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MELANOCORTIN-1-RECEPTOR (MCR-1) GENE POLYMORPHISMS ASSOCIATED WITH THE CHICKEN E LOCUS ALLELES

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Abstract:

The melanocortin 1-receptor (MC1-R) gene has been associated with E locus phenotypes in chickens. Variant alleles of the E locus are important for accurate down color sexing and also for the inhibition of unwanted tissue pigmentation in broilers. PCR (polymerase chain reaction) based tests for various replacement substitutions found in the published E allele sequences gave unexpected results when tested against known alleles of the E locus. To resolve these issues and gain a better understanding of how replacement substitutions in the MC1-R gene are affecting E locus phenotypes, a number of accessions of the e^+, e^b, wheaten (e^{Wh} and e^y), and the birchin (E^R) alleles were PCR cloned and sequenced. The published e^+ allele sequence is likely to be a wheaten sequence. All three new wheaten sequences produced an inferred amino acid sequence that was identical to the published e^+ sequence, but all three of our new e^+ sequences had a Thr143Ala polymorphism. All E alleles sequenced except for the new wheaten alleles have Thr143, indicating that the Ala143 mutation may be associated with restriction of black pigment in the feathers. The two recessive e^b sequences had the Lys92 mutation associated with extended black in both chickens and mice, but they also had a His215Pro substitution. The Pro215 mutation is a likely candidate to attenuate the Lys92 mutation producing a brown instead of a black female. The Leghorn birchin allele had the Lys92 mutation, but the Fayoumi birchin allele did not. Instead, the Fayoumi sequence had a Leu133Gln substitution.

Andrew Ellett and Ronald Okimoto

Introduction.

Feather pigmentation studies on the molecular level have shown that melanocortin 1-receptor (MC1-R) gene polymorphisms are responsible for various *E* locus alleles of the chicken (1,2). The chicken *E* locus had been tentatively mapped to chromosome 1 (3,4,5), but PAMSA (PCR amplification of multiple specific alleles) tests for specific single nucleotide polymorphisms within the MC1-R gene indicate that the MC1-R gene is on one end of the E30 linkage group of the East Lansing reference population (6). The E30 linkage group is thought to be one of the microchromosomes and is not associated with genetic markers found on chromosome 1. Functional MC1-R is a G-protein coupled receptor and, when activated by a-melanocyte-stimulating hormone, controls the synthesis and distribution of the two major melanin pigment types (7). An abundance of eumelanin causes brown to black pigment while more phaeomelanin results in yellow to red coloration. The alleles associated with the eumelanin abundant phenotypes are usually dominant to those displaying more phaeomelanin (8). In chickens, a number of *E* locus alleles have been identified and described (9). Phenotypic descriptions of some of the *E* alleles are given in Figure 1 and 2.

Eumelanin is not only deposited in feathers but also in the eye, skin and connective tissue of poultry (9). Dark tissue

