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Phylogeny and regulation of four lipocalin genes clustered in the chicken genome: evidence of a functional diversification after gene duplication

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Abstract

A novel lipocalin gene is here reported that represents the fourth member of a cluster we have identified in the chicken genome. This cluster also includes Chondrogenesis-Associated Lipocalins β and γ (CALβ, CALγ) and Extracellular Fatty Acid Binding Protein (Ex-FABP). The new gene codes for a 22-kDa secreted protein with three cysteine residues and a series of sequence features well conserved in the lipocalin family. All the genes in the cluster are structurally similar presenting comparable exon/intron boundary positions and exon sizes. A phylogenetic analysis indicates the monophyletic grouping of these genes, and their relationship with the lipocalins α-1-microglobulin (A1mg), complement factor 8γ chain (C8GC), prostaglandin D synthase (PGDS), and neutrophil-gelatinase-associated lipocalin (NGAL). The new cluster gene appears to be the ortholog of the mammalian C8GC and was thus named Ggal-C8GC. This orthology also suggests that this lipocalin was present in the ancestor common to reptiles and mammals.

In addition to other expressing tissues, Ex-FABP, CALβ and CALγ genes are highly transcribed in chondrocytes at late stages of chondrogenesis during endochondral bone formation and/or upon inflammatory stimulation. Here, we show that they are also transcriptionally induced when chondrocytes are subjected to various biological events as cell quiescence, cell shape transition, and hormonal stimulation. By contrast, Ggal-C8GC transcripts are only barely detectable in chondrocytes, but are more abundant in liver, kidney, brain, heart, skeletal muscle and particularly in skin. Moreover, no expression induction was observed neither during chondrocyte differentiation, nor upon any of the stimulations mentioned above. This indicates that the Ggal-C8GC gene was co-opted for a novel function after the duplication events that gave rise to the cluster.

The peculiar coordinated regulation of Ex-FABP, CALβ and CALγ, and the apparent divergent role of Ggal-C8GC suggest that these gene duplications may have been maintained during evolution by a sub-functionalization mechanism where some common function(s) are shared by several members of the cluster and some other specialized function(s) are unique to other members.

Keywords: Gene structure; Evolution; Transcription regulation

Abbreviations: CAL, chondrogenesis-associated lipocalin; Ex-FABP, extracellular fatty acid binding protein; A1mg, α-1-microglobulin; C8GC, complement factor 8γ chain; PGDS, prostaglandin D synthase; NGAL, neutrophil-gelatinase-associated lipocalin; A1gp, α-1-acid glycoprotein; Gd, glycodelin; LPS, lipopolysaccharides; SCR, structurally conserved regions; FCS, fetal calf serum; T3, triiodothyronine; COLL II, type II collagen; COLL X, type X collagen; LBP, local bootstrap proportions; kDa, kilodalton; nt, nucleotide; RT-PCR, reverse transcriptase-polymerase chain reaction; ERBP, epididymal retinol binding protein; βLG, β-lactoglobulin; MUP, mouse urinary protein; OBP, odorant binding protein; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

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1. Introduction

The lipocalin protein family encompasses a large number of polypeptides with a characteristic cup-like folding. A protein is included in the lipocalin superfamily on the basis of criteria as an overall sequence identity of more than 20% with the closest relative, the presence of at least two of the three structurally conserved lipocalin motifs (Structurally Conserved Regions or SCRs) and a protein length of about 200 residues (Flower et al., 1993; Flower et al., 2000). Additional features are a common β-barrel structure formed by eight anti-parallel β-sheets and a ligand-binding pocket where a specific hydrophobic molecule is bound. Despite these structural similarities, the lipocalins are involved in various biological processes such as proliferation and differentiation, growth and repair within the nervous system, chemosensory signaling, fertility and reproduction, acute systemic inflammation and immunomodulation, and tumor invasion (Flower, 1996).

Another peculiarity of the lipocalin family is the organization of several genes in cluster on a given chromosome, as shown for human, rat and mouse genomes (Chan et al., 1994; McFadyen et al., 1999). In *Homo sapiens*, for instance, many lipocalin genes were found at bands 32–34 of chromosome 9 long arm (9q), whereas in *Mus musculus* two clusters were found on chromosome 2 (band B to band C1, and bands F3 to G) and on chromosome 4 (bands B to C). In some cases, a biological significance has been attributed to gene clustering as it holds functionally related proteins within the same locus. In human, the so-called "immunocalins" (A1gp/α1-acid glycoprotein, Almg/α1-microglobulin, Gd/glycodelin, NGAL/neutrophil gelatinase-associated lipocalin and C8GC/complement factor 8γ chain) illustrate this concept: this subset of proteins, all encoded by the q32–34 region of chromosome 9, groups lipocalins that all share anti-inflammatory properties (Logdberg and Wester, 2000).

The cluster organization, together with the conserved exon/intron distribution and arrangement found in different phyla, also indicates that the lipocalins probably evolved by successive rounds of tandem gene duplications (Salier, 2000). A recent phylogenetic analysis of the family evidenced several evolutionary related clades subsequently used to make a phylogenetic tree rooted with the eubacterial lipocalins (Ganfornina et al., 2000). In this analysis, a clade encompasses prostaglandin D synthase (PGDS), NGAL and the quiescence-specific p20K, whose functions and tissue distributions seem to be unrelated.

In recent publications, we have reported that lipocalin gene clustering has occurred also in the chicken genome (Pagano et al., 2002, 2003). Indeed, three lipocalin genes, Chondrogenesis-Associated Lipocalins β and γ (CALβ, CALγ), and Extracellular-Fatty-Acid-Binding-Protein (Ex-FABP), are located in sequence within the same genomic locus, CALγ being in 5′ position, CALβ in intermediate position and Ex-FABP at the 3′ end of the cluster. When compared to other proteins in the GenBank database, all three lipocalins show the highest overall amino acid similarity with prostaglandin D synthase. Expression studies both at the mRNA and protein levels have further suggested that the cluster could be coordinately regulated at the transcription level. An analysis of the tissue distribution revealed that Ex-FABP, CALβ and CALγ have similar expression patterns during chicken embryo development (Pagano et al., 2002, 2003). In addition, focusing on chondrocyte differentiation during endochondral bone formation, we demonstrated that the synthesis of all three lipocalins is stage-specific, the proteins being barely detectable in proliferating undifferentiated pre-chondrogenic cells but highly expressed in pre-hypertrophic and fully differentiated hypertrophic chondrocytes. Furthermore, a similar co-regulation of expression was observed in at least another biological context such as the response to inflammatory stimuli where the synthesis of all three lipocalins was specifically and highly induced in chondrocytes treated with lipopolysaccharides (LPS). Taken together, these findings suggest that the chicken cluster is composed of lipocalins that are coordinately regulated and possibly functionally related. As to this function, we recently showed that at least Ex-FABP is a constitutive survival protein that acts in preventing apoptosis (Di Marco et al., 2003).

