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The Cysteine-rich Protein Family of Highly Related LIM Domain Proteins*

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Here we describe a family of closely related LIM domain proteins in avian cells. The LIM motif defines a zinc-binding domain that is found in a variety of transcriptional regulators, proto-oncogene products, and proteins associated with sites of cell-substratum contact. One type of LIM-domain protein, called the cysteine-rich protein (CRP), is characterized by the presence of two LIM domains linked to short glycine-rich repeats and a potential nuclear localization signal. We have identified and characterized two evolutionarily conserved members of the CRP family, CRP1 and CRP2, in chicken and quail. Expression of the genes encoding both CRP1 and CRP2 is differentially regulated in normal *versus* transformed cells, raising the possibility that members of the CRP family may function in control of cell growth and differentiation.

A number of genes are specifically and rapidly up-regulated in response to growth factor stimulation (1). The expression of these primary response, or immediate early, genes is independent of new protein synthesis and requires only the activation of pre-existing transcriptional regulators (2). The primary response genes encode proteins including transcription factors, proto-oncogene products, and other regulatory proteins that facilitate the transition of the cell from an arrested to a proliferative growth state and stimulate differentiation (3–5). The first cysteine-rich protein family member to be described (referred to here as CRP1)[1] was shown to be encoded by the primary response gene, *CSRP1*, that exhibits serum induction coordinate with c-*myc* expression (6, 7).

From analyses of both human and chicken cDNA, genomic DNA, and protein sequences (6–9), it has been determined that CRP1 contains two copies of a specific amino acid sequence motif termed LIM. The LIM motif displays the consensus amino acid sequence $CX_2CX_{16-23}HX_2CX_2CX_2CX_{16-21}CX_{2-3}$(C/H/D) (8). Spectroscopic studies of LIM domains derived from a number of different proteins have revealed that the LIM domain specifically coordinates two zinc ions (10–13). The solution structure of a LIM domain derived from chicken CRP1 has been solved by two-dimensional NMR and illustrates that the LIM domain is itself a bipartite structure with spatially distinct modules focused around each metal binding site (14). Interestingly, although the LIM domain has been clearly demonstrated to function in specific protein-protein interactions (15–17), the tertiary fold of one zinc-binding module within the LIM domain is essentially identical to that found in well characterized DNA-binding zinc fingers (14). It remains to be determined whether the structural features of the LIM domain reflect a biologically significant ability to associate with nucleic acids as well as proteins.

The LIM motif was first identified in three developmentally regulated transcription factors, *C. elegans* Lin-11, rat Isl-1, and *C. elegans* Mec-3, from which the term LIM is derived (18, 19). The LIM domain is often found in association with obvious functional domains, such as a DNA-binding homeodomain (18, 19) or a kinase domain (20). However, the LIM domain may also represent the primary sequence element in a protein. Examples of such "LIM only" proteins include CRP1 (6–9), the cysteine-rich intestinal protein (21), and rhombotin (22–25). Interestingly, although the LIM-only proteins lack DNA-binding homeodomains, they may also function in the regulation of cell growth and differentiation. For example, rhombotin-2 is a proto-oncogene product that is required for erythroid differentiation during mouse development (26) and overexpression of rhombotin genes in the thymus of transgenic mice results in T-cell acute lymphoblastic leukemia (27, 28).

CRP1 has been purified to homogeneity from chicken smooth muscle, and many of its biochemical and biophysical properties have been characterized (9). Binding studies have revealed that CRP1 interacts directly with another LIM protein called zyxin (8, 9). Both zyxin and CRP1 are localized at sites of membrane-substratum contact in association with the actin cytoskeleton (9). Together these proteins are postulated to perform a regulatory or signaling function at the adhesive membrane (8, 9, 15).

Recently, the level of a quail transcript that encodes a LIM domain protein with a high degree of structural similarity to CRP1 was shown to be dramatically reduced in avian fibroblasts transformed by retroviral oncogenes or chemical carcinogens (29). The amino acid sequence of the predicted quail protein was 79.8% identical to that of human CRP1 (29), and the protein was therefore postulated to represent the quail homologue of human CRP1. Subsequently, the complete amino acid sequence of chicken CRP1 was deduced from a cDNA clone and shown to be 90.6% identical to human CRP1 (9). Because

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[1] The abbreviations used are: CRP, cysteine-rich protein; pBS, pBluescript KS; RACE, rapid amplification of cDNA ends; kb, kilobase(s); MLP, muscle LIM protein.

the quail protein, although obviously closely related to CRP1, was significantly less similar to human CRP1 than was chicken CRP1, we postulated that the quail protein represented a new member of the CRP family, a CRP2. In this report, we demonstrate that both chicken and quail display multiple genes encoding CRP proteins. We have characterized two members of this gene family, *CSRP1* and *CSRP2*, in both species.

EXPERIMENTAL PROCEDURES

Cells and Viruses—Chicken or quail embryo fibroblasts were prepared as described previously (30). The quail embryo fibroblast nonproducer line Q8 transformed by the v-*myc* oncogene of avian retrovirus MC29 and the quail embryo fibroblast producer line MH2-A10 transformed by the v-*myc*/v-*mil* oncogenes of the avian retrovirus MH2 have been described before (30, 31). The Rous sarcoma virus mutant *ts*LA29 encoding a temperature-sensitive v-Src protein was a kind gift of John A. Wyke (32). Infection of quail embryo fibroblasts with MH2(MHAV) virus released from MH2-A10 cells and transfection with *ts*LA29 cloned proviral DNA were carried out as described previously (30, 33).

Isolation and Characterization of Quail CSRP1 cDNA—Duplicate plaque lifts of a quail embryo fibroblast cDNA library prepared in λgt10 (29) were screened by hybridization to quail *CSRP2* cDNA (29) or to chicken *CSRP1* cDNA (9) probes. The probes were prepared with the Multiprime DNA labeling system (Amersham Corp.). Hybridization was carried out for 16 h at 37 °C in buffer H containing 50% (v/v) formamide, 6 × SSC (1 × SSC is 150 mM sodium chloride, 15 mM sodium citrate), 5 × Denhardt's solution (0.1% (w/v) Ficoll, 0.1% (w/v) bovine serum albumin, 0.1% (w/v) polyvinylpyrrolidone), 5 mM EDTA, 0.5% (w/v) SDS, and 100 μg/ml sheared, denatured salmon sperm DNA. Following hybridization, the filters were washed once for 20 min at 55 °C in 2 × SSC, 1 mM EDTA, and 0.1% (w/v) SDS and then twice for 20 min at 50 °C in 0.4 × SSC, 1 mM EDTA, and 0.1% (w/v) SDS. A screen of 3×10^5 recombinant phage led to the identification of about 210 phage positive for both probes. Comparison of the hybridization signals obtained with the quail *CSRP2* and chicken *CSRP1* probes revealed that about 40 of these recombinant phage harbored cDNA inserts of quail *CSRP1*. Two of these phage were purified using standard procedures, and their cDNA inserts were subcloned into the *Eco*RI-site of the plasmid vector pUC19. Nucleotide sequences of plasmid DNAs were directly determined by the dideoxynucleotide chain termination method, using a T7 sequencing kit (Pharmacia Biotech Inc.). Both clones were positively identified by partial sequence comparison with the reported sequence of chicken *CSRP1* cDNA(9). The complete nucleotide sequence of one clone (clone 7) was determined by usage of the two internal *Bgl*II and the unique internal *Pvu*II restriction sites.

