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Identification of the Syrian hamster *cardiomyopathy* gene

Vincenzo Nigro[1,*], Yasushi Okazaki[2], Angela Belsito[1], Giulio Piluso[1], Yoichi Matsuda[3], Luisa Politano[4], Giovanni Nigro[4], Carlo Ventura[5], Ciro Abbondanza[1], Anna Maria Molinari[1], Dario Acampora[6], Masahiko Nishimura[7], Yoshihide Hayashizaki[2] and Giovanni Alfredo Puca[1]

[1]Istituto di Patologia Generale e Oncologia, Facoltà di Medicina, Seconda Università degli Studi di Napoli, Larghetto S. Aniello a Caponapoli, 2, 80138 Napoli, Italy, [2]Genome Science Laboratory, Tsukuba Life Science Center, The Institute of Physical and Chemical Research (RIKEN), 3-1-1 Koyadai, Tsukuba, Ibaraki 305, Japan, [3]Laboratory of Animal Genetics, School of Agricultural Sciences, Nagoya University, Chikusa, Nagoya 464-01, Japan, [4]Dipartimento di Internistica Clinica e Sperimentale, Facoltà di Medicina, Seconda Università degli Studi di Napoli, Piazza Miraglia, 80138 Napoli, Italy, [5]Istituto di Chimica Biologica, Facoltà di Medicina, Università degli Studi di Sassari, viale S. Pietro 43/b, 07100 Sassari, Italy, [6]International Institute of Genetics and Biophysics, via Marconi 12, 80121 Napoli, Italy and [7]Institute for Experimental Animals, Hamamatsu University School of Medicine, 3600 Handa-cho, Hamamatsu 431-31, Japan

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The BIO14.6 hamster is a widely used model for autosomal recessive cardiomyopathy. These animals die prematurely from progressive myocardial necrosis and heart failure. The primary genetic defect leading to the cardiomyopathy is still unknown. Recently, a genetic linkage map localized the cardiomyopathy locus on hamster chromosome 9qa2.1–b1, excluding several candidate genes. We now demonstrate that the cardiomyopathy results from a mutation in the δ-sarcoglycan gene that maps to the disease locus. This mutation was completely coincident with the disease in backcross and F_2 pedigrees. This constitutes the first animal model identified for human sarcoglycan disorders.

INTRODUCTION

For at least three decades, the cardiomyopathic Syrian hamster (BIO14.6) has constituted a widely studied animal model of hypertrophic cardiomyopathy and muscular dystrophy (1,2). These animals develop widespread muscle cell necrosis in the myocardium as well as in the skeletal muscle (3,4). Hypertrophy of the remaining cardiomyocytes is followed by heart dilatation leading to ultimate failure. Several genes which cause human hypertrophic cardiomyopathy including those coding for β-myosin heavy chain, α-tropomyosin, cardiac troponin T, and β-myosin-binding protein C were excluded (5,6). Thus, despite a very large number of studies, the *cardiomyopathy* gene has not yet been identified.

In the last few years, research has concentrated upon dystrophin (7) and the dystrophin-associated glycoprotein complex, since a similar muscle cell necrosis occurs in Duchenne/Becker muscular dystrophy (DMD/BMD) and severe childhood autosomal recessive muscular dystrophies (SCARMD) (8,9 and references therein). The characterization of the dystrophin-associated complex has greatly improved our understanding of muscular dystrophies (10–14). Dystrophin is a subsarcolemmal rod-shaped protein which associates with several other proteins and glycoproteins to form the dystrophin–glycoprotein complex. This is composed of two main subcomplexes: dystrophin–dystroglycan and sarcoglycan. Dystrophin–dystroglycan functions as a bridge across the muscle membrane from actin to merosin (15).

Sarcoglycan (SG) has been described as a complex of four transmembrane glycoproteins: a 50 kDa (A2, 50DAG, adhalin, α-SG), a 43 kDa (A3b, 43DAG, β-SG), a 35 kDa (A4, 35DAG, γ-SG) (8,15,16) and a 35 kDa (δ-SG) (17). Mutations of each *SG* gene have been proved to cause autosomal recessive human limb-girdle muscular dystrophies (LGMD2) (18); the LGMD2C (19), LGMD2D (20,21), LGMD2E (22,23) and LGMD2F (24,25) are caused by γ-, α-, β- and δ-*SG* gene defects, respectively. In all forms, the primary loss of one SG component involves the full complex. A triplet of 59 kDa proteins, known as syntrophins (A1), a 25DAP (A5) and the human homologues of the *Torpedo* 87 kDa (dystrobrevins, A0), also seem to be involved in complexes with dystrophin (15).