In this paper, we report the identification of a novel chicken lipocalin gene located right upstream CALγ, thus representing the fourth member of the genomic cluster. This gene is structurally similar to the three others, but differs in its expression, being not specifically induced during chondrogenesis. A tissue expression analysis showed a very active transcription of the gene in skin while a moderate amount of transcripts was observed in muscle and heart. We also present further evidences that the expression of Ex-FABP, CALβ and CALγ is coordinately modulated in chondrocytes under hormonal stimulation or in states of cell stress (quiescence and cell shape transition), while the fourth new member of the cluster is not. A phylogenetic analysis is also reported, showing the relationships of the chicken cluster proteins to other members of the lipocalin family. Orthology of the new gene with the mammalian C8GC is proposed, which led to name it Ggal-C8GC, and the position of the cluster members within the lipocalin tree is discussed as a first input for the identification of their function.

2. Materials and methods

2.1. Genomic clones

Isolation and characterization of the genomic clones pGD15 and BAC bw093F21 containing the genes for Ex-FABP (#AF121346), CALβ (#AF438198) and CALγ (#AY082334) have been previously reported (Pagano et al., 2002, 2003; Giannoni et al., 2004). The same BAC genomic clone was here further analyzed to search for additional member(s) of the lipocalin cluster: the region upstream the

promoter of the CALγ gene was sequenced on both strands by the dideoxynucleotide chain termination method (Sanger et al., 1977) using the Thermo Sequenase ™ Kit (Amersham Life Science).

The sequences were then computer-analyzed for structure, function and homology determinations (Grail version 3.1, Protein Predict, GeneScan, MegAlign, NetPhos, Ipsort Prediction, Signal PV1.1, Prot Param Tools, 3D-pssm and SwissProt Database).

Transcription factor responsive elements present on the promoter of all genes of the cluster were identified using TESS/Transfac database version 4.0.

2.2. Monolayer and suspension cell culture

Methods for chondrocyte isolation and culture have been extensively described elsewhere (Castagnola et al., 1986). Briefly, primary cells were obtained from 6-day chicken embryo tibia (stage H.H. 29–30) by repeated digestions with trypsin and collagenase. Dedifferentiated chondrocytes were obtained by culturing the primary cells for 3 weeks in monolayer. To analyze the early phase of re-differentiation, the expanded cells were transferred into suspension culture on dishes coated with 1% agarose and maintained as such for a time period ranging from 4 to 144 h. Hypertrophic chondrocytes were obtained by maintaining the suspension cultures up to 4 weeks. Culture medium was Coon's modified Ham's F-12, supplemented with 10% fetal calf serum (FCS).

2.3. Cell quiescence

Dedifferentiated chondrocytes expanded for 3 weeks in monolayer were plated at a density of 35×10^3 cells/cm^2 (sub-confluence). Quiescence was induced by transferring the cells in F12 medium depleted of serum. Quiescent cells were analyzed for gene expression profile (Real-Time Quantitative RT-PCR as below) at regular time intervals ranging from 2 to 9 days.

2.4. Cell stimulation by thyroid hormones

Dedifferentiated chondrocytes expanded for 3 weeks in monolayer were plated at a density of 35×10^3 cells/cm^2 (sub-confluence). The cells were then stimulated for 24, 48 and 72 h with triiodothyronine (T_3, 10^{-8}M) and analyzed thereafter for gene expression profile (Real-Time Quantitative RT-PCR as below).

2.5. Total RNA preparation and real-time quantitative RT-PCR

Total RNA was prepared from tissues of 12-day chicken embryos (stage HH. 38; skeletal muscle heart and skin) and from chondrocytes cultured for the time and in the conditions indicated. The extraction was performed according to the guanidinium–isothiocyanate procedure of Chomczynski and Sacchi (1987). Levels of mRNA were measured by Real-Time Quantitative RT-PCR using the PE ABI PRISM 7700 Sequence Detection System (PE Applied Biosystems). Expression of the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) of type II and type X collagens (COLL II and COLL X) and of Curly Protein (CURP) were measured in parallel as endogenous control (GAPDH), chondrogenic differentiation markers (COLL II and COLL X) and negative control (CURP). The sequences of forward and reverse primers and of the TaqMan™ fluorogenic probes, as designed by the Primer Express 1.5 software, are shown in Table 1. All probes were located at the junction between two exons. During PCR amplification, the 5′ nucleolytic activity of Taq polymerase cleaves the probe, separating the 5′ reporter fluorescent dye from the 3′ quencher dye. Threshold cycle, Ct, which correlates inversely with the target mRNA levels, was measured as the cycle number at which the reporter fluorescent emission increases above a threshold level. Relative transcript levels were determined from the relative standard curve constructed from stock cDNA dilutions, and divided by the target quantity of the calibrator according to the manufacturer's instructions.

2.6. Phylogenetic analysis

The amino acid sequences of the chicken cluster proteins were added to a database that includes a set of 134 lipocalins present in chordates. The proteins were designated by a functional label after an abbreviated species name. The protein sequences were aligned with ClustalX version 1.8 (Thompson et al., 1997) using the Gonnet series matrix and a gap penalty mask based on the lipocalins secondary structure (Gutierrez et al., 2000). Manual corrections helped to refine the final alignment. The sequence of the grasshopper protein Lazarillo (#U15656) was included in the alignment for rooting purposes. The lipocalin database and alignment are available from the authors upon request.

Tree building was carried out under the maximum-likelihood principles following the steps previously described (Ganfornina et al., 2000). This procedure includes the production of a maximum-likelihood distance matrix; a neighbor-joining tree reconstruction using the distance matrix; and a topology search seeking for a higher likelihood value (for details, see Ganfornina et al., 2000). Local bootstrap proportions (LBP) were calculated under 1000 replicates to evaluate branch support.

3. Results

3.1. Identification of a fourth gene within the chicken lipocalin cluster

In previous reports, we have shown that the genomic clone pGD15 and the overlapping genomic BAC clone bw093F21

Table 1
Primers and TaqMan probes used in the Real-Time Quantitative RT-PCR reactions

GAPDH	forward 5′ -AAAGTCGGAGTCAACGGATTTG-3′ reverse 5′ -TGTAAACCATGTAGTTCAGATCGATGA-3′ probe 5′ -VIC-CGTATTGGCCGCCTGTCACCA-TAMRA-3′
Type II collagen	forward 5-GAGGGCAACAGCAGGTTCAC-3′ reverse 5′ -TTCTGCGACCGGTACTCGAT-3′ probe 5′ -FAM-CGGCTGCACAAAACACACTGGC-TAMRA-3′
Type X collagen	forward 5-AGGCAGTGCTGTCATTGATCTCATGGA-3′ reverse 5′ -TCAGAGGAATAGAGACCATTGGATT-3′ probe 5′ -FAM-TCAAGTGTGGCTCCAGCTGCCAAA-TAMRA-3′
Ex-FABP	forward 5′ -AGAAAATGGGAGACAACCTTCAAG-3 reverse 5′ -CACCCTGGTTGCAAAGATCACT-3′ probe 5′ -FAM-CTACTACTCAGAGGAAGCCGAGAAAACGGTA-TAMRA-3′
CAL β	forward 5′ -TCCCTGTGCAGCTGGACTTC-3′ reverse 5′ -GTTGGAAACAGCAGCCATCA-3′ probe 5′ -FAM-GTGCCACATCCCTGCAAATTTCTTGGT-TAMRA-3′
CAL γ	forward 5-CTGTCACTGCAGATGGCAACAT-3′ reverse 5′ -CGTGGGTTGGTGTAGCTGAAC-3′ probe 5′ -FAM-CCTCTTCTCGCACTGTTCACCCTTGG-TAMRA-3′
C8GC	forward 5-AGCCAAGGCCTCTCTGTCAA-3′ reverse 5′ -AGTAGTGGGTCACATCCTCACTCA-3′ probe 5′ -FAM-CTACGGCCGCAGCAGCCAGCT-TAMRA-3′
CURP	forward 5′ -CCGGGAGATCGCGCA-3′ reverse 5′ -CTCATGGAGCAGAGAGATGTGC-3′ probe 5′ -FAM-TGCTGTCCGAAGGGTACAGCGTGG-TAMRA-3′

