Regulator of Complement Activation (RCA) Locus in Chicken: Identification of Chicken RCA Gene Cluster and Functional RCA Proteins

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Regulator of Complement Activation (RCA) Locus in Chicken: Identification of Chicken RCA Gene Cluster and Functional RCA Proteins

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A 150-kb DNA fragment, which contains the gene of the chicken complement regulatory protein CREM (formerly named Cremp), was isolated from a microchromosome by screening bacterial artificial chromosome library. Within 100 kb of the cloned region, three complete genes encoding short consensus repeats (SCRs, motifs with tandemly arranged 60 aa) were identified by exon-trap method and 3’ or 5’-RACE. A chicken orthologue of the human gene 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 2, which exists in close proximity to the regulator of complement activation genes in humans and mice, was located near this chicken SCR gene cluster. Moreover, additional genes encoding SCR proteins appeared to be present in this region. Three distinct transcripts were detected in RNA samples from a variety of chicken organs and cell lines. Two novel genes named complement regulatory secretory protein of chicken (CRES) and complement regulatory GPI-anchored protein of chicken (CREG) besides CREM were identified by cloning corresponding cDNA. Based on the predicted primary structures and properties of the expressed molecules, CRES is a secretory protein, whereas CREG is a GPI-anchored membrane protein. CREG and CREM were protected host cells from chicken complement-mediated cytolysis. Likewise, a membrane-bound form of CRES, which was artificially generated, also protected host cells from chicken complement. Taken together, the chicken possesses an regulator of complement activation locus similar to those of the mammals, and the gene products function as complement regulators. The Journal of Immunology, 2005, 175: 1724–1734.

The complement (C) system recognizes foreign cells and targets them for clearance and immune cytolysis (1). Host cells in contrast are protected from autologous C attack by membrane-associated C regulatory proteins (2). It is generally accepted that the C susceptibility in host cells is regulated mainly at C3 step. In humans, the C regulatory proteins consist of tandemly arranged domains named short consensus repeats (SCRs) and their genes cluster in a region designated as the regulator of C activation (RCA) locus. Each SCR consists of 60–70 aa, including 4 highly conserved cysteines (3, 4). The cysteines form disulfide bonds, folding the SCRs into a rigid triple-loop structure (3, 4). Although the proteins in the RCA family vary in size, they share significantly similar in three-dimensional structures due to conserved amino acids at specific locations. Considering their similarities and configurations, RCA locus might have been expanding by repeating gene duplications. The structural and functional properties of these proteins have been extensively studied in humans and rodents, whereas the RCA proteins or even locus have not been identified yet in nonmammalian vertebrate.

In humans, two soluble forms, factor H and C4b-binding protein (C4bp), and four membrane forms, CR1 (CD35), CR2 (CD21), decay-accelerating factor (DAF) (CD55), and membrane cofactor protein (MCP) (CD46), have been identified as C regulatory proteins (4, 5). Genes for all these regulators, except for factor H, were mapped to the RCA locus, 1q32 (4, 5). This locus is in close proximity to the 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 2 (PFKFB2) gene in mammals (6). Factor H gene is also mapped to the long arm of chromosome 1 but outside of the RCA

7 Abbreviations used in this paper: SCR, short consensus repeat; RCA, regulator of C activation; C4bp, C4b-binding protein; DAF, decay-accelerating factor (CD55); MCP, membrane cofactor protein (CD46); PFKFB2, 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 2; CREM, (formerly Cremp) C regulatory membrane protein of chicken; SBP1, sand bass protein 1; BAC, bacterial artificial chromosome; ORF, open reading frame; BLAST, Basic Local Alignment Search Tool; CRES, C regulatory secretory protein of chicken; CREG, C regulatory GPI-anchored protein of chicken; FISH, fluorescence in situ hybridization; CH3, Chinese hamster ovary; mCRES, membrane form of CRES; TM, transmembrane; GVB, gelatin veronal buffer; LHR, long homologous repeat.
locus. All human counterparts of these proteins are identified in mice. However, the mouse RCA locus is split into the two regions presumably through gene translocation. However, the RCA locus is conserved between humans and mice (7–9).

From an evolutionary point of view, we first identified chicken Crem (here designated regulatory membrane protein of chicken (CREM)) as its C regulatory system (10). This is a nonmammalian membrane-anchored C regulatory protein similar to MCP and DAF (11). That was the first report on the SCR-containing C regulatory protein in oviparous animals. However, no other SCR protein with C regulatory function has thus far been identified in chicken. In fish (Sand bass), an SCR-containing C regulator named sand bass protein 1 (SBP1) was cloned. SBP1 binds to both rainbow trout C3b and human C4b (12) and serves as a cofactor for factor I (12). However, SBP1 is unlikely to be a homologue of any member of human RCA because neither gene cluster of SCR-containing proteins nor a human homologue of a fish gene PFKFB2 was identified near the SBP1 locus. SBP1 would be a putative structural homologue of huFactor H (13). In contrast, a jawless fish, *Lampetra japonica* (lamprey) (14), and puffer fish (H. Os humble and T. Seya, unpublished data) possess an additional SCR-containing protein similar in size to huC4bp near the PFKFB2 gene. However, no gene clusters of SCR-containing proteins have been identified in the relevant regions of the fish and lamprey genomes to our database knowledge, suggesting that the RCA gene cluster is expanded in terrestrial animals.