Genes encoding the dystrophin-associated proteins are now considered good candidates for the hamster cardiomyopathy. In particular, adhalin (α-SG) deficiency has been revealed in the cardiomyopathic hamster muscle before the onset of myocytolysis (26,27). However, two independent studies have indicated that the α-SG deficiency is secondary, the first showing that the α-*SG* mRNA and cDNA sequences are normal (28), and the second physically mapping the α-*SG* gene to a chromosomal region outside the disease locus (5). In the latter report, we constructed a genetic linkage map of the Syrian hamster using

*To whom correspondence should be addressed

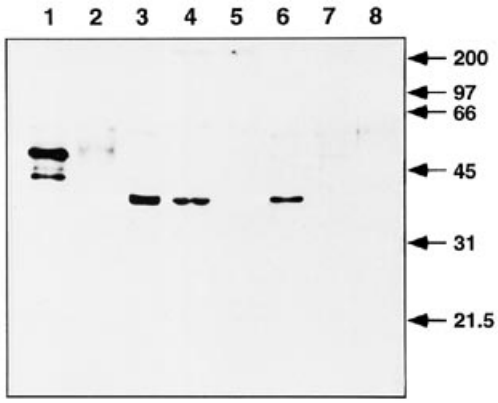

Figure 1. Western blot analysis. 1, GST–δ-SG fusion protein; 2, GST–γ-SG fusion protein; 3, human pectoral muscle; 4, Syrian hamster cardiac muscle; 5, BIO14.6 cardiac muscle; 6, Syrian hamster leg muscle; 7, BIO14.6 leg muscle; 8, BIO14.6 paravertebral muscle.

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hamster   MPQEQYSHH RSTMPSSEGP HIYKVGIYGW RKRCLYFFVL LLMILILVNL  49
human     M------T-- -----G-V-- QV--------- ---------- ----------

          AMTIWILKVM NFTIDGMGNL RITEKGLKLE GDSEFLQPLY AKEIQSRPGN  99
          ---------- ---------- ---------- ---------- ----------

          ALYFKSARNV TVNILNDQTK VLTRLVTGPK AVEAYGKKFE VKTVSGKLLF  149
          ---------- ---------- ---Q-I---- ---------- ----------

          SADDNEVVVG AERLRVLGAE GTVFPKSIET PNVRADPFKE LRLESPTRSL  199
          ---N------ ---------- ---------- ---------- ----------

          VMEAPKGVEI NAEAGNMEAT CRSELRLESK DGEIKLDAAK IKLPRLPRGS  249
          ---------- ---------- --T------- ---------- -R-----H--

          YTPTGTRQKV FEVCICANGR LFLSQAGTGS TCQINTSVCL 289
          ---------- --I-V----- -------A-- ----------
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Figure 2. Amino acid homology between deduced Syrian hamster (GenBank accession No. Y08838) and human (GenBank accession No. X95191) δ-*SG* gene product. Dashes indicate amino acid identity with the Syrian hamster sequence.

backcross progeny and assigned the cardiomyopathy locus to the centromeric region of hamster chromosome 9qa2.1–b1 (5). Afterwards, the β- and γ-*SG* genes were also checked for mutations, with no result (29).

In this study, we investigated whether the δ-*SG* gene is the primary cause for the cardiomyopathy of BIO14.6 hamsters using molecular and genetic analyses.

RESULTS

The δ-SG protein is absent from BIO14.6 muscle

We analysed the skeletal and cardiac muscle from cardiomyopathic BIO14.6 hamster compared with the control F1B strain by Western blot. A rabbit polyclonal serum directed against the human δ-SG (17) specifically recognized only one 35 kDa band from the hamster muscle. This band appears to be identical to that observed using fresh homogenates from human or mouse muscle. The immunoreactivity was totally competed out with the fusion protein used to raise the serum, but not with the control proteins. In the BIO14.6 strain, we saw no 35 kDa band corresponding to the δ-SG in either cardiac or skeletal muscle (Fig. 1).

