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Identification of a Mutation in Porcine Ryanodine Receptor Associated with Malignant Hyperthermia

Author(s): Junichi Fujii, Kinya Otsu, Francesco Zorzato, Stella De Leon, Vijay K. Khanna, Janice E. Weiler, Peter J. O'Brien and David H. MacLennan

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- 9. NMR measurements were performed on a GN-500 spectrometer. The majority of the measurements were made with 5 mM kistrin at pH 2.2 and 20°C. Preliminary analysis of the two-dimensional NMR spectra indicates that there is no significant conformational shift between pH 7 and pH 2.2 at 20°C (M. Adler and G. Wagner, unpublished results); the structure published here should be comparable to that at neutral pH.
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- in preparation.11. NOEs are effects arising from dipole-dipole interactions between protons that are close in space. Such interactions usually give rise to cross peaks in NOESY spectra if the protons are separated by less than  $\sim 5$  Å. The symbols *i*, *j*, and *i* + *a* are used as indices for the two residues connected by an NOE. NOEs with values of i to i + 2 indicate turns or helices.
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   P. Carter *et al.*, unpublished results.
   We performed the calculations as follows using the program Dspace (Hare Research, Inc., Woodinville, WA). The structures were first embedded and then minimized with a conjugate gradient against a con-straint file based on the NOEs alone. The 1-4 constraints that enforce the 40 angle constraints were then introduced, and the penalties that regulate the integrity of the covalent structure (1-2, 1-3, and 1-4 distance bounds) were increased tenfold over the NOE penalty functions. After some further minimization, an exhaustive round of simulated annealing was performed, followed by minimization. At this point, the structures with no NOE violations in excess of 0.7 Å were selected and subjected to 40 rapid rounds of simulated annealing followed by minimization. Roughly 1 out of 20 structures had no NOE violations over 0.5 Å. (Two-thirds of the structures had no violations greater than 1 Å). There were on average two violations of the covalent structure over 0.1 Å, and some structures had no violations this large. A large number of the residual violations centered around C45. This residue has a disulfide bond to C64 and appears in roughly the third position of a tight bend. The disulfide bond itself passes under (and sometimes over) residues R56-C57 (Fig. 3). This geometry apparently stretches the disulfide bond and causes distortion in the covalent geometry of the flanking residues. This problem has yet to be resolved and it may in part stem from spin diffusion effects causing overinterpretation of the NOEs to the methyl groups of I44.
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## Identification of a Mutation in Porcine Ryanodine **Receptor Associated with Malignant Hyperthermia**

JUNICHI FUJII,\* KINYA OTSU, FRANCESCO ZORZATO,† STELLA DE LEON, VIJAY K. KHANNA, JANICE E. WEILER, PETER J. O'BRIEN, DAVID H. MACLENNAN<sup>‡</sup>

Malignant hyperthermia (MH) causes neurological, liver, and kidney damage and death in humans and major economic losses in the swine industry. A single point mutation in the porcine gene for the skeletal muscle ryanodine receptor (ryr1) was found to be correlated with MH in five major breeds of lean, heavily muscled swine. Haplotyping suggests that the mutation in all five breeds has a common origin. Assuming that this is the causal mutation for MH, the development of a noninvasive diagnostic test will provide the basis for elimination of the MH gene or its controlled inclusion in swine breeding programs

H is an inherited myopathy in which skeletal muscle contracture with attendant hypermetabolism and elevation in body temperature are triggered by inhalational anesthetics and skeletal muscle relaxants (1, 2). The syndrome occurs in one out of 15,000 anesthetized children and one out of 50,000 anesthetized adult humans; can cause neurological, liver, or kidney damage; and is frequently fatal. In swine homozygous for the defect, MH can also be triggered by stress; thus the disease is referred to as the porcine stress syndrome. Major economic losses in the swine industry result from the development of pale, soft, exudative pork that arises from postmortem manifestation of the disease in MH susceptible (MHS) animals (1).

