Evaluation of a short interspersed nucleotide element in the 3' untranslated region of the defective dystrophin gene of dogs with muscular dystrophy

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Objectives—To determine the distribution of a 231-base pair (bp) element in the dystrophin gene 3' untranslated region (UTR) in a colony of Golden Retrievers with muscular dystrophy and other unrelated dogs and to estimate the frequency of recombination for the canine dystrophin gene.

Animals—77 dogs from the Golden Retriever Muscular Dystrophy (GRMD) colony at the Murdoch Veterinary School and 30 unrelated dogs from the Murdoch University Veterinary Clinic.

Procedure—Samples of blood or hair from dogs were used for amplification of DNA, using primers to the canine dystrophin 3' UTR.

Results—The DNA from affected dogs generated a larger PCR product than that obtained from clinically normal dogs. Products were cloned and sequenced, and the difference in size was found to be attributable to a 231-bp short interspersed nucleotide element (SINE). The SINE was found in all affected dogs in the colony but not in most unaffected puppies in the colony. Eighteen of 19 dogs in the colony were heterozygous for the GRMD mutation, and 7 of 30 unrelated dogs also were heterozygous for the SINE.

Conclusion and Clinical Relevance—Evidence of recombination between the GRMD mutation and the SINE was observed in only 4 dogs (2 sets of littermates) in the GRMD colony. Incidence of this SINE in a few unrelated dogs suggests that this particular insertion into the dystrophin gene may have been a recent event. The SINE in the dystrophin 3' UTR did not have an apparent influence on dystrophin mRNA concentrations. (*Am J Vet Res* 2001;62:1964–1968)

Personnel in our laboratory group investigate genetic therapies for the treatment of people with **Duchenne muscular dystrophy (DMD)**, a progressive muscle-wasting disease affecting 1 in 3,500 boys[1] that is caused by a lack of functional dystrophin. The muscle-specific isoform comprises 3,685 amino acids and forms a bridge between the cytoskeleton and the extracellular matrix via interactions at the amino terminal with actin and the carboxyl terminal with a membrane-associated dystrophin-glycoprotein complex.[2,3] Duchenne muscular dystrophy essentially is a disease of attrition in which damage to muscle fibers is followed by cycles of degeneration and regeneration until regenerative capacity is exhausted.[4] The disease ultimately results in death of affected people during the late teens or early twenties from respiratory or cardiac failure.

Several models of DMD in animals have been developed. The most commonly used are *mdx* mice and Golden Retrievers affected with muscular dystrophy. Muscular dystrophy in Golden Retrievers appears to be an appropriate model of the human disease because of similarities in clinical severity and pathologic characteristics.[5] The condition in affected Golden Retrievers results from a point mutation at the intron 6-exon 7 splice acceptor site; thus, skipping of exon 7 disrupts the reading frame with termination of translation in exon 8.[6]

We have evaluated a number of approaches to use in ameliorating the disease in affected Golden Retrievers.[7,8] We have searched for alternatively processed (revertant) dystrophin transcripts,[9] the results of which could play a role in therapeutic approaches that use antisense oligonucleotides.[10,11]

The full-length canine dystrophin cDNA of the muscle-specific isoform has been sequenced.[a] Previously, amplification of a portion in the 3' **untranslated region (UTR)** of the dystrophin gene from a clinically normal and a dystrophic dog yielded **reverse transcriptase-polymerase chain reaction (RT-PCR)** products that differed in size by approximately 200 **base pairs (bp)**. Analysis of the DNA sequence revealed this difference was attributable to a **short interspersed nucleotide element (SINE)** in the 3' UTR of the allele from the dystrophic dog. Little is known about the role of SINE. Although most SINE may not be functional, the potential exists for these sequences to contribute to a gene or regulatory element.[12] The objectives of the study reported here were to determine the distribution of the SINE in Golden Retrievers with muscular dystrophy and in unrelated dogs, to estimate the rate of recombination between the mutation and the SINE, and to determine whether the SINE has any biological effects on dystrophin transcripts.

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Materials and Methods

Animals—Samples of blood and hair were obtained from 77 dogs that were members of the **Golden Retriever Muscular Dystrophy (GRMD)** colony at the Murdoch University Veterinary School. Samples also were obtained from 30 unrelated dogs examined at the Murdoch University Veterinary Clinic.

Preparation of DNA—A commercially available kit[b] was used to extract DNA from samples of blood and hair roots obtained from dogs. Extraction was performed in accordance with manufacturer's instructions. The DNA was eluted in 30 µl of the supplied elution buffer, and 5 µl of this solution was used in DNA amplification reactions.

