RESEARCHREPORT

ANIMAL SCIENCE

pork checkoff

Title: Identification of quantitative trait nucleotides (QTN) for pork tenderness – **NPB #08-105**

Investigator: Dan Nonneman

Institution: USDA/ARS, U.S. Meat Animal Research Center, Clay Center, NE

Date Submitted: June 15, 2009

Industry Summary

The identification of predictive DNA markers for pork quality would allow U.S. pork producers and breeders to more quickly and efficiently select genetically superior animals for production of consistent, high quality meat. Improvement in the genetic potential of swine populations to efficiently convert feed into lean, palatable pork will provide long-term economic returns to the producer. By predicting cooked meat tenderness genetic potential, such technology could be used by packers to ensure product quality and more efficiently determine optimal use of pork. The objectives of this proposal were to identify the causative sequence variation in calpastatin that likely affects tenderness in commercial-level pig populations and to develop definitive DNA markers that are predictive of pork tenderness for use in marker assisted selection programs. Because the calpastatin gene lies under QTL for tenderness in different resource populations and markers in calpastatin are highly associated with tenderness traits, we resequenced the calpastatin gene in pigs with divergently extreme shear force values in order to identify all possible mutations that could affect tenderness. We tested these polymorphisms in our research population and samples of industry pigs for association with objective measures of tenderness. From this study we have identified 5 genetic markers that were highly associated with pork tenderness in all of the populations studied representing 2,826 pigs from 4 distinct populations. These markers should be predictive of pork tenderness in industry populations.

Scientific Abstract

Genome scans have identified QTL for tenderness on pig chromosome 2. The QTL have been fine-mapped to the calpastatin locus. In this study we genotyped one hundred thirty polymorphisms in promoter regions and evaluated these with previous markers and changes in amino acids. We identified five SNPs that were consistently highly associated with tenderness measures across four different populations of industry-relevant pigs. Five polymorphic sites demonstrated binding to nuclear extracts in electrophoretic mobility shift assays and two of these were allele-specific in their ability to bind nuclear proteins. At the polymorphic sites that bound protein, the SNPs were not in linkage disequilibrium with each other and may independently affect calpastatin expression.

These research results were submitted in fulfillment of checkoff-funded research projects. This report is published directly as submitted by the project's principal investigator. This report has not been peer-reviewed.

For more information contact:

National Pork Board • PO Box 9114 • Des Moines, IA 50306 USA • 800-456-7675 • Fax: 515-223-2646 • pork.org

Introduction

Pork tenderness is a highly heritable trait (Suzuki et al., 2005) and several QTL have been identified (Edwards et al., 2007; Rohrer et al., 2006; Malek et al., 2001). One QTL in particular, found in at least three different commercial-type pig populations resides on chromosome 2 positioned over the calpastatin gene (Rohrer et al., 2006; Meyers et al., 2007; Stearns et al., 2005, Meyers and Beever, 2008). Calpastatin is the specific endogenous inhibitor of calpains, calcium-dependent proteases responsible for postmortem tenderization of meat (Koohmaraie, 1992). Linkage and haplotype association analyses (Meyers et al., 2007, Ciobanu et al., 2004) provide further evidence that calpastatin is a likely candidate affecting tenderness in this region. We also have analyzed SNPs flanking this region and in the calpastatin gene in Duroc-Landrace and Duroc-Landrace-Large White populations and found that markers in the calpastatin gene were more significantly associated with slice shear force than the flanking markers (Lindholm-Perry et al., 2009). However, the significance of markers in calpastatin differed by marker and population (Rohrer et al., 2007). Although we have found markers that are predictive of shear force in these populations, identifying the causative mutation in calpastatin would provide a definitive marker for tenderness in all U.S. swine populations.

Objectives

1. Identify single nucleotide polymorphisms (SNP) in the calpastatin gene by sequencing divergently selected boars in a phenotyped Duroc-Landrace-Large White composite population. We have found several SNPs in calpastatin, some of which code for different amino acids that are significantly associated with trained sensory panel tenderness ratings and slice shear force in different research populations. However, because the most significant SNP differ among populations it seems these SNPs are not in complete linkage disequilibrium with the causative mutation(s). Sequencing boars with the most extreme phenotypes should allow us to identify the genetic variation(s) responsible for the variation in tenderness.
2. Evaluate identified single nucleotide polymorphisms for associations with slice shear force in a large group of animals from the phenotyped composite population.
3. Verify SNP variation and validate SNP effects in commercial-level pigs obtained from the industry.

Materials and Methods

USMARC Resource Population

The phenotyped animals were from a multigenerational population developed at the U.S. Meat Animal Research Center (USMARC) by mating Yorkshire-Landrace composite females to either Duroc or Landrace boars selected from the industry. Twelve boars of each breed were randomly assigned a sire code and mated to Yorkshire-Landrace females (n=220). This population (2001-born animals) was further developed by mating female descendants (n=10/boar) to either a Duroc or Landrace-sired boar; Duroc-sired animals were mated to Landrace-sired animals. Subsequent matings were random except that matings of the same sire line code were avoided. Animals phenotyped from this population were selected to represent all of the sires being used in the current generation. The phenotyped animals included 227 2001-born barrows, 532 2005-born gilts, 120 2006-born barrows and 120 2008-born barrows and gilts (n=999). In addition, 163 barrow and gilts were harvested from an experiment using females from this population and mated to terminal cross sires (n=3). All animals were harvested at the USMARC abattoir and phenotypes were collected as described in Rohrer et al., 2006.

The longissimus was removed from pork carcasses and frozen at $^{-}20$°C after aging. Tenderness as slice shear force was assessed at either day 7 or 14 postmortem according to Shackelford et al. (2004). Briefly, chops were thawed until an internal temperature of 5°C was reached. Belt grill cooking was conducted with a Magigrill (model TBG-60; MagiKitch'n Inc., Quakertown, PA). Belt grill settings (top heat = 163°C, bottom heat = 163°C, preheat = 149°C, gap between platens = 2.16 cm, and cook time = 5.8 min) were designed to achieve a final internal temperature of 71°C for 2.54-cm-thick longissimus chops. Immediately after cooking, a

1-cm-thick, 5-cm-long slice was removed from each chop parallel to the muscle fibers. The slice was acquired by first cutting across the width of the longissimus at a point approximately 2 cm from the lateral end of the muscle. Using a sample sizer, a cut was made across the longissimus parallel to the first cut at a distance 5 cm from the first cut. Using a knife that consisted of two parallel blades spaced 1 cm apart, two parallel cuts were simultaneously made through the length of the 5-cm-long chop portion at a 45° angle to the long axis of the longissimus and parallel with the muscle fibers. Each sample was sheared once with a flat, blunt-end blade using an electronic testing machine (model 4411; Instron Corp.). The crosshead speed was set at 500 mm/min. Table 1 is a summary of the slice shear force values collected including the mean values, ranges and standard deviation for each group of animals in the population used in this study.

