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Chicken Ornithine Transcarbamylase Gene, Structure, Regulation, and Chromosomal Assignment: Repetitive Sequence Motif in Intron 3 Regulates This Enzyme Activity[1]

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Ornithine transcarbamylase (OTC) is one of the urea cycle enzymes. While the chicken is a uricotelic animal, it has measurable OTC activity in its kidney. OTC activity is highly variable within and between chicken breeds. Chicken OTC may have some physiological significance because of its significant activity in the kidney. We cloned the OTC cDNA from chicken kidney and found 77% homology between the deduced amino acid sequence of the mature protein and that of mammals. The chicken OTC gene spans 26 kb, consists of 10 exons and 9 introns, and utilizes the same exon-intron boundaries as the human gene. The 5′-flanking region contains a putative TATA box and two potential regulatory sites, but neither the 5′-flanking region nor the splice sites correlated with variation in OTC activity. In intron 3, two polymorphic sites were found: one comprising a deletion of 401 nucleotides; and the other was a length and sequence polymorphic region located 8 bases upstream from the deletion. The latter polymorphism provides an explanation for phenotypic variation in OTC. Linkage analysis has suggested reassignment of the chicken OTC gene from the suggested Z chromosome to chromosome 1q.

Key words: chicken, chromosomal localization, gene regulation, gene structure, ornithine transcarbamylase.

Avian species are uricotelic in terms of nitrogen excretion and do not have a functional urea cycle. Nonetheless, Tamir and Ratner (*1*) found activities of several urea cycle enzymes in chicken tissues. Their finding of an absence of carbamyl phosphate synthetase I (CPS I) in chicken supported the contention of no functional urea cycle in chickens (*1*). Since then little attention has been paid to the physiological role of the urea cycle enzymes other than arginase in chicken tissues.

In ureotelic animals, ornithine transcarbamylase (OTC: EC 2.1.3.3) catalyses the second step of the urea cycle, in which citrulline is synthesized from carbamyl phosphate and ornithine. It is located exclusively in the mitochondrial matrix of liver and intestinal epithelial cells. OTC deficiency in humans is one of the causes of inherited hyperammonemia. Complementary DNAs of OTC from human (*2, 3*), rat (*4, 5*), mouse (*6*), and frog (*7*) have already been cloned and sequenced. The human OTC gene consists of 10 exons which span 72 kb (*3*) and is located on chromosome Xp 21.1 (*8*). Promoter and enhancer elements have been identified in the rat OTC gene (*9-11*).

Chickens express OTC activity in the kidney at a level of about one-thousandth that of rat liver (*1*). This activity is highly variable within and between breeds of chickens (*12, 13*). We have maintained chickens expressing high OTC activity since 1972, and some enzymatic characteristics of this enzyme and its tissue activity have been described previously (*12, 14*). For example, OTC purified from kidney is a homotrimer of 36-kDa subunits, has kinetic properties similar to the mammalian enzyme, and is located in the mitochondria (*14*). Only in chickens expressing genetically high OTC activity is this enzyme induced by egg yolk feeding (*15, 16*). Both variation and induction of this enzyme are controlled genetically, and the gene coding for this enzyme was believed to be autosomal (*16, 17*), although the chicken OTC gene had been mapped to the short arm of the Z chromosome, near the centromere (*18*). Thus in contrast with enzymological studies, OTC gene structure, regulation and location have not been reported in chicken.

Chicken OTC reportedly has no physiological role since

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Abbreviations: C/EBP, CCAAT enhancer binding protein; cM, centi-Morgan; CPS, carbamyl phosphate synthetase; FISH, fluorescent *in situ* hybridization; LA PCR, long and accurate polymerase chain reaction; LOD, logarithm of odds; NF-κB, nuclear factor kappa B; OTC, ornithine transcarbamylase; SRY, sex-determining region Y gene product; SSCP, single strand conformation polymorphism.

its absence does not affect survival of chicks (*12*). A dispensable gene is ignored by natural selection and may, therefore, accumulate all manner of randomly sustained mutational base substitutions, insertions and deletions. For example, the nonfunctional human gene for L-gulono-γ-lactone oxidase, which catalyses the terminal step of L-ascorbic acid biosynthesis, has accumulated a large number of mutations in the absence of selection pressure (*19*). On the other hand, it has also been reported that a dispensable gene would not easily disappear from the genome of higher animals (*20*). As avian species have evolved independently of ureotelic mammals for 250 million years (*21*), it is interesting to examine how and why the OTC gene has been maintained in chicken.

In this report, we describe the nucleotide sequence of chicken OTC cDNA, exon-intron structure in comparison with human OTC gene, a regulatory region in intron 3, and chromosomal reassignment of the gene.

EXPERIMENTAL PROCEDURES

Chickens—White Leghorn B strain chickens, which express high kidney OTC activity, have been maintained in our laboratory since 1972 (*15*). The Barred Plymouth Rock breed, which expresses low OTC activity, was used as a reference breed for comparisons of OTC gene structure and activity. White Leghorn S46, MA, and BL strain chicks used for comparison of OTC activity between genotypes were maintained in the National Livestock Breeding Center, Okazaki station. For linkage analysis, White Leghorn (N strain)×Fyoumi (Fam strain) backcross chicken family was constructed at the Nippon Institute of Biological Science.

RNA Isolation—Kidneys were dissected from 6-week-old White Leghorn B strain chicks in which OTC activities had been induced by feeding egg yolk for 2 weeks (*15*). These were frozen immediately after dissection in liquid nitrogen. Total RNA was prepared from 2 g of frozen kidney by the guanidium thiocyanate-CsCl method (*22*). Poly(A)$^+$ RNA was purified by oligo (dT)-cellulose column chromatography for cDNA synthesis.

