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Protocol for the Sequence Analysis of Ryanodine Receptor Subtype 1 Gene Transcripts from Human Leukocytes

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Background: **The search for novel mutations in the ryanodine receptor subtype 1 (*RYR1*) gene causing malignant hyperthermia and central core disease is hampered by the fact that the gene contains 106 exons. Searching for novel mutations in complementary DNA (cDNA) requires an invasive muscle biopsy. Accordingly, an alternate source of *RYR1* cDNA was sought for sequence analysis.**

Methods: **Leukocytes were isolated from human blood and used for extraction of RNA and reverse transcription of messenger RNA into cDNA. A detailed protocol was developed in which overlapping fragments of *RYR1* cDNA were amplified by polymerase chain reaction in a series of steps and used for double-strand sequencing.**

Results: **The sequences of full-length leukocyte *RYR1* cDNA obtained from four human blood samples were shown to be identical to the sequence of a human muscle *RYR1* cDNA. The incidence of aberrant splicing was more pronounced in the blood-derived cDNAs, but this could be minimized by adequate sample preparation. Protocols to sequence alternatively spliced products were also developed. Several silent nucleotide polymorphisms were detected, and minor revisions were made to the *RYR1* sequence.**

Conclusions: **Because there are no differences in *RYR1* transcript structure between muscle and leukocytes, aside from those that may be ascribed to RNA splicing aberrations during processing, leukocytes seem to be an adequate substitute tissue for screening the *RYR1* gene for previously undiscovered mutations in families with malignant hyperthermia or central core disease.**

SEVERAL muscle diseases have been associated with mutations in Ca^{2+} release channels of the sarcoplasmic reticulum, referred to as ryanodine receptors.[1–9] The ryanodine receptor subtype 1 (*RYR1*) gene[10] on chromosome 19q13.1[11] is linked to malignant hyperthermia (MH) and central core disease (CCD). More than 50 individual mutations in *RYR1* have been detected in patients with MH and/or CCD to date[12–16] and are mapped to three compact regions of the sequence, referred to as MH/CCD domains. However, there is no prevalence of particular mutations in all populations, and many mutations have been found only in single families. A comprehensive scan of the 106 small exons of the *RYR1* gene is expensive and time consuming, so MH/CCD domains have been investigated intensively. Sequencing of the spliced *RYR1* messenger RNA (mRNA) from muscle is less laborious, but involves an invasive muscle biopsy. Although muscle is usually available if an *in vitro* contracture test has been performed within the recent past, this is not the case for many patients. Characterization of the entire spectrum of MH-causative mutations in humans is a prerequisite for streamlined genetic analysis,[17,18] based on the detection of single-nucleotide polymorphisms, made possible by the completion of the human genome sequence.

The expression of *RYR1* in cultured B lymphocytes has been demonstrated previously.[19] Although the concentration of this protein in blood is very low, we succeeded in developing a protocol for the amplification and sequencing of human *RYR1* complementary DNA (cDNA) starting from a standard 7-ml blood sample using reverse transcription, followed by amplification by the polymerase chain reaction (RT-PCR). This noninvasive procedure permits a rapid search for new mutations. The principles of the protocol might have wider application because they should be suitable for mutation screening of any low-abundance mRNA expressed in leukocytes.

Materials and Methods

Blood Collection and Conservation

Following institutional ethics approval (University of Toronto, Toronto, Ontario, Canada) and individual informed consent, blood samples were collected in 7-ml tubes containing potassium-EDTA (Vacutainer; Becton Dickinson, Franklin Lake, NJ) and placed on ice. The sample was diluted into 20 ml ice-cold erythrocyte lysis solution containing 150 mM NH_4Cl, 10 mM KH_2CO_3, and 0.1 mM EDTA in a 50-ml polypropylene tube with a conical bottom, mixed three times by gentle inversion, and kept on ice for at least 10 min. Lysis was monitored by a visible decrease in turbidity. The suspension was centrifuged for 10 min at 1,500*g*, and the supernatant was carefully discarded. For storage and shipment at room temperature, the pellet was suspended gently in 1 ml RNALater (Ambion Inc., Austin, TX), and transferred into a screw-cap 1.5-ml tube.

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Readers interested in this topic should also see the article presented on page 297 of this issue (Loke JCP, Kraev N, Sharma P, Du G, Patel L, Kraev A, MacLennan DH: Detection of a novel ryanodine receptor subtype 1, mutation (R328W) in a malignant hyperthermia family by sequencing of a leukocyte transcript. ANESTHESIOLOGY 2003; 99:297–302).

