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Remarkable synteny conservation of melanocortin receptors in chicken, human, and other vertebrates

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Abstract

The melanocortin receptors (MCR) belong to the superfamily of G-protein-coupled receptors that participate in both peripheral and central functions, including regulation of energy balance. Genomic clones of the five chicken (GGA) MCRs were isolated and used to find the chromosomal location of each of the loci. The genes encoding MC2R and MC5R mapped to the middle part of the long arm of chromosome 2 (GGA2q22–q26) and MC4R proximally on the same chromosome arm, close to the centromere (2q12). This arrangement seems to be conserved on chromosome 18 in the human (HSA18). The MC1R and MC3R genes mapped to different microchromosomes that also appear to share homology with the respective human localization. The conserved synteny of the MC2R, MC5R, and MC4R cluster in chicken (GGA2), human (HSA18), and other mammals suggests that this cluster is ancient and was formed by local gene duplications that most likely occurred early in vertebrate evolution. Analysis of conserved synteny with mammalian genomes and paralogon segments prompted us to predict an ancestral gene organization that may explain how this family was formed through both local duplication and tetraploidization processes.

Keywords: Chicken; Human; MCR; Comparative mapping; Evolution; Genome duplications

Gene duplications occur by individual and/or block duplications. The former often leaves the new gene in close proximity to the parent gene, while the latter contributes to duplication of whole or partial chromosomal regions. Large-scale duplications, including polyploidizations, are believed to be an important mechanism in the evolution of the vertebrate genomes [1]. Two rounds of large-scale duplications are proposed to have occurred in early vertebrate ancestry [2,3], resulting in up to four copies, in mammals, of each gene, which originate from a common ancestor gene in a cephalochordate. This is now known as the "2*R* hypothesis" or the "one-to-four model." The resulting repetitive blocks of genes have led to the construction of maps that contain paralogous chromosomal regions, or paralogons [2–5], in humans. Several common syntenic regions in the human and zebrafish genomes support this hypothesis [6] and conserved synteny has also been found between mammals and chicken [7,8]. However, the 2*R* hypothesis has also been challenged by some researchers [9,10], as the number of members of vertebrate gene families does not display fourfold excess over invertebrate genes. To obtain further insight into the mechanism of gene family evolution, we are currently analyzing vertebrate gene families, one of which is the melanocortin receptors (MCRs).

α-Melanocyte-stimulating hormone (α-MSH) and adrenocorticotropin hormone (ACTH) are the endogenous agonist ligands within the melanocortic system. In a unique fashion, this system also has endogenous antagonist ligands