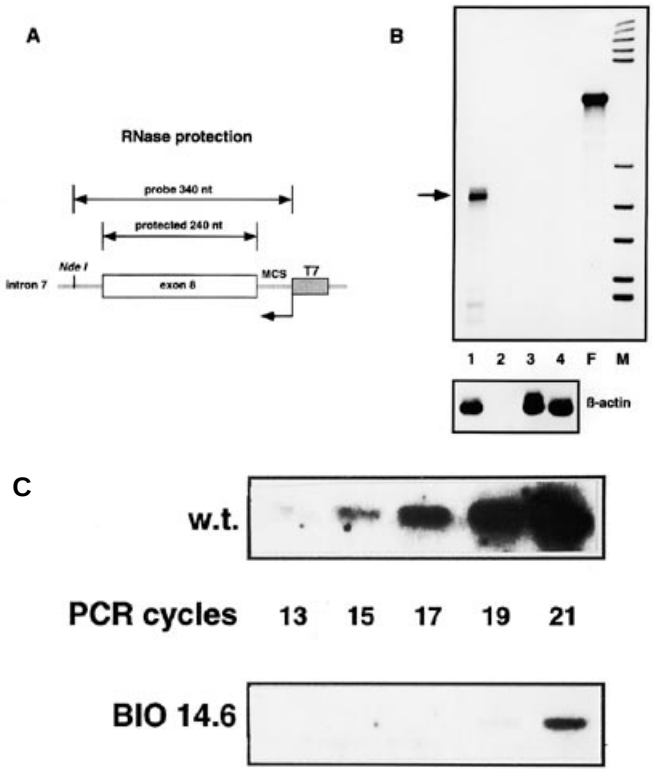

Figure 3. δ-SG RNA quantitative assays. (**A**) A 340 nucleotide antisense RNA probe corresponding to exon 8 of hamster δ-*SG* was labelled using [α-^{32}P]GTP and T7 RNA polymerase. The probe contained 55 nucleotides of multiple cloning site (MCS) and 45 nucleotides of intron 7. (**B**) The arrow indicates the protected RNA fragment of 240 nucleotides that is visible after RNase digestion of the normal hamster skeletal muscle sample (lane 1). Lane 2 represents a yeast RNA control; lane 3, BIO14.6 skeletal muscle RNA; lane 4, BIO14.6 cardiac muscle RNA. F indicates the undigested 340 nucleotide free RNA probe. M indicates the radiolabelled molecular weight markers (587, 540, 504, 458, 434, 267, 234, 213, 192 and 184 nucleotides). (**C**) Quantitative RT-PCR amplification of cardiac muscle cDNAs. cDNA was amplified using the primers 190F (ATTGATGGAATGGGGAACTTAAGAA, exon 3) and 520R (AGACTGTG-CCTTCAGCTCCTAAGACT, exon 6) by the indicated cycles of PCR and hybridized with a ^{32}P-labelled internal oligo (433F, TCTGGAAAATTGCT-CTTTTCTGCGGATG, exon 5). Counting of the 331 bp bands showed an ~1:16 ratio every four cycles. In the experiment shown, after 21 cycles 1 084 000 counts were measured from the wild-type (w.t.) Syrian hamster and 41 600 from the BIO14.6. The autoradiogram was exposed for 2 h.

δ-*SG* mRNA expression is severely reduced in BIO14.6 muscle

Non-degenerate primer pairs were designed from the human cDNA sequence (X95191) and used in PCR to amplify overlapping fragments of the hamster δ-*SG* coding sequence (17). These sequences were assembled into an open reading frame (ORF), similar to the human sequence (Fig. 2).

We next amplified the δ-*SG* cDNA from the BIO14.6 templates using hamster-specific primer pairs. Interestingly, we obtained faint δ-*SG* RT-PCR products from the BIO14.6 compared with the control hamster. RNase protection assay was then performed using a 340 nucleotide probe encompassing part of the eighth exon of hamster δ-*SG* (Fig. 3A). In the experiment shown, no δ-*SG* protection was obtained by either skeletal or cardiac muscle BIO14.6 RNA (Fig. 3B, lanes 3–4). However, after longer