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Isolation of Total RNA from Pelleted Leukocytes

All plastic ware was DNAase/RNAase-free, and all pipette tips contained aerosol filters to prevent contamination of the RNA sample. Reagent grade water, certified RNAase-free, was purchased from Fluka (Buchs, Switzerland). For isolation of total RNA, the leukocyte suspension in RNA-Later was centrifuged at 1,500 × *g* for 2 min. The pellet was homogenized in 1 ml Trizol reagent (Invitrogen, Ontario, Canada), using 15–20 strokes of a 5-ml syringe attached to an 18-gauge needle. A clear, brownish, nonviscous solution indicated successful cell lysis and chromosomal DNA fragmentation. The solution was transferred into a 1.5-ml Eppendorf tube, 0.2 ml chloroform was added, and the sample was mixed by gentle inversion and centrifuged for 5 min at 14,000 rpm in an Eppendorf (Eppendorf AG, Hamburg, Germany) centrifuge (model 5417C). The clear upper phase (approximately 0.5 ml) was transferred into another 1.5-ml polypropylene tube, and RNA was precipitated by the addition of 0.5 ml isopropanol with 50 μg glycogen (Roche Diagnostics, Laval, Quebec, Canada) as carrier. The pellet was washed with 300 μl ethanol (70% v/v) and air dried. Finally, the pellet was allowed to dissolve in 30 μl RNAase-free water overnight at room temperature and subsequently stored at −20°C. The yield of total RNA was approximately 5–10 μg. Extensive mixing was avoided at all steps following leukocyte lysis to prevent shearing of the *RYR1* transcript.

Isolation of RNA from Muscle

RNA was isolated from frozen muscle biopsies (100–400 mg) by homogenization directly in Trizol at 50°C, essentially using the manufacturer's recommendations, or by the guanidinium-acid phenol method.[20] PolyA$^+$ RNA was isolated from 100–250 μg total RNA using oligo-dT20 latex beads (Oligotex kit; Qiagen, Mississauga, Ontario, Canada), according to the protocol of the manufacturer, eluted in 50 μl elution buffer, and stored at −20°C.

RT-PCR Amplification of RYR1 *mRNA*

For cDNA synthesis, two solutions were prepared in 0.2-ml thin-wall PCR tubes on ice. Tube 1 contained 5 μl RNA preparation, estimated to contain 1 μg total RNA. Tube 2 contained 4 μl 5X reaction buffer (250 mM Tris-HCl, pH 8.3, 375 mM KCl, 15 mM $MgCl_2$), 2 μl dithiothreitol (0.1 M), 2 μl deoxynucleotide triphosphate (10 mM), 1 μl random nonamers (100 pmol; New England Biolabs, Beverly, MA), 6 μl saturated trehalose (Sigma Aldrich Canada, Ontario, Canada),[21] and 1 μl Expand reverse transcriptase (Roche Diagnostics). Tube 1 was placed into a thermocycler (PCR Sprint Thermo Hybaid, Middlesex, United Kingdom) for 2 min at 70°C before 4 μl denatured RNA was added to tube 2 and mixed by gentle pipetting. The cDNA synthesis was performed at 45°C during 1 h. The cDNA can be stored at 4°C for several weeks.

Aliquots of the cDNA synthesis reaction were used in either a single-stage PCR (using polyA$^+$ RNA or total RNA from muscle) or a two-stage nested PCR (using total RNA from leukocytes) to amplify 1- to 2.5-kb fragments of the *RYR1* coding sequence. The reaction mixtures with final volume of 50 μl containing 250 μM deoxynucleotide triphosphate, 0.3 μM primers, 1X reaction buffer 1 (supplied with the enzyme), and 5% dimethyl sulfoxide (DMSO) were assembled on ice and overlaid with 40 μl oil. The cycling program was started at 95°C, paused to allow the addition of 2-μl aliquots of cDNA to each tube, and, after 2 min at 95°C, paused again while 1 μl Expand Long Template polymerase mixture (Roche Diagnostics Canada) was added to each tube. The cycling program was resumed after this "hot-start" process. The reactions were subjected to 45 cycles of 15 s at 95°C and 2 min at 70°C, followed by a 70°C hold. During the first stage of cycling, the second-stage reaction mixes were assembled (where applicable). They had the same composition as the first-stage mix, except for a different, nested primer pair. At the end of the first stage, second-stage tubes were placed into the heating block, and the program was resumed. As soon as the block reached 95°C, 1- to 2-μl aliquots of the first-stage reactions were added into the corresponding second-stage reaction mixtures, followed by 1-μl aliquots of the Expand enzyme. The same cycling program was performed, using the hot-start method, for another 45 cycles. Each reaction was resolved on an 18-mm-wide slot of a 1% agarose gel, and the main band of appropriate length was excised and purified on a Qiagen Gel Extraction kit (Qiagen Verto, NV, The Netherlands). The primers for the PCR amplification are listed in table 1.

Cloning of PCR Products

Where indicated, 5-μl aliquots of the purified PCR products were cloned into Bluescript-Xcm vector[22] according to established procedures.

