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学位論文題目	メダカFTZ-F1による*P*-450アロマターゼ遺伝子の転写調節
論文審査委員	主　査　教　授　諸橋　憲一郎 教　授　上野　直人 教　授　野田　昌晴 教　授　長濱　嘉孝

論文内容の要旨

In nonmammalian vertebrates, estradiol-17β a major estrogen in all vertebrates, is responsible for the growth of oocytes, vitellogenesis. The cytochrome P-450 aromatase (P-450arom) is an important steroidogenic enzyme which converts testosterone to estradiol-17β. In contrast to well-known physiological functions of estradiol-17β, very little is known about the molecular mechanisms of P-450arom activation in ovarian follicles during oocyte growth.

The medaka, *Oryzias latipes*, under the conditions of 26°C, 14 hours light and 10 hours dark, usually spawn within 1 hour of the onset of light for several consecutive days. The majority of vitellogenesis and meiotic maturation of individual oocytes occurs within 48 hours; germinal vesicle breakdown and ovulation being completed at 6 and 1 hour before spawning, respectively. Thus, medaka follicles provide an excellent model for understanding the hormonal regulation of follicular growth and maturation. Previous studies in our laboratory involving incubations of isolated medaka ovarian follicles with gonadotropins, forskolin and dbcAMP which are known to raise the cellular level of cAMP, and/or actinomycin D suggest that P-450arom activity is transcriptionally regulated. The aim of this study is to elucidate the regulatory mechanism of time-specific *P*-450arom transcription responsible for estradil-17β production by medaka ovarian follicles during active vitellogenesis.

Promoter analysis of the medaka *P*-450arom gene identified putative orphan nuclear receptor binding sites (Ad4-1, Ad4-2). To clone orphan nuclear receptors from medaka ovarian follicle, RT-PCR was performed using degenerate primers designed against highly conserved amino acid sequences in the DNA binding region of orphan nuclear receptors. Seven distinct fragments encoding putative RXR, Ad4BP/SF-1, TAK1, PPARα, PPARγ, COUP-TF and Rev-erb were amplified. RNase protection assays using cRNA probes prepared against these seven fragments were used to investigate the expression profile of each fragment in medaka ovarian follicles during various stages of oogenesis; total RNAs from follicles collected at 35, 29, 23, 11 and 5 hours before the onset of light as well as the previtellogenic stage were used. The results showed that only the Ad4BP/SF-1 homologue gave a clear expression profile which correlated well with *P*-450arom expression.

Based upon these data, an attempt was made to clone a full length cDNA enconding the Ad4BP/SF-1 homologue by library screening and 5' RACE. The 1,458 bp open reading frame of this cDNA is predicted to encode a 486 amino acid polypeptide. Sequence analysis showed high homology of this cDNA clone to *Ad4BP/sf-1* and *Ftz-*

*F1*s from various species. The DNA binding region, FTZ-F1 box, and AF-2 domain showed almost 100% homology with Ad4BP/SF-1 and LRH-1/FTF. Therefore, we designated the medaka clone as medaka *Ftz-F1* cDNA (*mdFtz-F1*).

There are two groups of FTZ-F1 family proteins. One, the Ad4BP/SF-1 group, is mainly expressed in steroidogenic tissues. The other is the LRH-1/FTF group which is expressed in liver. Despite a close similarity of the primary structure of mdFTZ-F1 to that of either FTZ-F1 group, tissue distribution analysis indicating that *mdFtz-F1* transcripts are highly expressed in ovary and testis with weak signals in brain, spleen and kidney, suggest that mdFTZ-F1 functions similar to Ad4BP/SF1.

To investigate whether mdFTZ-F1 can specifically bind to putative orphan nuclear receptor binding sites of P-450arom gene, gel shift assays were performed. mdFTZ-F1 translated in reticulocyte lysate bound the Ad4-1 and Ad4-2 sites. The band disappeared in the presence of 50 times excess cold competitor, demonstrating the ability of mdFTZ-F1 to specifically bind to the Ad4-1 and Ad4-2 sites on the *P*-450arom promoter.

A series of transfection experiments were performed to investigate the mdFTZ-F1 function the *P*-450arom promoter in living cells. In these experiments, *P*-450arom promoter-reporter constructs having the *P*-450arom promoter region upstream of firefly luciferase and an *mdFtz-F1* expression vector which is driven by the SV40 promoter in CV-1 mammalian cells were used. Luciferase activity increased when the *mdFtz-F1* expression vector were co-transfected. He also prepared deletion reporter constructs to identify the essential region for the transcriptional activation by mdFTZ-F1. Luciferase activity dropped when the Ad4-1 and Ad4-2 sites were deleted. To further investigate the importance of the Ad4-1 and Ad4-2 sites, these sites were mutated such that mdFTZ-F1 could not bind to them. Single or double mutation of the Ad4-1 and Ad4-2 sites caused decreased reporter activity. These results suggest that the *P*-450arom promoter can be positively regulated by specific binding of mdFTZ-F1 to the Ad4-1 and Ad4-2 sites.

