IDENTIFICATION OF DIFFERENTIALLY EXPRESSED GENES IN LINES OF JAPANESE QUAIL DIVERGENTLY SELECTED FOR HIGH AND LOW FOUR-WEEK BODY WEIGHT

by

IVAN W. MOTT

(Under the direction of Robert Ivarie)

ABSTRACT

Poultry production is of worldwide economic importance as a valuable source of high protein food. Understanding the genetic mechanisms that control growth and muscle mass deposition may lead to production of animals that produce larger, leaner cuts of meat for human consumption, facilitate selective breeding schemes that would increase genetic heterozygosity while maintaining desirable growth traits, as well as contribute to general knowledge regarding muscle biology and disease. Decades of selective breeding have produced lines of fast-growing, high muscle bearing chickens that are found in today's broiler industry. Growth-selected lines of Japanese quail have been developed as a model system to aid in the study of poultry breeding and selection issues. These lines have been selected for high and low 4-week body weight.

Myostatin is a negative regulator of muscle mass and mutations in this gene give rise to two independently derived European breeds of cattle that have larger, leaner muscle fibers than other cattle breeds with *wild type* myostatin. This work analyzes the expression pattern of myostatin in different lines of growth-selected chickens and quail to determine if it has been selected against during decades of selective breeding. It is shown here that with respect to myostatin there have been no mutations in the DNA coding sequence, no changes in transcript levels, and no differences in the protein levels between the control and selected lines.

DNA array analysis was used to identify genes that were differentially expressed between lines of Japanese quail. Random anonymous quail cDNA clones (4,704) were robotically spotted to nylon membranes and screened using isotopically-labeled whole quail embryo cDNA from the different lines. Three cDNAs were identified as having substantial differences in expression. Expression levels were confirmed by Northern blotting, and nucleotide sequences were obtained and annotated. Analysis was also done using CyDye-labeled proteins and two-dimensional polyacrylamide gel electrophoresis to identify four proteins that were differentially expressed in liver from the different quail lines.

INDEX WORDS: Growth-selected poultry, Broilers, Japanese quail, Myostatin, cDNA array, Expression analysis, Proteomics

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# DEDICATION

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# CHAPTER 1

# INTRODUCTION

## *Growth-Selected Poultry*

Production of muscle-bearing agricultural animals is of worldwide economic importance and a valuable source of high protein food. Preference for non-red meat has driven increases in poultry consumption to where it now leads all meat sources in per capita consumption. According to the United States Department of Agriculture (USDA), the United States produced 8.39 billion broiler chickens worth $16.8 billion in 2001 and the market is expanding at 7% annually. Since the 1950's, traditional selective breeding methods have produced highly inbred broiler lines mostly controlled now by a few large multinational companies. Separate male and female lines are crossed to produce hybrid vigor in production broilers that grow incredibly fast, reaching the meat market at about 5 weeks of age compared to 26-30 weeks to reach sexual maturity. However, as with most highly inbred populations undesirable traits appear in these flocks such as reduced disease resistance, hatchability, loss of libido and general health (Allen and Fetterer, 2002; Jones, 2002; Liu, 2002; Notter, 1999; Fussell, 1998). Identification of the genes for quantitative trait loci (QTL) that contribute to the fast growth rates of broiler chickens would allow out-crossing of breeder stock to improve other desirable traits while allowing the retention of fast growth rates through marker-assisted selection. Understanding the genetic mechanisms that control muscle mass deposition may lead to

breeding schemes that produce animals that more efficiently produce heavier, leaner cuts of meat for human consumption. Additionally, further insight into genetic factors that regulate muscle deposition and maintenance would have broader implications in understanding the general biology of human health and disease such as muscular dystrophies.

Chickens have a relatively short generation time (7 months) compared to other meat-producing animals (bovine, 20-24 months; ovine, 11-12 months; and swine, 9-10 months) (Hafez, 1993). However, Japanese quail (*Coturnix japonica*) have a generation time as short as 3 months. This shorter generation time along with reduced food, water and housing requirements has led to their use as a model system for studying the genetics of growth-selected poultry (Marks, 1996). Three lines of Japanese quail (L, H, and P) have undergone long-term selection for decreased or increased four-week body weight (Marks, 1996; Anthony et al., 1996). The importance of these lines is that the random bred control (C) line has also been kept for comparison. The P line has been selected for high four-week body weight for > 110 generations. Currently, P line birds are nearly three-fold larger (251 g) than the C line birds (88 g) at four-weeks of age. Two other lines, L and H were later divergently selected for low (54 generations) and high (52 generations) four-week body weight, respectively. The L line individuals average less than 63 g and H line average more than 190 g at four-weeks of age.

The dramatic increase in P line body weight is primarily pleiotropic in nature resulting in increased size of all body organs except brain. At hatch, quantitation of components of chicks as a percentage of total mass showed that the only differences were in plumage, wings, and liver, all of which were relatively heavier in the C line. At nine

weeks of age, the major differences were in the relative percentages of the pectoralis (P 24%, C 19%), head (P 2.8%, C 3.4%), and brain (P 0.18%, C 0.39%) (Ricklefs and Marks, 1985). Muscles from the heavy lines have increased mass due to both hyperplasia (increase in muscle fiber number) and hypertrophy (increase in fiber size) (Burke and Henry, 1999). To date, no genetic factors have been identified that contribute to the increased or decreased growth rates in these lines of quail, including any that would explain the increased muscle mass.

*Myostatin Is a Negative Regulator of Muscle Mass*

The identity of a muscle-specific growth factor was realized with the discovery of GDF-8, later termed myostatin (McPherron and Lee, 1997a). Myostatin belongs to the transforming growth factor-β (TGF-β) super-family of signaling ligands. Its mRNA expression was specific to skeletal muscle and the protein is synthesized as a 376 amino acid, 52 kiloDalton (kD) propeptide that is processed at a putative proteolytic processing site (RSRR) to produce a 15 kD mature protein. When a mouse knockout model was created it displayed a remarkable double-muscled phenotype with 2-3-fold increase in muscle mass as compared to wild type littermates. This increase in muscle mass was due primarily to hyperplasia but also hypertrophy. Myostatin also decreases adipogenesis (Kim et al., 2001; Lin et al., 2002) and suppresses body fat accumulation in adult mice (McPherron and Lee, 2002), but is primarily recognized as a negative regulator of muscle mass. Myostatin has been detected in all mammalian and avian species investigated and its protein sequence is highly conserved (McPherron and Lee, 1997a and 1997b).

How myostatin affects muscle mass deposition at the cellular and molecular levels is of great interest. Myogenesis is a process involving induction of precursor cells that originate in transient structures called somites in paraxial mesoderm. These cells give rise to myoblasts that later fuse into multinucleated myotubes that mature into muscle fibers. Expression of four basic helix-loop-helix (bHLH) transcription factors known as myogenic regulatory factors (MRFs: MyoD, Myf-5, myogenin, and MRF4) has been shown to control this process. Mouse knockout experiments have elucidated the genetic hierarchy and suggested a model for how these genetic factors function. Two somewhat redundant factors, MyoD and Myf5, are the first to be expressed in somites and induce the formation of proliferating myoblasts (Braun et al., 1992; Rudnicki et al., 1992; Rudnicki et al., 1993). Coinciding with myogenin expression, myoblasts withdraw from the cell cycle and become terminal cells that differentiate and fuse into multinucleated myotubes (Hasty et al., 1993; Nabeshima et al., 1993) that undergo maturation upon subsequent expression of MRF4 (Rawls, 1998).

In chick embryos, myostatin has been shown to inhibit expression of Myf5 and MyoD (Amthor et al., 2002). In murine C2C12 myoblasts, myostatin also down-regulates MyoD expression (Langlet et al., 2002) and myostatin is a downstream target of MyoD (Spiller et al., 2002), suggesting a feedback loop that helps maintain a proper pool of proliferating myoblasts. Other studies using cultured C2C12 myoblasts have shown that myostatin negatively regulates muscle mass by inhibiting myoblast proliferation (Rios et al., 2001; Taylor et al., 2001), as well as inhibition of DNA synthesis and protein synthesis (Taylor et al. 2000). Myostatin expression also appears to induce p21

expression while decreasing Cdk2 protein activity which blocks myoblasts at the $G_1$- to S-phase of the cell cycle (Thomas et al. 2000).

The discovery of myostatin as a muscle-specific regulator fueled a search in other organisms for alterations in myostatin signaling. Mutations, changes in transcript levels and changes in detectable myostatin protein have all been identified in various organisms under varying physiological conditions. The double-muscling phenotype in cattle known as muscle hypertrophy (*mh)* has been maintained for over a century in Belgium blue and Piedmontese cattle, two independently derived European cattle breeds. The *mh* locus has been mapped (Charlier et al., 1995) and myostatin immediately became a candidate gene for the doubled-muscle phenotype. Three independent reports (Grobet et al., 1997; Kambadur et al., 1997; McPherron and Lee, 1997b) showed that the myostatin gene was located at the *mh* locus and that there were deletion (Belgium Blue) and point mutations (Piedmontese) in the myostatin genes that lead to inactive myostatin protein. Muscle hypertrophy was also observed and mapped in the compact (*cmpt*) line of mice selected for increased muscle density (Vargo et al., 1997); a deletion mutation in myostatin was shown to be cause of the *cmpt* mutation (Szabo et al., 1998). These results indicate that increased muscling in meat bearing agricultural animals can be the result of mutations in the myostatin gene.

Altered myostatin expression also plays a role in several other phenotypes involving muscle mass. Stunted growth in the youngest of porcine litters results in runt piglets that have a detrimental economic impact on pork producers. Ji et al. (1998) have shown that myostatin mRNA levels are 65% higher in *longissimus* muscle tissue of runt piglets compared to their healthy littermates. In humans, HIV-infected men with muscle-

wasting disease have elevated levels of myostatin-immunoreactive protein circulating in their serum (Gonzalez-Cadavid et al., 1998). In muscle regeneration studies, myostatin protein expression is elevated at the time of injury and reduced during muscle regeneration (Kirk et al., 2000). Wehling et al. (2000) found that myostatin protein levels are elevated in atrophied hind limbs of rats that have undergone muscle unloading. While investigating sexual dimorphism in mice, McMahon et al. (2003) have shown that muscles from larger-bodied male mice have 40-60% lower myostatin protein levels than female mice even though myostatin mRNA levels were not different. Although initial studies indicated that myostatin expression was skeletal-muscle specific, Sharma et al. (1999) have shown that myostatin was also expressed in cardiac tissue and that myostatin protein levels were increased after induced myocardial infarctions. These studies have shown a general inverse correlation between healthy muscle growth and maintenance and myostatin expression.

Chapter 2 of this work reports the analysis of the sequence of myostatin in broiler chickens, compared to that in White leghorn layers, to determine if selection for fast-growing, heavy muscled chickens has resulted in deleterious mutations as was found in double-muscled cattle and *cmpt* mice. Chapter 2 also reports the relative expression levels and pattern of myostatin transcript expression as well as protein expression levels. It was determined that there were no differences in myostatin expression between these lines of chickens. Additionally, the availability of the L, P, and C lines of Japanese quail, as well as the controlled manner in which they have been bred, provided an even better model of growth-selected poultry to investigate the role, if any, that myostatin played in

their development. Like the finding in chickens, chapter 2 also confirms that there were no differences in myostatin signaling between these lines of quail.

*Myostatin Receptor Is Likely a Type-1 Transmembrane Protein*

Altered myostatin signaling would have been a promising explanation for increased muscling in growth-selected poultry. Although myostatin itself is not altered in these birds, other components of the pathway may be, including its receptor or other downstream targets. Myostatin is a member of the TGF-β superfamily including a signal sequence for secretion, a proteolytic processing site that releases the mature C-terminus from the pro-region, and a conserved pattern of cysteine residues (McPherron and Lee, 1997a) that is highly conserved among family members as it is involved in formation of the cysteine knot which stabilizes the TGF-β dimer (Sun et al., 1995). One of these conserved cysteine residues is lost by a missense mutation in Piedmontese cattle (Kambadur et al., 1997; McPherron and Lee, 1997b). Therefore, it is presumed that myostatin will function at the molecular level as do other members of this superfamily.

TGF-βs are processed at the proteolytic site by proteases (Dubois et al. 1995) which release the active C-terminus that is secreted from the cell (Derynck et al., 1985; Gentry et al., 1988). Different combinations of ligand hetero-dimerizations give rise to large variation in biological effects (Massague, 1998; Massague et al., 2000b). Dimerized ligand cooperatively binds the receptor, also a heterodimer of type-1 and type-2 receptors (Cheifetz et al., 1988). The type-2 receptors contain constitutively active kinase domains that phosphorylate the type-1 receptor upon the cooperative binding of the ligand (Massague, 1998). Activated type-1 receptors activate SMAD proteins via

phosphorylation that translocate to the nucleus of the cell where they act as transcription activators (Massague, 2000a).

Some insight has been gained into the involvement of myostatin in this signaling pathway. TGF-β circulates in the blood serum as a latent form where the mature protein is associated with the propeptide (Gentry, 1987). Additionally, follistatin is known to negatively regulate TGF-β by binding to it (de Winter et al., 1996). Myostatin circulating in blood was shown to be in the latent form that can be activated by acid treatment (Zimmers et al. 2002), and Hill et al. (2002), have reported that myostatin is in a complex containing both the myostatin propeptide and the follistatin-related gene (FLGR). Although a novel receptor has not been identified through which myostatin signals, Lee and McPherron (2001) reported that myostatin binds to the activin receptor IIB (ActRIIB) in vitro. Furthermore, generation of transgenic mice expressing a dominant-negative form of ActRIIB resulted in a phenotype similar to the myostatin null mice. This experiment, although compelling, does not eliminating the possibility that myostatin acts through a yet unknown receptor and that the dominant negative ActRIIB simply binds all available myostatin, effectively eliminate its biological activity. Myostatin specifically induces SMAD3 phosphorylation, and a dominant-negative SMAD3 rescues the myostatin-induced repression of MyoD expression (Langley et al., 2002). SMAD3 physically interacts with MyoD and inhibits myogenic differentiation (Liu et al., 2001).

*A Genetic Trap For Type-I Transmembrane Proteins*

Identification of the myostatin receptor(s) is important in elucidating the myostatin signaling pathway. Typical approaches for the isolation of TGF-β receptors

that bind a known ligand have utilized expression screening (Mathews et al., 1991; Lin et al., 1992). This type of screen is very labor-intensive and involves the screening of complex cDNA expression libraries transfected into COS cells. In particular, the libraries are subdivided into separate pools of approximately 1000 recombinants, and each pool of cDNA is transfected into COS cells where transient expression is screened by incubating the cells with $^{125}$I-labeled ligand. Once a positive cell is identified in a transfected pool, the process is repeated with successive divisions of the cDNA pool until a pure cDNA clone is identified. Alternatively, related family members have been isolated via PCR with degenerate primers to the highly conserved kinase domains of the receptor (Tsuchida et al., 1993).

An expression screen as described above had been undertaken to no avail suggesting that traditional methods might not be fruitful in the identification of a novel myostatin receptor. However, myostatin was screened for its ability to bind known TGF-β receptor family members and was shown to have an affinity for ActRIIB (Lee and McPherron, 2001). We therefore sought to develop a novel approach that would combine ligand-binding screening with genetic selection using a signal sequence trap to identify receptor proteins that bind myostatin. Kojima and Kitamura (1999) developed a signal sequence trap that isolates a cDNA containing a signal sequence in mammalian cell culture. Chapter 3 of this work describes the modification of the method for selection of type I transmembrane proteins followed by screening their ability to bind a ligand of interest.

*Identification of Differentially Expressed Genes by cDNA Array Analysis*

Transcriptional regulation is one way phenotypic variation is manifested. Differential gene expression can be measured by one of a variety of methods such as northern blots or quantitative reverse transcription-polymerase chain reaction (RT-PCR) among others. Analysis of arrayed cDNA is an effective way to screen up to genome-wide expression patterns to identify genes that are differentially expressed. Several types of DNA arrays have been developed including macroarrays which contain relatively low density DNA spots on a solid support such as nylon membranes vs. microarrays which can contain tens of thousands of DNAs on glass slides. DNA arrayed on membranes or glass slides (probe) is hybridized to cDNA (target) labeled from RNA sources under differing experimental conditions. Target samples that are differentially labeled with different colorimetric dyes are pooled and hybridized to a single array containing the immobilized probe. Alternatively, if the target are isotopically labeled ($^{32}P$ or $^{33}P$), they are hybridized to separate, identically printed membranes. Differences in steady-state transcript levels are determined by differences in signal intensities.

Microarrays have been used to assess differences in gene expression in many organisms and under different experimental conditions. In yeast, DNA arrays have been used to assess differences between strains (Shalon et al., 1996) and to identify expression changes after temperature shock (Lashkari et al., 1997) and on different carbon sources (Lashkari et al., 1997). Gene arrays were used to compare expression profiles between activated and resting murine T cells (Teague et al., 1999) and to characterize expression patterns of signaling pathways (Fambrough et al., 1999; Madhani et al., 1999). They have been used to identify genes involved in human disease states such as inflammatory

disease (Heller et al., 1997) and cancer (DeRisa et al., 1996; Trent et al., 1997) and in hematopoietic differentiation (Tamayo et al., 1999). In poultry, arrays have been used to identify induced host genes in chicken fibroblasts infected with oncogenic Marek's disease (Morgan, et al., 2001) as well as candidate genes underlying Marek's disease resistance in resistant and susceptible chicken lines (Liu, H.-C. et al., 2001).