Industry F2 Population
This population was composed of fifty F2 Duroc-Landrace litters produced from 4 F1 boars and 50 F1 sows (Rohrer et al., 2006). A total of 370 animals phenotyped for slice shear force on days 2 and 7 postmortem were included in this study.

OSU National Pork Board Industry Samples
A total of 905 loin samples were obtained from 3 different plants. A subset of loins (n=227) were enhanced to improve tenderness. Samples were selected to represent the range of color, pH and marbling. At day 7 postmortem, chops were cooked at 4 different temperatures (145, 155, 165 and 175° C) and Warner-Bratzler Shear force (WBSF) was measured.

Babcock Genetics Industry Samples
Samples obtained from Babcock Genetics from 3 different lines were evaluated for pork quality by Dr. Ken Prusa, at Iowa State University. Chops (total of 535) were aged for 10 days and instrumental tenderness was measured using an Instron Star probe.

Identification of Single Nucleotide Polymorphisms
Primer pairs for amplification of calpastatin from genomic DNA were designed from porcine sequences obtained from BAC subclone sequences and those deposited in GenBank (Accession number EU137105) using Primer 3 (Rozen and Skaletsky, 2000) (code available at http://www-genome.wi.mit.edu/genome_software/other/primer3.html). Primer sets were obtained from IDT (Integrated DNA Technologies, Coralville, IA). PCR was performed in a PTC-225 DNA engine (MJ Research Inc., Watertown, MA) using 0.5 U Hot Star *Taq* polymerase (Qiagen, Valencia, CA); 1x supplied buffer; 1.5 mM $MgCl_2$; 200 µM dNTPs; 0.8 µM each primer; and 100 ng genomic DNA of eight animals representing different sire lines in 25 µl reactions. Five microliters of the PCR reaction was electrophoresed in 1.5% agarose gels to determine quality of amplification, and the remainder was prepared for sequencing after treatment with 0.1 U exonuclease I (USB, Cleveland, OH). Sequencing reactions were precipitated with 58% isopropanol and sequenced with an ABI 3730 capillary sequencer (Applied Biosystems, Foster City, CA). Bases were called with Phred and assembled into contigs with Phrap. Polymorphisms were identified using Polyphred and assessed using Consed (http://www.phrap.org).

SNP Genotyping
Multiplex assays for use in the Sequenom MASSARRAY® system were designed using MASSARRAY® Assay Design software (Sequenom, San Diego, CA). Assays were designed for approximately 30 SNP per multiplex group. Each amplification primer had a 10-base tag added to ensure that the amplification primer masses were outside the range of the allele masses. Amplicon lengths were 90-120 bp. Reaction conditions were performed as suggested by Sequenom iPLEX chemistry.

Association Analyses

The data were analyzed separately for each population. Pedigreed populations were analyzed using the QTL Association option of Mendel v8.0.1 (Lange et al., 2001) which uses full pedigree. The statistical model included harvest date and line as fixed effects.

The OSU National Pork Board Industry Samples were analyzed with PROC MIXED of SAS 9.2 (SAS Institute, Cary, NC). Loin was treated as a random effect, regression coefficients for final cooked temperature and cooking time were fitted along with the fixed effects of processing plant and enhancement treatment.

Linkage Disequilibrium (LD) Analysis
Linkage disequilibrium (r^2) was estimated for the SNP using Haploview 4.0 software (Barrett et al; 2005; http://www.broad.mit.edu/mpg/haploview/index.php). Haplotype blocks were based on pairwise LD values.

Identification of transcription factor binding sites
Transcription factor binding sites were identified in the sequence using Cister (Frith et al., 2001) and the cis-element matrices from TRANSFAC (Wingender et al., 2000).

Nuclear Extracts
Nuclear extracts from pig skeletal muscle and heart were prepared using reagents from the NE-PER extraction system (Pierce, Rockford, IL). The nuclear extract was aliquotted at a concentration of 5-8µg/µl and frozen at -80°C until use.

Polymorphic Oligonucleotide Duplexes
For each polymorphic site, allele-specific oligonucleotides and the complementary strand of 25 bp or greater were synthesized (IDT) with the polymorphic base in the center flanked by 12 bp. If the polymorphic site was an indel, the insertion was centered in the oligo. Oligos were labeled with biotin using a 3'-end DNA labeling kit (Pierce, Rockford, IL). Equal amounts of oligos and complementary oligos (0.1µM) were annealed by heating in a boiling water bath and allowed to cool to room temperature over 1 hour. Annealed duplex oligonucleotides were stored at -20°C until use.

Electrophoretic Mobility Shift Assays
Electrophoretic mobility shift assays (EMSA) were performed using the Lightshift Chemiluminescent EMSA kit (Pierce). Binding reactions contained 100 fmol of labeled probes, 0.25-1 µg of poly (dI-dC) and 10-12 µg of nuclear extract in the supplied buffer. Reactions were preincubated at room temperature for 10 minutes before the addition of probe and incubated for 30 minutes on ice. Reactions were loaded on 5% native polyacrylamide gels in 0.5X Tris borate/EDTA buffer and electrophoresed on ice at 100V for one hour. The gel products were transferred to positively charged nylon membranes at 380mA for 30 minutes with cold 0.5X Tris borate/EDTA buffer. The membranes cross-linked at 120mJ/cm^2 (Stratagene, La Jolla, CA) before biotin detection by chemiluminescence. The membranes were exposed to a Chemimager (AlphaInnotech, San Leandro, CA) for band detection.

Results

Animal Populations
The number of animals with slice shear force, Warner-Bratzler shear force and Instron star probe measurements collected from four different populations are shown in Table 1. A total of 2,826 phenotyped animals in four populations were used in this study.

Resequencing and SNP Discovery
We sequenced about one third of the calpastatin gene (over 38,000 base pair) in twelve animals representing the most tender and tough slice shear force values. These animals also differ in their genotypes at the most predictive markers for slice shear force (Lindholm-Perry et al., 2009; Figure 1). This sequence includes the complete coding region and all the predicted mRNA splice sites and regulatory (promoter) regions. One hundred ninety-four single nucleotide polymorphisms (SNP) were identified in this sequence. These SNP were targeted for genotyping on the Sequenom MassArray system.

Promoter Predictions
Two hundred twelve transcription factor binding sites were identified in the calpastatin sequence (Figure 2). These sites tended to be clustered in the previously reported promoter regions (Meyers and Beever, 2008). Thirty-one of these SNP were found in transcription factor binding sites and alternate alleles are predicted to alter or obliterate transcription factor binding.