Polymerase chain reaction—The PCR was performed in 25-µl volumes containing 50 ng of each primer,[c] 5 µl of DNA sample, 67 m*M* Tris-HCl (pH 8.8), 16.6 m*M* $(NH_4)_2SO_4$, 0.45% TritonX-100, 0.2 µg of gelatin/ml, 3.0 m*M* $MgCl_2$, 300 µ*M* of each **deoxyribonucleoside triphosphate (dNTP)**, and 1.1 units of *Taq* DNA polymerase.[d] Reaction products were overlaid with 25 µl of paraffin oil and then amplified as follows: 3 minutes at 94 C for the first cycle only and 15 seconds for each cycle thereafter, 55 C for 6 minutes for 20 cycles, 94 C for 15 seconds, and 53 C for 6 minutes for another 20 cycles.

Detection of GRMD mutation—The GRMD mutation was determined as described elsewhere.[13]

Extraction of RNA—Samples of muscle (30 mg) from clinically normal and affected GRMD dogs were chopped and homogenized in lysis buffer,[e] and the RNA was extracted in accordance with the manufacturer's protocol. The RNA was eluted from the column by use of two 30-µl aliquots of nuclease-free water.

Reverse transcriptase-polymerase chain reaction—The RT-PCR was performed on 200 ng of total RNA, using a commercially available system[f] with final concentrations of 3.0 m*M* $MgCl_2$ and 300 µ*M* of each dNTP. Primers used were DS2222 (5'-AACCAAAGTGAGGTAGAAATAGC-3') and DS2229 (5'-TACAACAAAGAGGATTAGACAG-3'). Reactions were incubated at 25 C for 2 minutes, 48 C for 30 minutes, and then cycled as described previously. Reaction products were fractionated on 2% agarose gels in tris-acetate buffer.

Generation of 3' UTR sequence—Use of primers DS2222 and DS2366 (5'-CCCTGGAGCCTGAAACCAGG-3')[g] resulted in amplification of a fragment of approximately 1.2 **kilobases (kb)** in affected dogs and 1.0 kb in clinically normal dogs. These products were cloned, and 8 clones derived from clinically normal and affected GRMD dogs were analyzed by use of PCR, using universal M13 primers. Recombinants containing inserts of the expected (ie, normal) or large (ie, GRMD) size were used to inoculate 5 ml of Luria-Bertani broth containing 50 µg of ampicillin/ml. Cultures were allowed to incubate overnight at 37 C with shaking before bacteria were pelleted and the plasmid purified, using a centrifugation miniprep kit[h] in accordance with manufacturer's instructions. Sequencing of DNA[i] was performed on 0.5 µg of plasmid, using M13 primers. Sequencing reactions were precipitated with ethanol, washed with 70% ethanol, and fractionated on an automatic DNA sequencer.[j]

Determination of distribution of inserted sequence—Samples of DNA from Golden Retrievers bred at the GRMD colony and unrelated dogs of several breeds examined at the Murdoch University Veterinary Clinic were tested. The PCR was performed as previously described, using primers DS2229 and DS2222.

Results

Amplification of the central portion of the 3' UTR of the canine dystrophin gene resulted in products of differing lengths when DNA and RNA from clinically normal and affected dogs were used as the template. Direct sequencing of the 3' UTR amplification products was initially undertaken using a primer-walking approach. The 3' UTR sequences from a number of dogs were found to be identical except for the insertion of > 200 bases in the GRMD allele. Several primer sets were used to amplify across the insert; however, when smaller products were generated (< 500 bp), the shorter (ie, normal) allele from heterozygous dogs was preferentially amplified with little or no product amplified from the longer 3' UTR. This preferential amplification was not as evident when larger PCR products were generated, presumably because of a decrease in the relative size differences of the products (data not shown).

Dystrophin transcripts from muscle of clinically normal, GRMD carrier, and affected dogs from the GRMD colony were analyzed by use of RT-PCR, which yielded products of 1.9 kb for affected dogs and 1.7 kb for most clinically normal dogs. Comparison of the amount of RT-PCR products obtained from transcripts with and without the SINE indicated that the SINE did not have an apparent influence on the amount of the