In a preliminary screen, Chapter 4 describes the use of arrayed cDNAs to identify differentially expressed genes that potentially contribute to the high and low weight quail phenotypes. From an embryonic day 7 quail library 4,704 anonymous random clones were robotically arrayed onto nylon membranes to serve as the probe and isotopically labeled embryonic cDNA synthesized from the different lines of quail was used as target to identify differentially expressed genes. Analysis of the DNA array using labeled target cDNA from P and C line embryos revealed three cDNAs that displayed vastly different levels of expression. After confirmation of differential expression by Northern blot, the clones were sequenced and annotated and the possible role of these genes is discussed. Over this limited array the success in identifying differentially expressed cDNA was 0.08% (3/4704).

*Comparative Proteomic Analysis*

Proteomics is the analysis of gene expression at the protein level. Unlike DNA array and expressed sequence tag (EST) projects that sample a fraction of the expressed genome, proteomics samples all the proteins present in a given cell type or tissue provided that they are sufficiently abundant to be detected. Proteomic analysis has been used to identify differential protein expression in a wide array of organisms under

differing circumstances. Two-dimensional gel analysis has been used to study heat shock responsive changes in both gram positive and negative bacteria (Rosen et al. 2002), identify proteins involved in glucose starvation in *Bacillus subtilis* (Bernhardt et al. 2003), analyze stage-specific proteins from the cocoa pathogen, *Phytophthora palmivora* (Shepherd et al. 2003) and identify proteins that are induced and repressed during metal stress in the yeast *Saccharomyces cerevisiae* (Hu et al. 2003). In the rat, differentially expressed proteins were identified in sensory ganglia after induced inflammation (Friso, et al. 2001). Using proteomics, Ou et al. (2001) identified a potential cause of food-induced anaphylaxis associated with eating the Chinese delicacy *Collocalia spp*. bird's nests — a serine protease inhibitor homolog, similar to ovoinhibitor, a Kazal-type serine protease inhibitor that is a dominant allergen found in the egg white of chickens.

The first "proteomics" description of proteins that were induced or repressed in response to a glucocorticoid hormone in hepatoma cells used 2-dimensional polyacrylamide gels to separate proteins from $^{35}$S-methionine-labeled cell cultures with and without hormones (Ivarie et al. 1978). The first dimension of separation (isoelectric focusing) is accomplished by equilibrating the samples on an immobilized pH gradient. The second dimension separates the proteins according to molecular weight by electrophoresis in polyacrylamide gels. In early experiments, samples were analyzed on separate gels and these images were superimposed to identify differences. Gel to gel variation creates inconsistencies and necessitates large numbers of replications to confirm experimental results. Experimental procedures have been improved to allow the analysis of different samples on the same gel. (Goldman et al. 1983; Luo et al. 1993; Bernhardt et al. 1999). Since then, advances have led to the development of fluorescence two-

dimensional differential gel electrophoresis (2-D DIGE) technology (Unlu, et al. 1997; Tonge, et al. 2001) that has greatly simplified and improved the process by allowing two protein samples to be separately labeled with one of several mass- and charge-matched fluorophores (Cy2, Cy3, or Cy5). The samples are mixed prior to separation by 2-D gel electrophoresis, eliminating gel-to-gel variation by allowing the differentially labeled proteins to be analyzed on the same gel.

Chapter 5 of this work describes the use of two-dimensional differential gel electrophoresis to identify proteins that have been altered in liver of growth-selected lines of Japanese quail. Three proteins have been found with altered levels of expression in the low line of quail, and a class of proteins in the low line that exhibit a mobility shift on the gel, suggesting either an altered post-translational modification such as lack of glycosylation or a deletion mutation with respect to the other lines.

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# CHAPTER 2

# EXPRESSION OF MYOSTATIN IS NOT ALTERED IN LINES OF POULTRY EXHIBITING MYOFIBER HYPER-AND HYPOPLASIA[1,2]

[1] The nucleotide sequence data reported in this paper have been submitted to GenBank (Submission Mail Stop K710, Los Alamos National Laboratories, Los Alamos NM 87545) nucleotide sequence database and have been assigned the Accession Number AF407340.

[2] Mott, I. and R. Ivarie. 2002. Poultry Science 841:799-804. Reprinted here with permission of publisher

# ABSTRACT

Decades of selective breeding has yielded lines of poultry with substantial myofiber hyperplasia, yet little is known about what genes have been altered during the course of selection. Myostatin is a strong negative regulator of muscle mass in mice and cattle, and may have been one of many genetic factors contributing to increased myofiber deposition in growth-selected lines of poultry. To test this hypothesis, the sequence and expression patterns of myostatin were analyzed in growth-selected lines of chickens and quail. The sequence of broiler myostatin cDNA, amplified via RT-PCR from embryonic muscle RNA, contained no missense mutations in the coding sequence when compared to that of White Leghorn layers although two silent single nucleotide polymorphisms (SNP) were found. Northern analysis of myostatin transcripts from embryonic pectoralis and quadriceps showed no significant differences in expression levels between broiler and layer muscle RNA. However, levels of myostatin transcripts were greatly reduced in muscles of post-hatch chicks compared to embryonic muscle. Myostatin protein was also present in both broiler and layer embryonic muscle at similar levels. No significant polymorphisms or differences in RNA expression levels were found in embryonic muscles of divergently selected lines of Japanese quail. These results indicate that intense artificial selection in these growth-selected lines of poultry has neither silenced the expression of myostatin nor created null alleles via mutation in the lines analyzed.

(*Key words:* myostatin, broiler, layer, quail, myofiber hyperplasia)

# INTRODUCTION

Myostatin (GDF-8) belongs to the TGF-β superfamily of signaling molecules. Expression of the gene is restricted to the developing myotome in somites and to embryonic and adult skeletal muscle in mice (McPherron and Lee 1997a). Transgenic mice bearing disrupted myostatin alleles have 2 to 3 times the skeletal muscle mass as wild type littermates suggesting that myostatin negatively regulates muscle mass. Myostatin is highly conserved and homologous genes have been found in every vertebrate examined including mice, human, and aves.

Myostatin has been shown to be an important regulator of muscle deposition in mammals. Two independently derived double-muscled cattle breeds, Belgian Blue and Piedmontese, have mutations in their myostatin alleles (Grobet et al., 1997; Kambadur et al., 1997; McPherron and Lee, 1997b). These mutations map to the same chromosomal location as the muscle hypertrophy (*mh*) locus (Charlier et al., 1995) implying that disruption of myostatin signaling in these cattle leads to increased muscle mass. Selection for increased muscle density in mice gave rise to the *compact* (*Cmpt*) allele, a deletion in the N-terminus of myostatin (Szabo et al., 1998). In addition to mutations in the myostatin gene, mRNA expression levels have also been shown to negatively correlate with muscle mass and deposition. For instance, HIV-infected men with muscle-wasting disease have elevated levels of myostatin in their serum (Gonzalez-Cadavid et al., 1998) and in longissimus muscle tissue, myostatin mRNA levels were 65% higher in low birth-weight runt piglets vs. normal littermates (Ji et al., 1998).

Poultry breeders have been artificially selecting heavily muscled chickens since the 1950s. Body weight in poultry, as in other species, is a polygenic trait, and selection to yield today's broilers has produced chickens with fast growth rates and increased muscle deposition. When compared to a lightly muscled line of chickens such as White Leghorn layers, broilers not only have substantially increased muscle mass, but an increase in skeleton and other organs suggesting that pleiotropic growth factors are responsible for the increased growth rates (Aberle et al., 1983; O'Sullivan et al., 1992). In addition to chickens, lines of Japanese quail have been genetically selected from a control C line for increased (P line) and decreased (Line 54) growth rates. After more than 100 generations of selection for high body weight at 4 wk of age, the P line quail showed significant myofiber and myonuclear hyperplasia with nearly a three-fold increase over the control line (Fowler, et al., 1980; Campion, et al., 1982; Ricklefs, et al., 1985). By contrast, 54 generations of selection for low body weight at 4 wk of age in line 54 quail resulted in substantial myofiber hypoplasia. It is not known what genetic factors specifically contribute to this increased and decreased muscle mass in poultry.

Because of its large effects on muscling, we have asked whether altered expression of myostatin contributes to myofiber hyper- or hypoplasia in growth-selected lines of poultry. We report here on the coding sequence, RNA transcript levels and protein expression levels of myostatin in growth-selected lines of chickens and quail. The results indicate that myostatin expression is not altered in the broiler chickens analyzed or in growth-selected lines of Japanese quail, nor has the gene been inactivated by mutation.

# MATERIALS AND METHODS

*Poultry Lines*

Broiler eggs were obtained from an Arbor Acres/Peterson Meat cross[3]. Eggs from White Leghorn layers and lines 54, C, and P Japanese Quail (*Coturnix japonica*) were obtained from the Southern Regional Poultry Genetics Laboratories at the University of Georgia. Eggs were incubated at 39.5° C at 50-60% humidity with hourly rocking through 60°. All animals were cared for in accordance with the University's Animal Care and Use Committee policy, and were killed by cervical dislocation.

*RNA Isolation*

RNA was extracted from various tissues by homogenization with a polytron in RNA STAT-60[4] according to the manufacturer's protocol. Muscle weight prior to pooling were: broiler D10 pectoralis = 20.907 +/- 3.250 g (mean +/- SE) vs layer D10 pectoralis = 5.120 +/- 0.511 g, and broiler D10 quadriceps = 12.660 +/- 2.236 g vs layer quadriceps = 3.757 +/- 0.232 g. Tissues from 3 embryos or chicks were pooled before RNA extraction. RNA concentrations were measured by absorption at $O.D._{260}$ in a Spectronic Genesys 2 spectrophotometer[5], and stored at -80° C until use.

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[3] Harrison Poultry, Bethlehem, GA 30620.
[4] Tel-Test, Inc. Friendswood, TX 77546.
[5] Spectronic Instruments, Rochester, NY 14625.

*RT-PCR and Sequencing of Myostatin cDNA*

Myostatin cDNA was amplified by the Titan One Tube RT-PCR System according to the manufacturer's protocol[6] using primers Mstnf1 (5'-CGCGGATCCAAAGGCAAAAAGCTGCAGTG-3) and Mstnr2 (5'-CGCGGATCCTTTCAAAGATGGATGAGGGG-3') with *Bam*HI sites at their 5' ends. Cycles were as follows: 50° C for 30 min, followed by 10 cycles (94° C for 30 sec, 55° C for 30 sec, 68° C for 90 sec), and 25 cycles (94° C for 30 sec, 55° C for 30 sec, 68° C for 90 sec plus 5 sec elongation per cycle), and 68° C for 7 min. Mstnf1, Mstnr2, and two other internal myostatin primers, Mstnr4 (5'-TTGCAGCACTGTCTTCACATC-3') and Mstnf5 (5'-CCAGATATACTGGAATTCGATCTTTG-3') were used to sequence both strands of six independent myostatin RT-PCR products from broiler RNA. Sequencing was done using Big-Dye Terminator reactions at ½ dilution on a ABI/PE 3700 sequencer. DNA sequence contigs were assembled using Sequencher 3.0 software and aligned with the published White Leghorn myostatin cDNA sequence (GenBank accession number AF019621). Quail myostatin cDNA (GenBank accession number AF407340) was amplified using chick primers MSTNf1 and MSTNr3 (5'-ATGGATTCCCGACCGAAACT-3') as described above.

*Northern Analysis of Myostatin Transcripts*

A template for synthesis of a myostatin riboprobe was prepared by cloning a RT-PCR amplified White Leghorn myostatin cDNA into the *Bam*HI site of pBluescriptKSII. The resulting plasmid, pBS-WL-Mstn, was linearized with *Nhe*I and used to synthesize a

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[6]Roche Molecular Biochemicals, Indianapolis, IN 46250.

1,230 nucleotide antisense myostatin DIG-labeled riboprobe using T7 RNA polymerase according to the manufacturer's protocol[6]. RNA samples were electrophoresed on 1% agarose/2% formaldehyde gels and transferred to nylon membranes. Hybridization and DIG luminescent detection were carried out according to the manufacturer's protocol[6].

Quantitative analysis of myostatin mRNA levels was determined by densitometry. Bands (n=3) representing myostatin were scanned and normalized to background on the autoradiograph. Student's t-test was used to compare band intensities between broiler and layer samples. To compare band intensities of different tissues and their intensity over time, the Kruskal-Wallis test was used.

*Antibody Production and Protein Detection*

A 16 amino acid epitope from the C-terminal fragment of myostatin (amino acids 350-375) was synthesized as a MAP peptide (PDM072) and used to immunize rabbits for the production of polyclonal antibodies. Myostatin reactive antibodies were purified by column chromatography over Sepharose conjugated to PDM072 (Harlow and Lane, 1999).

Tissues were lysed by homogenization with a polytron in ice-cold RIPA buffer (1X PBS, 1% v/v Nonidet P-40, 0.5% wt/vol sodium deoxycholate, 0.1% wt/vol SDS, Complete™ protease inhibitor cocktail[6]). After centrifugation at 10,000 x g for 10 min at room temperature, supernatants were stored at –70 °C until use. Recombinant myostatin expressed in cultured chicken whole embryo fibroblasts (WEF) were use as controls in western blotting. Full-length chicken myostatin cDNA was cloned into the eukaryotic expression plasmid pCMV to create pCMV-MSTN. Vector alone (pCMV) or pCMV-

MSTN were transfected into WEFs in 6-well dishes and conditioned medium was collected 48 h after transfection. Samples (100 μg tissue lysate, 10 μl of conditioned WEF medium ) were electrophoresed on 12% SDS polyacrylamide gels, transferred to Immobilon-P membranes[7], incubated with anti-myostatin primary antibody (1:1000), and goat anti-rabbit-POD conjugated secondary antibody[6] and detected by ELC™ according to manufacturer's protocol[8].

## RESULTS AND DISCUSSION

### *Equivalent Muscle Specific Expression of Myostatin in Broilers and Layers*

In mice, expression of myostatin is limited to muscle and the developing myotome (McPherron and Lee, 1997a). To determine whether the same holds true in chickens, RNA was extracted from heart, brain, liver and pectoralis of E10 broiler and White Leghorn embryos, and analyzed by Northern blotting. As shown in Figure 2.1, expression was limited to skeletal muscle.

Although differences in embryonic body weight and muscle content of growth-selected poultry can be detected in ovo (Marks, 1975; Lilja et al., 1985; Lilja et al., 1987; Lilja et al., 1991), the major period of muscling occurs after hatch. We therefore examined the levels of myostatin expression in both embryonic and post-hatch muscles of broilers and White Leghorn layers at days 10 and 18 of development and at 10 days post. At D10,

[7] Millipore, Bedford, MA 01730.
[8] Amersham Pharmacia Biotech, Piscataway, NJ 08855.

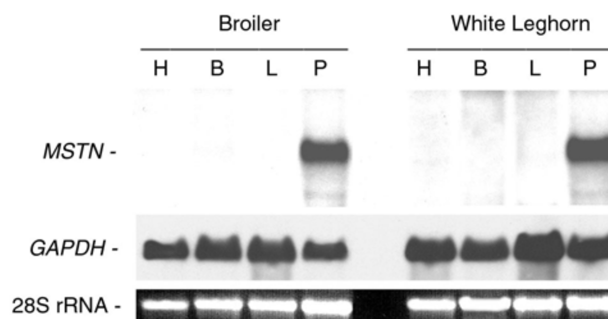


**FIGURE 2.1.** Northern blot analysis of myostatin mRNA expression in E10 chicken tissues. Total RNA (5 µg) from broiler and White Leghorn heart (H), brain (B), liver (L), and pectoralis (P) tissues was probed with DIG-labeled anti-sense myostatin (MSTN) or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) riboprobes. Levels of 28S rRNA was also used as a measure of the uniformity of the RNA sample concentrations.

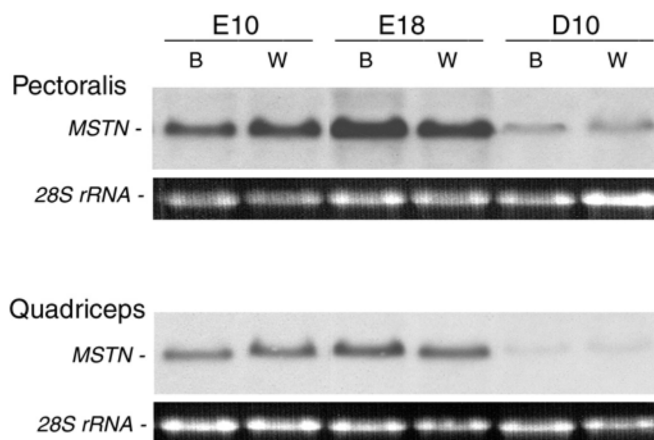


**FIGURE 2.2.** Myostatin mRNA expression in embryonic and post-hatch pectoralis and quadriceps of broiler and layer chickens**.** A representative autoradiograph of a Northern blot showing myostatin expression in embryonic day 10 (E10) embryonic day 18 (E18), and post-hatch day 10 (D10) tissues from broiler (B) and White Leghorn (W) chickens is shown. A DIG-labeled anti-sense riboprobe was used to detect myostatin (MSTN) transcripts in 5 μg total RNA from pectoralis and quadriceps. Levels of 28S rRNA was also used as a measure of the uniformity of the RNA sample concentrations.

pectoralis and quadriceps of broilers weighed 4.1-fold and 3.3-fold more than layer pectoralis and quadriceps. As illustrated in Figure 2.2, the level of myostatin mRNA varied during the course of embryonic development and post-hatch growth with greater levels in E18 RNA and greatly reduced levels after hatch when secondary myofiber deposition is accelerated. At any one time, myostatin expression was higher in pectoralis than in quadriceps. It also appeared, qualitatively, that no differences in the levels of myostatin transcripts were detected between broilers and layers at any of these times. To confirm this conclusion, myostatin transcript band intensities were measured by densitometry (Figure 2.3). As judged by Student's T-test, there was no statistically significant difference in the levels of myostatin transcripts between broilers and layers measured at the same times. However, there were significantly higher levels of myostatin mRNA in pectoralis than in quadriceps at each time point. The lower levels of myostatin expression in quadriceps may be important to allow faster embryonic development of the leg muscles, which are required for mobility at hatch.