contain at least three lipocalin genes organized in sequence from 3′ to 5′ as Ex-FABP, CALβ and CALγ (Pagano et al., 2002, 2003; Giannoni et al., 2004). The BAC clone was here further sequenced in 5′ direction upstream the CALγ promoter. An analysis with GENESCAN algorithms of the 2974 bp retrieved a novel lipocalin coding region. This new gene thus represents the fourth member of the cluster. The full genomic sequence has been deposited to GenBank™ Data Base with accession number AY307111.

As predicted by 3D-pssm program, the protein encoded by this gene shows the typical lipocalin secondary structure including three α-helices, eight anti-parallel β-sheets and the diagnostic lipocalin domain NLSGELVGRWFLV (Fig. 1A). All these properties are well-known characteristics of all members of the lipocalin family (Flower et al., 2000). The protein also contains three cysteine residues and a predicted signal peptide with the most likely cleavage site between residues 21 and 22. These features are consistent with a secreted molecule with no other hydrophobic or highly charged region. The molecular weight is 22 332 Da (20 210 after signal peptide cleavage) and the isoelectric point 6.85 (6.2 without signal peptide). The sequence also shows several potential sites of posttranslational modifications (Fig. 1B).

A BLASTp analysis (non-redundant database) indicated that the protein has 45% identity (77/170) with human C8GC and 42% (72/170) with the mouse ortholog. The gene was thus named Ggal-C8GC. The alignment of the protein sequence with the other members of the cluster and other closely related lipocalins further supports the assignment of this gene to the lipocalin family (Fig. 1C).

3.2. Gene structure of the chicken lipocalin cluster

As depicted in Fig. 2, a very similar gene structure is shared by the cluster members, three out of four being organized in six exons and five introns. Only Ggal-C8GC revealed a small difference in the C-terminal part of the coding region that includes a seventh very short exon. A strong similarity was also observed in exon/intron boundary positions as well as in exon sizes. These features are consistent with the generation of the lipocalin cluster as a consequence of tandem-repeated gene duplications from an ancestral member by unequal crossing-over during meiosis.

3.3. Phylogenetic relationships of the chicken cluster proteins

The cluster of chicken lipocalins represents paralogs of other chordate lipocalins. Basic pairwise comparisons of protein sequences can help to propose orthologous relationships for the chicken lipocalins. In order to confirm this orthology and to reveal the evolutionary relationships