Genotyping and Association
Assays were designed for 184 SNPs. About 10 SNP were intractable due to the repetitive nature and their position in the sequence; these genotypes could be inferred from other SNPs in the research population. Additionally, two deletions were genotyped by fragment size on agarose and polyacrylamide gels. Of these SNPs, 18 were monomorphic (sequence artifacts), 6 were heterozygous (sequence differences in repetitive elements) and 34 would not amplify or give reliable genotypes. One hundred thirty markers were analyzed in the USMARC Resource Population (Figure 3). Thirty-seven markers were significant ($p<0.01$) in this population. These markers and three others that were previously significant in the resource population were assayed in the other three populations (Table 2). Six of these markers were highly significant in all four populations (67831_429-430, 66602_392, 67853_270, 67855_230, 67855_289 and 77013_98, Table 2). The linkage disequilibrium patterns were similar in the different populations and several of the significant SNPs were in high LD with each other (Figure 4). The allele frequencies are shown in Table 3. These SNPs were more significantly associated with shear force and Instron force than three SNPs that change amino acids (41646_874, Ser/Asn; 41650_892, Lys/Arg; and M20160_638, Ser/Arg) reported previously (Ciobanu et al., 2004).

Electrophoretic Mobility Shift Assays
Electrophoretic mobility shift assays were designed for sixteen polymorphic sites, including the six SNPs that were the most significant in all populations (Table 4). Five sites demonstrated a gel shift when probes were incubated with nuclear extract from either muscle or heart. Two of these sites, a Sp1 site around nucleotides 12979 and 12980, and Mef-2/TATA sites with SNPs at positions 49223 and 49228 were allele-specific in binding nuclear proteins (Table 4 and Figures 5-8). Competition of nuclear protein binding could be demonstrated using 100-fold excess of the unlabeled allele-specific probe (Figure 5 and Figure 8).

Discussion

This study confirms the presence of a QTL for pork tenderness on chromosome 2 in other populations of industry-relevant pigs. QTL for tenderness had previously been found on SSC2q near the centromere (Stearns et al., 2005; Meyers et al., 2007; Rohrer et al., 2006). Rohrer et al. (2006) identified QTL in a Duroc-Landrace population for slice shear force at day 7 and day 2 postmortem at 60 (near the centromere) and 79 cM, respectively, with calpastatin mapping under the distal QTL. Meyers et al. (2007), showed evidence for two QTL for tenderness in a Berkshire-Duroc cross with one QTL being positioned at calpastatin and the other located more distal. The most significant associations were found within the calpastatin gene (Meyers et al., 2007; Meyers and Beever, 2008; Lindholm-Perry et al., 2009) and associations with several markers suggested the causative mutation(s) were regulatory. In this study we genotyped one hundred thirty polymorphisms in promoter regions and evaluated these with previous

markers and changes in amino acids. We identified five SNPs that were consistently more significant across four different populations of industry-relevant pigs. Five polymorphic sites demonstrated binding to nuclear extracts and two of these were allele-specific in their ability to bind nuclear proteins. The SNPs at the sites that bound nuclear proteins were not in high linkage disequilibrium with each other and may independently affect calpastatin expression.

Acknowledgements

Materials identified as OSU-NPB samples were derived from pork *longissimus thoracis* samples representing National Pork Board funded research project numbers 06-139 and 07-005 entitled 'Effects of Pork Quality and Cooked Temperature on Consumer and Trained Sensory Perception of Eating Quality in Non-enhanced and Enhanced Pork Loins'. Projects 06-139 and 07-005 were managed through The Ohio State University Research Foundation, 1960 Kenny Road, Columbus, OH 43210-1063 with subcontract awards with the Texas Agricultural Experiment Station, 2147 TAMSU, College Station, TX 77843, the Iowa State University Office of Research Assurances, 1138 Pearson Hall, Ames, IA, 50011, and the USDA, ARS, Northern Plains Area Natural Resources Research Center, 2150 Center Ave., Bldg. D, Suite 300
Fort Collins, CO 80526-8119. Principal investigators for projects 06-139 and 07-005 were Drs. Steven J. Moeller (The Ohio State University), Rhonda K. Miller (Texas A&M University) and Henry Zerby (The Ohio State University).

We also thank Babcock Genetics for providing DNA and tenderness data for evaluation in this study.

References

1. Suzuki, K., Irie, M., Kadowaki, H., Shibata, T., Kumagai, M., Nishida, A. 2005. Genetic parameter estimates of meat quality traits in Duroc pigs selected for average daily gain, longissimus muscle area, backfat thickness, and intramuscular fat content. J. Anim. Sci. 83:2058-65.

2. Edwards, D.B., Ernst, C.W., Raney, N.E., Doumit, M.E., Hoge, M.D., Bates, R.O. 2008. QTL mapping in an F2 Duroc x Pietrain resource population: II. Carcass and meat quality traits. J. Anim. Sci. 86:254-66.

3. Rohrer, G.A., Thallman, R.M., Shackelford, S., Wheeler, T., Koohmaraie, M. 2006. A genome scan for loci affecting pork quality in a Duroc-Landrace F population. Anim. Genet. 37:17-27.

4. Stearns, T.M., Beever, J.E., Southey, B.R., Ellis, M., McKeith, F.K., Rodriguez-Zas, S.L. 2005. Evaluation of approaches to detect quantitative trait loci for growth, carcass, and meat quality on swine chromosomes 2, 6, 13, and 18. II. Multivariate and principal component analyses. J. Anim. Sci. 83:2471-81.

5. Malek, M., Dekkers, J.C., Lee, H.K., Baas, T.J., Prusa K., Huff-Lonergan, E., Rothschild, M.F. 2001. A molecular genome scan analysis to identify chromosomal regions influencing economic traits in the pig. II. Meat and muscle composition. Mamm. Genome. 12:637-45.

6. Meyers, S.N., Rodriguez-Zas, S.L., Beever, J.E. 2007. Fine-mapping of a QTL influencing pork tenderness on porcine chromosome 2. BMC Genet. 8:69.

7. Meyers, S.N. and Beever, J.E. 2008. Investigating the genetic basis of pork tenderness: genomic analysis of porcine *CAST*. Anim. Genet. 39:531-43.

8. Koohmaraie, M. 1992. The role of Ca(2+)-dependent proteases (calpains) in post mortem proteolysis and meat tenderness. Biochimie. 74:239-45.

9. Ciobanu, D.C., Bastiaansen, J.W., Lonergan, S.M., Thomsen, H., Dekkers, J.C., Plastow, G.S., Rothschild, M.F. 2004. New alleles in calpastatin gene are associated with meat quality traits in pigs. J. Anim. Sci. 82:2829-39.