*The Myostatin Gene Is Not Inactivated By Mutation In Arbor Acres/Peterson Broiler Offspring*

Although the myostatin gene is expressed normally in broilers as compared to layers at the transcript level, the myostatin gene may have been inactivated by mutation in the coding sequence during the course of artificial selection in the Arbor Acre and Peterson pedigree lines. To test this, myostatin cDNAs were amplified by RT-PCR from E10 broiler pectoralis and sequenced, and their sequences compared to the published sequence of White Leghorn myostatin. No mutations were detected that would have changed the

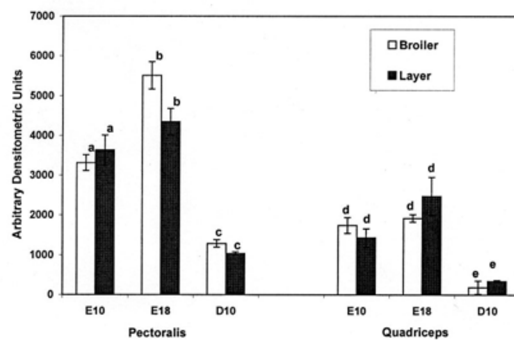


**FIGURE 2.3.** Quantitative measurements of mRNA levels in broiler and layer chicken muscle. Bands (n=3) from Northern blots representing myostatin expression in embryonic day 10 (E10) embryonic day 18 (E18), and post-hatch day 10 (D10) muscles were analyzed by densitometry and values (mean +/- SE) are expressed in arbitrary densitometric units. Open bars, broiler; filled bars, layer. $P<0.05$.

coding sequence of the gene. However, two silent mutations were found in the third position of the R65 codon (C254G) and the K78 codon (G293A). Whether these single nucleotide polymorphisms arose in one or the other of the parent lines is unknown. Regardless, the broiler alleles encode wild-type myostatin protein.

*Myostatin Protein Levels Are the Same In Embryonic Muscle of Broilers and Layers*

The foregoing results implied that transcription of the gene and processing of its transcripts have not been altered detectably by artificial selection for body weight and carcass composition in the two parental breeder lines. To determine whether the level of the myostatin polypeptide in embryonic muscles was different between the broilers and layers, E10 muscle extracts were subjected to polyacrylamide/SDS gel electrophoresis and analyzed by Western blotting using an affinity-purified rabbit anti-myostatin polyclonal antibody. Pre-immune serum served as a control. The ability of the antibody to recognize the secreted form of myostatin was also assessed by transfecting whole embryo fibroblasts from Hamilton and Hamburger stage 16 chick embryos (Kuwana et al., 1996) with and without a plasmid containing myostatin cDNA fused to the CMV promoter. Medium from each transfection was then analyzed by Western blotting and the results are illustrated in Figure 2.4. A 14 kDa band was detected of similar intensity in the protein extracts from E10 pectoralis of broilers and layers that was absent from the pre-immune serum control blot. Furthermore, the anti-myostatin antibody detected a 14 kDa band in the medium from whole embryo fibroblasts transfected with the expression vector, but not in the medium from mock-transfected control cells. Taken together, these

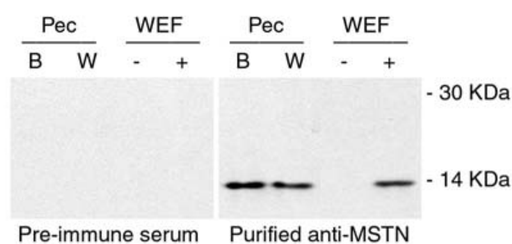


**FIGURE 2.4.** Western analysis of myostatin protein in chicken tissues. Total protein (100 µg) from boiler (B) and White Leghorn (W) pectoralis (Pec) was electrophoresed on 12% polyacrylamide gel along with 10 µl of conditioned media from chicken whole embryo fibroblasts (WEF) that were transfected with vector only (-) or vector containing myostatin (MSTN) cDNA (+) as negative and positive controls, respectively.

results indicate that similar levels of myostatin protein occur in E10 pectoralis of broilers and layers analyzed.

*Myostatin Expression In Divergent Lines of Growth-Selected Japanese Quail*

In the foregoing experiments, the control random-bred population from which the two broiler lines had been selected was not available for analysis necessitating the use of layers as a “control”. For this reason, we analyzed Mark’s lines of Japanese quail divergently selected for body weight at 4 wk post hatch because the control, random-bred C line from which the lines were derived was available for comparison (Marks et al., 1975; Fowler et al., 1980; Lilja, et al., 1987; Marks et al., 1988; Lilja et al., 1991). At the time muscles were sampled, the rapidly growing P line was in its 110th generation of selection while the slow growing line 54 was in its 54th generation of selection. To determine the relative myostatin transcript levels, RNA extracted from E10 pectoralis and quadriceps of P, L, and C lines was analyzed by Northern blotting (Figure 2.5). As was found for broilers and layers, no detectable differences were seen among the three quail lines in the level of myostatin expression.

To determine whether the quail myostatin (qMSTN) gene has undergone an inactivating mutation in the P line, quail myostatin cDNA was amplified by RT-PCR from total pectoralis RNA using primers for chick myostatin. Two SNPs (C928A and C1114T) in the coding sequence were detected in the P line, both of which are silent changes. The C1114T mutation was also detected in line 54 suggesting that the nucleotide change occurred in the control line before the divergence of the P and 54 quail. However, not enough animals were analyzed to confirm this possibility. Regardless,

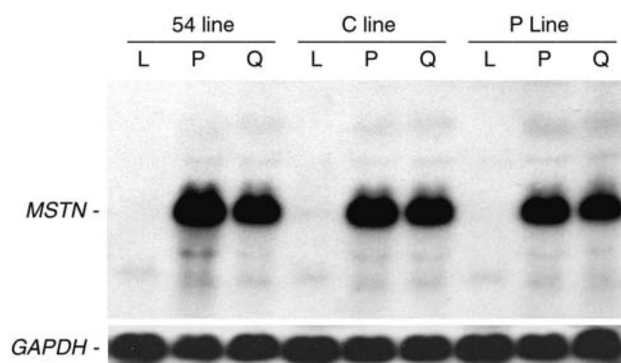


**FIGURE 2.5.** Myostatin mRNA expression in tissues of growth-selected lines of Japanese quail. A representative autoradiograph of Northern blot analysis of myostatin mRNA extracted from E10 quail tissues is shown. Total RNA (5 μg) from 54, C, and P line quail liver (L), pectoralis (P), and quadriceps (Q) was probed with DIG-labeled anti-sense chicken myostatin (MSTN) or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) riboprobes.

no inactivating mutation in the myostatin gene has occurred during the growth selection of these Japanese quail.

*On the Unaltered Levels of Myostatin Expression In Growth-Selected Poultry*

We have shown here that no difference in the expression pattern of the myostatin gene was detectable at the transcript and polypeptide levels between broilers and layers. Furthermore, no inactivating mutation was detected in the alleles expressed in the Arbor Acres/Peterson parent line cross. A similar result at the sequence and transcript level was seen in Mark's quail lines. These observations indicate that transcriptional and post-transcriptional control of the gene's expression has not been a target in broiler selection experiments. It is still possible that other steps in the myostatin signaling pathway have been altered during selection. One candidate would be the myostatin receptor(s) assuming other members of the TGF-β receptor superfamily do not mediate the effects of myostatin. It has recently been reported that the mouse activin type IIB receptor (ActRIIB) can bind myostatin and that a dominant negative ActRIIB induces increased muscling in transgenic mice (Lee and McPherron, 2001).

Our results also show that during the course of embryonic muscle development, the levels of myostatin change. The level of myostatin expression in posthatch muscle growth was substantially reduced compared to levels of myostatin expression in embryonic muscle, which may reflect that muscle growth and differentiation need to be kept in check in ovo where embryo growth is volume-restricted. At hatch, muscle growth is accelerated and correlates with down-regulation of myostatin expression. This is the result expected for a negative regulator of muscle growth and differentiation. Since

recombinant myostatin inhibits proliferation of murine C2C12 myoblasts (Rios et al., 2001; Taylor et. al., 2001), perhaps high levels of myostatin in embryonic muscle slows myotube formation by reducing the number of myoblasts available for fusion, while low levels in post-hatch muscle allows greater myoblast proliferation and differentiation.

Finally, one unanswered question is why the myostain gene has not been genetically inactivated during the course of intense selection by breeders of these broiler lines. Naturally occurring mutations in the gene in both cattle and mice exhibit complex inheritance patterns. In cattle, two mutations arose independently (Mennisier, 1982) in the 19th century in the Piedemontese and Belgium Blue lines and were eventually fixed. Both traits appear to be incompletely dominant. In mice, the Compact gene is of intermediate dominance in males but fully recessive in females (Varga et al., 1997). Yet in genetically engineered mice, targeted disruption of the gene is recessive to wild type (Lee and McPherron, 2001). Regardless, any inactivating mutation in chickens that would cause excessive growth in ovo is likely to be detrimental because of the restricted volume of the egg. As shown here, chicken myostatin expression is unaltered and the gene is wild type in analyzed broiler breeder lines and this may imply that the gene functions in a different manner in birds than in mammals although its high conservation suggests otherwise. This issue will eventually be solved once methods are available to disrupt the gene in birds via homologous recombination.

## ACKNOWLEDGMENTS

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# CHAPTER 3

# A TRANSMEMBRANE TRAP ISOLATES DOMAINS NECESSARY FOR FUSION PROTEIN NEEDED TO DEVELOP A TRANSMEMBRANE RECEPTOR TRAP[1]

[1] Mott, I. and R. Ivarie. To be submitted to *BioTechniques*

# ABSTRACT

Myostatin is a ligand belonging to the TGF-β superfamily of signaling molecules that negatively regulates muscle mass and is highly conserved across many vertebrate species. It is not known what receptor myostatin uses to transduce its signal; whether it signals through a previously identified receptor, or one that has not yet been identified. Several schemes have been developed to identify new signaling molecules, both ligands and receptors, by identifying cDNAs that contain a signal sequence for secretion. All secreted and transmembrane proteins contain signal sequences that target them to the secretory pathway. The presence of a series of one or more hydrophobic translocation transfer/stop sequences in addition to a signal sequence causes proteins to become embedded in the cellular membrane. This report describes a model system for a receptor trap that selects only type I transmembrane proteins containing intact extracellular domains, such as a potential myostatin receptor. The system involves modification of the signal sequence trap described by Kojima et al. (1999) that allows for the selection of cDNAs containing both signal sequences and a transmembrane domain. Furthermore, it would provide a means of ligand interaction screening due to the presence of intact natively conforming extracellular domains displayed on the surface of Ba/F3 cells. An intermediate transmembrane trap (TMT) was utilized to develop the receptor trap and results from the TMT are reported here.

# INTRODUCTION

Transmembrane receptors, integral membrane proteins and secreted ligands play important roles in cell-cell signaling, and regulation of such cellular activity as proliferation, differentiation, apoptosis, migration and polarity. All of these processes are critical to the development and homeostasis of multicellular organisms. One common feature of these proteins is the need to pass through or integrate into the cellular membrane. A signal sequence at the 5' end of the mRNA (Blobel et al., 1975; Simon et al., 1987) that codes for a signal peptide supplies the needed information for assembly of the translocation apparatus at the rough ER (Gilmore, 1991). Coding sequences that contain only a signal sequence will translocate to the lumen of the ER and will subsequently be secreted from the cell (Blobel et al., 1975; Crowley et al., 1993). While peptides containing a single stop-transfer peptide will form single pass transmembrane proteins (High et al., 1992; High et al., 1993; Singer et al., 1990; Thrift et al., 1991; von Heijne, 1988), combinations of start-and stop-transfer signals will form multipass membrane proteins (Engelman et al., 1986; Hartmann et al., 1989; Kyte et al., 1982; Wessels et al., 1988). There are two general classes of transmembrane proteins: type I transmembrane proteins that span the membrane one or more times resulting in an extracellular N-terminus, and type II transmembrane proteins which contain a dual signal sequence/anchoring domain that results in membrane bound protein with an embedded N-terminus and an extracellular C-terminus (Hartmann et al., 1989).

Several strategies have been employed to isolate signal sequences of secreted and transmembrane proteins. The first such Signal Sequence Trap (SST), developed by

Tashiro et al., (1993), functioned by allowing signal peptide-containing cDNAs to direct a signal sequence-deficient CD25 to the cell surface for antibody staining. This method involved time-consuming screening and was inefficient due to low detection levels of antibody staining. A subsequent SST using yeast (Kleinet al., 1996) overcame the screening problem by allowing genetic selection of signal sequence containing cDNAs. Yeast utilize the enzyme invertase, which is secreted through the cell membrane, to metabolize sucrose into glucose and fructose, thus allowing them to grow on medium containing only sucrose as an energy source. For this system, cDNAs were cloned into a vector containing the invertase gene lacking a signal sequence, and the fusion library was then expressed in yeast mutants lacking invertase. Cells containing a clone in which a signal sequence from a cDNA was fused in frame to the invertase coding sequence were able to grow on sucrose medium. However, as noted by Kojima et al. (1999), some mammalian signal sequences may not function in yeast. Therefore, Kojima et al. developed a SST based on the constitutively active $MPL^{M}$ receptor (Onish et al., 1996) that confers IL-3 independent growth of IL-3 dependent Ba/F3 cells. As shown in Figure 3.1, the method exploits the requirement of the $MPL^{M}$ receptor to be inserted in the membrane with the C-terminus exposed to the cytoplasmic side of the membrane. The minimal extracellular (E) transmembrane (T) and intracellular (I) domains were determined experimentally (Kojima et al. 1999). Only cDNAs that contain a signal sequence cloned in frame with the ETI domain will confer IL-3 independent growth. Those lacking signal sequences may be translated but are not positioned in the membrane. To overcome the relative difficulty in transfecting cDNA libraries into mammalian cells, Kojima et al. used a retroviral packaging cell line to produce high titer

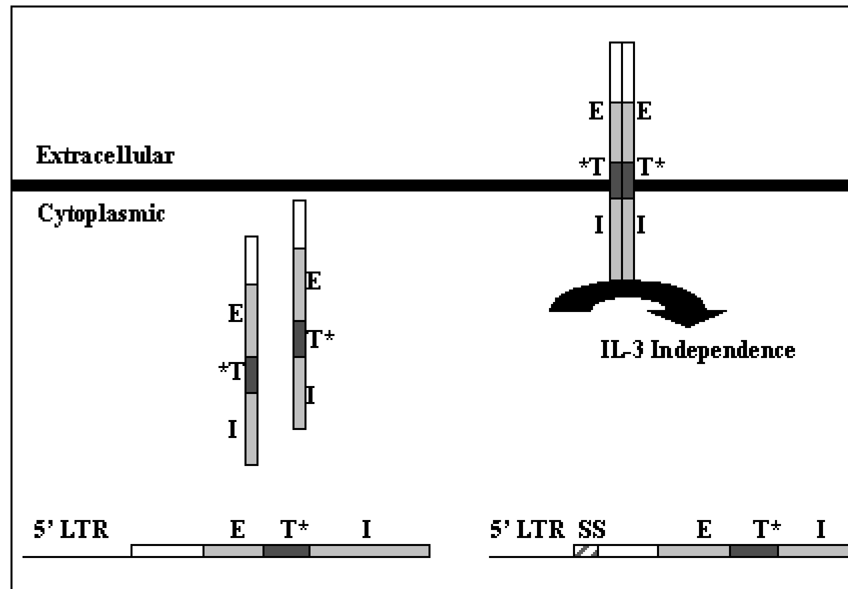


**FIGURE 3.1**. How signal sequence trapping works via the $MPL^M$ receptor. The extracellular (E), transmembrane (T), and intracellular (I) domains of the $MPL^M$ receptor were the minimal components necessary for the trap to function. If a signal sequence (SS) is cloned in-frame upstream of the ETI domains, a fusion protein is synthesized that integrates into the cell membrane in the correct topological orientation that confers IL-3-independent growth to Ba/F3 cells. If a SS is lacking, protein will be synthesized, but will not integrate into the membrane, and the cell cannot grow in the absence of IL-3.

viral supernatant for infection into the Ba/F3 cells. After infection of the Ba/F3 cells, they are grown in the absence of IL-3 where only cells receiving a signal sequence containing MPL$^{M}$ will grow. PCR is used to amplify the integrated DNA containing the signal sequences for sequencing and annotation. Several cDNAs encoding secreted, type I and type II transmembrane, GPI anchored and integral transmembrane proteins were discovered using this method, validating the system as an approach to isolate novel signal peptide containing genes.

