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cDNA Cloning of Constitutive Androstane Receptor (CAR) Gene of Japanese Quail

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Summary

The constitutive androstane receptor (CAR) belongs to the nuclear receptor class 1, subfamily I (NR1I3). The previous studies suggest the importance of the CAR gene for physiological mechanisms of the vertebrates, especially metabolism of steroid hormones. The present study determines the DNA sequence of a full length cDNA of Japanese quail (*Coturnix japonica*) CAR by the rapid amplification of cDNA end (RACE) method (Genbank accession No.AB104462). The cDNA consists of 1365bp, which is 25bp longer than chicken CAR (Genbank accession No.AF276753). The A/B domain of the CAR gene was highly variable and the revealed sequence difference between mammals and birds. The fifteen amino acids deletion of mammals CAR and six amino acids insertion of chicken CAR were found in the D domain in comparison to Japanese quail CAR. No insertion or deletion were found in the DNA binding domain (C domain) and the ligand binding domain (E domain) among Japanese quail and other animals. Expression of Japanese quail CAR was detected in duodenum, liver, and kidney using RT-PCR method.

Keywords: nuclear receptor, constitutive androstane receptor, steroid hormone, Japanese quail

Introduction

The nuclear receptor class 1 superfamily includes receptors for many biological regulators, such as thyroid hormone, retinoids and vitamin D. These receptors control expression of many genes as heterodimers with 9-cis-retinoic acid receptors (RXRs). The DNA-binding domain of the nuclear receptor class 1 proteins and RXRs bind to the 5' upstream region of regulated genes.

A new orphan nuclear receptor has been isolated by Bases *et al.*[1] and identified that the expression of the gene is highest in the liver. The receptor, constitutive androstane receptor (CAR), belonging to the nuclear receptor class 1, subfamily I (NR1I3), forms heterodimers with RXR. These bind to retinoic acids-response elements (RAREs). Two androstane metabolites, androstanol and androstenol, were the ligands of CAR. The heterodimer, with these ligands, decrease expression of the target genes[2]. The CAR translocates to the mouse liver nucleus in response to Phenobarbital (PB) and other PB-type inducers[3]. The heterodimer with 1,4-bis[2-(3,5-dichlorpyridyloxy)] benzene (TCPOBOP) as a ligand, increases the expression of the cytochrome

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P450 enzyme gene[4].

The CAR protein might function in the domestic animals. These ligands, such as Phenobarbital and TCPOBOP, may be accumulated in the soil. If these substances are taken to the domestic animals with the feed, the metabolism of steroid hormones may be disturbed. Handschin *et al.*[5] isolated a chicken xenobiotic-sensing orphan nuclear receptor (CXR) gene, which is related to human and mouse CAR gene. Hence, it can be inferred that CXR gene is the same as chicken's CAR gene.

Since CAR gene of birds only been reported by Handschin *et al.*[5], it is necessary to examine CAR gene of birds more in detail. This study sequenced the full length cDNA of Japanese quail (*Coturnix japonica*) CAR by the rapid amplification of cDNA end (RACE) method. The mRNA expression of Japanese quail CAR was analyzed in duodenum, liver, pancreas, kidney and testis of mature male Japanese quail using RT-PCR method.

Materials and Methods

In this study, one Japanese quail bird was used for the extraction of total RNA. The bird was belong to the 82th generation of RR line in Saga University[6] and was a healthy mature male. The bird was bred under the same condition of other RR line quails up to 30 weeks at age and the total RNA was extracted from the duodenum, liver, pancreas, kidney and testis tissues of the bird using the QuickPrep Total RNA Extraction Kit (Amersham Biosciences). This experiment was performed in accordance with the guideline for animal experiment, Saga University.

The RT-PCR was used to obtain the partial cDNA segment of Japanese quail CAR using Ready-To-Go RT-PCR beads (AmershamBiosciences). The RT-PCR analysis for 200ng of total RNA derived from liver was done with the primer pair, qc1f/r, qc3f/r and qc4f/r, under an initial denaturing step at 95℃ for 5 min, 35 cycles were done in the following manner: 30sec at 95℃, 30sec at annealing temperature of Table 1, and 1 min at 68℃. The primer pairs were designed

Table 1. Primers used in this study listed from 5' to 3' and annealing temperature.

