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ALTERNATIVE SPLICING IN TURKEY SKELETAL MUSCLE RYANODINE RECEPTOR αRYR

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Introduction

The term "pale, soft, exudative (PSE)" is a descriptor for a meat quality defect, primarily pork and turkey, that has an abnormal light color, often flaccid consistency, poor water-holding capacity, and substantially reduced cook yield (De Smet et al., 1996). PSE meat is the result of denaturation of myofibrillar proteins caused by the combination of high carcass temperature and low muscle pH during the early stage of conversion of muscle to meat. The combination of high body temperature and acidic muscle pH in the meat animal mainly is attributed to a disorder of skeletal muscle Ca^{2+} regulation which leads to hypermetabolism and accelerated glycogenolysis during muscle contraction.

A dysfunctional homotetrameric Ca^{2+} release channel protein in skeletal muscle known as ryanodine receptor 1 (RYR1) is believed to contribute to abnormal Ca^{2+} regulation. A point mutation, $Arg^{614}Cys$, identified in RYR1 has been correlated with the development of PSE pork (Fujii et al., 1991). In addition, more than 20 missense mutations of human RYR1 have been identified (Jurkat-Rott et al., 2000). The overall physiological effect of the mutations in RYR1 is an elevation of resting muscle Ca^{2+} levels as a result of increased Ca^{2+} permeabilities of the mutant channels (MacLenna, 2000). Although a strong correlation has been shown between the mutated RYR1 and the development of PSE pork, little is known about the molecular mechanism of PSE turkey meat development.

The similarities of postmortem biochemical changes between PSE turkey and PSE pork including increased rates of postmortem pH decline, ATP depletion, and glycolysis (Pietrzak et al., 1997) suggest that the occurrence of PSE turkey is likely caused by a dysfunctional RYR in turkey skeletal muscle. However, the mechanisms of Ca^{2+} regulation in avian skeletal muscle are more complex than in mammalian species. These include a co-expression of two RYR isoforms (αRYR and βRYR; Ottini et al., 1996) and expression of different alternatively spliced transcript variants (ASTVs) of αRYR in turkey skeletal muscle (Chiang et al., 2004). Turkey skeletal muscle αRYR and βRYR have been cloned and sequenced in our laboratory. During cloning, at least eight ASTVs of αRYR have been identified. This paper describes our current research of turkey skeletal muscle αRYR and discusses the significance of the research in identifying the molecular mechanism regulating turkey meat quality.

Objectives

To determine the primary structures of ASTVs of turkey skeletal muscle αRYR and to correlate the changes of the primary structure of αRYR with differences in turkey meat quality.

Methodology

Cloning of turkey skeletal muscle αRYR and βRYR cDNAs

Total RNA was extracted from turkey pectoralis major muscle using Trizol reagent (Invitrogen). First strand cDNA was synthesized as follows: a 50 μl reverse transcription reaction contained 10 μg of RNA, 0.1 mM of first-strand primer, 9% glycerol, 1× first-strand buffer, 10 mM DTT, 0.5 mM dNTP mix, 30% saturated trehalose, and 600 U of SuperScript II RNase H^- reverse transcriptase (Invitrogen). First strand cDNA synthesis was followed the method of Carninci and Hayashizaki (1999) with modifications.

PCR amplification of the resulting first-strand cDNA was conducted using Advantage cDNA polymerase (BD Biosciences). For highly GC-rich regions, amplification was carried out using the Extended Long Template PCR system (Roche). Depending on the melting temperature of the primers, the annealing temperature used during amplification was set in the range of 58º-65ºC. The PCR reaction was started with an initial denaturation step at 94ºC for 1 min, followed by 35 cycles consisting of 94ºC for 10 sec., 58º to 65ºC for 15 sec., and 70ºC for 1-2 min. Reaction was terminated by a final extension at 70ºC for 10 min. Degenerate PCR primers used in the reverse transcription or PCR reaction were designed based on the conserved regions of the RYR sequences among different species. Other turkey RYR specific primers were designed from the sequence of the established turkey RYR cDNA sequence. PCR products were cloned and nucleotide sequences were determined from both strands. In each PCR reaction, all the amplified PCR fragments were cloned and sequenced and these fragments were used to identify ASTVs.

Analysis of cDNA sequences

Nucleotide sequences data from individual clones were assembled into a full-length contig based on overlapping sequences between the adjacent clones. Comparisons of sequence identity among turkey RYRs and published RYR sequences were performed using the UW-GCG program. The splice junction of each ASTV was identified by comparing the sequence of the spliced transcript variant and the full length cDNA sequence.