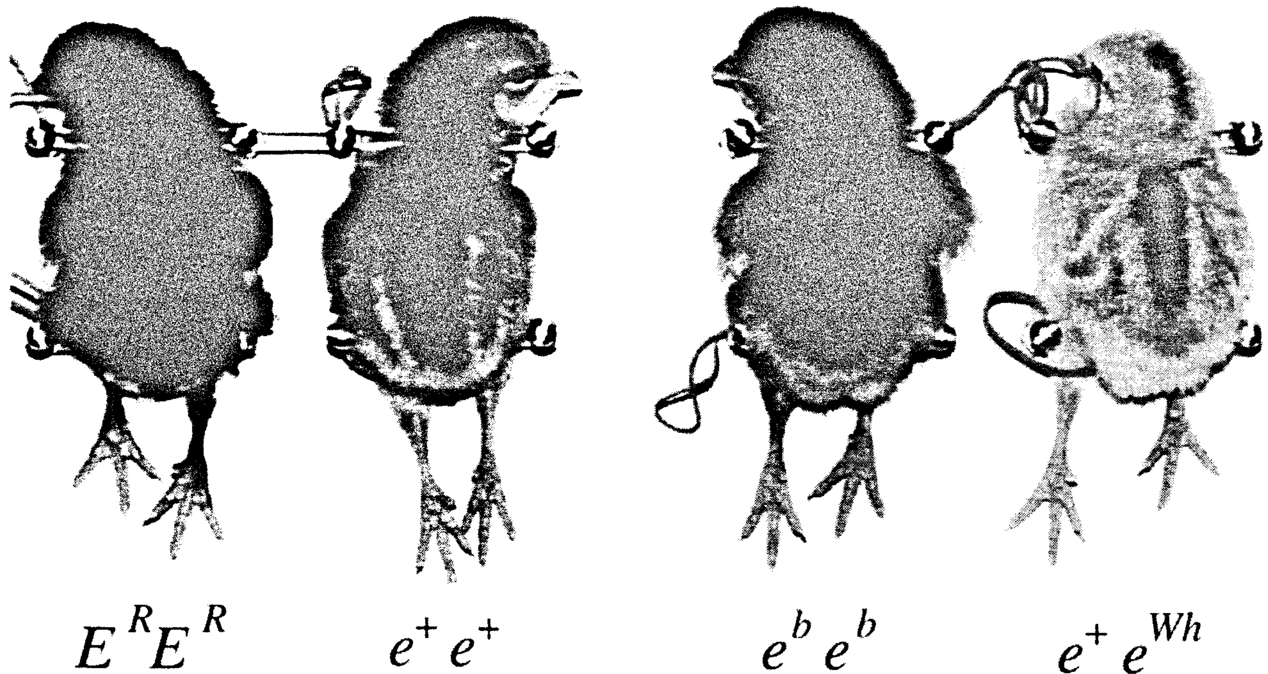

Figure 1. Representatives of various E *allele down types.* E^R *is usually dominant to the other alleles and produces a black downed chick. Wild-type* (e^+) *is dominant to* e^b *and produces a chipmunk striped down with alternating tan, black and dark brown stripes. The brown or partridge* (e^b) *allele produces a dark brown down with some light striping often seem in some chicks. The dominant wheaten* e^{Wh} *allele usually produces a clean yellow down with no striping when homozygous, but is incompletely dominant to some alleles such as the* e^+e^{Wh} *chick down depicted in the figure.*

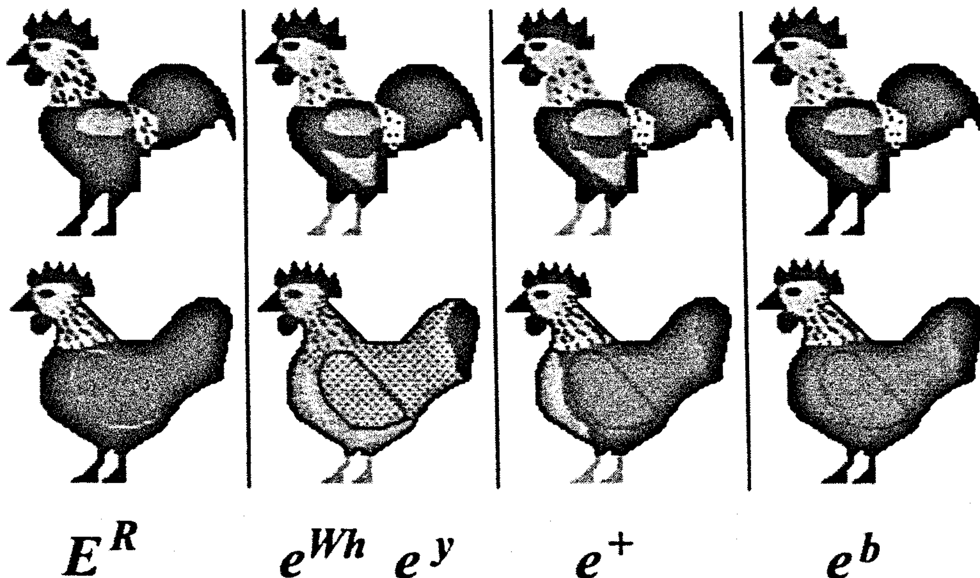

Figure 2. Adult phenotypes of the various E *alleles. Females display the most differences in adult feather color caused by the various* E *alleles. The most dominant extended black* (E) *allele is not shown, but produces males and females that are all black or nearly all black. Female birchin* (E^R) *birds are black except in the head and hackle, while males are nearly wild-type, but lack the red in the primary flight feathers of the wing. Wheaten females lack almost all black pigment and show mostly browns and salmon pigmentation. Males are wild-type in coloration, but unlike all other* E *alleles the wheatens produce a cream not gray feather undercolor (the fluff at the base of the feather next to the body is a light cream color instead of gray). Wild-type* (e^+) *females have a salmon colored breast with a brown stippled back. Brown* (e^b) *females are brown stippled over their entire bodies. Males are not distinguishable from wild-type.*