Our interest was to isolate cDNAs coding for type I transmembrane proteins such as those that belong to or are related to the TGF-β receptor superfamily More specifically, we wanted to develop a novel way to identify the receptor(s) for myostatin (McPherron and Lee, 1997). While expression screening has been an effective means of identifying receptor proteins when a known ligand is available (Mathews et al., 1991; Lin et al., 1992), it is labor-intensive and requires screening large numbers ($>10^{6}$) of cDNAs to get sufficient coverage of genes expressed in the source tissue. Closely related receptors have also been identified using degenerate PCR (Tsuchida et al., 1993) but this is unlikely to identify all receptors. The Kajima SST would be especially useful in that the power of genetic selection eliminates all cDNAs lacking signal sequences, ultimately reducing the number of cloned cDNAs that must be screened for ligand binding. However, the downside to this method is the necessity of isolating genomic DNA from each Ba/F3 clone followed by PCR amplification and sequencing to determine the value of the cDNA.

An approach that selects only for type I transmembrane receptors would be useful for discovery of receptor cDNAs that bind orphan ligands by 1) reducing the numbers of

clones that require sequencing by eliminating unwanted secreted and type II transmembrane cDNAs from the pool of clones and 2) allowing the direct screening of clonal cells for specific ligand-binding affinity. An analysis of the characteristics of the cDNAs isolated by the Kajima SST revealed some interesting findings that led to our development of such a method. First, multiple type II transmembrane proteins were isolated, suggesting that anchoring of the N-terminus of the fusion protein (Figure 3.2C) did not inhibit the function of the C-terminal MPL. Secondly, of the 29 Type I transmembrane proteins isolated, none of the cloned fragments contained transmembrane coding regions. As they discussed, it is likely that inclusion of a transmembrane domain in the fusion protein would create a two-transmembrane protein that would cause the C-terminal MPL to be outside the cell (Figure 3.2D) thus excluding such cDNAs from selection. Those transmembrane protein cDNAs that were isolated likely lacked complete extracellular domains that would be needed in order to achieve the native conformation required for ligand binding. Supporting this hypothesis is the fact that three conserved cysteine residues involved in tertiary conformation of the extracellular domain of receptors belonging to the TGF-β superfamily cluster near the membrane (Wrana et al. 1994). Disruption of ligand binding renders such constructs ineffective in direct ligand-binding screens.

To develop a functional receptor trap as shown schematically in Figure 3.3, we designed a vector that selects for cDNAs encoding intact extracellular and TM domains for TGF-β like receptors. The final gene product of such a trap will contain multiple (three or more) transmembrane regions that are inserted into the membrane such that the $MPL^M$ receptor is in the proper orientation to confer IL-3 independent growth of Ba/F3

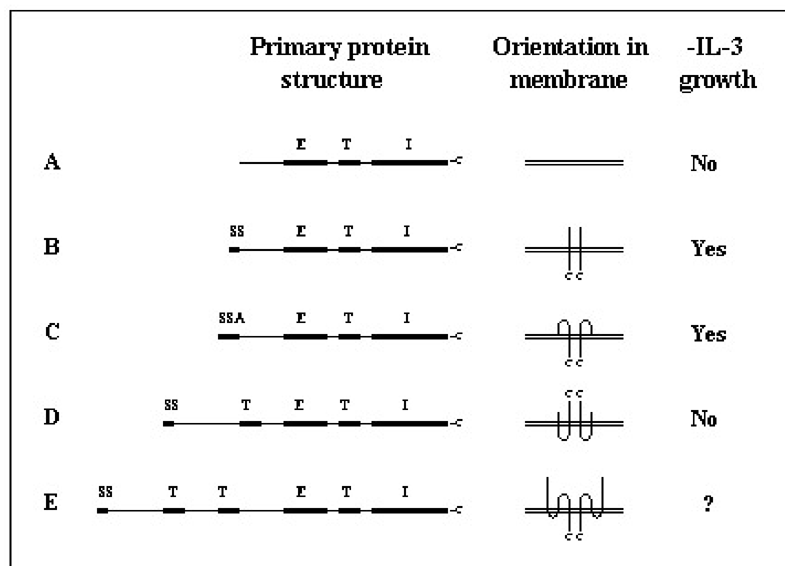


**FIGURE 3.2.** Signal sequence trap constructs and proposed topology in the cell membrane. The primary protein structure of A-E all contain the minimum extracellular (E), transmembrane (T), and intracellular (I) domains of the $MPL^M$. It is assumed that the C-terminus of the ETI domains must be cytoplasmic (down in the figure) for it to confer IL-3-independent growth in Ba/F3 cells. Constructs lacking a signal sequences will not confer growth (A). Signal sequence-containing constructs (B) will confer growth as long as they lack an additional T domain (D). Constructs containing type II transmembrane protein signal sequence/anchor domains (C) also confer growth. A construct containing the signal sequence and the T domain of a type I transmembrane protein may confer correct topology in the membrane (E) if a third T domain is also present.

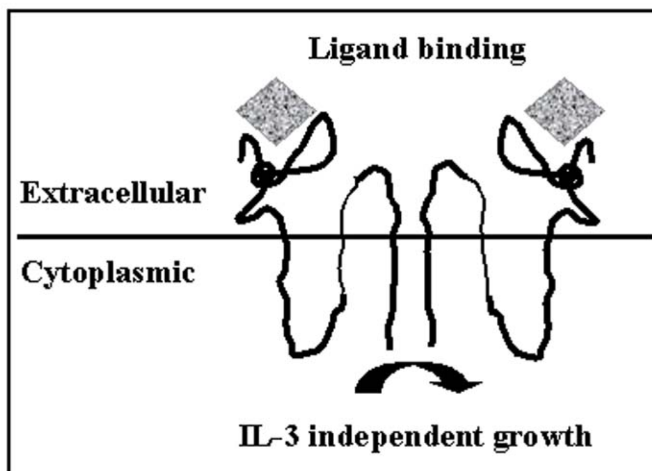


**FIGURE 3.3.** Genetic trap for transmembrane proteins. The transmembrane protein trap contains the coding sequence for two transmembrane regions, one of which is in the C-terminal MPL receptor domain and the other is a short sequence crossing back through the membrane (thin ribbon). A fusion library created by cloning random cDNA upstream of the two transmembrane regions in the trap is introduced into and screened in Ba/F3 cells. Those recombinant proteins containing signals and a transmembrane domain provide correct membrane topology (C-terminus of the MPL must be cytoplasmic) and confer IL-3-independent growth to Ba/F3 cells. IL-3-independent Ba/F3 clones are then directly screened for the ability to bind a ligand of interest.

cells (Figure 3.2E) and contains a complete extracellular domain in its native conformation.

Modification of the Kajima SST vector to include an additional transmembrane (TM) domain with a 5’→ 3’ intracellular to extracellular orientation immediately upstream of the MPL should allow selection of fusion proteins containing cDNAs coding for transmembrane proteins, such as TGF-β like receptors. Clonal Ba/F3 cells containing such a construct can then be screened for their ability to bind a labeled ligand of interest. In this work we describe the construction of a transmembrane trap (TMT) vector as an intermediate step to select for a functional transmembrane region needed to create the receptor trap.

## MATERIALS AND METHODS

### *Transmembrane Trap (TMT) Construction*

The plasmid pCMV5/ActRII-B2 containing the cDNA for ActRIIB (Nucleotide accession number NM 007397) was kindly provided by Dr. Joan Massague (Cell Biology Program, Memorial Sloan-Kettering Cancer Center, New York, NY 10021). The sequence coding for the N-terminal 167 amino acids of ActRIIB containing the start codon, signal sequence, extracellular ligand binding domain, and transmembrane domain was PCR amplified in two fragments in order to eliminate a *Bst*X1 restriction site at nucleotide 232. The 5' fragment (219 bp) was amplified using the primers ActRII-F-BamHI (5'-CGGGATCCGAAAATGGGAGCTGCT-3') and ActRIIBST-Rev (5'-/5Phos/TGTGCCCGA GCTGTT-3') while the 3' fragment (301 bp) was amplified using

primers ActRIIBST-For (5'ATCGAGCTTGTGAAGAAGGGCT-3') and ActRII-rev2 (5'ATGGATCCTGGGAGGTTTCCGATGA). Both amplifications used Clontech Advantage™ PCR mix with 25 cycles of 94° C for 30 sec, 55° C for 30 sec, 68° C for 60 sec. PCR products were gel-purified, ligated, and subjected to a second round of PCR using primers ActRII-F-BamHI and ActRII-rev2 (both of which contain *Bam*HI restriction sites) using the same cycling parameters. The resulting 520 bp fragment was gel purified and cloned into the *Bam*HI site of the pMX-SST vector to create pMX-TMT.

*TMT Library construction and screening*

Experimental cDNA was prepared from chick whole embryo E7 total RNA using the SMART cDNA synthesis kit (Clontech). Full length cDNAs were divided into three aliquots and truncated by restriction digests with the 4-base pair cutters—*Rsa*I, *Alu*I and *Rsa*I+*Alu*I. Digests were inactivated, mixed fragments gel-purified. cDNA fragments of 0.1 – 0.4 kb in length were ligated to *Bst*XI adapters (Invitrogen), purified by gel electrophoresis, and ligated into pMX-TMT. Production of virus, infection of Ba/F3 cells, and selection for IL-3 independent clones was carried out as described (Kojima et al., 1999). Total RNA was extracted from selected clones using RNA STAT-60 (Tel-Test, Inc.) and cDNA was amplified by RT-PCR using the Titan One Tube™ RT-PCR system (Roche) according to the manufacturer's protocol. Briefly, total RNA (1 μg) was cycled with the primers pMXSSTFor (5'-GGGGGTGGACCATCCTCTA-3') and pMXSSTETI (5'-CGTCGACTCAAGGCTG-3') at 50° C for 30 min, 94° C for 2 min, followed by 35 cycles of 94° C for 30 sec, 55° C for 30 sec, 68° C for 90 sec. RT-PCR products were purified using a PCR Purification Kit (Qiagen, Valencia, CA 91355).

Sequencing was done using Big-Dye Terminator reactions at 1/8 dilution on a ABI/PE 3700 sequencer.

## RESULTS AND DISCUSSION

### *A Transmembrane Trap*

To develop the receptor trap, a cDNA fragment coding for a transmembrane domain needs to be cloned in frame to the $MPL^M$ cDNA. Attempts were made to clone a transmembrane domain from the G-protein coupled receptor, GPR37 (Marazziti et al., 1998) into the pMXSST vector. These experiments were unsuccessful at promoting IL-3 independent growth in Ba/F3 cells (data not shown). Therefore, a transmembrane trap (TMT) was constructed that would allow the power of genetic selection to isolate a functional fragment of cDNA. It traps cDNA fragments capable of fusing a known TGF-β like receptor cDNA (activin receptor type II B (ActRIIB)) to the $MPL^M$ cDNA receptor in a way that promotes IL-3 independent growth of Ba/F3 cells. As shown in Figure 3.4, the TMT consisted of the cDNA coding for the N-terminal domain of ActRIIB, including the complete extracellular and transmembrane domains, followed by BstX1 cloning sites, and the cDNA for the C-terminal $MPL^M$ in the vector pMX-TMT. IL-3-independent clones emerging from selection after introduction of the library into BaF3 cells should contain fusion proteins containing three TM domains, one from the ActRIIB, one in the selected cDNA, and one from the $MPL^M$.

Ba/F3 cells were transfected with and without TMT cDNA and challenged for growth in the absence of IL-3. Figure 3.5 shows a typical clonal population of Ba/F3 cells

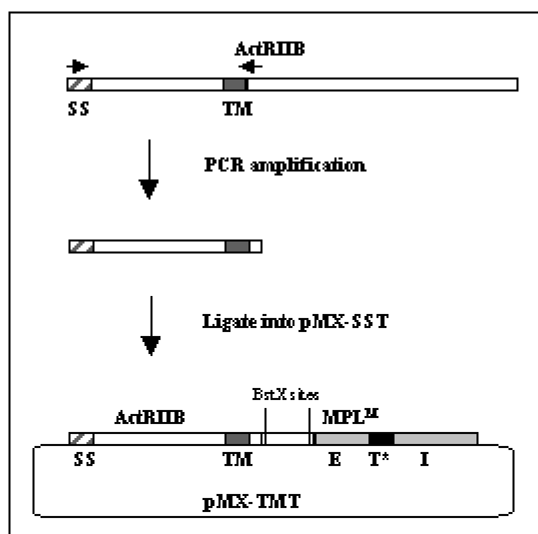


**FIGURE 3.4.** Construction of a transmembrane trap (TMT). A 520 bp fragment coding for the signal sequence (SS) and the transmembrane (TM) domains of ActRIIB was amplified by PCR and cloned into the *BamHI* site of pMX-SST. A cDNA library containing small fragments was then cloned into the *BstXI* sites. Selection for dual fusion proteins was carried out in Ba/F3 cells.

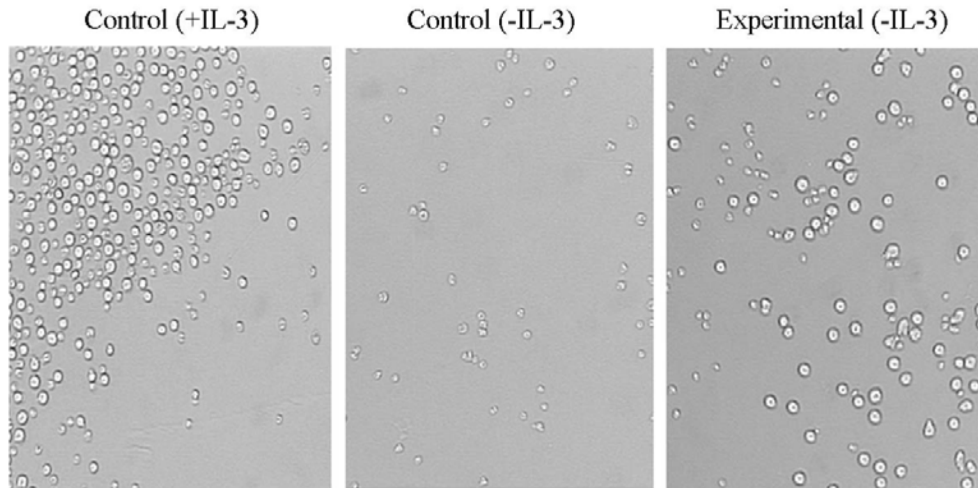


**FIGURE 3.5.** TMT containing Ba/F3 cells grow clonally. Control Ba/F3 cells were grown in the presence (+) or absence (-) of IL-3 for 7 days. Experimental cells were infected with virus particles containing transmembrane trap (TMT) cDNA fusion library clones followed by growth in culture medium lacking IL-3 for 7 days.

growing in culture medium lacking IL-3. Positive control cells were maintained in the presence of IL-3 and negative control cells were infected with pMX-TMT vector only. Infected cells were plated in a 96-well plate. Six wells developed clonal populations of cells that were then expanded into 6-well dishes for RNA extraction. Primers flanking the 5' ActRIIB and 3' $MPL^M$ domains were used via RT-PCR to amplify cDNA from experimental Ba/F3 IL-3-independent clone total RNA. Amplified DNA was purified and sequenced. Multiple Ba/F3 clones, including TMT3-29 (Figure 3.6), contained identical 170 bp cDNA inserts that codes for 57 amino acids. Interestingly, they were the only cells that continued to proliferate after multiple passages in the abcence of IL-3. As shown in Figure 3.7, hydropathy analysis of the entire predicted fusion protein revealed three hydrophobic regions: the first comes from ActRIIB, the second from the selected 170 bp cDNA fragment and the third from the $MPL^M$. To test the functionality of the clone further, the 170 bp PCR-amplified 3-29 cDNA fragment was digested with *BstXI*, gel-purified and cloned into both the pMX-TMT and pMX-SST vectors. As predicted, the pMX-TMT-3-29, but not the vector alone or pMX-SST-3-29, gave rise to IL-3-independent clones.

