Identification of a Chicken β1 Integrin DNA Polymorphism by Denaturing Gradient Gel Electrophoresis

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Abstract

Vertebrate skeletal muscle development is, in part, regulated by myoblast-extracellular matrix (ECM) interactions mediated by the transmembrane integrin family of heterodimeric receptors. Previous investigations have demonstrated a precise spatial and temporal distribution of muscle specific integrins during development, but they have not addressed the possibility of integrin allele variation, DNA polymorphisms. Using denaturing gradient gel electrophoresis (DGGE), commercial DeKalb Delta White Leghorn chickens were screened for a β1 integrin DNA polymorphism. We have identified a β1 integrin DNA polymorphism that is characterized by three electrophoretic alleles *a*, *b*, and *c*.

Key words: β1 integrin, DNA polymorphism, denaturing gradient gel electrophoresis.

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Skeletal muscle differentiation is a complex process involving cell proliferation, differentiation, migration, cell-cell signaling, fusion of myoblasts, and subsequent development of myofibers. Each of these developmental stages is complex but it is not clear how they interact to govern myofibrillar muscle formation or function. However, as muscle differentiates it is clear that myogenic cells interact with the extracellular matrix (ECM). Recent research has indicated a major role for cell surface integrin receptors in this process by linking ECM-cell and cell-cytoskeleton interactions [10, 13].

The integrins are a widely expressed family of heterodimeric cell surface receptors. The β1 family of integrin receptors is specific for several ECM molecules: e.g., collagens types I, II, and IV, laminin, and fibronectin [1, 5, 8, 9, 15, 18]. Neff et al. [14] and Decker et al. [4] through the development of the CSAT monoclonal antibody were the first to demonstrate the importance of the β1 integrin in cell adhesion. Blocking the myoblast β1 integrin by the binding of the CSAT antibody reversibly blocks myoblast differentiation into myotubes [13]. Muscle β1 integrins are also important in myoblast fusion [16], located at points of adhesion and migration [11, 12] myotendon junctions [3, 17] and neuromuscular junctions [3].

Although significant data has been generated concerning the role of the β1 integrin in muscle development, there has not been any reports on whether allele variation, DNA polymorphisms, exist for this gene and if these polymorphisms may be linked to muscle development. The objective of the present study was to identify β1 integrin DNA polymorphisms in a commercial avian line that exhibited phenotypic differences in skeletal muscle function as measured by exhaustion score analysis (the ability of a bird to right itself from a supine position). Screening genes like the β1 integrin for DNA polymorphisms is important in uncovering useful genetic markers which may be potentially linked to skeletal muscle differentiation.

Materials and Methods

DeKalb Delta White Leghorn chickens were purchased at hatching and raised at the Storrs Agricultural Experiment Station at the University of Connecticut.

Exhaustion score

Exhaustion scores were measured at 5-6 months of age. The birds were placed in a supine position up to 30 times. The number of times the bird righted itself from the supine position was recorded as the exhaustion score. DeKalb Delta birds were separated into high score and low score pens based on exhaustion scores. High score birds ranged in score from 21-30 whereas low score birds had a maximum score of 11. Birds with scores in the range of 12-20 were not used in this study.

Blood DNA extraction

Total genomic DNA was extracted from approximately 2 ml of blood drawn from the wing vein and transferred to an Oakridge tube containing 500 µl of 3.8% sodium citrate. The DNA was extracted in 10 ml of lysis buffer (10 mM NH_4HCO_3/0.1 mM EDTA, pH 8.0/15 mM NH_4) and incubated on ice for 15 min followed by centrifugation at 8,000 x g for 10 min in a Sorval SS-34 rotor. The supernatant was carefully decanted and the pellet resuspended in 5 ml of lysis buffer followed by centrifugation at 8,000 x g for 10 min in a Sorval SS-34 rotor. This procedure was repeated 2 to 3 more times until the pellet was almost completely devoid of visible hemoglobin. The supernatant was decanted and the pellet was resuspended in 4.5 ml of 7.5 mM NaCl/25 mM EDTA pH 8.0. To the resuspended solution, 500 µl of a 10 mg/ml proteinase K solution was added followed by incubation with gentle agitation overnight at 37°C.

