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淇河鲫生长激素全长cDNA的克隆与序列分析*

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摘 要 采用RT-PCR法和3', 5' RACE (Rapid amplification of cDNA ends)法从淇河鲫脑垂体RNA克隆出生长激素(Growth hormone, GH) cDNA. 此cDNA全长1 191 nt [含Poly(A) 14 nt], 其5'端非编码区长55 nt, 阅读框(Open reading frame, OAF)长633 nt, 3'端非编码区长503 nt. 由其推导的GH前体由210个氨基酸组成, 其中氮端前22个氨基酸为信号肽部分. 氨基酸序列比较表明, 淇河鲫与同目的鲫鱼、鲤鱼、团头鲂和斑马鱼的同源性分别为98.6%、96.2%、91.5%和88.6%; 与不同目的胡子鲇和鳗鲡的同源性分别为74.6%和49.5%; 与哺乳类的家鼠和人等的同源性低于40%. 图3 参30

关键词 生长激素cDNA; RT-PCR; RACE; 淇河鲫
CLC Q785 : Q959.468.05

Cloning and Sequencing of Full Length Growth Hormone cDNA from *Carassius auratus gibelio var**

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Abstract The full length cDNA encoding growth hormone of *Carassius auratus gibelio var* was cloned from pituitary RNA with RT-PCR, 3'and 5' RACE (Rapid amplification of cDNA ends). The GH cDNA, about 1 191 nt (nucleotide) long [including a 14 nt poly(A) tail], consisted of a open reading frame of 633 nt long, and 5' and 3' untranslated regions of 55 nt and 503 nt long, respectively. The DNA sequence analysis showed that the pregrowth hormone peptide of 210 aa deduced from CagvGH cDNA included a putative signal peptide (22 aa) located in its N-terminal. Homological comparison among CagvGH aa and other species growth hormones showed that the homogeneities were 98.6%, 96.2%, 91.5%, 88.6%, 74.6% and 49.5% compared with those of *Carassius auratus, Cyprinius carpio, Megalobrama amblycephala, Danio rerio, Clarias batrachus* and *Anguilla japonica*, respectively, but less than 40% compared with those of *Mus musculus* and *Homo sapiens*. Fig 3, Ref 30

Keywords Growth hormone cDNA; RT-PCR; rapid amplification of cDNA ends; RACE; *Carassius auratus gibelio var*
CLC Q785 : Q959.468.05

生长激素(Growth hormone, GH)是脊椎动物脑垂体细胞合成和分泌的一种单链多肽激素. 鱼类成熟的GH由173~188个氨基酸组成, 相对分子质量(M_r)为20~22×10^3. 在鱼类中, 同一目的GH氨基酸组成有80%以上相同, 而在不同目之间, GH就有较大的差异, 大约只有49%~68%的相同, 表现出明显的种属特异性[1]. 鱼类GH具有促进鱼类生长, 加快蛋白质的合成, 提高食物转化效率, 提高鲑鳟鱼类的渗透压调节能力等的作用, 被认为是最有效的生长促进剂之一, 具有重要的应用价值. 早期应用各种动物的垂体抽提物或纯化的生长激素对鱼体进行处理, 能诱导鱼的快速生长[2, 3]; 随后证实, 通过基因重组技术在原核生物中表达得到的重组GH对鱼体的生长也具有显著的促进作用[4]. 此后, 多种鱼类的GH基因被克隆并在原核中得到表达, 同时, 构建了多种全鱼GH重组基因并开展了转基因鱼研究[5], 目前, 国内外已有几十种鱼类的GH基因被分离和克隆[6~20]. 在国内, 对鱼类GH的研究多集中在草鱼、鲤和鲇等少数几种鱼类[21~24], 而对淇河鲫的研究尚未见报道.