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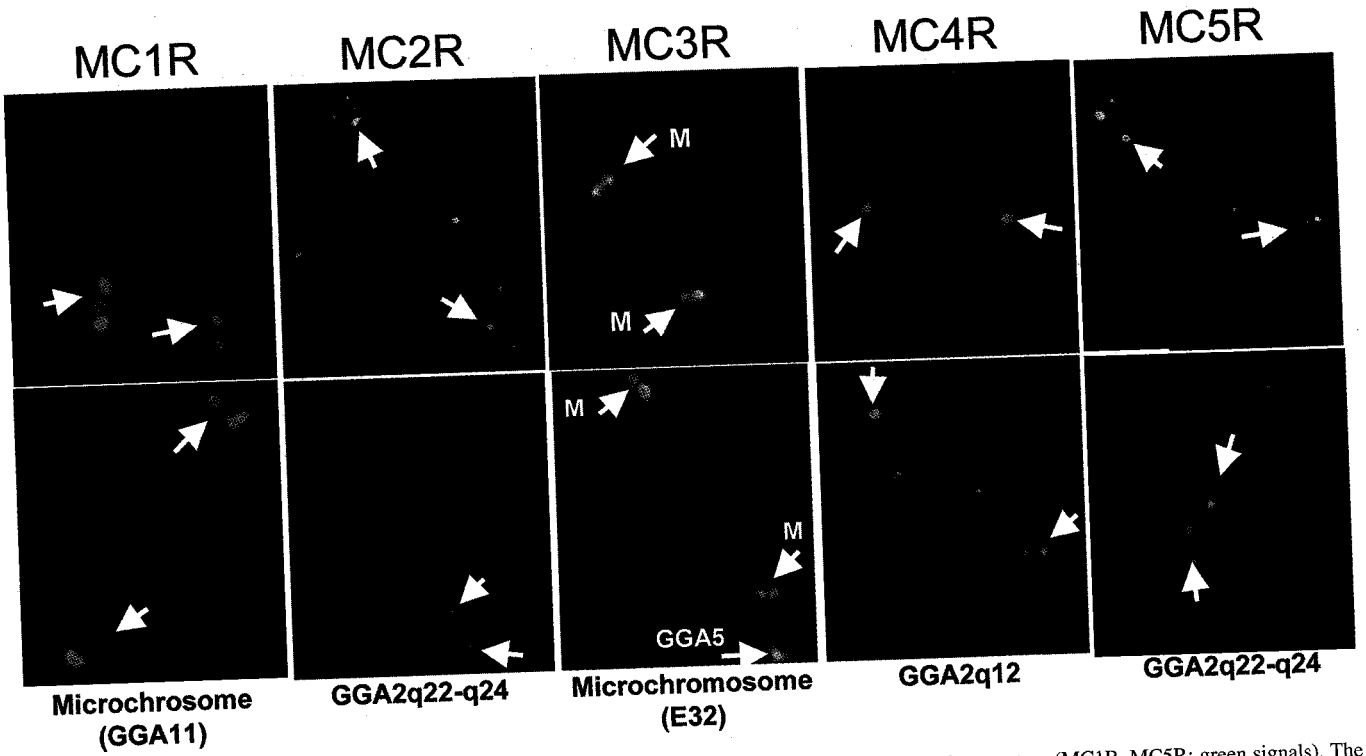

Fig. 1. Partial chicken metaphase spreads showing (arrows) chromosomal location of individual melanocortin receptors (MC1R–MC5R; green signals). The gene is specified on the top of each pair of images, while the map location is indicated below. MC1R and MC3R show cohybridization with digoxigenin-labeled (red) BAC clones containing markers ADL0232 or MCW0097 and BMP7 or LEI0346, respectively.

in the form of agouti and agouti-related peptide. At present, five MCR subtypes are known to mediate the effects of these ligands [11]. These are seemingly all found in most mammals and also in the chicken [12]. At least two of the subtypes are found in fishes, too [13].

The melanocortin 1 receptor (MC1R) is expressed mainly in the skin and has a role in skin and hair/fur pigmentation in most mammals, as clearly shown by several inactivation and activation mutations within this receptor gene [14]. In the chicken, mutations in the MC1R correlate with feather pigmentation [15]. This receptor also mediates the anti-inflammatory effect of the MSH peptides. The MC2R is exclusively found in the adrenal gland in mammals and mediates the effect of ACTH on steroidoneogenesis. The MC2R has a pharmacological profile that is different from that of the other MCRs because it binds only to ACTH and not the MSH peptides. The MC3R and MC4R are important for the central regulation of the energy balance in mammals. Activation of the MC4R causes anorexia while blockage or inactivation of the MC4R causes overeating and obesity. The MC5R is expressed in a variety of tissues and plays a role in exocrine gland secretion in mice [for review on MSH and MCRs see 11,16].

Fairly little is known about the evolutionary origin of the MCR gene family. The chicken lineage diverged from the lineage leading to mammals some 300–350 million years ago. The chicken MCRs are found in a much wider range of tissues compared with mammals, but their physiological impact is still unclear. Moreover, very little is known about the physical location of MCRs in the chicken genome or how that relates to the position of the MCRs in the mammalian genomes. Hence, to understand the origin of the genes in this receptor family, the chromosomal locations of the five chicken MCRs were determined by FISH mapping. Additionally, phylogenetic and paralogon analyses were carried out to delineate the putative mechanisms of how this gene family may have arisen.

Results and discussion

Large genomic clones containing each of the five chicken MCR genes were used for FISH mapping on the chicken (GGA) chromosomes. The MC1R locus mapped to a microchromosome, MC2R and MC5R to the middle part of the long arm of chromosome 2 (GGA2q22–q26), and MC4R proximally on the same chromosome arm close to the centromere (GGA2q12). The MC2R and MC5R signals overlapped and their relative order could not be deduced. Last, hybridization signals with the MC3R probe were observed consistently on two different chromosomes, viz., GGA5 (5q21–q23) and a microchromosome, with a predominance of signals on the latter. Partial metaphase spreads showing hybridization signals for individual probes are presented in Fig. 1 and the schematic diagrams depicting the chromosomal locations are presented in Fig. 2.