pigmentation of broilers is a problem for the poultry industry because consumers mistakenly associate the dark coloration with contamination or poor health of the bird. The *E* and E^R alleles are most responsible for the epidermal coloration while e^+ and e^b result in more pigmented abdominal skin fascia (9). Most broiler chickens have white plumage that masks the E allele phenotypes affecting feather and tissue coloration which are segregating in a population. In addition, many poultry markets around the world differ in their preference for various skin and plumage color (9), making *E* locus phenotype selection important for an international breeding company. Another important application for the accurate identification of the MC1-R alleles involves the correct sex determination of day-old chicks. Down color sexing is used extensively in industry for sex determination and certain alleles of the *E* locus are used for the most accurate sexing (9).

The MC1-R gene of the chicken has been cloned, sequenced and the amino acid sequence of some alleles (*E*, e^+, and e^y) has been published (1,2). Using the DNA sequence of the published alleles, PAMSA primers were developed and tested on the DNA isolated from birds with known *E* alleles. We obtained unpublished results showing incongruities in the amino acid sequence at position 92. Several polymorphisms in the published sequences could not be verified. In order to address this, several accessions of the various alleles were PCR cloned, sequenced, and compared to the previously published sequences. The ambiguities were resolved and new mutations were identified that could explain the phenotypes of the various alleles.

Line/	Allele	Amino Acid Positions								
		33	37	71	92	133	143	213	215	244
1	e^+	Cys	Asp	Met	Glu	Leu	Thr	Arg	His	Leu
		TGC	GAC	ATG	GAG	CTG	ACC	CCG	CAC	CTG
2	e^+	...	...	...	...	...	...	Cys	...	...
								TGC		
3	e^+	...	...	...	...	...	...	Cys	...	...
								TGC		
4	E?	...	...	Thr	Lys	...	...	Cys	...	...
				ACG	AAG			TGC		
5	E?	...	...	Thr	Lys	...	...	Cys	...	...
				ACG	AAG			TGC		
6	E?	...	...	Thr	Lys	...	...	Cys	...	...
				ACG	AAG			TGC		
7	e^b	...	...	Thr	Lys	...	...	Cys	Pro	...
				ACG	AAG			TGC	CCC	
8	e^b	...	...	Thr	Lys	...	...	Cys	Pro	...
				ACG	AAG			TGC	CCC	
9	E^R	...	...	...	Lys	...	...	...	...	...
					AAG					
10	E^R	...	...	...	...	Gln	...	...	...	...
						CAG				
11	e^{Wh}	...	...	...	...	...	Ala	...	...	...
							GCC			
12	e^{Wh}/e^y	...	...	...	...	...	Ala	...	...	...
							GCC			
13	e^{Wh}/e^y	...	...	...	...	...	Ala	...	...	...
							GCC			
14	e^+?	...	...	...	...	...	Ala	...	...	...
							GCC			
15	e^y?	Trp	Gly	...	...	...	Ala	...	...	Pro
		TGG	GGC				GCC			CCG

Figure 3. Polymorphisms in the chicken MC1-R gene delineating mutants. Accessions are number coded and specified below. Codons and amino acids are listed for the new e^+ *sequence at the top and under selected amino acid positions. Codons and amino acids are shown for substitutions and dots indicate identical sequences. Sequences 4, 5, 14, and 15 are taken from Takeuchi* et al *(2). 1 – Richardson's Red Jungle Fowl* (e^+)*, 2 – San Diego Zoo Red Jungle Fowl* (e^+)*, 3 - Welsummer* (e^+)*, 4 – Takeuchi Rock Cornish* (E)*, 5 – Takeuchi Plymouth Rock* (E)*, 6 – Black Australorp* (E)*, 7 – Smyth Brown line* (e^b)*, 8 – Commercial broiler* (e^b)*, 9 – ADOL Line 0* (E^R)*, 10 – Fayoumi* (E^R)*, 11 – New Hampshire Red* (e^{Wh})*, 12 – Buff Minorca* (e^{Wh}/e^y)*, 13 – Rhode Island Red* (e^{Wh}/e^y)*, 14 – Takeuchi Brown Leghorn* (e^+)*, 15 – Takeuchi Nagoya Cortin* (e^y)*. A ? indicates uncertain allele designation.*

Materials and Methods.

Chicken breeds and lines and their associated *E* locus alleles are given in Figure 3. DNA was isolated from whole blood using Qiagen, blood kits or IsoQuick, DNA isolation kits.