Isolation and Characterization of Chicken CSRP2 cDNA—A chicken embryo cDNA library cloned into the *Eco*RI site of the expression vector λgt11 (Clontech Laboratories Inc., Palo Alto, CA) was screened by hybridization to chicken *CSRP1* DNA (9) or to quail *CSRP2* cDNA (29). Duplicate plaque lifts were probed with a chicken *CSRP1* probe and a quail *CSRP2* probe. Both the chicken *CSRP1* and quail *CSRP2* probes were labeled with ^{32}P by the random primer method according to an established procedure (Stratagene, La Jolla, CA). In the initial screen of 1.5×10^6 recombinant phage, hybridization and washing conditions were as described above for the isolation of quail *CSRP1* cDNA. Of the 34 positive phage that were identified by hybridization to quail *CSRP2*, 32 were also identified by cross-hybridization with chicken *CSRP1*. Comparison of the signals obtained with the chicken *CSRP1* and quail *CSRP2* probes revealed that 26 of these recombinant phage harbored cDNA inserts that hybridized weakly with the *CSRP1* probe and more strongly with the *CSRP2* probe. Seven of these phage were isolated based on their ability to hybridize to a quail *CSRP2* probe as follows: hybridizations were performed at 45 °C in 5 × SSPE (1 × SSPE is 150 mM sodium chloride, 10 mM sodium phosphate pH 7.4, 1 mM EDTA), 5 × Denhardt's solution, 0.5% (w/v) SDS, and 250 μg/ml yeast tRNA (Boehringer Mannheim). These hybridizations were followed by two room temperature washes in 2 × SSPE and 0.1% (w/v) SDS, one 15-min wash at 45 °C in 1 × SSPE and 0.1% (w/v) SDS, and one 10-min wash at 45 °C in 0.1 × SSPE and 0.1% (w/v) SDS. Two chicken *CSRP2*-containing clones were positively identified by partial sequence comparison with the reported sequence of quail *CSRP2* (29). Sequence analysis of phage DNA was conducted by the method of dideoxynucleotide chain termination sequencing of polymerase chain reaction products (Life Technologies, Inc.) using primers directed against regions flanking the *Eco*RI cloning site of λgt11. The positively identified chicken *CSRP2* cDNA inserts were then cloned into the *Eco*RI site of the plasmid vector pBluescript KS (pBS; Stratagene, La Jolla, CA) to generate pBS-*CSRP2*-TM1 and pBS-*CSRP2*-TM6. Sequencing of both strands of these inserts was performed by the method of double-stranded DNA sequencing using the dideoxy chain termination method (34) and Sequenase II (U.S. Biochemical Corp.), or by dideoxynucleotide chain termination sequencing of polymerase chain reaction products (Life Technologies, Inc.). Ambiguities were resolved using a modification of the dideoxynucleotide chain termination method (35).

Chicken genomic DNA containing a *CSRP2* fragment was isolated by the polymerase chain reaction using primers corresponding to nucleotides 354–374 and 558–577 of *CSRP2*-TM1 cDNA. Sequencing of the genomic DNA fragment was conducted using methods described above.

Identification of the 5′ End of Chicken CSRP2 cDNA—The 5′ end of chicken *CSRP2* was identified using two methods. First, sequences corresponding to the 5′ end of *CSRP2* mRNA (referred to as *CSRP2*-5′RACE) were isolated using a modified primer extension 5′-RACE technique (Clontech Laboratories, Inc., Palo Alto, CA) (36). The poly(A)$^+$ mRNA utilized in this procedure was purified from approximately 2×10^7 chicken embryo fibroblasts using a commercially available protocol (Qiagen Inc., Chatsworth, CA). All primers were synthesized using an Applied Biosystems DNA synthesizer, model 380 B. In addition, a chicken embryo fibroblast λgt11 cDNA library (37) was screened using methods described above. Four clones containing the 5′ end of chicken *CSRP2* were identified and analyzed by DNA sequencing, confirming the results obtained with the 5′RACE procedure.

Southern Blot Analysis of Quail and Chicken Genomic DNA—High molecular weight quail genomic DNA was isolated from quail embryo fibroblasts following a proteinase K/phenol extraction protocol (31). Equal amounts of quail genomic DNA or chicken genomic DNA (Promega, Madison, WI) were digested with *Eco*RI, *Bam*HI, or *Pst*I. The digests were separated according to size by electrophoresis through agarose (10 μg/lane) and transferred to Hybond™-nylon membrane (Amersham Corp.) using standard techniques (38). Sequences corresponding to the coding regions of chicken and quail *CSRP1* and *CSRP2* cDNAs were labeled with [^{32}P]dCTP as described above and used as hybridization probes. Hybridizations were performed for approximately 16 h at 65 °C in 5 × SSPE, 5 × Denhardt's solution, 0.5% (w/v) SDS, and 250 μg/ml yeast tRNA (Boehringer Mannheim). For high stringency washes, the filters were incubated twice for 10 min at room temperature in a solution containing 2 × SSPE and 0.1% (w/v) SDS, once for 15 min at 65 °C in 1 × SSPE and 0.1% SDS and once for 10 min at 65 °C in 0.1 × SSPE and 0.1% (w/v) SDS.

Northern Blot Analysis of Quail and Chicken RNA—Preparation of total cellular RNA and selection of poly(A)$^+$ RNA were performed essentially as described previously (29). For Northern analysis, 2-μg portions of poly(A)$^+$ RNA or 30-μg portions of total RNA from normal or transformed chicken or quail embryo fibroblasts were separated by electrophoresis on 1% (w/v) agarose, 2.2 M formaldehyde gels as described (29). Hybridizations were carried out for 16 h at 37 °C in 35 ml of buffer H using ^{32}P-labeled insert DNAs from chicken or quail *CSRP1* and *CSRP2* cDNA clones or from a quail *GAPDH* cDNA clone (39) as probes. After hybridization, the filters were washed once for 20 min at 55 °C in 2 × SSC, 1 mM EDTA, and 0.1% (w/v) SDS and then twice for 20 min at 50 °C in 0.4 × SSC, 1 mM EDTA, and 0.1% (w/v) SDS.

Characterization of the Two Chicken CSRP1 Transcripts—Two *CSRP1* transcripts are observed by Northern analysis. The originally reported chicken *CSRP1* cDNA sequence corresponds to the longer transcript, *CSRP1.1* (9). Reexamination of the collection of *CSRP1* cDNAs isolated in the initial library screen revealed a cDNA that corresponded to the shorter *CSRP1* transcript. This cDNA insert was subcloned into the *Eco*RI restriction site of pBS to generate pBS-*CSRP1.2*. The sequence of *CSRP1.2* cDNA was determined on both strands as described above. The sequences of the 3′-untranslated regions of *CSRP1.1* and *CSRP1.2* were compared using the program "Bestfit" (40).

Comparisons of Amino Acid Sequences—Alignments of pairs of amino acid sequences were performed by the method of Myers and Miller (41), and multiple sequence alignments were performed by the method of Higgins and Sharp (42). Both algorithms were used as parts of the PC/Gene program package (IntelliGenetics Inc., Mountain View, CA).

Nomenclature—The first CRP family member to be identified (6–9) is referred to here as CRP1. The genes encoding the CRPs are referred to with the symbols *CSRP1, CSRP2,* etc. It was necessary to use the four-letter gene designation *CSRP* because the gene symbol *CRP* has already been assigned to an unrelated gene. The nomenclature used to describe avian proteins and genes in this report conforms to that first developed for the human proteins and genes (43).

RESULTS

As described above, previous work had suggested that multiple *CSRP* family members might be represented in the vertebrate genome. Here we have explored the possibility that two avian species, the chicken and the quail, express multiple forms of CRP. In this paper, the cysteine-rich protein that was originally described in human (6, 7) and chicken (8, 9) is referred to as CRP1. The closely related gene product that was originally described in quail (29) is here referred to as CRP2 to indicate that we consider it to be a member of the same family. The *CSRP* gene symbols refer to genes that encode members of the CRP family of proteins.