A

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aatcaagaccaggcagggcagagcacagagggacagtgggggattccacttggaacaggc  -515
aaggtacatagagaccacattgaactgggagagaaaagtaaaacgtgacttgtctattgc  -455
atccagtacccagagcaaagcctgtgtggagaatggctggaaaatccaagtcgtatcgag  -395
aaacagccttctttctttctttctgtgagtgtgagcccagcagacgccatgagcgcagta  -335
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agttgtgcggttggaaaatgtgactggggcagctcctgagtgcaggcagggcctgctcac  -95
atgggcgggctaagtggccgcctccttcagaacctctcagctttccccaggaccacgggg  -35
actctcatccgccactctgtgaaacagaggagag- intron 1 -ATGCCTCAG....
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B

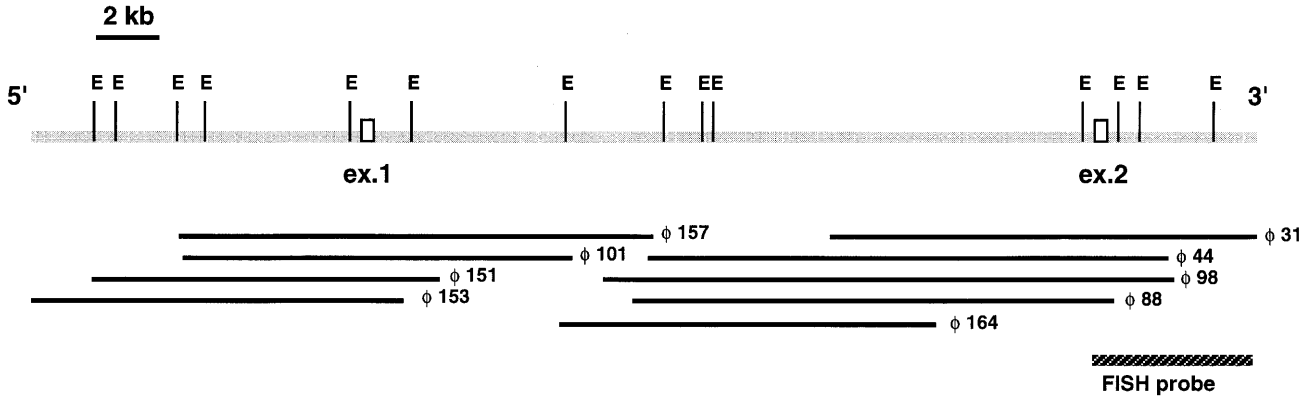

Figure 4. (**A**) Sequence of the genomic region containing the first exon and part of the putative promoter (GenBank accession no. Y08840) of the Syrian hamsterδ-*SG* gene. The 5′ ends of the longest RACE products (indicated by the arrow) may define the transcriptional start site of the δ-*SG* gene. Primer extension performed on wild-type muscle RNA confirmed the possible start of transcription indicated by the longest RACE products. (**B**) Partial genomic map of the Syrian hamster δ-*SG* gene. λ phage clones are indicated as bold lines. The probe used in FISH experiment is described. E, *Eco*RI; ex., exon.

exposure, a faint protection was visible in BIO14.6 heart RNA, but not in skeletal muscle (not shown). These findings were constant using different RNA probes and template preparations and were confirmed by Northern blot (not shown).

We then estimated the amount of δ-*SG* mRNA in BIO14.6. A low number of PCR cycles was performed and products were blotted and hybridized to an internal primer. Results were quantified using a Phosphor Image analyser and normalized. The δ-*SG* mRNA level was always seen as <3–5% in heart (Fig. 3C), while it was negligible in skeletal muscle.

We next performed 5′ rapid amplification of cDNA ends (RACE) (30) using reverse nested oligonucleotide primers located at the 5′ end of the hamster cDNA. The δ-*SG* cDNA contains an ~200 bp sequence correctly spliced to the second exon (Fig. 4a). No difference was observed using either cardiac or skeletal muscle cDNA templates. We obtained the same data performing the RACE with different nested primers and confirmed the sequence co-linearity with genomic DNA clones. The putative first exon only comprises the 5′-untranslated region (5′ UTR) with two in-frame stop codons, whereas the second exon begins with the first AUG. In contrast, in the human transcript, the start codon is located at the end of the upstream exon. The full ORF encodes a predicted 289 amino acid protein, having 95% amino acid identity with the human cDNA (Fig. 2). In comparison, the hamster α-, β- and γ-SG have 88, 95 and 83% amino acid identity with the respective human sequences (29). Sequence comparison between the BIO14.6 and normal cardiac muscle cDNA revealed a different 5′ UTR, but no mutation in the coding region.