It is difficult to identify precisely the chicken chromosome to which the MC1R locus maps. Nevertheless, avail-

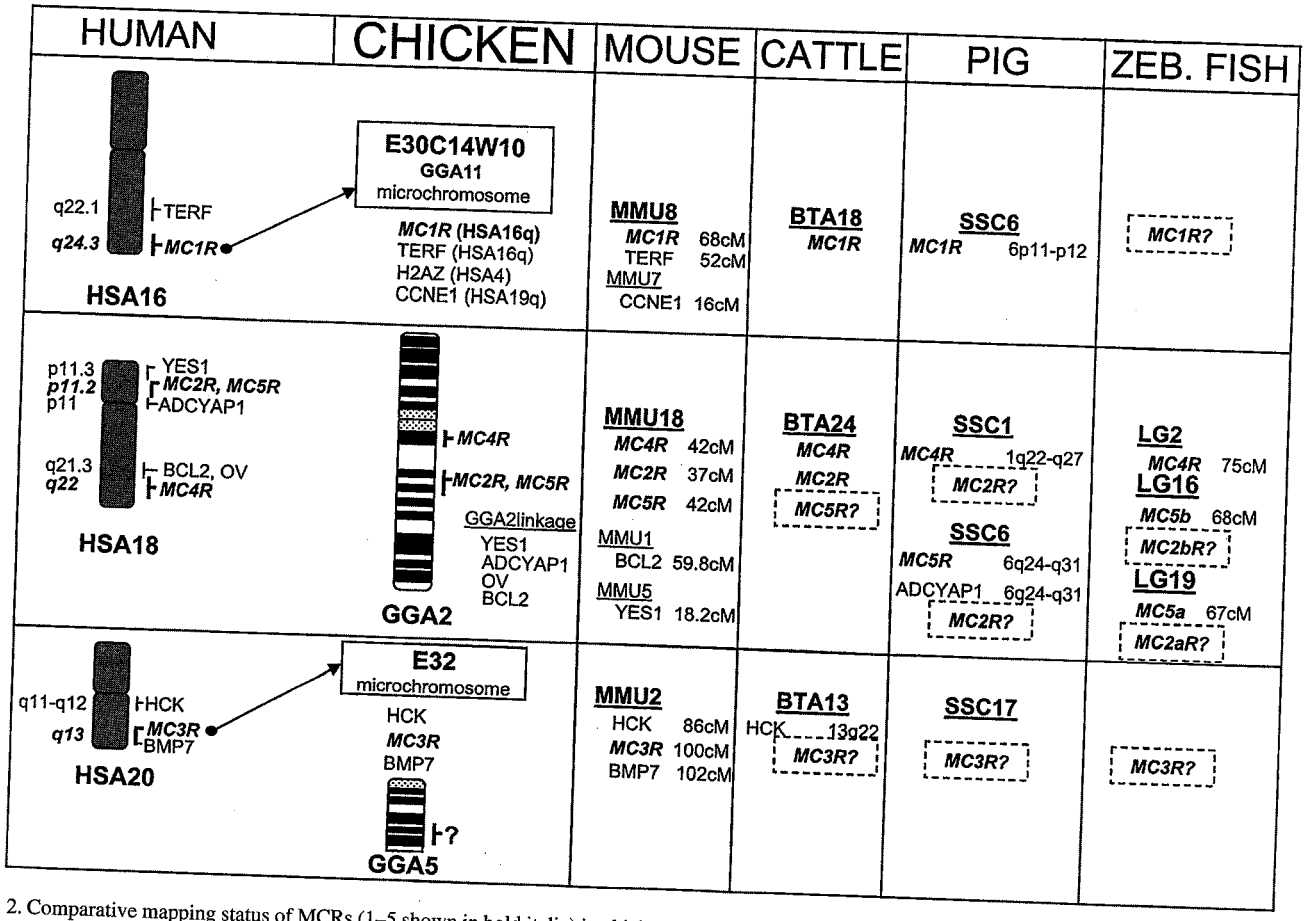

Fig. 2. Comparative mapping status of MCRs (1–5 shown in bold italic) in chicken (GGA), different mammals, and zebrafish. The chicken locations are either assigned to a linkage group or shown on idiograms. Below each of the chicken linkage groups, genes hitherto mapped are presented. Where available, comparative locations of these genes are shown in human (HSA), mouse (MMU), cattle (BTA), pig (SSC), and zebrafish. Based on comparative data, the likely locations of some of the MCRs in cattle and pig are predicted (dotted boxes). In mouse and zebrafish, the relative order of the loci is obtained from the genetic map. Hence the locations are indicated in centimorgans (cM). Mapping data for all loci were retrieved from the respective databases.

able comparative mapping information (http://poultry.mph.msu.edu) can be used to propose the likely chromosome. In humans, MC1R maps to the terminal part of HSA16q (Fig. 2). This human chromosome corresponds to parts of two chicken microchromosomes that harbor linkage groups E30C14W10 (referred to as GGA11 at http://www.thearkdb.org/anubis) and E35C18W14, respectively. The chromosome bearing the former linkage group shows equivalence to parts of HSA16q (in addition to HSA4q and HSA19q), while that bearing the latter hitherto only to HSA16p. If synteny conservation for loci on individual arms of HSA16 is assumed in chicken, the MC1R would be expected on GGA11. This likelihood further increases because TERF, a locus that maps close to MC1R in humans as well as mouse, also maps to this chicken chromosome. Additional support for this can be drawn from the fact that the conserved synteny between HSA16q and HSA19q loci observed in the vast majority of