In the present study, we report the identification of a gene cluster of SCR-containing chicken proteins. Three proteins identified in this cluster exerted host cell-protective activity against chicken C. Based on the structural and functional analyses of these SCR proteins, we concluded that the two loci of chicken and human RCA evolved from a common prototype. This is the first report on analysis of the nonmammal RCA locus and proteins.

**Materials and Methods**

**Isolation of chicken bacterial artificial chromosome (BAC) clones**

Chicken BAC libraries were screened with the full-length CREM cDNA. The properties of the established BAC libraries and the method for the isolation of BAC clones by four-dimensional PCR with the cDNA-derived primer set were described previously (15). We successfully obtained four CREM-positive DNA clones with 90–160 kb. Based on the information of the human RCA locus, we presumed that the chicken RCA and PFKFB2 loci were localized near or overlapping the genes of Crem (CREM), the human CD46 (MCP) analogue (11). The largest clone was found to contain a putative RCA locus because it covered many SCR-encoding exons judging from the results determined by the exon-trapping method described below.

**Exon trapping**

The exon-trapping methods were described in the manufacturer’s exon trapping manual (Invitrogen Life Technologies). Briefly, the BAC clone containing the chicken CREM gene was cut with *Pst*I and the exon-trapping library was made by inserting those *Pst*I fragments into *Pst*I site of pSPL3 (Invitrogen Life Technologies)-modified vector. The library was transferred into COS-7 cells using LipofectAMINE 2000 reagents (Invitrogen Life Technologies). After 48 h of incubation, total RNA was extracted using TRIzol (Invitrogen Life Technologies). Reverse transcription reactions were performed using vector-specific primer, SA2. The primary PCR was conducted using the primers, SA2 and SD6. To remove the fragments that contain no exon, the PCR products were cut with *Bst*XI, which degraded exon-deficient fragments. Secondary PCR was performed with SD2 and SA4 primers, using ExTaq polymerase (Takara Shuzo). A total of 105 independent clones was isolated by this technique. The primer sequences were listed in Table I. The amplified cDNA fragments were cloned into *gEM*-T easy vector using the TA cloning method. Basic Local Alignment Search Tool (BLAST) search analysis revealed that seven clones were similar to human CR1, three to CR2, two to DAF, six to MCP, three to polymeric IgR, and two to PFKFB2. The other clones neither showed similarity to any known genes nor *Escherichia coli* genome vector sequences.

**Cloning of C regulatory secretory protein of chicken (CRES) and C regulatory GPI-anchored protein of chicken (CREG)**

Using the chicken expressed sequence tag (accession no. BG713462) sequence, the clone of clone no. 54 isolated by exon trapping showed similarity to human CR1/CR2. We performed nested PCR on chicken thymus cDNA library using the vector-specific and CRES primes, PCR2 and PCRR. The library was obtained as described earlier (16). We obtained partial cDNA fragments that did not contain 5′- or 3′-end of open reading frame (ORF). To isolate these sequences, mRNA was prepared from the total RNA of chicken DT40 cells using a mRNA Purification kit (Amersham Biosciences). The cDNA was made from this mRNA using a Marathon kit (BD Clontech). Using the primers, GSP2-CR2 and NGSP2-CR2, 5′-RACE, and the primers, GSP1-CR2 and NGSP2-CR2, 3′-RACE was performed. The primer sequences are shown in Table I. To confirm the CRES sequence, we performed RT-PCR using mRNAs from DT40 or chicken liver as a template. We found the cDNA containing full-length ORF and several other cDNA fragments lacking the SCR4 region, which are probably derived by alternative splicing (data not shown).

Based on the clone no. 100 sequence, which shows similarity to human DAF, the 5′- and 3′-RACE were performed with the primers GSP1–100 and NGSP1–100 for 5′-RACE and GSP2–100 and NGSP2 for 3′-RACE, using the cDNA from DT40 cells as the template. Three independent RT-PCR amplions using the cDNA of DT40 or chicken liver as a template were sequenced and translated. The predicted protein consisted of seven SCRs with the GPI anchor, which we designated as CREG.

**Isolation of mRNA and RT-PCR**

Total RNA was extracted from chicken tissues and cell lines with TRIzol reagent (Invitrogen Life Technologies). Four micrograms of total RNA were reverse transcribed by *RnaseH*- reverse transcriptase (Promega) and then subjected to PCR cycle of cDNA amplification using ExTaq polymerase (Takara Shuzo). PCR was performed as follows: denaturation at 94°C for 2 min and 30 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 30 s. The products were separated on 0.7% agarose gel and stained with ethidium bromide.

