**ANNUAL REPORT**

**Regional Research Project NC-131**

**1995**

**PROJECT TITLE:** Factors Regulating Protein Synthesis, Degradation, and Growth in Skeletal Muscle

**PROJECT PERIOD:** 10-1-94 to 9-30-95

**COOPERATING AGENCIES AND PRINCIPAL LEADERS:**

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<tr>
<th>State</th>
<th>Institution</th>
<th>Principal Leader(s)</th>
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<tr>
<td>Alabama</td>
<td>Auburn University</td>
<td>Mulvaney, D.R.</td>
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<td>Arizona</td>
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<td>Goll, D.E. Allen, R.E.</td>
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<tr>
<td>California</td>
<td>University of California at Davis</td>
<td>Bandman, E. Ashmore, C.R.</td>
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<td>Indiana</td>
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<td>Illinois</td>
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<td>Robson, R.M Stomer, M.H.</td>
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<td>Michigan</td>
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<td>Bergen, W.G.</td>
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<td>Hathaway, M.R. White, M.E.</td>
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<tr>
<td>Minnesota</td>
<td>University of Minnesota</td>
<td>Gerrard, D.E.</td>
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<tr>
<td>Missouri</td>
<td>University of Missouri, Columbia</td>
<td>Jones, S.J. Zeece, M.G.</td>
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<tr>
<td>New York</td>
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<tr>
<td>Ohio</td>
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<td>USDA/ARS</td>
<td>Beltville Agricultural Research Center</td>
<td>Czerwinski, S.M</td>
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<tr>
<td>USDA/ARS</td>
<td>Roman L. Hruska U. S. Meat Animal Research Center</td>
<td>Koohmaraie, M.</td>
</tr>
<tr>
<td>Washington</td>
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<td>Dodson, M.V.</td>
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<tr>
<td>Wisconsin</td>
<td>University of Wisconsin, Madison</td>
<td>Greaser, M.L.</td>
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*Denotes voting member of the Technical Committee

**PROGRESS OF THE WORK AND PRINCIPAL ACCOMPLISHMENTS:**

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**Objective 1:** To identify and characterize the molecular mechanisms that regulate the synthesis, assembly and degradation of proteins in skeletal muscle.

In studies on protein synthesis, research at the Michigan Station has focused on development of methods to measure expression of skeletal muscle actin in muscle cell cultures and growing food animals. A specific alpha-actin cDNA probe has been prepared, which hybridizes with the skeletal but not the cardiac actin mRNA in mice, rats, pigs, sheep, and cattle. Investigators at the Indiana Station are examining the regulation of alpha-actin expression in porcine skeletal muscle. A 5.2 kb porcine genomic fragment containing the complete coding region of porcine alpha-actin along with 5′ and 3′ noncoding regions was cloned and sequenced. Transfection of reporter gene constructs containing the alpha-actin promoter into C2C12 myogenic cells demonstrated that the promoter was functional. Additional constructs were used to identify three enhancer-like regions within the porcine alpha-actin gene.

Research at the California Station focused on the biochemistry and molecular biology of the chicken myosin heavy chain multigene family. Monoclonal antibodies to specific sites on the myosin rod have been used to determine the specific regions of the molecule that have important functions in the assembly of myosin into filaments. Two monoclonal antibodies that bind different regions within 150 amino acids of the C-terminus inhibit aggregation in low salt, suggesting that these regions are important in assembly. Studies are in progress on characterizing the assembly properties of expressed myosin LMM regions that contain different mutations. In addition, a 6 kb cDNA encoding the full length adult pectoral muscle myosin heavy chain has been prepared and is being transferred into a baculovirus shuttle vector containing the myosin light chains 1 and 2 in order to express a functional myosin that can be used for further studies on assembly and function.