10. Lindholm-Perry A.K., Rohrer G.A., Holl J.W., Shackelford S.D., Wheeler T.L., Koohmaraie M., Nonneman D. Relationships among calpastatin single nucleotide polymorphisms,calpastatin expression and tenderness in pork longissimus. Anim Genet. 2009 Apr

11. Rohrer, G.A., Holl, J., Perry A., Nonneman, D., Shackelford, S.D., Wheeler, T., Koohmaraie, M. 2007. Update on genetic markers for pork quality. National Swine Improvement Federation Conference. Kansas City, MO.

12. Lange, K., Cantor R., Horvath S., Perola M., Sabatti C., Sinsheimer J., Sobel E. (2001) Mendel Version 4.0: A complete package for the exact genetic analysis of discrete traits in pedigree and population data sets. American Journal of Human Genetics **69** (Supplement), A1886.

13. Frith, M. C., Hansen U. and Weng, Z. 2001. Detection of cis-element clusters in higher eukaryotic DNA Bioinformatics 17:878-889.

14. Wingender, E., Chen, X., Hehl, R., Karas, H., Liebich, I., Matys, V., Meinhardt, T., Pruss, M., Reuter, I. and Schacherer, F. 2000. TRANSFAC: an integrated system for gene expression regulationNucleic Acids Res. 28: 316-319.

Table 1. Animals and phenotypes used for SNP associations.

Population	Number of Animals[1]	Phenotype[2]	Mean ±S.E.	Range
USMARC	1042	Slice Shear Force	13.47 ± 3.61	6.4 – 31.9
Duroc-Landrace F2	370	Slice Shear Force	14.39 ± 4.72	6.79 – 33.79
OSU-NPB	905	WBSF	2.47 ± 0.85	0.88 – 7.02
Babcock	509	Starprobe	5.71 ± 1.02	3.7 – 10.54

[1] Animals with phenotypes available.
[2] Slice shear force (SSF), Warner-Bratzler Shear Force (WBSF) were measured in kilograms at 7 days postmortem. Instron Starprobe was measured in kilograms at 10 days postmortem.

Table 2. Marker associations in research and industry populations.

Marker	Position	+ Allele (- Allele)	USMARC + Effect	USMARC P-Value	Duroc-Landrace F2 + Effect	Duroc-Landrace F2 P-Value	OSU-NPB + Effect	OSU-NPB P-Value	Babcock + Effect	Babcock P-Value
67831_429	12979	A (G)	0.398	0.00015			0.1311	0.00042	0.0242	0.69649
67831_430	12980	G (T)	0.4553	0.00002	0.1077	0.05439	0.0797	0.00153	0.3576	0.03386
67831_623	13173	G (A)	0.4239	0.00015	0.1335	0.01956	0.0867	0.00059	0.0345	0.43468
71530_499	13830	A (C)	0.4067	0.00027	0.1073	0.04636	0.0794	0.00138	0.0363	0.54577
71532_367	14479	T (C)	0.3921	0.00003	0.0655	0.24242	0.0850	0.00019	0.1247	0.00275
67839_239	24162	T (C)	0.3719	0.00060	0.0377	0.40598	0.0565	0.00538	0.0239	0.67424
67839_249	24172	A (G)	0.2963	0.00086	0.0809	0.14024	0.0553	0.01453	0.1014	0.01531
67849_578	39290	G (A)	0.3271	0.00027	0.0737	0.12190	0.0492	0.0143	0.0745	0.04236
77005_145	45241	T (G)	0.3841	0.00380	0.1156	0.04996	0.0838	0.00099	0.0960	0.02455
67851_470	45329	T (C)	0.4084	0.00004	0.0772	0.16334	0.0990	0.00036	0.2629	0.00029
66602_392	48191	C (A)	0.3133	0.00098	0.1634	0.00161	0.1062	0.0000018	0.0624	0.09248
67853_270	48309	A (G)	0.3733	0.00012	0.1690	0.00107	0.1076	0.0000015	0.1960	0.000004
67853_459	48499	G (C)	0.3354	0.00317	0.0606	0.25933	0.0944	0.02885	0.1093	0.08376
67855_127	48596	T (C)	0.4510	0.00001	0.0755	0.17719	0.0948	0.00026	0.1275	0.00156
67855_230	48699	G (T)	0.3317	0.00052	0.1842	0.00036	0.1057	0.0000029	0.1886	0.00001
67855_289	48759	G (A)	0.3853	0.00307	0.1668	0.00122	0.1057	0.000002	0.0957	0.01335
67857_306	49223	G (A)	0.2042	0.00426	0.1187	0.01307	0.0487	0.01528	0.0057	0.89283
77013_98	49228	G (A)	0.3909	0.00222	0.1653	0.00079	0.0948	0.000002	0.1023	0.00883
67859_67	49450	G (A)	0.2124	0.00753	0.1168	0.01618	0.0060	0.79554	0.0627	0.31418
70000_338	49478	G (T)	0.3116	0.00411	0.1107	0.02384	0.0490	0.03530	0.0168	0.62237
67859_255	49480	T (G)	0.4156	0.00016	0.1188	0.04106	0.1161	0.00151	0.0725	0.25550
67859_353	49636	T (G)	0.3465	0.00015	0.1184	0.01485	0.057	0.00702	0.0005	0.99100
70000_689	49734	A (G)	0.3788	0.00001	0.0896	0.06471	0.0817	0.00006	0.0775	0.02624
67861_456	49830	G (C)	0.4177	0.00122	0.0674	0.15847	0.0724	0.00049	0.0422	0.26109
67865_149	50284	T (C)	0.4309	0.00004	0.0931	0.10878	0.0852	0.00074	0.0832	0.04893
77015_228	57827	C (G)	0.3729	0.00075	0.1067	0.02830	0.0612	0.00327	0.0019	0.96307
41642_192	72735	T (C)	0.4412	0.00010	0.1268	0.03093	0.0877	0.00058	0.0080	0.89023
41642_408	79518	G (A)	0.3937	0.00043	0.1034	0.08489	0.0949	0.0002	0.0103	0.86393
41646_595	79734	T (C)					0.1068	0.00004		
41646_874	82232	G (A)	0.3536	0.00006	0.1067	0.02834	0.0701	0.00039	0.0455	0.30552
69886_124	84561	G (T)	0.3692	0.00002	0.1041	0.03302	0.0479	0.01443	0.0523	0.20031
69886_260	84699	C (T)	0.3436	0.00006	0.1230	0.01319	0.0407	0.03775	0.0027	0.94848
69890_414	91532	A (G)	0.4155	0.000001	0.1076	0.0274	0.0577	0.00332	0.0438	0.22084
41650_892	91885	G (A)	0.5024	0.00002	0.1040	0.04478	0.0675	0.00098	0.0437	0.34351
41650_975	91968	T (C)	0.5019	0.00090	0.1279	0.03132	0.0925	0.00044	0.0817	0.16994
41658_290	104138	T (C)	0.4445	0.00001	0.0773	0.16341	0.0974	0.00023	0.1559	0.00147
41658_293	104141	T (C)	0.3466	0.00054	0.0798	0.19501	0.0913	0.00025	0.0161	0.78020
23795_1	104200	C (T)	0.1208	0.17395	0.1078	0.00472	0.0663	0.00096		
69894_322	111801	A (D)	0.4445	0.00041	0.1156	0.04996	0.0411	0.18573	0.0420	0.21578
M0160_638	114652	C (A)			0.0751	0.24811	0.0089	0.69387		

[1]USMARC marker number refers to forward sequencing primer followed by position in contig.
[2]Position in GenBank Accession number EU137105.