**Construction of chicken RCA map**

The BAC clone was cut with indicated restriction enzymes and separated on 1% agarose gel in TAE buffer by pulse-field gel electrophoresis apparatus using Genofield (Atto Bioscience); the voltage was DC 40 V and AC 294 V, and the frequency modulation was from 0.30 Hz (start) to 0.60 Hz (end) in the linear setting, and the run time was 900 min. The DNA fragments were transferred to the Hybond-N (Amersham Biosciences) and southern hybridized with indicated probes.

**Protein domain structure and homology analyses**

The domain structures of chicken proteins were predicted using Simple Modular Architecture Research Tool program (http://smart.embl-heidelberg.de/). Putative GPI anchor site was predicted using big PI predictor (http://mendel.imp.univie.ac.at/sai/gpi/gpi_server.html) (17). Signal peptide was predicted by SignalP program (http://www.cbs.dtu.dk/services/SignalP/) (18). Homologies between chicken and human proteins were examined by BLAST search analysis. SCR domain homology was determined by comparing the SCR domains of chicken proteins with those of human proteins using blastn program in National Center for Biotechnolog Information BLAST server and Genetyx-Mac version 11.2.1 (Gene-tyx) maximum matching program.

**Chromosome preparation and in situ hybridization**

Fluorescence in situ hybridization (FISH) method was used for chromosomal assignment of chicken RCA genes. Preparation of R-banded chicken metaphase was described previously (19, 20). The results were consistent with those of CREM (11), indicating that the genes were mapped in close proximity to the CREM gene.

**Ab, cells, human proteins, and serum**

Fresh chicken and human sera were obtained from each species by standard methods (11, 21). All samples were stored at −80°C immediately after collection until use. Chinese hamster ovary (CHO) cells were obtained from American Type Culture Collection. RK13 cells (derived from the rabbit kidney) were obtained from Riken Cell Bank (Wako Pure Chemical). CHO cell clones expressing human MCP (CHO/MCP) were established as described in a previous report (22). CHO and RK13 cells were...
of the exon-intron boundaries, respectively.

phoresis in a 1.0% (w/v) agarose gel. RNAs were transferred onto a Hy-

TRIzol Reagent (Invitrogen Life Technologies) and separated by electro-

method established in our laboratory (11). Briefly, RK13 cells (1 /H11003

Rabbit anti-CRES and anti-CREG polyclonal Abs were produced by the

(FACS)

Rabbit anti-CRES and anti-CREG Abs and flow cytometry (FACS)

Table 1. Primers used in this study

<table>
<thead>
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<th>Primer Name</th>
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<tr>
<td>SA4</td>
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</tr>
<tr>
<td>SD2</td>
<td>GTGAACCTCACGTGGACAGC</td>
<td>Exon trapping</td>
</tr>
<tr>
<td>SA2</td>
<td>ATTCCTGATGTAATTTTGGAC</td>
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</tr>
<tr>
<td>SD6</td>
<td>TTCTGGTCACCTGCCAAACC</td>
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</tr>
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<td>PCR2</td>
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<td>5'-RACE (CREG)</td>
</tr>
<tr>
<td>PCRR</td>
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<td>5'-RACE (CREG) Exon/Intron (CREG)</td>
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<td>GSP2-CR2</td>
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<td>5'-RACE (CREG) Exon/Intron (CREG)</td>
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<td>NGSP2-CR2</td>
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<td>5'-RACE (CREG)</td>
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<tr>
<td>GSPI-CR2</td>
<td>GCACCTCGCTTGGGACAGTGC</td>
<td>3'-RACE (CREG)</td>
</tr>
<tr>
<td>GSPI-CR2</td>
<td>CGACCAAGAGTGACATCGGACAC</td>
<td>5'-RACE (CREG)</td>
</tr>
<tr>
<td>GSPI-100</td>
<td>ATCAGGATACAGCGTGGGCTCG</td>
<td>5'-RACE (CREG)</td>
</tr>
<tr>
<td>GSP2-100</td>
<td>CCTTTGGACAGAGTCACTTC</td>
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</tr>
<tr>
<td>GSPI-2-100</td>
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<td>3'-RACE (Exon/Intron (CREG))</td>
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<td>CRL1 SCR2F</td>
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<td>Exon/Intron (CREG)</td>
</tr>
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<td>CGGAGTTCTTCTTGGT</td>
<td>Exon/Intron (CREG)</td>
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<td>2GSP1-100</td>
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<td>Exon/Intron (CREG)</td>
</tr>
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<td>Exon/Intron (CREG)</td>
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<td>Exon/Intron (CREG)</td>
</tr>
<tr>
<td>chCRL2-F3</td>
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<td>Exon/Intron (CREG)</td>
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<tr>
<td>chCRL2-R3</td>
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<td>Exon/Intron (CREG)</td>
</tr>
<tr>
<td>100R1</td>
<td>GCCACACAGAAAGGAGATG</td>
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<tr>
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</tr>
<tr>
<td>chCRL2-F6</td>
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</tr>
<tr>
<td>chCRL2-R3UTR</td>
<td>ATTCGAGGTGTTCTTCTCC</td>
<td>Exon/Intron (CREG)</td>
</tr>
</tbody>
</table>

a “Exon trapping,” “5'- or 3'-RACE,” or “Exon/Intron” represents the primers used for exon-trapping screening, RACE reactions to isolate chicken proteins, or determination of the exon-intron boundaries, respectively.

maintained in Ham’s F-12/10% FCS and DMEM/10% FCS, respectively. These cells were transfected with cDNAs in expression vectors by the usual method. For RNA and protein blot analysis, total RNAs and proteins were obtained from various tissues and stored at −80°C until use.