Investigators at the Wisconsin Station have characterized cDNA sequences for two different regions of the giant myofibrillar protein titin, which is thought to have an important role in assembly and maintenance of the myofibrillar structure. The first region is a 5.4 kb cDNA from a region of rabbit cardiac titin located near the Z-line. The sequence of this cDNA provided additional information about the properties of the repeating domains in this region of the titin molecule. Also, a protein fragment expressed from this cDNA could be phosphorylated in vitro by the proline-directed ERK-1 kinase. The second region is a 2.3 kb region from the kinase domain of rabbit skeletal and cardiac muscle titins. Sequence comparisons of the cDNAs from this second region suggest that there are no major tissue specific differences in this region of titin.

Several studies on other proteins involved in the organization, attachment, and assembly of myofibrils were done at the Iowa Station. First, the specific arginine residues in the muscle intermediate filament (IF) protein desmin that are modified by the muscle arginine-specific ADP-ribosyltransferase were identified, and shown to be in the head region of the molecule. Previous studies demonstrated that ADP-ribosylation blocks the ability of desmin to assemble into 10-nm filaments in vitro. Treatment of modified desmin with the specific hydrolase removed the ADP-ribose and restored assembly properties of the desmin, demonstrating reversibility. Further, desmin was labeled in chick myotube membrane preparations incubated with labeled NAD, suggesting that ADP-ribosylation could modulate desmin assembly in vivo. Second, studies on the actin-attachment site protein talin, which is suggested to play a role in attachment of the muscle cytoskeleton to the cell membrane, demonstrated that the interaction of talin with actin is sensitive to pH and ionic strength, and thus, attachment in the
cell could be modified by the microenvironment at attachment sites. Third, cDNAs coding for the protein synemin were isolated and sequenced. Synemin is colocalized with desmin IFs around Z-disks of myofibrils, where it may function in IF attachment. Sequencing demonstrated that synemin contains the IF protein rod domain, and thus is itself an IF protein. This may provide a mechanism for synemin attachment to desmin IFs.

Researchers at the Ohio Station have characterized extracellular matrix proteins synthesized by sheep skeletal muscles during development. Proteoglycans and collagen from longissimus and biceps femorus muscles from animals of different ages as well as proteoglycans synthesized by primary cultures derived from these muscles were analyzed. Results demonstrate that synthesis of proteoglycans and collagen by skeletal muscle is developmentally regulated. Studies are also in progress on characterization of extracellular matrix development of chicken pectoral muscle from chickens with the Low Score Normal (LSN) genetic weakness. Results of these latter studies suggest that the LSN muscle weakness is associated with changes in characteristics of both collagen and proteoglycans.

In the area of regulation of muscle proteolysis, investigators at the US-MARC unit completed several studies related to calpastatin, the endogenous inhibitor of the calpain proteases. Studies were done to examine the possible relationship between restriction fragment length polymorphisms (RFLPs) at the bovine calpastatin locus with either calpastatin activity and/or meat tenderness. No relationship was found between these RFLPs and meat tenderness, although the relationship between calpastatin activity and tenderness is well-established, suggesting the need for development of other calpastatin-based methods to predict tenderness. Second, an enzyme-linked immunosorbent assay (ELISA) was developed for quantitation of calpastatin in muscle. Third, C2C12 mouse myoblasts were transfected with an inducible vector containing an insert coding for specific regions of calpastatin, thus providing a model system to examine the function of calpastatin and calpains in myoblast development. Finally, cloning and sequencing of the bovine calpastatin gene is in progress.

Investigators at the Arizona Station used protease digestion of purified calpains to obtain information about the structure of the calpain molecules in solution. In the absence of Ca\textsuperscript{2+}, both trypsin and chymotrypsin digestion produced a limited number of fragments, and both cleaved the \(\mu\)- and m-calpain molecules in similar places, providing evidence to suggest that the N-termini of the 80 kDa and 20kDa subunits and the boundaries between domains II and III and between III and IV in the 80 kDa subunit are all located on the surface of the molecule. Digestion in the presence of Ca\textsuperscript{2+} was 5-10 times faster, suggesting that Ca\textsuperscript{2+} causes a substantial opening of the molecule, with only the Ca\textsuperscript{2+} binding domains remaining in a compact conformation. Studies on phosphorylation of the \(\mu\)-calpain molecule are in progress.

