

Quantification of Splice Variants Using Molecular Beacon or Scorpion Primers

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Uncovering the relationship between the generation of alternative transcripts and cellular processes is of crucial importance in the exploration of a gene's biology. The description and quantification of the spatio-temporal splicing pattern can be one method to select the most interesting transcripts for future studies. Fluorescence-based real-time quantitative RT-PCR has recently revolutionized the possibilities for transcriptional quantification studies. In this report, Molecular Beacon and Scorpion probes have been tested as new possibilities for determining the expression level of alternative transcripts. We validated these systems by analyzing alternative splicing of exons 6, 15, and 16 of the calpain 3 gene with tissues containing large variation in the ratio of the different transcripts. We determined conditions that demonstrated that boundary probes are useful tools and good alternatives to boundary primers, when developing a system to quantify specific transcripts. We suggest that the choice of a quantification system should depend in part on the structure and base composition of the gene and may have to be determined experimentally. © 2002

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One of the conclusions driven by the availability of the huge amount of sequence data generated in recent years is that the gene number present in a genome does not seem to correlate with the complexity of an organism. It is becoming clear that a part of the complexity resides in the proteome. Alternative splicing is one of the means to enhance the protein's catalogue through the generation of a diversity of transcripts. In particular, recent data suggest that the transcripts from 35 to 59% of human genes are alternatively

spliced and this is likely to be a significant underestimate (1). In addition several examples have demonstrated that one gene could encode hundreds or even thousands of different mRNAs (2–4). Therefore as a result the number of proteins in the proteome can exceed the number of genes by literally orders of magnitude. Uncovering the relationship between the generation of such a large number of isoforms and cellular processes is going to be a huge task. One of the means is to obtain a description of the spatiotemporal splicing gene pattern and its correlation with biological events. Therefore, there is a need for robust and reliable methods for such evaluations.

Fluorescence-based real-time quantitative RT-PCR, combining amplification, detection, and quantification, has recently revolutionized the possibilities for quantitating alternative transcripts (5, 6). This method encompasses several alternatives that can be used to discriminate different isoforms through the design of specific boundary-spanning probes or primers: use of DNA-binding dye (SYBR green), of Taqman probes, of two hybridization probes, of Molecular Beacon (7), or of Scorpion primers (8). It should be noted that, up to now, not all of these techniques have been used in alternative splicing studies. (i) SYBR green is a DNA-binding dye that emits fluorescence when bound to double-stranded DNA. To detect specific variants, it is then necessary to use boundary-spanning primers. (ii) Taqman probes are oligonucleotides complementary to a target sequence and labeled at one end with a fluorophore and at the other end with a fluorogenic quencher. The cleavage of the probe during PCR due to the 5'-exonuclease activity of the *Taq* polymerase results in an increase in fluorescence. (iii) When using two hybridization probes, one of the probes carries at its 3' end a fluorogenic donor and the other one a fluorogenic acceptor at its 5' end. They are chosen in such a way

that they hybridize head-to-tail in the target sequence allowing a fluorescence resonance energy transfer phenomenon to occur. (iv) Molecular Beacons are DNA hybridization probes flanked by a hairpin loop that holds a fluorophore and a quencher in close proximity. Specific binding of the probe to its target opens up the structure, producing a fluorescent signal. (v) Scorpion primers are primers flanked by a probe associated with a fluorophore and a quencher. The probe corresponds to a sequence in the vicinity of the primer and is held in a hairpin loop configuration until the hairpin is opened up by hybridization during amplification.