The defect in MH appears to be hypersensitive gating of the Ca<sup>2+</sup>-release channel (ryanodine receptor) of the skeletal muscle sarcoplasmic reticulum (3); channel opening is facilitated, and closing is inhibited (3-6). Alterations in the Ca<sup>2+</sup> dependence of ryanodine binding (7) and tryptic digestion patterns (8) for the ryanodine receptor from MHS swine have been observed. Molecular genetic studies also associate an altered ryanodine receptor with MH. The skeletal muscle ryanodine receptor (ryr1) gene, which is expressed only in fast- and slowtwitch skeletal muscle (9-11), has been localized to human chromosome 19q13.1 (12) and linked to mutations causing MH in humans (13). The porcine MH (hal) locus

(14) and ryr1 (15) have been localized to pig chromosome 6p11-q21 in a region that is related to human chromosome 19q13.1, suggesting linkage between porcine MH and ryr1. We have identified a single point mutation in ryr1 that is correlated with, and likely to be causative of, MH in five lean, heavily muscled breeds of swine.

In a sequence comparison of full-length ryanodine receptor cDNAs from both an MHS Pietrain pig and an MH normal (MHN) Yorkshire pig (16), we found no evidence for a deletion or an internal stop codon. Instead we found 18 single nucleotide polymorphisms between the two animals (Table 1). One of the polymorphisms, (replacement of C at nucleotide 1843 from the MHN animal with a T in the cDNA from the MHS animal) (Table 1 and Fig. 1) led to an alteration in amino acid sequence from an arginine at position 615 in the MHN animal to a cysteine in the MHS animal.

In order to provide evidence that the single amino acid alteration is causative of MH, we carried out analysis of the association of the substitution of T for C at nucleotide 1843 with the MH susceptibility status of 182 pigs in six breeds (17). We performed blood or muscle biopsies for blind DNA-based analysis (18). Genomic DNA was isolated from these biopsies and a 74-bp sequence between nucleotides 1811 and 1884 (Fig. 1) was amplified by the polymerase chain reaction (PCR). Two oligonucleotides (18) were used as differential hybridization probes to detect the presence of the C and T alleles in the various breeds (Fig. 2A). Because the mutation deletes a Hin P1 site and creates a Hgi AI site (Fig. 1), we also used loss of the Hin P1 site in the PCR product as an analytical test (Fig. 2B).

Study of the correlation between phenotypic and genotypic diagnosis is complicated by the lack of a well-defined phenotypic test for the N/n genotype (heterozygous MH) and by the fact that a low percentage of

J. Fujii, K. Otsu, F. Zorzato, S. de Leon, V. K. Khanna, D. H. MacLennan, Banting and Best Department of Medical Research, University of Toronto, C. H. Best Institute, Toronto, Ontario, M5G 1L6, Canada. J. E. Weiler and P. J. O'Brien, Department of Pathology, Ontario Veterinary College, University of Guelph, Guelph, Ontario, N1G 2W1, Canada.

<sup>\*</sup>Present address: Department of Biochemistry, Osaka University Medical School, Osaka, Japan. †Present address: Istituto di Patologia Generale, Universita di Ferrara, Ferrara, Italy ‡To whom correspondence should be addressed.

animals reacting to the commonly used halothane challenge test are of the N/n genotype, whereas a low percentage of those that do not react are of the n/n genotype (homozygous MH) (1, 19, 20). The T/T allelic pair was found at nucleotide 1843 in each of 48 Pietrain animals from an inbred herd. We have shown that all breeding stock used in development of this herd were n/n when progeny were tested with the halothane challenge test or the halothanecaffeine contracture test (19, 20). Of these 48, 11 were diagnosed in this study as n/n in a halothane-caffeine contracture test and 6 in the test for hypersensitive gating of the  $Ca^{2+}$ -release channel (3, 21).

The C/C allelic pair was found in 36 of 43 and the C/T allelic pair in 7 of 43 Yorkshire animals from a closed herd inbred for 10 vears for halothane resistance but originating in a commercial stock in which 0.78% had been found to be positive for the halothane challenge test (17). Of the animals

Table 1. Polymorphisms noted in the fulllength cDNA sequences (16) that encode ryanodine receptors from N/N Yorkshire and n/ n Pietrain swine. The nucleotide column identifies polymorphisms that were found by sequencing (the nucleotides 1,843, 4,332, and 13,878 were the only nucleotides confirmed by alternate tests); amino acid numbers that are potentially altered are given in parentheses. Alteration of nucleotide 1,843 deletes a Hin P1 site (GCGC to GTGC) and creates a Hgi AI site (GCGCTC to GTGCTC); of 4,332 creates a Ban II site (GAACCC to GAGCCC); and of 13,878 deletes an Rsa I site (GTAC to ATAC) in the MH haplotype. The Yorkshire and Pietrain columns identify the nucleotide polymorphisms (italic) within their respective codons and the effect of the polymorphism on the amino acid encoded. Nucleotides encoding MHN and MHS ryanodine receptors are numbered positively in the 5' to 3' orientation, beginning methioni beginning