Construction and Screening of a Chick cDNA Library and DNA Sequencing—Chick kidney cDNA synthesis and a cDNA library construction were performed by use of cDNA Synthesis System Plus and cDNA Cloning System λgt11 (Amersham) respectively, according to the directions supplied. Approximately 6×10^6 clones were transferred to GeneScreen Plus® (Du Pont Company) and screened with a ^{32}P-labeled probe corresponding to the 810-bp fragment of rat OTC cDNA (*5*) kindly provided by Prof. M. Mori (Kumamoto University Medical School, Kumamoto). Hybridization was achieved by incubation overnight at 60°C. Membranes were washed at room temperature in 2× SSC for 15 min, at 60°C in 2×SSC/0.1% SDS for 30 min, and at room temperature in 2×SSC/0.1% SDS for 30 min, then autoradiographed. Positive clones were purified and the cDNA inserts were isolated and subcloned into M13-mp18 (Stratagene) for complete sequencing. Single-stranded M13 DNA was prepared, and each strand was sequenced by the dideoxynucleotide method (*23*). Sequences were analyzed on 6% Hydrolink long ranger (Bioprobe Systems, Montreui), 8 M urea sequencing gels, with 0.6×TBE (54 mM Tris borate, 1.2 mM EDTA) as running and gel buffers.

Primers—Primers used in this study are listed in Table I. The primers were purchased from Life Technologies and Takara Biomedicals. The primers to amplify introns were designed from chicken OTC cDNA sequence assuming the same exon-intron structure as the human OTC gene (*3*).

Structural Organization of the Chicken OTC Gene—The size and position of introns in the chicken OTC gene were determined by comparing the size of the products obtained

TABLE I. **Sequence of primers.** The sequences of primers used in polymerase chain reactions and sequencing are listed. Primers C1 and C2 are included in the TaKaRa LA PCR *in vitro* cloning kit and the 5′-flanking region was amplified by the nested-PCR method.

Primer	Direction[a]	Target[b]	Nucleotide sequence
1	S	Intron 1	5′-TCCAACTTCA TTTCTGTAAA CTCTATCTAC AGG-3′
2	A	Intron 1	5′-TTTGTCCTTT ATCCTTTGTT TCAAATCTGA GGC-3′
3	S	Intron 2	5′-TCGTGACCTC CTTACTCTAC AGAACTACAC AGC-3′
4	A	Intron 2	5′-TGTTCTTGTG CTTCTCTTCT CAAAAATCAT GGC-3′
5	S	Intron 3	5′-GCCATGATTT TTGAGAAGAG AAGCACAAGA AC-3′
6	A	Intron 3	5′-AATGTCTTGT TTTGTAAGGA AGGAAGAATG TC-3′
7	S	Intron 4	5′-TCTTCCTTCC TTACAAAACA AGACATTCAC CTG-3′
8	A	Intron 4	5′-CAGCTAAAAT CTGGAGAGGA TGGTATAAAT CAG-3′
9	S	Intron 5	5′-TGGCTCGAGT TTACAAGCAT AATGATCTGG ACC-3′
10	A	Intron 5	5′-CTTTGCAGCA CTTGTCATGA TGGAGTGGAG GAC-3′
11	S	Intron 6, 7	5′-AAACAATGTC CTCCACTCCA TCATGACAAG TGC-3′
12	A	Intron 6, 7	5′-CCCATGCTTA TCCATGTATC CGTAACTAAG ACG-3′
13	S	Intron 8	5′-TCTTAGTTAC GGATACATGG ATAAGCATGG GGC-3′
14	A	Intron 8	5′-TACTTCATCA TCAACTTCTT CGGGTTTTCT GGG-3′
15	S	Intron 9	5′-CCAGAAAACC CGAAGAAGTT GATGATGAAG-3′
16	A	Intron 9	5′-TGTAGGCATT TGTAGCTGTG GTGAGTAATC-3′
17	A	5′-flanking	5′-GCATCTTCTT GCACACCTTT TCTCTCAGTC CTG-3′
18	A	5′-flanking	5′-GATAGAGTTT ACAGAAATGA AGTTGGATGG AGC-3′
C1	S	5′-flanking	5′-GTACATATTG TCGTTAGAAC GCGTAATACG ACTCA-3′
C2	S	5′-flanking	5′-CGTTAGAACG CGTAATACGA CTCACTATAG GGAGA-3′
19	S	401-bp region	5′-CTACTTGCCC AACATACCAG AGACAGAC-3′
20	A	401-bp region	5′-CATGAAGTTC CACACAAGAC AGCACCTC-3′
21	S	Genetic diagnosis	5′-CCACAGCCTG AGACATAAAA CT-3′
22	A	Genetic diagnosis	5′-CTGGAGAGAC AGGATAGATT AC-3′

[a]S and A show sense and antisense, respectively. [b]Target shows the region being amplified by these primers.

by long and accurate polymerase chain reaction (LA PCR: Takara Biomedicals) amplification using genomic DNA of White Leghorn B strain as a template. All PCRs were carried out in a DNA thermal cycler (Perkin Elmer). The PCR reactions were carried out in a final volume of 50 μl containing 25 pmol each of primer, 200 ng of chicken genomic DNA, 200 μM each of four deoxynucleoside triphosphates, 1.25 units of TaKaRa Ex Taq™ (Takara Biomedicals). The reaction conditions were 94°C, 1 min for 1 cycle; 94°C for 30 s and 68°C for 3-15 min for 30 cycles; 72°C, 10 min for 1 cycle; and the block temperature was held at 4°C. Amplified products were purified by use of a QIAEXII Gel Extraction Kit (QIAGEN) after electrophoresis on 1% agarose gel, then directly sequenced for determining the exon-intron boundary using the Silver Sequence™ DNA Sequencing system (Promega).

Identification of the 5′-Flanking Region of the OTC Gene—Identification of the 5′-flanking region was performed by use of an LA PCR *in vitro* cloning kit (Takara Biomedicals). Chicken genomic DNA was digested with *Pst*I restriction endonuclease, and ligated to *Pst*I cassette. One microliter of this ligation mixture was used as a template. The first PCR reactions were carried out in a final volume of 50 μl containing 10 pmol each of primers C1 and 17 (Table I), 1 μl of this ligation mixture, 400 μM each of four deoxynucleoside triphosphates, 2.5 units of TaKaRa LA Taq™. The reaction conditions were 94°C, 1 min for 1 cycle; 94°C for 30 s and 68°C for 5 min for 30 cycles; 72°C, 7 min for 1 cycle; and the block temperature was held at 4°C. One microliter of this PCR reaction mixture was then used for reamplification with primers C2 and 18 (Table I) under the conditions for the first PCR reaction. Primers C1 and C2 are included in the kit. After electrophoresis on 1% agarose gel, the PCR products were purified from the gel and sequenced as above. Potential transcription factor-binding sites within the 5′-flanking region were searched for using TFSEARCH program. TFSEARCH program searches for highly correlated sequence fragments *versus* TFMATRIX transcription factor binding site profile database in TRANSFAC databases by GBF-Braunschweig (*24*).