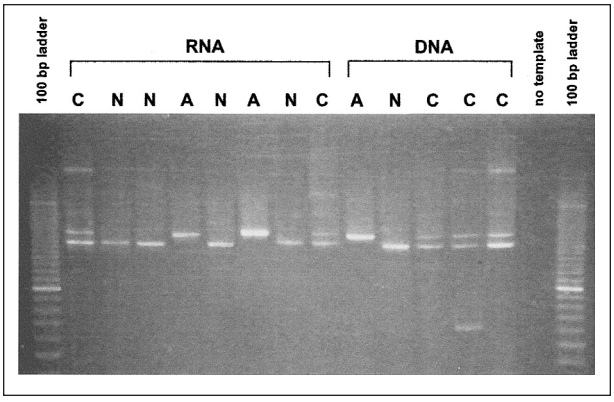

Figure 1—Amplification of the region containing a short interspersed nucleotide element (SINE) of canine dystrophin mRNA and genomic DNA in samples obtained from affected (A; approx 1.9 kilobases [kb]), clinically normal (N; approx 1.7 kb), and carrier (C; 1.9 and 1.7 kb) dogs. Outer lanes contain ladders of 100-base pair markers.

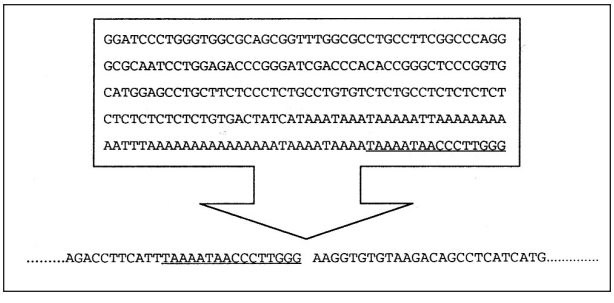

Figure 2—Results obtained when primers DS2366 and DS2222 were used to amplify a fragment from the dystrophin 3' untranslated region (UTR) of Golden Retrievers affected with muscular dystrophy. The SINE (A) contained an extra 231 BP and was inserted at the position indicated in the sequence found in most clinically normal dogs (B). Notice that the 16-base repeat (underlined) is found at both ends of the SINE.

dystrophin transcript. There was a slight excess of the shorter (ie, normal) transcript, compared with that of the transcript carrying the SINE; however, this bias was attributed to preferential amplification during the PCR, because the same pattern was observed when genomic DNA was used as the template under identical cycling conditions (**Fig 1**).

Sequence of the insert could not be determined by analysis of direct DNA sequencing of PCR products. It was necessary to clone the GRMD PCR products and then sequence 4 independent recombinant plasmids derived from clinically normal and affected dogs. An insertion of 231 bp was evident after position 1384 of the canine dystrophin 3' UTR (12716 of the canine dystrophin cDNA sequence). The precise site of insertion cannot be determined, because the sequence TAAAATAACCCTTGGG, which is found in the normal canine 3' UTR, is detected at both ends of the inserted element and is likely to have been involved in the original integration event (**Fig 2**).

Search of databases revealed that 131 bases of the SINE reported here had > 94% homology to a highly repetitive canid SINE.[14] Overall architecture of this SINE in the GRMD dystrophin 3' UTR was typical of that described by Minnick et al.[14]

Samples of DNA from dogs in the GRMD colony were assessed for GRMD status and evidence of the SINE. When detected, the SINE in the canine dystrophin 3' UTR generally correlated with disease status of that dog. In our GRMD colony, carrier dogs were heterozygous for the SINE, and affected dogs were homozygous (or hemizygous in the case of affected males) for the insertion. Clinically normal dogs did not have the SINE.

Of the 77 dogs (116 dystrophin alleles) in the GRMD colony that were tested, all but 1 (a carrier female) with the GRMD allele had the SINE. Three clinically normal female dogs of the GRMD colony were heterozygous for the SINE. From these observations, the recombination rate between the GRMD mutation and the SINE is 3.45% (95% confidence intervals, 0.1 to 6.7%). Recombination across the human dystrophin gene has been estimated to be 10 to 12%.[15]

Seven of 30 unrelated dogs that were tested had the SINE in their dystrophin 3' UTR. This included 1 of 4 Greyhounds, 1 Golden Retriever, 1 of 2 Boxers, and 4 of 5 Rottweilers (**Table 1**).

Gene sequences of various canid species were evaluated. There appears to be little sequence conservation between these repeats, although there is evidence of a pattern of polyadenylation-rich motifs (**Table 2**).[16-25]

Table 1—Distribution of the canid short interspersed nucleotide element (SINE) in the dystrophin 3' untranslated region (UTR) in dogs that were members of a Golden Retriever Muscular Dystrophy (GRMD) colony and dogs that were not related

Dogs	No.	Normal allele	GRMD allele	SINE
GRMD				
Male (affected)	16	0	16	16
Female (affected)	12	0	24	24
Female (carrier)	19	19	19	18
Male (clinically normal)	22	22	0	0
Female (clinically normal)	8	16	0	3
Unrelated dogs				
Male	15	15	NA	2
Female	15	30	NA	5

NA = Not applicable.