Name	Primer sequence	Annealing temperature (℃)
qc1f	gatccagcaggtcatcaaattc	60.0
qc1r	gagaggtcctggatgtgtaggat	
qc3f	ttcacccggagctgccccataa	65.0
qc3r	aagacgtcgggcagcacatcct	
qc4f	gtggggatgaggaaggacat	57.7
qc4r	tccttgatggtgaagcagtg	
qc3raceN	tgcccatcgacgaccagatctctct	63.0
QC-RT	tcgttgaacaccgtg	
QC-S1	aggtcatcaagttcgccaag	56.0
QC-A1	tctctggtgggcgctgatga	
QC-S2	agatctcgctgctcaaaggc	56.0
QC-A2	ctgcgcttggccttagttat	
qBACT2F	gttaccaactgggatgatatgg	60.0
qBACT2R	agggagttcatagctcttctcc	

from the DNA sequence of chicken CAR gene (Genbank accession No.AF276753). These RT-PCR products was checked in the 1.2% agarose gel and the products was purified using the MagExtractor PCR & Gel Clean up (Toyobo Co., Ltd.) and directly sequenced using ABI BigDye Terminator Cycle Sequencing Ready Reaction Kit v3.0 and ABI PRISM 310.

The 3'-Full RACE Core Set (TaKaRa Holdings, Inc.) was used to obtain the 3' full length segment of Japanese quail CAR mRNA. The ss-cDNA for 3' RACE was synthesized from total RNA with the oligo dT-3 sites adaptor primer supplied by the kit. The 3' RACE segment of Japanese quail CAR mRNA was amplified with the 3 sites adaptor primer and the gene specific primer qc3raceN (Table 1) which was designed from the partial cDNA sequences of Japanese quail CAR (Fig 1). After an initial denaturing step at 94 ℃ for 1 min, 35 cycles were done in the following manner: 30sec at 94 ℃, 30sec at 63 ℃, and 2 min at 72 ℃. Electrophoresis of the PCR product was done in the 1.2% agarose gel and the 700bp major band was purified using the MagExtractor PCR & Gel Clean up (Toyobo Co., Ltd.). The purified PCR product was subcloned to TA-vector, pT7Blue (Novagene, Inc.) using the DNA Ligation Kit Ver.2 (TaKaRa Holdings, Inc.): five clones were sequenced using ABI BigDye Terminator Cycle Sequencing Ready Reaction Kit v3.0 and ABI PRISM 310.

The 5'-Full RACE Core Set (TaKaRa Holdings, Inc.) was used to obtain the 5' full length segment of Japanese quail CAR mRNA. The ss-cDNA for 5' full RACE was synthesized from the total RNA with the gene specific primer, QC-RT. Concatenation was performed according to the manufacture's protocol. The 5' full RACE was done with the first PCR primer set, QC-S1 and QC-A1, and the nested PCR primer set, QC-S2 and QC-A2. After an initial denaturing step at 94 ℃ for 1 min, 35 cycles were done in the following manner: 30sec at 94 ℃, 30sec at 56 ℃, and 2 min at 72 ℃. The electrophoresis of the PCR product was done in the 1.2% agarose gel and the 470bp major PCR product was purified and subcloned; then, five clones were sequenced.

The DNA sequence of Japanese quail CAR full length mRNA (Genbank accession No.AB104462) was determined from DNA sequences of the RT-PCR products, 3'RACE clones and the 5'RACE clones. Alignment of the DNA sequences of chicken CAR (Genbank accession