Size Heterogeneity and Determination of Polymorphic Sites in Intron 3 of the Chicken OTC Gene—Size heterogeneity was observed in the PCR products containing intron 3 of the chicken OTC gene. Alleles of different sizes were easily detectable on 1% agarose gel and segregated following simple Mendelian genetics.

The PCR products containing intron 3 were amplified using genomic DNA of White Leghorn B strain and N strain as a template. Each product was digested with *Pst*I restriction endonuclease, and polymorphic fragments between them were subcloned into Bluescript SK$^-$ (Stratagene). The obtained clones were purified and sequenced. The primers were synthesized to amplify and to sequence the polymorphic sites.

Genotyping and Comparisons of OTC Activity between White Leghorn Chickens—Sequencing of the size heterogeneity region in intron 3 showed new length and sequence polymorphism. Genotyping of this region was performed by PCR-SSCP. The PCR reactions were carried out in a final volume of 10 μl containing 5 pmol of primers 21 and 22 (Table I), 40 ng of chicken genomic DNA, 200 μM each of four deoxynucleoside triphosphates, 0.25 unit of TaKaRa Ex Taq™. The reaction conditions were 94°C, 1 min for 1 cycle; 94°C for 30 s, 60°C for 30 s, and 72°C for 30 s for 30 cycles; 72°C, 7 min for 1 cycle; and the block temperature was held at 4°C. After the reaction, these products were diluted 10 times with a solution containing 95% formamide, 10 mM EDTA (pH 7.5), 0.05% bromophenol blue, and 0.05% xylencyanol, heat-denatured for 5 min at 80°C, and typed by 6% PAGE and silver staining. To examine the relationship between genotypes and OTC activity, White Leghorn S46, BL, and MA strain DNAs were genotyped by PCR-SSCP on the polymorphic region.

Chickens which were homozygous for each allele (HH, LL, and NN as shown in Table III) were selected as parents and the six chick groups encompassing all genotypes were constructed by specific mating. OTC activity of 2-day-old chick kidney was measured as described (*17*). One unit of OTC was defined as the amount of enzyme forming 1 μmol of citrulline per hour at 37°C. Statistical analysis was carried out by Least-Squares Analysis of Variation (*25*).

Gene Mapping of OTC—A backcross family was constructed using inbred lines of chickens (White Leghorn N strain and Fyoumi Fam strain). A total of 129 backcross chicks were used to map the OTC gene. White Leghorn N strain and Fyoumi Fam strain are homozygous for 4.8 and 5.2 kb products, respectively. The presence or absence of the Fyoumi-specific products was chased in backcross chicks. Seven microsatellite markers used in this study were ADL0101, ADL0134, ADL0150, ADL0353, LEI-0071, LEI0088, and MCW0061, on which information was obtained from the East Lansing genetic map (through the Internet: http://poultry.mph.msu.edu/). Backcross chick DNAs were typed for these microsatellite markers as follows. The PCR reactions were carried out in a final volume of 10 μl containing 5 pmol of ^{32}P end-labeled forward primer, 5 pmol of reverse primer, 40 ng of chicken genomic DNA, 200 μM each of four deoxynucleoside triphosphates, 0.25 unit of TaKaRa Ex Taq™. The reaction conditions were 94°C, 1 min for 1 cycle; 94°C for 30 s, 53-60°C for 30 s, and 72°C for 30 s for 30 cycles; 72°C, 7 min for 1 cycle; and the block temperature was held at 4°C. These products were then typed by using PAGE and autoradiography. The segregation data were analyzed by Mapmaker (*26*). All markers were grouped using threshold linkage criteria of logarithm of odds (LOD) scores of 3.0 and a linkage group determined maximum likelihood order and genetic distance. Genetic distance was calculated from recombination fractions by means of the Kosambi mapping function (*27*) and was represented as centi-Morgan units.

RESULTS

Isolation and Characterization of Chicken OTC cDNA—Eight clones were detected by screening about 6×10^6 independent clones from a λgt11 cDNA library of chick kidney, using a radiolabeled 810-bp *Eco*RI fragment of rat OTC cDNA as a probe. One of the eight, CKO101, covered the entire OTC mRNA, and was sequenced. As shown in Fig. 1, the nucleotide sequence of CKO101 spanned 1,297 bp from its 5′ end to the poly(A)$^+$ tail. The first 84 bases of the cDNA clone were the 5′-untranslated region, while the last 151 bases (1147-1297) were a termination codon and the 3′-untranslated region. A termination codon and the 3′-untranslated region contained the polyadenylation signal AATAAA located 21 bp upstream from the site of poly-