A

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MAAPRALLLLSLLLAAPQGRGQRPPPPHNPLEKVV   35
TEGNLSLGELVGRWFLVGVASRCSYLAENSHRLEA   70
TAMTVAVPDGQSLAISTFRKLDGQCWEIRQRYVPE  105
GAHRRFSVRGRGYNSKMEVVVGEADPRSYAIIYYQ  140
DSQGLSVKLYGRSSQLSNAIVDKFEQRARAVGLSE  175
DVTHYFPTYGFCDSADDFHILDETEL            201
```

B

Post-translational modification sites	Position	Sequence
N-Glycosilation site	39-42	NLSL
cAMP and cGMP Phosphorylation Site	109-112	RRfS
Protein Kinase C Phosphorylation Site	65-67	ShR
	87-89	TfR
	112-114	SvR
	146-148	SvK
Casein Kinase II Phosphorylation Site	41-44	SlgE
	120-123	SkmE
	189-192	SadD
N-Myristoylation Site	80-85	GQslAI

C

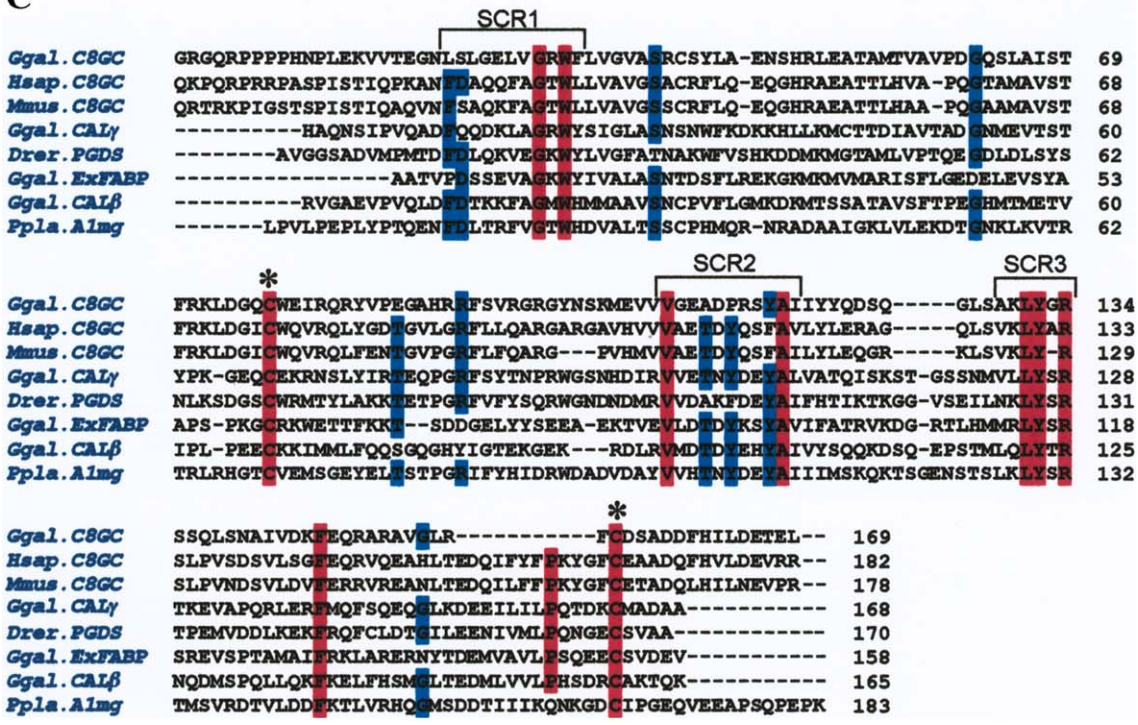
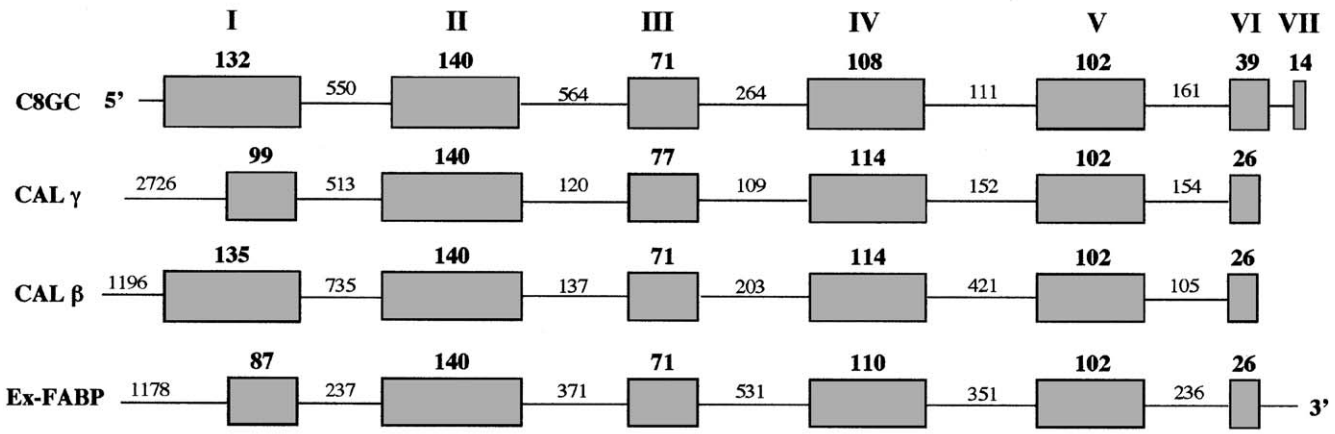


Fig. 1. Panel A: Deduced primary structure of the fourth member, Ggal-C8GC, of the chicken lipocalin cluster. The site of cleavage of the signal peptide is denoted by the arrowhead. The main lipocalin domain is boxed while the three cysteine residues are underlined. The α-helices and the β-sheets regions are shown in red and blue, respectively. Numbers on the right refer to amino acid position. Panel B: The main sites of posttranslational modification, predicted with "very high probability of occurrence", are listed. Panel C: Multiple sequence alignment among the cluster lipocalins and their closest homologues. Fully conserved residues are evidenced by red boxes. Partially conserved residues are shown by blue boxes. Structurally Conserved Regions are indicated (SCRs). The two cysteines involved in the lipocalin β-barrel formation and maintenance are indicated by the asterisk.

among these lipocalins, one has to infer a phylogenetic tree based on multiple sequence alignment. Using the methods described above, we thus built a phylogenetic tree of 134 chordate lipocalins, including the four chicken proteins considered in this work (Fig. 3). The insect lipocalin Lazarillo (Same.Laz in Fig. 3) was used as a sister group for tree rooting. The four lipocalins clustered in the chicken genome appear grouped within a monophyletic clade of chordate lipocalins that includes α-1-microglobulins, C8GC, PGDS and NGAL. In this clade, CALβ locates in a basal position; therefore, no particular orthology with a known lipocalin can be assigned. However, from this



	Exon	5' Junction Intron sequence	Exon sequence	3' Junction Exon sequence	Intron sequence
C8GC	1	5' UTR	ATGGCC-	-GGCGAG	gtgagg ccc-
	2	-tgctgtgcag	CTGGTG-	-GAAGCT	gtgaggatg-
	3	-ccctgcacag	GGATGG-	-TGAGAG	gtgagcacag-
	4	-tccgccatag	GCCGTG-	-TCTACG	gtgggtggc-
	5	-tcccccatag	GCCGCA-	-ATATGG	gtgagtgcc-
	6	-ctctacacag	GGTTCT-	-TCGATG	ggtgccccg-
		-tcctttccag	AAACGG-	-CTGTAG	atggaggca-

	Exon	5' Junction Intron sequence	Exon sequence	3' Junction Exon sequence	Intron sequence
CAL γ	1	5' UTR	ATGCAA-	-GACAAG	gtgatcgagc-
	2	-ttggtgcag	CTTGCA-	-CCCCAA	gtacgggtgg-
	3	-tgctctccag	GGGTGA-	-CCCACG	gtgagcctct-
	4	-atctccgcag	GCTGGG-	-TCTACA	gtacgtcctc-
	5	-ctttccgcag	GCCGCA-	-AGACGG	gtaagggtgg-
	6	-ctttctgcag	ACAAGT-	-GCCTAG	3' UTR

	Exon	5' Junction Intron sequence	Exon sequence	3' Junction Exon sequence	Intron sequence
CAL β	1	5' UTR	ATGCAG-	-AAGAAG	gtaatggcaa-
	2	-ctcgctgcag	TTTGCA-	-CCCGCT	gtgagtcccg-
	3	-ccccttccag	GCCAGA-	-GCACAG	gtatgttggg-
	4	-gatcttttag	AAAAGG-	-TCTACA	gtgggtgtct-
	5	-ccacctgcag	CAAGGA-	-ACTCGG	gtgagtgcca-
	6	-cactgaacag	ATCGGT-	-AAGTGA	3' UTR

	Exon	5' Junction Intron sequence	Exon sequence	3' Junction Exon sequence	Intron sequence
Ex-FABP	1	5' UTR	ATGAGG-	-AGCGAG	gtgacctgcg-
	2	-ctacttccag	GTTGCA-	-CCCCAG	gtaagtgcca-
	3	-tgccttgcag	CCCAAA-	-ACTCAG	gtgagtggga-
	4	-ccttttttcag	AGGAAG-	-TCTACA	gtacgtggcc-
	5	-ttgccttcag	GCAGAA-	-GCCAGG	gtcagtctga-
	6	-attttcacag	AGGAAT-	-GTGTAG	3' UTR

Fig. 2. Structural organization of the clustered lipocalin genes. Within the genomic cluster, the genes are distributed from 5′ to 3′ as Ggal-C8GC, CALγ, CAL β and Ex-FABP. With the exception of Ggal-C8GC, whose coding sequence is spanned by seven exons, the coding region of each gene is composed of six exons (boxed and numbered in roman) and five introns. The respective exon and intron sizes (in nt) are indicated. The region comprised between the first exon of one given gene and the last exon of the previous one corresponds to the sequence that includes its promoter. The table underneath lists for each single gene all the sequences of the exon/intron junctions.

analysis, we can propose that CALγ is the chicken ortholog of PGDS, a result supported by the PGDS-based cloning of an identical chicken sequence (#AB077952). Ex-FABP appears also included in the PGDS group, but as a divergent member related to the fish PGDS. Ex-FABP had previously been proposed to be the chicken ortholog of NGAL, the mammalian duplicate of PGDS (Descalzi Cancedda et al., 2001). However, none of the chicken cluster proteins appear to be related to NGAL in our tree. The phylogenetic analysis strongly suggests the orthology of the newly identified chicken cluster member with the mammalian C8GC, herein named as chicken C8GC (or Ggal-C8GC). In a previous phylogenetic analysis of the lipocalin family (Gutierrez et al., 2000), we hypothesized that C8GC originated as a duplicate of A1mg during mammalian evolution. However, the new Ggal-C8GC places the duplication event further back in time, before mammals split from the reptilian lineage.