淇河鲫(*Carassius auratus gibelio var*)属硬骨鱼纲Osteichthyes鲤形目Cypriniformes鲤科Cyprinidae鲫属*Carassius*, 是自然三倍体雌核发育鲫鱼, 产于河南省淇河, 以生长快、味道美和效益高等优点而久负盛名, 具有良好的开发前景. 只有掌握其生长发育规律并加以应用, 才能在生产实践中提高其生长速度, 缩短养殖周期, 为市场提供优质产品, 促进淇河鲫养殖业的健康持续发展. 本试验采用RT-PCR和RACE方法克隆出淇河鲫生长激素cDNA全长, 并进行序列分析, 将推导出的氨基酸序列与其他动物生长激素进行了同源比较分析, 为进一步的蛋白表达和转基因研究工作奠定基础.

1 材料与方法

1.1 试验鱼以及PCR引物和酶

试验用淇河鲫来源于河南省鹤壁市淇县淇河鲫原种场; 本试验所用引物由大连宝生物公司合成; 逆转录酶、Taq酶、T4连接酶、克隆用pMD19-T载体和大肠杆菌(*Escherichia coil*) DH5a由TaKaRa公司生产.

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1.2 淇河鲫脑垂体RNA的提取和cDNA第一链的合成

淇河鲫垂体总RNA的提取：在实验室活体解剖，取垂体约50~100 mg，放入2 mL离心管中，加1 000 μL TRIzol，立即用解剖剪剪碎，至出现粘丝为止，氯仿抽提，异丙醇沉淀，加适量无RNA酶水溶解总RNA. 以1.5 μL总RNA为模板，采用引物Olig dT和逆转录酶在20 μL反应体系中合成第一链.

1.3 淇河鲫GH cDNA部分片段的扩增

以2 μL cDNA作模板，用引物P1 (5'ATG GCT AGA GTA TTA GTG CTA TTG TC3')和P2 (5'CTA CAG GGT GCA GTT GGA ATC C3')进行PCR扩增. PCR反应条件为：50 μL反应体积中含有5 μL 10×PCR Buffer (Mg^{2+} Free)、6 μL dNTP Mix (2.5 mmol/L)、4 μL $MgCl_2$ (25 mmol/L)、1 μL rTaq (5 u/μL)、1 μL P1 (20 pmol/L)、1 μL P2 (20 pmol/L)、2 μL cDNA；反应液在94 ℃预变性5 min，然后94 ℃变性30 s、55 ℃退火30 s、72 ℃延伸30 s，共30个循环，最后72 ℃保温2 min.

1.4 淇河鲫GH cDNA 3'端(下游区)和5' 端(上游区)片段的扩增

淇河鲫GH cDNA 3'端的扩增采用TaKaRa 3'-Full RACE Core Set进行，cDNA第一链合成具体操作见试剂盒说明. 以引物P1和3' RACE Out Primer为引物，以垂体cDNA为模板，进行第一轮 PCR 反应，反应条件同“1.3”. 再以此扩增产物为模板，采用套式引物 P3 (5'CGA CTT GAA AAT GGG CAT C3')和3' RACE Inner Primer进行套式PCR. 两次PCR条件相同.

淇河鲫GH cDNA 5'端的扩增采用TaKaRa 5' RACE Core Set进行，cDNA第一链合成(引物为磷标记的特异性引物P4 [5'(P)GGT GCC TCA ATG TA3']、Hybrid RNA的分解和连接反应等具体操作见试剂盒说明. 用引物P5 (5'CTG TTG CCT GAG GAG CGC A3')和P6 (5'AAC TAA CCG GCA CCA CCG AC3')进行第一轮PCR反应；再以此扩增产物为模板，用引物P7 (5' TGA GGA GCG CAG ACA GCT GAG TA 3')和P8 (5' GGC ACC ACC GAC AAT AGC ACT 3')进行第二轮 PCR反应；以获得生长激素cDNA 5'端，两次PCR条件同“1.3”.

1.5 PCR产物的克隆和DNA测序及序列分析

PCR产物经纯化后直接克隆到pMD19-T (TaKaRa)载体上，其具体操作见厂家说明. DNA测序由大连宝生物公司完成，采用DNAstar和Clustal软件进行序列分析.