DNA Sequence Analysis

The gel-purified PCR products were sequenced (see tables 1–3 for the primer sequences) using a Big Dye Terminator Sequencing Kit (Perkin-Elmer, Foster City, CA). The reaction mixture in a 0.2-ml thin-wall tube contained 0.2 pmol gel-purified PCR product, 10 pmol sequencing primer, and 4 μl sequencing kit reagent mixture in 10 μl. All reactions contained 5% DMSO. The reactions were subjected to denaturation for 5 min at 95°C and 25 cycles of 15 s at 95°C and 4 min at 60°C. Each fragment was sequenced with a set of six to eight primers placed approximately 650 bases apart on each strand (fig. 1) and having a predicted annealing temperature of 60°–63°C (internal primers) or 68°–72°C (for 5' and 3' terminal primers). The reactions were purified according to the kit manufacturer's recommendations and resuspended in 3 μl deionized formamide. One-third of the reaction mixture was analyzed on the ABI 377 sequencer (Applied Biosystems, Foster City,

Table 1. The List of Primers Used to Amplify the Complete Ryanodine Receptor Subtype 1 Transcript in 10 Overlapping Fragments

Fragment/Stage	Forward Primer 5′–3′	Reverse Primer 5′–3′
1		
1	TCTGTCCAGCATGCGTGTACTCCTCGCAGT	GCCCTGGTCCTCGGTGAGCGCTAGGTACT
2	TCTGTCCAGCATGCGTGTACTCCTCGCAGT	CTGATTCTCAGTGGCTCCAGCCTCCAGA
2		
1	GTCACGTCCTCCGCCTCTTTCATGGACAT	GACACCCTGCACAGGGCAGCCGTTGA
2	CTATGAGGGGGGAGCTGTGTGCACTCAT	AGGTCCAGGCAGCAGCTGATCACGTCTTCA
3		
1	GTACTTTGAGGTGATGGTGGACGAGGTGAC	GAGGCTGCTGTGGACGTTGCCTTGTTCA
2	TCCATTTCTGACAGCTCAGGCCACCCACTT	ATGGTCACCGTCACGACCCGGACCTTG
4		
1	CGGAGAATGAGAAGGATGCCACCACCGA	AGGAGGCTGAACATGGCCCGCACCA
2	AGCTCGGGGCTCTGCACGAAGTCCTCTT	GCTTCTTATTCAAGGCCAAGAAGGTCGCCA
5		
1	CTGTCCTCTCCCTGAAGAGATTCGACAGGAT	CCGCAAACTTGTTAATGAAGGAGTCCAGCTTCT
2	GACCTGCTGGCACACTGTGGAATTCAGCTA	CGGGATGATCACATTGAGGGTCTCCACAG
6		
1	CCTACCCACGGGCTGGGCCAACTTC	GCGCTTGAAGTTGTGGGACTTGGACCAGTA
2	GTCACCTCAGAGGAGGAGCTGCACCTCACA	GAAGATCTCGCCCACCATCCTGAACAGCTC
7		
1	GCACAGCCCATTGTGAGCCGTGCAC	GATGTCGCGTGCTGGCTCCTGGAAG
2	GGAGCTCCTGCAGTCCCACTTCATCCCAAC	CGGTTGGCGAACTCTTCGCAGTTGATCATT
8		
1	GCTGTCGCTACTAGAAGGGAACGTGGTGAACGGCA	TCCGGGACAGGTAGTTCAGGAACTTCACCCTCTG
2	ATGGTGGACATGCTCGTGGAATCCTCATCCAATGT	ACCTCCAGTTCTCCCCAGAATTCGCCTCCA
9		
1	CCACACCCGAGGGCTCTCCCATCCTCAAGA	CAGTACTAGGTCACCTCTCCCCCAGCTGCCTT
2	AGTGGAGGAGGAGCTCCCGCCAGAGCCAGA	GGGCTTGCTGTGAGAATAAGGCACTTGAGGT
10		
1	ACACTAGAGATCACAGCCCACAATGAGCGCAA	CAGTACTAGGTCACCTCTCCCCCAGCTGCCTT
2	ACACTAGAGATCACAGCCCACAATGAGCGCAA	GGGCTTGCTGTGAGAATAAGGCACTTGAGGT

CA). Sequence data were analyzed with Sequencher 3.1 (Gene Codes, Ann Arbor, MA).

Rapid Amplification of cDNA Ends Experiments

Rapid amplification of cDNA ends was performed essentially according to Frohman *et al.*[23] Briefly, 0.5 μg deoxyadenosine-tailed cDNA was annealed to 5 pmol Not-dT20 anchor primer (Amersham-Pharmacia Biotech, Piscataway, NJ) and extended for 10 min at 45°C in a PCR reaction mixture. Amplification was then continued for 45 cycles of 15 s at 95°C and 2 min at 70°C in 5% DMSO, using an *RYR1* exon 9–specific primer and an anchor-specific primer. The 1-kb resultant product was sequenced using an internal antisense primer located 380 bases downstream of the start codon.