The association of the chicken lipocalins in a genomic cluster is in agreement with the relationships established in our tree between these proteins and the lipocalins that are also organized in clusters in mammalian chromosomes (reviewed by Salier, 2000). To further confirm this idea, we set out to test the existence of other chicken lipocalins whose sequence similarity to known clustered mammalian lipocalins could help to resolve their orthologous relationships. We BLAST-searched the chicken EST databases of the Roslin Institute and the University of Delaware with exemplars of well-supported clades belonging to the lipocalin chromosomal cluster of chordates. We found several EST clones displaying significant similarity to be considered the chicken orthologs of A1mg (#UDELPATPK0050B9F) and A1gp (#UDELPCO1CPK0001M16). However, no significant similarities were found when searching with the protein sequences of NGAL, epididymal retinol binding protein (ERBP), β-lactoglobulin (βLG), Mouse Urinary Protein (MUP) or the chemoreception lipocalin Odorant Binding Protein (OBP). Thus, in addition to the four proteins examined in this work, we could expect to find the chicken A1mg and A1gp in the cluster. Attempts to identify these genes with primers based on the EST sequences are in progress.

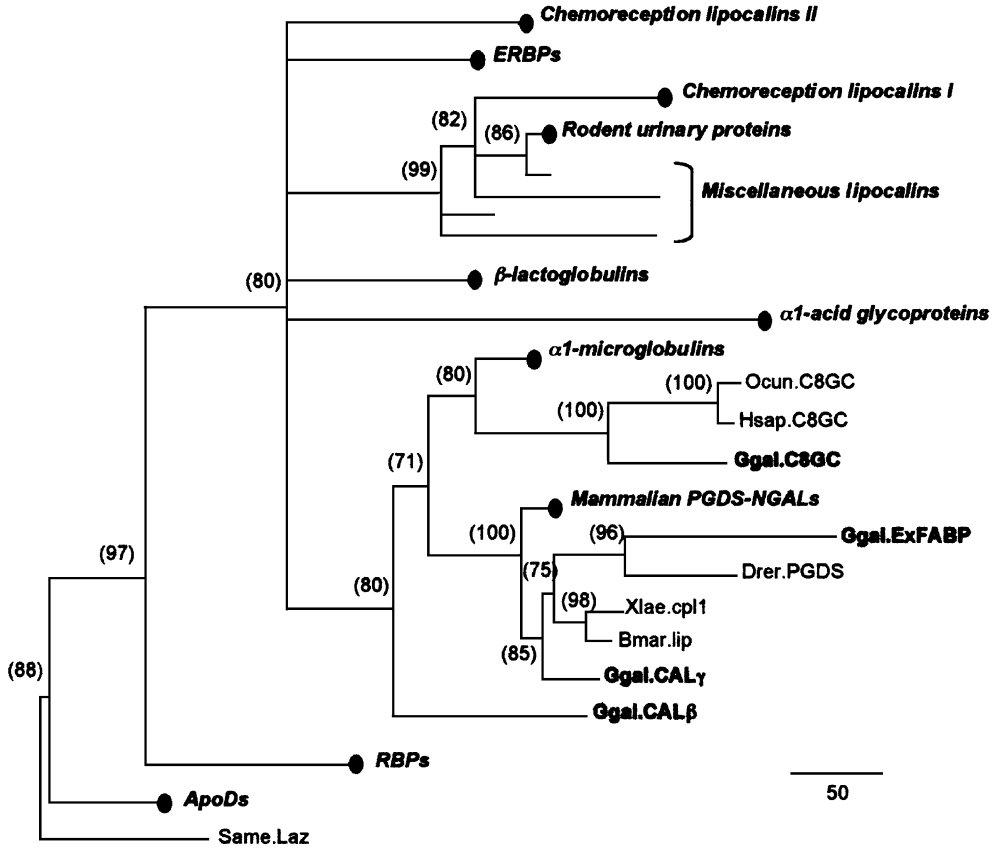


Fig. 3. Phylogenetic tree of chordate lipocalins rooted with the grasshopper Lazarillo sequence as an outgroup. Proteins are named using an abbreviated species name followed by a functional label (see Ganfornina et al., 2000). LBP values are indicated at each node. The lipocalin clades not containing chicken sequences are resumed to their main clade node (shown as a black dot), and the LBP evaluating these nodes are shown in parentheses. The chicken cluster lipocalins are highlighted in bold. Polytomies reflect nodes with LBP values ≤70. The scale bar represents branch length (amino acid substitutions/100 residues).

3.4. Expression pattern of the Ggal-C8GC gene

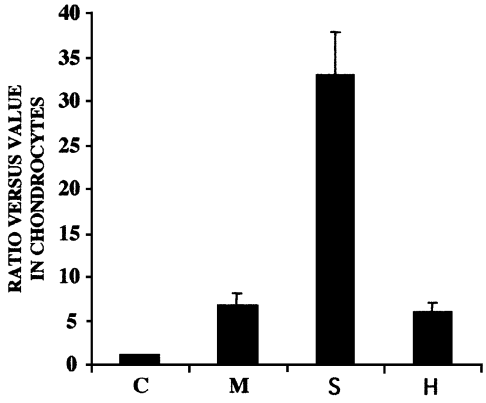
In view of its genomic location close to the chondrogenesis-related lipocalins Ex-FABP, CALβ and CALγ, the expression of Ggal-C8GC was assessed by Real-Time RT-PCR in chondrocytes cultured in vitro at different stages of differentiation. At variance with the other lipocalins of the cluster, the level of Ggal-C8GC transcripts was similar both in dedifferentiated and in hypertrophic chondrocytes (data not shown). The absolute level of transcription of Ggal-C8GC was also very low, which suggests that cartilage is not a major site of expression of this lipocalin. The tissue distribution of Ggal-C8GC was thus measured in a 12-day-old chicken embryo and the values of Real-Time RT-PCR obtained were normalized to GAPDH and expressed relative to the level of expression in dedifferentiated chondrocytes. Fig. 4 shows that Ggal-C8GC transcripts were ≅6 times more abundant in skeletal muscle and in heart, and ≅30 times more abundant in skin than in chondrocytes. In addition, we found several clones in the chicken EST databases currently available that indicate the expression of Ggal-C8GC also in liver (clone ID 603593790F1), kidney/adrenal (clone ID 603604428F1), ovary (clone ID 603214349F1), small intestine (clone ID 603612056F1) and brain/cerebellum (clone ID 604169824F1).

3.5. Co-regulation of the lipocalin cluster

To further document the coordinated transcriptional modulation of lipocalin genes seen during chondrogenesis and in response to inflammatory agents (Pagano et al., 2002, 2003), we assessed the expression profile of the whole cluster in other defined biological settings such as hormonal stimulation and induction of cell stress.

3.5.1. Induction of lipocalin expression upon cell quiescence

The p20K protein, a homologue of Ex-FABP, is known to be induced in heart mesenchymal fibroblasts rendered quiescent by serum starvation (Kim et al., 1999). In order to assess a possible overexpression of the chicken cluster lipocalins as a consequence of quiescence, we analyzed by Real-Time Quantitative RT-PCR their transcript levels in dedifferentiated fibroblast-like chondrocytes cultured in vitro in the absence of serum for 2, 4, 6 and 9 days. Results obtained in serum-free conditions were normalized to their counterparts in FCS (Fig. 5). The amount of Ex-FABP transcripts was



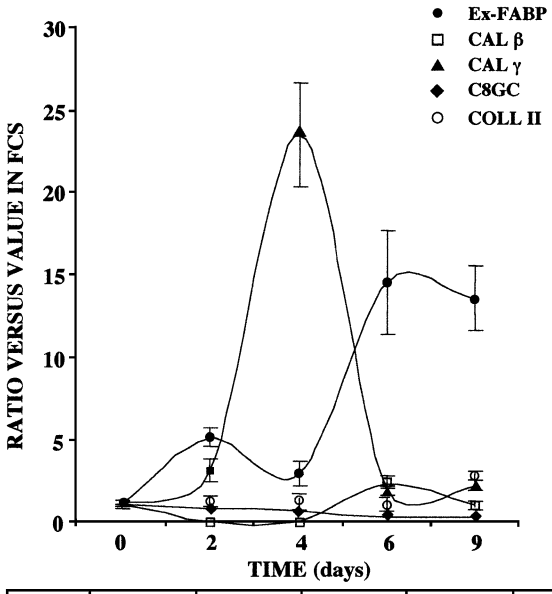
TISSUE	C8GC VALUE
Chondrocytes (C)	1.0 ± 0.16
Muscle (M)	6.82 ± 1.28
Skin (S)	33.06 ± 4.74
Heart (H)	6.02± 1.08

Fig. 4. Tissue distribution of Ggal-C8GC as measured by Real-Time Quantitative RT-PCR on total RNA extracted from 12-day-old chicken embryo and from cultured dedifferentiated chondrocytes. Results are presented graphically and numerically. Ggal-C8GC mRNA quantities are expressed as an *n*-fold difference relative to chondrocytes. The values are means (±standard deviation) of three independent determinations. For reference and normalization, GAPDH gene expression was measured from the same RNA samples. M, skeletal muscle; H, heart; S, skin; C, dedifferentiated chondrocytes.

strongly increased (about 14-fold) in the late period of quiescence (6 and 9 days). CALγ was also readily induced, reaching the maximum level (about 23-fold) after 4 days of serum deprivation and then decreasing to the basal level after 6 days. CALβ was transcribed at a very low level, barely detectable in the early phase of quiescence. Nevertheless, this gene was transiently increased by 2.3-fold after 6 days of serum deprivation. By contrast, the amount of Ggal-C8GC transcripts was not increased at any time of serum starvation, confirming a peculiar expression pattern independent from the other members of the cluster. As a control, the level of expression of type II collagen (COLL II), a typical marker of chondrocytes analyzed in parallel, was not significantly modified upon cell quiescence. Thus, a Go-specific expression of lipocalins is here evidenced for three components of the cluster, CALγ being expressed at an earlier stage (4 days) than CALβ and Ex-FABP (6 and 9 days).