In our study, we evaluated the use of Molecular Beacon and Scorpion probes to quantify the alternative spliced isoforms of calpain 3. This protein is a calcium-binding cysteine protease whose defects have been associated with limb-girdle muscular dystrophy type 2A (9). This gene gives rise to a complex range of spliced isoforms affecting mostly two specific regions of the protein that are implicated in the regulation of proteolytic activity and subcellular localization (10). As preliminaries for future functional studies, we wanted to develop quantification systems for the two main splicing events of calpain 3. We were not able to find any boundary spanning primers that worked even in conventional PCR. Therefore, we tried boundary-spanning probes. We were able to bypass the competition problem inherent in the strategy of spanning probes by using an increased amount of primers and demonstrated the usefulness of Molecular Beacon and Scorpion technologies in quantification assays of alternative transcripts. We suggest that there is no universal strategy for detecting alternative splicing and a choice can be made between boundary probes or primers or between the different chemistries (Molecular Beacon, Scorpion, Taqman, and SYBR green). The choice could depend, among others, on the sequence composition of the boundary region.

MATERIALS AND METHODS

RNA Isolation and cDNA Synthesis

Total RNAs were isolated from frozen skeletal muscles (tibialis anterior and quadriceps) and retinas of 129Sv or transgenic mice using TRIzol Reagent (Invitrogen, Gröningen, The Netherlands) and the Fast-Prep apparatus (Bio 101, Vista, CA). Four transgenic lines were used: C3 Tg-7.2 and C3 Tg-7.9 for the complete calpain 3 cDNA and ex6-Tg-41.1 and ex6-Tg-37.3 for the minus exon 6 calpain 3 cDNA (M. Spencer *et al.*, submitted for publication). One microgram of total RNA was reverse-transcribed to single-stranded cDNA with 50 U of Superscript II Reagent (Invitrogen) and

random hexamers. Reverse transcription (RT)¹ was performed in a 20- μ l final volume and the sample was incubated at 42°C for 60 min. In addition, multiple-tissue cDNAs containing cDNA from heart, brain, lung, and skeletal muscle were purchased from Clontech (Palo Alto, CA).

Cloning of Exons 6–15–16 Spliced Isoforms

Calpain 3 PCR products were subcloned into the pASCII plasmid in *Escherichia coli* XL1 Blue. Subclones were subjected to sequence analysis using internally specific primers and specific primers of the plasmid, by the dideoxy method on Applied Biosystems sequencers. Plasmids were named as follows: pA6- for skipping of exon 6, pA15–16- for skipping of exons 15–16, pA6–15–16- for skipping of exons 6–15–16, and pA-CAPN3 for complete calpain 3 cDNA.

Primer and Probe Design

Primers were designed using the OLIGO 4.0 software (Primer Analysis Software; Table 1). Primers with minimal secondary structures and T_m close to 60°C were selected. Three pairs of primers and three reverse primers, respectively, for exon 6 skipping and exons 15–16 skipping quantification were purchased from Genosys (Cambridge, UK). These couples were tested on serial dilutions of pA6- or pA15–16- for low cycle threshold (Ct)¹ values and amplification efficiency close to 100%. The specificity of the PCR amplifications was assessed by agarose gel electrophoresis and sequencing of the amplicons. Primers retained are listed in Table 1.

Molecular Beacon for quantification of exon 6 skipping was designed with DNA Mfold software (<http://www.ibc.wustl.edu/zuker>) and purchased from Stratagene (La Jolla, CA; Table 1A). Predictive T_m of the specific part of the probe is 63°C, i.e., 5° higher than the primer T_m's. In this way, fluorescence signal can be detected only when Molecular Beacon's target recognition is perfect. The Scorpion probe for quantification of exons 15–16 skipping was modeled using the DNA Mfold software and purchased from Oswel-Eurogentech (Southampton, UK, and Liege, Belgium; Table 1B). The principle of the Molecular Beacon and Scorpion primer mechanisms is diagrammed in Fig. 1.

Quantification of global calpain 3 mRNA level was performed using two sets of primers and probes located in exon 3 and exons 4–5, which are not spliced (Table 1C).

Real-Time Quantitative PCR

PCR amplifications were performed using 9 μ l of a 1/20 dilution of each reverse transcription reaction

¹ Abbreviations used: T_m, melting temperature; Ct, cycle threshold; RT, reverse transcription.