Results

Amplification Strategy for the Complete RYR1 *Transcript*

Our initial intent was to test the feasibility of sequencing the three MH/CCD domains: exons 1–19 (MH1), 38–47 (MH2), and 98–106 (MH3) from leukocyte mRNA, as shown in figure 1. Screening of 12 blood samples with this primer set was shown to be reproducible, so a set of primers was designed to amplify the entire 15-kb transcript using an overlapping series of fragments averaging approximately 2 kbp in length (fig. 1). Although one-step amplification of 1- to 2-kb segments of *RYR1* mRNA from total muscle RNA posed no problem, amplification from leukocyte RNA under the same conditions failed. Consequently, a nested, two-round PCR approach was adopted, which was developed into a reproducible procedure.

To boost the sensitivity and specificity of the PCR amplification, primers were designed[24–26] with an annealing temperature of 68°–72°C to allow fast cycling between 95°C and 70°C. Presumably, this modification decreased the gradual loss of polymerase activity during the reaction, effectively increasing the reaction yield. The inclusion of DMSO, a two-enzyme DNA polymerase mixture, a hot-start PCR, a 70°C hold step between the nested PCR stages, and a two-step cycling profile all contributed to the success of the procedure.

When a number of samples from both blood and muscle were processed to the stage of a complete DNA sequence, it was found that standard Big Dye Terminator chemistry produced ambiguous band patterns or an abrupt transition to a very low peak intensity in certain regions of sequence. These problems could be alleviated by modifications of sequencing chemistry and by procedures taking account of alternative splicing.

Table 2. Internal Primers Used to Obtain Complete Double-Strand Sequences of Fragments 1–9

Fragment Name	Primer sequence 5′–3′
1	CTGCGCTGGTCATCACTGTCAGCA
	CCAGCCTCCACCGTGTTAGCCAGCATC
	GAGCCCACTAGCAACGCGCAGA
2	GGAGTGGGAGCCACCTGCGCT
	CCCATCGAGGGCGTTATCCTGA
	GTCCTCATTGAGAGTCCAGAGGTTCTGAAC
	GGTTGGAGCGTACAGCCACACCAT
	AGGCTTCGCAGCTTGCTCTGCTTCT
3	CTGCCCTGTGGACACTGTCCAGAT
	CCTCCTGGGCTACGGCTACAACAT
	ATGAGGTATCCCGAGTGGACGGCA
	CGGAAGTGCTGGTGGAACTGGACT
	ATCTCGCCTGTGGTGACTGCTTCA
	GAAGTCCACAAGACACGGGTGCAG
4	CGCCATGTTCCAAAGCGAGCGCAA
	GGGAGTTGGAGTCACCACTTCGCT
	GTGCCACCTGCTGGAGTATTTCTGTGACCA
	TTTATGAGGAGGCCATAGCGGCTC
	CCCAGCATCCTCAGTGCCTTGTC
	GCCTCGTCTCCACCTGCAGGAAGT
5	GGTCATGGAGGTCATGGTCAACGTC
	GAGCACTTTGGTGAGGAACCGCCT
	CTGCATACCGTGTACCGCCTGTC
	GCGCAACAGGTGCTGCAGCATC
	TGGTGCACAGCGTCCGAGCAGGT
	CGCTGGTTCTGCCGGCTGATTC
6	AGGCCATGGCAGAACAACTGGCAGA
	AACTTGCTGCTCTCGTCCGCCA
	CTGGAGCGGCTCATGGCAGACA
	TGCATAGCATGGGCAGCGTGA
	GACTTCATCACTGTCCTGGCATCCA
	GGTAATTTTCTGCCAGTTGTTCTGCCA
7	GGTACTCTGTGCAGACGTCACTGATCGT
	TGCACCAGTTGGTCCTGCACTTCA
	GGAGAGAAGGTCATGGCGGATGA
	CAAAGGCATTGAGATCCAGGACGCT
	CTCCAGGTGGCAGCTCTTTGCCAT
	TGTTGTCAGCAGTCAGGAAGGACATGT
8	AGCGCATCTACTTCGAGATCTCAGAGACCA
	GACGGTGACCGAGCTCCTGGCA
	CACCGCCTCCTCCTCGTCTCCA
	GATCTGCGCGGCGATCTGCATC
9	TGAAGATGACCCCGAACTTCCAGA
	CGAGCTCTACAGGGTGGTCTTCGAC
	CGTCGATGATCAGACCCTGGATGA