Fig. 1. Nucleotide and deduced amino acid sequences of part of the exon in which the single Arg<sup>615</sup> to cysteine mutation was located (in the box). The forward primer used in PCR amplification corresponded to the sequence of nucleotides 1811 to 1834, which are overlined, whereas the reverse primer was complementary to nucleotides 1861 to 1864, which are also overlined. The alteration of the normal sequence GCGCTC starting at nucleotide 1842 to the mutant sequence GTGCTC led to the deletion of a Hin PI site and the creation of a Hgi AI site in the mutant sequence. A second polymorphism (C in Yorkshire or G in Pietrain at position 1878) is also noted. Abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F; Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.

studied, nine were diagnosed as N/N by the halothane-caffeine contracture test and found to be C/C in the DNA-based test. Two had intermediate halothane-caffeine contracture test results and were tentatively diagnosed as N/n and shown to be C/T in the DNA-based test. The finding of a T allele frequency of 8.1% in this inbred herd was correlated with the earlier finding of 8.8% for the allele in the Yorkshire population from which the herd was derived and 9.1% on the basis of our present halothanecaffeine contracture test results for 11 animals (17). Of 14 animals derived from crosses of Pietrain with Yorkshire, all had the C/T genotype, as did five proven halothane-resistant Landrace animals resulting from the mating of animals from inbred halothaneresistant and halothane-sensitive lines. A single Poland China pig, diagnosed as n/n by both breeding and halothane-caffeine contracture testing, had the T/T genotype.

We also applied the DNA-based test to 71 animals from commercial herds in which a low frequency of halothane-susceptible animals was known to exist, but in which the N/n and N/N animals were not distinguished phenotypically. We found 11 C/T and 21 C/C genotypes among 32 Durocs, resulting in a frequency for the T allele of 17.2%, where the predicted frequency was 14.4%, and a T allele frequency of 9.1% in 11 Poland China, where the T allele frequency was predicted to be comparable to that in Yorkshires (8.8%). We found no T alleles in 20 Hampshire pigs, correlating with the fact that MH Hampshires have never been reported in Canada. We also

Table 2. Haplotype and genotype analyses of the ryr1 gene in six swine breeds. PCR-amplified products that surround the mutation at nucleotide 1,843 (Hin PI) or polymorphic sites at nucleotides 4,332 (Ban II) and 13,878 (Rsa I) were analyzed for the presence (+) or absence (-) of appropriate restriction endonuclease sites (18). On the basis of the results of Hin P1 digestion, animals were grouped as T/T (proven n/n); C/T (probable N/n); and C/C (probable N/N). The Hin  $PI^-$  Ban II<sup>+</sup> Rsa I<sup>-</sup> haplotype was identical for the two T/T chromosomes in each individual in each breed, and therefore chromosomes were counted individually in the T/T row. The haplotypes of the probable C/T and C/C chromosomes differed among individuals and breeds and were counted as chromosome pairs. PC, Poland China.