TABLE 1

(A) Primer and Molecular Beacon Probe Sequences for Splicing of Exon 6

	Name	Sequence
Forward primer 1	Exon6-1.F	TGAGGAAAGCTATCGAGAGAGG
Forward primer 2	Exon6-2.F	ATGCTCCGAGTGACATGTACAA
Forward primer 3	Exon6-3.F	TTCACAGGAGGGGTGACAGAG
Reverse primer 1	Exon6-1.R	CCAACCCACAGGCCATTC
Reverse primer 2	Exon6-2.R	ATGCCCTTTCACCAACCCAC
Reverse primer 3	Exon6-3.R	CCCACAGGCCATTCTTGTTT
Molecular Beacon probe	Exon6-MB	CGGTGGCTGCTCCATTGACACAATTGTTTCGACCG

(B) Primer and Scorpion Sequences for Splicing of Exons 15–16

	Name	Sequence
Reverse primer 1	Exon15–16-1.R	CTGCGATCTGCCTGAAGATGT
Reverse primer 2	Exon15–16-2.R	TGCGATCTGCCTGAAGATG
Reverse primer 3	Exon15–16-3.R	GCGATCTGCCTGAAGATGT
Scorpion probe	Exon15–16-Scorp	CCGCGGGCCCTTGGCACTGGCCGCCGCGG76CTCTGAGGAAGCTGAAAATACAAT

(C) Primers and Probes for Quantification of Global Calpain 3 mRNA Level (Exons 4–5 and Exon 3)

	Name	Sequence
Exons 4–5 probe	M884CAPN3.P	TGCCAAGCTCCATGGCTCCTATGAAG
Exons 4–5 forward primer	M811CAPN3.A	ACAACAATCAGCTGGTTTTTCACC
Exons 4–5 reverse primer	M954CAPN3.M	CAAAAAACTCTGTCACCCCTCC
Exon 3 probe	M427CAPN3.P	CGGCTGCTTTTTCCGAGTTATACCCCATGAT
Exon 3 forward primer	M402CAPN3.A	CATCGCCTGCCTGACCC
Exon 3 reverse primer	M485CAPN3.M	ATCCCTGCGTAGTTTTTCAGTGAA

Note. (A) and (B) selected primers are indicated in boldface.

product or of a 1/10 dilution of Clontech cDNAs. Standard Reaction buffer contains 1× Taqman buffer, 5 mM MgCl₂, 200 nM primers (forward and reverse), 0.5 units of AmpliTaq Gold DNA polymerase (Applied Biosystems, Foster City, CA), and 100 nM Taqman probe in a final volume of 18 μl.

Various primer and probe concentrations were used to adjust the conditions of amplification. Primers for quantification of exon 6 skipping were used at 0.375, 0.75, 1.1, and 1.5 mM and Molecular Beacon at 50, 100, 150, and 200 nM concentrations. Scorpion probe and reverse primer for exons 15–16 skipping quantification were used at 325 and 750 nM concentrations.

Standard amplification conditions consisted of an AmpliTaq Gold activation step at 95°C for 10 min followed by 40 cycles of two steps: 15 s of denaturation at 95°C and 60 s of annealing at 60°C. For quantification experiments using Molecular Beacon or Scorpion probe, 40 cycles of three steps including 15 s of denaturation at 95°C, 30 s of annealing at 55°C, and 30 s of elongation at 72°C were used. PCRs were performed on an ABI Prism 7700 sequence detector (Perkin–Elmer Applied Biosystem), allowing automatic data collection of the fluorescence emission. The mRNA level of each sample was determined as an average from duplicate data.