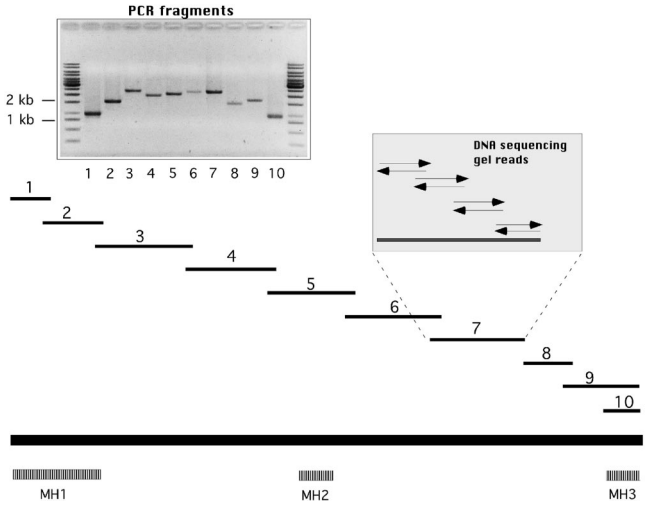

Fig. 1. Strategy for RNA-based analysis of the human ryanodine receptor subtype 1 gene. Three regions of high occurrence of mutations associated with malignant hyperthermia susceptibility are shown as *filled bars* designated MH1, MH2, and MH3. The messenger RNA is shown as a *solid bar* with *lines* above it, indicating polymerase chain reaction (PCR) fragment positions. The *caption* above one of the fragments illustrates the general scheme of primer positioning, with sequencing gel reads shown as *arrows* in two directions. An example of gel-purified PCR fragments is shown at the upper left.

Modifications of the Sequencing Chemistry

The quality of gel reads, especially in the MH1 region, was greatly improved with the use of a modified Big Dye Terminator chemistry on an ABI 377 sequencing instrument. Specifically, the problem of a band pattern being absent from a particular primer or an abrupt loss of signal intensity in specific areas was alleviated by the use of a two-step cycling profile in the presence of 5% or 10% DMSO in the sequencing protocol. Composite data from the two complementary strands were found to be reliable and required only minor manual editing when the length of the gel read did not exceed 700 bases. Any remaining areas of ambiguous base calls were shown to reflect alternative or erratic splicing of the original mRNA template.

Structure of the RYR1 *Transcript in Leukocytes*

Using the strategy outlined above, complete *RYR1* transcript sequences were obtained from one muscle sample and four blood samples from unrelated individuals. In limited areas of every sample, accurate base determination was seen only up to a point, beyond which two or more sequence profiles were superimposed. This phenomenon was observed regardless of whether the sample originated from muscle or blood. However, although in muscle samples this phenomenon was limited to the sequences of exons 70 and 83, in blood samples additional problem sequencing areas were present in a

Table 3. Flanking Primers Used to Sequence Skipped Exons

Exon	Forward	Reverse
9	GTCACGTCCTCCGCCTCTTTCATGGACAT	GCCCTGGTCCTCGGTGAGCGCTAGGTACT
26	CCTCCTGGGCTACGGCTACAACAT	CGGAAGTGCTGGTGGAACTGGACT
31	GCTTCTTATTCAAGGCCAAGAAGGTCGCCA	GAAGTCCACAAGACACGGGTGCAG
70	TCAACAACATGTCCTTCCTGACTGCTGACA	CGCTTCTTCTTGGTGCGTTCCTGGT
83	ATGCCTTTGAGAGACAAAACAAGGCCGA	GGAATCGGAACAGGTCTTGTGTGAATTCATC
94	AGTGGAGGAGGAGCTCCCGCCAGAGCCAGA	TGAAGATGACCCCGAACTTCCAGA

Table 4. List of Splicing Aberrations Observed during Sequencing of the Four Ryanodine Receptor Subtype 1 Transcripts Isolated from Leukocytes

Exon	L1	L2	L3	L4
9	IS	IS	IS	IS
26	PT	C	C	C
31	IS	IS	IS	IS
51/52	PIR	C	C	C
61	CS	CS	C	C
70	IS	CS	CS	CS
83	IS	IS	CS	C
94	IS	IS	IS	IS

Abbreviations: The columns represent data from leukocyte RNA from four individuals, L1–4.

C = correct exon inclusion; CS = complete exon skipping; IS = incomplete exon skipping; PIR = partial intron retention; PT = partial exon truncation.

quasi-random fashion (table 4). These observations suggested that alternative splicing, previously demonstrated in two specific areas of the *RYR1* transcript,[27] might be occurring in other areas of the *RYR1* transcript expressed in leukocytes.

Among the alternative splices that we observed, only the skipping of exons 9, 70, and 83 would retain the reading frame. All others were predicted to disrupt the reading frame, resulting in a severely truncated protein. Because it is unlikely that these truncated transcripts are produced *in vivo*, we believe that exon skipping is an artifact produced in processing of the blood sample, a recently recognized phenomenon.[28,29] The extent of skipping of exon 31 that was observed in most blood RNA samples was shown to increase with the time that the blood sample was stored and be minimal if the blood was stored on ice immediately on being drawn (fig. 2).

Because immediate storage on ice may not always be possible in a clinical setting, we designed additional primer pairs that flanked the relevant exons, so that they would amplify short (150–600 bp) DNA fragments. The products that included the spliced exon were then isolated by polyacrylamide or agarose gel electrophoresis and sequenced (fig. 3).