Intragenic deletion of the first exon sequence in the BIO14.6 hamster

We screened a Syrian hamster genomic DNA λ phage library using the δ-*SG* cDNA as a probe. Like the human gene, the hamster δ-*SG* gene spans a very large genomic region, since most introns are exceedingly larger than single λ phage clones (size 9–23 kb). We isolated several overlapping clones in the promoter

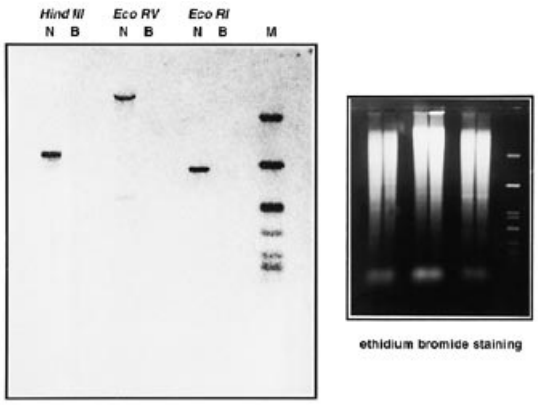

Figure 5. Southern blot analysis of DNA from control Syrian hamster (N) and BIO14.6 (B). DNA (10 µg) was digested with *Hin*dIII, *Eco*RV or *Eco*RI prior to 0.8% agarose gel electrophoresis. The blot was hybridized with the radiolabelled 2.0 kb *Eco*RI genomic fragment containing the first exon and the putative promoter of the hamster δ-*SG* gene. The ethidium bromide staining is shown for direct visualization of the equality of the DNA quantity in the gel. A second probe (at lower specific labelling) recognizes the following molecular weight markers (M): 5000, 2258, 1204, 794, 517, 396 bp.

region and constructed an *Eco*RI restriction map covering >40 kb (Fig. 4B). The 5′ UTR and the putative promoter were mapped to a 2.0 kb *Eco*RI fragment, 24 kb from the second exon, while the minor promoter was located upstream. We used these sequences as probes in Southern blotting. In the experiment, DNA was cut with *Hin*dIII, *Eco*RV or *Eco*RI. The first exon-containing probe did not recognize the specific bands from BIO14.6 DNA (Fig. 5).

Localization of the δ-*SG* gene by FISH analysis

To see if the defect in δ-SG protein and reduction in δ-*SG* mRNA expression level could be the primary cause of the cardiomyopathy, we performed fluorescence *in situ* hybridization (FISH) analysis. Cloning of the δ-*SG* gene and the probes used are described in Materials and Methods and Figure 4B. FISH analysis revealed that the δ-*SG* gene is located on chromosome 9qa2.1–b1 where the Syrian hamster cardiomyopathy locus has been mapped (5) (Fig. 6).

Linkage analysis using backcross and F_2 pedigrees

We had previously constructed a whole genetic map of the Syrian hamster using 72 backcross progeny (5). (ACN×BIO14.6) F_1 was mated to BIO14.6 and the backcross progeny were produced. The phenotypes of progeny (cardiomyopathic or normal) were at an