Standard Curves and Estimation of Ratios between Spliced and Global Calpain 3 mRNA Populations

Serial dilutions of plasmids pA6- and pA15–16- were used to determine the standard curves of Molecular Beacon and Scorpion probe quantification systems, respectively. Standard curves were determined using linear regression analysis of the Ct values relative to plasmid copy numbers. For the quantification experiments on biological samples, each of the different sets of primers and probes designed to quantify calpain 3 splicing events was used in the same serial dilution of pA6–15–16-, allowing direct comparison of their expression levels in the samples. Proportions of spliced transcripts were directly obtained with the ratio of results provided by the splicing event quantification system and by the global calpain 3 mRNA quantification system.

Normalization of Quantitative PCR

To account for variations due to RNA extraction and the RT reaction, the measured levels of the different calpain 3 mRNAs were correlated with those of TFIID mRNA. TFIID is a transcription factor that has been used as an endogenous control (11). Results were expressed as the ratio of the mRNA level of each gene of

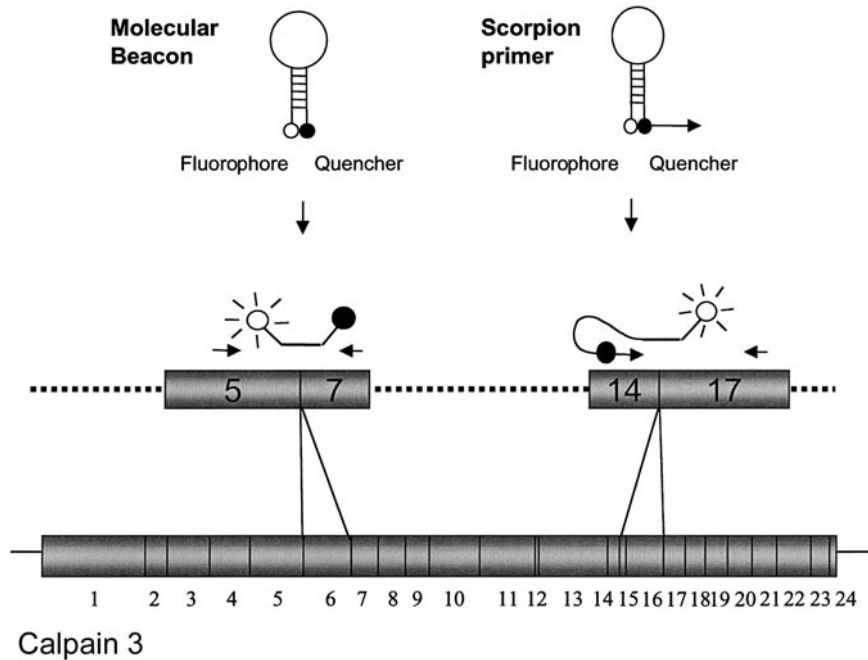


FIG. 1. Principle of the detection of calpain 3 splicing events by Molecular Beacon and Scorpion probes. (Top) Schematic representation of Molecular Beacon and Scorpion primer molecules. In the absence of target, these molecules do not emit fluorescence, as the quencher is close to the fluorophore. (Middle) Mechanisms of detection of the splicing out of calpain 3 exon 6 by Molecular Beacon and of calpain 3 exons 15 and 16 by Scorpion primer. Hybridization of the probe sequence separates the quencher from the fluorophore, restoring fluorescence. (Bottom) Representation of the complete calpain 3 mRNA with its 24 exons.

interest (calpain-3 global transcript population and splicing events) to the mRNA level of TFIID.

RESULTS

Validation of Quantification Systems Using Molecular Beacon and Scorpion Probe as Relevant Tools to Quantify Splicing Events

After having determined primers giving the best results for quantification, i.e., low Ct values and amplification efficiency close to 100%, we tested the specificity of Molecular Beacon and Scorpion probes on a high-copy-number (10^6 copies) plasmid containing a complete cDNA isoform of calpain 3 (pA-CAPN3). No signal was detected in these assays, indicating good specificity of the probes (data not shown).