mammalian species [24] is found on GGA11 and not on the chromosome bearing linkage group E35C18W14 (see Fig. 2). These indications suggest that MC1R is located on GGA11. Confirmation for this was obtained by double-color FISH experiments that unambiguously showed consistent hybridization of biotin-labeled MC1R on the same chromosome as digoxigenin-labeled ADL02232 and MCW0097. The latter are known to be present on GGA11 (Fig. 1).

The localization of MC2R, MC4R, and MC5R in chicken closely matches that in humans [e.g., 25]. The overlapping signals of MC2R and MC5R on GGA2 and the presence of MC4R on the same chromosome, but at a different location, almost mimics the map location of the three loci in the two species (Fig. 2). MC4R is also mapped to mouse chromosome 18 (MMU18) and the tight linkage between MC2R and MC5R is consistent with our findings in the chicken. In cattle MC5R has not yet been mapped. Nevertheless, synteny between MC2R and MC4R suggests that this locus is also present on BTA24. In pigs, however, it seems that synteny between the three MCR loci is broken. MC4R maps to SSC1 while MC5R maps to SSC6, where MC1R is also located. It is highly likely that MC2R also maps to this chromosome, in close vicinity of the MC5R (SSC6q24–q31). Overall, synteny and spatial conservation

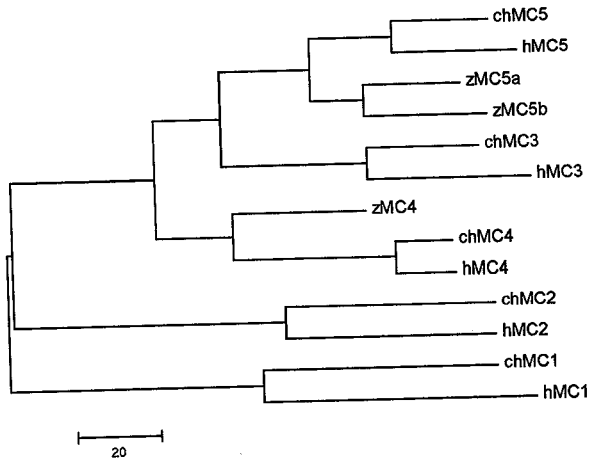

Fig. 3. Maximum parsimony (MEGA2)-based phylogenetic analysis of the MCR family using full-length amino acid sequences of each of the receptors. Stability of the nodes was assessed using a bootstrapping method with 100 replicas and all sites were treated as informative. The accession numbers are listed under Material and methods. Note: ch, chicken; h, human; z, zebrafish.

of the three MCRs in evolutionarily highly divergent species (chicken and human) indicates that this arrangement is most likely ancestral.