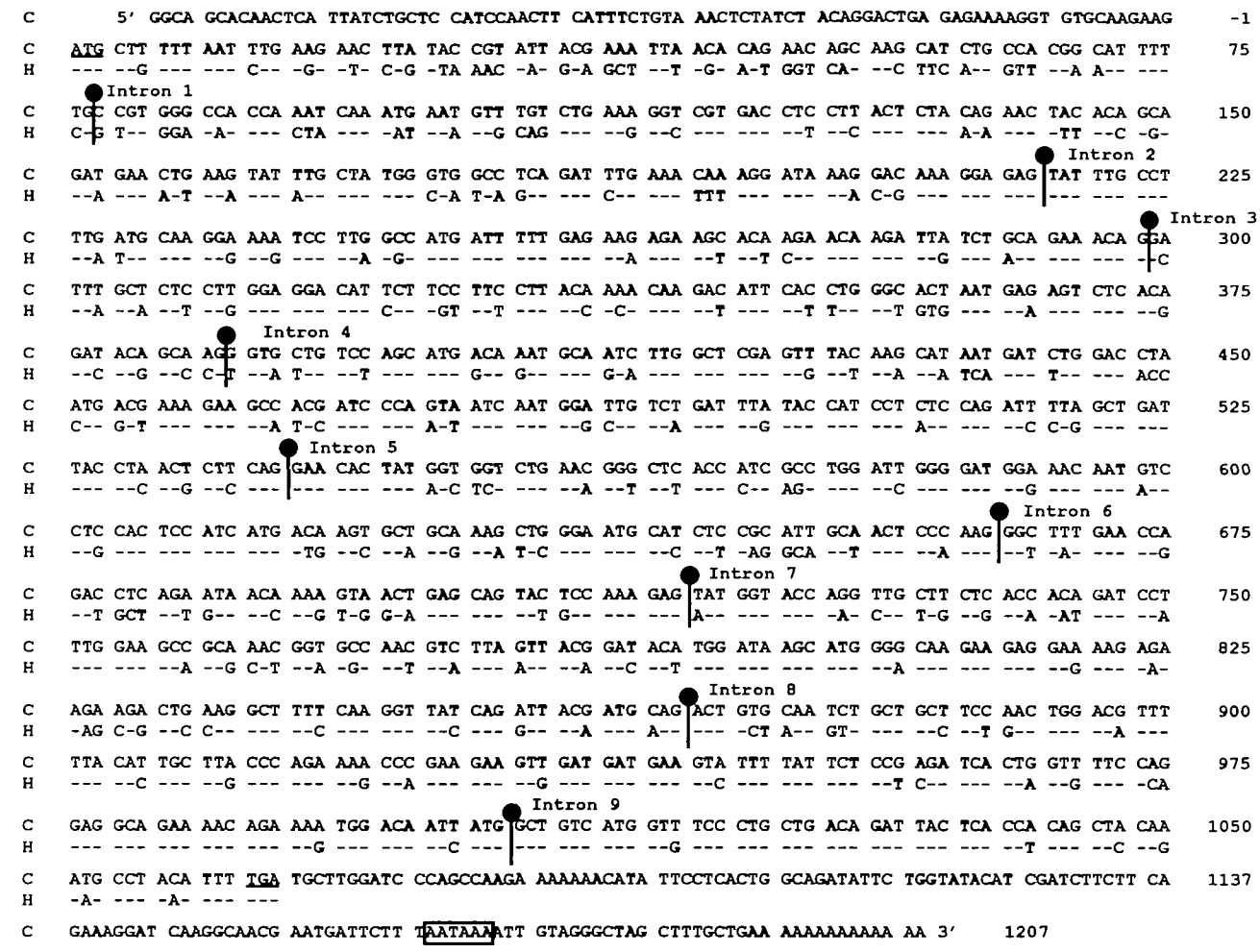

Fig. 1. **Complementary DNA sequence of chicken OTC and intron positions of the gene compared with human OTC.** C and H represent chicken and human, respectively. Position +1 starts at the first nucleotide of the initiation codon. The initiation and stop codons are indicated by underlines. Open box indicates the poly A signal AATAAA. The nucleotide sequence of the human OTC coding region is taken from Hata *et al.* (*3*). Dashes are identical nucleotides to the chicken sequence. Intron positions are shown by vertical lines (|) with intron numbers. Intron positions of the chicken OTC gene are from Table II and those of human OTC gene are from Hata *et al.* (*3*).

adenylation.

The cDNA contained an open reading frame of 1,062 bp. The precursor amino acid sequence deduced from the cDNA (Fig. 2) specified a protein of 40,316 Da molecular mass. Correspondence between N-terminal sequence data (not shown) and the deduced sequence of residues 33-38 (MNVCLK) suggested that a 32 amino acid leader sequence must be present in the nascent protein. The translated sequence suggested a mature protein of 36.4 kDa molecular mass, which is in good agreement with the previous SDS-PAGE data (*28*).

Comparison of Amino Acid Sequence of OTC—Figure 2 compares the deduced amino acid sequence of chick OTC cDNA with the published OTCs from human (*2*), rat (*5*), and mouse (*6*). Mature chicken OTC has 322 amino acid residues, the same number as mammalian OTCs, and exhibited 78.3, 77.6, and 77.6% identity to OTCs from rat, mouse, and human, respectively. Putative binding sites for the two OTC substrates, carbamyl phosphate (90-94) and ornithine (300-305), were completely conserved among these species (*29*). The leader sequence of chicken OTC exhibited 34.4, 37.5, and 34.4% identity to OTC leader sequences of rat, mouse, and human, respectively. Leader sequences of these species were cleaved off at the same position, and were relatively rich in basic and hydroxylated residues and devoid of acidic residues, as is characteristic of leader sequences of mitochondrial targeted proteins (*30, 31*). The conserved arginine-23 plays a critical role in targeting precursor OTC to mitochondria (*32*). Thus, the deduced amino acid sequence of chicken OTC supports previous conclusions based on enzymological data, that chicken OTC is very similar to mammalian OTCs (*14*).

Organization of the Chicken OTC Gene—The positions of introns within various genes are highly conserved between species. To define positions of introns and their boundaries in the chicken OTC gene, the human OTC gene was chosen as the reference, and primer sets for each corresponding intron were designed (Table I). Regions including introns were amplified by LA PCR using genomic DNA of White Leghorn B strain as a template. Both ends of each PCR product were sequenced. The nucleotide sequences obtained were compared with the corresponding cDNA sequences and the intron positions determined. As shown in Table II and Fig. 3, the chicken OTC gene

comprised 10 exons and 9 introns. The 10 exons ranged in size from 54 bp (exon 7) to 194 bp (exon 10), and the 9 introns from 102 bp (intron 7) to 7 kb (intron 5). The chicken OTC gene spanned 26 kb. As shown in Table II, all of the splice donor and acceptor sites complied with the GT/AG rule (*33*).

Mutations in exon-intron boundaries sometimes disturb mRNA splicing and thus affect the amount of gene product (*34, 35*). In this experiment, we compared nucleotide sequences of exon-intron boundaries between Barred Plymouth Rock and White Leghorn B strain genes, since these breeds differ in the level of expression of OTC activity of kidney. No sequence differences were observed between these two breeds.

Comparison with the Human OTC Gene—The deduced amino acid sequences of mature OTCs exhibit a striking homology between chickens and mammals. This finding may suggest that chicken OTC has some physiological role even though the urea cycle is non-functional in these animals. To obtain further evidence, the structural features were compared between chicken and human genes.

Figure 1 shows that the position of introns in the coding sequence of chicken and human OTC. It is apparent that all exons in the chicken gene were interrupted precisely at the same position as in the human OTC gene.