Results & Discussion

Alternatively Spliced Transcript Variants (ASTVs) of Turkey Skeletal Muscle αRYR

The complete full length cDNA sequences for αRYR and βRYR from turkey skeletal muscle have been successfully cloned and sequenced in our laboratory. There are 15,150 bps in the open reading frame of the αRYR cDNA sequence which encode a protein of 5,050 amino acid residues and a deduced molecular mass of 565,120 Da. There are 14,604 bps in the open reading frame of the βRYR cNDA sequence which encode a protein of 4,868 amino acid residues and a deduced molecular mass of 552,778 Da. The αRYR and βRYR isoforms share 66% amino acid sequence identity. Major sequence variations in the primary structure of the two isoforms were identified in three divergent regions (DRs).

Analysis of full length cDNA sequences of αRYR and βRYR revealed at least eight ASTVs of αRYR, but no ASTV was identified in βRYR. The eight ASTVs of αRYR are clustered in three different regions of αRYR (Fig 1). Since each of these regions is characterized by a high frequency of alternative splicing, we refer to these regions as alternative splicing regions 1-3 (ASRs 1-3). Interestingly, the locations of ASR2 and ASR3 coincide with DR2 and DR1 of αRYR, respectively. ASR1 encompasses two ASTVs (AS-81 and AS-193; Chiang et al., 2004). ASR2 encompasses five ASTVs, AS-1043, AS-401, AS-454, AS-370 and AS-268. With the exception of AS-401, which represents the removal of exon 28, the other four ASTVs occur at aberrant alternative splice junctions. Unlike the conventional 5'/3' splice junctions identified by the GT/AG consensus sequences, the alternative splice junctions of AS-1043, AS-454, AS-370 and AS-268 are characterized by polycytosine (poly-C) tracts for both the 5' and 3' splice junctions. We were unable to determine the exact cytosine residue for the splicing junction of these ASTVs because the sequences at the 5' and 3' junctions were indistinguishable (Fig. 2). The splicing observed within the poly-C tract is novel and does not match to any of the published nonconforming splicing sites (Senapathy et al., 1990; Jackson, 1991). ASR3 resides in the C-terminal region of αRYR. One transcript variant, AS-816 was identified and this transcript variant generates a deletion of 272 amino acids encoded by exon 91 of αRYR.

The observation that ASR2 and ASR3 are contained within DR2 and DR1, respectively, of turkey skeletal muscle αRYR suggests that sequence variation in DR2 and DR1 can arise via nucleotide substitution and by alternative splicing.

Functional effects of alternative splicing on αRYR

The biological determinants of the alternative splice sites in the ASRs of αRYR are not clear. Developmental stage and environmental stresses such as temperature changes can regulate the alternative splice pattern of pre-mRNA (Futatsugi et al., 1995; Takechi et al., 1994). As we analyzed the expression of ASTVs in the ASR1 of αRYR, we found that birds treated with different levels of heat stress expressed different patterns of full length, AS-81 and AS-193 transcript variants (Fig 3). This suggests that the expression

pattern of the full length and the ASTVs of αRYR is a dynamic process which may result in differential channel activity.

Although it is commonly thought that RYR exists as a homotetrameric channel under a normal physiological condition, heterotetrameric channels containing a mixture of RYR3 spliced variant and wild type subunits have been reported (Jiang et al., 2003). The heterotetrameric RYR3 channel shows altered channel activity. All eight αRYR ASTVs, except AS-81 and AS-816, would cause frame-shifts which introduce premature stop codons in the open reading frame. Thus, these transcript variants would not encode a complete functional channel protein. AS-81 and AS-816 result in continuous open reading frames and may encode subunits with deletions of 27 and 272 amino acid residues, respectively. The αRYR subunits encoded by AS-81 and AS-816 could combine with subunits encoded by the same ASTV to form a homotetrameric channel. Alternatively, subunits encoded by AS-81 or AS-816 could combine with subunits encoded by different ASTVs and/or wild type subunits to form a heterotetrameric channel. Channels formed by subunits which are encoded by AS-81 or AS-816 are expected to show a different channel function than the wild type. This is based on the structural and functional analysis of mammalian RYR showing that the ASRs of turkey αRYR are localized in the functional domains of αRYR. For example, the 27-amino-acid-residue deletion generated by AS-81 in ASR1 resides within the cytoplasmic clamp domain which has been proposed to serve as part of the protein-protein contact site of RYR1 with dihydropyridine receptor (DHPR; Wu et al., 1997; Baker et al., 2002). Deletion in the ASR1 of αRYR could alter the interaction between αRYR and DHPR and the lack of interaction between these two proteins which, in turn, could modulate Ca^{2+} release via both αRYR and βRYR (O'Brien et al., 1995).

ASR3 resides in part of the transmembrane domain of αRYR (Krogh et al., 2001) where the Ca^{2+} sensor of RYR has been located (Du et al., 2000). A deletion of 272-amino-acid-residue generated by AS-816 in ASR3 is therefore expected to affect the kinetics of Ca^{2+} diffusion through αRYR.