Tissue RNA blotting analysis

Total RNAs (20 μg) were extracted from various chicken tissues using TRIzol Reagent (Invitrogen Life Technologies) and separated by electrophoresis in a 1.0% (w/v) agarose gel. RNAs were transferred onto a Hybond N+ membrane (Amersham Biosciences). The blot was prehybridized for 30 min at 68°C and hybridized for 1 h at 80°C in ExpressHybridization buffer (BD Biosciences/Clontech) with 32P-labeled full-length ORF of chicken RCA cDNAs as a probe. The membrane was washed and exposed to x-ray film at ~80°C.

Rabbit anti-CRES and anti-CREG Abs and flow cytometry (FACS)

Rabbit anti-CRES and anti-CREG polyclonal Abs were produced by the method established in our laboratory (11). Briefly, RK13 cells (1 × 106) were transiently transfected for 48 h with a pFlag CMV- (CREG) or CREG-HisX6 construct using LipofectAMINE Plus reagent (Invitrogen Life Technologies). Transfected RK13 cells were collected in 10 mM EDTA-PBS and suspended in 0.5 ml of PBS after washing three times with PBS. The RK13 cell suspensions were then mixed and emulsified with 0.6 ml of Freund’s complete adjuvant (Difco) and used for immunization of rabbits. Immunization was performed for 4 days at 7-day intervals, and the rabbits were boosted before drawing the blood. IgG was precipitated with 33% ammonium sulfate, dialyzed against PBS (11), and stored at −30°C until use. These monospecific Abs recognized only the relevant proteins. FACS analysis was performed as described previously (22). Cells were treated with the above Abs, washed three times, and tagged with FITC-labeled second Abs. FACS analysis was used for working out the RCA LOCUS IN THE CHICKEN.

Protein blot analysis

Various chicken tissues were solubilized in lysis buffer (0.02 M Tris-HCl (pH 7.4) containing 1% (v/v) Nonidet P-40, 0.14 M NaCl, 0.01 M EDTA, 1 mg/ml iodoacetamide, and 1 mM PMSF) using a potter type homogenizer. After incubation at 4°C for 30 min, each lysate was centrifuged at 15 000 rpm at 4°C for 30 min. The supernatant was collected, and protein concentration was measured using a protein assay kit (Bio-Rad). Fifty micrograms of total cellular proteins (extracted from 50 mg of tissue) were resolved by SDS-PAGE (7.5% gel) and transferred to polyvinylidene difluoride membranes. CRES, CREG, and CREM were visualized using an ECL detection system (Amersham Biosciences) with rabbit Abs (2 μg/ml) and a HRP-linked goat anti-rabbit secondary Ab (1 μg/ml) (BioSource International).

Generation of stable CHO transfectants expressing CREG, CREM, or artificial membrane form of CRES (mCRES)

The cloned CRES cDNA was ligated with the DNA sequence of the transmembrane (TM) and cytoplasmic portion of MCP (CD46) and placed in the XhoI/NotI site of pEFBOS, the method as described previously (14).
CHO cells were transfected with the expression plasmid using Lipo- 
fectAMINE (Invitrogen Life Technologies). CHO clones expressing a 
mCRES were established through limiting dilution by G418 selection (0.7 
mg/ml) (14) and screened by flow cytometry using anti-CRES Ab. CHO 
clones expressing CREM were established as described previously 
(11). CHO cell clones expressing CREG were obtained by transfection of 
CHO cells with the CREG cDNA in mammalian expression vector 
pCXN-2 (11). Stable transfectants were selected by 0.6 mg/ml G418 (In-
vitrogen Life Technologies). Selected CHO cells were assessed for CREG 
expression by immunoblotting and flow cytometry using anti-CREG Ab.