These results suggest that the TMT3-29 sequence represents a functional transmembrane domain that fuses the extracellular domain of the ActRII receptor and $MPL^M$ so that IL-3-independence is conferred. Perhaps the extracellular domain of ActRII in this fusion peptide is in its native conformation because it contains sequence from the N-terminus through the transmembrane region. It is expected, therefore, that this protein can bind activin, and be detected in an activin binding assay. Replacing the ActRIIB sequence with a random cDNA library should allow the selection of similar

```
   1 ccgaacatgacggcgccctgggcggccctcgcccttctctggggatcgctgtgcgccggttccgggcgaggggag   75
     ---------+---------+---------+---------+---------+---------+---------+-----
           MetThrAlaProTrpAlaAlaLeuAlaLeuLeuTrpGlySerLeuCysAlaGlySerGlyArgGlyGlu

  76 gctgagactcgggagtgcatctactacaacgccaactgggagctggagcgcaccaaccagagcggcctggagcgc   150
     ----+---------+---------+---------+---------+---------+---------+---------+
     AlaGluThrArgGluCysIleTyrTyrAsnAlaAsnTrpGluLeuGluArgThrAsnGlnSerGlyLeuGluArg

 151 tgcgagggggaacaggacaagcggctgcactgctacgcctcgtggcgcaacagctcgggcaccgagctggtgaag   225
     ---------+---------+---------+---------+---------+---------+---------+-----
     CysGluGlyGluGlnAspLysArgLeuHisCysTyrAlaSerTrpArgAsnSerSerGlyThrGluLeuValLys

 226 aagggctgctggctagatgacttcaattgctacgacaggcaggagtgtgtggccaccgaggagaacccccaggtg   300
     ----+---------+---------+---------+---------+---------+---------+---------+
     LysGlyCysTrpLeuAspAspPheAsnCysTyrAspArgGlnGluCysValAlaThrGluGluAsnProGlnVal

 301 tacttctgctgctgcgaaggcaacttctgcaacgagcgcttcacccacttgccggagcctgggggcccagaagtc   375
     ---------+---------+---------+---------+---------+---------+---------+-----
     TyrPheCysCysCysGluGlyAsnPheCysAsnGluArgPheThrHisLeuProGluProGlyGlyProGluVal

 376 acgtacgagccacccccgacagcccccaccctgctcacggtgctggcctactcgctgctgcccattggaggcctc   450
     ----+---------+---------+---------+---------+---------+---------+---------+
     ThrTyrGluProProProThrAlaProThrLeuLeuThrValLeuAlaTyrSerLeuLeuProIleGlyGlyLeu

 451 tctctcatcgtcctgctggccttctggatgtatcgtcatcggaaacctcccaggatccccagtgtgctggaaaga   525
     ---------+---------+---------+---------+---------+---------+---------+-----
     SerLeuIleValLeuLeuAlaPheTrpMetTyrArgHisArgLysProProArgIleProSerValLeuGluArg

 526 cgtgcctcagcattcaggggcacatacctcgttgattgcctgtcagaatgtgtgtgcgagattccatggacagcc   600
     ----+---------+---------+---------+---------+---------+---------+---------+
     ArgAlaSerAlaPheArgGlyThrTyrLeuValAspCysLeuSerGluCysValCysGluIleProTrpThrAla

 601 attgcagcccaggggctcccggggctcctgtggttgttagaggacctcaccggtgccactggggacccctgcagc   675
     ---------+---------+---------+---------+---------+---------+---------+-----
     IleAlaAlaGlnGlyLeuProGlyLeuLeuTrpLeuLeuGluAspLeuThrGlyAlaThrGlyAspProCysSer

 676 aaagccctgccagaacgtggtctttccagcacagtggcggccgccctggagctgcgcccgcgatctcgctaccgt   750
     ----+---------+---------+---------+---------+---------+---------+---------+
     LysAlaLeuProGluArgGlyLeuSerSerThrValAlaAlaAlaLeuGluLeuArgProArgSerArgTyrArg

 751 ttacagctgcgcgccaggctcaacggccccacctaccaaggtccctggagctcgtggtcggacccaactagggtg   825
     ---------+---------+---------+---------+---------+---------+---------+-----
     LeuGlnLeuArgAlaArgLeuAsnGlyProThrTyrGlnGlyProTrpSerSerTrpSerAspProThrArgVal

 826 gagaccgccaccgagaccgcctggatctccttggtgaccgctctgcatctagtgctgggcctcaacgccgtcctg   900
     ----+---------+---------+---------+---------+---------+---------+---------+
     GluThrAlaThrGluThrAlaTrpIleSerLeuValThrAlaLeuHisLeuValLeuGlyLeuAsnAlaValLeu

 901 ggcctgctgctgctgaggtggcagtttcctgcacactacaggagactgaggcatgccctgtggccctcacttcca   975
     ---------+---------+---------+---------+---------+---------+---------+-----
     GlyLeuLeuLeuLeuArgTrpGlnPheProAlaHisTyrArgArgLeuArgHisAlaLeuTrpProSerLeuPro

 976 gacctgcaccgggtcctaggccagtaccttagggacactgcagccctgagcccgcccaaggccacagtctcagat   1050
     ----+---------+---------+---------+---------+---------+---------+---------+
     AspLeuHisArgValLeuGlyGlnTyrLeuArgAspThrAlaAlaLeuSerProProLysAlaThrValSerAsp
```

```
1051  acctgtgaagaagtggaacccagcctccttgaaatcctccccaagtcctcagagaggactcctttgcccctgtgt  1125
      ---------+---------+---------+---------+---------+---------+---------+-----
      ThrCysGluGluValGluProSerLeuLeuGluIleLeuProLysSerSerGluArgThrProLeuProLeuCys

1126  tcctcccaggcccagatggactaccgaagattgcagccttcttgcctggggaccatgcccctgtctgtgtgccca  1200
      ----+---------+---------+---------+---------+---------+---------+---------+
      SerSerGlnAlaGlnMetAspTyrArgArgLeuGlnProSerCysLeuGlyThrMetProLeuSerValCysPro

1201  cccatggctgagtcagggtcctgctgtaccacccacattgccaaccattcctacctaccactaagctattggcag  1275
      ---------+---------+---------+---------+---------+---------+---------+-----
      ProMetAlaGluSerGlySerCysCysThrThrHisIleAlaAsnHisSerTyrLeuProLeuSerTyrTrpGln

1276  cagccttgagtcgac  1290
      ----+---------+
      GlnPro
```

**FIGURE 3.6.** cDNA sequence and the deduced protein sequence of TMT3-29. The first 502 nucleotides code for the N-terminus of ActRIIB and contain the signal sequence (22-58) and transmembrane domain (409-480). Nucleotide residues 701-1281 code for the MPL$^M$ and include a transmembrane domain (853-915). The trapped cDNA 3-29 (boldface) is nucleotides 521-700 and include a predicted transmemrane domain (553-639). Amino acid residues composing the signal peptide and the transmembrane regions and the 12-nucleotide BstXI restriction endonuclease cloning sites (509 and 691) are underlined.

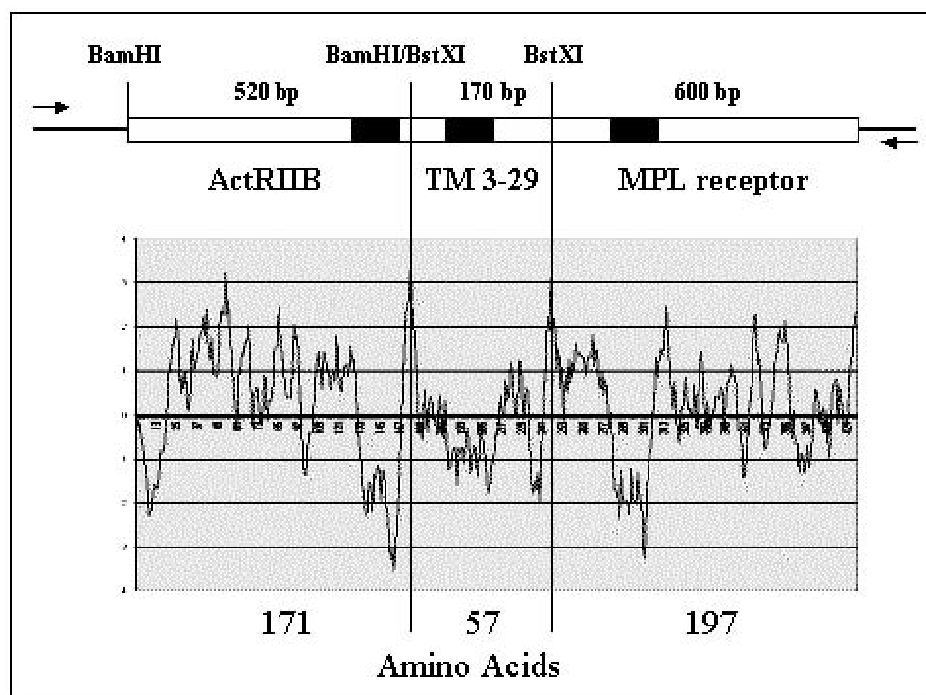


**FIGURE 3.7.** Hydropathy plot of TMT clone 3-29. Arrows indicate location of primers used to amplify fragment from total RNA using RT-PCR. Kyte-Doolittle (averaged over a window of 7 residues) hydropathy values were determined for predicted amino acid sequence and the plot is aligned with the domains defined in the construct design.

transmembrane proteins with intact extracellular domains that could be screened for their ability to bind ligands of interests. IL-3 independent Ba/F3 clones rising from such experiments can be maintained in 96 well plates for screening.

Our purpose in pursuing the development of a receptor trap was to develop a novel approach to identify the myostatin receptor(s). However, after it was reported that ActRIIB binds myostatin *in vitro*, and that transgenic mice expressing dominant-negative ActRIIB phenocopy myostatin null mice (Lee and McPherron, 2001) we felt that it was likely that myostatin signals endogenously through ActRIIB. As a consequence, the receptor trap was never experimentally tested to demonstrate proof of concept. Regardless, the transmembrane trap was shown to be successful in isolating a necessary domain that link two transmembrane domains into a single fusion protein in a way that its topology in the membrane allowed functionality of the $MPL^{M}$. This implies that it should successfully screen for transmembrane proteins when the ActRII cDNA is replaced by randomly primed cDNA.

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# CHAPTER 4

# cDNA ARRAY ANALYSIS OF LINES OF JAPANESE QUAIL DIVERGENTLY SELECTED FOR FOUR-WEEK BODY WEIGHT[1,2]

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[1] The nucleotide sequence data reported in this paper have been submitted to the NCBI nucleotide sequence database (http://www.ncbi.nlm.nih.gov/BankIt/) and have been assigned Accession Numbers AY353854 (EB1), AY353856 (F3-15), and AY353855 (D10-15).

[2] Mott, I. and R. Ivarie. To be submitted to *Poultry Science*

# ABSTRACT

Decades of divergent selection for 4-week body weight has produced three lines of growth-selected Japanese quail. P line quail have been selected for >110 generations for 4-wk post hatch bodyweight and are nearly 3-fold larger than the randomly bred control line. The H line has been selected for high 4-wk body weight for 52 generations and the L line has been selected for low 4-wk bodyweight for 54 generations. To identify differentially expressed genes that may play a role in defining the differences in these lines, a DNA array containing 4704 random anonymous cDNAs was screened using isotopically-labeled cDNA from the different quail lines. Array analysis yielded three differentially expressed cDNAs that were confirmed by Northern blot analysis. The 35-kD quail EB1 protein, previously unidentified, was shown to have elevated transcripts in the L line and decreased transcripts in the H and P lines, when compared to the C line. Two other cDNAs are novel sequences also expressed at higher levels in the L line and lower levels in the H and P lines. These limited findings suggest that anonymous cDNA array analysis is a productive means to identify differentially expressed genes in growth-selected poultry.

(*Key words*: Japanese quail, growth-selected poultry, DNA array, differential expression, EB1)

# INTRODUCTION

Japanese quail (*Coturnix japonica*) lines have been divergently selected for high and low 4-wk post hatch body weight (Marks, 1996; Anthony et al., 1996). The importance of these lines is that the random bred control line has also been kept for comparison. The P line has been selected for high four-week body weight for > 110 generations. Currently, P line birds are nearly threefold larger (251 g) than the C line birds (88 g) at four-weeks of age. Two other lines, L and H were later divergently selected for low (54 generations) and high (52 generations) four-week body weight, respectively. The L line individuals average less than 40 g and H line average more than 190 g at four-weeks of age.

The dramatic increase in P line body weight is primarily pleiotropic resulting in increased size of all body organs except for brain. At nine weeks of age, the major differences in these lines were in the relative percentages of the pectoralis (P 24%, C 19%), head (P 2.8%, C 3.4%), and brain (P 0.18%, C 0.39%) (Ricklefs and Marks, 1985). Muscle comprises 40% of birth weight in vertebrates. The increase in muscles mass in these lines (Burke and Henry, 1999) came largely from hyperplasia (increase in muscle fiber number) and to a lesser extent hypertrophy (increase in fiber size). Anatomical responses due to selection under varying diets (Ricklefs and Marks, 1985), the relationships between egg weight, hatch weight and growth rates (Marks, 1975), survival rates (Aggrey, 2002), and satellite cell content of semimembranous muscles (Campion et al., 1982) of the different lines have been documented. Surprisingly, expression of myostatin, a negative regulator of muscle mass, is not altered in these lines of quail (Mott

and Ivarie, 2002). However, no specific genetic factors have been identified that contribute to the increased or decreased growth rates in these lines of quail, including any that explain the increased muscle mass.

DNA macro- and microarrays have been used to assess differences in gene expression in many organisms and under different experimental conditions. In yeast, DNA arrays have been used to assess general differences between strains (Shalon et al., 1996) and to identify expression changes after temperature shock (Lashkari et al., 1997) and responses on different carbon sources (Lashkari et al., 1997). Gene arrays were used to compare expression profiles between activated and resting murine T cells (Teague et al., 1999) and to characterize expression patterns of signaling pathways (Fambrough et al., 1999; Madhani et al., 1999). They have been used to identify genes involved in human disease states such as inflammatory disease (Heller et al., 1997) and cancer (DeRisa et al., 1996; Trent et al., 1997) and in hematopoietic differentiation (Tamayo et al., 1999). In poultry, arrays have been used to identify host genes in chicken fibroblasts induced by infection with oncogenic Marek's disease virus (Morgan, et al., 2001) as well as candidate genes underlying Marek's disease virus resistance in resistant and susceptible chicken lines (Liu, H.-C. et al., 2001).

To identify genes that are differentially expressed in these growth-selected lines of quail, we have performed a preliminary screen of arrayed cDNAs. A DNA array containing 4704 random unidentified clones from a control line quail whole embryo cDNA library was screened by hybridization with isotopically-labeled cDNA from P and C lines. Subsequent Northern blot analysis was performed on all 4 lines to confirm the

results of two of the most profoundly differentially expressed cDNAs on the array, an EB1 homolog, and a novel sequence.

## MATERIALS AND METHODS

### *RNA and Sample Preparation*

Eggs from lines L, C, H, and P quail (*Coturnix japonica*) were obtained from the Southern Regional Poultry Genetics Laboratory at the University of Georgia. Eggs were incubated at 37.5°C and 55% humidity with rocking through 60° for specified times. Tissues from 3 embryos for each sampling were pooled and snap-frozen in liquid nitrogen, ground to fine powder, and stored in liquid nitrogen until use. Total RNA was extracted from samples using RNA STAT-60[3] according to the manufacturer's protocol. RNA concentrations were measured by absorption at an optical density of 260 nm in a Spectronic Genesys 2 spectrophotometer[4], and were stored at -80° C until use. Isolation of mRNA was carried out using MACS mRNA Isolation Kit[5] according to the manufacturer's protocol.

### *E8 C Line Quail cDNA Library Construction*

Control quail eggs were incubated for 8 days, after which total RNA was extracted from whole embryos. Synthesis of cDNA from total RNA (1 μg) was carried

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[3] Tel-Test, Inc. Friendswood, TX 77546
[4] Spectronic Insterments, Rochester, NY 14625
[5] Miltenyi Biotec, Auburn, CA 95602

out according to the LD-PCR protocol in the Smart™ cDNA Library Construction Kit[6]. Size selected cDNA (0.1–4.0 kb) was ligated into λ TriplEx2 phage and packaged using Gigapack™ Packaging Extracts[7] according to the manufacturer's protocol. The cDNA library contained 1.9 x $10^6$ recombinants and was amplified according to the manufacturer's protocol[8], and stored at –70° or 4°C until use.

*cDNAs Arrays*

Phage from the E8 C line quail λ library were converted to plasmids in BM25.8 cells (Clontech Laboratories), according to the manufacturer's protocol, then plated on LB plates containing carbenicillin and grown overnight at 37°C. Colonies (4,704) were robotically picked to 49 96-well plates containing 180 µl freezing medium (LB supplemented with 36 mM $K_2HPO_4$, 13.2 mM $KH_2PO_4$, 1.7 mM sodium citrate, 0.4 mM $MgSO_4$, 6.8 mM $(NH_4)_2SO_4$, and 4.4% glycerol), grown overnight without shaking at 37°C, robotically arrayed in a 7 X 7 pattern on Hybond™-XL nylon membranes[9], overlaid on LB agar containing ampicillin, and then grown 8 h at 37°C. After incubation, membranes were stored at 4°C until treatment for 5 min each on saturated heavy chromatography paper[10] with10% SDS, denaturation solution (1.5 M NaCl, 0.5 N NaOH), neutralization solution (1.5 M NaCl, 0.5 M Tris·HCl pH 7.4), and 2X SSC. Membranes were then air-dried and baked at 80°C for 2 h. Prior to pre-hybridization,

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[6] Clontech Laboratories, Inc., Palo Alto, CA 94303
[7] Stratagene, La Jolla, CA 92037
[8] Clontech Laboratories, Inc., Palo Alto, CA 94303
[9] Amersham Pharmacia, Piscataway, NJ 08855-1237
[10] Fisher Scientific, Suwanee, GA 30024

membranes were washed (2X SSC, 0.1 % SDS) overnight with gentle agitation and the cell debris was wiped off.

*cDNA Probe Synthesis and Array Hybridization*

Filters were incubated in pre-hybridization buffer (0.5 M sodium phosphate, pH 7.2, 7 % SDS, 1 mM EDTA, 1% BSA) overnight at 65°C. Labeled cDNA probe was prepared using the Strip-EZ™ RT kit[11] according to the manufacturer's protocol. Briefly, separate cDNA synthesis reactions from P and C line mRNA (250 ng) were primed with random decamers and incubated with 20 μCi ($\alpha$-$^{32}$P)-dATP[12] at 37°C for 90 min. Reactions were stopped by addition of 10X stop buffer (2 M NaOH, 2 mM EDTA), and fraction of each reaction was TCA-precipitated to quantitate labeled cDNA probes. Probe (1.0 X $10^6$ cpm/ml pre-hybridization buffer) was hybridized to membranes at 65°C overnight in Autoblot glass tubes and hybridization oven[13]. Membranes were washed twice with wash buffer (0.25X SSPE, 0.25% SDS) at 65°C for 30 min, and once with 2X SSC at room temperature before being exposed to Kodak imaging film[14] or phosphoimager for 24-48 h.

