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Genomic organization and analysis of the 5′ end of the porcine ryanodine receptor gene (*ryr1*)

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In this study we describe the isolation of genomic clones of the 5′ region of the porcine ryanodine receptor gene, a candidate for malignant hyperthermia in pigs and humans. The recombinants were isolated from a porcine liver, genomic DNA library in phage EMBL3A after screening with PCR amplified DNA fragments. The exon/intron structure of the ryanodine receptor gene was determined by DNA sequencing. Based on the sequence data it was possible to develop a simple test for the detection of malignant hyperthermia susceptible and normal pigs.

Ryanodine receptor; Malignant hyperthermia; Stress syndrome; Halothane; Volatile anaesthetics; *Sus scrofa domestica*

1. INTRODUCTION

Sensitivity to volatile anesthetics, i.e. halothane, commonly known as malignant hyperthermia (MH), has been reported in a variety of species, e.g. man [1,2], swine [3–5], horse [6], dog [7], cat [8], rabbit [9], and *Caenorhabditis elegans* [10,11]. Malignant hyperthermia (MH) in swine is an autosomal recessive disorder with variable penetrance. In swine MH is part of an adaptations incompetence, the so-called porcine stress syndrome (PSS). MH is a consequence of abnormal increases in myoplasmic Ca^{2+} which is released from the sarcoplasmic reticulum. Linkage analyses have shown that the gene responsible for halothane sensitivity is located within a group of closely related markers [5].

By in situ hybridization with the glucosephosphate-isomerase, the 'halothane' gene has been assigned to porcine chromosome 6p12-q22 [12]. However, recently it has been reported that the ryanodine receptor gene (*ryr*) coding for a calcium release channel located in the terminal cisternae of the sarcoplasmic reticulum might be a candidate for predisposition to malignant hyperthermia [13,14]. The ryanodine receptor gene has been assigned to the same region of porcine chromosome 6p11-q21 [15]. By comparison of the ryanodine receptor cDNAs of malignant hyperthermia susceptible (Pietrain) and normal (Yorkshire) pigs, it was possible to identify a probable causative mutation within the ryanodine receptor gene [16]. This mutation has now been analysed and detected in different breeds (Pietrain, Yorkshire, Poland China, Duroc, Canadian and British Landrace, Hampshire), supporting the view that it is the causative mutation in porcine malignant hyperthermia [17].

We have used PCR amplified DNA fragments of the porcine ryanodine receptor gene to screen a genomic DNA EMBL3A library. Recombinant phages were isolated and sequenced to determine the exon/intron structure of the gene. Based on our sequence information and previous results [16] we were able to develop a test for the detection of malignant-hyperthermia-susceptible and normal pigs. DNA-based detection of the MH status was performed in 405 pigs of German Landrace and compared with phenotypes and genotypes determined by conventional halothane challenge testing and haplotyping.

2. MATERIALS AND METHODS

Two lines of pigs (German Landrace breed), a stress-resistant selection line, and a stress-susceptible test line, have been selected by the halothane challenge test of progeny from matings between phenotypically halothane negative sow and holathane positive boars [18]. Additionally MHS (malignant-hyperthermia-susceptible; genotype nn) and MHN (malignant-hyperthermia-negative; genotypes Nn, NN) pigs were blood-typed to confirm the genotypes of individual animals. Four serological markers (phosphohexose-isomerase; H-blood group; α_1B-glycoprotein; phosphogluconate-dehydrogenase) which have been assigned to the *hal* linkage group [5] on porcine chromosome 6 [12] were determined. Furthermore pigs were examined using an *Xba*I RFLP detected with the porcine GPIcDNA [19,20] which is informative in approximately 70–80%.

DNA was isolated from lymphocytes or tissue samples after lysis and incubation at 58°C in proteinase K lysis-buffer according to standard protocols [21].

The porcine genomic DNA library was constructed from *Sau*3AI partially digested liver DNA and screened essentially as described [22,23]. PCR amplified fragment RYAHUM56 (*Eco*RI/*Hin*dIII) was a gift of A. Burny, V. Claes Jr., and M. Chaput (State Faculty of Agronomy and Department of Molecular Biology, University of Brussels, Gembloux, Belgium) and has been cloned in pBluescript KS+.

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Recombinant EMBL3A phages λRYR56-15 and λRYR56-18 were digested with appropriate restriction enzymes (*Bam*HI, *Bgl*II, *Pst*I) and fragments subcloned into pGEM-4Z (Stratagene). Recombinant plasmids were used to transform *E. coli* XL1-blue (Stratagene). All subclones were sequenced using the T7 sequencing kit (Pharmacia).