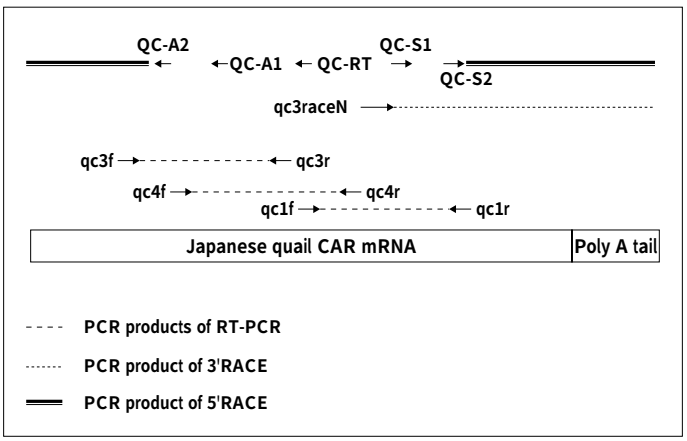

Fig. 1 Illustration of PCR primers and PCR products for Japanese quail CAR cDNA cloning.

No.AF276753) and Japanese quail CAR (this study) was computed by CLUSTAL W (Ver.1.82)[7]. The multiple alignment of the amino acid sequences of mouse CAR1 (Genbank accession No.NM_009803), human CAR (Genbank accession No.NM_005122), chicken and Japanese quail CAR was also computed by CLUSTAL W. The phylogenic tree of the amino acid sequences of Japanese quail CAR, chicken CAR, mouse CAR1, human CAR, mouse pregnane X receptor (PXR) (Genbank accession No.AF031814), human PXR (Genbank accession No.AF061056), Xenopus PXR (Genbank accession No.AF305201) and human vitamine D receptor (VDR) (Genbank accession No.AF026260) as an outgroup was estimated using neighbor-joining method[8] with 1000 times bootstrap analysis.

One tube RT-PCR analysis was performed to study the expression pattern of Japanese quail CAR mRNA using Ready-To-Go RT-PCR beads (AmershamBiosciences). The 600ng total RNA extracted from duodenum, liver, pancreas, kidney and testis was used as a template of the RT-PCR. Analysis was done with the primer pair, qc1f and qc1r. After RT reaction at 42 ℃ 30 min, the PCR analysis was performed under an initial denaturing step at 95 ℃ for 5 min, 35 cycles were done in the following manner: 30sec at 95 ℃, 30sec at 60 ℃, and 1 min at 68 ℃. One tube RT-PCR for β actin gene was also performed as a positive control. The positive control RT-PCR analysis for 200ng of the same total RNA sample was done with the primer pair of qBACT2F and qBACT2R at the same temperatures. Electrophoresis of 1.2% agarose gel was performed to check the mRNA expression.

Results and Discussion

The RACE method determined the nucleotide sequence of the full length Japanese quail CAR mRNA (Genbank accession No.AB104462). The mRNA consists of 1365bp, which was 25bp longer than chicken CAR (Genbank accession No.AF276753). The length of 5' untranslated region (5'UTR), coding region and 3' untranslated region was 130bp, 1158bp and 77bp, respectively. The length of 5'UTR was 84bp longer than that of chicken CAR mRNA (Fig. 2). The 6bp insertion, 6bp deletion, 7bp and 11bp deletion in 24bp region were found in the Japanese

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Quail CAR      GGGGGGGACGGAAACCAAAGGGAGAACGGGGACAAACCCCACAACGTGGGGACATTAGAG
Chicken CAR    ------------------------------------------------------------

Quail CAR      GGGACACGATCATAGAGGGGACACCACAACCACAGAGGGGACACAACAGCCATAGAGCTG
Chicken CAR    ------------CGTGGGGAC---ACAACCGCAGCGGTGACAC--CGGGCACAG-----
                          * ******   ****** *** ** *****  * * ** **

Quail CAR      ACACTACAGCCATGTCCCTGTGCAGCCCCTCGGACACAGACAGCGCCGTGCTGCAGTGTG
Chicken CAR    -CATCCCAGCCATGTCCCAGTCCAGCCCCTCGGACCCGGACAGCCCCGGGGCGCAGCGTT
                **   ************ ** ************ * ****** *** *  **** **
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Fig. 2 Alignment of 5' untranslated region and the head part of coding region of Japanese quail and chicken CAR mRNA.

The underlined ATG is the start codon of the mRNAs.

* indicates a common base to Japanese quail and chicken.