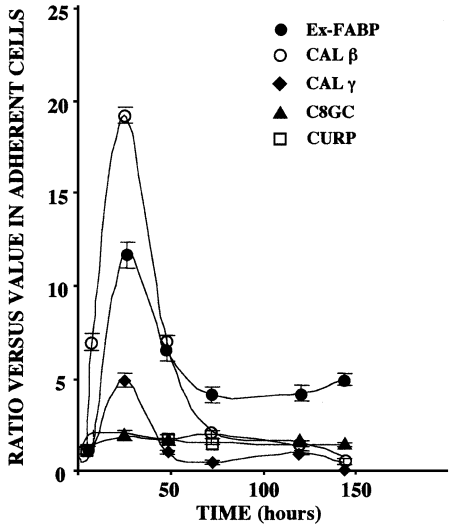
3.5.2. Induction of lipocalin expression upon cell shape modification

During the process of chondrogenesis where Ex-FABP, CALβ and CALγ genes are up-regulated (Descalzi Cancedda et al., 1988; Pagano et al., 2002, 2003), cell condensation represents a necessary step for the induction of chondrocyte differentiation. In this phase, the role of cell shape and the establishment of cell to cell contacts are essential (Benya and Shaffer, 1982; Glowacki et al., 1983; Tacchetti et al., 1992). In particular, the transition from adherent to suspension culture also implies of profound reorganization of the cytoskeleton, a phenomenon known to trigger chondrogenesis (Zanetti and Solursh, 1984; Benya et al., 1988; Loty et al., 1995). We thus investigated the effect of cell spreading inhibition and cell remodeling on the cluster expression. Dedifferentiated chondrocytes grown in monolayer were seeded on dishes coated with agarose and cultured in suspension for up to 144 h. Total RNA samples were collected at different time intervals and subjected to Real-Time RT-PCR quantification. Results obtained in suspension culture were normalized to their counterpart in monolayer culture (Fig. 6). The analysis evidenced a clear transient induction of Ex-FABP, CALβ and CALγ, but not of Ggal-C8GC. Indeed, Ex-FABP, CALβ and CALγ showed an increase in transcription level after 24 h of suspension culture (11.7-, 19.2- and 4.9-fold, respectively) and quickly returned to the basal level thereafter. The expression of Ggal-C8GC was increased at the most by twofold, as seen also for CURP used as negative control, being a gene totally unrelated to the cluster (CURP, recently cloned in our laboratory, is the chicken homologue of



TIME.	Ex-FABP	CAL β	CAL γ	C8GC	COLL II
0 d	1.00 ± 0.1	1.00 ± 0.27	1.00 ± 0.1	1.00 ± 0.07	1.00 ± 0.1
2 d	5.12 ± 0.6	N.D.	3.12 ± 0.7	0.78 ± 0.14	1.36 ± 0.2
4 d	2.94 ± 0.8	N.D.	23.44 ± 3.1	0.67 ± 0.06	1.25 ± 0.2
6 d	14.52 ± 3.1	2.30 ± 0.54	2.02 ± 0.1	0.35 ± 0.04	0.99 ± 0.1
9 d	13.56 ± 2.0	1.03 ± 0.12	2.23 ± 0.3	0.32 ± 0.05	2.70 ± 0.3

Fig. 5. Up-regulation of the cluster genes upon cell quiescence, as estimated by Real-Time Quantitative RT-PCR. Results are presented graphically and numerically. RNA was extracted from dedifferentiated chondrocytes rendered quiescent by serum deprivation. The levels of mRNA transcripts at the various time intervals during starvation are expressed for each gene as an *n*-fold difference relative to the value before serum depletion (0 day). The values are means (±standard deviation) of three independent determinations. For reference and normalization, the expression of the housekeeping GAPDH gene was measured from the same RNA samples. Transcription rate of type II collagen (COLL II) was measured in parallel as a control with respect to an overall effect of quiescence on the cells. N.D., not detectable.



TIME	Ex-FABP	CAL β	CAL γ	C8GC	CURP
0 h	1.00 ± 0.13	N.D.	1.00 ± 0.07	N.D.	1.00 ± 0.04
1 h	0.87 ± 0.05	N.D.	0.48 ± 0.08	1.00 ± 0.03	1.35 ± 0.08
4 h	0.92 ± 0.08	1.00 ± 0.37	0.61 ± 0.08	1.90 ± 0.22	1.18 ± 0.14
8 h	1.50 ± 0.13	7.70 ± 0.54	1.14 ± 0.06	2.04 ± 0.16	1.41 ± 0.24
24 h	11.70 ± 0.69	19.2 ± 3.59	4.93 ± 0.31	2.06 ± 0.17	1.82 ± 0.19
48 h	6.50 ± 0.58	7.00 ± 0.67	1.00 ± 0.08	1.73 ± 0.11	1.63 ± 0.25
72 h	4.10 ± 0.42	2.20 ± 0.46	0.46 ± 0.07	1.94 ± 0.19	1.54 ± 0.11
120 h	4.20 ± 0.42	1.50 ± 0.30	1.00 ± 0.13	1.43 ± 0.21	1.47 ± 0.17
144 h	5.00 ± 0.32	0.80 ± 0.02	0.50 ± 0.15	1.40 ± 0.05	1.45 ± 0.08

Fig. 6. Up-regulation of the cluster genes upon cell shape modification, as estimated by Real-Time Quantitative RT-PCR. Cell shape modification was induced by transferring dedifferentiated chondrocytes from monolayer to suspension culture. RNA was extracted from adherent cells and at various time intervals after transfer to suspension culture. The levels of mRNA transcripts are expressed for each gene as an *n*-fold difference relative to the value determined in the adherent cells (0 h). The values are means (±standard deviation) of three independent determinations. For reference and normalization, GAPDH gene expression was measured from the same RNA samples. Transcription rate of CURP was measured in parallel as a control with respect to an overall effect of quiescence on the cells. N.D., not detectable.

the murine gene BF383754; unpublished results). The moderate fluctuation of Ggal-C8GC transcription during cell shape modification was thus considered as nonspecific.

3.5.3. Induction of lipocalin expression upon hormonal stimulation

As shown above, two members of the cluster are phylogenetically related to prostaglandin D synthase. Reports in the literature have shown that in human and rat, PGD2 synthase is transcriptionally targeted by thyroid hormones (Garcia-Fernandez et al., 1997; White et al., 1997). We thus assessed the induction of the cluster by T3 in vitro. Dedifferentiated chondrocytes cultured in monolayer were stimulated with 10^{-8} M T3. The transcription level of the genes was measured by Real-Time Quantitative RT-PCR after 24, 48 and 72 h of treatment and compared to control unstimulated cultures (Fig. 7). The transcription level of both Ex-FABP and CALγ was significantly up-regulated after 24 h of T3 treatment, showing a respective 3.7- and 3.1-fold increase. Thereafter, the two genes gradually returned to the basal level of expression within 72 h. A different time-course of induction was observed for CALβ that reached the highest increase of expression (3.2-fold) at 72 h of T3 treatment.