[3]The allele with a positive effect on shear force is shown and the allele with the negative effect is in parentheses. The most significant markers are highlighted in yellow.
[4]Estimated additive effect of positive allele in kilograms.
Empty cells imply the marker was not genotyped or analyzed.

Table 3. Allele frequencies of significant SNPs in different populations.

Marker	Positive Allele[1]	USMARC	F2	OSU-NPB
67831_429/430	A/G	0.195	0.220	0.192
67851_470	T	0.217	0.292	0.182
66602_392	C	0.641	0.894	0.644
67853_270	A	0.709	0.892	0.641
67855_230	G	0.715	0.882	0.639
67855_289	G	0.727	0.893	0.648
67857_306	G	0.506	0.310	0.392
77013_98	G	0.724	0.842	0.639

[1]The positive allele is associated with greater instrumental force.

Table 4. Predicted polymorphic transcription factor binding sites.

Marker	Position	TF Binding Site	Gel Shift	Allele-specific
77003_101-116	5661	NF-1, Sp1	No	No
67831_429	12979	Sp1	Yes	Yes
67831_430	12980	Sp1	Yes	Yes
67839_249	24172	GATA	No	No
67841_556	25587		Yes	No
67849_578	39290	Myf	No	No
66602_392	48192	Ets	Yes	Slight
67853_270	48309	LSF	No	No
67853_287	48328	NF-1	No	No
67853_459	48499	ERE	No	No
67855_230	48699		No	No
67855_289	48758	AP-1	Yes	Slight
67857_306	49223	TATA	Yes	Yes
77013_98	49228	Mef-2	Yes	Yes
37628_571	49420	Sp1	No	No
67865_149	57827	LSF	No	No

Table 4. Summary of EMSA results for 19 polymorphic sites residing in potential transcription factor binding sites. SNPs 67831_429 and 67831_430 and SNPs 67857_306 and 77013_98 are contained within the same probes. The SNPs 67831_429 and 67831_430 are in complete linkage disequilibrium with each other.

Figure 1. Associations of SNPs around calpastatin with slice shear force.

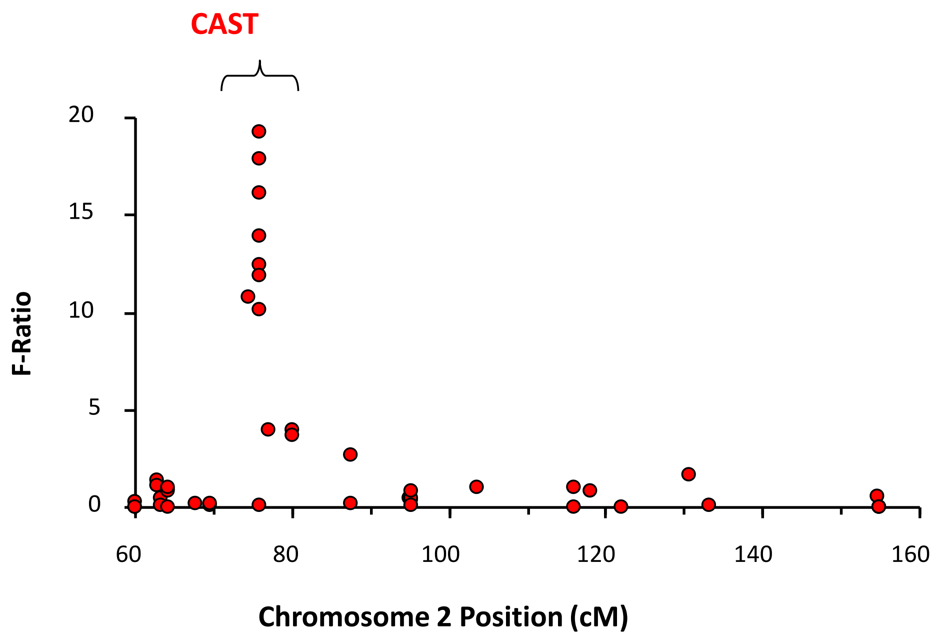


Figure 1. Association of SNPs on SSC2q around calpastatin with slice shear force. Marker F-ratios are plotted by their genetic position on the chromosome. The most significant markers are located in the calpastatin gene. Taken from Lindholm-Perry et al., 2009.

Figure 2. Predicted Promoter Regions in Pig Calpastatin

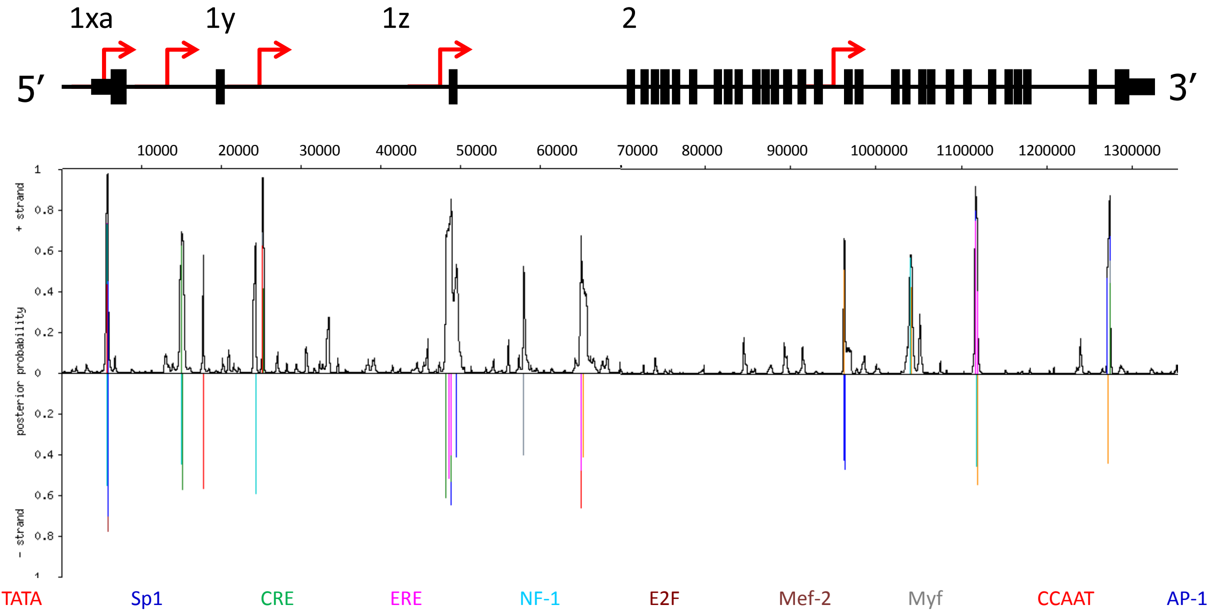


Figure 2. Promoter regions and transcription factor binding sites were predicted in calpastatin using Cister. Sites are shown by position in the gene and the probability is indicated by magnitude on the Y-axis. The gene organization is shown above.