The resulting viscous liquid was phenol/chloroform extracted two times. To precipitate the DNA, 2 volumes of 95% EtOH and 1/10 volume 3 M NaAc, pH 5.8 were added at room temperature for 10 min. The suspension was centrifuged for 30 min at 10,000 x g in a Sorval SS-34 rotor. The resulting pellet was washed with 5 ml of 70% EtOH two times. The DNA pellet was dried and resuspended in 3 to 5 ml of TE (10 mM Tris/1 mM EDTA pH 8.0). The concentration for the resuspended material was determined spectrophotometrically by the OD 260 reading.

Embryo DNA extraction

Pectoral muscle from 16 day old embryos was removed and stored at -70°C. In a disposable sterile 15 ml tube, 0.5 to 1.0 gm of minced tissue was placed in 5.0 ml of STE (10 mM Tris / 100 mM NaCl / 1 mM EDTA pH 8.0) containing 2% SDS and 200 µg/ml proteinase K and incubated overnight at 55°C. After adding an equal volume of phenol/chloroform: isoamyl alcohol 24:1, the tubes were mixed and centrifuged at 5,000 x g for 10 min in a Sorval SS-34 rotor. The aqueous phase was transferred to a new tube and extracted with an equal volume of chloroform:isoamyl alcohol 24:1 and centrifuged to separate the phases at 5,000 x g in a Sorval SS-34 rotor. The aqueous phase was transferred to a new tube and a 1/2 volume 7.5 M NH_3Ac was added and incubated at room temperature for 10 min. The samples were then centrifuged at 10,000 x g for 10 min in a Sorval SS-34 rotor. An equal volume of 100% EtOH was added and incubated at -20°C for 30 min and the DNA was removed by spooling. The DNA was washed twice by dipping the glass rod into 70% EtOH and then allowed to air dry. The tip of the glass rod containing the DNA was broken off into a 2 ml nunc freezer tube and resuspended in 1.0 ml TE and stored at -70°C.

Denaturing Gradient Gel Electrophoresis (DGGE)

Instead of using the more traditional experimental approach of restriction fragment length polymorphism (RFLP) analysis, we used denaturing gradient gel electrophoresis (DGGE) which does not rely on restriction endonuclease recognition site polymorphisms or a large nucleotide insertion or deletion. DGGE, originally described by Fischer and Lerman [7] functions by separating DNA fragments according to their melting domain behavior. Single base modifications will alter DNA melting domains and change the migration of DNA through a polyacrylamide gel matrix. This property makes DGGE methodology extremely sensitive and increases the potential of identifying unknown DNA polymorphisms.

Genomic DNA samples were digested with Hae III. The restriction digested samples were electrophoresed on a 20-80% linear gradient polyacrylamide gel based on the method of Fischer and Lerman [7] in a CBS Scientific DGGE-2000 apparatus. The stock denaturant mixes were: 0% denaturant mix containing 6.5% acrylamide in 1 X TAE (40 mM Tris acetate / 1 mM EDTA pH 8.0); 100% denaturant mix containing 6.5% acrylamide, 40% formamide, and 7 M urea in 1 X TAE. Gradient gels were poured with a Hoefer Scientific 50 ml gradient maker. The mixes were combined in the appropriate ratio to create the desired denaturing gradient conditions. The gels were electrophoresed for 17 h, 65°C at 60 volts in a 1 X TAE running buffer with constant circulation.

Blotting and probing of denaturing gradient gels

After electrophoresis, all gels were incubated in 50 mM NaOH for 10 min followed by 500 mM Tris-HCl, pH 8.0 for 10 min and then equilibrated for 10 min in transfer buffer (20 mM Tris-HCl, pH 8.0/1 mM EDTA pH 8.0). The gel was then electrophoretically transferred to Schleicher and Schuell Nytran in a Hoefer Scientific transblot for 2 h at 0.8 mAmp at 4°C. The blots were exposed to a short wave ultraviolet source for 2-3 min, air dried, and baked in a vacuum oven at 80°C for 45 min and subsequently hybridized to a randomly labeled [6] β1 integrin probe generously provided by Dr. Richard Hynes [19]. Filters were hybridized by standard conditions in 50% formamide and then washed after 16 to 18 h hybridizations at 42°C until reaching final conditions of 0.1 X SSPE (1 X SSPE = 18 mM NaCl, 10 mM sodium phosphate, 1 mM EDTA, pH 7.7).