Discussion

Short interspersed nucleotide elements are abundant in mammalian genomes.[12] A highly repetitive element specific to carnivores of the superfamily Canoidea was originally identified in the dog (*Canis familiaris*) by Minnick et al.[14] Those researchers used hybridization studies to examine the distribution of this element in representative species from a number of families belonging to the order Carnivora, including foxes, bears, raccoons, ferrets, and domestic cats. They concluded that the element was restricted to canids and hypothesized that closely related canids such as wolves and coyotes would share this SINE. A recent search of databases confirmed detection of 1 or more descendants of this SINE in a number of canine genes, including introns 7 and 8 of the retinal guanylate cyclase E gene,[26] intron 2 of the cGMP-gated channel α-subunit gene,[16] intron 1 of the tyrosinase-related protein 2 gene,[17] and the 3' UTR of the factor IX gene.[18] There are 5 copies of this SINE in the canine chymase gene.[27] In addition, others have identified this SINE in a number of other carnivores including harbor seals (*Phoca vitulina concolour*),[28] red foxes (*Vulpes vulpes*),[29] giant and red pandas (*Ailuropoda melanoleuca* and *Ailurus fulgens*, respectively),[19] and mink (*Mustela vison*).[28,30]

Insertion of these elements is believed to require reverse transcription of class-III genes encoding small

Table 2—Results of nucleotide sequences of repeats flanking the canid SINE for the study reported here and in other studies

Genome analyzed	Nucleotide sequence
Canis familiaris dystrophin 3' UTR	TAAAATAACCCTTGGG
Other studies	
Canis familiaris DLA-53 pseudogene[20]	TAAAATAAATTTTA
Canis familiaris cGMP-gated channel a subunit (intron 2)[16]	TTAAAATCAATTAATT
Canis familiaris tyrosinase-related protein[17]	TAAACTACTCCAG
Canis familiaris tRNA-derived SINE[21]	AAAAAAGAAAT(+N)CATTTGT
Canis familiaris blood clotting Factor IX 3' UTR[18]	AAAGG(C/T)ATGAGTCA
Canis familiaris 5' to pancreatic colipase gene[22]	AGAACAGATAACTTG
Canis familiaris interferon ω 5' UTR[23]	AAGAAAG
Canis familiaris retinoblastoma gene 5' UTR[24,25]	AAAAGATTA
Potos flavus transthyretin intron 1[19]	AAAG(G/A)ATAGATCCTT(C/T)CAG

Imperfect repeats are indicated; differences are shown in parentheses.

cytoplasmic RNA (RNA polymerase-III transcripts).[31,32] The basic architecture of these elements appears well conserved in many cases in which, in addition to the direct repeats flanking the insertion, there are putative A and B boxes for RNA polymerase-III binding and a $(TC)_{3\ to\ 16}$ repeat at the 3' end, which is followed by a region of polyadenylation.[14] This canine dystrophin SINE consists of a 16-base repeat at the 5' and 3' ends, the 131-base SINE, followed by $(TC)_8$ and a 56-base region with adenines interspersed with thymines.

Most of the aforementioned canid SINE were flanked by direct repeats of up to 16 bases. Although there appears to be little sequence conservation among these repeats, there is a pattern of motifs rich in adenine (Table 2). In some cases, the flanking repeats were not obvious (intron 8 of the retinal guanylate cyclase E gene[26]) or reduced to a few bases (intron 2 of the tyrosinase-related protein 2 gene[17]). In others, these repeats varied from 7 to 16 bases, as found in intron 2 of the cGMP-gated channel α-subunit gene[16] and the canine dystrophin 3' UTR. It seems plausible that the more recently the SINE was integrated at a particular site, the longer or more perfect the flanking repeat. For example, the SINE in intron 8 of the retinal guanylate cyclase E gene does not have obvious flanking repeats and may have been inserted long before the SINE in intron 7 in the same gene, which has an imperfect 13-base repeat.[26] The dystrophin 3' UTR SINE, with a perfect 16-base repeat, is not found in all dogs, further supporting the idea that this insertion is a comparatively recent event in canine evolution.