Breed

inning with residue 1 of the ATG initiator thionine; amino acids are numbered inning with the same methionine.							-						Total
				Hin PI	Ban II	Rsa I	Pietrain	Yorkshire	PC	Duroc	Landrace	Hampshire	10(a)
						T/T							
Nucleotide (amino acid)		Valation	D'	-	+	-	18/18		2/2		4/4		24/24
		TOrkshire	rictrain		C/T								
·				+/-	+/-	+/-		6/9	5/6	8/10	4/11		23/36
276	(92)	TCC (Ser)	TCA (Ser)	+/-	+/+	+/-			1/6				1/36
,843	(615)	CGC (Arg)	TGC (Cys)	+/-	-/	+/-							
,878	(626)	CTG (Leu)	CTC (Leu)	+/-	+/-	+/+							
,942	(1,314)	GGT (Gly)	GGC (Gly)	+/-	+/+	+/+							
,332	(1,444)	GAA (Glu)	GA <i>G</i> (Glu)	+/	-/-	+/+							
,365	(1,455)	CCC (Pro)	CCT (Pro)	+/	+/-	-/-		1/9		1/10	1/11		3/36
,738	(2,246)	CAA (Gln)	CA <i>G</i> (Gln)	+/-	+/+	-/-		2/9		1/10	6/11		9/36
,563	(2,521)	CAT (His)	CAC (His)	+/-	-/-	-/-							
809	(2,603)	GTC (Val)	GTT (Val)		·	•		$C_{\ell}$	'C				
,811	(2, 937)	GCG (Ala)	GCA (Ala)	+/+	+/-	+/-			1/4	3/8		5/20	9/45
063	(3,021)	ACT (Thr)	ACA (Thr)	+/+	+/+	+/-			'	'			•
456	(3, 152)	CAG (Gln)	CAA (Gln)	+/+	-/-	+/-							
471	(3,157)	GTC (Val)	GTA (Val)	+/+	+/-	+/+		2/12					2/45
822	(3, 274)	ACG (Thr)	ACA (Thr)	+/+	+/+	+/+		•					
982	(3, 328)	CTG (Leu)	TTG (Leu)	+/+	-/	+/+		4/12	3/4	4/8	1/1	2/20	14/45
121	(3,707)	ACT (Thr)	ACC (Thr)	+/+	+/	-/-		•	,	1/8	•		1/45
171	(4,057)	TTT (Phe)	TTC (Phe)	+/+	+/+	_'/_		6/12		'		13/20	19/45
878	(4.626)	GTG (Val)	GTA (Val)	+/+	-/-	_/_		,				,	1
,		/	,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,		'	,							

Haplotype (genotype)

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276 1,843 1,878 3,942 4,332 4,365 6,738 7,563 7,809 8,811 9,063 9,456 9.471 9,822 9,982 11,121 12,171 13.878

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analyzed eight Landrace pigs that reacted to halothane, where N/n and n/n could not be distinguished phenotypically, and found five C/T and three T/T genotypes. Estimates of allele frequency based on phenotypic analyses were highly correlated with the genotypic analysis (P < 0.0001).

Our study illustrates that the same mutation in the ryr1 gene that is associated with MH in the Pietrain breed is also associated with MH in each of four other genetically distinct breeds of swine and is absent from a herd of Hampshires in which MH is absent. This suggests either that the mutation has been introduced into MH-susceptible breeds from a single common source or that the mutation has recurred and been selected for in each of these breeds. To see if the mutation is associated with a specific haplotype in all breeds, we analyzed the cosegregation of the mutation at nucleotide 1,843 with a polymorphism at nucleotide residue 4,332 (Table 1), which alters a Ban II site in a 160-bp exon in the ryanodine receptor gene (Fig. 2C), and with a polymorphism at nucleotide 13,878 (Table 1), which alters an Rsa I site in a 261-bp exon (Fig. 2D). By analogy with the human gene (22), the Ban II site is about 35 kbp and the Rsa I site 145 kbp downstream of the Hin P1 site.

The Hin P1 cleavage site, incorporating nucleotide 1843, is absent in inbred Pietrain DNA, the Ban II cleavage site is present, and the Rsa I cleavage site is absent, so that the

Fig. 2. (A) Detection of the  $\operatorname{Arg}^{615}$  to cysteine mutation by oligonucleotide hybridization. Autoradiographs show the binding of the two specific oligonucleotide probes to the 74-bp PCR-amplified product identified in Fig. 1 from n/n Pietrain, N/N Yorkshire, or heterozygous N/n individuals from Pietrain-Yorkshire crosses (left) and of individuals from Yorkshire, Poland China, Duroc, and Landrace herds (right). Oligonucleotide probe 17-C detected the MHN DNA sequence and oligonucleotide probe 15-T detected the MHS DNA sequence. Both probes bound heterozygote sequences. (B) Detection of the C to T muMH Pietrain haplotype is Hin P1<sup>-</sup> Ban II<sup>+</sup> Rsa I<sup>-</sup>. Data presented in Table 2 show that all diagnosed n/n individuals from Pietrain. Landrace, and Poland China breeds carried only the Hin P1<sup>-</sup> Ban II<sup>+</sup> Rsa I<sup>-</sup> haplotype. The Ban  $\Pi^{-/-}$  and Rsa  $I^{+/+}$  allelic pairs were excluded from all Hin P1+/- individuals, tentatively identified as N/n, so that all Hin P1<sup>+/-</sup> individuals in Yorkshire, Duroc, Landrace, and Poland China breeds contained a potential Hin P1<sup>-</sup> Ban II<sup>+</sup> Rsa I<sup>-</sup> haplotype. In Hin P1<sup>+/+</sup> individuals, tentatively identified as N/N in each of the six breeds, the Hin P1<sup>+/+</sup> genotype coexisted with several combinations of the Ban II and Rsa I polymorphisms, including Ban II<sup>+/+</sup> Rsa  $I^{-/-}$ , the genotype that was found for these two polymorphisms in all Hin P1<sup>-</sup> animals, and the Ban  $II^{-/-}$  Rsa  $I^{+/+}$ genotype, excluded from the Hin  $P1^{+/-}$  genotype. On the basis of these results, a common origin for the chromosome containing the T allele and, by association, a common ancestry for all of the MH animals in all five breeds are indicated.