Calcein release cytotoxicity assay

The method for the cytotoxicity assay using a fluorescent tracer was de-
scribed previously (11). Briefly, the intact or transfected CHO cells (2 
× 10^4 cells/well) were seeded in 96-well plates. After they attained 90% 
confluence, the cells were loaded with a fluorescent dye, calcein-AM (Mo-
lecular Probes), by incubation with 10 μM calcein-AM in serum-free 
Ham’s F-12 medium for 30 min at 37°C. The cells were then incubated 
with 50 μl of 400 μg/ml rabbit anti-CHO cell Ab (22) in PBS for 30 min 
at 4°C. The Ab-sensitized CHO cells, which are known to be susceptible to 
lysis by the human alternative pathway (4, 22), were suspended in Ca^{2+}/ 
Mg^{2+}-containing medium (gelatin veronal buffer, GVB). These cells 
were subsequently incubated with 50 μl of various concentrations (typi-
cally 10%) of human or chicken serum diluted in GVB^+/− for 60 min at 
37°C with gentle shaking. In some cases, chicken serum (1 ml) was mixed 
with intact CHO cells (1 × 10^5) at 4°C for 15 min (11, 22) and used as 
natural Ab-absorbed serum. The plates were centrifuged at 1500 rpm for 5 
min, and the fluorescence intensities of 100-μl aliquots of the supernatants 
were measured using a fluorescence plate reader with excitation at 488 nm 
and emission at 514 nm. Percent cytotoxicity was calculated as described 
previously (22). The experiments were performed three times in triplicate.

Results

Identification and mapping of the RCA locus genes in the chicken

Several lines of evidence suggested that CREM is the chicken 
homologue of MCP (CD46) (11). The CREM gene was mapped to 
chicken microchromosome 26 (11, 23). We surmised that the chicken possesses the RCA locus that involves the CREM gene. 

Four genomic clones containing the CREM gene were isolated from the chicken BAC library by PCR with the CREM cDNA-
derived primer set. Several exons encoding SCRs were obtained 
from the BAC clones by the exon trap method and mapped within 
the 100 kb. We identified CREM and two other novel genes, CRES 
and CREG, in the 100-kb chicken SCR-rich locus (Fig. 1). Re-
striction analysis shows that these were single copies in the puta-
tive RCA locus. FISH analysis indicated that their genes are 
mapped near the CREM gene (data not shown). Their exons were 
arranged based on the RFLP and Southern blot analyses and com-
parable to those of human RCA proteins, C4bp, DAF, CR2, CR1, 
and MCP (Fig. 2). In regard to their configurations, CRES, CREG, 
and CREM seemingly correspond to C4bp, CRY/DAF, and MCP, 
respectively. Three cDNA fragments coding the putative SCR pro-
teins were obtained by RT-PCR, confirming the expression of these 
genes. Clustering of SCR protein genes, the order of the gene orga-
nization, and the identification of PFKFB2 gene at close proximity to

FIGURE 1. Pulse-field gel analy-
sis of chicken BAC DNA containing 
CREM. The figure shows the ethidium bromide (EtBr) staining of a 
gel and the subsequent hybridization of the Southern blots (lower panels) 
with corresponding 32P-labeled cDNA probes. Restriction enzymes 
abbreviations are as follows: Nr, 
NrdI; P, PvuII; No, NotI; C, CpoI; M, 
MluI; and S, SfiI. Molecular markers 
are indicated on the EtBr panel.

FIGURE 2. Structures of chicken BAC clones of SCR-rich protein 
genes. The code for the restriction enzymes is given in the legend to Fig. 
1. Human RCA locus is aligned with the putative RCA locus of chicken. 
Notice that the PFKFB2 gene is linked on the CRES-containing fragment.
FIGURE 3. Sequences of the exon-intron junctions of CRES, CREG, and CREM. Exons are boxed. Closed boxes indicate translated regions. The ag-gt consensus sequences for splicing are conserved. The sizes of introns are indicated. A, CRES; B, CREG; and C, CREM.
this locus suggested this BAC fragment to be the RCA locus of chicken.

Genomic and primary structures of the chicken RCA proteins

Genomic structures, including the exon-intron boundaries, were determined with these three chicken RCA genes (Fig. 3). SCR2 of CRES, SCR2 and SCR6 of CREG, and SCR2 of CREM were encoded by split exons similar to the functionally essential exons of the human C regulatory proteins (Fig. 3, A–C). Furthermore, the amino acid similarities of the split exon-encoded SCRs to those of corresponding functional SCRs of human proteins were relatively high at 43% (Fig. 4, A and B). The divisions in their coding

FIGURE 4. Complete amino acid sequences of CRES and CREG. Deduced amino acid sequences of CRES (A) and CREG (B) are shown under the nucleotide sequences. Asterisks indicate the stop codons. The signal sequences are underlined. The nucleotide sequence of CRES contained both the polyadenylation signal (double underlined) and poly(A) sequence. The circled Gly in CREG C-terminal region is the predicted GPI anchor modification site (see Materials and Methods). The nucleotide sequences have been registered in the European Molecular Biology Laboratory Data Library/GenBank/DNA Data Bank of Japan databases with the accession nos. AB074567 (CRES) and AB109024 (CREG). C, The outlines of the CRES and CREG primary structures are shown. Incomplete SCR are not numbered. Circle, SCR motif; square, TM domain; and hexagon, cytoplasmic tail. Model of CREM was delineated according to the published primary structure (11). D, Each SCR sequence of CRES and CREG was compared with that of human SCR proteins using an National Center for Biotechnology Information BLAST search. Regions with high homology scores are shown as red and orange.
regions occur at similar positions. Thus, it is likely that the split exons in the chicken SCR proteins serve as functionally active domains.