PCR analysis of pigs was performed using a biomed Thermocycler 60 with 30 cycles of denaturation at 94°C (40 s), annealing at 68°C (40 s), and polymerization at 72°C (40 s). PCR products were digested with *Hha*I and electrophoresed on 2% agarose gels. PCR primers RYR56.1 (5'-GTGCTGGATGTCCTGTGTTCCCT-3') corresponding to positions 1795–1817 and RYR56.2 (5'-CTGGTGACATAGTTGATGAGGTTTG-3') corresponding to positions 1904–1928 (numbers refer to the positions in the ryanodine receptor cDNA beginning with 1 at the ATG initiator methionine) were synthesized on a DNA Synthesizer 380A (Applied Biosystems).

3. RESULTS

Initially a 840 bp PCR amplified *Eco*RI/*Hin*dIII fragment of the porcine ryanodine receptor cDNA designated pBS-RYAHUM56 (positions 2490–3330) was used to screen 1.2×10^6 recombinant EMBL3A phages from a porcine genomic DNA library. Clones which lit up where enriched and rescreened. Finally, two clones designated λRYR56-15 and λRYR56-18 were isolated and analysed further. In the following analyses we focussed on the characterization of λRYR56-15, because it hybridized approx. 2-fold stronger to the 840-bp PCR fragment than λRYR56-18. λRYR56-15 was digested with *Bam*HI and *Bgl*II and resulting fragments were subcloned into pGEM-4Z or further reduced in size with appropriate restriction enzymes for sequence analysis. Fig. 1 outlines the structure and restriction map of λRYR56-15. Sequences of 5' and 3' splice sites are depicted in Fig. 2 and display the characteristic consensus motifs of splice junctions.

Based on our sequence data we wanted to investigate whether the putative causal C to T mutation at position 1843 as described by Fujii et al. [16] would also be detectable in German Landrace breeds. Therefore we analysed 405 halothane challenge tested, haplotyped, and progeny tested pigs. As shown in Table I all animals with the DNA-based genotype C/C or C/T were phenotypically non-reactors. Four animals that had been determined to be homozygous non-reactors (NN) from progeny testings were shown to be heterozygous (C/T) from DNA-based analysis. Four animals that had been determined to be heterozygous non-reactors (Nn) from progeny testings were shown to be homozygous non-reactors (C/C) from DNA-based analysis. One animal which had been classified to be a heterozygous non-reactor (Nn) from progeny testings was shown to be a homozygous reactor (T/T) by DNA-based analysis.

4. DISCUSSION

In this study we describe the isolation of two recombinant EMBL3A phages, designated λRYR56-15 and λRYR56-18, which harbour approx. 19 kb of porcine genomic DNA, respectively. They are located at the 5' end of the porcine ryanodine receptor gene. λRYR56-15 was almost completely sequenced in order to determine the exon/intron structure of this region. It was shown to contain ca. 2,500 bp of coding DNA corresponding to at least 10 exons. Two regions between positions 1443–1675 and 2170–2403 have not been mapped in detail, because they are located on a 5-kb and 4-kb *Bam*HI subfragment, respectively. Therefore they were only partially sequenced. Unfortunately λRYR56-15 lacks the complete 5' end of the ryanodine