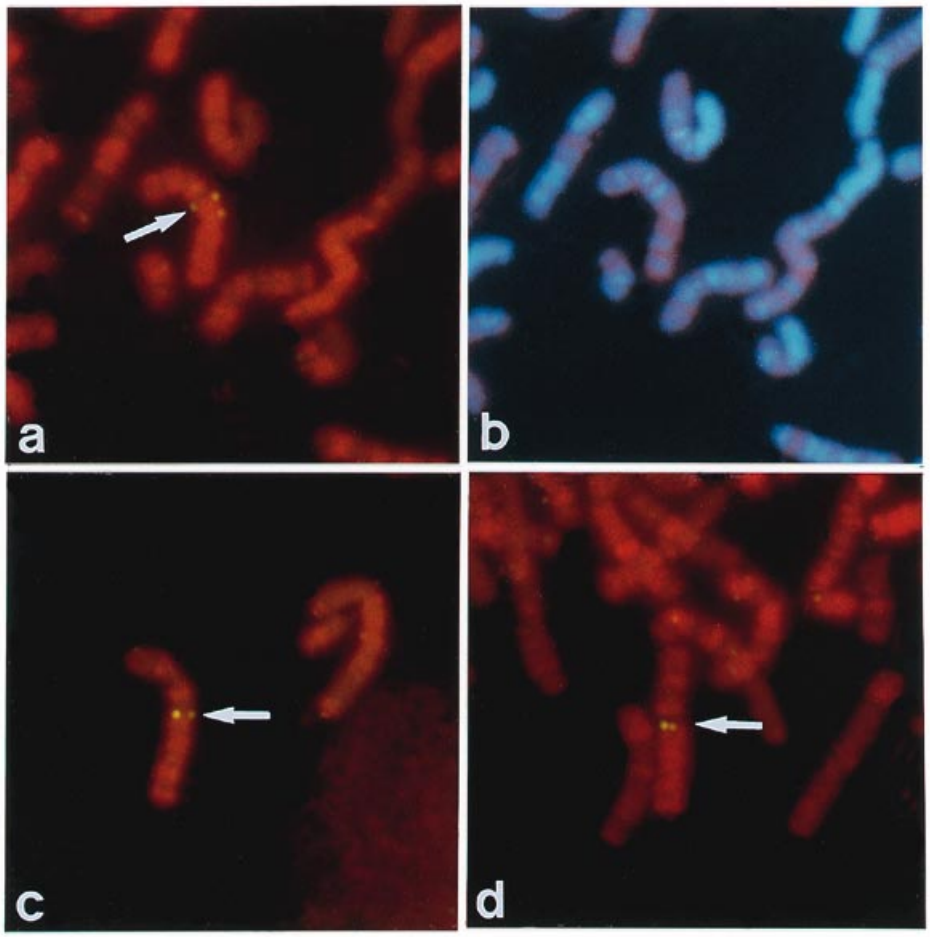

Figure 6. Chromosomal localization of the δ-*SG* gene with the direct R-banding FISH method using a 5.3 kb genomic DNA fragment (encompassing the second exon and part of the second intron) inserted in pBS (SK^+) as a biotinylated probe. The hybridization signals are indicated by arrows (A, C and D). The signals are localized to the proximal end of the qb1 band of chromosome 9. The metaphase spreads were photographed with Nikon B-2A (A, C and D) and UV-2A (B) filters. R-band and G-band patterns are demonstrated in (**A**, **C** and **D**) and (**B**), respectively.

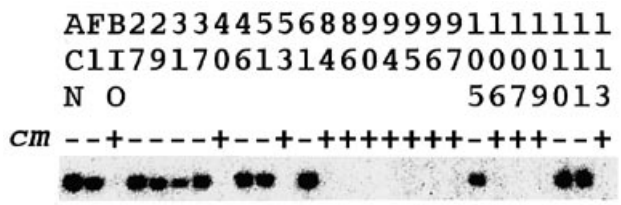

Figure 7. Southern blot analysis of backcross pedigree using a 2.0 kb *Eco*RI genomic DNA fragment containing the first exon of the δ-*SG* gene as a probe. This panel shows only a part of the backcross progeny. BIO and F1 represent the BIO14.6 strain and (ACN×BIO14.6) F_1, respectively. The number refers to the backcross pedigree. Five µg of genomic DNA digested with *Eco*RI were loaded onto a 0.8% agarose gel and Southern blot analysis was performed. The symbol '+' indicates that the individual has cardiomyopathy (cm), whereas '–' indicates the absence of cardiomyopathy. The deleted band of 2.0 kb *Eco*RI fragment is completely coincident (no recombination) with the mutant phenotype in all the backcross (n = 72) and F_2 (n = 131) pedigrees.

~1:1 ratio, following the Mendelian rule. The linkage analysis using the (ACN×BIO14.6) F_1×BIO14.6 backcross pedigree (n = 72) and F_2 pedigree (n = 131) showed that the absence of 2.0 kb *Eco*RI fragment containing the first exon was completely coincident with the mutant phenotype (Fig. 7). This evidence strongly suggests that the δ-*SG* gene is the primary cause of the hamster cardiomyopathy.

DISCUSSION

Here we show that δ-SG protein is undetectable in the cardiomyopathic hamster muscle. This is associated with a strong reduction in δ-*SG* mRNA levels. Our findings indicate that a deletion occurs in the δ-*SG* regulatory sequences and first exon. To verify whether this is the primary cause of the BIO14.6 disease, we used genetic analyses. FISH localized the δ-*SG* gene to chromosome 9qa2.1–b1, where the cardiomyopathic locus has been mapped (5). Analysis with the backcross (n = 72) and F_2 (n = 131) pedigrees demonstrated that the deletion of the first exon is completely coincident with the mutant phenotype. These data demonstrate that the δ-*SG* gene is the primary causative gene for the Syrian hamster cardiomyopathy.