Knowledge of this molecular alteration associated with MH and with a specific ryr1 haplotype has allowed us to develop a simple, accurate, and noninvasive test for the altered ryr1 gene that will make it possible to eliminate this gene from each of these breeds if that is desired. The high frequency of MH susceptibility in Pietrain, Yorkshire, Poland China, Duroc, and Landrace breeds of



tation at position 1843 by Hin PI cleavage in the 74-bp PCR product from porcine genomic DNA (18). Cleavage of the product generates 41- and 33-bp fragments. Lanes 1 and 2, Hin PI digestion of one allele (Poland China); lanes 3 and 4, digestion of both alleles (Duroc); and lanes 5 and 6, lack of digestion (n/n Pietrain). Digestion of the same samples with Hgi AI produces a mirror-image digestion pattern. (**C**) Detection of the A to G polymorphism at position 4332 by Ban II cleavage of a 160-bp PCR product from porcine genomic DNA (18). Cleavage of the product generated 124- and 36-bp fragments. Lanes 1 and 2, Ban II digestion of one allele (Duroc); lanes 3 and 4, lack of digestion (N/N Yorkshire); and lanes 5 and 6, digestion of both alleles (n/n Poland China). (**D**) Detection of the G to A polymorphism at position 13,878 by Rsa I cleavage of a 227-bp PCR product from porcine genomic DNA (18). Cleavage of the product generated 111- and 116-bp fragments that move in the acrylamide gel as a single band. Lanes 1 and 2, digestion of one allele (N/n Yorkshire × Pietrain); lanes 3 and 4, digestion of one allele (N/n Yorkshire × Pietrain); lanes 3 and 4, digestion of both alleles (N/N Vorkshire).

swine, however, may result from the widespread selection for leanness and muscularity in breeding stock. A clear demonstration of an association between a defective *ryr1* gene and lean body mass might make it advantageous to reintroduce the defective *ryr1* gene selectively into breeding stock (1) to yield F1 generation N/n market animals that might all have lean body mass (1) and acceptable meat quality (23).

Several deductions can be made concerning the structure and function of the altered ryanodine receptor. An alteration has been observed (8) in the tryptic cleavage pattern of the ryanodine receptor in which a 99-kD tryptic peptide from MHS pigs replaces an 86-kD tryptic peptide from MHN pigs. This alteration is consistent with the deletion of an exposed tryptic cleavage site in the abnormal 99-kD fragment that would give rise to 86- and 13-kD tryptic fragments in the normal sequence. Because it is probable that the Arg<sup>615</sup> to cysteine mutation we have detected is responsible for this altered tryptic cleavage pattern, we suggest that Arg<sup>615</sup> is located on the surface of the  $Ca^{2+}$ -release channel.

The MHS Ca<sup>2+</sup>-release channel is opened by Ca<sup>2+</sup>, adenosine trisphosphate, and caffeine at concentrations an order of magnitude lower than those required for the MHN channel, and inhibition of the MHS Ca<sup>2+</sup> channel by Mg<sup>2+</sup> and Ca<sup>2+</sup> is dampened (1, 3, 6, 21). It is, therefore, possible that  $\operatorname{Arg}^{615}$  is involved in the binding of regulators of Ca2+-channel gating and that its alteration leads to hypersensitive channel gating. This postulate is supported by analogy to a homologous Ca<sup>2+</sup>-release channel, the inositol 1,4,5-trisphosphate (IP<sub>3</sub>) receptor (24); IP<sub>3</sub> binds to the NH<sub>2</sub>-terminal part of the IP<sub>3</sub> receptor in a region homologous to that of the ryanodine receptor (25). There is 31% sequence identity between the 39 amino acids surrounding Arg<sup>615</sup>. This homologous region is therefore a candidate site for ligand binding in both receptors.