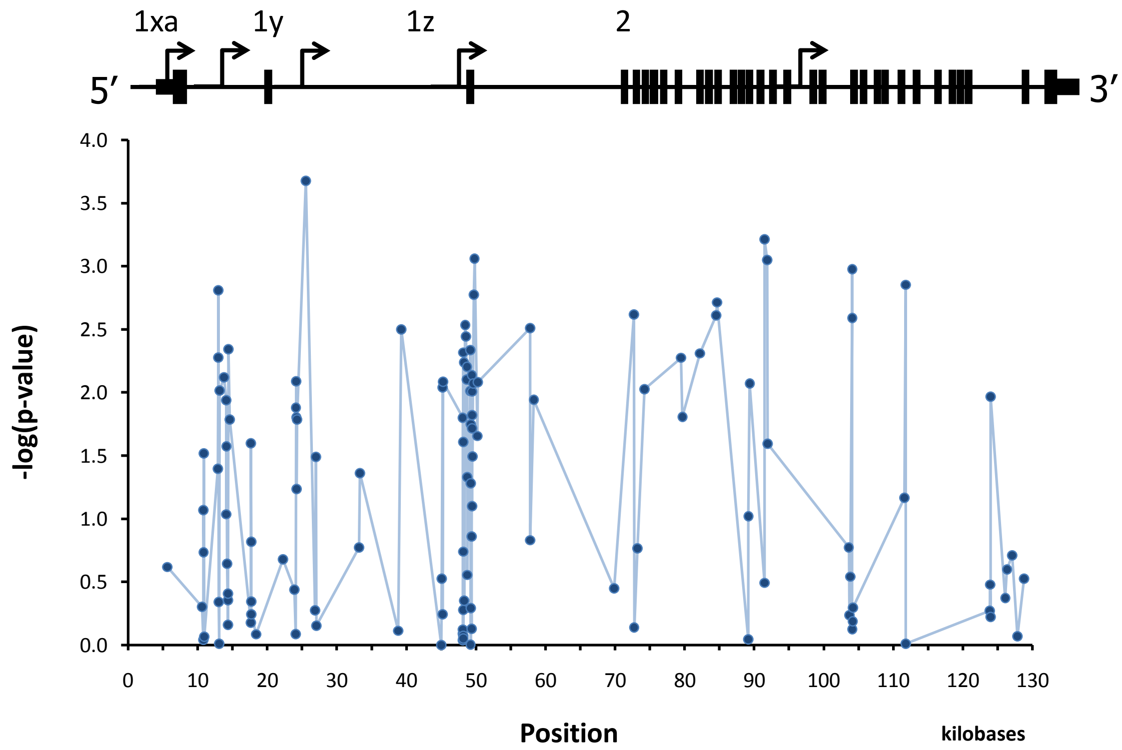
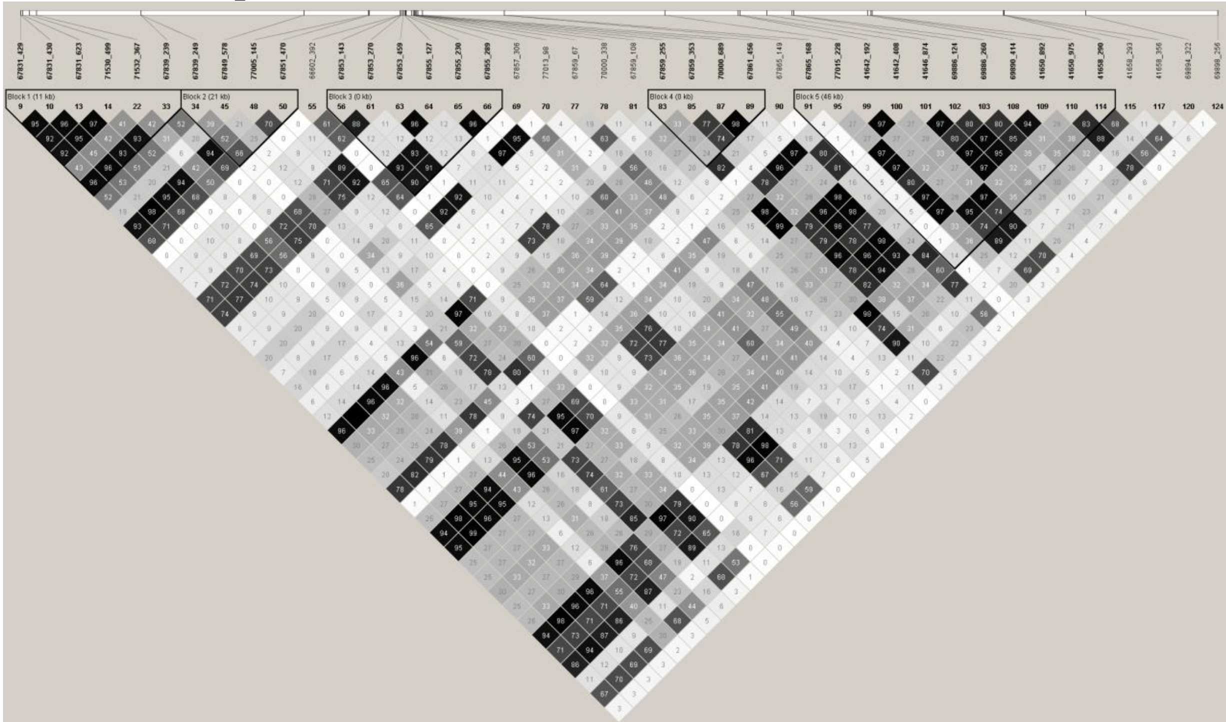


Figure 3. Marker associations of 130 SNPs genotyped in the USMARC population with slice shear force are plotted as –log(p-value) by their position in the calpastatin gene (GenBank Accession No. EU137105).

Figure 4. Linkage disequilibrium plot of markers in the calpastatin gene.