Polymerase Chain Reaction was carried out using Promega Taq polymerase that leaves A overhangs facilitating the ligation into pCR 2.1 plasmid vector (Invitrogen,). The 5' and 3' primer sequences were –MC1132 (5' AGCCTTTAT TTGGGAG CGCGA) and +MC-117 (5' TGCTGCGGGAGCACTGGT), respectfully, that amplified the entire coding region of the MC1-R gene. PCR product was then purified by gel electrophoresis and extracted using the dialysis-filter paper method of Girvitz *et al* (10). The purified products were ligated into pCR 2.1 plasmid vectors using the Invitrogen, TA cloning kit and transformed into competent *E. coli* cells from Invitrogen,. Four clones from each accession were subjected to plasmid purification using Qiagen, Plasmid Purification Protocol and Kit.

Plasmids were sent to University of Delaware Molecular facilities for DNA sequencing. Sequencing was accomplished using M13 forward and reverse primers on an automated PerkinElmer ABI 377 system. The DNA sequence was assembled and analyzed using the DNAStar, software package.

Results and Discussion.

PAMSA tests were designed using the methodology of Okimoto and Dodgson (11) to differentiate the Cys33Trp, Asp37Gly, and Leu244Pro polymorphisms between the published (2) MC1-R wild-type (e^+) sequence and the recessive wheaten (e^y). We were unable to confirm any of these polymorphism using our three wheaten lines (unpublished results). PAMSA tests designed to differentiate the Glu92Lys polymorphism confirmed that all lines with extended black or birchin black (E or E^R) had the Lys92 substitution. Interestingly, we found two non-black lines that were known to have the recessive e^b allele to also have the Lys92 mutation, and one E^R line (Fayoumi) did not have the Lys92 mutation. To resolve these issues, 12 additional MC1-R genes were PCR cloned and sequenced. The replacement substitutions found in these sequences are listed in Figure 3.

We were unable to confirm the e^y Cys33Trp, Asp37Gly, and Leu244Pro polymorphisms with three new wheaten sequences. In the literature, two of these lines (Rhode Island Red and Buff Minorca) have been found to segregate recessive wheaten (e^y) (9). All three new wheaten sequences had the same inferred amino acid sequences, and one new line (New Hampshire Red) has been confirmed to have dominant wheaten (e^{Wh}) by test mating (Figure 1 and unpublished data). It is possible that all three new wheaten sequences may represent dominant wheaten. The published e^+ sequence appears to be a misidentification (sample 14 Figure 3). The amino acid sequence of the published e^+ allele is identical to the three *new* wheaten sequences. Eggs were obtained from breeders and DNA was isolated from embryos with no down type confirmation of allele identification. None of the three new e^+ sequences have the Ala143 polymorphisms. Instead, they have Thr143 that is found in all sequences except wheaten sequences. Ala143 appears to be characteristic of wheaten and it appears that the published e^+ sequence may be a wheaten sequence. We were unable to confirm the existence of a recessive wheaten (e^y) allele. The Ala143 substitution at a conserved position is a likely candidate to cause the reduction of black pigmentation of the feathers seen in wheaten breeds.

The recessive e^b (brown) allele is likely to have been derived from the dominant E (extended black) allele. The two new e^b sequences have the same amino acid sequence as the published E sequences and the new Black Australorp (E or E^R) sequence with one additional substitution (His215Pro, Figure 3). The Lys92 mutation has been found in mice and causes an extended black phenotype by constitutive activation of the MC1-R (7). The Pro215 mutation occurs at a highly conserved position and may attenuate the constitutive activation of MC1-R producing the brown phenotype instead of black.

Previous test matings indicate that ADOL Line 0 has a birchin like E^R allele, and Malone and Smyth identified the Fayoumi as having the E^R allele. Both E and E^R alleles produce chicks with black down, but the extension of black is less in E^R birds than in birds with the most dominant extended black (E) allele. The amino acid sequence comparisons indicate that there are two distinct birchin alleles found in chickens. Both ADOL Line 0 and the Fayoumi differed from the published E sequences. Line 0 had the Lys92 mutation of the other E alleles, but lacked the Thr71 mutation. The Fayoumi allele lacked the Lys92 mutation, but had a novel Gln133 mutation. The Gln133 mutation (found in the third transmembrane region) may be another mutation that causes the constitutive activation of the MC1-R. Pig and Fox have been found to have extended black alleles with mutations in the third transmembrane region (8), and various *in vitro* mutations in the third transmembrane region have been found to constitutively activate the MC1-R (7). If sequences 4, 5 and 6 (Figure 3) are representitives of the dominant E allele, the Thr71 mutation that they all share, but that is not found in wild-type, birchin (E^R) and wheaten, may enhance the constitutive activation of the MC1-R.