2 结果与讨论

2.1 淇河鲫生长激素全长cDNA的克隆与序列分析

根据鲫鱼GH基因序列(GenBank检索号：AF069399)设计了一对特异性引物P1和P2，以淇河鲫垂体cDNA为模板，经过30个循环扩增出一条约630 nt的单一DNA条带(图1-A, Lane 1). 经琼脂糖凝胶电泳分析，此片段大小与其他鱼类生长激素区间的DNA片段大小相一致. 将此PCR产物克隆到pMD19-T载体上，筛选阳性克隆，再经限制性内切酶酶切分析确认阳性克隆，将阳性克隆进行双向测序，确认此片段大小为633 nt，经BLAST在Genbank上查询，证实此克隆片段为生长激素cDNA部分片段.

为了获得淇河鲫生长激素cDNA的全部信息并对其进行全面的分析，进一步克隆了其生长激素cDNA的3'和5'端. 采用引物P1和3' RACE Out Primer，以cDNA为模板扩增出了约1 100 nt左右的片段(图1-B, Lane 2). 为了增加PCR扩增特异性，采用套式引物P3和3' RACE Inner Primer，以此扩增产物为模板扩增出约740 nt的单一DNA片段(图1-C, Lane 3)，经克隆和DNA序列分析，确认此克隆片段为完整的淇河鲫生长激素cDNA 3'端.

同时，根据淇河鲫生长激素cDNA部分片段序列设计基因特异引物 P4、P5、P6、P7、P8，以实验“1.2”所剩余的总RNA为模板合成第一链，经过Hybrid RNA的分解和连接反应后，以连接反应液稀释物(用TE buffer稀释10倍)为模板，采用引物P5、P6合成特异基因的cDNA，凝胶电泳后，目的条带模糊，然后以基因特异引物P7和P8进行套式PCR，得到160 nt左右的片段(图1-D, Lane 4)，经克隆和序列分析，证实此克隆片段为淇河鲫生长激素cDNA 5'端.

通过3次扩增与克隆，获得了淇河鲫生长激素全长cDNA (图2)，将其命名为CagvGHc. 克隆的CagvGHc全长1 191 nt (nucleotides)[含 poly(A)]，5'非编码区长55 nt，含有在真核生物中高度保守的Kozak序列[25]，其序列形式为CAG^{-45} TGG^{+4}；克隆的5'非编码区有55 nt长，3' poly (A)尾14 nt，说明所提取的RNA质量是很好的，克隆的GH cDNA可以认为是全长. 开放

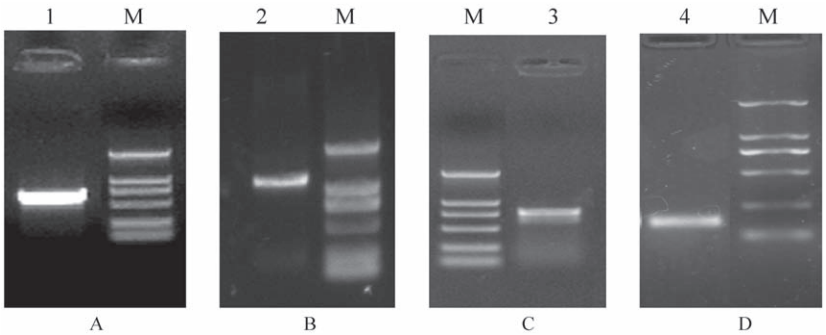

图1 琼脂糖凝胶电泳结果

Fig. 1 Agrose gels (1.5%)

A. Results of RT-PCR with primers P1 and P2; B. The first PCR results of 3' RACE; C. The second PCR results of 3' RACE; D. The second PCR results of 5' RACE. Lane M: DNA ladder marker 2000; Lane 1. RT-PCR product with primers P1 and P2; Lane 2. The first RT-PCR product of 3'RACE; Lane 3. The second PCR product of 3'RACE; Lane 4. The second PCR product of 5' RACE