USMARC Population



OSU-NPB Population

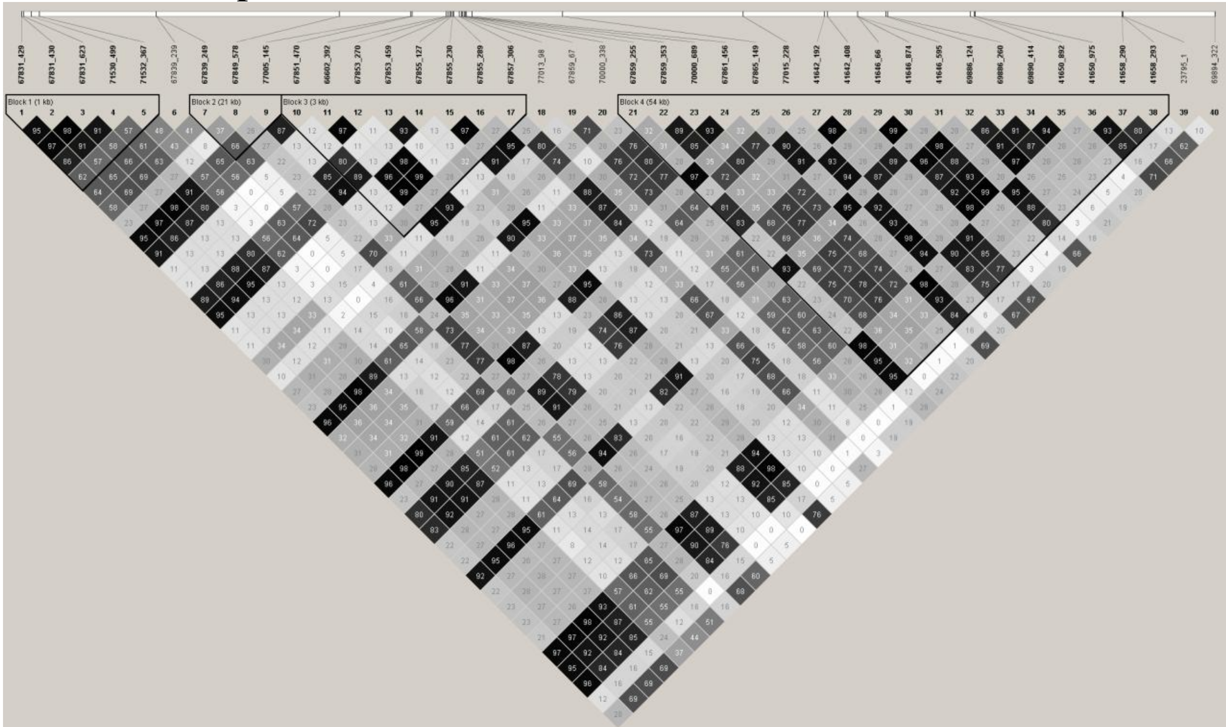


Figure 4. Linkage disequilibrium (LD) in r^2 values of markers in the calpastatin gene in USMARC and industry pig populations. Darker shading represents greater LD. Although the LD blocks are slightly different, the general pattern for LD is similar. The most significant markers associated with shear force are located in or around block 3.

Figure 5. Sp1 site at nucleotide 12979-12980 (markers 67831_429 and 67831_430)

AACCAA*GGGGGAGGGGTGG*GTTAAG
AACCAA*GGGGGGTGGGTGG*GTTAAG

Probe	AG	AG	GT	GT	AG	AG	AG	AG
Nuclear Extract	-	+	-	+	-	+	+	+
Competitor (100x)	-	-	-	-	-	-	GT	AG

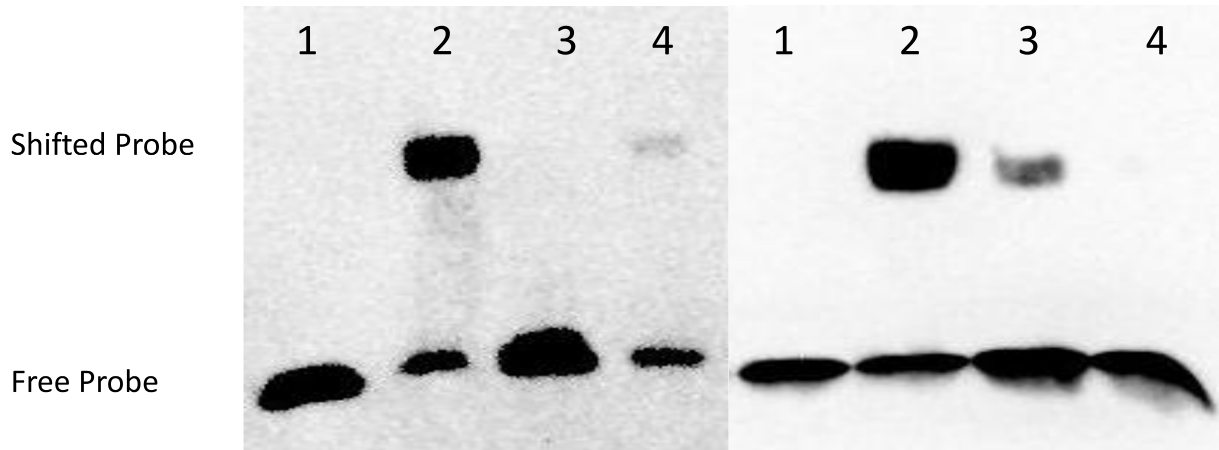


Figure 5. Gel shift of polymorphic Sp1 site at nucleotides 12979 (A/G) and 12980 (G/T) in the presence of nuclear extract from muscle. The AG and GT probes represent different alleles and the binding site is shown underlined in probe sequences above. The AG probe binds transcription factor Sp1 while the GT probe does not. The A and G alleles are associated with greater shear force. Binding can be competed with the addition of specific unlabeled probe.