The residual δ-*SG* transcription in BIO14.6 cardiac muscle is produced by a weak alternative promoter and first exon (Y08839) located upstream from the deleted region. This promoter is also used in the normal hamster heart (unpublished data).

The BIO14.6 hamster has been a historical model for cardiomyopathy. Now it is the first animal model reported for a sarcoglycanopathy, which represents a documented example of an *SG* gene mutation associated with an autosomal recessive cardiomyopathy. In most human sarcoglycanopathies, cardiac function is spared (31,32). In the case of dystrophin gene mutations, in contrast, cardiac damage is very common and a major factor in reducing life expectancy, particularly in BMD patients and in female carriers of DMD/BMD (33–35). Interestingly, an α-SG negative staining has been observed in the heart transplanted from a 13-year-old boy with severe cardiomyopathy (36). Since no α-*SG* gene mutation has been reported for that patient, it may be interesting to investigate whether δ-*SG* gene mutations also lead to human cardiomyopathies.

The observation that SG defects affect cardiac muscle less than dystrophin mutations has also been correlated with the different expression pattern of dystrophin mRNA. It may be noteworthy that β-SG and δ-SG, resembling dystrophin, are expressed in skeletal, cardiac and smooth muscle, while β-SG is also expressed in non-muscle cells. SG proteins in skeletal muscle are found together in equimolar ratio (16,37). In other tissues, whether the four SG components are associated in a standard functional array is still unknown. One can hypothesize that the differences in the tissue-specific expression may affect different arrangements of these SGs. This might explain why the hamster δ-*SG* mutation mainly affects the heart function even if the δ-SG protein is absent in both skeletal and cardiac muscle. In LGMD2F, skeletal muscle is more involved, but cardiac symptoms might be mitigated by limitation of heart work load in patients confined to wheelchairs. Finally, the existence of LGMD2F patients with prevailing cardiac damage cannot be excluded.

Interestingly, the mutation described is a deletion of putative regulatory sequences in a gene mainly involved in muscular dystrophy. This situation is comparable with the one described by Muntoni *et al*. (38,39) in X-linked dilated cardiomyopathy. This condition is, in fact, associated with a deletion in the dystrophin muscle promoter and first exon. Recently, a transcriptional enhancer in this same region of the human dystrophin gene has been identified (40). It will be interesting to determine in both conditions whether the deleted regions contain cardiac- and/or stage-specific regulatory sequences.

Our results lend significant support to a specific role for δ-SG in the cardiac dystrophin-associated complex. However, the function of δ-SG within the complex remains obscure. Further studies on SG expression in human cardiomyopathies are necessary and a still larger use of this hamster model may have an impact on therapy.

MATERIALS AND METHODS

Animals

BIO14.6 cardiomyopathic Syrian hamsters were purchased from Bio Breeders, Fitchburg, MA. Male F1B control golden Syrian hamsters were obtained from Charles River (Italy). Animals were sacrificed in accordance with institutional guidelines. Backcross progeny were produced from BIO14.6 (B) and an inbred strain ACN (A) using (ACN–BIO14.6) F_1 females×BIO14.6 males (ABB, n = 72). (ACN×BIO14.6) F_2 progeny (n = 131) were also produced and genotyped. Phenotype was confirmed by the measurement of serum creatine phosphokinase (CPK) level at 60 and 90 days of age, and by the microscopic examination of heart, tongue and skeletal muscle at 90 days of age.

Western blotting

Induction and purification of fusion proteins and antibody production against δ-SG were performed as described (17).

Hamster frozen muscular tissue was crushed in a pre-cooled mortar. Approximately 50 mg of sample were added to 200 µl of loading buffer [8 M urea, 125 mM Tris–HCl pH 6.8, 4% SDS, 100 mM dithiothreitol (DTT) and 0.001% bromophenol blue], boiled for 3 min and centrifuged to remove SDS-insoluble proteins. Samples were run on 11% SDS–PAGE and transferred to nitrocellulose sheets. Membranes were incubated for 2 h at room temperature with rabbit antiserum diluted 1:4.000 in phosphate-buffered saline (PBS) with 3% non-fat milk, 0.05% Tween-20 and 0.05% NP-40. After washing, membranes were incubated for 1 h with peroxidase-conjugated anti-rabbit IgG diluted 1:10 000

in PBS with 0.5% non-fat milk, 0.05% Tween-20 and 0.05% NP-40. Immunoreactive bands were visualized by ECL (Amersham).