It is not clear that agents such as halothane or succinylcholine, which trigger MH episodes, act directly on the channel or that their action would be affected directly by the mutation. An alternative explanation for their action in MH is that anesthetics or neuromuscular and endocrine responses to stress raise the intracellular concentrations of physiological channel-gating agents to the point where they would trigger opening of the hypersensitive MHS Ca2+-release channel, but not of the MHN Ca<sup>2+</sup>-release channel (1, 3). Once opened, the MHS channel would be unresponsive to Ca<sup>2+</sup>- and Mg<sup>2+</sup>induced closing (3, 6), thereby inducing muscle contracture, hypermetabolism, and hyperthermia.

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- I. Harbitz et al., Genomics 8, 243 (1990). Polyadenylated [poly(A)<sup>+</sup>] RNA was isolated [D. H. MacLennan and S. de Leon, Methods Enzymol. 16. 96, 570 (1983)] from skeletal muscle from a proven N/N Yorkshire pig and from a proven n/n Pietrain pig. Complementary DNA was synthesized with the cDNA Synthesizing System supplied by Bethesda Research Laboratories (BRL), and we constructed cDNA libraries in  $\lambda$  gt10 (BRL) and  $\lambda$  ZAP (Stratagene) according to the manufacturer's instructions. We primed n/n Pietrain poly(A)<sup>+</sup> RNA (10  $\mu$ g) for cDNA synthesis with 100 ng of oligo(dT)<sub>17</sub>, a 24-nucleotide primer complementary to nucleotides 1243 to 1266 of the ryanodine receptor from human skeletal muscle (10) and 18 nucleotide primers complementary to nucleotides 3499 to 3516, 4900 to 4917, and 9123 to 9140 of the ryanodine receptor from rabbit skeletal muscle (9). We primed N/N Yorkshire  $poly(A)^+$  RNA (10  $\mu$ g) with the same set of oligonucleotides, plus 24 nucleotide sequences complementary to human nucleotides 4,000 to 4,023, 5,859 to 5,882, and 11,099 to 11,122. We used restriction endonuclease fragments from ryanodine receptor cDNA of human skeletal muscle as probes to screen porcine cDNA libraries [T. Maniprobes to screen porcine CDNA horards [1. Man-atis, E. F. Fritsch, J. Sambrook, *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Labora-tory, Cold Spring Harbor, NY, 1982)]. We used PCR [R. K. Saiki et al., *Science* 239, 487 (1988)] to isolate nucleotides 12,502 to 12,567 in Pietrain cDNA and nucleotides -24 to 448 in Yorkshire cDNA. All cDNAs were subcloned into the Bluescript vector (Stratagene) for sequence analysis [F. Sanger, S. Nicklen, A. R. Coulson, *Proc. Natl. Acad. Sci.* U.S.A. 74, 5463 (1977)] with the T7 sequencing kit (Pharmacia). Five independent PCR products were sequenced for the detection of potential artifactual sequences.
- 17. Pietrain and Yorkshire swine were obtained from closed herds with inbreeding programs controlled for more than 10 years. Selection of Pietrain breeders was based on production of offspring positive for

either the halothane challenge test or the halothanespecific contracture (HSC) test and the caffeine-specific contracture (CSC) test, the reference tests for diagnosis of MH susceptibility (1, 2, 19). Selection of Yorkshire breeders was based on negative halothane challenge tests and on the production of offspring negative for this same test. Swine were considered MH-susceptible if a 1-g increase in mus-cle tension occurred with less than 4 mM caffeine in the CSC test or if more than a 0.5-g increase in tension developed with 5% halothane in the HSC test; normal if a 1-g increase in muscle tension occurred with more than 7 mM caffeine and 5% halothane produced less than a 0.2-g increase in tension; and heterozygous if between 5 and 6 mM caffeine produced a 1-g increase in tension (26). To confirm the presence of a functional defect in the ryanodine receptor in MHS swine, we performed microassays for hypersensitive gating of the Ca<sup>2+</sup>-release channel (3, 21) on isolated skeletal muscle sarcoplasmic reticulum. Half-maximal Ca release occurred at less than 2 mM caffeine for all Pietrains and at greater than 7 mM caffeine for all Yorkshires. The estimate of the allele frequency of 8.8% for 24 Duroc swine unrelated to swine known to react to halothane is made on the basis of the assumption that the allele frequency is similar to that of Ortario Yorkshire swine [D. C. Seeler, W. M. McDonell, P. K. Basrur, *Can. J. Comp. Med.* 47, 284 (1983)]. This estimate was modified to 31.3%, however, for a group of eight Durocs, six of which were the offspring of two that had produced both offspring that reacted to halothane and offspring that did not react to halothane and were therefore heterozygotes. The combined T allele frequency for 32 Durocs was estimated to be 14.4%.