At the Nebraska Station, serum from sheep in which acidosis was induced by rumen glucose administration was assayed for effects on muscle cell proliferation and protein turnover in C2C12 myogenic cells in culture. Serum from the acidotic sheep reduced protein turnover, proliferation, and protein synthesis in the cultured cells. Investigators at the Nebraska Station have also developed a capillary electrophoresis method for determination of 3-methylhistidine, which could be used to measure protein turnover in cell cultures.

**Objective 2:** To determine the molecular mechanisms that regulate the proliferation, differentiation
and maturation of cells in muscle tissue.

Investigators at the South Dakota Station are continuing their studies on satellite cell function using clonal cultures of turkey skeletal muscle satellite cells. Early and late developing clones of turkey satellite cells, which differ in the time that they take to become confluent in culture, were isolated from the same muscle from a single animal. Examination of the effect of growth factors on proliferation of the two clones, in a serum-free defined medium, demonstrated that the early clone was more responsive to all growth factors tested, including FGF, IGF-I, PDGF-BB, and insulin. Early clones were also more responsive to the differentiation-suppressing effects of TGF-β. Studies are in progress to examine differences in growth factor receptors. These studies provide new information about differences between populations of satellite cells. In combination with the Missouri Station, studies are in progress to examine IGF expression during differentiation on a single cell basis. Also, the South Dakota and Ohio Stations are collaborating on the study of satellite cells derived from chickens with the LSN genetic muscle weakness Pure populations of satellite cells from LSN birds and appropriate controls have been developed, and characterization of extracellular matrix proteins from the cell cultures is in progress.

Studies on satellite cells are also the focus of work reported by the Washington Station. In studies directed toward development of a defined myogenic and adipofibroblast cell co-culture system, a defined fusion medium for sheep satellite cells has been developed and used to examine differences in IGF expression between different clones, a method has been established for isolation of sheep-derived adipofibroblasts, and co-cultures using 3T3-L1 cells as an adipocyte reference have been characterized. Results demonstrated that sheep satellite cells do not express detectable levels of IGF-I. Characterization of growth factor effects on equine satellite cells demonstrated that FGF and IGF-I stimulated both proliferation and differentiation, TGF-β and GH inhibited both proliferation and differentiation, and EGF, NGF and PDGF had no effect. Finally, the effect of substratum on the growth of trout satellite cells was characterized.

Researchers at the Arizona Station examined the influence of growth factors on expression of myogenic regulatory factors in satellite cells. Initial studies on satellite cells from adult rats showed that the time course of expression followed the previously described pattern in that MyoD is detected first, followed by MRF4 and myf5, followed by myogenin. Labeling with bromodeoxyuridine demonstrated that MRF4 and myf5 are expressed in proliferating cells, as is MyoD. Studies on the influence of various growth factors on myogenin expression demonstrated that hepatocyte growth factor (HGF) is a potent stimulator of satellite cell proliferation and myogenin expression. In cell cultures, HGF selectively affects satellite cells, because fibroblasts do not have HGF receptors. Effects of HGF on satellite cells have not been described, and thus, HGF provides a previously undiscovered mechanism for regulation of satellite cell proliferation.

At the Wisconsin Station, investigators examined effects of in vitro and in vivo irradiation on subsequent growth of turkey satellite cells in vitro. Results demonstrated that a 25 Gy dose does not abolish satellite cell division in the turkey pectoralis.

Researchers at the Nebraska Station have characterized a variety of different media formulations to determine the best media to support growth and differentiation of clonal cultures of bovine fetal muscle cells.
Characterization of the expression of growth factors receptors and growth-factor binding proteins in muscle is also a major part of the work proposed under this objective. At the Missouri Station, investigators have used in situ hybridization to examine expression of the type-I and type-II IGF receptors during muscle development in cattle. During early embryonic development, the type-II receptor was expressed at higher levels in more anterior somites, while expression of the type-I IGF receptor was dispersed equally throughout the embryo.

