32h[9]。银鲫独特的生殖方式暗示其具有特殊的胚胎发育调控机制[16, 19]。因此, 我们推测雌核生殖的多倍体银鲫具有不同于有性生殖的二倍体斑马鱼的染色质成分, 从而使得合子型*Cglbh-b*的表达比斑马鱼*lbh-b*的表达更早开始。*Cglbh-b*基因的表达模式提示该基因在早期胚胎发育过程中具有重要作用, 说明它可能参与调控脑和视网膜的发育及分化。至于*Cglbh-b*是否具有与斑马鱼*lbh-b*相似或不同的功能, 仍有待研究。

*Cglbh-b*在17个所检测的成体组织中, 主要表达于垂体、端脑、卵巢和视网膜(图 2A), 也与斑马鱼*lbh-b*类似[9]。在鱼类繁殖过程中, 外界因素如光照、温度、营养物质及水流状况等严重影响着鱼类产卵质量[40]。光调控的神经内分泌系统(Photoneuroendocrine)是由视网膜接收光信号进而调节"脑-垂体-性腺"轴, 最终调控鱼类的繁殖行为[41]。"下丘脑-垂体-性腺"轴在调控鱼类生殖发育过程中发挥着关键作用[42]。*Cglbh-b*大量表达于垂体、脑、卵巢和眼睛, 同时银鲫雌性个体垂体中*Cglbh-b*的表达显著高于雄性(图 4A), 且在卵子成熟过程中, *Cglbh-b*在垂体中的表达上调 (图 4B)。这种独特的表达模式一方面暗示该变化可能是由于注射促性腺激素释放激素和多巴胺的混合物引起的, 另一方面该基因可能与鱼类雌性个体的卵巢分化及排卵等过程密切相关, 其具体生物学功能及与光调控的神经内分泌系统之间的关系仍有待进一步的研究。

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CLONE AND EXPRESSION ANALYSIS OF TRANSCRIPTION COFACTOR *LBH-B* in GIBEL CARP

LI Wen-Hua[1, 2], WANG Yang[1], LI Zhi[1], GUI Jian-Fang[1] and ZHOU Li[1]

(1. *State Key Laboratory of Freshwater Ecology and Biotechnology*, *Institute of Hydrobiology*, *Chinese Academy of Sciences*, *Wuhan* 430072, *China*; 2. *Key Laboratory of Fujian Molecular Medicine*, *Huaqiao University*, *Xiamen* 361021, *China*)

Abstract: LBH (limb-bud and heart) is a novel high-conserved transcription cofactor in vertebrates involved in embryonic development and pathogenesis of some human disease. We screened the *lbh-b gene* that was abundantly expressed in the pituitary of gibel carp, and cloned the *lbh-b* (*Cglbh-b*) cDNA sequence of *lbh* gene family from gibel carp cDNA library by RACE-PCR. The full-length of *Cglbh-b* cDNA was 1526 bp with a 549 bp long open reading frame (ORF) coding a 182 amino acid protein. Bioinformatics analysis showed that CgLbh-b protein shared high homology (>68%) with other vertebrate LBH and is one of intrinsic disordered proteins. *Cglbh-b* was abundantly expressed in pituitary, telencephalon, ovary and eye of adult gibel carp. The expression of *Cglbh-b* in female pituitaries was 4.66 times higher than that in male pituitaries, and *Cglbh-b* in pituitary was up-regulated during the process of oocyte maturation. During the early embryonic development, maternal mRNA of *Cglbh-b* was detected in embryos from 4-cells stage to gastrula stage, and its transcripts were synthesized at bud stage. The results of whole mount *in situ* hybridization showed that *Cglbh-b* was distributed on the brain and eyes of embryos from 2 days post fertilization (dpf) to 3dpf. These results suggest that *Cglbh-b* may play important roles in brain and retina development, and reproductive regulation in oocyte maturation of gibel carp.

Key words: Gibel carp; *lbh-b*; Embryogenesis; Oocyte maturation