Identification and Characterization of Quail CSRP1 cDNA—CSRP1 had been identified unequivocally in humans (6, 7) and chickens (8, 9), but not yet in quail, in which the *CSRP2* gene was originally identified (29). A chicken *CSRP1* cDNA probe (9) was used to screen a quail cDNA library in an effort to identify the quail sequences that are most closely related to *CSRP1*. 3×10^5 recombinants were screened, and about 40 clones carrying sequences that hybridized strongly with the chicken *CSRP1* probe were identified. A 927-base pair cDNA clone (clone 7) was isolated, and its complete nucleotide sequence was determined (Fig. 1). The *underlined ATG* is believed to represent the site of translational initiation based on comparison with the known initiation codon in chicken *CSRP1* (8, 9). A single polyadenylation signal (*AATAAA*) is found in the 3′-untranslated region. The single open reading frame encodes a polypeptide of 192 amino acids with a calculated molecular weight of 20,385 and an unmodified isoelectric point (pI) of 8.57. The predicted protein displays two copies of the LIM motif with the sequence $CX_2CX_{17}HX_2CX_2CX_2CX_{17}CX_2C$ (Fig. 1). One of the hallmarks of a CRP family member that distinguishes it from other LIM proteins is the presence of a short glycine-rich repeat (GPKG(Y/F)G(Y/F)G(M/Q)GAG) (9) that extends from the C-terminal sequence of each LIM domain. As is characteristic of both human and chicken CRP1, the quail CRP1 protein also exhibits this glycine-rich sequence (Fig. 1). In addition, the quail protein exhibits a potential nuclear targeting signal at amino acid sequence positions 64–69 (KKYGPK) that is also conserved in CRP1 from other species (6–9). The predicted amino acid sequence of quail CRP1 is absolutely identical to chicken CRP1 and shares 90.6% identity with human CRP1. With 76.6% identity, quail CRP1 is more distantly related to quail CRP2. These results clearly demonstrate that *CSRP* genes represent a multigene family.

Identification and Characterization of Chicken CSRP2 cDNA—A quail *CSRP2* cDNA probe (29) was used to screen a chicken embryo cDNA library in an effort to identify sequences that are most closely related to *CSRP2*. 1.5×10^6 recombinants were screened, and 26 strongly reactive clones were identified and isolated. The cDNA inserts carried in seven of the initial phage isolates were characterized further; two are described in detail here. One clone referred to as *CSRP2*-TM1 contained a 783-base pair insert with an open reading frame that encodes 192 amino acids but lacks a translation initiation codon (Fig. 2*A*). Another clone isolated in this initial screen, designated *CSRP2*-TM6, contained a deletion of two nucleotides corresponding to positions 530 and 531 in the composite *CSRP2* sequence shown in Fig. 2*B*. This deletion resulted in the generation of an in-frame stop codon (TGA) that disrupted the open reading frame of *CSRP2* within the second LIM domain, causing the elimination of 27 C-terminal amino acid residues. None of the other clones that were analyzed exhibited this two-base pair deletion. Nevertheless, in order to evaluate the possible significance of this sequence heterogeneity, we isolated and sequenced chicken genomic DNA in this region and found that the chicken genome contained sequences that were compatible with the *CSRP2*-TM1 cDNA, but not the *CSRP2*-TM6 cDNA. Consequently, we conclude that the *CSRP2*-TM1 cDNA accurately reflects the sequence information present in the genome, and the two-base pair deletion observed in *CSRP2*-TM6 is likely to reflect an artifact introduced during the generation or amplification of the cDNA library.

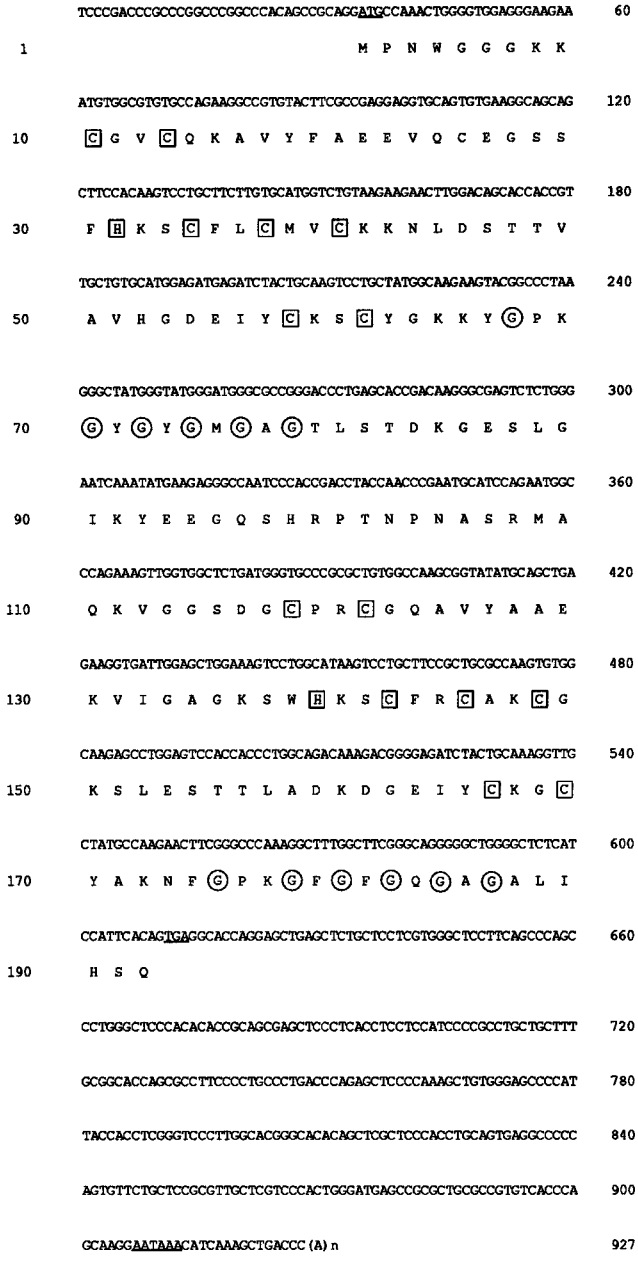

FIG. 1. **Nucleotide sequence of quail *CSRP1* cDNA and deduced amino acid sequence of the quail CRP1 protein.** The nucleotide and amino acid sequence positions are *numbered* in the *right* and *left margin*, respectively. Translational start and stop codons and the polyadenylation signal are *underlined*. The conserved cysteine and histidine residues that define the two LIM domains are *boxed*. Glycine residues that occur in the glycine-rich repeat following each LIM domain are *circled*. The sequence is deposited in the EMBL data base under the accession number Z28333.

In multiple *CSRP2* cDNAs that were characterized, none extended the 3′ end beyond what was observed for *CSRP2*-TM1. Likewise, none of the clones isolated in the original screen contained any useful 5′ sequence beyond what was found in *CSRP2*-TM1. Therefore, in order to identify cDNA clones that contained the 5′ end of the coding sequence and the