Researchers at the Illinois Station are examining the function of the IGF binding proteins (IGFBPs) in muscle development. These investigators have characterized the effect of zinc on the binding of IGF-I and II to IGFBP-3 and 5. Zinc lowers binding affinity of the IGFs for these BPs, suggesting that zinc may modulate IGF activity. In separate studies, muscle cell lines stably transfected to over-expression of porcine IGFBP-1 or 5 showed an increase in confluent cell density but not doubling times. Studies using muscle cell lines to examine regulation of IGFBP expression demonstrated that agents that alter intracellular cAMP modulate IGFBP secretion.

Investigators at the Minnesota Station have isolated and sequenced a cDNA clone coding for porcine IGFBP-5, and at the Illinois Station, a cDNA clone for bovine IGFBP-5 has been isolated. These cDNAs provide useful tools for analysis of IGF binding protein expression in porcine or bovine cells and tissues.

Objective 3: To characterize the effects and mode of action of hormones and growth factors on growth of muscle tissue.

Studies under this objective focus on understanding the effects of hormones, growth factors, and other growth promotants on overall muscle growth, and includes studies designed to examine mechanisms regulating muscle growth in whole animals. Researchers at the Minnesota Station examined possible mechanisms for the effects of a combined trenbolone acetate and estradiol implant on growth of feedlot steers. Implants improved feedlot performance and stimulated carcass protein accretion, and increased serum concentrations of both IGF-I and IGF-II. Implantation also increased the mitogenic activity of the sera when assayed using a muscle satellite cell culture system. The growth factor responsiveness of satellite cells isolated from the semiimembranosus muscle of implanted and control steers was also examined. A combination of IGF-I and bFGF resulted in a 19% increase in proliferation of satellite cell cultures from the implanted steers over cultures from control steers. In combination, these alterations may be partially responsible for the positive effects of the implants. A separate study at the Minnesota Station demonstrated a 24.8% increase in serum IGF-I concentration and a 59% increase in serum IGFBP-3 concentration, in comparison to controls, in pigs fed an antimicrobial (ASP-250). This increased IGF-I concentration may be involved in the enhanced growth observed in response to antimicrobials.

Investigators at the US-MARC conducted a comprehensive characterization of the effects of the callipyge gene in sheep on indices of muscle growth and meat tenderness. Results demonstrated that callipyge generally increased muscle growth and decreased fat, but did not affect carcass or organ weights. Activities of m-calpain and calpastatin, but not µ-calpain, were higher in muscle from callipyge animals. Calpastatin activity was not altered in liver, lung, brain and kidney, and, in muscle, the increase in activity was generally proportional to the effect of callipyge on muscle weight. Callipyge also negatively affected meat tenderness. These results suggest that the affects of callipyge
on muscle growth are due to decreased proteolysis, mediated at least in part by calpastatin.

At the Illinois Station, IGF-I, IGF-II and IGFBP levels were quantitated in serum of normal and callipyge sheep. Results showed little difference between control and callipyge animals during growth, suggesting that serum levels of the components of the IGF system are not involved in the development of the muscle hypertrophy characteristic of the callipyge sheep.

Investigators at the Alabama Station reported continued progress on characterization of a porcine model utilizing maternal treatment with porcine somatotropin (pST) during early gestation to alter muscle development in embryos. Maternal treatment during the gestational window of day 28-40 resulted in altered patterns of expression of myogenic regulatory genes and increases in embryo number, weight, and size. Treatment of gilts from day 15-30 of gestation demonstrated a greater improvement in progeny carcass traits. These results support the hypothesis that embryonic development is sensitive to manipulation of the sow, and this system provides a model for examination of events controlling early embryonic growth.