Figure 3 compares schematically the OTC gene structures of chicken and human. In contrast to the conservation of the exon-intron boundaries, most introns of the chicken OTC gene are smaller than the corresponding introns of the human gene, except that intron 5 of the chicken OTC gene (7 kb) is larger than the corresponding human intron (1.9 kb). Total length of the gene in chicken was 1/3 the length of the human gene as a result of size differences between chicken and human introns.

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                ←···  Leader Sequence  ···→
C  MLFNLKNLYR ITKLTQNSKH LPRHFCRGPP NQMNVCLKGR DLLTLQNYTA  50
R  --S--RI-LN KAA-RKAHTS MV-N-RY-K- V-SQ-Q---- -----K-F-G
M  --S--RI-LN NAA-RKGHTS VV---WC-K- V-SQ-Q---- -----K-F-G
H  -----RI-LN NAAFRNGHNF MV-N-RC-Q- L-NK-Q---- -----K-F-G
                          ▲

C  DELKYLLWVA SDLKQRIKDK GEYLPLMQGK SLAMIFEKRS TRTRLSAETG 100
R  E-IQ-M--LS A---F---Q- ------L--- --G------- ------T---
M  E-IQ-M--LS A---F---Q- ------L--- --G------- ------T---
H  E-I--M--LS A---F---Q- ------L--- --G------- ------T---

C  FALLGGHSSF LTKQDIHLGT NESLTDTARV LSSMTNAILA RVYKHNDLDL 150
R  -------P-- L-T------V ---------- -----D-V-- ----QS---I
M  -------P-- L-T------V ---------- -----D-V-- ----QS---T
H  -------PC- P-T------V ---------- ----AD-V-- ----QS---T

C  MTKEATIPVI NGLSDLYHPL QILADYLTLQ EHYGGLNGLT IAWIGDGNNV 200
R  LA------IV ----------I ---------- ----S-K--- LS-------I
M  LA---S--IV ----------I ---------- ----S-K--- LS-------I
H  LA---S--I- ----------I ---------- ---SS-K--- LSCF-----I

C  LHSIMTSAAK LGMHLRIATP KGFEPDLRIT KVTEQYSKEY GTRLLLTTDP 250
R  -----M---- F----QA--- --Y---PN-V -LA---A--N ----SM-N--
M  -----M---- F----QA--- --Y---PN-V -LA---A--N --K-SM-N--
H  -----M---- F----QA--- --Y---ASV- -LA---A--N --K----N--

C  LEAANGANVL VTDTWISMGQ EEEKRRRLKA FQGYQITMQT VQSAASNWTF 300
R  ----R-G--- I---------- -D--KK--Q- -----V--K- AKV---D---
M  ----R-G--- I---------- -D--KK--Q- -----V--K- AKV---D---
H  ----H-G--- I---------R ----KK--Q- -----V--K- AKV---D---

C  LHCLPRKPEE VDDEVFYSPR SLVFQEAENR KWTIMAVMVS LLTDYSPQLQ 350
R  ---------- ---------- ----P----- ---------- -------V--
M  ---------- ---------- ----P----- ---------- -------V--
H  ---------- ---------- ----P----- ---------- ----------

C  MPTF  354
R  K-K-
M  K-K-
H  K-K-
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Fig. 2. **Alignment of the deduced amino acid sequences of chicken, rat, mouse, and human OTC.** C, R, M, and H represent chicken, rat (*5*), mouse (*6*), and human (*2*) OTC. Dashes indicate identities to chicken sequence. Leader sequence is indicated by ←•→. Triangle (▲) shows arginine-23, which has a critical role in targeting of precursor OTC to mitochondria (*32*). Putative binding sites for two OTC substrates, carbamyl phosphate (90-94) and ornithine (300-305), are indicated by underlines (*29*).

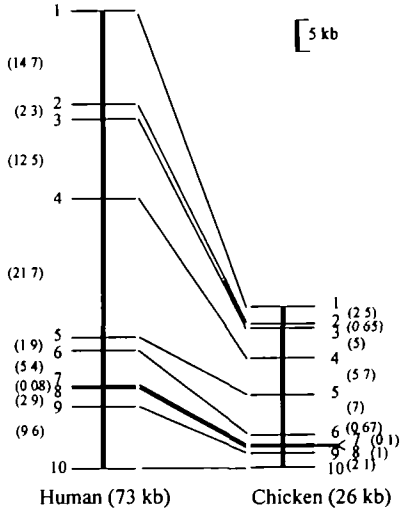

Fig. 3. **Comparison of structural organization of the OTC genes between chicken and human.** Horizontal lines with numbers and vertical lines show exons and introns, respectively. Figures in parentheses show the intron size. Structural organization of the chicken OTC gene is from Fig. 1, while that of the human OTC gene is from Hata *et al.* (*3*). Total sizes of the human and chicken OTC gene are about 73 and 26 kb, respectively.

TABLE II. **Nucleotide sequences around exon-intron boundaries of the chicken OTC gene.** Exon sequences are shown in uppercase letters and intron sequences in lowercase letters.

Exon No.	Exon size (bp)	Exon	Intron	Exon
1	>161	GGCATTTTTG	gtaagcttat ·····	ttttcacag CCGTGGGCCA
2	139	CAAAGGAGAG	gtaaaatgct ·····	tattgtccag TATTTGCCTT
3	82	GCAGAAACAG	gtaaattgga ·····	ctccttgcag GATTTGCTCT
4	88	ATACAGCAAG	gttggtaaga ·····	catcccccag GGTGCTGTCC
5	154	AACTCTTCAG	gtaccaatat ·····	cttttggcag GAACACTATG
6	123	AACTCCCAAG	gtataaaagc ·····	cttttggcag GGCTTTGAAC
7	54	CTCCAAAGAG	gtatgagccc ·····	ttcacattag TATGGTACCA
8	150	TACGATGCAG	gtaaaaattc ·····	tgatctacag ACTGTGCAAT
9	138	GACAATTATG	gtaaaaaaac ·····	cctcctctag GCTGTCATGG
10	194			

5′-Flanking Region of the OTC Gene—The 5′-flanking region of the chicken OTC gene was amplified by use of an LA PCR *in vitro* cloning kit and sequenced. Figure 4 shows that nucleotide sequence of this region contained a putative TATA box, TATAAG, located 120 bp upstream from the first nucleotide of the initiation codon. The potential transcription factor-binding sites were searched for within this region using the TFSEARCH program. Figure 4 also shows a potential binding site for CCAAT/enhancer binding protein (C/EBP: −226−−210) and a potential binding site for nuclear factor kappa B (NF-κB: −516−−507). The nucleotide sequence of this region was compared between White Leghorn B strain and Barred Plymouth Rock chickens, showing no replacement, insertion, or deletion. It was concluded that this region cannot account for the variation in OTC activity between breeds of chickens.