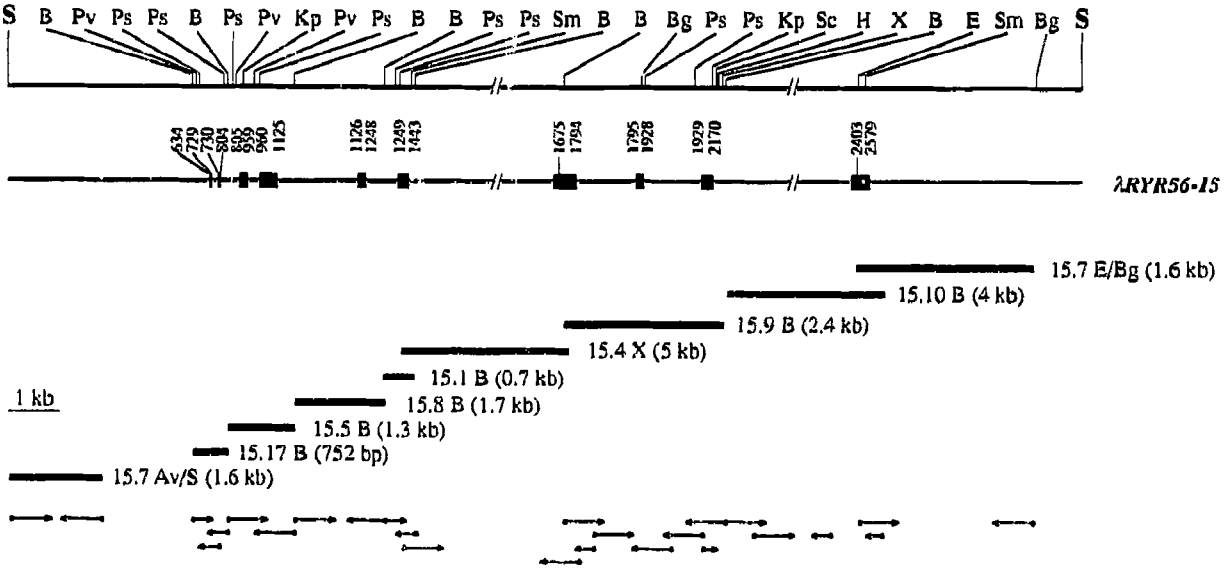

Fig. 1. Structure and restriction map of λRYR56-15. The diagram outlines the structure of the ryanodine receptor gene (*ryr1*) at the 5' end. Boxes indicate exons and thin lines introns. Numbers refer to the positions in the ryanodine receptor cDNA beginning with 1 at the ATG initiator methionine. The arrows at the bottom of the diagram outline the sequencing strategy of the different sublones (bars). Av, *Ava*I; B, *Bam*HI; Bg, *Bgl*II; E, *Eco*RI; H, *Hin*dIII; Kp, *Kpn*I; Ps, *Pst*I; Pv, *Pvu*II; Sc, *Sac*I; Sm, *Sma*I; S, *Sal*I; X, *Xba*I.

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            634      729
CTCTGTCCAG GCTAT..CGCAG GTCTGGGCTG
            730      804
ACGTGCACAG ACTTG..ATCAG GTAGGGGGGC
            805      959
CCCTCTGCAG CTGGA..CCAAG GTCGGTGGG
            960     1125
ACCCCTGCAG GAGAA..AGAAG GTGGGTGTCT
           1126     1248
         AG GCCAT..TCAAG GTAGCCACCT
           1249     1443
GGTCCCACAG GGGCC..AGGAG GTGAGGCCAG
                    1794
                  ..ACAAG GTGGGCCTGT
           1795     1928
TGACCCCTAG GTGCT..ACCAG GTCTGGCCCC
           1929     2170
TCTCCTGCAG CATCC..GACAG GTACCTGGAC
                    2579
                  ..GTCAG GTACTCTCTG
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Fig. 2. DNA sequences of splice sites of the 5′ end of the porcine ryanodine receptor gene (*ryr1*). Each line represents the 3′ splice site of an intron followed by the appropriate exon and the 5′ splice site of the subsequent intron. Conserved bases are shown in boldface letters. Numbers refer to the positions of the exons in the ryanodine receptor cDNA beginning with 1 at the ATG initiator methionine.

receptor gene, so that an exact numbering of the exons was not yet possible. Within the coding region λRYR56-15 shows 85–95% homology to the human and rabbit skeletal muscle ryanodine receptor gene cDNAs (*ryr1*). However, λRYR56-18 is only approx. 60–70% homologous to λRYR56-15. It also shows no significant homology to the cardiac isoform of the ryanodine receptor gene. Therefore we believe that λRYR56-18 may be part of a second skeletal muscle ryanodine receptor gene (*ryr2*).

Based on the sequence data it was possible to develop a test for the detection of malignant hyperthermia susceptible pigs. PCR primers were selected so that the 134-bp exon harbouring the C to T mutation causative for malignant hyperthermia in pigs [16,17] was amplified. After digestion with *Hha*I this fragment will be reduced to a 84-bp and 50-bp fragment if the unmutated allele is present. From our analyses we conclude that in German Landrace breeds the same mutation as it was found in other breeds is causative for malignant hyperthermia. The discordance between the results obtained from DNA-based analysis and halothane challenge test as well as progeny testings can be explained by the known inaccurary of the latter ones. This DNA-based test will be used to distinguish the genotypes of susceptible (nn) and normal (Nn and NN) pigs in further breeding programmes.

Table 1

Comparison of genotypes from DNA-based analysis (PCR), phenotypes from halothane challenge testing, and genotypes from progeny testing in the detection of MH susceptibility in pigs.

Genotype from DNA-based analysis	Number of animals	Phenotype from halothane test		Genotype from progeny test
		R	NR	
C/C	214	–	194[b]	117[d]
C/T	153	–	133[c]	46[e]
T/T	38	32[a]	4	18[f]

[a]2 nd; [b]20 nd; [c]20 nd; [d]102 nd; [e]93 nd; [f]20 nd; R, reactor; NR, non-reactor; nd, not determined.

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