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1    AAAACACTCAGAAGCTTTTAACAAAGTCTGCAAGAGTTGGTCTACCCTGAGCGAA
56   ATG GCT AGA GTA TTA GTG CTA TTG TCG GTG GTG CCG GTT AGT TTG TTG GTG AAC CAG GGG
       M   A   R   V   L   V   L   L   S   V   V   P   V   S   L   L   V   N   Q   G
116  AGA GCA TCA GAT AAC CAG CGG CTC TTC AAT AAC GCA GTC ATT CGT GTA CAA CAC CTG CAC
       R   A   S   D   N   Q   R   L   F   N   N   A   V   I   R   V   Q   H   L   H
176  CAG CTG GCC GCT AAA ATG ATT AAT GAC TTT GAG GAC AGC CTG TTG CCT GAG GAG CGC AGA
       Q   L   A   A   K   M   I   N   D   F   E   D   S   L   L   P   E   E   R   R
236  CAG CTG AGT AAA ATC TTC CCG CTG TCT TTC TGC AAT TCT GAC TAC ATT GAG GCA CCC ACT
       Q   L   S   K   I   F   P   L   S   F   C   N   S   D   Y   I   E   A   P   T
296  GGA AAA GAT GAA ACA CAG AAG AGC TCT ATG TTG AAG CTC CTT CGC ATT TCC TTC CGC CTC
       G   K   D   E   T   Q   K   S   S   M   L   K   L   L   R   I   S   F   R   L
356  ATT GAG TCT TGG GAG TAC CCC AGC CAG ACC CTG AGC GGA ACC GTC TCA AAC AGC CTG ACC
       I   E   S   W   E   Y   P   S   Q   T   L   S   G   T   V   S   N   S   L   T
416  GCC GGG AAC CCC AAC CAG ATC ACT GAG AAG CTG GCC GAC TTG AAA ATG GGC ATC AAT GTG
       A   G   N   P   N   Q   I   T   E   K   L   A   D   L   K   M   G   I   N   V
476  CTC ATT AAG GGA AGT CTC GAT GGT CAA CCA AAC ATA GAT GAT AAC GAC TCC CTA CCA CTG
       L   I   K   G   S   L   D   G   Q   P   N   I   D   D   N   D   S   L   P   L
536  CCT TTT GAG GAC TTC TAC TTG ACC ATG GGG GAG AAC AAC CTC AGA GAG AGC TTT CGT CTG
       P   F   E   D   F   Y   L   T   M   G   E   N   N   L   R   E   S   F   R   L
596  CTG GCT TGT TTT AAG AAG GAC ATG CAC AAG GTC GAA ACC TAC CTG AGG GTT GCA AAT TGC
       L   A   C   F   K   K   D   M   H   K   V   E   T   Y   L   R   V   A   N   C
656  AGG AGA TCC CTG GAT TCC AAC TGC ACC CTG TAG
       R   R   S   L   D   S   N   C   T   L   *
689  AGGGCGCCGATGTATTGCTAGTCAATGCCTGCAAATCTAAAACCAGTTTAATCCTTAAAACTTCCTTAAGTTATTATGAT
769  CTGGTCTTATATATACAGGAATTGTCAACCAATTGCATGGCTATGCCCGTCTTTTCTTTTTCCCTCCTATATTTAAACAT
849  TGTCTACCACTATTTTATTTATTCTTCTCATAAGGGAATGCTCTTAAATTAAAGCTGTTCCTTTAAAAAGGATGGATCTG
929  ATTTCACAGTGGTGCTTATGGCAATATATTTTCAAATGTGGCCAGATTGGCCTTAGTCACAGAGCTATTAATGTGTACAA
1009 TATTATTGCTCAAAAAGACTAGATTAGTATTACGTTAAAATTGTAATCTAAATGGATAGACTCACTCTTGTATACATATT
1089 CGTTTGTCTCATATATTATGCTCTTATTATTAGCGCATCTTATTCTCAAGTTCTGTGTCTTTCTTCATTAAAGTTTAAAA
1169 ATTGCATCCAAAAAAAAAAAAAA 1191
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图2　淇河鲫生长激素全长cDNA序列及其推导的氨基酸序列
(位于核苷酸下面)

Fig. 2　Nucleotide sequence of *Carassius auratus gibelio var* growth hormone cDNA and the deduced amino-acid sequence
(Presented below the nucleotide sequence)
The termination coden is indicated by "*", a putative poluadenylation signal is underlined, the nucleotide sequence data reported here have been submitted to the GenBank and EMBL nucleotide sequence databases)

阅读框(ORF)长633 nt, 3' 端非翻译区长503 nt, 在poly(A)上游23 nt处有ATTAAA序列, 为真核生物mRNA前体3'剪切和加poly(A)尾的信号[26, 27].