Size Heterogeneity in Intron 3—As shown in Fig. 5, amplification of intron 3 gave products of 5.2 and 4.8 kb. This size heterogeneity in intron 3 was examined using several domestic chicken breeds and jungle fowl. The large product was predominantly observed in domesticated chicken breeds, while the small product was predominant in jungle fowl. Only two domesticated chicken breeds, White Leghorn and Black Minorca, had the small product (data not shown).

Determination of Polymorphic Sites—To reveal the cause of size heterogeneity in intron 3, we attempted to determine the nucleotide sequence of intron 3 and its flanking regions. As shown in Fig. 6B, the size heterogeneity of intron 3 occurred by unique insertion/deletion of 401 bp. We found that the small product correlated actually with no OTC activity, suggesting that this region may have a role in regulation of the enzyme activity. We then sequenced around this 401-bp deletion/insertion. As shown in Fig. 6, A and C, there are three different sequences located 8 bp upstream from the 401-bp deletion/insertion. This polymorphic region consisted of different repetitive numbers of the unit "AAAAAC," designated H, L, and N alleles. H and L alleles produce only the large products, while N allele produces both large and small products.

Length and Sequence Variation in Intron 3 and OTC Activity—To reveal the relationships between allelic types and OTC activity, three different parents homozygous for H, L, and N alleles were selected by genotyping within the White Leghorn chicken strains of Okazaki station. These parents were then mated to produce six different genotypes

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-784                    GTAGATGAGGGAGTGAAGGTATCCGCATTGGCCA
-750  CCACAATCATAGAACCATACAATCATTAAGGTTGGAGAAGACCTCTAAGA
-700  TCACCTACCTAGTCCAGCCATCAACCCATCCCCACCATGCCCACTGCCCA
-650  TGGCCCTCAGTGCCACATCCACATCCACACTGTTCTTAAACACTTCCAGG
-600  GATGGTGACTCCACCACCTCCCTGGTCAGCCTGTGCCACTGCCTCACTGC
-550  TCTTTTGGAGAAGAAATTGTTCCTATAAACAAATGGAAACTCCCCTGGCA
                                   *1
-500  CAATGTGAAGCCATTCACTTTTGTCCTATCACTGTTACCCAGGAGAAGAG
-450  GCTGACCTCCACCTCACCGCATCCTCCTTCCAACTCCAACTCCTCCAACT
-400  TCCAGGGTCCCTAATGGTCCTGGCCCCCAATGCCAATCTGCTCTGCTCAT
-350  GCTGTTCTATGGTGATTCTTTCACCTTTTTCTCCTAGGACAAATATTTCA
-300  CCAACAGGAAATTGCTGCAAAATGATATTGACACAGTGGTAATATGATGA
-250  TCTGACACAGAAGAGCACAAACTGAGAGTTGTGTAATGGACCCTGTGAAG
                              *2
-200  AGTCTTTATCTGTAGAATTCGACCAAAGAAAGATTCTGTTTCGGCCCCAG
-150  CCAGGCTCGGCCCACACTGGTAAAATCAAGTATAAGATTGTCCTTTGCAC
-100  TCCACTCTTGTTGCACGGCAGCACAACTCATTATCTGCTCCATCCAACTT
                    *3
 -50  CATTTCTGTAAACTCTATCTACAGGACTGAGAGAAAAGGTGTGCAAGAAG
  +1  ATGCTTTTTAATTTGAAGAACTTATACCGTATTACGAAATTAACACAGA-
```

Fig. 4. **Nucleotide sequence of the 5′-flanking region of the chicken OTC gene.** The first nucleotide of initiation codon is numbered as +1. Open box shows a putative TATA box. Potential binding sites for C/EBP and NF-κB are shown by *1 and *2, respectively. *3 shows the 5′-terminus of the CKO101 clone (Fig. 1).

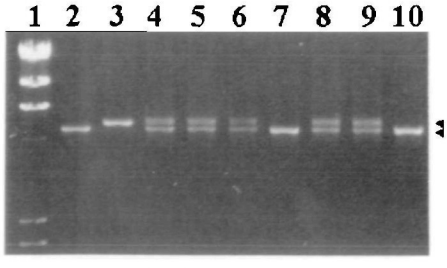
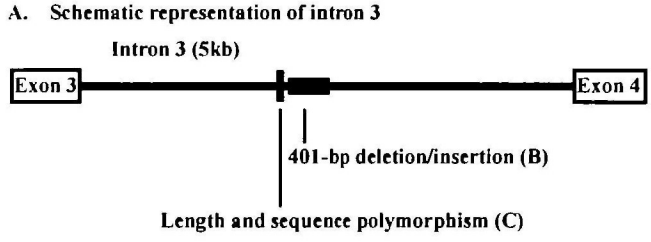


Fig. 5. **Size heterogeneity in intron 3.** The region including the intron 3 of the OTC gene was amplified using primers 5 and 6 (Table I) in chickens. Arrowheads indicate 5.2 kb (large) and 4.8 kb (small) PCR products, respectively. Lanes 2, 6, 9, and 10 are from male samples, and lanes 3, 4, 5, 7, and 8 are from female samples. Lane 1 indicates lambda DNA *Hin*dIII digests.



B. 401-bp deletion/insertion

```
ATTAAAAAAACAAAAACAAAAACAAAAAACAACCAAAAAACTATCAGGag
taaagctgtggtaatgagacagtaatctatcctgtctctccagtatggat
ttgtccaatacagtttgctgaagataccggggttgatatgaacacttagc
gtgtggctttgggaatcaccagattgtgacaacaaagaggattacatctt
ccagtttaaggctgcactccagcatgcgctaatttgctgagcaggtgcct
gcatgtttttagaagcaccagctattttctttatactgataatctggatg
aaagaatctaaaacacagattctcctcctgtaactgtaaacaaaactaga
gaacaggctcctgtatttccagatgggaagttttggatggaagtacattc
ccaggaaatgcatggatggccagttcatgagaagaaactcaacacattaA
GACCTTGCT
```

C. Length and sequence polymorphism