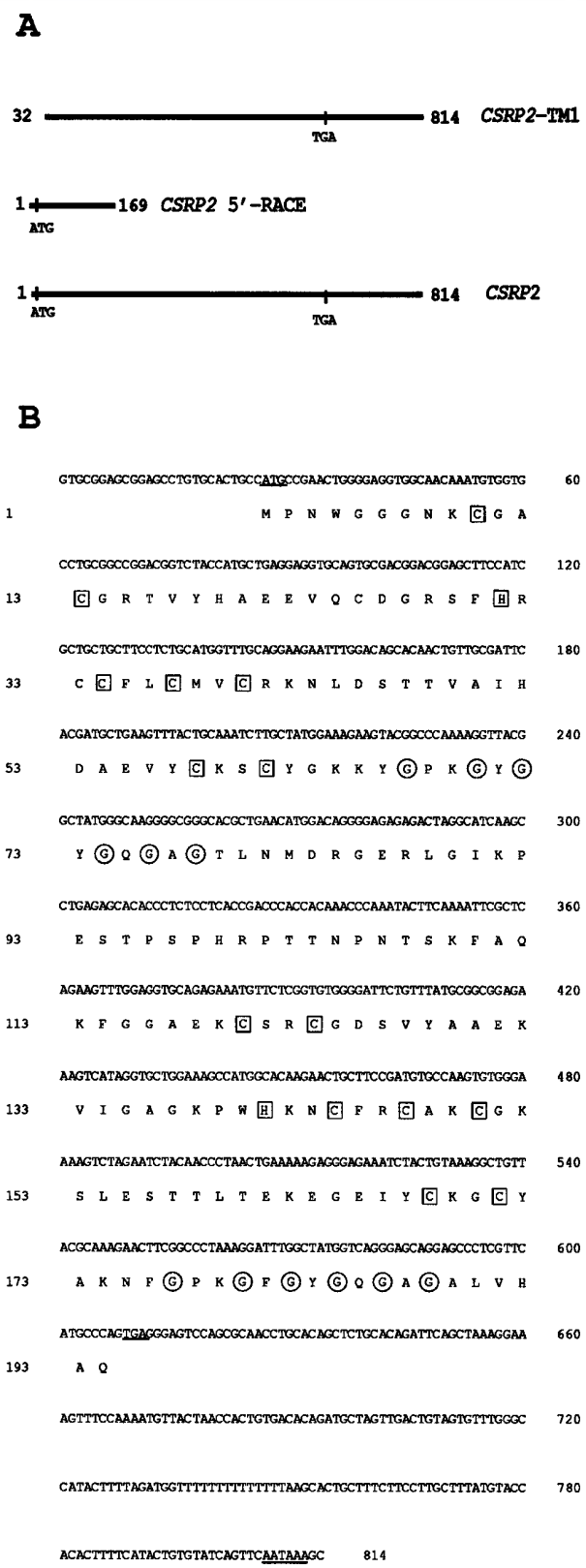

FIG. 2. **Nucleotide sequence of chicken *CSRP2* cDNA and deduced amino acid sequence of the chicken CRP2 protein.** *A*, a schematic representation of the isolated *CSRP2* cDNA clones used to generate the composite *CSRP2* cDNA. *B*, the nucleotide (*numbered* on the *right*) and deduced amino acid (*numbered* on the *left*) sequences of the composite cDNA clone (*CSRP2*) are shown. The translational initiation codon, stop codon, and the polyadenylation signal are *underlined*. Cysteine and histidine residues that contribute to the LIM consensus motif are *boxed*. Glycine residues that are found in the repeat adjacent to each LIM domain are *circled*. The sequence is deposited in the EMBL data base under the accession number X84264.

5′-untranslated region corresponding to the *CSRP2* mRNA, we used two strategies. First, we employed a modified primer extension-5′-RACE technique (36) to identify the 5′ end of the *CSRP2* cDNA; a representative cDNA clone derived from this screen is referred to as *CSRP2*-5′-RACE (Fig. 2*A*). In addition, we screened an independently generated chicken embryo fibroblast cDNA library (37) with the quail *CSRP2* probe. Four clones that were identified in this screen displayed 5′ extensions of the cDNA insert that contained the remainder of the coding sequence as well as some of the 5′-untranslated region as observed in the *CSRP2*-5′-RACE cDNA.

The nucleotide and deduced amino acid sequences of the composite *CSRP2* cDNA derived from the fusion of *CSRP2*-TM1 with the product of the 5′-RACE is shown in Fig. 2*B*. The initiation codon, termination codon, and polyadenylation signal are *underlined*. The predicted chicken CRP2 protein is 194 amino acids in length with a calculated molecular weight of 20,925 and a predicted unmodified pI of 8.68. As in the case of other CRP family members, chicken CRP2 displays two LIM domains with associated glycine-rich motifs and a potential nuclear localization signal. The metal-coordinating cysteine and histidine residues that contribute to the LIM consensus are *boxed* in Fig. 2*B*. The chicken CRP2 amino acid sequence is 76.6% identical to chicken CRP1 and 99.5% identical to quail CRP2, with only a single conservative amino acid substitution at residue 95 distinguishing the chicken and quail CRP2 homologues.

Expression of CSRP1 and CSRP2 in Normal and Transformed Avian Fibroblasts—CSRP2 was originally identified in quail cells by a differential screen for genes whose expression was altered in quail embryo fibroblasts that were transformed with the retroviral oncogene v-*myc* (29). Subsequent analysis revealed that *CSRP2* expression was dramatically reduced in quail cells that were transformed with a variety of oncogenes including v-*myc*, v-*src*, and v-*myc*/v-*mil* or in cells that were derived from a methylcholanthrene-induced quail fibrosarcoma (29). These studies raised the possibility that the expression of a *CSRP* family member could be important for the regulation of controlled cell growth and differentiation.

We have extended these studies here to evaluate whether *CSRP1* and *CSRP2* behave in a similar manner when cells are challenged with a transforming factor. The levels of *CSRP1* and *CSRP2* mRNAs in normal chicken embryo fibroblasts, normal quail embryo fibroblasts, and transformed quail embryo fibroblasts were evaluated by Northern analysis (Fig. 3). In chicken embryo fibroblasts, the *CSRP1* probe recognizes two transcripts, a minor species of 1.4 kb and a major species of 1.0 kb, but only a single transcript of 1.0 kb is detected in quail embryo fibroblasts (Fig. 3, *A* and *C*). The *CSRP2* probe hybridizes to a single transcript of 0.9 kb that is present in both chicken and quail embryo fibroblasts (Fig. 3, *B* and *D*). *CSRP1* expression is strongly decreased in the v-*myc*-transformed quail embryo fibroblast line Q8 (Fig. 3, *A* and *C*). However, longer exposures of the autoradiographs (not shown) reveal the presence of residual *CSRP1* mRNA in transformed cells. In contrast, there is a nearly complete loss of *CSRP2* transcripts in such cells (Fig. 3, *B* and *D*), in confirmation of our previous results. The level of mRNA encoding the enzyme glyceraldehyde-3-phosphate dehydrogenase was used as an internal control for the quality and amount of mRNA present in each lane (39).

In order to rule out the possibility that the suppression of *CSRP1* and *CSRP2* expression occurs only in long term transformed cell lines such as Q8, we analyzed their expression in a conditional transformation system and during the process of the initial establishment of oncogene-induced transformation.

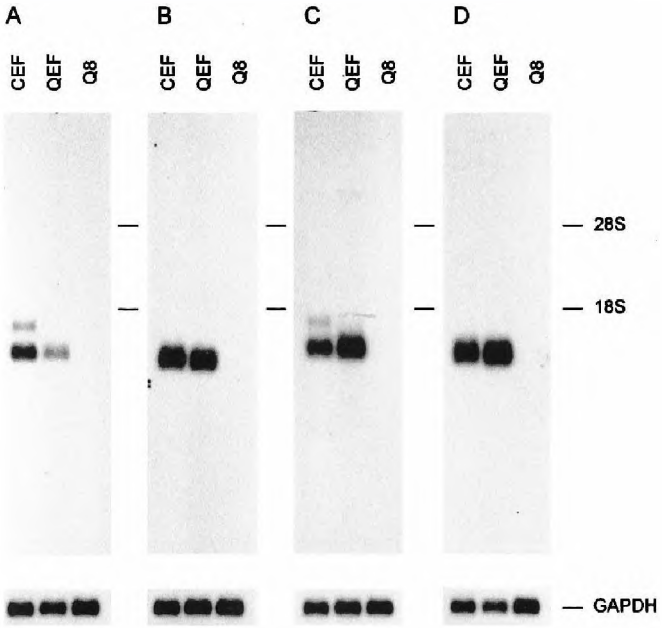

FIG. 3. **Expression of *CSRP1* and *CSRP2* mRNAs in normal and transformed avian fibroblasts.** Northern blot analyses of poly(A)$^+$ RNA (2 μg/lane) from chicken embryo fibroblasts (*CEF*), quail embryo fibroblasts (*QEF*), and the v-*myc*-transformed quail fibroblast line Q8 (*Q8*) are shown. RNAs were hybridized with a ^{32}P-labeled chicken *CSRP1* probe (4.1×10^7 cpm) (*A*), a chicken *CSRP2* probe (4.6×10^7 cpm) (*B*), a quail *CSRP1* probe (6.0×10^7 cpm) (*C*), or a quail *CSRP2* probe (5.4×10^7 cpm) (*D*). The autoradiographs were exposed for 4.5 h (*A* and *B*) or 2.5 h (*C* and *D*) using intensifying screens. The positions of ribosomal RNAs are indicated in the *margin*. Hybridization with a ^{32}P-labeled quail glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) probe (4.9×10^7 cpm; 4-h exposure) was used as an internal control.

