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学位論文題目	Regulatory mechanism of the POU family transcription factor that governs the brain formation in zebrafish embryos （ゼブラフィッシュの脳形成を制御する POU 型転写因子遺伝子の発現調節）
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論文の内容の要旨

It is well established that the development of the vertebrate brain starts with the induction of the neural plate, which is patterned along the anterior-posterior (AP) axis and then regionalized into different brain compartments. The boundary region in the neural plate between the prospective midbrain and the hindbrain, which is called the midbrain-hindbrain-boundary (MHB), forms the isthmus and serves as a signaling center for the development of the midbrain and cerebellum. The position of MHB along the anterior-posterior axis in the neural plate depends on the interaction of *Otx2* and *Gbx2* that are expressed in the future fore/midbrain and hindbrain, respectively. At the expression boundary of these two homeobox genes, *fgf8, pax2/pax2a* and wnt1 are independently induced around the end of gastrulation to establish MHB, leading to activation of the downstream genes including en and other MHB genes. This gene cascade/network, which is known in all the vertebrates examined, is considered to promote and maintain the formation of the MHB/isthmic region.

Zebrafish *pou2,* which was originally identified as a gene for a novel POU transcriptional factor with structural features of both class III and V of the family. Takeda et al., (1994) reported that *pou2* is maternally expressed ubiquitously in the whole blastoderm in early embryos and that its zygotic expression becomes confined to the prospective midbrain and hindbrain by the end of epiboly and finally disappears in the brain during early somitogenesis. Based on the syntenic relationship and sequence comparisons, zebrafish *pou2* is now considered the orthologue of *Oct 3/4 /pou5f1*, which is required to maintain pluripotency of early embryonic cells and ES cells.

pou2 is disrupted in *spiel-ohne-grenzen* (*spg*) mutant embryo that show defects in the MHB/isthmus and hindbrain (Schier et al. 1996; Belting et al. 2001; Burgess et al. 2002). Indeed, it is considered that *pou2* functions specifically in the AP patterning of the neuroectoderm, in which it is necessary to establish the MHB and the hindbrain primordium. Genetically, *pou2* is necessary to activate expression of regulatory genes involved in brain development, especially *pax2a*, which is considered to form the MHB and primordial hindbrain. Expression of *pou2* in the embryonic brain overlaps that of *pax2a* around MHB, and *pax2a* expression is down-regulated in *spg* embryos, whereas

pou2 expression is not affected in mutant embryos that have a defect in *pax2a*, showing that *pou2* is required for activation of *pax2a* in the prospective MHB. On the other hand, since *spg* embryos show normal expression of *otx2* and *gbx1*, which is involved in the establishment of MHB in zebrafish embryos, it seems that *pou2* is required not for establishment, but for maintenance of MHB. In addition to the defects in MHB, *spg* embryos show abnormal morphology, size and boundary of rhombomeres in the hindbrain, showing that *spg/pou2* is also involved in hindbrain segmentation. During hindbrain development, respective rhombomeres are specified by combinations of *Hox genes* (Hox code), as well as by additional transcription factor genes that are under regulation by *Hox genes*, such as *krox20* and *velentino* (*val)*/*mafB*. In the establishment of rhombomeres, pou2 is assumed to function upstream to *krox20* and *val*, leading to the formation of a gene network required for hindbrain formation. Although the role of *Oct 3/4* in brain formation is not known at present in mouse, *Oct 3/4* is expressed broadly in the neural plate in embryos as well, and *Oct 3/4* mRNA overexpression in *spg* embryo restores the expression of *pax2a*, raising a possibility that *Oct3/4* is also involved in the development of the brain in mouse development.

In spite of the essential role played by *pou2* in zebrafish brain development, however, the regulatory mechanism governing its unique expression pattern is not known. A preliminary functional analysis of the flanking DNA of *pou2* was previously conducted by Okuyama and Yamasu (2003, unpublished) via the promoter analysis, revealing that the upstream 6.5-kb DNA region drives the GFP expression transiently and broadly at early somite stages in the brain including the prospective midbrain and hindbrain, reminiscent of the endogenous expression of *pou2*. In the present study, to understand the regulatory mechanism of the brain formation, especially in the midbrain and hindbrain, I intended to further examine the regulatory mechanism of *pou2* in the brain by extending the previous promoter analysis.

First, to precisely know the regulatory function of the upstream 6.5-kb DNA, I established a transgenic fish line that harbors a GFP construct in which the GFP gene (*egfp*) is under regulation by the 6.5-kb DNA (GFP-6.5), and examined GFP expression in embryos via fluorescence and in situ hybridization. GFP-6.5 was expressed transiently in the midbrain and hindbrain, as well as in the medial stripe of the neural plate and tail bud, thus closely recapitulating *pou2* expression in the brain. Therefore, it was concluded that this upstream DNA contains the main part of the regulatory activity of *pou2* expression in the brain. The GFP construct (GFP-2.2) under the regulation of the region from -2.2 to the ATG codon (-2.2/ATG) showed similar transient expression in injected embryos. I further showed by the reporter assay that most of this activity resides in the upstream 2.1-kb DNA from -2.2 to -0.1 (-2.2/-0.1).