Since thyroid hormones are well-known inducers of chondrocyte differentiation (Burch and Lebovitz, 1982; Burch and Van Wyk, 1987; Quarto et al., 1992), the level of type X collagen (COLL X) expression was measured in parallel as an index of the differentiation stage of the cells. At any time point, type X collagen transcription level was not significantly modified as compared to unstimulated cells. Therefore, the increased expression of the three lipocalin genes is a direct effect of T3 treatment and not an indirect consequence of an overall differentiation process induced by the hormone. Again, no effect of T3 was observed on the expression level of Ggal-C8GC that remained unchanged as previously observed in conditions of cell shape modification and cell quiescence.

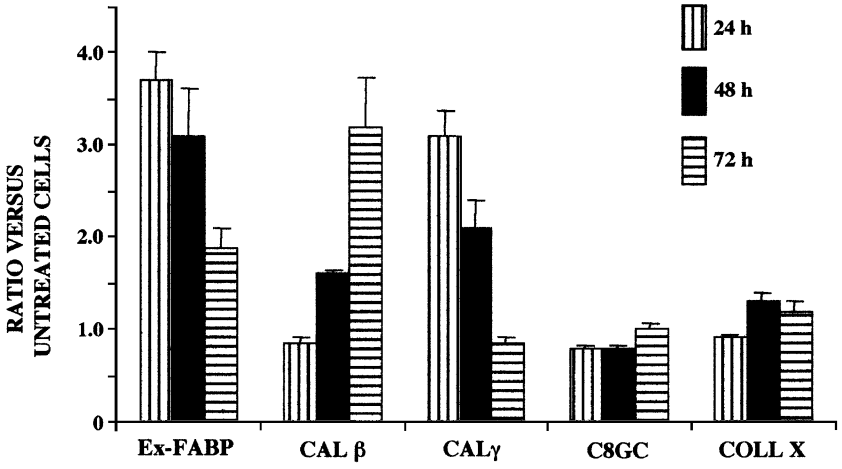
3.6. Analysis of the promoter sequences of the cluster lipocalins

Taken together, our expression results support the concept of a coordinated regulation of all the genes of the cluster, but Ggal-C8GC, in several biological processes. To further document this observation, we searched for consensus sequences for key transcription factors within the 5′ regulatory region of each gene. The main factors we have identified are listed in Table 2. As expected, Ex-FABP, CALβ and CALγ genes share common sequences as, for instance, for AP-1, NF-kappa-B, Cbfa1, thyroid hormones and C/EBP. Besides osteogenesis that is linked to Cbfa1, the biological events we have studied involve one of these factors: stimulation by T3 through the specific nuclear receptor, induction during chondrogenesis through activation of AP-1 as demonstrated for Ex-FABP (Giannoni et al., 2004), stimulation by LPS possibly through NF-kappa-B (Pagano et al., 2002, 2003) and regulation in quiescence state through C/EBPβ as shown for p20K (Kim et al., 1999). Concerning Ggal-C8GC gene, its promoter region could not be analyzed in details since the insert of the BAC genomic clone available terminated in 5′ direction 107 base pairs upstream the ATG starting codon. Therefore, whether the peculiar expression profile of Ggal-C8GC is due to a different set of regulating transcription factors awaits further investigation.

4. Discussion

4.1. Evolutionary considerations on the chicken lipocalin cluster

The present study focuses on the structural, functional and phylogenetic characterization of a lipocalin cluster



T3 treatment	Ex-FABP	CAL β	CAL γ	C8GC	COLL X
24 h	3.7 ± 0.31	0.84 ± 0.06	3.1 ± 0.26	0.8 ± 0.03	0.9 ± 0.03
48 h	3.1 ± 0.52	1.6 ± 0.03	2.1 ± 0.30	0.8 ± 0.01	1.4 ± 0.10
72 h	1.9 ± 0.21	3.2 ± 0.51	0.85 ± 0.06	1.0 ± 0.07	1.3 ± 0.11

Fig. 7. Up-regulation of the cluster genes upon hormonal stimulation by T3, as estimated by Real-Time Quantitative RT-PCR. Dedifferentiated chondrocytes grown in monolayer were stimulated for 24, 48 and 72 h with 10^{-8} M T3. RNA was extracted from stimulated and unstimulated cells. The levels of mRNA transcripts are expressed for each gene as an *n*-fold difference relative to the value determined in unstimulated cells. The values are means (±standard deviation) of three independent determinations. For reference and normalization, the expression of the housekeeping GAPDH gene was measured from the same RNA samples. Transcription rate of type X collagen (COLL X) was measured in parallel as a control with respect to an overall effect of T3 on the cells.

localized in the chicken genome. This cluster is composed of genes that encode proteins like Ex-FABP, CALβ and CALγ that are secreted during chondrogenesis in the pericellular zone surrounding the hypertrophic chondrocytes (Descalzi Cancedda et al., 1988; Pagano et al., 2002, 2003). At variance, the fourth member of the cluster herein reported appears to be barely expressed in chondrocytes.

The chicken lipocalins of the cluster are related to a monophyletic clade of chordate lipocalins that includes A1mg, C8GC, PGDS, and NGAL (see Fig. 3). In this lipocalin group, the phylogenetic analysis strongly suggests the orthology of both CALγ and Ex-FABP with PGDS, and that of the fourth lipocalin here reported with C8GC. Moreover, the tree position of Ex-FABP close to the fish PGDS suggests a duplication of PGDS-like genes in birds. The length of the branches leading to the CALγ and Ex-FABP suggests that the ancestral avian PGDS was more similar to the CALγ protein present in contemporary birds. However, if Ex-FABP is the chicken ortholog of NGAL, as suggested by (Descalzi Cancedda et al., 2001), the sequence and functional divergence of NGAL from a PGDS ancestral protein would have occurred only in the mammalian lineage. CALβ, on the contrary, stands alone in the tree without relating to a known orthologous group. However, its position at the base of the clade grouping the chordate-clustered lipocalins suggests a protein sequence divergence possibly due to functional specialization. The finding of the chicken C8GC is very significant for our knowledge of the evolutionary pathway followed by lipocalins, as it pushes back to the reptile-mammal common ancestor the duplication that probably gave rise to C8GC from an ancestral A1mg-like lipocalin.

The gene structure of the chicken lipocalins also shed light to the evolution of lipocalins, as all of the chicken proteins have their coding sequence included in six exons. This exon–intron arrangement is conserved between Ex-FABP, CALβ and CALγ, and other chordate relatives, but the mammalian C8GCs so far reported show a seventh exon coding for part of the C-terminal end of the protein. The presence of seven exons in Ggal-C8GC is in agreement with the exon–intron arrangement observed for the coding sequence of A1mg (Lindqvist et al., 1999), further supporting their relationship in the lipocalin phylogeny.