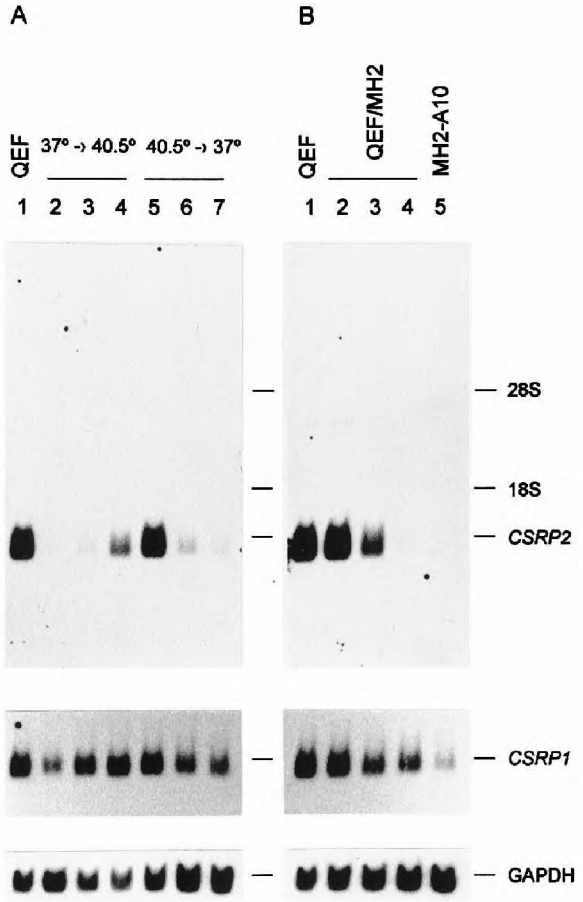

FIG. 4. **Correlation between *CSRP* gene expression and cell transformation.** *A*, a Northern blot analysis of total RNAs (30 μg/lane) from the following cellular sources is shown: quail embryo fibroblasts (*lane 1*); quail embryo fibroblasts transformed by the temperature-sensitive protein product of the v-*src* oncogene of *ts*LA29 and kept at 37 °C (*lane 2*) or shifted to 40.5 °C for 1 day (*lane 3*) or 2 days (lane 4); quail embryo fibroblasts transformed by *ts*LA29 at 37 °C and then shifted and kept at 40.5 °C (*lane 5*) or shifted back to 37 °C for 1 day (*lane 6*) or 2 days (*lane 7*). *B*, a Northern blot analysis of total RNAs (30 μg/lane) from the following cellular sources is shown: quail embryo fibroblasts (*lane 1*); quail embryo fibroblasts at day 7 (*lane 2*), day 16 (*lane 3*) or day 29 (*lane 4*) postinfection with the avian retrovirus MH2 carrying the two oncogenes v-*myc* and v-*mil*; the MH2-transformed quail fibroblast line MH2-A10 (*lane 5*). RNAs from both filters (*A* and *B*) were first hybridized with a ^{32}P-labeled quail *CSRP2* probe (3.4×10^7 cpm), and the autoradiograph was exposed for 7.5 h using an intensifying screen. The filters were stripped and then hybridized with a ^{32}P-labeled quail *CSRP1* probe (6.7×10^7 cpm), and the autoradiograph was exposed for 15 h. The positions of ribosomal RNAs are indicated in the *margin*. Hybridization with a ^{32}P-labeled glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) probe (4.9×10^7 cpm; 8-h exposure) was used as an internal control.

In quail embryo fibroblasts transfected with proviral DNA from a temperature-sensitive mutant of Rous sarcoma virus, expression of *CSRP2* is strongly induced upon shift of the cells from the permissive to the nonpermissive temperature and strongly reduced again upon shift from the nonpermissive to the permissive temperature (Fig. 4*A*). Thus, suppression of *CSRP2* expression directly correlates with the morphologically transformed state of the cells at the permissive temperature. Likewise, in quail embryo fibroblasts freshly infected with the avian MH2 retrovirus and passaged three times until complete morphological transformation of the culture was observed, complete suppression of *CSRP2* expression and full spread of virus-induced transformation coincide (Fig. 4*B*). Collectively, these data are a strong indication of a direct correlation between cell transformation and the suppression of *CSRP2* expression. The level of transcripts encoding CRP1 is also negatively correlated with the degree of cell transformation; however, the changes in *CSRP1* mRNA levels are not as great as we observe for *CSRP2* transcripts (Fig. 4, *A* and *B*). The distinct changes in the levels of *CSRP1* and *CSRP2* transcripts in response to transformation indicate that the abundance of *CSRP1* and *CSRP2* transcripts is independently regulated; our results do not distinguish whether transcript level is controlled at the level of transcription, mRNA degradation, or both.

We have investigated whether the two transcripts in chicken cells that hybridized with the *CSRP1* probe are derived from a single gene or represent unique transcripts from closely related genes. Inspection of the previously reported sequence of chicken *CSRP1* cDNA (9) revealed that two potential polyadenylation recognition motifs were present: an AATAAA sequence at nucleotides 942–947 and a CACTG recognition element (44) at nucleotides 1312–1316. Utilization of both of these signals would be predicted to give rise to mRNA transcripts of approximately 1.4 and 1.0 kb, consistent with what is observed by Northern analysis (Fig. 3). Characterization of a number of *CSRP1* cDNA clones isolated from a chicken embryo fibroblast cDNA library revealed that two classes of cDNAs were present. The two classes were indistinguishable in their coding sequences and varied only in their 3′-untranslated regions, in particular with respect to the position of the poly(A) tail (Fig. 5). In the large sized minor transcript, referred to as *CSRP1.1* mRNA, the CACTG site appears adjacent to the poly(A) tail in a 3′-untranslated region of 668 nucleotides, whereas the small sized major transcript, referred to as *CSRP1.2* mRNA, appears to result from the alternative use of the AATAAA signal to generate a 3′-untranslated region of only 310 nucleotides excluding the poly(A) tail.