Single-nucleotide Polymorphisms in the RYR1 *Transcript*

The sequence of the *RYR1* gene contains a number of single-nucleotide polymorphisms. Two reference sequences of the *RYR1* transcript have been generated by the Genbank Reference Sequence Project.‖ One was compiled from data available in the literature (Genbank entry NM_000540); the other was compiled by *in silico* splicing of genomic sequences generated by the Human Genome Project (entry XM_009145). Both of these entries are generated from cloned, single-strand sequences, so that part of the allelic information is lost. In this study, the *RYR1* sequence was generated by direct sequencing of one muscle transcript and four independent transcripts from leukocytes. Consequently, heterozygosity was defined for a number of alleles (table 5). Although most of the sites of discrepancy between the reference sequences and ours seem to exist as silent allelic single-nucleotide polymorphisms, two specific sites may represent previous sequencing errors. In comparison with published consensus sequences, these represent a transversion at position 5495 and a GC→CG inversion at position 7647–7648, which were found in all individuals sequenced in this study (table 5).

Point of Initiation of RYR1 *Transcription*

The region upstream of the start codon, as it is currently defined, is unsuitable for interaction with amplification primers because of its extremely guanine-plus-cytosine–rich base composition. However, previous analyses of human *RYR1* cDNAs may not have provided adequate documentation of the 5' region. In our rapid amplification of complementary ends experiments, we were able to extend the transcription start in muscle by seven bases, the transcript now starting with the sequence 5'-AGTTCCA/TCTACCTCG- (a slash denotes the previously assigned start). This longer sequence agrees better with data available for mouse, which has a longer 5' end (fig. 4). The fact that there are three noncomplementary cytidine residues in the sequence is consistent with their addition to the end of the transcript during reverse transcription.[30] This supports the view that this is the authentic 5' end of the mRNA template.

This additional sequence was used to design a primer that would permit amplification of a fragment across the start codon that would be suitable for sequencing. Attempts to perform rapid amplification of cDNA ends on RNA from leukocytes, using the same set of *RYR1* gene-specific primers, were unsuccessful. Thus, the 5'-un-

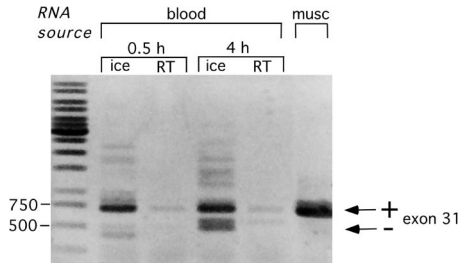

Fig. 2. The effect of storage temperature on exon 31 skipping in ryanodine receptor subtype 1 transcripts was analyzed by amplifying the area of the transcript corresponding to exon 31 at different times following blood collection. A fresh muscle sample frozen immediately in liquid nitrogen was used for comparison. The samples processed at room temperature show a severe decrease in yield of the 750–base pair product. Storage, even on ice for 4 h, enhanced skipping of exon 31, resulting in an increase in the amount of the 500–base pair product, which interferes with automated sequencing. RT = room temperature.

‖ Genbank Reference Sequence Project. Available at: http://www.ncbi.nlm.nih.gov/RefSeq/. Accessed June 24, 2003.

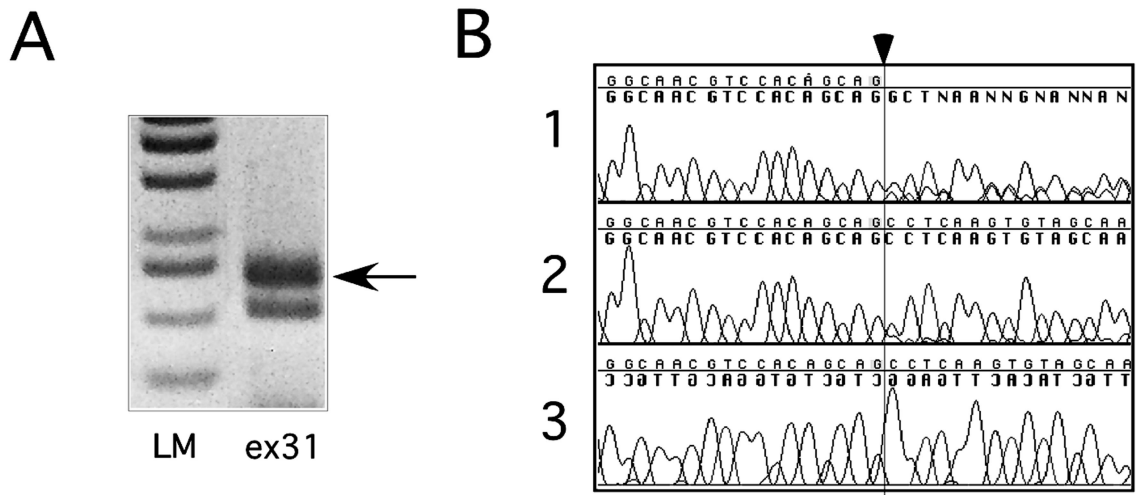

Fig. 3. An example of the resolution of polymerase chain reaction fragments, resulting from partial exon 31 skipping in ryanodine receptor subtype 1 messenger RNA. (*A*) Fragments amplified from a pair of primers, flanking exon 31, which were resolved on 1% agarose gels. The band excised for sequencing is shown by an *arrow*. (*B*) Sequencing gel traces obtained from a 2-kb fragment, which includes all of exon 31 (*line B1*); *lines B2* and *B3* show sequences of complementary strands from a gel-purified shorter subfragment. Note the ambiguous base calling in line 1, which starts at the left border of exon 31. The sequences of other skipped exons were rescued by a similar approach (not shown).

translated region of the *RYR1* transcript in leukocytes might be different from that in muscle.