The new sequences were used to develop PAMSA tests that can be used to genotype animals in commercial populations. With white feathers masking the E locus phenotypes, these molecular tests can be a valuable tool for breeding companies having to use inefficient test crosses to identify unwanted segregating alleles.

Among vertebrates the wheaten (Thr143Ala) mutation, the e^b (combination of Glu92Lys and His215Pro, and the Fayoumi birchin (Leu133Gln) are unique mutations that have not previously been analyzed pharmacologically. The Ala143 mutation of the wheaten allele is particularly interesting because it is a dominant or partially dominant allele. All other MC1-R alleles that decrease the amount of black eumelanin pigmentation are recessive. Dominant restriction of eumelanin does not fit the models of MC1-R function proposed by Lu *et al* (7) and Robbins et al (12). It may be that a closely linked modifier gene is affecting the expression of what should be a recessive allele. Carefoot (13) concluded that wheaten could act as a dominant or recessive allele due to modifier genes, but this conclusion was based on chicks produced from a single hen.

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Dr. Carl Shmidt of the University of Delaware for sequencing. We greatly appreciate Dr. Shmidt for his assistance.

Faculty Comments on Mr. Ellett's Research:

Ronald Okimoto, Mr. Ellett's faculty mentor, describes the importance of the research as follows:

> My laboratory has been working on the melanocortin 1-receptor (MC1-R) gene for around two years. This is an important gene in the control of melanin biosynthesis. The MC 1 -R gene is important to the broiler industry because it is associated with tissue pigmentation as well as the feather color. Tissue pigmentation is not a desired trait due to consummer preference for processed chicken products. One bird with tissue pigmentation that slips by for further processing can ruin large batches of chicken nuggets and breast patties. The melanin pigment is harmless, but the consumer associates the flecks of pigment with contamination or poor health of the animal.
>
> Andy Ellett's cloning and sequencing work, along with our previous work, has allowed us to create specific molecular tests for the various alleles of the E locus. We can now type a blood sample and tell the genetics of the animal at this locus. This is important because all broilers have white feathers. The epistatic nature of white masks the expression of the E locus alleles. Without these molecular tests the breeders would have to test mate their animals to color testing lines. Test mating is not an economic or efficient means of idenfying the animals segregating unwanted alleles.
>
> Andy's paper deals with the basic science of the MCI-R gene. DNA was isolated from individual chickens with the desired E locus alleles. A PCR (polymerase chain reaction) primer set was designed to amplify and clone the entire coding region of the MC 1 -R gene for each allele. These clones were sent to an outside facility for DNA sequencing. The amino acid sequences were inferred from the DNA sequence and compared. This process allows us to clear up some misidentifications in the literature and to correlate the sequences with the phenotypes associated with the various alleles to deduce the effect amino acid substitutions have on the function of this receptor. We can compare our mutations to those described in mammals to get a better understanding of the functional nature of different regions of the protein. MCI-R is a model system for the study of other melanocortin-receptors, and the melanocortin-receptors have been associated with cancer, obesity, and related behavioral traits.

Dr. Douglas Rhoads, Assoc. Professor, Biological Sciences, made the following comments:

> I have read the manuscript and am familiar with the subject and the research efforts Dr. Okimoto. This area of research is of prime importance to the poultry industry as down and feather color are associated with consumer estimations of meat quality, and provide a crucial means for sex-sorting chickens at hatch. The impact of feather coloration on the industry is probably measured in the hundreds of millions of dollars annually. Despite this, Dr. Okimoto and his collaborators, including Mr. Ellett, are some of the first to perform truly detailed analyses of the underlying genetics. Previous work by others had been less than complete.
>
> Mr. Ellett's manuscript details the systematic examination of allelic variation in the gene which is believed to be mainly responsible for color determination. Ellett now provides sequences for most of the major alleles which are used in breeding programs. The results are surprising in light of the differences (and lack thereof) between wildtype and the wheaten allele. Therefore, this manuscript provides some key new information and suggests that other genes may be involved. Regardless, the documentation of the allele variations and establishment of easy PCR based screens for the alleles will be of great interest to the industry, to chicken basic research and to research in other animals. Although highly technical in nature the paper is well written and the figures are adequate. This paper easily exemplifies the high quality of research which undergraduates are performing at the University of Arkansas.