Dystrophinopathies in the 2 most studied models of DMD in animals (*mdx* mice[33] and GRMD dogs[6]) result from point mutations. Apart from the report of 1 complete deletion of the dystrophin gene in a naturally occurring mutation in a German Shorthaired Pointer,[34] the only other mutation that has been characterized is the deletion of the muscle promoter in a cat.[35] The most common defects in the human dystrophin gene are genomic deletions of 1 or more exons, with most recombinations identified around introns 7 and 44.[15,36]

Consequences of the insertion of the SINE into the canine dystrophin gene have not been established conclusively. This SINE carries sequence motifs that have been implicated or associated with polyadenylation (AAUAAA or AUUAAA) and mRNA stability (AUUUA). It was possible that the GRMD transcript was further down regulated as a result of the SINE in the 3' UTR through a shorter-than-normal 3' UTR or AU-rich elements involved in mRNA turnover. However, we have examined RNA from clinically normal (no SINE), GRMD affected (with SINE), and heterozygous dogs and could not detect differences in transcript amounts that could be attributed to sequence motifs in the SINE.

[a]Sequence submitted under GenBank accession No. AF070485.
[b]DNA Direct system II, Dynal, Oslo, Norway.
[c]Oligonucleotides, Geneworks, Adelaide, Australia.
[d]*Taq* DNA polymerase, Biotech International, Perth, Australia.
[e]RNeasy, Qiagen, Hilden, Germany.
[f]Titan One Tube system, Roche Molecular Biochemicals, Mannheim, Germany.
[g]DS2366, Geneworks, Adelaide, Australia.
[h]QIAprep spin miniprep kit, Qiagen, Hilden, Germany.
[i]Prism dye terminator chemistry, ABI, Foster City, Calif.
[j]373A DNA sequencer, ABI, Foster City, Calif.

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Correction: Effects of dietary fat and l-carnitine on plasma and whole blood taurine concentrations and cardiac function in healthy dogs fed protein-restricted diets

In the article "Effects of dietary fat and l-carnitine on plasma and whole blood taurine concentrations and cardiac function in healthy dogs fed protein-restricted diets" (*AJVR*, Oct 2001, pp 1616–1623), the table on page 1618 should appear as follows:

Table 1—The effect of 3 protein-restricted diets on plasma and whole blood taurine concentrations in dogs

	Low fat diet		High fat plus carnitine diet		High fat diet	
Time (mo)	Plasma taurine* (mean ± SEM [range])	Whole blood taurine† (mean ± SEM [range])	Plasma taurine* (mean ± SEM [range])	Whole blood taurine† (mean ± SEM [range])	Plasma taurine* (mean ± SEM [range])	Whole blood taurine† (mean ± SEM [range])
0	70.5 ± 5.2 (62–96)	ND	68.8 ± 8.5 (41–86)	254.2 ± 20.9 (188–299)	66.8 ± 7.4 (47–82)	258.3 ± 20.9 (208–339)
6	60.2 ± 5.6[a] (46–85)	229.2 ± 15.1 (169–277)	50.8 ± 11.9 (31–95)	196.8 ± 27.5 (116–263)	30.7 ± 6.5[b] (14–49)	196.7 ± 37.6 (105–328)
12	42.7 ± 8.3 (18–68)	196.2 ± 23.3[a] (144–293)	36.6 ± 12.5 (9–76)	112.4 ± 28.4[b] (28–181)	22.0 ± 5.9 (13–45)	135.8 ± 25.4 (51–187)
18	55.7 ± 12.8 (21–98)	102.3 ± 17.0 (63–166)	54.2 ± 17.4 (18–116)	122.2 ± 29.7 (54–223)	29.5 ± 4.6 (14–53)	135.7 ± 42.0 (52–281)
24	28.0 ± 6.2 (5–45)	120.0 ± 19.2 (75–204)	27.0 ± 9.7 (5–52)	93.4 ± 29.1 (30–181)	24.8 ± 4.9 (7–55)	122.7 ± 23.3 (45–189)
30	65.3 ± 21.3 (12–162)	171.2 ± 45.9 (53–387)	34.4 ± 12.3 (9–78)	98.8 ± 19.9 (67–173)	36.5 ± 10.4 (12–81)	126.5 ± 19.8 (53–227)
36	37.5 ± 9.7 (2–64)	113.2 ± 20.7 (24–153)	33.8 ± 8.6 (6–52)	103.4 ± 27.1 (24–188)	14.2 ± 4.7 (5–28)	110.3 ± 26.6 (35–200)
48	59.1 ± 19.8 (12.1–142.4)	115.5 ± 19.9 (39.0–175.6)	58.7 ± 12.2 (24.2–87.9)	115.1 ± 11.3 (78.0–146.3)	23.0 ± 3.5 (15.2–33.3)	80.0 ± 20.3 (29.3–136.6)

ND = Not done.
*Reference range for heparinized plasma taurine = 41–97 nmol/ml. †Reference range for heparinized whole blood taurine = 155–347 nmol/ml.
[a,b]Different superscripts in the same row indicate significant ($P < 0.05$) difference between the diet groups indicated.