To further narrow down the effective *cis*-elements in the -2.2/-0.1 region, the regulatory activity of this region was examined by the reporter assay after externally introducing systematic deletions from the 5'-end and 3'-end. The results showed that external deletions from either end gave rise to gradual decrease of the regulatory activities, suggesting that there are multiple cis-elements in the -2.2/-0.1 region. Fragmentation of this region led to drastic reduction in the regulatory activities, leading to a possibility that the multiple elements function in a co-operative manner.

Since the promoter analysis showed that the 2.1-kb DNA is the effective regulator of *pou2* in the brain, and that it consists of multiple *cis*-elements, I searched for consensus binding sequences for transcription factors in this region, finding four possible binding sequences (octamer sequences, Poct1, Poct2, Poct3 and Poct4) for POU family transcription factors and one DR2-type retinoic acid responsive element (DR2-RARE). The identification of four octamer sequences prompted me to examine their role in the regulatory activity of the -2.2/-0.1 region. A series of electrophoretic mobility shift assay (EMSA) demonstrated that all these octamer sequences were bound by Pou2 that was synthesized in *vitro*. In all cases, binding was competed efficiently by unlabeled probes, and their competition

activities were disrupted when the octamer sequences were disrupted by base substitution. These results showed that all four octamer sites are the real binding sites of Pou2. Furthermore, a DNA fragments (4xPoct) containing all the octamer sites yielded a distinct DNA protein complex, suggesting that the binding of Pou2 with the -2.2/-0.1 DNA is a highly co-operative reaction. This is consistent with the results of the reporter analysis showing cooperativity of the regulatory function of -2.2/-0.1.

I further sought to evaluate the role of the four octamer sites in the enhancer activity of -2.2/-0.1 *in vivo* by introducing base substitutions into the octamer sites within GFP-2.2. Injection of the mutated GFP-2.2 constructs into zebrafish embryo showed that the disruption of any octamer site abolished the expression of GFP-2.2 in the brain region, demonstrating that all the octamer sites are essential components of the -2.2/-0.1 enhancer and that they work cooperatively to regulate the transcription in the brain. Furthermore, co-injection of *pou2* mRNA up-regulated the GFP-2.2 expression in the whole blastoderm at the shield stage and in the whole embryonic body at early somite stages. Meanwhile, knockdown of endogenous *pou2* with anti-*pou2* morpholino down-regulated GFP-2.2 expression in the brain region. Taken these gain-of-function and loss-of-function studies together, it is concluded that *pou2* regulates itself in a positive manner, forming an autoregulatory loop.

Besides the reduction of expression in the brain, deletion of any of the octamer sequences in GFP-2.2 led to ectopic expression in the posterior embryonic region including the tail bud. This expression was disrupted by further deletion of the intervening sequence between Poct2 and Poct3 (IS2). Thus, it is likely that IS2 drives transcription in the posterior region, which is endogenously suppressed by POU-family proteins. Since *pou2* is not expressed in the posterior region, this suppressive effect is probably exerted by different POU factors.

As mentioned above, a DR2-RARE sequence (P2-RARE) was identified within the upstream 2.1-kb DNA. Since retinoic acid (RA) is known to suppress pou2 in zebrafish embryos, I addressed the possible involvement of this RARE in the regulation of pou2. First, I found that RA efficiently suppresses the transient expression of GFP-2.2 in injected embryos, showing that the *pou2* responsiveness to RA is conducted by the -2.2/ATG region. When the RARE was disrupted, the expression of GFP-2.2 expanded laterally in the posterior embryonic region in late gastrulae. Furthermore, this expression was unaffected by externally administered RA. The ectopic expression due to RARE deletion was canceled by further deletion of IS2. In EMSA, the RAR/RXR, which is a receptor complex for RA, was shown to bind to P2-RARE in -2.2/-0.1. This binding was competed by an excess amount of unlabeled P2-RARE, and the competing activity was lost when P2-RARE was disrupted by base substitution, showing that the association of RAR/RXR with P2-RARE is specific. Taken together, RA affects *pou2* expression at the transcriptional level through the -2.2/-0.1, and the suppressive action of RA is mediated via the P2-RARE. It is probable that RA/P2-RARE functions in embryos to suppress *pou2* expression in the posterior region that is also driven by IS2.

Based on the present study, I assume that the zygotic expression of *pou2* is activated ubiquitously by maternal Pou2, and is probably maintained during epiboly by an autoregulatory loop. Furthermore, down-regulation of *pou2*, which is seen during epiboly in the posterior portion, seems to be due to the RA signal that is known to emanate from the posterior blastoderm margin.