The Complexity of the CSRP Gene Family—Southern blot

```
CSRP1.1  TGA  GGCACCAGGAGCCGGGCTCTGCTCCCCCTGGGCTCCTGCAGCCCAGCCCT 50
         stop ||||||||||||||||||||||||||||||||||||||||||||||||||
CSRP1.2  TGA  GGCACCAGGAGCCGGGCTCTGCTCCCCCTGGGCTCCTGCAGCCCAGCCCT 50

              GGGCTTCCACACGCAGCAGCGAGCTCCCTCACCTCCTCCATCCCCGCCTG 100
              ||||||||||||||||||||||||||||||||||||||||||||||||||
              GGGCTTCCACACGCAGCAGCGAGCTCCCTCACCTCCTCCATCCCCGCCTG 100

              CTCGTTCGCGGCACCAGCGCCTTCCCCTGCCCCGACCCAGCGCTCCCCGA 150
              ||||||||||||||||||||||||||||||||||||||||||||||||||
              CTCGTTCGCGGCACCAGCGCCTTCCCCTGCCCCGACCCAGCGCTCCCCGA 150

              AGCTGCGGGAGCCCCATTGCCACCACGGGTCCCCGGCACGGGCACACAGC 200
              ||||||||||||||||||||||||||||||||||||||||||||||||||
              AGCTGCGGGAGCCCCATTGCCACCACGGGTCCCCGGCACGGGCACACAGC 200

              TCGCTCCCACCCGCAGTGAGGCCCGCAGCGTTCCGCTCCACGTCGCCCGT 250
              ||||||||||||||||||||||||||||||||||||||||||||||||||
              TCGCTCCCACCCGCAGTGAGGCCCGCAGCGTTCCGCTCCACGTCGCCCGT 250

              CCCGCCGGGATGAGCCGCGCTGCGCCGCGTCACCGAGCAAAGGAATAAAC 300
              ||||||||||||||||||||||||||||||||||||||||||||||||||
              CCCGCCGGGATGAGCCGCGCTGCGCCGCGTCACCGAGCAAAGGAATAAAC 300

              ATCAAAGCTGACCCAAAGCATTGCCCGAGGGGTTGTTCCCCAGCGGGGGT 350
              ||||||||||||
              ATCAAAGCTGA(A)n      3'end of CSRP1.2 cDNA

              GCGCGGTTGAAGCAGGAGCTCAGGATGGAGCGGGAGGACGGGGAGCGGTG 400


              CGGTCGCAGCTCGCAGTTAAAGCGGAGGGGCGCTCCCTGCGGCTCTGCAG 450
              CCCGCTCTGCCCCCCCCGTGTGACCATTTCCAAAGCAGCGTTTCCTTCCT 500
              GCCCTATCCAAACCCATCGGCGCTGCTCCGCGGTGCTGCTCCGGGCAGCC 550
              GGTGCCCAGCAGTGCTAACAGCTCAGAGCTTCTCACCTCCTCCCGGGTTG 600
              GGTATTTTTTTGTGCTGGATTCTGATCCGCCAGCCTGGCAGCTCCCCAGG 650
              TTTAATTTGTTTTCACTG(A)n      3'end of CSRP1.1 cDNA
```

FIG. 5. **Differential polyadenylation of chicken *CSRP1* transcripts.** The two chicken *CSRP1* transcripts observed by Northern analysis (*cf.* Fig. 3, *A* and *C*) result from alternative polyadenylation. The 3′-untranslated regions of the longer transcript (*CSRP1.1*) and the shorter transcript (*CSRP1.2*) are shown. The sequences corresponding to polyadenylation recognition signals (AATAAA and CACTG) are *underlined*.

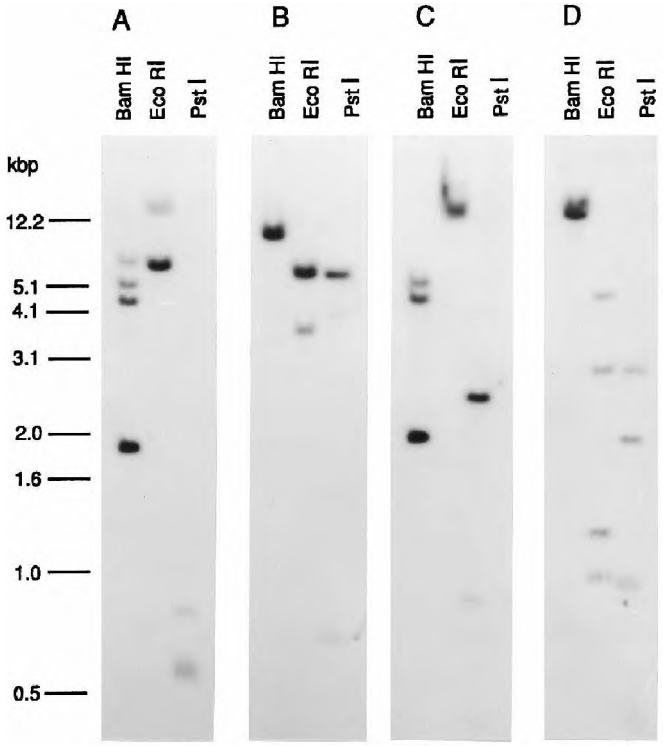

FIG. 6. **Chicken and quail *CSRP1* and *CSRP2* genes represent distinct genetic loci.** Chicken (*A* and *B*) and quail (*C* and *D*) genomic DNA (10 μg/lane) were digested with *Bam*HI, *Eco*RI, or *Pst*I, and the digests were analyzed by Southern blotting using ^{32}P-labeled chicken *CSRP1* (*A*), chicken *CSRP2* (*B*), quail *CSRP1* (*C*), or quail *CSRP2* (*D*) cDNA clones as hybridization probes. The positions of DNA size markers are indicated in the *margin*.

analysis was used to confirm that *CSRP1* and *CSRP2* sequences are derived from distinct genes. When chicken genomic DNA was digested with *Bam*HI, *Eco*RI, or *Pst*I and probed at high stringency with either a chicken *CSRP1* probe (Fig. 6*A*) or a *CSRP2* probe (Fig. 6*B*), a simple pattern of nonoverlapping bands was detected. When the same blots were probed at lower stringency (not shown), substantial cross-hybridization of *CSRP1* and *CSRP2* sequences was observed, indicating that *CSRP1* and *CSRP2* represent distinct but related genes in the chicken. Similar experiments using quail genomic DNA confirmed that *CSRP1* and *CSRP2* are distinct genes that display cross hybridization under low stringency conditions (Fig. 6, *C* and *D*, and data not shown).

Interestingly, low stringency Southern blots also revealed the presence of some minor cross-hybridizing DNA fragments that were not detected in the high stringency screens with either the *CSRP1* or *CSRP2* probes (not shown). These bands may represent sequences derived from more distantly related *CSRP* family members. Indeed, a recent report describes another avian gene, *MLP*, that encodes a protein with characteristics similar to CRP1 and CRP2 (45). The muscle LIM protein (MLP) exhibits two LIM domains with the spacing found in CRP1 and CRP2 proteins, adjacent glycine-rich repeats, and a potential nuclear localization signal, the three structural hallmarks of CRP family members (see below). Searches of the EMBL/GenBank data bases revealed that CRP1 and CRP2 are the most closely related sequences to MLP that have been reported to date. Because of the significant sequence relationships and similarities in global protein organization between MLP and the two CRP family members described here, it is likely that MLP and CRPs are evolutionarily related. We suggest that it would be appropriate to refer to MLP as CRP3 to indicate its relationship to members of the CRP family of proteins.