Isolation of hamster δ-*SG* cDNA and genomic sequences

Total cardiac and skeletal muscle RNA from Syrian hamster and BIO14.6 was isolated and reverse transcribed according to standard procedures (41). Low stringency PCR amplifications were performed in a Robocycler Gradient 96 (Stratagene), which allows the simultaneous use of 12 different annealing temperatures using a 10 µl reaction, 3 µM of each primer, 3.5 mM dNTPs, 5 mM $MgCl_2$, 100 mM KCl and 0.5 U AmpliTaq polymerase (Perkin Elmer). All the other PCR amplifications were performed in a thermal cycler PTC-100 (MJ Research) as described (42). Most of the PCR products generated by these primer pairs were specific. Screening of the genomic library was performed according to standard procedures (41). We screened ~500 000 plaques of Golden Hamster λ FIX II library (Stratagene). Duplicate sets of filters were hybridized in 6× SSC and 40% formamide at 42°C with the ^{32}P-labelled 0.8 kb probe(10^8 c.p.m.). Washes were carried out at 67°C in 0.15× SSC 0.1% SDS. More than 100 positive clones were classified according to the hybridization with different δ-*SG* oligonucleotides. Hyperfilm MP (Amersham) was used for most applications. We confirmed the cDNA sequence and the exon–intron boundaries at the same positions of the human δ-*SG* gene.

RACE

5′ RACE was performed from 1 µg of total RNA from skeletal or cardiac muscle. Reverse transcriptase (BRL) was from the semi-random oligo 5′-NNNNNNSS-3′. After treatment with terminal transferase (BRL) in the presence of dATP, nested amplifications were performed starting from specific reverse primers located at positions: 290R (CGGGACTGGATTTCTTTGGCGTAC), 193R (CAATTGTGAAGTTCATGACTTTGAGAATC), 160R (TGGTCATGGCCAAGTTCACCAGGAT), 90R (CCAGCCATAAATCCCCACTTTGTA) or 24R (AGAGTACTGTTCCTGAGGCATCTC) of the cDNA sequence together with standard RACE primers. The following buffer (JD) was used for all PCR amplifications: 30 mM Tris, 8 mM HEPES, 8% glycerol, 1% dimethylsulphoxide (DMSO), 2 mM DTT, 5 mM $MgCl_2$, 20 mM potassium L-glutamate (Fluka), 60 mM ammonium acetate (Fluka), 0.002% gelatin.

FISH analysis

The direct R-banding FISH method was used for chromosomal assignment of the δ-*SG* gene. Preparation of R-banded chromosomes and FISH were performed as described by Matsuda *et al.* (43), and chromosomal identification was followed as in Li *et al.* (44).

Genomic DNA clones inserted in pBS (SK+) vector were used as biotinylated probes. The probes were labelled by nick-translation with biotin-16-dUTP (Boehringer Mannheim). The labelled DNA fragments were ethanol precipitated with a 100-fold excess of sonicated whole genomic DNA, salmon sperm DNA and tRNA and then denatured at 75°C in 100% formamide. The denatured probe was mixed with an equal volume of hybridization solution to make a final concentration of 50% formamide, 2× SSC, 10% dextran sulphate and 2 mg/ml bovine serum albumin (BSA, Sigma). Each 20 µl mixture containing 250 ng of labelled DNA was put onto a denatured slide, covered with Parafilm and incubated overnight at 37°C. The slides were washed for 20 min in 50% formamide in 2× SSC at 37°C and in 2× SSC and 1× SSC for 20 min each at room temperature. After rinsing in 4× SSC, they were incubated under coverslips with fluoresceinated avidin (Vector Laboratories) at a 1:500 dilution in 1% BSA/4× SSC for 1 h at 37°C. After washing, the slides were stained with 0.75 mg/ml propidium iodine. Excitation at a wavelength of 450–490 nm (Nikon filter set B-2A) and near 365 nm (UV-2A) was used for observation of R-banded and G-banded chromosomes, respectively. Kodak Ektachrome ASA100 films were used for microphotography.

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