Discussion

Constraints of Blood-based Mutation Detection Procedures

A clinically relevant blood-based mutation detection procedure is placed under logistical constraints: successful *RYR1* analysis requires routine, reproducible sample collection, which is as simple, effective and inexpensive as possible; steps involving isolation of RNA and amplification of DNA must be performed in a laboratory equipped to use molecular biologic techniques; DNA sequence analysis is best performed in a dedicated sequencing facility to minimize overall cost. This study demonstrates that sequence-based mutation analysis is feasible for the low-abundance *RYR1* transcript found in

Table 5. List of Single Nucleotide Polymorphisms, Detected during Direct Ryanodine Receptor Subtype 1 Transcript Sequencing

			Sequence Source							
Exon	Position	Effect	HGP	LS	db_SNP	M1	L1	L2	L3	L4
7	573	SIL	C	C	T	C	C	C	C	C
7	594	SIL	A	G		R	R	R	R	G
9	742	ALT	G (Gly)	G	A (Arg)	G	G	G	G	G
11	1077	SIL	T	C		Y	Y	Y	C	Y
15	1668	SIL	G	A		G	A	R	R	R
19	2286	SIL	C	T		Y	Y	T	C	C
24	2943	SIL	G	A		A	R	A	G	G
24	2979	SIL	C	C		C	Y	C	C	T
26	3456	SIL	C	C		C	Y	C	T	T
35	5625	SIL	A	G		A	A	A	A	A
34	5495	ALT	G (Gly)	C (Ala)		G	G	G	G	G
36	5988	SIL	C	T		C	C	C	C	C
47	7584	SIL	C	C	T	C	C	C	C	C
48	7647	SIL	G	C		G	G	G	G	G
48	7648	ALT	C (Leu)	G (Val)		C	C	C	C	C
49	7872	SIL	C	C	T	C	C	C	C	C
53	8337	SIL	G	G		G	G	G	G	A
82	11547	SIL	G	A		G	G	G	G	G
89	12147	SIL	A	A		A	A	A	R	A

Reference sequences of the *RYR1* transcripts were taken from the Genbank entries XM_009145 (column HGP), NM_000540 (column LS) and from Genbank SNP database (db_SNP). Other columns contain experimentally derived data from patients, each designated by a four-digit number. Other columns contain experimentally derived data from patients, designated by M1 (muscle RNA) and L1– L4 (leukocyte RNA). SIL denotes a functionally silent base substitution. ALT denotes a substition altering the amino acid, in which case the amino acid change is indicated in parentheses.

A = adenosine; C = cytosine; G = guanosine; R = A or G heterozygote; T = thymidine; Y = C or T heterozygote.