The probe containing the MC3R-specific DNA consistently hybridized to GGA5 as well as a microchromosome. As far as is known, no homology has yet been detected between GGA5 and HSA20, the chromosome on which MC3R maps in the humans. However, HSA20 genes have been mapped to one of the microchromosomes that carries the linkage group E32 (Fig. 2). It is, therefore, highly likely that the chicken MC3R gene maps to the E32-containing microchromosome. Cohybridization of the MC3R probe with BAC clones for two E32 markers (BMP7 and LEI0346) showed distinct red and green signals on a single pair of microchromosomes, thus verifying our observations (Fig. 1). The signal observed on GGA5 might be attributed to either chimerism in the genomic phage clone used as probe or cross-hybridization to a sequence closely resembling MC3R. To investigate the latter possibility, the coding region of the MC3R probe was used for Southern hybridization on chicken genomic DNA digested with three restriction enzymes. Two of the digests showed a single hybridization band each, while the third showed two bands due to *Hin*dIII cleavage in the middle of the gene, thus clearly indicating that the probe binds only to a single gene in the chicken genome.

The conserved synteny between the MC2R/MC5R cluster in chicken (GGA2), human (HSA18), and mouse (MMU18) suggests that these receptors arose from a common ancestor by a local gene duplication event. It is, however, intriguing that the MC2R and MC5R are not among the most closely related within the MCR family. Fig. 3 illustrates the phylogenetic relationship between the chicken, the human, and the recently cloned zebrafish MCRs (see [13]). The phylogenetic tree shows that the MC2R and MC5R are evolutionarily quite distant. Thus the duplication that generated the MC2R and MC5R genes is likely to have occurred very early, probably before the putative tetraploidizations. The analysis also clearly shows that MC3R and MC4R are more closely related to the MC5R than the MC2R. Assuming that MC2R and MC5R indeed arose from a common ancestor through early local duplication, the MC3R and MC4R genes might have arisen later from the MC5R-linked ancestor, thus explaining the close relationship of the two with the latter. Alternatively, if the genomes underwent tetraploidization, it would appear likely that the MC5R ancestor gene generated both MC3R and MC5R during the polyploidizations. The MC4R is, however, likely to have been the result of a later local duplication from the MC5R. This fits nicely with the fact that these two closely related genes are found together on HSA18 and MMU18, as well as GGA2. In this scenario, absence of all the duplication products arising from MC2R (except MC1R) can be attributed to subsequent losses during genome evolution. Although the phylogenetic analysis is not conclusive about the MC1R, it seems likely that the MC1R and MC2R arose from a common ancestor that was paralogous to the ancestor of the MC3/5/4R gene.

It is interesting in this context that the MC2R and MC1R seem to have distinct physiological functions (i.e., regulation of steroid production and pigmentation, respectively) which are quite different from the MC3R, MC4R, and MC5R. The latter have all been linked to the regulation of energy balance and are all expressed in the *brain*. Compared to this, the MC2R and MC1R are expressed only in the *periphery*. Intriguingly, all the human MCRs are found in the same paralogon and belong to the strong 8q/16q/18/20q paralogon [5]. This paralogon contains paralogues of cadherins and also NFATC and SALL transcription factors found on the same three human chromosomes (HSA16, HSA18, and HSA20). This further strengthens the likelihood of the scenario described above, whereby both local duplications and chromosomal duplications or tetraploidization may have been responsible for the expansion of this gene family. The findings thus bear significance in developing links between this human paralogon and the corresponding segments in the chicken genome. Eventually, the observations may lead to the creation of a complete paralogon map also for the chicken.

In summary, we have shown that there is remarkable synteny conservation of MCR genes—among themselves and in relation to other genes—between chicken and evolutionarily distantly related vertebrate species. The specific conserved chromosomal positioning of MC2R and MC5R, and the analysis of the human paralogons, predicts an ancestral gene organization that may explain how this family has been formed through both local duplication and tetraploidization processes. The fact that all the MCR genes belong to a specific human paralogon together with the conserved synteny provides support to the hypothesis of two large-scale genomic duplications, or the 2*R* hypothesis.