Relationships Among CRP Family Members—An alignment of the amino acid sequences of human, chicken, and quail CRP1, chicken and quail CRP2, and chicken MLP/CRP3 is shown in Fig. 7. Each protein is between 192 and 194 amino acids in length, and each displays two LIM domains. In addition, there is absolute conservation of length in every finger-like projection found in the LIM domains of CRP family members; that is, all 12 of the individual LIM domains found in the six CRP family members display exactly 17 amino acid residues between the second and third, and between the sixth and seventh, metal-coordinating residues. In contrast, other LIM proteins may display anywhere from 16 to 23 residues in these positions (8). Moreover, in CRP family members, each LIM domain is proximal to a glycine-rich repeat that closely conforms to the sequence originally found in human CRP1 (6). A potential nuclear targeting signal (KKYGPK) is found at the same position (amino acid residues 64–69) in all CRP proteins. Its sequence is almost completely conserved, with only one chemically conservative substitution of an arginine for a lysine residue at position 64 in the case of MLP/CRP3. The distance between the two LIM domains is also highly conserved in all six CRP proteins, varying only from 56 to 59 residues. This conservation in the relative positions of the LIM domains is particularly striking given the heterogeneous spacing between LIM domains found in other proteins (Fig. 8). For example, within a single protein, two LIM domains may be separated by as few as 7 (8) or as many as 68 (46) amino acids. Finally, the sequence conservation of the CRP family members is high (Fig. 7, Table I). Human, chicken, and quail CRP1s share more than 90% amino acid sequence identity. The CRP2 sequences are the next most closely related sequences to CRP1, exhibiting 76–80% identity to the CRP1 sequences. MLP/CRP3 retains between 63 and 67% sequence identity to CRP1 and CRP2 proteins. Collectively, the common features described above

```
                        +  +                    +  +  +  +
c CRP1          MPNWGGGKKCGVCQKAVYFAEEVQCEGSSFHKSCFLCMVCKKNLDSTTVAVHGD    54
q CRP1          MPNWGGGKKCGVCQKAVYFAEEVQCEGSSFHKSCFLCMVCKKNLDSTTVAVHGD    54
h CRP1          MPNWGGGKKCGVCQKTVYFAEEVQCEGNSFHKSCFLCMVCKKNLDSTTVAVHGE    54
c CRP2          MPNWGGGNKCGACGRTVYHAEEVQCDGRSFHRCCFLCMVCRKNLDSTTVAIHDA    54
q CRP2          MPNWGGGNKCGACGRTVYHAEEVQCDGRSFHRCCFLCMVCRKNLDSTTVAIHDA    54
c MLP/CRP3      MPNWGGGAKCGACEKTVYHAEEIQCNGRSFHKTCFLCMACRKALDSTTVAAHES    54
                ******* *** *   ** *** ** * ***  ***** * * ******* *

                   +  +     -  - - - - -
c CRP1          EIYCKSCYGKKYGPKGYGYGMGAGTLSTDKGESLGIKYEEG-QS-HRPT-NPNA   105
q CRP1          EIYCKSCYGKKYGPKGYGYGMGAGTLSTDKGESLGIKYEEG-QS-HRPT-NPNA   105
h CRP1          EIYCKSCYGKKYGPKGYGYGQGAGTLSTDKGESLGIKHEEA-PG-HRPTTNPNA   106
c CRP2          EVYCKSCYGKKYGPKGYGYGQGAGTLNMDRGERLGIKPEST-PSPHRPTTNPNT   107
q CRP2          EVYCKSCYGKKYGPKGYGYGQGAGTLNMDRGERLGIKPESS-PSPHRPTTNPNT   107
c MLP/CRP3      EIYCKTCYGRKYGPKGVGFGQGAGCLSTDTGDHLGLNPAPGSPKPARPSTPTNP   108
                * *** *** ****** * * *** *  * *  **           **    *

                         +  +                    +  +  +  +
c CRP1          SRMAQKVGGSDGCPRCGQAVYAAEKVIGAGKSWHKSCFRCAKCGKSLESTTLAD   159
q CRP1          SRMAQKVGGSDGCPRCGQAVYAAEKVIGAGKSWHKSCFRCAKCGKSLESTTLAD   159
h CRP1          SKFAQKIGGSERCPRCSQAVYAAEKVIGAGKSWHKACFRCAKCGKGLESTTLAD   160
c CRP2          SKFAQKFGGAEKCSRCGDSVYAAEKVIGAGKPWHKNCFRCAKCGKSLESTTLTE   161
q CRP2          SKFAQKFGGAEKCSRCGDSVYAAEKVIGAGKPWHKNCFRCAKCGKSLESTTLTE   161
c MLP/CRP3      SKFAKNMVDVDKCPRCGKSVYAAEKIMGGGKPWHKTCFPCAICGKSLESTNVTD   162
                *  *         * **   ******  * ** *** ** ** *** ****

                      +  +     -  - - - - -
c CRP1          KDGEIYCKGCYAKNFGPKGFGFGQGAGALIHSQ   192
q CRP1          KDGEIYCKGCYAKNFGPKGFGFGQGAGALIHSQ   192
h CRP1          KDGEIYCKGCYAKNFGPKGFGFGQGAGALVHSE   193
c CRP2          KEGEIYCKGCYAKNFGPKGFGYGQGAGALVHAQ   194
q CRP2          KEGEIYCKGCYAKNFGPKGFGYGQGAGALVHAQ   194
c MLP/CRP3      KDGELYCKVCYAKNFGPKGLGFG-GLTQVEKKE   194
                * ** *** ********** * * *
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FIG. 7. **Comparison of the amino acid sequences of CRP family members.** An alignment of the amino acid sequences of chicken (*c*) CRP1 (9), quail (*q*) CRP1 (Fig. 1), human (*h*) CRP1 (6), chicken CRP2 (Fig. 2), quail CRP2 (29), and chicken MLP/CRP3 (45) is shown. Residues conserved in all sequences are marked by *asterisks below* the *bottom line*. The conserved Cys and His residues of the LIM domains are indicated by *crosses*, and the conserved glycine residues of the glycine-rich repeats are marked by *dashes above* the *top line*.

illustrate that the architecture of CRP family members is conserved and is distinct from that of other LIM proteins.

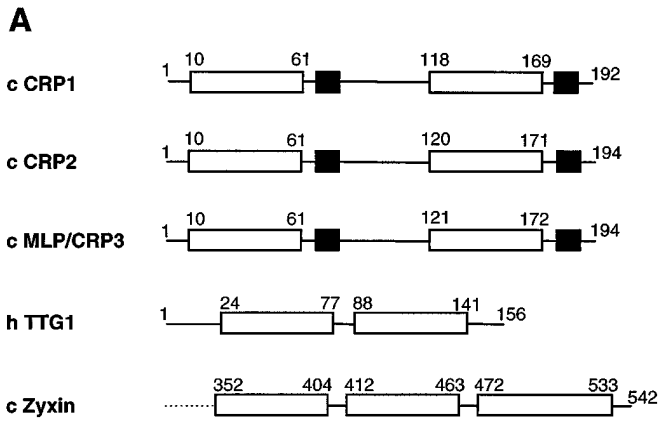
DISCUSSION

The CRP Family of LIM Only Proteins—The discoveries of quail (29) and chicken (8, 9) genes encoding cysteine-rich LIM-domain proteins (CRPs) that were closely related to each other and yet showed significantly different degrees of homology to a human protein of similar structure (6, 7) had raised the possibility that the two avian genes were not homologous counterparts of each other but rather represented closely related members of a multigene family. The results presented in this paper provide direct proof for this suggestion. For each of the two genes originally identified, quail *CSRP2* and chicken *CSRP1*, the homologous counterpart in the other avian species was isolated. The *CSRP1* and *CSRP2* genes specify mRNAs of different size that are related in their protein-coding sequences but largely unrelated in their untranslated regions. We have also obtained preliminary evidence[2] that the human genome contains a homologous counterpart of *CSRP2* in addition to the originally described human *CSRP1* gene (6, 7). Further evidence for a multigene family specifying CRP-type proteins comes from the recent discovery of rat and chicken MLPs with structures strikingly similar to CRP1 and CRP2 proteins (45). Comparisons of a number of LIM-proteins (Fig. 8) reveal the conserved structural elements of the CRP family members that set them apart from other LIM only proteins. Both the abundance and spacing of the metal-coordinating residues (cysteine and histidine) are absolutely conserved in all individual LIM domains of all CRP-type proteins identified so far. Furthermore, the spacing between the two LIM domains is nearly identical in all CRP proteins, and the location and sequence of glycine-rich repeats and potential nuclear localization signals are shared among all of these proteins. In addition to this highly conserved domain topography, the overall sequence identities between different types of CRPs are further indications of the close evolutionary relationships of these proteins (Fig. 7, Table I). We therefore conclude that avian and mammalian species contain in their genomes a multigene family specifying different but very closely related CRP proteins. We suggest a uniform nomenclature for these proteins, CRP1 and CRP2 for the proteins described here and previously (6–9, 29) and CRP3 for the recently described muscle-specific LIM protein, MLP (45). It should be noted that a recently described rat LIM protein that is also referred to as a cysteine-rich protein (46) exhibits a general domain topography that is similar to the CRP family members. However, both the differences in spacing within and between the two LIM domains and low overall sequence identities distinguish the rat protein from members of the CRP family.