Table 2
Key transcription factors and corresponding consensus sequences identified on the cluster gene promoters

Factor	Consensus sequence	Ex-FABP	CALβ	CALγ	C8GC
AP-1 (c-jun)	TGAGCTCA	+	+	+	+
NF-kappaB	GGGGCTTTCC	+	+	+	N.D
Cbfa1	AACCAC	+	+	+	N.D
T3R alpha	AGG(T/A)C(A/G)	+	+	+	N.D
T3R beta	TTACTT	–	+	–	N.D
C/EBPβ	TTCCGTAAG	+	+	+	N.D

The consensus sequences for key transcription factors potentially regulating the cluster genes were identified by screening the promoter sequences with TESS/Transfac database version 4.0. The symbols (+) and (–) refer, respectively, to presence and absence of the indicated consensus sequence on the given promoter. N.D. (not determined) indicates that these consensus sequences could not be traced due to the limited portion of C8GC promoter available on the BAC genomic clone.

With respect to gene expression, we have mentioned the very low level of expression of Ggal-C8GC in chondrocytes. However, Ggal-C8GC is expressed in liver, kidney, brain, and muscle, and at a high level in skin. This also agrees with the orthologous relationship of the chicken protein with mammalian C8GC, which has been reported to be expressed in liver, kidney and brain (Trojer et al., 1999; Pontoglio et al., 2001; Hosokawa et al., 2003). Nevertheless, the expression patterns of the cluster lipocalins share some commonalties: they are all expressed in liver; Ex-FABP and CALβ are expressed in heart and skeletal muscle; and CALγ and Ggal-C8GC are specifically transcribed in brain and skin.

The results gathered from the phylogenetic analysis and database searches reveal six lipocalins in chicken that appear organized in chromosomal clusters in mammals (Salier, 2000). Along with Ggal-C8GC, three of these lipocalins (Ex-FABP, CALβ and CALγ) are also grouped in a single chromosomal cluster in chicken. This supports the view that a group of lipocalins that had originated by gene duplication were already organized in a single cluster in the common ancestor of mammals and reptiles (Gutierrez et al., 2000). To further extend this proposal to the remaining lipocalins, the localization of the chicken orthologs of A1mg and A1gp in the same chromosomal cluster needs to be proven.

The relationships observed among the sequence, gene structure, chromosomal position, expression pattern and, possibly, function suggest an origin of the chicken cluster by a tandemly repeated gene duplication. The preservation of the duplicate genes might have been due to a process of subfunctionalization of regulatory or protein coding regions (Force et al., 1999), where some common functions are shared by several cluster members and other functions and/or expressing tissues are unique for some proteins of the cluster.

4.2. Functional considerations on the chicken lipocalin cluster

Structurally, Ggal-C8GC shares with the three other proteins of the cluster the typical lipocalin domain, the three α-helices and the eight β-sheets composing the β-barrel folding. The amino acid sequence has 45% of identity with human C8GC. In particular, it contains an unpaired cysteine residue in position 58 (Cys 39, after signal peptide cleavage). This cysteine is also present on the mammalian C8GC (Cys 40) and is important for the binding of the protein to the rest of the complement complex: it has been demonstrated that Cys 40 of the human C8 gamma is linked to Cys 164 of C8 alpha, between the LDL-receptor and the membrane-inserting region of C8 alpha (Haefliger et al., 1991). Thus, also in chicken, Ggal-C8GC may be functionally involved in the formation of an active C8 complex.

Our previous and present findings of a coordinated transcriptional regulation of Ex-FABP, CALβ and CALγ genes, together with conserved consensus sequences for specific mediators, support the concept that these lipocalins may act in concert as a whole protein system sharing a related function. It should be noted that most of the biological phenomena we have examined (cell shape transition during phenotype differentiation, growth arrest in G0 of cell cycle, cell inflammatory reaction) represent physiological contexts where some level of cellular stress occurs. The induction by T3 may be seen in the same light in the context of bone formation. Endochondral ossification is indeed a process of profound tissue remodeling including metalloproteinase activation and degradation of the cartilaginous extracellular matrix, neovascularization, invasion by proliferating osteoblasts and deposition of bone matrix. This succession of events occurs at the level of the growth plate where T3 plays an essential role as a differentiation inducer. The T3-mediated activation of the cluster may thus correlate with a function of the lipocalins in the remodeling of cartilage into bone. The identification on the gene promoters of the osteogenic transcription factor Cbfa1 also keeps in line with the hypothesis of an involvement of the cluster in the control of bone formation. Therefore, three lipocalins of the cluster could be considered as a coordinated complex of polypeptides acting as acute phase response proteins, as previously suggested for Ex-FABP (Descalzi Cancedda et al., 2001).

At the present time, little is known about the mechanism of action of this group of proteins, and the elucidation of their function(s) will remain hypothetical until all their extracellular interactors are identified. Nevertheless, the fact that some of the cluster proteins appear to be orthologs of other well-known lipocalins suggests that an essential role has made this biological protein system to be preserved along chordate evolution.

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