*Sequencing*

Plasmids containing cDNAs were prepared for sequencing using Qiagen Plasmid Maxi or Mini Kits[15]. Cycle sequencing was done using ¼ dilution Big-Dye Terminator v3.1 with the following program in a Genamp® 9600 thermal cycler: 96 °C for 1 min, 25

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[11] Ambion, Austin, TX 78744
[12] Amersham Biosciences, Piscataway, NJ 08855
[13] Bellco Glass Inc., Vineland, NJ 08360
[14] Eastman Kodak Company, Rochester, NY 14650
[15] Qiagen, Valencia, CA 91355

cycles at 96 °C for 10 sec, 50 °C for 5 sec, 60 °C for 4 min. Purified reactions were analyzed on a ABI/PE 3700 sequencer.

*Northern Blot Analysis of Selected Genes*

DIG-labeled probes were synthesized from *BamH1* linearized plasmids using T7 RNA polymerase or by PCR according to the manufacturer's protocol[16]. RNA samples were electrophoresed on 1% agarose/2% formaldehyde gels and transferred to nylon membranes. Hybridization and DIG luminescent detection were carried out according to the manufacturer's protocol[15].

Quantitative analysis of transcripts was determined by densitometry. Bands (n=3) representing cDNAs were scanned and normalized to background on the autoradiograph. Student's t-test was used to compare band intensities between the lines of quail.

## RESULTS AND DISCUSSION

To identify cDNAs that are differentially expressed in the growth-selected lines of Japanese quail (*Coturnix japonica)*, arrayed cDNAs were screened with $^{32}$P–labeled cDNAs synthesized from either P or C line RNA from whole embryo RNA. Typical results are shown in Figure 4.1. Analysis of the blots revealed three highly differentially expressed cDNAs (C2-08, F3-15 and D10-15) all of which were down-regulated in the P line quail as shown in Figure 4.2. The selected cDNAs were retrieved from the library for sequencing and further characterization on Northern blots to confirm differential expression.

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[16] Roche Applied Science, Indianapolis, IN, 46250

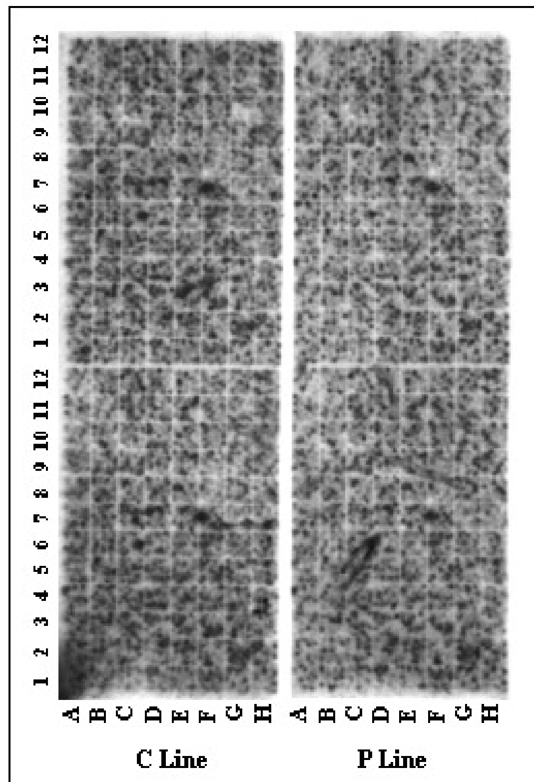


**FIGURE 4.1.** A representative low resolution autoradiograph of cDNA arrays screened with $^{32}$P-labeled cDNA from E14 whole quail embryos. Random cDNA clones (4704) grown in 49-96-well plates were arrayed in duplicate (vertical strips) onto nylon membranes in a 7 X 7 pattern. Each coordinate (for example, all A1 positions) contains the same complement of 49 different cDNAs (each A1 well from all 49 plates). The vertical strips were hybridized with labeled cDNA from control quail (left) or P line quail (right). Washed membranes were exposed to Kodak imaging film. Differentially expressed cDNA was identified by manual inspection of the spot intensities on the developed film.

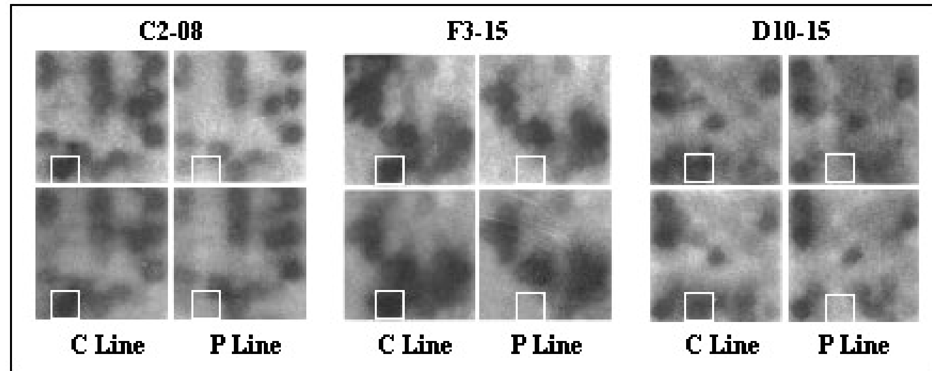


**FIGURE 4.2.** Differentially expressed cDNAs identified on a cDNA array. Screening the array of 4704 random clones resulted in the identification of three differentially expressed cDNAs (enclosed by squares), corresponding to cDNA in the C2 well of plate 08 (C2-08), F3 well of plate 15 (F3-15), and D10 well of plate 15 (D10-15). Each panel contains the duplicate coordinates (top and bottom) from each experiment. All three cDNAs display lower levels of expression in the P line quail.

*Quail EB1 Is Differentially Expressed in Growth-Selected Quail Embryos*

As shown in Figure 4.3, Northern analysis identified C2-08 as a 2300 nucleotide transcript expressed at significantly higher levels in L line, and lowest levels in H and P line, when compared to control quail. The cDNA clone was sequenced to produce a 1,038 base sequence that contains a 789 base open reading frame (nucleotides 106-894) that codes for a 263 amino acid protein with 87% identity (Figure 4.4) to human and mouse APC-binding protein EB1. Human EB1 is a 35-kDa, mildly acidic leucine zipper protein first discovered in a yeast two-hybrid screen as a protein that interacts with the human adenomatous polyposis coli (APC) tumor suppressor protein (Su et al. 1995).

EB1 proteins have been identified in many organisms from yeast to humans (Tirnauer and Bierer, 2000). The budding yeast EB1 homolog BIM1p has been the most studied and has been shown to localize at the plus ends of microtubules where it increases dynamic instability (Tirnauer et al., 1999), facilitates orientation of the spindle toward the bud site (Korinek et al., 2000; Lee et al., 2000), and indirectly participates in a checkpoint delaying cytokinesis until mitotic exit (Muhua et al., 1998). Interestingly, over-expression of Mal3, the fission yeast EB1 homolog, led to severe growth inhibition and abnormal cell morphology while Mal3 mutants had abnormally short, often faint cytoplasmic microtubules (Beinhauer et al., 1997). In *Drosophila*, EB1 is important in ensuring proper dynamics and positioning of the mitotic spindle (Rogers et al., 2002) and in directing the axis of cell division (Lu et al., 2001).

In vertebrates, EB1 localizes to the growing (plus) ends of microtubles, and targets APC to the tips of microtubles under restricted conditions (Mimori-Kiyosue et al., 2000a,b). Loss of heterozygosity at the APC tumor suppressor locus is an early event in

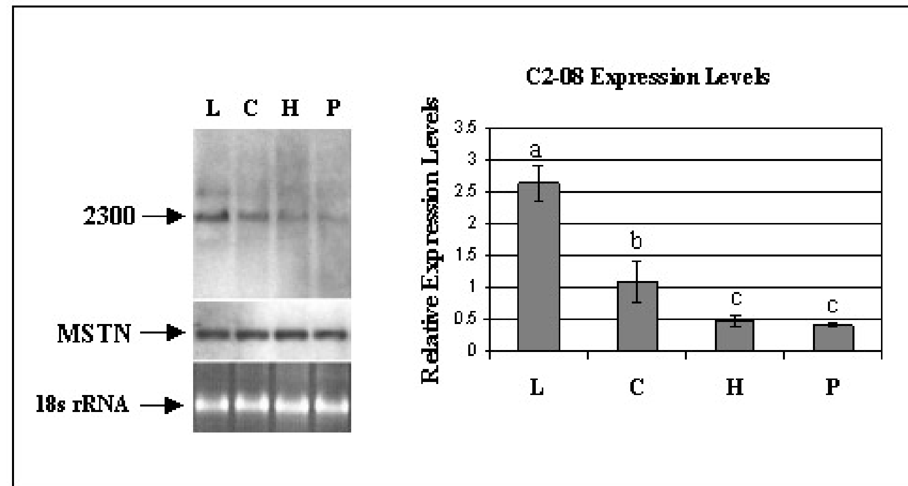


**FIGURE 4.3.** Northern analysis of C2-08 expression in whole quail embryos. Left, DIG-labeled DNA probe was used to detect C2-08 transcripts in total (2 μg) RNA extracted from whole embryo homogenate from L (low), C (control). H (high), and P line quail. Ethidium bromide stained 18s rRNA and DIG-detected myostatin (MSTN) were used as loading controls. Right, bands (n=3) from Northern blots representing C2-08 were analyzed by densitometry and relative values (mean +/- SE) are expressed in arbitrary densitometric units. $^{a\text{-}c}P<0.05$.

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human    1 ~~~~~~~~~~~~~~~~MAVNVYSTSVTSDNLSRHDMLAWINESLQLNLTKIEQLCSGAAY
mouse    1 ~~~~~~~~~~~~~~~~MAVNVYSTSVTSDNLSRHDMLAWINESLQLNLTKIEQLCSGAAY
quail    1 ~~~~~~~~~~~~~~~~MAVNVYSTSVTSDNLSRHDMLAWINESLQLTLTKIEQLCSGAAY
frog     1 ~~~~~~~~~~~~~~~~MAVNVYSTSVTSDNLSRHDMLAWINESLQLNLTKIEQLCSGSVY
rat      1 MPPTPDIELRVGRCWGMAVNVYSTSVTSENLSRHDMLAWVNDSLHLNYTKIEQLCSGAAY
fly      1 ~~~~~~~~~~~~~~~~MAVNVYSTNVTSENLSRHDMLAWVNDCLQSQFSKIEELCTGAAY
worm     1 ~~~~~~~~~~~~~MGYQVVNVYTTASSADNLSRHEMLMWVNDCLQAHFTKIEQLHTGAGY

human   45 CQFMDMLFPGSIALKKVKFQAKLEHEYIQNFKILQAGFKRMGVDK...............
mouse   45 CQFMDMLFPGSIALKKVKFQAKLEHEYIQNFKILQAGFKRMGVDK...............
quail   45 CQFMDMLFPGSVALKKVKFQAKLEHEYIQNFKVLQAGFKRMGVDK...............
frog    45 CQFMDMLFPGAVVLKKVKFQAKLEHEYIHNFKLLQASFKKMGVDK...............
rat     61 CQFMDMLFPGCVHLRKVKFQAKLEHEYIHNFKVLQAAFKKMGVDKVGVCTPGVHEELCFP
fly     45 CQFMDMLFPNSVPVKRVKFRTNLEHEYIQNFKILQAGFKKMSVDK...............
worm    48 CLFTDFLFPDSIQLKKVKWNSRLELDWLSNWKLVQTTWKNLGVEK...............

human   90 .........IIPVDKLVKGKFQDNFEFVQWFKKFFDANYDGKDYDPVAARQGQ..ETAVA
mouse   90 .........IIPVDKLVKGKFQDNFEFVQWFKKFFDANYDGKEYDPVAARQGQ..ETAVA
quail   90 .........IIPVDKLVKGKFQDNFEFVQWFKKFFDANYDGKEYDPVAARQGQ..ET.VA
frog    90 .........IIPVDKLVKGKFQDNFEFVQWFKKFFDANYDGKDYDPVAARQGQ..ETAPA
rat    121 GEEDDPPARIIPVEKLVKGKFQDNFEFIQWFKKFFDANYDGKDYNPLLARQGQ..DVAPP
fly     90 .........IIPIDKLVKGRFQDNFEFLQWFKKFFDANYDGRDYDASAVREG.......A
worm    93 .........VIPVDKLIKGKFQDNFEFLQWFKKLFDANYDGHEYDPMQARNGEGLPTEGG

human  139 PSLVA........PALNKPKKPLTSSSA..............................AP
mouse  139 PSLVA........PALSKPKKPLGSSTA..............................AP
quail  138 PNLVA........PVMNKPKKPLGTGSA..............................AP
frog   139 PVLSA........PVLNKPKKPLGSGNTDDSFAGGSEGKASRTDPTPGLKKMA...KIAP
rat    179 PNPGD........QIFNKSKKLIGTAVPQRTSPTGPKNMQTS.......GRLS...NVAP
fly    134 PMGFG........SGAVKSLPGTAASGVSSSYRRGPSATTRPAMTSAVKPTVS...KVLP
worm   144 PAAGASAKTPSRMPARSVPQKPVTTMRTPAATPAAPPTRPTPSRSSAAPRATAPTPTAAP

human  161 QRPISTQRTAA.APKAGPGVVRKNPGVGN.G..DDEAAELMQQVNVLKLTVEDLEKERDF
mouse  161 QRPIATQRTTA.APKAGPGMVRKNPGVGN.G..DDEAAELMQQVKVLKLTVEDLEKERDF
quail  160 QRPIVAQRTPA.TPKGGTGMVKK....AA.G..DDESAGLIEQINVLKLTVEDLEKERDF
frog   188 QRTVPVQRTTV.SNKPAQGISKKPATVGN.G..EDESAELIQQINVLKITVEDLEKERDF
rat    221 ..PCILRKNPP.SARNG.G........HE.A..DAQILELNQQLLDLKLTVDGLEKERDF
fly    183 RTNNAAPASRINACANSTGTVKKNDVSNSVN..NQQIEEMSNQVMDMRINLEGLEKERDF
worm   204 PKTCAPPVRSASTVAAAAAAPPGVDMATFNKLKEEELEEVTRQLTESDNVIASLEKERDF

human  217 YFGKLRNIELICQE.NEGENDPVLQRIVDILYATDEGFVIPD....EGGPQEEQEEY
mouse  217 YFGKLRNIELICQE.NEGENDPVLQRIVDILYATDEGFVIPD....EGGPQEEQEEY
quail  212 YFGKLRNIELICQE.NEGENDPVLQRIVEILYATDEGFVIPD....EGAPQEEQEEY
frog   244 YFGKLRNIELICQE.NEGESDPVLQRIIEILYATDEGFVIPD....EGAPPEDQEEY
rat    266 YFSKLRDIELICQE.HESENSPVISGIIGILYATEEGFAPPEDDEIEEHQQEDQDEY
fly    241 YFSKLRDIEILCQEADDAEAHPIIQKILDILYATEDGFAPPDD.....APPED.EEY
worm   264 YFSKLRTIEVICQD.NESIGNVEVNRVLEVLYETEEGFAPPEDEANGGAEAEEF~~~
```

**FIGURE 4.4.** Amino acid sequence alignment of human (GenBank database accession no. Q15691), mouse (NP_031922), quail (AY353854), Xenopus (BAB84522), rat (XP_233925), Drosophila (NP_610233), and Caenorhabditis (NP_507526) EB1. Quail EB1 is 87% identical to the human and mouse orthologs ($P = 7 \times 10^{-37}$).

most colon cancers and heritable mutations in APC lead to familial adenomatous polyposis (FAP) colon cancer syndrome (Kinzler et al., 1996). Most mutations in APC cause truncations that lack the EB1 binding domain (Polakis, 1997), raising the question about the role of EB1 in APC functions.

Relatively little is known about the function of EB1 proteins in vertebrates, therefore, it is difficult to speculate about the role it may play in the development of growth-selected quail. However, the negative correlation between quail EB1 mRNA expression and bodyweight is striking. Given that over-expression of the EB1 homolog in fission yeast led to growth inhibition (Beinhauer et al., 1997), it is plausible that over-expression during avian embryogenesis, as reported here in the L line, could lead to reduced numbers of proliferating precursor cells ultimately resulting in smaller organs and animals. The role EB1 plays in regulating the function of APC is also of interest. Normal EB1 expression levels may facilitate the function of the tumor suppressor APC given that loss of APC function via truncation of the C-terminal EB1 binding domain leads to tumor formation via uncontrolled cell replication. Therefore, reduced expression of EB1 could potentially reduce the effectiveness of APC leading to increased proliferation of precursor cells in the H and P lines which have reduced levels of EB1 transcripts.

*Novel cDNAs Are Differentially Expressed in High and Low Body Weight Quail*

Northern analysis of the F3-15 clone confirms that a 1800 base transcript (Figure 4.5) was expressed at highest levels in L line, and lower levels in H and P lines when compared to control quail embryos, similar to EB1. One of several small open reading

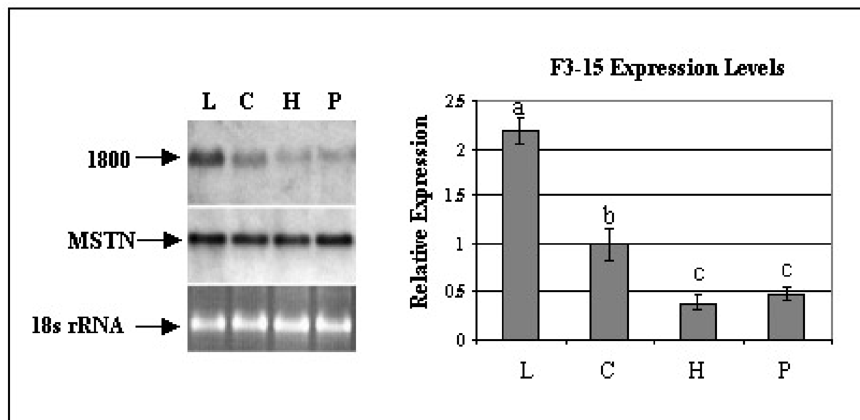


**FIGURE 4.5.** Northern analysis of F3-15 expression in whole E8 embryo RNA. Left, DIG-labeled DNA probe was used to detect F3-15 transcripts in total (2 μg) RNA extracted from whole embryo homogenate from L (low), C (control). H (high), and P line quail. Ethidium bromide stained 18s rRNA and DIG-detected myostatin (MSTN) were used as loading controls. Right, bands (n=3) from Northern blots representing C2-08 were analyzed by densitometry and relative values (mean +/- SE) are expressed in arbitrary densitometric units. $^{a\text{-}c}P<0.05$.

frames in this cDNA is shown in Figure 4.6. This 108 amino acid peptide contains no conserved domains but has 82% identity to annotated mouse and human protein sequences.

D10-15 was differentially expressed between P and control quail (Figure 4.2) with the lower expression levels detected in the P line. D10-15 mRNA codes for a 133 amino acid hypothetical peptide that contains five ValGlyGln repeats (Figure 4.7) that give it 38% identity to *Plasmodium falciparum* phosphatidylcholine-sterol acyltransferase precursor protein. It is not possible to speculate on how these two cDNAs might contribute to differences in body weight in these quail lines until these proteins and their functions are identified and characterized in other model organisms.

In this report, it has been demonstrated that anonymous cDNA array analysis is an effective tool to identify differentially expressed genes in growth-selected quail embryos. Three genes of 4,704 were identified in an initial screen of whole embryo RNA. Additional genes may be found by screening these arrays with other tissue- or stage-specific populations of RNA. Additional arrays printed with anonymous cDNAs may be expected to yield one differentially expressed cDNA for every 1,500 random sequences, based on our limited findings.