```
N-allele: ATT AAAAAAACAAAAACAAAAACAAAAAACAACCAAAAAA CTA
L-allele: ATT AAAAAAAC------AAAAACAAAAAACAACCAAAAAA CTA
H-allele: ATT TAAAAAACA-----AAAAACAAAAAAACCCCAAAAAG CTA
```

Fig. 6. **Schematic representation of the OTC intron 3 in which polymorphisms are observed (A), nucleotide sequences of 401-bp deletion/insertion (B), and length and sequence polymorphism (C).** A: Open box, closed box, and vertical line show exon, 401-bp deletion/insertion, and length and sequence polymorphism, respectively. Length and sequence polymorphism is located in 8 bp upstream from 401-bp deletion/insertion. B: Lowercase letters represent the nucleotide sequence of a 401-bp deletion/insertion. C: This region consists of three alleles named H, L, and N. Genotyping of this polymorphism was performed by PCR-SSCP. Dashes are gaps in the sequence.

TABLE III. **Relationships between genotypes and OTC activity.** Least-square means of OTC activity for each genotype and the statistical significance of differences in OTC activities between genotypes, are estimated by analysis of variance.

Genotype[a]	No. of chicks[b]	OTC activity (units)[c]
HH	7	222.4[d]
HL	7	105.3[e]
HN	7	99.2[e]
LL	6	44.6[f]
LN	6	14.5[g]
NN	6	1.1[g]

[a]Genotype was determined by PCR-SSCP using length and sequence polymorphism (see "EXPERIMENTAL PROCEDURES"). [b]White Leghorn chicks of all genotypes were produced by Mr. Hirooka at National Livestock Center, Okazaki Station. [c]Means with different superscripts are significantly different from each other ($p<0.01$).

as shown in Table III. Offspring of each genotype were phenotyped on the basis of kidney OTC activity. Table III shows a clear relationship between genotype and OTC activity. The genotype HH chicks showed the highest value of all genotypes, while the genotype NN chicks gave very low activity. The ranking of each genotype with respect to OTC activity was as follows: HH>HL>HN>LL>LN>NN, and differences were statistically significant ($p<0.01$) between each pair except for HL-HN and LN-NN. These results suggested that each allele made a unique contribution to OTC activity and that this allelic polymorphism controls variation of OTC activity in chicken.

Linkage Analysis of the OTC Gene—In a previous report, the chicken OTC gene was mapped to the short arm of the Z chromosome (*18*). However, as shown in Fig. 5, size heterozygotes of intron 3 was observed in both sexes. This finding suggested that the chicken OTC gene was located on an autosome. For this reason preliminary chromosomal localization of the OTC gene was performed by fluorescent *in situ* hybridization (FISH) using part of the chicken OTC gene as a probe and metaphase spreads. These results suggested that the chicken OTC gene was located on the long arm of chromosome 1 (data not shown).

Using the 401-bp deletion/insertion in intron 3 as a genetic marker, genetic linkage analysis was made employing seven known genetic markers on 129 progeny of backcross chicks (White Leghorn N strain×Fyoumi Fam strain). Seven known genetic markers used in this experiment were microsatellite markers assigned to chromosome 1q in chicken. Six of seven markers were polymorphic between parents. Using these six markers, the Fyoumi-specific allele was chased in backcross chicks. These segregation data were analyzed by "Mapmaker." As shown in Fig. 7, 2-point LOD scores indicated that OTC gene was significantly linked to three genetic markers, ADL0353, LEI0088, and MCW0061 (LOD=16.14, 10.48, and 3.05, respectively). The maximum likelihood order and genetic distance calculated from recombinant fraction was (ADL-0353) 13.6 cM (OTC) 21.9 cM (LEI0088) 16.9 cM (MCW-0061). The order of these microsatellite loci also corresponded to that in the East Lansing reference population. Thus we concluded that OTC locus is located on chromosome 1q in chicken.

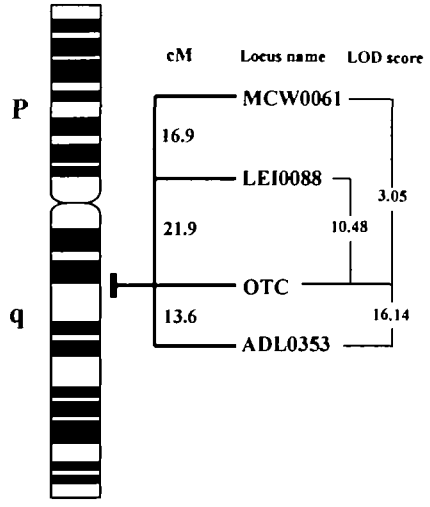


Fig. 7. **Genetic linkage map for the chicken chromosome 1q showing the OTC gene and other markers.** Using the 401-bp deletion/insertion in intron 3 as a genetic marker, genetic linkage analysis was made employing microsatellite markers on 129 progeny of backcross chicks (White Leghorn N strain×Fyoumi Fam strain). Microsatellites obtained from East Lansing genetic map homepage (http://poultry.mph.msu.edu/) have already been mapped to chromosome 1q for the East Lansing reference population, and here show microsatellites significantly linked to OTC locus (LOD score>3.0). Maximum likelihood order and genetic distance was obtained by Mapmaker (*26*). Genetic distance was calculated by means of the Kosambi mapping function (*27*).

DISCUSSION

Chicken OTC is believed to have a redundant physiological role, since absence of this activity does not affect the survival of chicks (*12*). This activity is about one-thousandth that of rat liver (*1*). Genes that lack a physiological role are ignored by natural selection and are free to accumulate random base substitutions, insertions and deletions. In this study we have described the nucleotide sequence of cDNA and exon-intron structure of the chicken OTC gene.

Chicken OTC cDNA shows no insertions or deletions that could explain its low enzyme activity. In addition, the chicken OTC cDNA demonstrates conserved putative binding sites for the two OTC substrates, carbamyl phosphate and ornithine, and leader sequences for importation into mitochondria. All exon-intron boundaries of the OTC gene were completely conserved between chicken and human, and the sequences of all splice donor and acceptor sites in the chicken OTC gene complied with the GT-AG rule regardless of activity.

One feature of chicken OTC is the highly variable activity within and between chicken breeds. Here, we attempted to determine the genetic basis of activity. A putative TATA box was present in the 5′-flanking region of the chicken OTC gene. Even though both promoter (*9, 10*) and enhancer (*9, 11*) elements have already been identified in the rat OTC gene, a TATA box was not identified in the regulatory region. A unique feature of the putative TATA box (TATAAG) of the chicken OTC gene is the existence of G at the 6th position. Wobbe and Struhl (*36*) analyzed the DNA sequence requirements for TATA element function by assaying the transcriptional activities of 25 promoters including the 18 single-point mutants of the consensus