The expression profile of *mdFtz-F1* transcripts during oogenesis was analyzed by RNase protection assay. The expression profile of *mdFtz-F1* correlated with the *P*-450arom expression profile which also supports the hypothesis that mdFTZ-F1 may regulate *P*-450arom.

To investigate whether there is specific binding activity to the Ad4-1 and Ad4-2 in medaka ovarian follicles, gel shift assays using nuclear extracts prepared from medaka ovarian follicles at the time (23 hours before the onset of light) when the transcript level of *mdFtz-F1* peaks were performed. Three bands appeared when the nuclear

extracts were incubated with the radio-labeled Ad4-1 or Ad4-2. One of these bands appeared at the same position as the band which appeared when reticulocyte-expressed *mdFtz-F1* was incubated with Ad4-1 or Ad4-2. These three bands disappeared simultaneously in the presence of 50 times excess of cold competitor. When a nuclear extract from medaka ovarian follicles at the time of oocyte maturation was incubated with the radio-labeled Ad4-1 or Ad4-2, no specific band appeared. These results suggest that there is specific binding activity to the Ad4-1 and Ad4-2 *in vivo* at the time of vitellogenesis. Furthermore, this binding activity disappears concomitant with oocyte maturation.

In summary, the results of this study suggest a potential role of mdFTZ-F1 on the time-specific transcriptional regulation of *P*-450arom in medaka ovarian follicles. This is the first investigation with respect to the transcriptional regulation of *P*-450arom in ovarian follicles during oogenesis. Further study of other transcription factors which synergistically act with mdFTZ-F1 or modifications of mdFTZ-F1 such as phosphorylation will reveal the molecular detail of the intracellular signaling pathways which regulate *P*-450arom transcription in response to gonadotropin.

論文の審査結果の要旨

卵形成は、卵巣における最も重要な機能である。この過程は種々のホルモンによって制御されるが、特に卵巣で生合成される雌ホルモンが重要な役割を担う。一方、これら性ステロイドは、多くの動物種において２次的性成長の進行を促すホルモンとしても知られる。魚類においても、このような性ステロイドの機能は維持されているが、これに加え雌ホルモンである Estradiol 17-β は遺伝的性を逆転させることが可能であることも知られている。従って、雌性ホルモン合成酵素である P-450 aromatase の遺伝子発現調節機構を明らかにすることは、卵巣の機能発現と生殖腺の性決定機構の解明には不可欠であると考えられる。

申請者、渡辺正忠はメダカ P-450 aromatase の遺伝子発現調節機構を、以下の実験によって明らかにした。申請者が所属する研究室では、既にメダカ P-450 aromatase の遺伝子が単離されており、遺伝子上流域には核内レセプター型転写因子の認識配列が見い出されていた。そこで、申請者は RT-PCR 法によって、メダカ卵巣に発現する７種のオーファンレセプターに対する cDNA の断片を単離した。更にその中で、最も重要であると思われる mdFTZ-F1 について、その全長をクローニングし、その構造を決定した。本因子の発現はステロイドホルモン産生組織である生殖腺の他に、脳や脾臓、卵母細胞にも認めらるが、その詳細は明らかされるところまでは至っていない。本因子の卵形成過程における発現様式を RNase protection 法にて検討した結果、卵黄形成期には高い発現を示すが、卵成熟後期にはその発現が著しく低下することを見い出している。これは、卵形成過程における Estradiol 17-β の産生量や P-450 aromatase の発現量の推移と良い相関を示すものであり、 mdFTZ-F1 が P-450 aromatase 遺伝子の転写を調節していることを強く示唆するものであった。そこで、メダカ P-450 aromatase 遺伝子の上流域を用い、mdFTZ-F1 の機能を調べたところ、メダカ P-450 aromatase 遺伝子の上流には２箇所の mdFTZ-F1 結合配列が存在することをゲルーシフト法などから明らかにした。更に、ルシフェラーゼ遺伝子をリポーターに用い、これらの配列が実際に mdFTZ-F1 依存的にメダカ *P*-450 aromatase 遺伝子の転写を活性化することも見い出している。

本研究は、卵巣における P-450 aromatase の遺伝子発現が、mdFTZ-F1 によって制御されることを魚類の卵形成における特徴を生かしつつ、in vitro と in vivo の両面で証明したものであり、本研究分野に大きく貢献するものであると判断された。従って、審査委員会は本博士論文に対して、合格の判定を下した。

また、学位論文として提出された研究結果について、口答発表させた後、審査委員が論文内容について試問した。更に、関連分野の一般知識、及びその背景となる基礎知識についても、口答試問により審査した。これらの質問に対する申請者の応答は、いずれも的確なものであった。また、提出された学位論文は日本語で書かれていたが、申請者は既に国際誌に２つの論文（うち１報は in press）を発表しており、英語に関する能力についても充分であると思われる。これらの結果をもとに、審査委員会は申請者の持つ研究能力及び学力は学位取得に値するものと判定した。