Genomic DNA was isolated from fresh pig blood or frozen pig muscle [S. A. Miller, D. D. Dykes, H. F. Polesky, *Nucleic Acids Res.* 16, 1215 (1988)]. For 18 analysis of the nucleotide 1843 mutation, a 74-bp sequence was amplified with 100 ng of the forward primer 5'-GTTCCCTGTGTGTGTGTGCAATGGTG-3', which corresponds to porcine ryr1 cDNA nucle-otides 1811 to 1834, and 100 ng of the reverse primer 5'-ATCTCTAGAGCCAGGGAGCAAGT-TCTCAGTAAT-3' in which the last 24 nucleotides were complementary to *ryr1* cDNA nucleotides 1861 to 1884. For analysis of the Ban II polymorphism at nucleotide 4332, a 160-bp sequence was amplified by PCR with the forward primer 5'-TACTATTACTCGGTGAGGGTCTTC-3', which corresponds to  $r\gamma r1$  cDNA nucleotides 4297 to 4320, and the reverse primer 5'-TGCTGTGGAT-GTTGCCCTGCTCAT-3', which is complementary to ryr1 cDNA nucleotides 4433 to 4456. For analto ryr1 cDNA nucleotides 4435 to 4450. For anal-ysis of the Rsa I polymorphism at nucleotide 13,879, a sequence of 227 bp was amplified by PCR with the forward primer 5'-ATGACATG-GAGGGTTCAGCAGCCG-3', which corresponds to ryr1 cDNA nucleotides 13,763 to 13,786, and the reverse primer 5'-CTTGAGGCAGITG-TAGCCTATGAT-3', which is complementary to ryr1 cDNA nucleotides 13,966 to 13,989. The ryr1 cDNA nucleotides 13,966 to 13,989. The primers were annealed at 52°C for 2 min, extended

at 72°C for 3 min, and denatured at 95°C for 40 s for 35 cycles, with a final cycle of 7 min for extension in a DNA thermal cycler (Perkin-Elmer Cetus). The Mg<sup>2+</sup> concentration was 1.5 mM for amplification of the mutant sequence and 1 mM for amplification of the Ban II and Rsa I sequences in the PCR reaction buffer (Perkin-Elmer Cetus). For hybridization analysis of the MH mutation, we applied 50 to 100 ng of DNA in duplicate to BA85 nitrocellulose filters with a Minifold II Slot Blot apparatus (Schleicher and Schuell, Dassel, Germany), following the manufacturer's instructions. Hybridization with the Truckeotide (nt) sequence 5'-TGGCCGTGCGC TCCAAC-3' (probe 17-C) and the 15-nt sequence 5'-GGCCGTGTGCTCCAA-3' (probe 15-T), end-labeled with <sup>32</sup>P, were at 45°C in 6× SSC (0.90 M sodium chloride and 0.090 M sodium citrate), 1× Denhart's solution, and 0.1% SDS. Filters were washed (with shaking) six times with 50 ml of 6× SSC and 0.1% SDS over the course of 30 min and once with 500 ml of the same solution at 61°C (probe 17-C) and 55°C (probe 15-T) for 20 min. After x-ray film was exposed to the filters, diagnosis was made on the basis of differential probe binding. Digestion of the PCR products with Hin PI, Hgi AI, Ban II, or Rsa I was carried out with standard protocols. Sequence analysis of three to eight sub-cloned 74-bp PCR products from each of the het-erozygous Yorkshire, Poland China, Duroc, and Landrace individuals confirmed the presence of the C to T change at position 1843 in each breed and its absence in a homozygous normal Hampshire animal.

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