sequence TATAAA, and reported that the TATAAG sequence had about 50% activity of the consensus element. A single-base transition in the basal promoter may explain why chicken OTC activity is low. However, this hypothesis can only be confirmed by an *in vivo* transfection experiment that can compare this region with the canonical TATA box. In addition potential binding sites for C/EBP (−226−−210) and for NF-κB (−516−−507) were found in this region by TFSEARCH program. Binding sites for C/EBP were identified in a region 11 kb upstream of the rat OTC gene (*9, 11*). NF-κB activates the gene for nitric oxide synthase. Nitric oxide synthase converts arginine into both nitric oxide and citrulline and constitutes a NO-citrulline cycle that can be regarded as a bypass of the urea cycle (*37*). However, as no significant replacement, insertion, or deletion was observed in this region in regard to the White Leghorn B strain and Barred Plymouth Rock breed, we concluded that the 5′-flanking region can not account for variation in activity.

Some introns contain regulatory sequences such as enhancers or silencers. In this study we found deletions, as well as length and sequence polymorphism in intron 3, which correlates with variation of activity. This length and sequence polymorphism is mainly due to the number of repeats of a unit "AAAAAC," and this was assigned into three alleles designated H, L, and N. Each allele has a different effect on activity, and genotypes described by these alleles cover almost all the range of activities found in chickens. Recently regulation of expression by allelic polymorphism has been reported in the human insulin gene (*38*), the human histo-blood group ABO genes (*39*) and others. In the case of the insulin gene, the polymorphic region consists of minisatellites containing the binding sites for transcription factors. Both the number and sequence variation of minisatellites regulate transcriptional activity (*38*). In the chicken OTC gene, the region of repetitive "AAAAAC" may be a transcription factor-binding site, because AT-rich regions and "AAACAAA" are unique SRY protein binding sites (*40*). We have already found binding proteins to this region using chick kidney nuclear extracts (data not shown). It is possible that sequence variation in this regulatory region alters the binding of transcription factors and leads to variation in activity. It is yet to be proven that derived allelic polymorphism directly regulates this activity.

The OTC locus has been mapped to the sex chromosome of both eutherian mammals (*8*) and chicken (*18*). This represents the first evidence of a mammalian X-linked gene being sex-linked in avian species and supported the hypothesis that mammalian X and avian Z chromosomes evolved from the same ancestral linkage group (*41*). However, size heterozygosities in intron 3 of the chicken OTC gene were observed in both sexes. This finding strongly suggests that the chicken OTC gene is located on an autosome. To clarify this fact, we performed both FISH and linkage analysis. Both experiments showed that the OTC gene is located on chromosome 1q of chickens. The difference between the result of Dominguez-Steglich and Schmid (*18*) and our findings may be due to use of human OTC cDNA as a probe in their experiment. Our data support previous enzymatic studies suggesting autosomal inheritance of OTC activity (*12, 17*). Furthermore this finding is consistent with previous reports that genes located on the mammalian X chromosome are located on an autosome in birds: *e.g.* phosphoglycerate kinase (*42*), glucose-6-phosphate dehydrogenase (*43*), hypoxanthine phosphoribosyltransferase (*44*), and dystrophin (*45*). Two chicken Z-linked genes, creatine kinase (*46*) and iron responsive element binding protein (*47*), are autosomally linked in mammals.

Recently it has been reported that the genes with little or no function do not easily disappear from the genomes of higher organisms (*20, 48*). This is thought to be because DNA polymerase of higher animals is more accurate and more efficient in editing than it should be for the removal of non-functional genes. Therefore the average half-life of such genes was estimated as high as 47.2 million years in salmon (*20*). It is difficult to apply this hypothesis to conservation of the OTC gene, because avian species have been evolving independently of mammals as ureotelic animals for 250 million years (*21*). δ-Crystallin may provide one clue as to why the chicken OTC gene has been conserved. δ-Crystallin is the dominant structural protein in lenses, is present only in birds and reptiles, and exhibits one of the urea cycle enzymes, argininosuccinate lyase activity. Piatigorsky *et al.* (*49*) and Matsubasa *et al.* (*50*) reported the unexpected evolutionary relationship between δ-crystallin and argininosuccinate lyase, and the apparent duplication during evolution and the homology between chicken δ 2-crystallin and argininosuccinate lyase. Thus if the OTC gene became redundant and dispensable but was subsequently endowed with a novel function during evolution, this could explain why it has been conserved in the chicken genome. In future, it may be reported that a new gene exhibits high homology to the OTC gene in chicken, but at present it has no known homologues. From these results we speculate that chicken OTC may have an as yet unidentical physiological role at some other developmental stage or in another tissue. For this reason, we speculate that chicken OTC has been conserved under some unknown selection pressure.

More recently it has been reported that rainbow trout, a typical ammonotelic fish, has maintained OTC and CPS III activities (*51*). Although the functions of these activities in rainbow trout, like chicken, remain unknown, a deduced amino acid sequence of trout CPS III cDNA has about 70% identity to both shark CPS III and mammalian CPS I (*52*). This discovery suggested that the urea cycle enzymes may have been maintained regardless of ammonia excretion types. Chickens do not express CPS I (*1*). However, if the urea cycle itself has some physiological roles like the arginine-synthesis pathway in plants and *Neurospora crassa*, it is possible that the homologue of the mammalian CPS I gene may be conserved in the chicken genome.

We have suggested that the OTC gene has some physiological roles in chicken and that polymorphism within the OTC gene controls variation of activity. To excrete nitrogen, uricotelic animals utilize the purine biosynthetic pathway, while ureotelic animals use the arginine biosynthetic pathway. Thus, animals adapted to the environment by using basal metabolic pathways. We speculate that OTC, one of the arginine biosynthetic enzymes, was conserved for this reason in chickens. In the future we expect to resolve the question of a physiological role for OTC in chicken.

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