It is logical to expect the regulation of Ca^{2+} fluxes in turkey muscle will be altered as the αRYR channel formed by different subunits encoded by ASTVs rather than the wild type. The changes of the Ca^{2+} fluxes can affect postmortem metabolism and glycogenolysis rate of muscle, and that influence the meat quality.

Conclusions

1. Eight ASTVs of αRYR were identified and they are clustered in three ASRs of primary structure of αRYR. ASR2 and ASR3 overlap with DR2 and DR1 of αRYR.
2. The expression of AS-81 and AS-193, which carry deletions in the ASR1 of αRYR, was regulated by heat stress.
3. The expression of ASTVs of αRYR is expected to affect αRYR channel activity and which will, in turn, influence turkey meat quality.

References

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Tables and Figures

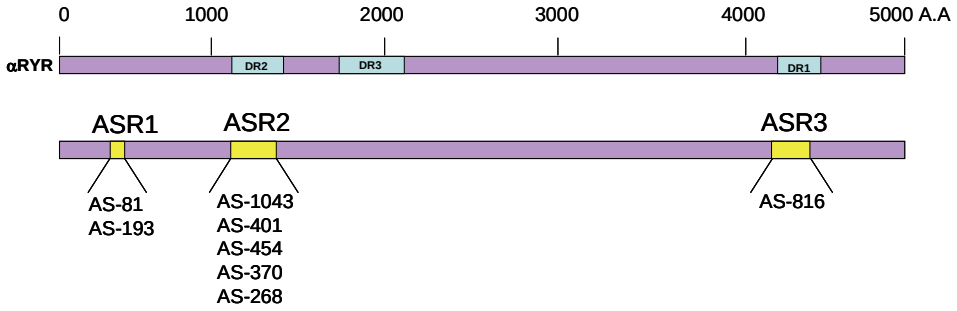

Figure 1. Alternative splicing regions of turkey αRYR. Eight alternatively spliced transcript variants of turkey skeletal muscle αRYR isoform are clustered in three alternative splicing regions 1-3 (ASR1-3). ASR2 and ASR3 coincide with the DR2 and DR1 of αRYR.

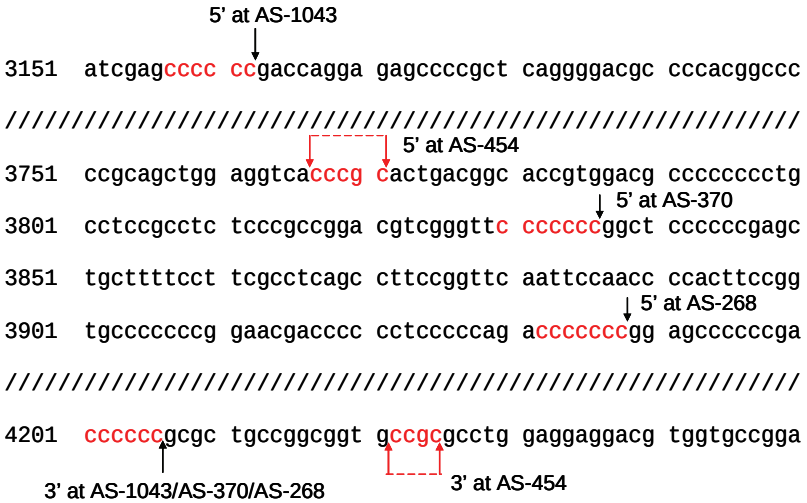

Figure 2. Alternative splicing of turkey αRYR within polycytosine tracts in the ASR2 region. The putative 5' and 3' alternative splice junctions of each transcript variant are indicated by arrows. The two possible splice sites at 5' and 3' junctions of AS-454 are indicated by the arrows connected by a dotted line. The slashed lines are added to replace the omitted nucleotides.

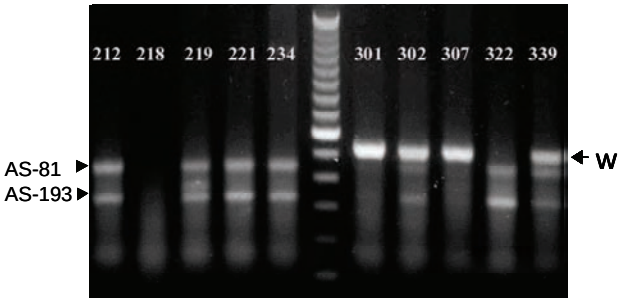

Figure 3. Expression of wild type (W), AS-81 and AS-193 transcript variants under two different heat stress levels. The 100-bp DNA marker is in the center of the gel image. Five individual birds on the left side of the marker are heat stressed two days less than the birds on the right side of the marker. The identification number of each bird is given at the top of the gel.