Researchers at the New York Station examined the effects of abomasal casein infusion on nitrogen balance, skeletal muscle IGF-I mRNA abundance, and circulating IGF-I in growing steers. Results demonstrated an increase in serum IGF and an increase in muscle IGF-I mRNA that reached a maximum 7 days after infusion and declined to control values after 14 days. These results suggest that enhanced amino acid availability may modulate skeletal muscle protein synthesis through a paracrine or autocrine IGF-I response. In a separate study, it was shown that supplementation of the diet of growing lambs with fishmeal increased N balance and efficiency of N use for growth.

USEFULNESS OF FINDINGS:

Results of the research done during the past year by the NC-131 Committee have provided substantial new, fundamental information about the mechanisms regulating protein synthesis, degradation and growth in skeletal muscle. The studies summarized herein have contributed significantly to our level of knowledge in several areas. Examples include: characterization of the role of proteolysis and the calpain/calpastatin system in regulation of muscle growth; examination of the mechanisms for the organization and assembly of muscle proteins; analysis of the factors that control initiation, proliferation, and differentiation of satellite cells; and characterization of the function and regulation of the IGFs in relation to control of muscle cell proliferation and differentiation. In addition, a number of novel and important experimental tools and model systems have been developed for future studies needed to determine the molecular mechanisms that regulate muscle growth and differentiation, including a number of specific cDNA probes, various assay methods, and several important satellite cell culture models. Development of these new tools is necessary for further research in this area, and these various methods and reagents will be widely used by numerous investigators in this field, as well as by the NC-131 Committee for the next five-year project. Overall, the NC-131 Project has continued to provide important basic information about the control of muscle differentiation and growth, particularly in meat animal species. A basic understanding of the molecular mechanisms that regulate muscle growth is clearly necessary for the future design of methods for improving the efficiency of muscle growth in meat animals.

WORK PLANNED FOR NEXT YEAR:
This is the final year for this five year NC-131 project (1990-1995). Plans for the next year are described in the revised proposal that has been approved for the period Oct. 1, 1995 - Sept. 30, 2000.

**PUBLICATIONS FOR THE YEAR:**

**Refereed Journal Papers**

**Published Full-Length Articles (40):**

**Arizona**


**California**


**Illinois**


**Indiana**


**Iowa**


**Minnesota**


**New York**


**Ohio**

growth of meat animals. *Basic Appl. Myology* 5:81-86.


**South Dakota**


**USDA - Clay Center**


Shackelford, S.D., T.L. Wheeler and M. Koohmaraie. 1995. Relationship between shear force and


**Washington**


**Wisconsin**


Published Abstracts (32):

California


Illinois


Indiana


Iowa


Ho, C.-Y., M.H. Stromer, M.S. Mayes and R.M. Robson. 1995. Electrical stimulation of bovine


**Minnesota**


**Missouri**


**New York**


**Ohio**


**South Dakota**


Yong, Y., D.C. McFarland, J.E. Pesall and K.K. Gilkerson. Variation in response to growth factor stimuli in satellite cell populations. (Submitted).

**Washington**


**Wisconsin**


Accepted Full-Length Articles (19):

Illinois


Minnesota


**Missouri**


**Nebraska**


**Ohio**


**South Dakota**


**Washington**


**Submitted Full-Length Articles** (14):
Illinois


Indiana


Missouri


Nebraska


South Dakota

Dodson, M.V., D.C. McFarland, A.L. Grant, M.E. Doumit and S.G. Velleman. Invited review:
Extrinsic regulation of domestic animal-derived satellite cells. (Submitted).

Ernst, C.W., D.C. McFarland and M.E. White. Expression of insulin-like growth factor II (IGF-II), IGF binding protein-2 and myogenin during differentiation of myogenic satellite cells derived from the turkey. (Submitted).

Washington


Non-Refereed Publications

Proceedings, Book Chapters, etc. (25):

Arizona


California


Iowa


Nebraska


New York


Ohio


UK, p. 188-189.


USDA - Clay Center


Washington


Wisconsin


Ph.D. Theses (2):

Iowa


New York

APPROVED:

Dr. Ted W. Huiatt, Chair of Technical Committee, 1995-96 Date

Dr. Robert Steele, Administrative Adviser Date