Alternatively spliced transcripts of the calpain 3 gene seem to be in the minority in skeletal muscles (10). In order to set up optimal conditions for the quantitative detection of the underrepresented transcripts using the Molecular Beacon and Scorpion probe systems, we first studied the influence of an excess of complete form on the quantification parameters (Ct values and standard curves). For this purpose, we performed amplification on dilution series of pA6-15-16-diluted either in water or in skeletal muscle cDNAs. cDNA of a skeletal muscle giving a Ct value of 25 for global calpain 3 expression level was used in these

assays. This Ct value corresponds roughly to a copy number of 10^5 estimated by comparison to a dilution series of plasmid (data not shown).

Our results show a strong inhibition of the detection of low-copy-number plasmids diluted in skeletal muscle cDNAs (Fig. 2A). When the plasmid containing a spliced isoform is underrepresented, Ct values obtained with the dilution in muscle cDNAs are increased compared to the dilution in water. For example, a dilution of 1000 plasmid copies gives a Ct value of 34 when diluted in muscle cDNA and a Ct value of 32 when diluted in water. This phenomenon is amplified when the copy number is lower and results in a decrease in amplification efficiency from 100% for the dilution in water to 60% for the dilution in muscle cDNAs.

As a means to limit the competition from the full-length transcripts, we challenged our quantification systems with larger amounts of primers. We also tested larger amounts of Molecular Beacon and Scorpion primers. Using these conditions, we obtained a decisive improvement in the quantification signals. Quantification of plasmids diluted in water or in muscle cDNAs give similar Ct's and amplification efficiency values (Fig. 2B). At primer and Molecular Beacon concentrations respectively of 1.5 mM and 200 nM, we can now accurately detect 100 copies of exon 6 spliced tran-