图3是通过淇河鲫与其它脊椎动物生长激素的同源性比较而绘制的基因树, 结果表明, 淇河鲫与鲤形目的鲫鱼、鲤鱼、团头鲂、斑马鱼的亲缘关系较近, 与不同目的胡子鲇和鳗鲡的亲缘关系较远, 而与哺乳类家鼠和人的亲缘关系更远. 图3所反映的进化关系与根据传统的形态学和生化特征分类进化地位是相一致的.

2.2 不同鱼类生长激素氨基酸序列的比较分析

从CagvGHc序列推导出的淇河鲫生长激素前体由210个氨基酸组成, 其中酸性氨基酸占11.90%, 碱性氨基酸占11.43%, 疏水氨基酸占34.76%, 亲水氨基酸占29.52%; 等电点为 6.60, 相对分子质量为23.78×10^3.

翻译成氨基酸后与鲫鱼(*Carassius auratus*) (GenBank检索号: AF069399)、鲤鱼(*Cyprinius carpio*) (GenBank检索号: X13670)、团头鲂(*Megalobrama amblycephala*) (GenBank检索号: AF463498)、斑马鱼(*Danio rerio*) (GenBank检索号: AJ937858)、胡子鲇(*Clarias batrachus*) (GenBank检索号: AF416486)、鳗鲡(*Anguilla japonica*) (GenBank检索号: M24066)、家鼠(*Mus musculus*) (GenBank检索号: NM_008117)和人(*Homo sapiens*) (GenBank检索号: BC020760)的GH蛋白质前体的氨基酸序列进行比较, 相似性分别为98.6%、96.2%、91.5%、88.6%、74.6%、49.5%、38.4%和29.4%.

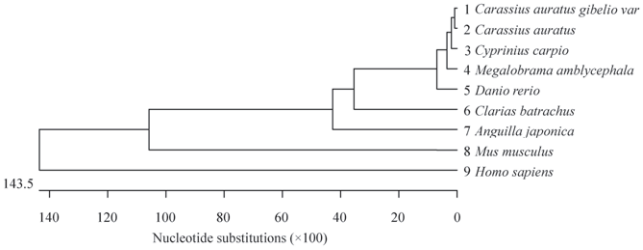

图3 GH系统进化树

Fig. 3　Maximum-parsimony tree for GH coding sequences

根据已知鱼类生长激素前体信号肽的氨基酸组成特点和序列保守性[11, 12], 推测淇河鲫GH信号肽为22个氨基酸, 从起始氨基酸(M)到第22位氨基酸(A)为信号肽部分, 成熟肽为188个氨基酸. 淇河鲫存在与鲤鱼GH氨基酸序列相同的潜在的N-糖基化位点(Asn-Xaa-Ser和Asn-Xaa-Thr)[28]. 在淇河鲫GH的成熟肽中, 有4个半胱氨酸(C), 根据已知鱼类GH的蛋白质结构得知, 这4个半胱氨酸的位置非常保守, 它们在成熟肽中形成两个二硫键(第49位与第161位, 第178位与186位), 对生长激素的正常折叠、维持空间结构以及发挥有效的生理功能有着重要的作用[1].

淇河鲫GH基因的克隆为GH的分子进化和同源比较研究提供了新的资料. 虽然鲫鱼味道美, 营养价值高, 但生长速度缓慢, 所以一般不作为主养鱼类, 通过转淇河鲫生长激素基因, 可望解决鲫鱼生长缓慢这一难题. 根据Rahman等[29]的观点, 转植生长激素基因与受体生长激素基因的同源性越高, 转植生长激素基因促生长的作用越明显, Nam等甚至通过自源转基因(Autotransgenic)获得了超大个体转基因鱼[30]. 因此, 淇河鲫生长激素基因的克隆也为选择和构建合适的转植基因重组子, 以及对转基因鱼中转植基因的检测等奠定了基础.

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