- indicates one base insertion.

quail CAR mRNA in comparison with chicken CAR mRNA. The 2 amino acids insertion, 2, 3 and 3 amino acids deletions and 21 amino acids substitutions were found in comparison with chicken CAR. Seven G bases are found at 5' end of Japanese quail CAR mRNA. It is necessary to accurately decide the 5' end of the mRNA comparing to the genome DNA sequence, since this poly-G might be an artifact.

The nuclear receptors have four domains, such as A/B domain, C domain, D domain and E domain. It is thought that the C domain is a DNA binding domain, the E domain is a ligand binding domain and the D domain is a hinge domain[9]. Figure 3 shows the multiple alignment of the A/B domain of the amino acid sequences among human, mouse, chicken and Japanese quail CAR mRNA. In this domain, 22 amino acids deletion of human, 12 amino acids deletion of mouse and 2 amino acids deletion of chicken were found in comparison with Japanese quail.

Figure 4 shows multiple alignment of the D domain of the amino acid sequences of human, mouse, chicken and Japanese quail CAR. The 15 amino acids deletion of mammals CARs and 6 amino acids insertion of chicken CAR were found in this domain in comparison to Japanese quail CAR. The function of the D domain is inferred to be related to the transcriptional control by the heterodimer of CAR and RXR. Differences between mammals and birds may indicate different systems of the transcriptional control, such as coactivators. No insertion or deletion were found in the DNA binding domain (C domain) and the ligand binding domain (E domain) among Japanese quail and other animals.

Figure 5 shows the 1000 times bootstrap neighbor-joining tree of the amino acid sequences of human CAR, mouse CAR1, chicken CAR, Japanese quail CAR, human PXR, mouse PXR, Xenopus PXR and human VDR. The phylogram of the tree was figured with human VDR as an outgroup; however, the neighbor-joining tree is an unrooted tree. The molecular genetic distances from the turning-point of CAR and

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Mouse CAR1      MTAMLTLETMASEEEYG-----------PRN
Human CAR       ---------MASREDE-------------LRN
Quail CAR       MSLCSPSDTDSAVLQCGTNVPDVPDVPEEPKV
Chicken CAR     MSQSSPSDPDSPGAQRCPNVTDVT--EEELKV
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Fig 3. Multiple alignment of the A/B domain of the amino acid sequences of human, mouse, chicken and Japanese quail CAR.
- indicates one amino acids insertion.

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Mouse CAR1      RKDMILSAEALALRRARQAQRRAEKA-SLQLNQQQKELVQILLGAHTRHVGPLFDQFVQF
Human CAR       RKDMILSAEALALRRAKQAQRRAQQT-PVQLSKEQEELIRTLLGAHTRHMGTMFEQFVQF
Quail CAR       RKDMIMSEEALCRRRALRLQRRLAH--PGGLTAEQQELIGILISAHQRTFDSSFSQFQHY
Chicken CAR     RKDMIMSEEALGRRRALRLQRRLAQAQPGGLTAEQQELISILIAAHKRTFDSSFSQFQHY
                ***** * ***  ***   ***       *   * **   *  ** *     * **

Mouse CAR1      KPPAYLFMHHRPFQ-PRGP--------------------VLPLLTHFADINTFMVQ
Human CAR       RPPAHLFIHHQPLP-TLAP--------------------VLPLVTHFADINTFMVL
Quail CAR       QPAVRLCIPGPCSQSPPGPTVPSC---P---CVDEDVLPDVFSILPHFADLSTFMIQ
Chicken CAR     QPAVRLCIPGPCSQSPPGPGVPSASLSPQLDCLDEDVLPDVFSILPHFADLSTFMIQ
                 *   *           *                    *     ****  ***
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Fig. 4 Multiple alignment of the D domain of the amino acid sequences of human, mouse, chicken and Japanese quail CAR.
* indicates a common amino acids between human, mouse, chicken and Japanese quail.
- indicates one amino acids insertion.