論文の審査結果の要旨

脊椎動物の脳形成においては、発生初期に形成される神経板の領域化が、その後の脳特有の高次構造の形成において不可欠であり、この過程を制御する遺伝子機構の解明は極めて重要な研究課題と言える。脳発生研究の優れたモデル動物とされる小型熱帯魚ゼブラフィッシュにおいて、これまでに多数の脳形成突然変異体が同定されているが、その一つに中脳から後脳にかけて広範に脳形成に異常を示す変異体 spiel ohne *grenzen (spg)* がある。近年、*spg* 突然変異の原因遺伝子がクラス V の POU 型転写因子である Pou2 をコードすることが報告された。この転写因子遺伝子（*pou2*）の発現は、未受精卵では広範に見られるが、その後、脳の領域化が進行する原腸形成後期から体節形成初期において、ゼブラフィッシュ胚の予定中脳、後脳領域に限局し、その後は脳領域から急速に消失する。他の脊椎動物でも、*pou2* 相同遺伝子の同様の発現が知られており、これらのクラス V POU 遺伝子の発現制御は、脊椎動物での脳形成制御機構を遺伝子レベルで解明する上で重要な課題である。Mst. Shahnaj Parvin 氏提出の「Regulatory mechanism of the POU family transcription factor that governs the brain formation in zebrafish embryos（ゼブラフィッシュの脳形成を制御する POU 型転写因子遺伝子 *pou2/pou5f1* の発現調節）」と題する学位論文は、ゼブラフィッシュ胚での脳形成における *pou2* の遺伝子発現制御機構の解明を通し、初期脳形成制御機構の解明に取り組んだ研究の成果をまとめたものである。

まず、Parvin 氏は、*pou2* の上流 6.5 kb 領域を GFP レポーター遺伝子につないだ上でゼブラフィッシュ生殖系列に導入して Transgenic 魚（Tg 魚）を作製した。得られた Tg 胚におけるレポーターの発現を、蛍光による GFP タンパク質の検出と Whole mount in situ hybridization による mRNA の検出により詳細に検討し、上流 6.5 kb が *pou2* の中脳・後脳領域における一過的な発現をほぼ忠実に再現しうることを明らかとした。このことは、脳での発現制御領域がこの上流 DNA 内にあることを示している。

この 6.5 kb 領域内の発現調節 cis-element を同定するため、Parvin 氏はこの領域に由来する各種 DNA 断片の転写調節活性を、導入胚におけるレポーター遺伝子の Transient 発現を指標に精力的に検討し、最終的に、遺伝子近位部 2.1 kb に主たる転写調節活性が存在することを示した。また、この 2.1 kb 領域をさらに断片化する、あるいは領域内に欠失を導入すると、転写調節活性が急激に減少することから、2.1 kb 領域内にある複数の領域が協調的に転写調節に働くことを示唆した。さらに、2.1 kb 領域内の塩基配列より POU 転写因子の結合配列（オクタマー配列）が 4 カ所予想されたため、これらを塩基置換により破壊した上、転写調節能への影響を検討した結果、これらのオクタマー配列が全て脳における転写活性化に不可欠であることを示した。一方、ゲルシフト解析により、Pou2 タンパク質が *in vitro* でこれら 4 カ所のオクタマー配列に特異的に結合することを確認した。

以上の結果は、*pou2* の発現が自己調節的に制御されていることを示唆する。Parvin 氏はこの可能性を検証するため、mRNA 導入による *pou2* の強制発現、または Morpholino antisense oligo による *pou2* のノックダウンを行なった胚において、2.1 kb 領域の転写調節能をやはりレポーターの発現により検討し、この 2.1kb 領域による転写調節が、胚内において pou2 自身により正に制御されていることを明らかとした。

さらに、Parvin 氏は、内在 *pou2* の発現及び 2.1 kb 領域の転写調節活性がレチノイン酸（RA）により抑制されること、RA 受容体複合体（RAR/RXR）の結合部位が 2.1 kb 領域内に存在し、この配列を破壊すると、2.1 kb 領域の RA 感受性が消失するとともに、転写活性化の起きる胚領域が後方で拡大することを示した。

以上の成果に基づいて、Parvin 氏は、ゼブラフィッシュ胚の中脳、後脳領域における *pou2* のダイナミックな発現調節が、ポジティブフィードバックによる転写活性化と後方からの RA シグナルによる転写抑制により説明しうることを明らかとした。

以上のように、Parvin 氏の提出した学位論文は、脊椎動物で広く知られるクラス V POU 転写因子の脳内におけるダイナミックな発現について、その制御機構の主要部分を明らかとした。この成果は、*pou2* を構成要素とする脳形成遺伝子ネットワークの全体像に関する今後の研究に大きく貢献すると考えられる。また、本論文の以上の内容は、発生生物学領域の国際誌において、査読付き論文として採択となっている。よって、本審査委員会は、この学位論文が博士（学術）の学位に十分ふさわしいと判断し、「合格」との判定を行った。