To identify cDNA clones covering full-length ORF for the chicken SCR proteins, we designed PCR primers based on the derived sequences (Table I). The primary structures of the three RCA proteins predicted from the RT-PCR products offered that CRES, CREG, and CREM consisted of 10 SCRs, 7 SCRs, and 5 SCRs with one SCR-like domain, respectively (Fig. 4 C). The first exons of all three genes contained signal sequences (Fig. 4; Ref. 11). CREG possessed a domain containing a GPI anchor-predicted site and CREM had a TM and a cytoplasmic tail in their C termini, respectively (Fig. 4; Ref. 11). Presence of the GPI anchor in CREG was confirmed by octylglucoside solubilization and phosphatidylinositol phospholipase C treatment (data not shown). No specific site for membrane attachment was identified in CRES, suggesting its secretory features.

Domain-to-domain comparison was performed with CRES and CREG vs human CR1, CR2, C4bp α-chain, DAF, and MCP (Fig. 4D). The sequential SCR2–4 structure of CRES was most similar to that of C4bp α-chain, which is the functional core (24, 25). The SCR2–4 of CRES was secondly similar to that of MCP, which is
again the functional core (26, 27). Other SCR sets of CRES had no marked similarity to SCR sets of human RCA proteins. Because CRES is a secretory protein consisting of 10 SCRs, it would be an orthologue of huC4bp. The sequential SCR1–4 structure of CREG periodically appeared in the structure of CR1 with significant similarity to SCR1–4, SCR8–11, SCR15–18, and SCR22–25, suggesting that CREG corresponds to one long homologous repeat (LHR) of huCR1 (3, 28).

**Tissue distribution of chicken SCR proteins**

Tissue distribution of mRNAs of CRES, CREG, and CREM were examined by Northern blot and RT-PCR analyses (Fig. 5, A and B). RNA blotting followed by hybridization with the full-length ORF of CRES or CREM as a probe revealed a single 3.8-kb band predominantly in the liver and widely distributed 3.0/2.2-kb bands among the other tissues examined (Fig. 5A). The trace messages of CREG were detected in various organs after long exposure of the film (data not shown). RT-PCR analysis also exhibited wide distribution of CREG in almost all tissues (Fig. 5B). Relative message levels of CREG were generally low compared with those of CREM. Clone no. 54 was also found to be a message with SCR-coding sequence (data not shown), but full-length cDNA could not be obtained with primers used (Table I).

**Protein expression of chicken RCA members**

To determine the tissue distribution and relative levels of CRES/CREG proteins, we produced polyclonal Abs against these proteins and performed immunoblotting analysis (Fig. 5C). In this analysis, the lanes contained 50 µg of proteins released from tissues. CRES was detected only in the serum and organs rich in plasma as a 50- to 70-kDa doublet band. Our findings suggest that CRES was synthesized mainly in the liver and then secreted into the systemic circulation. A two-band signal of CREG was detected in various organs by treatment of cells with octylglucoside. Preparations of cell lysates were incubated in 50 mM octylglucoside for 1 h at room temperature and then solubilized in 0.5% sodium deoxycholate. CREG was differentially spliced and/or glycosylated and expressed on the cell surface as a GPI-anchored protein (Fig. 5C). The molecular masses of CREM consisted of a 45-kDa major band and a 40-kDa minor band (Fig. 4C). The molecular masses of CREM were small as in CREG in the brain compared with other organs. CREM and CREG were distributed ubiquitously, except for the serum.

FACS analysis indicated that erythrocytes and large/small leukocytes were all CREG- and CREM positive (Fig. 5D). Erythrocytes altered morphologically if the cells were treated with anti-CREM Ab (Fig. 5D).

**Complement protection assay using CREM/CREG/mCRES-expressing CHO cells**

It is currently accepted that the chicken has the brusa of Fabricius where B lymphocytes are generated through gene conversion. IgY and IgN are effectors for C activation. Chicken possesses a structural and probably functional orthologue of human C3 (29). In our primary test, no chicken C-mediated cytolysis was virtually observed on intact CHO cells using chicken Ig-containing chicken serum, whereas chicken C was activated on rabbit IgG-sensitized CHO cells even by chicken serum preabsorbed with intact CHO cells (Fig. 6A). Therefore, we decided to use CHO cells or its transfecteds sensitized with rabbit Ab for C protection assay. To determine whether the chicken RCA proteins have the ability to protect host cells from attack by homologous C, we established CHO cell clones stably expressing CREG or CREM. Because CRES is a soluble protein, we generated a mCRES by attaching TM and cytoplasmic portions of MCP to the C terminus of CRES. We cloned a CHO subline expressing mCRES for this purpose (Fig. 6B).