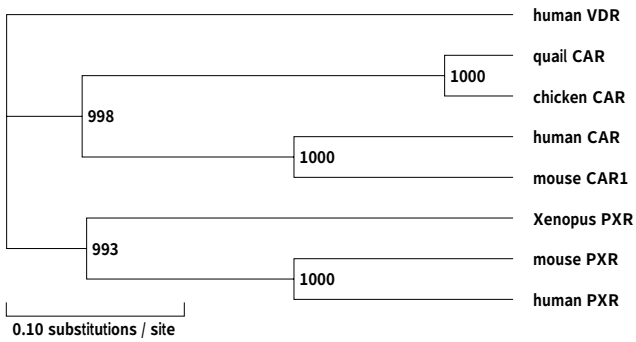

Fig. 5 Bootstrap neighbor-joining tree of the amino acid sequences of human CAR, mouse CAR1, chicken CAR, Japanese quail CAR, human PXR, mouse PXR, Xenopus PXR and human VDR.
The bootstrap values are indicated at the corresponding nodes.

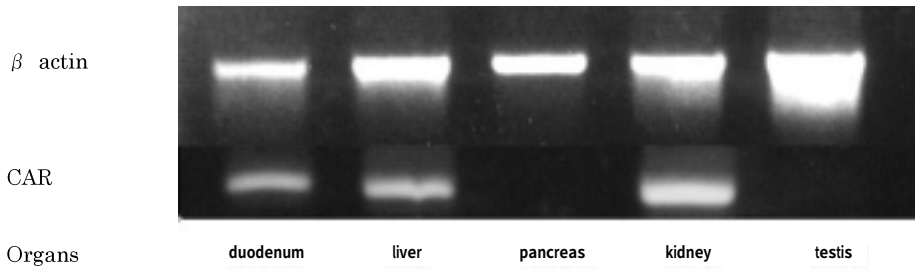

Fig. 6 Electrophoresis results of one-tube RT-PCR products for Japanese quail CAR and β actin.

PXR had almost equal length. The tree indicated that CAR and PXR gene had equal evolution rates of amino acid sequences.

Expression of Japanese quail CAR mRNA was detected in duodenum, liver, and kidney (Fig. 6). Handschin *et al.* [5] reported CAR expression of liver, kidney, small intestine and colon of chicken. Results of expression analysis for chicken and Japanese quail indicate that the mRNA of CAR was expressed mainly in main drug-metabolizing tissues, such as liver and kidney. Japanese quail CAR might have the function as an initial switch of the metabolism of the low-moleculer lipophilicity material including some drugs as human and mouse CAR.

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日本ウズラにおけるコンスティチューティブアンドロスタン受容体（CAR）の cDNA クローニング

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（動物資源開発学分野）

平成18年９月12日　受理

コンスティチューティブアンドロスタン受容体（CAR）は核内受容体クラス１のサブファミリーI に属しており，これまでの研究から，CAR 遺伝子は脊椎動物の生理機構，特にステロイドホルモンの代謝に関与する可能性が示唆されている．本研究では，日本ウズラの CAR の全長 mRNA の塩基配列を，肝臓より抽出した total RNA をテンプレートとした RACE 法を用いて決定した（Genbank Accession No. AB104462）．日本ウズラの CAR mRNA は1365塩基でニワトリの CAR よりも25塩基長かった．日本ウズラ，ニワトリ，ヒト，マウスの CAR のアミノ酸配列をマルティプルアライメントしたところ，A/B ドメインのアミノ酸配列は鳥類と哺乳類で大きく異なることが明らかとなった．また，日本ウズラの CAR の D ドメインは哺乳類の CAR よりも15アミノ酸長く，ニワトリの CAR の D ドメインよりも６アミノ酸短かった．近隣結合法を用いて脊椎動物における CAR と PXR のアミノ酸配列にもとづく分子系統樹を作成したところ，CAR と PXR の分岐から各遺伝子への遺伝的距離がおおよそ等しく，CAR と PXR が同様の進化速度を持つことが示唆された．各臓器から抽出した total RNA をテンプレートとした RT-PCR の結果，日本ウズラの CAR は十二指腸，肝臓，腎臓で発現しており，膵臓と精巣では発現が認められなかった．