Results and Discussion

Forty-six Dekalb Delta White Leghorn chickens were separated into high score and low score pens based on exhaustion score. High score birds ranged in score from 21-30 whereas low score birds had a maximum score of 11. Each of these of these birds were screened by DGGE for a β1 integrin DNA polymorphism using the β1 integrin cDNA probe, pCInt β1 [19]. An electrophoretic β1 integrin DNA polymorphism consisting of three electrophoretic alleles, *a, b,* and *c*, was identified. Figure 1 is a representative denaturing gradient gel blot containing genomic DNA samples extracted from high score and low score DeKalb Delta birds. Data summarized in Table 1, indicate several interesting phenomena. The high score DeKalb

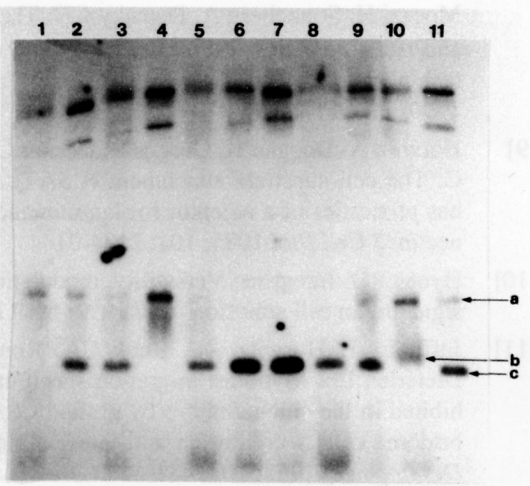

Figure 1. Autoradiography of a denaturing gradient gel blot of genomic DNA isolated from DeKalb Delta White Leghorn High Score and Low Score chickens. The blot was hybridized with the pCIntβ1 integrin cDNA clone. Twenty μg of genomic DNA was digested with Hae III and placed on the gel as follows: lanes 1-6, DeKalb Delta High Score DNA; and lanes 7-11, DeKalb Delta Low Score DNA.

Delta White Leghorns are 60.8% homozygous for possible β1 integrin allele arrangements. In contrast, the low scoring DeKalb Delta White Leghorn birds are 60.9% heterozygous. Of the six possible allelic arrangements, only *a/a, a/c, c/c,* and *a/b* were observed. Electrophoretic allele arrangements of *b/b* or *b/c* were never detected. It is possible that these allelic arrangements may be associated with lethality which will be addressed in future investigations.

These data suggest that homozygosity for β1 integrin electrophoretic alleles may afford some advantages in terms of skeletal muscle function. To further test this hypothesis, defined breeding crosses were established with the DeKalb Delta birds. The following crosses were established: DeKalb Delta low score *a/c* females crossed to a low score *a/c* male; and DeKalb Delta high score *a/c* females crossed to a high score *a/a* male. The data from these crosses are summarized in Table 2. As previously observed, a preferential distribution of electrophoretic variants was detected in defined breeding crosses. The DeKalb Delta low score *a/c* females crossed to a low score *a/c* male produced 70.8% heterozygous progeny. In contrast DeKalb Delta high score *a/c* females crossed to a high score *a/a* male produced 93.2% homozygous progeny. It is conceivable that different allelic arrangements of the β1 integrin could modify the organization of myofibrils which in turn may alter the response to load-bearing stress. In

Table 1. β1 Integrin electrophoretic allele arrangements.

Line	$\bar{x}$ Exhaustion Score	% Allele Arrangements						% Homozygous	% Heterozygous
		a/a	a/c	c/c	a/b	b/b	b/c		
DeKalb Delta Low Score n = 23	7.21	17.4	43.5	21.7	17.4	0	0	39.1	60.9
DeKalb Delta High Score n = 23	26.2	39.1	26.1	21.7	13.0	0	0	60.8	39.1

Table 2. β1 integrin electrophoretic allele arrangements for defined breeding crosses.

	cross	% Allele arrangements			% Homozygous	% Heterozygous
		a/a	a/c	c/c		
Low score n = 24	a/c♀ x a/c♂	8.3	70.8	20.8	29.1	70.8
High score n = 30	a/c♀ x a/a♂	66.6	6.6	26.6	93.2	6.6

support of this hypothesis, Menko and Boettiger [13] have suggested that the integrin receptor is part of a signal transduction pathway regulating myoblast gene expression; the α5β1 integrin receptor has been shown to be involved in the adhesion of muscle to the extracellular matrix and the organization of nascent fibrils [2, 12]. The data presented in this report is suggestive that the β1 integrin DNA polymorphism may modify myoblast-extracellular matrix interactions, this possibility will be considered in future investigations.

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