Chicken sera (5–20%) were used as C sources and rabbit anti-CHO Ab-mediated CHO damage was tested using these CHO clones expressing mCRES (Fig. 6B), CREG, or CREM (Fig. 6C). Intact CHO cells served as control. Cytotoxicity assay was performed with calcine-labeled sensitized CHO cells. The assay was performed at 39°C when using chicken serum (otherwise at 37°C). Rabbit IgG sensitization of CHO cells conferred susceptibility to chicken serum if the cells did not express chicken RCA protein(s). The results demonstrated that chicken serum (10%) damaged IgG-sensitized CHO cells, and the expression of CREG, CREM, or mCRES on CHO cells blocked chicken-serum-mediated cytotoxicity (Fig. 6, B and C), which is similar to the case of human serum-mediated cytotoxic studies using CHO cells expressing MCP or DAF (4, 22, 27). IgG-sensitized CHO cells also showed human C-mediated lysis and under the same conditions CHO/MCP blocked human C-mediated attack by 20% while cells expressing CHO/CREM or CHO/CREG barely blocked human C-mediated lysis (Fig. 6D). These results indicate that chicken RCA proteins exerted species specificity to block the attack by homologous C. However, by which mode the C pathways of the chicken is most efficiently blocked by each C regulator has not yet been identified in this study because Ab sensitization allows the cells to activate the classical (22), alternative (22), and possibly also lectin pathway (30). We currently hold that CREG, CREM, and CRES all serve as C regulators in the chicken body with different properties.

**Discussion**

We described here that CRES, CREG, and CREM compose chicken RCA locus, which we suggest evolved from a putative ancestral RCA locus common to the avian and mammalian. This is the first identification of the RCA locus and proteins in nonmammals. Chicken harbors a single RCA locus encoding multiple C regulatory proteins that correspond to the human. Human RCA is a single locus while the mouse RCA consists of two splits (7–9). Chicken RCA genes, CRES, CREG, and CREM, are located in a single locus. Clone no. 54 encoded an SCR, which is similar to SCR2 of huCR1. This, together with chicken genome draft search, suggests that clone no. 54 is a part of a putative CR1-like protein (T. Seya, unpublished data). The order of the genes, CRES (no. 54, CR1-like), CREG, and CREM, was principally overlapped with those of human genes, MCP, CR1, DAF, and C4bp. Their structural features were made up of the hybrid or mixed SCR combinations compared with the human counterparts. In mice, two contiguous genes, Mcry and Mcr2, are mapped to the 40 cM telomeric to C4bp on mouse chromosome 1 (9). Thus, they define a breakpoint in the large conserved linkage group between distal mouse chromosome 1 and human chromosome 1q32. This suggested that a translocation or inversion occurred within the mouse RCA gene family during the oviparous-to-mammalian evolution. The PFKFB2, which is not a member of the RCA group, was mapped on the centromeric to Mcry and Mcr2 (9), supporting this hypothesis. In addition, the soluble C regulator, Cres (or C4bp), is the most distal member of the conserved linkage group thus far identified. Hence, the gene clustering profile of the chicken but not mouse RCA appears to reflect a prototype of the human RCA locus.

In humans, the factor H gene is located at >7 Mb from the cluster of RCA gene family (1, 2). The fish and lamprey have factor H orthologues (12, 31–33), which are functional as C regulators. That is, sand bass has factor H-like protein SBP1 (12, 13),...
which serves structural and functional orthologues of huFactor H. Although sand bass has a putative additional SCR-containing protein, named sand bass cofactor related protein 1, it shares structural similarity with SBP1 (31), and their relationship is similar to that between factor H and its related proteins, factor H-related proteins (31). In the fish, no other RCA orthologues have been identified. However, in our database analysis, fish possesses soluble C4bp-like SCR proteins (H. Oshiumi and T. Seya, unpublished data), and the gene of this SCR protein is syntenic with fish PFKFB2. Nonetheless, this SCR protein did not grow into a multiple gene cluster in the fish (T. Seya, unpublished data), suggesting that the origin of the RCA locus consisted of a single gene encoding a soluble C regulatory protein with 6–10 SCRs and have evolved into multiple genes with differential functional profiling, i.e., intrinsic and extrinsic regulation and prevention of C consumption in blood plasma. Our findings favor the interpretation that SCR exon duplication and shuffling among RCA genes yielded the gene cluster of SCR proteins. Only the SCR of split exons were conserved as functional domains of these genes.

In humans, the RCA locus includes the six genes and two incomplete pseudogenes located within the 0.9-Mbp region. This contains the RCA genes C4bpα, C4bpβ, MCP, MCP-like, DAF, CR1, CR2, and CR1-like (2, 4, 5). In contrast, the chicken RCA locus was mapped within 0.1-Mbp in a microchromosome, which is 9-fold narrower than that of humans. Yet, putative corresponding genes were mapped within this region, suggesting that the non-coding regions, including the introns and intergenic regions, are small in chicken RCA compared with human RCA. Indeed, almost all introns of CRES, CREG, and CREM were shorter than 1 kbp (Fig 3). These are contrast to human introns, which are usually more than several kilobase pairs long. Chicken MHC is also 10-fold smaller compared with that of human (34). Thus, total immune-related locus would be compact in chicken. Our molecular analysis of the gene described in this investigation unequivocally predicts that the C-associated immune system and its responses in mammals were preserved in the avian through evolution. Further functional analyses of each SCR protein will give us more information about the relationship between the SCR proteins (35) and their roles in the C regulatory system of chicken.