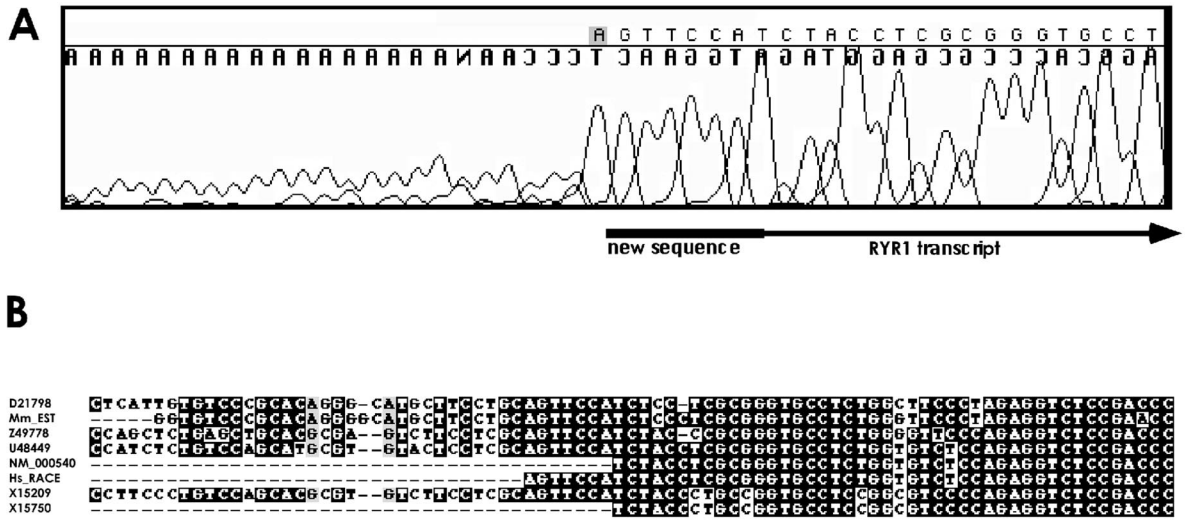

Fig. 4. Reassignment of the 5' end of the human ryanodine receptor subtype 1 transcript. (*A*) Portion of the sequencing trace showing the junction of the transcript end and the polyA tail attached during the rapid amplification of complementary ends (RACE) procedure. Between the polyA tail and the specific sequence are three noncomplementary cytidine residues that are appended during reverse transcription (see text). (*B*) Alignment of genomic and transcript sequences of ryanodine receptor subtype 1 (*RYR1*) genes of several mammals. All sequences, except mouse EST BB608312 (designated Mm_expressed sequence tag) and the sequence obtained in this work (Hs_RACE), are designated by their Genbank accession numbers.

leukocytes. In its current formulation, however, it is not suitable for high throughput application.

RT-PCR Amplification Protocol for Low-abundance Transcripts

Our protocol for successful amplification of the low-abundance, 15-kbp *RYR1* transcript begins with a nested "long" or "marathon relay" RT-PCR strategy, which generates a set of overlapping fragments with an average length of 2 kilobases. For cDNA synthesis, we use a genetically modified reverse transcriptase (Expand reverse transcriptase) with random nonamer primers in the presence of trehalose and, for the PCR step, we use the Expand Long Template enzyme mixture. A hot start and a 70°C hold step between the two stages of the nested PCR were found to be critical to specificity. The success of the procedure was highly dependent on the use of a high-quality thermocycler with a ramp speed in excess of 2°C/s and on two-step cycling profiles, manner of reagent addition, and reaction mixture composition. The optimized procedure permits sequencing of the entire transcript from a few micrograms of input RNA or less than a milligram of muscle and is therefore compatible with recently proposed novel tissue sources for physiologic diagnosis of MH, such as needle biopsies or cultured lymphoblastoid cells.[31–33]

The complete 15-kb *RYR1* transcript can be sequenced directly, except for areas where aberrant exon skipping occurs. Analysis of these areas may require individually optimized protocols. Modifications of the sequencing chemistry to deal with difficult-to-sequence GC-rich regions in the transcript made possible the automated sequencing of the *RYR1* transcript. Our data show that the *RYR1* transcript structure does not differ in muscle and peripheral blood cells, demonstrating that leukocytes represent an adequate substitute tissue for mutation searches in *RYR1*.

Revisions to the RYR1 *Transcript Structure*

In this study, a new reference sequence of the *RYR1* transcript was generated by combining sequence data from uncloned PCR products from five unrelated individuals. New single-nucleotide polymorphisms and two apparent sequencing errors (table 5) were discovered, together with revisions to the 5' end of the transcript. The 5' end and the start site of the human *RYR1* transcript[34] were derived by analogy to the published sequence of rabbit *RYR1*.[35] Using rapid amplification of cDNA ends technology, we were able to demonstrate that the human muscle polyA$^+$ mRNA is seven nucleotides longer at the 5' terminus than was suggested by previous data. Our results also increase the possibility that the porcine transcription start site, assigned experimentally by primer extension,[36] is based on a potential error resulting from strong secondary structure in this region.

Blood Sample Preparation, RNA Stability, and Splicing

Although our protocol for sample preparation for RNA-based *RYR1* sequence analysis is adequate for clinical use, splicing artifacts can occur if precautions are not taken. Studies of RNA splicing (for reviews see Hastings and Krainer[37] and Caceres and Kornblihtt[38]) revealed its regulation by extracellular cues. For example, on activation of B and T lymphocytes during the immune re-

sponse, a variant CD44 mRNA is synthesized, wherein inclusion of an additional exon requires activation of the MEK–ERK pathway.[39] We and others[28,29] were able to demonstrate that the extent of exon skipping increases during storage of samples. It is conceivable that aberrant splicing of RYR1 transcript occurs as a consequence of changes in the environment in which the leukocytes are maintained during blood storage at ambient temperature as well as a result of their relatively harsh treatment in the erythrocyte lysis solution.

However, because exon skipping was partial, correct sequences from these regions could be obtained with appropriate modifications of the strategy. In any case, the search for novel mutations by direct transcript sequencing requires threefold fewer primers (64 *vs.* 212) and sixfold fewer PCR reactions (18 *vs.* 106) than would be needed to obtain the same information from genomic DNA.

In summary, we have developed a method for direct sequencing of the full-length leukocyte *RYR1* cDNA. Our studies show that there are no differences in *RYR1* transcript structure between muscle and leukocytes aside from RNA splicing aberrations that can be overcome by appropriate sample preparation. As demonstrated in the accompanying article,[40] leukocytes seem to be an adequate substitute tissue for screening for novel mutations in the *RYR1* gene in families with malignant hyperthermia and central core disease.

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