Biochemical and Biological Functions of CRPs—Given the prominence of LIM domains in CRPs (Fig. 8), it is likely that the biochemical functions of CRP family members will largely be defined by their tandem LIM domains. The LIM domain has

[2] R. Weiskirchen and K. Bister, unpublished results.

been postulated to interact with both nucleic acids and proteins. Because a number of zinc-binding proteins are well defined transcription factors, the LIM domain was originally thought to function in DNA binding. Interestingly, the analysis of a chicken CRP1 LIM domain by two-dimensional NMR spectroscopy revealed that the tertiary fold of one zinc-binding module within the CRP LIM domain is essentially identical to that determined for well characterized DNA-binding zinc fingers (14). These structural studies illustrated that the tertiary fold of the LIM domain may be compatible with nucleic acid binding; however, a specific association between a LIM domain and nucleic acid has yet to be demonstrated.



B

c CRP1	LIM1,2:	C-X_2-C-X_{17}-H-X_2-C-X_2-C-X_2-C-X_{17}-C-X_2-C
c CRP2	LIM1,2:	C-X_2-C-X_{17}-H-X_2-C-X_2-C-X_2-C-X_{17}-C-X_2-C
c MLP/CRP3	LIM1,2:	C-X_2-C-X_{17}-H-X_2-C-X_2-C-X_2-C-X_{17}-C-X_2-C
h TTG1	LIM1:	C-X_2-C-X_{17}-H-X_2-C-X_2-C-X_2-C-X_{19}-C-X_2-D
	LIM2:	C-X_2-C-X_{18}-H-X_2-C-X_2-C-X_2-C-X_{18}-C-X_2-D
c Zyxin	LIM1:	C-X_2-C-X_{18}-H-X_2-C-X_2-C-X_2-C-X_{17}-C-X_2-C
	LIM2:	C-X_2-C-X_{16}-H-X_2-C-X_2-C-X_2-C-X_{18}-C-X_2-D
	LIM3:	C-X_2-C-X_{23}-H-X_2-C-X_2-C-X_2-C-X_{21}-C-X_2-C

FIG. 8. **Domain structure of selected LIM-proteins.** *A*, a schematic diagram of the structures of representative chicken (*c*) or human (*h*) LIM proteins including members of the CRP family is shown. LIM motifs and glycine-rich repeats are shown as *open* and *black* boxes, respectively. The amino- and carboxyl-terminal amino acid residues and the first and last residues of the LIM domains are *numbered*. Sources for the amino acid sequences are as follows: chicken CRP1 (9), chicken CRP2 (Fig. 2), chicken MLP/CRP3 (45), human TTG1 (22), chicken zyxin (8). *B*, the spacing of the eight metal-coordinating amino acid residues (shown in *boldface letters*) within all LIM domains of the proteins shown in *panel A* is compared. Among the members of the CRP family (CRP1, CRP2, MLP/CRP3), the spacing is absolutely conserved. Numbering of LIM domains refers to their order of appearance in these proteins relative to the amino terminus and does not reflect a structural classification.

On the other hand, there is growing evidence that LIM domains mediate specific protein-protein interactions. Direct evidence for a functional role of LIM domains in protein-protein interactions was recently provided for zyxin and CRP1, two interacting chicken LIM-proteins (8, 9, 15). In addition, a LIM protein has been shown to interact with tyrosine-containing tight turn motifs present in the cytoplasmic domain of the insulin receptor (17). LIM domains have also been implicated in homotypic, intermolecular interactions (16). Moreover, interactions of LIM domain proteins with proteins containing basic helix-loop-helix motifs known to be involved in protein dimerization have been demonstrated (47, 48). Thus, the LIM domain can clearly support specific associations with partner proteins. Proteins that display multiple LIM domains may serve as adaptor molecules or as scaffolds for the coordinated, localized assembly of multimeric protein complexes (15).

The detailed biological role of the CRP family of proteins is not well understood. In general, LIM domain proteins, in particular those that contain additional homeodomains, have been implicated in regulatory processes important for development and cellular differentiation (18, 19, 49). Many of the LIM-only proteins also appear to function in these broad processes. For example, the rhombotins were originally identified at chromosomal translocations and shown to be involved in tumorigenesis (22–24, 27, 28), and recent studies on their tissue-specific expression and *in vivo* function have revealed that rhombotins have essential roles in normal development (25, 26). For the CRP protein family, a proposed role in regulatory processes was most clearly confirmed for the MLP protein that was isolated from a subtracted cDNA library enriched in genes induced in skeletal muscle by denervation and then shown to be a positive regulator of myogenesis (45). For chicken CRP1, direct interaction with zyxin, an adhesion plaque protein, has been demonstrated, and it was postulated that both proteins may function as components of a signal transduction pathway that mediates adhesion-stimulated changes in gene expression (8, 9). The expression of the human *CSRP1* gene was shown to be induced as a primary response to serum in quiescent cells, with a serum induction profile similar to that of c-*myc*, and expression that continues, like that of c-*myc*, in logarithmically growing cells (7). This is in agreement with our results reported here and previously (29) on *CSRP1* and *CSRP2* expression in continuously growing normal avian fibroblasts. The strong suppression of *CSRP* genes, in particular of *CSRP2*, in all transformed cells tested may be connected with a regulatory function of CRP proteins in ordered cell growth. Although there is strong circumstantial evidence for the involvement of CRP

TABLE I
Primary structure relationships among CRPs

The percentages of identical and similar (in parentheses) amino acid residues were determined by alignments of pairs of CRP amino acid sequences of human (h), chicken (c), or quail (q) origin according to the method of Myers and Miller (41). Similarity groups were A,S,T; D,E; N,Q; R,K; I,L,M,V; F,Y,W (*cf.* Fig. 7 for references to the CRP amino acid sequences).

Protein	hCRP1	cCRP1	qCRP1	cCRP2	qCRP2	cMLP/CRP3
hCRP1	100	90.6 (3.7)	90.6 (3.7)	79.8 (8.8)	79.8 (8.8)	66.3 (10.4)
cCRP1	90.6 (3.7)	100	100	76.6 (9.9)	76.6 (9.9)	63.5 (9.9)
qCRP1	90.6 (3.7)	100	100	76.6 (9.9)	76.6 (9.9)	63.5 (9.9)
cCRP2	79.8 (8.8)	76.6 (9.9)	76.6 (9.9)	100	99.5 (0.5)	66 (11.3)
qCRP2	79.8 (8.8)	76.6 (9.9)	76.6 (9.9)	99.5 (0.5)	100	66.5 (10.8)
cMLP/CRP3	66.3 (10.4)	63.5 (9.9)	63.5 (9.9)	66 (11.3)	66.5 (10.8)	100

proteins in regulatory processes important for cell growth and differentiation, definitive characterization of their biological functions and distinction between the functions of the individual members of the CRP family of closely related LIM only proteins will depend on the elucidation of their biochemical functions and on the identification of their cellular targets.

Why Have So Many CRPs?—The existence of multiple, closely related members of the *CSRP* gene family may reflect an organism's need for functionally distinct CRP proteins or may be necessary for tissue-specific gene expression. It is often difficult to distinguish between these two possibilities, which are not mutually exclusive. It will be interesting to determine whether the individual members of the CRP family are functionally distinct or if a collection of *CSRP* genes has evolved to allow for tissue-specific or temporally regulated expression of CRP isoforms with equivalent biochemical functions.

Acknowledgments—We appreciate the participation of Todd Alder in the early stages of this project and the excellent technical assistance by Sabine Weiskirchen. The Human, Mouse, and Chicken Gene Nomenclature Committees have approved the use of the symbols *CSRP* (human and chicken) and *Csrp* (mouse) for the sequences encoding members of the CRP family of proteins.

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