After completion of our study, chicken genome draft sequence (36) was opened (www.ensemble.org/). Generally speaking, draft sequence contains ambiguous regions and incorrect sequences, which are repeatedly updated. Using the last update version, we conducted a BLAST search with cDNA sequences of chicken PFKFB2, CRES, CREG, and CREM and examined the positions of the genes on chicken genome (Fig. 7). Our conclusion is that the draft sequence supports our experimental data, and conversely, our data supports the draft sequence. However, we noticed that there are serious inconsistencies between our data and the chicken draft sequence (Fig. 7). A marked difference is that the genome region encoding CRES ORF is completely involved in the predicted gene region encoding chicken PFKFB2 cDNA. More precisely, draft sequence indicated that the PFKFB2 cDNA isolated by exon trapping is interrupted by two introns and thus consists of three exons. Pulse-field gel electrophoresis data (Fig. 2) did not support the results from the draft sequence. Considering that RCA locus contains many similar exons encoding SCR domains, the discrepancy seems to be explained by incorrect assembly caused by sequence similarity of this region. Prediction of exon/intron boundaries is usually very difficult without any experimental
data, and each prediction program often shows different results. These points, taken together with the unidentified structures predicted by the draft sequence (Fig. 7) located near the PFKFB2 but distinct from CRES, CREM, and CREM, reinforce the importance of our experimental data to convince the existence of chicken RCA. Correct assembling of the scaffolds in the draft sequence and annotation of the genes will be required to complete the RCA region of the chicken genome.

CRES was a secretory protein consisting of 10 SCRs; its SCRs 1–4 had a framework similar to that of the α-chain of C4bp. The functional SCR set of huC4bp α is SCR2–3, which contained a SCR encoded by a split exon similar to CRES (2, 31). Our preliminary data suggested that this protein served as a protease (presumably factor I-like)-cofactor for the cleavage of chicken C3b-like protein (T. Seya, unpublished data) that resembled one reported previously (29). Earlier studies by Kaidoh et al. (37, 38) suggested the presence of factor I-cofactor activity toward human C3b in birds, including the chicken. However, no divergent cation was required to cleave human C3b-like C3 by the serum protease. This SCR protein was similar to the human C regulatory system but dissimilar to the lamprey system (14). CRES may represent a soluble SCR-containing C regulatory protein that evolved to huC4bp.

CREG, a GPI-anchored membrane protein, consisted of seven SCRs with relatively high similarity to MCRY and a LHR of CR1 (7, 28). Therefore, the gene encoding “7 SCR” unit, designated LHR that compose CR1 and MCRY, seems to exist in the common ancestral animal genome. Likewise, GPI appears to have developed in SCR-containing proteins with host protection properties from C. So far, no CR1-like protein with seven SCRs has been reported, except CREG and MCRY. CREG has the ability to protect host cells from chicken C, and the species specificity between C and its regulators exists in the chicken C system. CREG may serve as the molecular predecessor for the previously reported functional entities of the self-protective C regulatory proteins in mammals. It may represent the earlier form of MCRY (7). Therefore, it is very likely that another yet to be further defined protein has opsonin activity through its ability to bind C3b deposited on foreign material in chicken. Possibly clone no. 54 may be a part of a bigger SCR protein, presumably chCR1.

C2, C3, factor B, MBL, and MASP have been identified as chicken C-related proteins (29, 39–41). Chicken has a system of gene conversion conferring B cell clonal variation on huge variation of Ig in the bursa of Fabricius (42). This means that chicken possesses multifarious C pathways similar to human. The RCA family could be expanded in coordination with the divergence of the C cascades. The tantalizing question is why multiple SCR proteins with differential structures and presumably functions diverged during the evolution from fish to birds. Shift of the lifestyle from the sea to the land might have been a crucial event for providing the sophisticated C regulatory system. Yet, what had happened at that stage needs additional investigation.

The pattern recognition systems of TLRs (43), phagocytosis receptors, and C-type lectins aiming at microbial and interspecies recognition to eliminate foreign materials are becoming clear with recent advance in studies on innate immunity. Acquired immune system appears to have emerged based on the necessity to precisely discriminate between self- and nonself-Ags, leading to immunological consolidation of individual identity. Coupling this to the change of innate-acquired interface, the C system evolved to adapt the two differential modes of immune system for foreign cell recognition and consequent elimination. Our hypothesis is that many RCA proteins were developed from a single C regulator with the primitive function, concomitantly evolving in higher vertebrates. Perhaps, the differential functional assignment to each RCA protein started before birds and mammals diverged from the fish. Additional phylogenetic studies using amphibians and reptiles and functional studies of each RCA protein in these lower vertebrates will test this hypothesis.

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Disclosures

The authors have no financial conflict of interest.

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