Material and methods

Probes. A genomic clone for the chicken MC1R was previously isolated from a Rock-Cornish DNA library cloned into Lambda GEM-11 (Promega, USA) using a mouse MC1R probe for screening [15]. Genomic clones for the remaining chicken MCR subtypes (MC2R, MC3R, MC4R, and MC5R) were isolated from a White Leghorn DNA library cloned into Lambda FIX II (Stratagene, USA) as described elsewhere (for MC2R [17]; for MC3R [18]; for MC4R and MC5R [12]). For double-color FISH, three BAC clones each containing markers ADL0232, MCW0097, and LEI0346 from the Wageningen library (clones BW010A05, BW029L10, and BW034H07, respectively) and one BAC clone containing BMP7 from the Texas A&M library (clone E021P7) were used.

Chromosome preparation, probe labeling, and in situ hybridization. Chicken metaphase chromosome spreads were prepared from short-term bone marrow cultures as described by Christidis [19]. The MCR1–5 λ clones were individually labeled with biotin-14–dATP and the BAC clones containing markers ADL0232, MCW0097, BMP7, and LEI0346 were individually labeled with digoxigenin (dig) by nick-translation, according to the manufacturers' instructions (BioNick labeling system, Life Technologies; Dig-Nick Translation Mix, Roche). For double-color FISH, biotin-labeled MC1R probe was cohybridized with dig-labeled BAC clones containing either ADL0232 or MCW0097. Similarly, the MC3R probe was cohybridized with dig-labeled BAC clones containing either BMP7 or LEI0346. The hybridization mixture contained 1 μg of labeled probe DNA, 10 μg chicken genomic DNA, 1 μg sonicated salmon sperm DNA, 10% dextran sulfate, 50% formamide, and 2× SSC. Overnight in situ hybridization, posthybridization washing, and signal detection with avidin–FITC (Vector) or anti-digoxigenin–rhodamine (Roche) was carried out as described elsewhere in detail [20]. The results were analyzed under a Zeiss Axioplan2 fluorescence microscope, and images were captured and processed using the CytoVision/Genus software version 2.7 (Applied Imaging). Chicken chromosomes were identified according to the recently proposed nomenclature [21].

Southern hybridization on genomic DNA. Ten micrograms of chicken genomic DNA was digested by three different enzymes, *Hin*cII, *Hin*dIII, and *Pst*I (Amersham Pharmacia Biotech), in a total volume of 100 μl, containing 1× appropriate restriction buffer and the enzyme. The reactions were incubated at 37°C overnight. Following this, enzymes were added again and the mix was incubated for 8 h. The restriction reactions were stopped by 10 min incubation at 70°C and the fragments were separated on a 1% agarose gel. The restriction products were transferred to a nylon filter overnight using 0.4 M NaOH. The filter was hybridized with a random-primed ^{32}P-labeled probe (Megaprime kit; Amersham Pharmacia Biotech) at 65°C in 25% formamide, 6× SSC, 10% dextran sulfate, 5× Denhardt's solution, and 0.1% SDS overnight. The coding region of the chicken MC3R receptor was used as probe. The filter was then washed five times in 0.2× SSC + 0.1% SDS for 1 h at 65°C and exposed to autoradiography film (Amersham Pharmacia Biotech).

Alignments and phylogenetic analysis. The full-length amino acid sequences of the MCRs were aligned using ClustalW (1.7) software [22] and edited manually after visual inspection. The sequences were retrieved from GenBank and have the following accession codes: *Homo sapiens* MC1R (NM_002386), MC2R (NM_000529), MC3R (XM_009545), MC4R (NM_005912), MC5R (XM_008685); *Mus musculus* MC4R (AF201662), MC5R (NM_013596); and *Gallus gallus* MC4R (AB012211), MC5R (AB012868). Phylogenetic trees were built using the Win32 version 2.1 of the MEGA2 software [23] using the maximum parsimony method with the Max-Mini Branch & Bound tree search algorithm. Stability of the nodes was assessed using a bootstrapping method with 100 replicas and all sites were treated as informative. The trees were plotted in Tree-Explorer.

Acknowledgments

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