```
1   MAGIETCGAG LAPVSSNSRE QRWERTTMNV EHEISLLVEE IRRLGTKNAD  50
51  GQVSVKFGVL FADEKCANLF EALVGTLKAA KRRKIVTYQG ELLLQGVHDN 100
101 VDIVLLQD 108
```

**FIGURE 4.6.** Amino acid sequence of a translated open reading frame (ORF) of F3-15 cDNA. The shown translated ORF is the largest of four small ORFs present in the F3-15 sequence and spans nucleotides185-508. The sequence has 82% identity ($P = 2 \times 10^{-56}$) to mouse (GenBank accession number XP125510) and human (GenBank accession: number: AAF28958) conceptual translations.

```
  1 MGWEVEVVGQ EVAIVGQELA LVGQEVALVG QEDEVGRSVT LVGQEVDYLG  50
 51 REVAVVWQNV ALVGQELALQ GNELALVGQE LELVGPGVFS SVGHGRAGDE 100
101 GAARGALRNG WCTWKAIGMV FPDLTATVIL IYY 133
```

**FIGURE 4.7.** Amino acid sequence of the D10-15 ORF. The D10-15 cDNA codes for a 133 amino acid hypothetical peptide (nucleotides 15-413) that contains seven VGQ repeats and has 38% identity (P = 2 x $10^{-5}$) to *Plasmodium falciparum* phosphatidylcholine-sterol acyltransferase precursor protein (GenBank accession number: NP_703950).

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# CHAPTER 5

# TWO-DIMENSIONAL GEL ANALYSIS OF TISSUES FROM DIVERGENTLY SELECTED LINES OF JAPANESE QUAIL[1]

[1] Mott, I. and R. Ivarie. To be submitted to *Proteomics*.

# ABSTRACT

Decades of divergent selection for 4-week body weight has produced two lines of growth-selected Japanese quail. The H line has been selected for high 4-wk body weight for 52 generations and the L line has been selected for low 4-wk bodyweight for 54 generations. A control (C) line of random-bred quail has also been maintained. To identify genetic factors that are expressed differentially among these lines, liver proteins from embryonic day 14 embryos were analyzed by 2-dimentional gel electrophoresis. Four proteins were differentially expressed. Proteins QE14Lp115i5.4 and QE14Lp25-i8.2 were expressed 4.2-fold and 2.4-fold greater in L line quail than in H or C lines, respectively while QE14Lp33i4.8 was expressed 2.2-fold less in the L line compared to H and C line which showed comparable expression. Although a band of proteins (QE14Lp40i7.1-8.3) did not quantitatively differ among the lines, a 3-4 kDa gel shift was observed in the L line. Presumably, this change in mobility reflects a deletion mutation or changes on post-translational modifications such as a lack of glycosylation.

(Key words*: Japanese quail, growth-selected poultry, proteomics, two-dimensional gel, differential expression)*

# INTRODUCTION

Japanese quail (*Coturnix japonica*) lines have been divergently selected for high and low 4-wk post-hatch body weight (Marks, 1996; Anthony et al., 1996). Compared to commercial poultry lines, the uniqueness of these lines is that the randombred control (C) line has also been kept for comparison. L line birds have been divergently selected for 54 generations for low 4-wk body weight while H line birds have been selected for 52 generations for high 4-wk body weight. Currently, 4-wk H line birds are nearly 2-fold larger (190 g) than C line birds (88 g), while L line birds are less than half (40 g) that of C line birds.

The change in body weight of these lines is primarily pleiotropic resulting in increased or decreased size of all body organs except for brain (Ricklefs and Marks, 1985). Muscle comprises 40% of birth weight in vertebrates and the relative increase in muscles mass in H line quail (Burke and Henry, 1999) comes largely from hyperplasia (increase in muscle fiber number) and to a lesser extent hypertrophy (increase in fiber size). Anatomical responses due to selection under varying diets (Ricklefs and Marks, 1985), the relationships between egg weight, hatch weight and growth rates (Marks, 1975), survival rates (Aggrey, 2002), and satellite cell content of semimembranous muscles (Campion et al., 1982) of the different lines have been documented. Surprisingly, expression of myostatin, a powerful negative regulator of muscle mass, is not altered in these lines of quail (Mott and Ivarie, 2002).

As discussed in Chapter 4, an initial screen of 4,704 arrayed anonymous cDNAs, three genes encoding EB1 and two uncharacterized proteins were found that are differentially expressed in these lines of quail. However, no other specific genetic factors

have been identified that contribute to the increased or decreased growth rates in these lines of quail, including any that explain the increased muscle mass.

As an alternative approach to identify genetic factors differentially expressed in different lines of growth-selected quail, gene expression at the protein level was analyzed via 2-dimensional polyacrylamide gel electrophoresis (2-D PAGE). Unlike cDNA array and expressed sequence tag (EST) projects that sample a fraction of the expressed genome, proteomics samples all the proteins present in a given cell type or tissue provided that they are sufficiently abundant to be detected. Proteomic analysis has been used to identify differential protein expression in a wide array of organisms under differing circumstances. Two-dimensional gels analysis has been used to study heat shock responsive changes in both gram positive and negative bacteria (Rosen et al. 2002), identify proteins involved in glucose starvation in *Bacillus subtilis* (Bernhardt et al. 2003), analyze stage-specific proteins from the cocoa pathogen, *Phytophthora palmivora* (Shepherd et al. 2003) and to characterize proteins that are induced or repressed during metal stress in the yeast *Saccharomyces cerevisiae* (Hu et al. 2003). In the rat, differentially expressed proteins were identified in sensory ganglia after inflammation was induced (Friso, et al. 2001). Using proteomics, Ou et al. (2001) found a potential cause of food-induced anaphylaxis associated with eating the Chinese delicacy *Collocalia spp*. bird's nests — a serene protease inhibitor homolog similar to ovoinhibitor, a Kazal-type serine protease inhibitor that is a dominant allergen found the egg white of chickens.

In this report, we used two-dimensional differential gel electrophoresis (2-D DIGE) (Unlu, et al. 1997; Tonge, et al. 2001) to analyze proteins expressed in the liver of L, C, and H line E14 quail embryos. Liver is critical for the production of enzymes that

allow feed to be converted to weigh gain in animals and changes in metabolic pathways could potentially affect overall bodyweight. Four proteins were differentially expressed in the L line with respect to the C and H lines. One protein was expressed in the L line, but not the C and H lines, two were expressed in the C and H lines, but not the L line, and one band of proteins displayed a mobility shift on the gel suggesting altered glycosylation or a deletion mutation in the L line.

## MATERIALS AND METHODS

### *Protein Extraction*

Eggs from lines L, C, and H line quail (Coturnix japonica) were obtained from the Southern Regional Poultry Genetics Laboratory at the University of Georgia. Eggs were incubated at 37.5°C and 55% humidity with rocking for 14 d. Tissues from 3 embryos for each sampling were pooled, snap-frozen in liquid nitrogen, and ground to fine powder. Protein was extracted from ground tissue samples by incubation in extraction buffer (30 mM Tris, pH 8.0, 4% CHAPS, 8 M urea) with rotation at 4°C for 1 h followed by centrifugation (60,000 x G for 4 h at 20°C) to remove insoluble debris. Protein extracts were washed and concentrated with three buffer exchanges of the extraction buffer using Microcon YM-10 centrifugal filter devices (Millipore).

### *Protein Labeling, 2-D Gel Electrophoresis, and Imaging*

Labeling and electrophoresis were carried out according to the manufacturer's protocol (Amersham Biosciences). Briefly, a sample of each extract (50 µg) was labeled with 200 pmol Cy2, Cy3, or Cy5 at 4ºC for 30 min before quenching with 10 nmol

lysine. Labeled protein extracts were combined and proteins were separated on pH 3-10 IPG strips in 8 M urea, 2% CHAPS, 0.5% IPG buffer, and 0.002% bromophenol blue using active rehydration at 30 V for 12 hr. IPG strips were first reduced in 6 M urea, 2% SDS, 30% glycerol, 50 mM Tris (pH 8.8), and 0.002% bromophenol blue with 64.8 mM DTT for 15 min at room temperature, and then alkylated in the above buffer replacing DTT with 135 mM iodoacetamide for 15 min at room temperature. Proteins were then separated on SDS polyacrylamide gels (8-15% gradient). Gels were fixed in 30% ethanol and 7.5% acetic acid overnight at room temperature. They were then imaged using the Typhoon 9400 (Amersham Biosciences) and analyzed for 2-fold or greater differences using DeCyder software (Amersham Biosciences). Gels were stained with Sypro Ruby (Molecular Probes), destained in 10% methanol and 6% acetic acid for 30 min at room temperature, imaged, and matched to the Cy images using DeCyder software.

*Protein Identification*

The list of spots for picking was created based on the Sypro image. Gel plugs (2 mm) were picked, washed, and digested with trypsin; the resulting peptides were extracted and spotted using the Spot Handling Workstation (Amersham Biosciences). In short, plugs were washed twice with 50 mM ammonium bicarbonate/50% methanol for 20 min at room temperature. Plugs were washed with 75% acetonitrile for 20 min at room temperature and dried at 40$^{o}$ C for 10 min. Plugs were incubated with 140 ng sequencing grade trypsin (Promega) at 37$^{o}$C for 1 h. Peptides were extracted twice with 50% acetonitrile/ 0.1% TFA for 20 min at room temperature. Approximately 25% of the

resulting peptides were spotted with partially saturated α-cyano-4-hydroxy-cinnamic acid (Sigma).

MS data were acquired on the 4700 Proteomics Analyzer (Applied Biosystems) using standard acquisition methods. MS spectra were calibrated using two trypsin autolysis peaks (1045.5 and 2211.1 m/z). Mass lists were submitted to NCBInr and SwissProt using Mascot (http://www.matrixscience.com/cgi/index.pl?page=/search_form_select.html) (Chordata Taxonomy used). Identifications were cross-examined using mass accuracy, molecular weight, and isoelectric point.

## RESULTS AND DISCUSSION

### *2D-PAGE analysis of E14 quail liver proteins*

We previously reported the identification of three differentially expressed cDNAs in E8 whole embryos from these lines (Mott and Ivarie, 2003). However, analysis of proteins from whole embryos via 2-D DIGE did not uncover any differentially expressed proteins (data not shown). We turned to an analysis of individual tissues such as embryonic liver. Growth and weight gain is largely dependent on the ability to utilize nutritional supplements. Avian embryos and their nutritional requirements are contained within the egg so analysis at this stage should only analyze genetic variation. Accordingly, eggs from H, C and L line quail were incubated for 14 days, after which embryos were sacrificed and liver proteins extracted. Proteins were labeled with 3 different CyDyes before separation by 2D-DIGE. Figure 5.1 shows typical results of an overlay image showing protein profiles from all three lines. Four proteins are identified in

Figure 5.1 that consistently exhibited differential expression in multiple gels. The proteins were named by combining the source of the protein sample (quail embryonic day 14 liver (QE14L)) with the molecular weight and isoelectric point, as determined by 2D-DIGE. These differences were reproducible in 6 replicate gels including 3 replicates of extracts labeled with the same combination of the three dyes and three in which the dyes were swapped. Interestingly, all of the differentially expressed proteins were in the L line, relative to the H and C lines.

*Unknown proteins are differentially expressed*

Three of the identified proteins displayed quantitatively different levels of expression. The largest protein, QE14Lp115i5.4, is a 115 kDA protein with a pI of 5.4 (Figure 5.2) that is expressed 4.2-fold greater in the L line than in the H and C line livers. As shown in Figure 5.3, QE14Lp33i4.8 is a 33 kDa protein with a pI of 4.8 that is expressed 2.2-fold higher in H and C line quail than L line. Furthermore a peak of expression was not detectable in the low line. QE14Lp25i8.2 is a 25 kDa protein with a pI of 8.2 that had 2.4-fold higher levels of expression in the L line than in the H and C lines (Figure 5.4). These differentially expressed proteins were picked from the gel and analyzed by MS. However, none were conclusively matched to proteins in the MS database, therefore, their identities remain unknown.