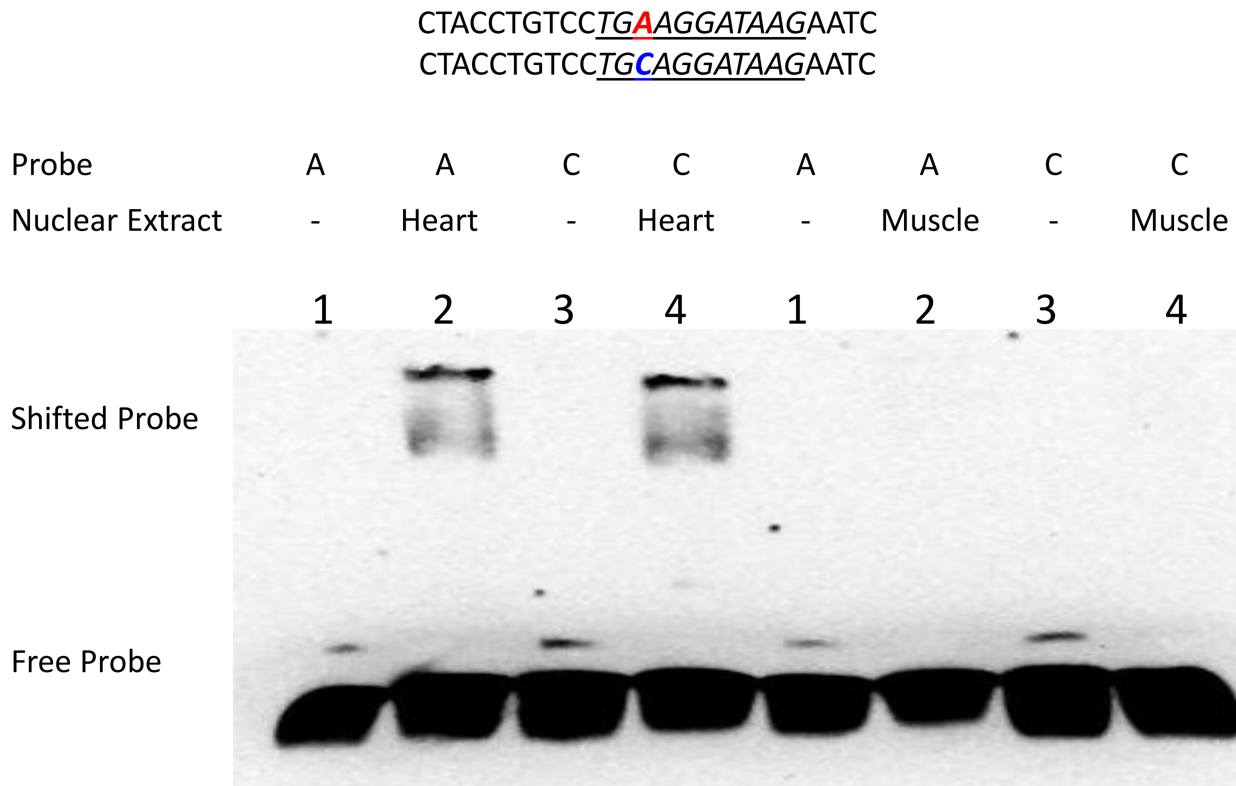


Figure 6. Gel shift at an Ets site around nucleotide 48192. The A and C probes represent different alleles and the binding site is shown underlined in probe sequences above. Probe was shifted in the presence of nuclear extract from heart, but not from muscle. The C probe showed slightly greater binding and is associated with greater shear force.

Figure 7. AP-1 site at nucleotide 48758 (67855_289 A/G)

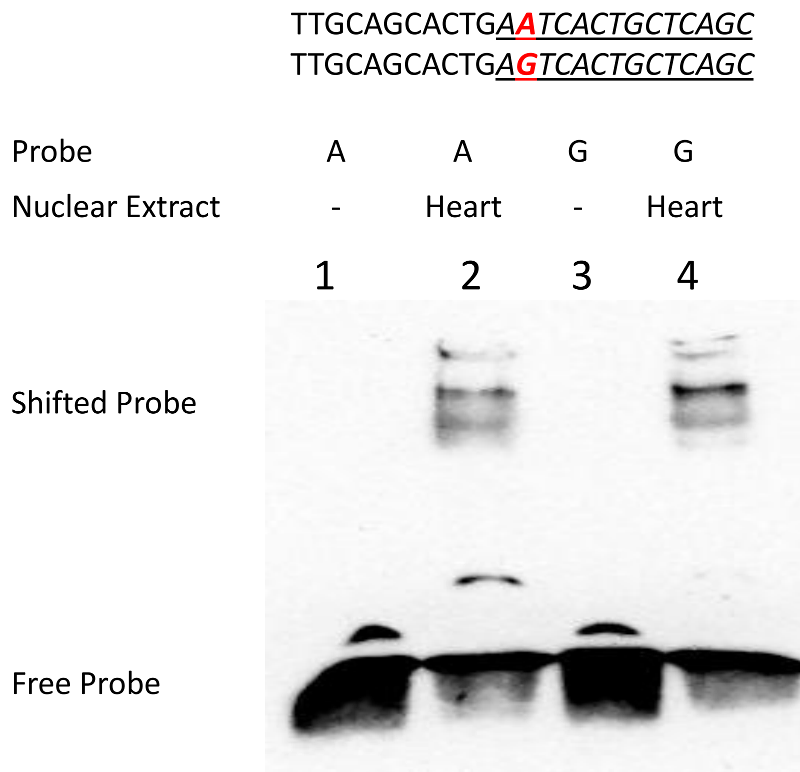


Figure 7. Gel shift at an AP-1 site around nucleotide 48758. The A and G probes represent different alleles and the binding site is shown underlined in probe sequences above. The G-probe showed slightly greater binding. The G allele is associated with greater shear force.

Figure 8. Mef-2/TATA sites at nucleotides 49223 + 49228

67857_306 A/G TATA GTTTAAAAAGCGGTATTTGGGGGTG
77013_98 A/G Mef-2 GTTTAAAAAGCGATATTTAGGGGTG

Probe (67857_306)	A	G	G	G
Nuclear Extract	+	+	+	+
Competitor (100x)	-	-	A	G
	1	2	3	4

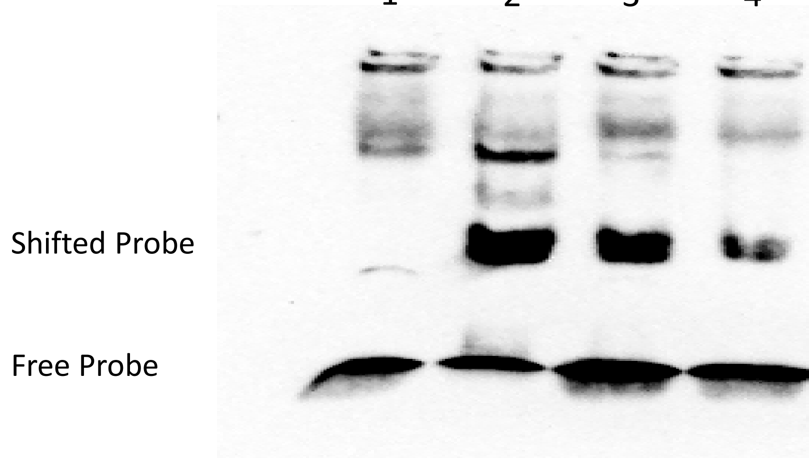


Figure 8. Gel shift of allele-specific probes surrounding a Mef-2 and TATA site at nucleotides 49223 (A/G) and 49228 (A/G). The respective binding sites are shown underlined in probe sequences above. The G allele probe is shifted by heart nuclear extract. The G alleles for both SNPs are associated with great shear force.