*Proteins exhibit gel mobility shift*

QE14Lp40i7.1-8.3 is a horizontal band of 40 kDa proteins ranging from pI 7.1 to 8.3 on the gel. In the L line, these proteins migrate further on the gel each coming from a

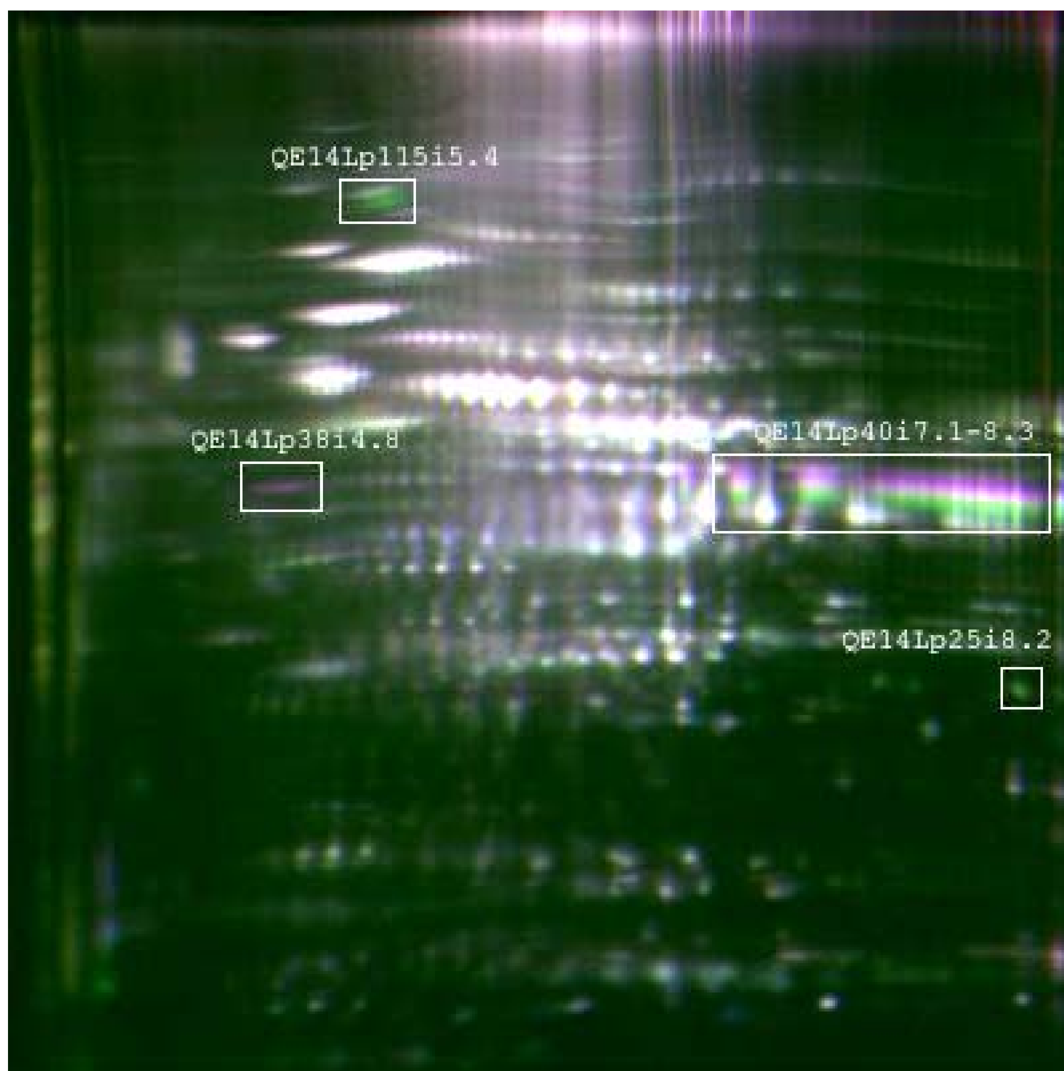


**FIGURE 5.1.** A representative overlay image of 2-D DIGE CyDye-labeled liver proteins from H (Cy3, red), C (Cy 5, blue), and L (Cy2, green) line quail. Differentially-labeled proteins were separated on a pH 3.0-10.0 gradient (left to right) followed by electrophoresis on 8-15% polyacrylamide gels. Differentially expressed proteins (white boxes) include proteins QE14Lp115i5.4 and QE14Lp25i8.2 that were expressed at higher levels in L line than in the C and H lines. QE14Lp33i4.8 is expressed 2.2-fold less in L line quail than in the C or H line. L line QE14Lp40i7.1-8.3 proteins display a 3-4 kDa gel shift with respect to C and H line. These proteins were differentially expressed in six replicate gels including three labeled as shown, and three with dye swapped (H [Cy2], C [Cy3], L [Cy 5]).

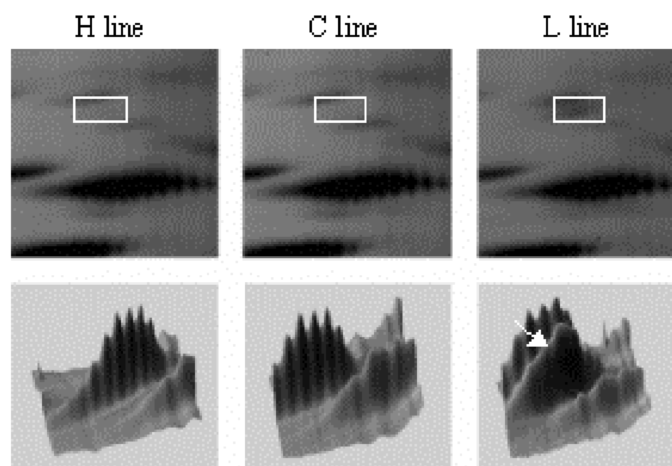


**FIGURE 5.2.** Images captured by DeCyder™ software show QE14Lp115i5.4 differential expression. QE14Lp115i5.4 is a 115 kDa protein with a pI of 5.4 (upper boxes). Peak volume was 4.2-fold higher in the L line, when compared to the H and C line (lower panel).

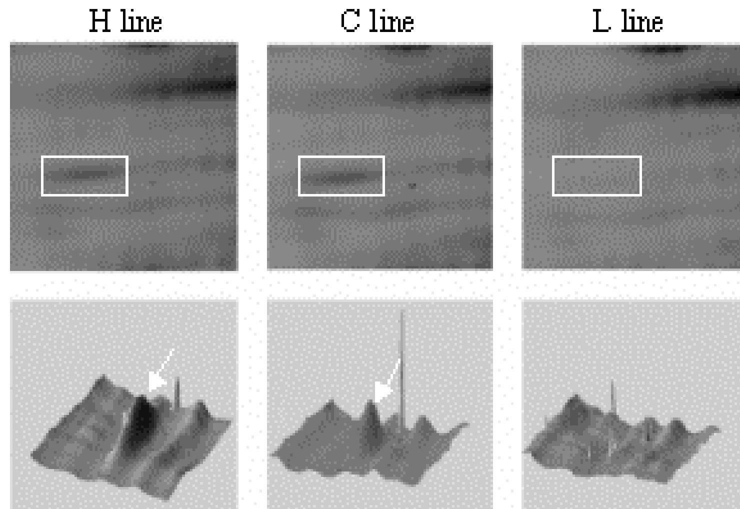


**FIGURE 5.3.** Images captured by DeCyder™ software show QE14Lp33i4.8 differential expression. QE14Lp33i4.8 is a 33 kDa protein with a pI of 4.8 (upper boxes). Peak volume was 2.2-fold less in the L line, when compared to the H and C line (lower panel).

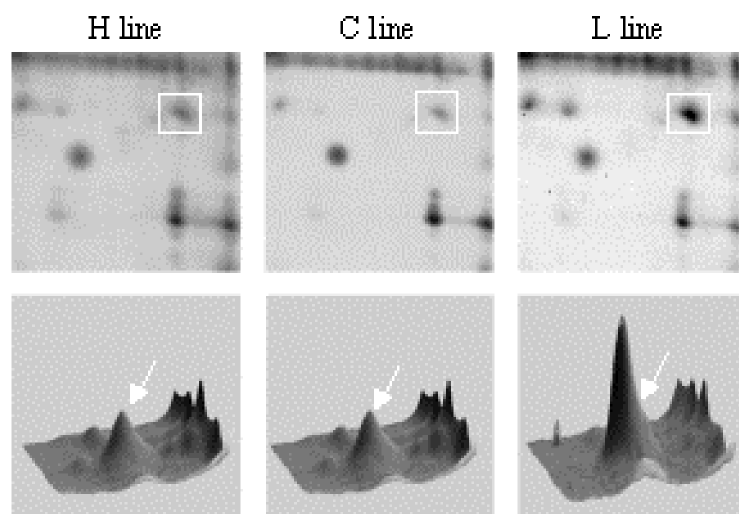


**FIGURE 5.4.** Images captured by DeCyder™ software show QE14Lp25i8.2 differential expression. QE14Lp25i8.2 is a 25 kDa protein with a pI of 8.2 (upper boxes). Peak volume was 2.4-fold greater in the L line, when compared to the H and C line (lower panel).

2-3 kDa reduction in molecular weight (figure 5.5). To ensure that the gel shift was not due to chemical differences in Cy2, a dye-swap was done on subsequent gels, all of which contained the same gel shift (data not shown). Multiple spots in this horizontal band were picked for MS analysis.  MS results match that of the chicken fructose-bisphosphate aldolase B (liver-type aldolase), a 39,670 dalton enzyme involved in fructose metabolism and gluconeogenesis in liver, kidney, and small intestine (Burgess and Penhoet, 1985).

Aldolase is a central enzyme in the conversion of glucose to pyruvate and the production of ATP. Specifically, aldolase B catalyzes the reversible cleavage of the 6-carbon fructose 1,6-bisphosphate to form the two three trios molecules dihydroxyacetone phosphate and glyceraldehyde-3-phosphate (Figure 5.6). Glycolysis is an essential biological process providing energy to growing and dividing cells. Alterations in enzymes involved with this process, including aldolase, could inhibit the ability of cells to obtain energy. For example, hexokinase is the first enzymatic reaction in glycolysis and arabidopsis plants with mutant hexokinase, *gin2,* experience reduced cell expansion in adult plants (Moore et al., 2003). Inhibition of aldolase may be one way that some mammals shut down glycolysis during hybernation (MacDonald and Storey, 2002). Over 21 heritable mutations in aldolase B have been characterized that lead to fructose intolerance in humans, including five deletion mutations (Tolan, 1995). The QE14Lp40i7.1-8.3 mobility shift observed in L line quail could be the result of a deletion mutation or altered posttranslational modifications such as a lack of glycosylation, both of which could reduce or eliminate the catabolic activity of aldolase B. Further experiments need to be carried out to confirm that aldolase B is the protein identified as

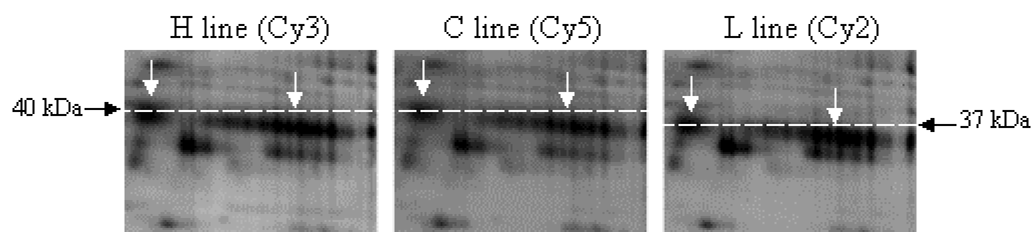


**FIGURE 5.5.** Images captured by DeCyder™ software show QE14Lp40i7.1-8.3 differential expression. In H and C lines, QE14Lp40i7.1-8.3 is a band of 40 kDa proteins with a pIs ranging from 7.1-8.3. In the L line, the proteins migrate as 37 kDa proteins.

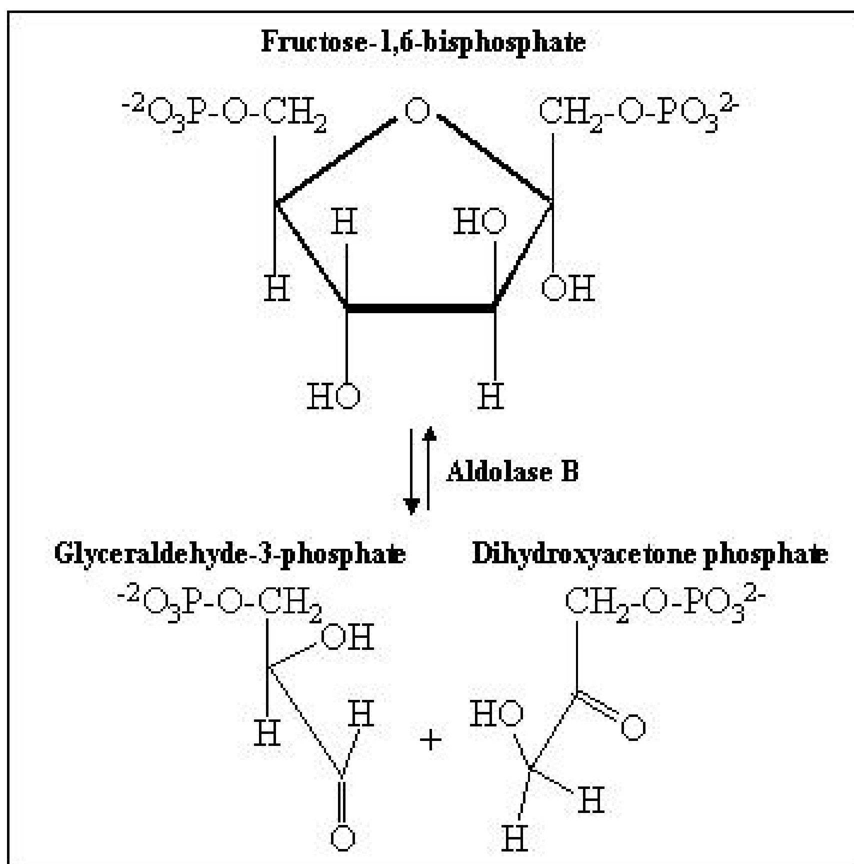


**FIGURE 5.5.** Aldolase B catalyzes a central reaction in the glycolytic pathway. This reaction is the reversible cleavage of fructose-1,6-bisphosphate to form two trios molecules, glyceraldehydes-3-phosphate and dihydroxyacetone phosphate. This is the fourth enzymatic reaction of glycolysis and occurs after glucose has been phosphorylated and converted to fructose-1,6-bisphosphate. The two trioses produced by aldolase are later converted to pyruvate while generating a net of two ATPs.

QE14Lp40i7.1-8.3. Western blot analysis could confirm the identity of aldolase B and the mobility shift in the L line and sequencing of the aldolase B cDNA from the various lines quail lines would identify any possibly deletion mutations in the L line. Detection of differentially glycosylated proteins in 2D gels can also be accomplished (Schulenberg, et al. 2003).

## CONCLUSIONS

We have reported here the results of a limited proteomic approach to identify differentially expressed genes that may be involved in body-weight determination in growth-selected lines of Japanese quail. We have shown that differences in liver protein expression can be detected by 2D-DIGE. One drawback to this method of analysis is the incomplete quail MS and protein databases. This makes identification of differentially expressed proteins much more difficult. To identify these proteins, it may be necessary to design degenerate probes from limited MS data that will allow the amplification of cDNA using PCR, or the screening of a cDNA library. Nevertheless, proteomics can be used to identify differentially expressed proteins from these quail lines and analysis of other tissues and stages of development may be fruitful.

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# CHAPTER 6

# DISCUSSION

Decades of divergent selection for 4-week body weight has yielded lines of Japanese quail with profound differences in size that are a useful model to study issues related to the growth-selected poultry industry. Large P line quail have undergone more than 110 generations of selection and are nearly 3-fold heavier than randomly bred C line control quail. H line quail have undergone 52 generations of selection and weigh twice that of the C line while the small L line quail have undergone 54 generations of selection and weigh less than half that of C line birds (Anthony et al., 1996; Marks, 1996). The quantitative traits that regulate body weight differences in these lines are unknown, and long generation times and lack of molecular markers make genetic mapping of the traits quite difficult.

In this work, several different methods have been utilized in an attempt to identify genetic factors that may contribute to body weight regulation in growth-selected poultry. The identification of myostatin, a negative regulator of muscle mass (McPherron and Lee, 1997a), led us to investigate the sequence and the expression of the myostatin gene in lines of chickens and quail. Mutations in myostatin have led to increased muscle mass in cattle and mice (Grobet et al., 1997; Kambadur et al., 1997; McPherron and Lee, 1997b; Szabo et al., 1998) and there is an inverse relationship between myostatin expression levels and muscle mass (Gonzalez-Cadavid et al., 1998; Ji et al., 1998; Kirk et al., 2000; Wehling et al. 2000; McMahon et al. 2003). Our analysis of the myostatin sequence, mRNA expression, and protein expression in broiler chickens and in the growth-selected quail have shown that myostatin expression had not been altered by intense growth selection in these lines. Furthermore, we showed that myostatin expression was negatively correlated with periods of rapid muscle deposition. For

instance, when muscle fibers are rapidly forming in E14 embryos, there was a moderate level of myostatin mRNA expression in the muscles which was followed by higher levels in E18 embryos which slow their growth due to space limitation of the egg. Furthermore, myostatin levels drop dramatically after hatch as rapid muscle deposition resumes. These results have been confirmed by Guernec et al. (2002).

Other elements in the myostatin pathway may be altered in these lines of quail. As elucidated in model systems such as the mouse and cell culture, analysis of their homologs in poultry should be informative. We attempted to develop a novel technique to identify receptor proteins that bind to ligands of interest with the specific goal of identifying the myostatin receptor(s). Further testing of the receptor trap discussed in Chapter 3 may provide such a tool to find novel growth-factor receptors that bind orphaned ligands. However, when myostatin was shown to act through ActRIIB (Lee and McPherron, 2001) we turned our attention to identifying differentially expressed genes in the lines of growth-selected Japanese quail.

Screening arrayed cDNAs has been a successful method of identifying differentially expressed genes in many different experimental organisms. In an initial experiment, we arrayed 4,704 random anonymous cDNAs from a library from C line E8 whole embryos onto nylon membranes. Membranes were then screened with $^{32}$P-labeled cDNA probes synthesized from P, C, and L line embryos. Three differentially expressed cDNAs were identified from these arrays and subsequently confirmed by Northern blot analysis of total RNA. Interestingly, all three of these cDNAs are expressed at the highest levels in L line and lowest levels in the H and P lines. Two of the cDNAs code for unknown “hypothetical” proteins. The third was an EB1 homolog, known to bind both

the plus ends of microtubules and the APC tumor suppressor protein. The success of our preliminary array experiments suggest that more differentially expressed genes may be identified by screening different tissues and/or stages of development, and by the creation of additional membranes.

The identification of differentially expressed genes using very limited numbers of arrayed cDNAs led us to the utilization of proteomics as a tool to monitor large numbers of expressed genes via the abundance levels of their encoded proteins. Differential CyDye-labeling of protein samples allowed the separation proteins from the H, C, and L lines on a single gradient polyacrylamide gel. Given that differential gene expression was detected in E8 whole embryos using cDNA arrays, we first analyzed E8 whole embryo protein by differential 2D-PAGE; however, no differentially expressed proteins were identified. Therefore, we analyzed individual tissues from E14 embryos to reduce the total number of different proteins present in the sample. Differences were observed in liver. Specifically, four proteins were identified in L line liver samples that have different expression patterns than the H and C lines. Two were expressed at higher levels in the L line, one was repressed in the L line, and a band of proteins displayed a mobility shift, consistent with a deletion mutation or reduced glycosylation. Although the lack of quail protein data in the MS database make identification of protein spots difficult, the band of proteins that underwent the mobility shift was identified as aldolase B. Further work will need to be done to identify and confirm the protein differential expression, such as use of degenerate probes to isolate cDNA that can be sequenced and western blotting.

In conclusion, we have identified multiple cDNAs and proteins through the use of cDNA array and proteomic analysis. These findings suggest that additional cDNA array

and proteomic analysis may lead to the identification of additional genes that may contribute to the regulation of body weight in the growth-selected Japanese quail.

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