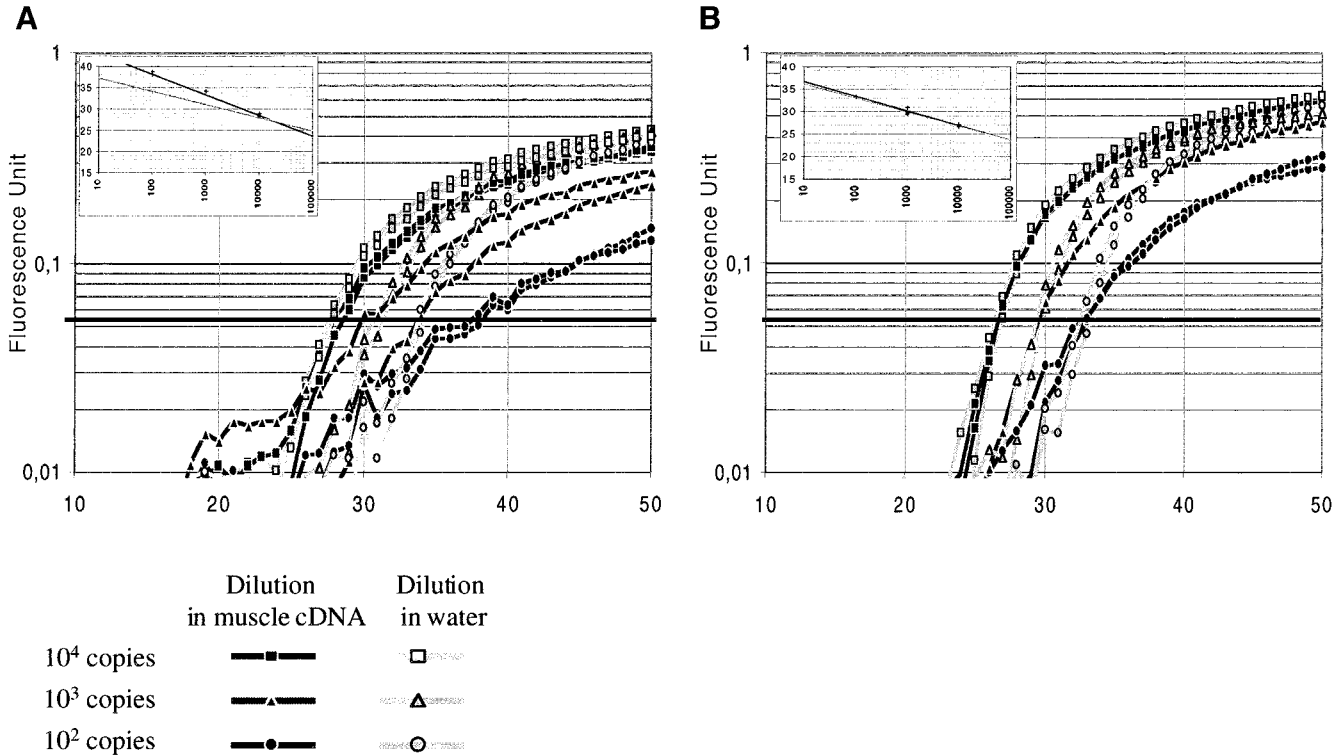


FIG. 2. Quantification of calpain 3 exon 6 splicing event using a Molecular Beacon. Quantification was assayed on serial dilutions of a plasmid containing calpain 3 isoform lacking exon 6. 10-fold dilutions were performed in water (light lines) and in a skeletal muscle cDNA containing the calpain 3 complete isoform in excess (dark lines). Results of the quantitative assays are presented as amplification curves for three plasmid dilutions (10^4 , 10^3 , and 10^2 copies) and as their associated standard curves (presented in the insets). The threshold, indicated as a black line, is set at 0.05. Results are shown for two different concentrations of primers and probes: 150 nM and 1 mM Molecular Beacon and primers (A) and 200 nM and 1.5 mM Molecular Beacon and primers (B). In (A), a strong inhibition of amplification is observed, i.e., higher Ct values and decreased amplification efficiency for plasmid diluted in skeletal muscle cDNAs compared to plasmid diluted in water. In (B), corresponding to the results obtained with higher concentrations of primers and molecular beacon, amplification efficiency for the dilution in muscle cDNAs is restored and gives results similar to those of the dilution in water.

scripts mixed with 10,000 copies of complete isoforms, representing a ratio of 1%.

Similar results were obtained for quantification of the exons 15–16 splicing event. At 750 nM concentration of reverse primer and Scorpion probe, it was possible to accurately quantitate up to 1% of spliced isoforms (Fig. 3). Of interest, the Scorpion detection system seems to be less sensitive to competition with complete isoforms in as much lower amounts of primers are needed to restore amplification efficiency (data not shown).

Determination of Expression Levels of Spliced Transcripts in Biological Samples

Having validated Molecular Beacon and Scorpion probes as adequate tools and having determined conditions of accurate quantification, we next evaluated the relative proportion of transcripts lacking exon 6 or exons 15–16 in different cDNAs isolated from tissue samples. Samples were chosen in order to test different

ranges of mRNA quantity of complete or spliced calpain 3 isoforms. We selected (i) tissues in which calpain 3 is expressed at low level (10), (ii) skeletal muscle in which calpain 3 is known to be predominantly expressed, and (iii) tissue from transgenic mice overexpressing complete or spliced calpain 3 transcripts (M. Spencer *et al.*, submitted for publication).

Among the different tissues tested, skeletal muscle is the predominant site of calpain 3 expression. In heart, brain, lung, and retina, calpain 3 is expressed 100- and 1000-fold less than in this tissue (Fig. 4A). Differences in the composition of transcript populations were observed. In skeletal muscle, transcripts lacking exon 6 or exons 15–16 are in the minority in comparison with the complete form (Figs. 4B, 4C, 4D, and 4E). Exon 6 splicing was also underrepresented in all the other tissues (Fig. 4D), whereas splicing of exons 15–16 shows a larger variability, being rare in retina but the major event in heart and in lung (Fig. 4E).

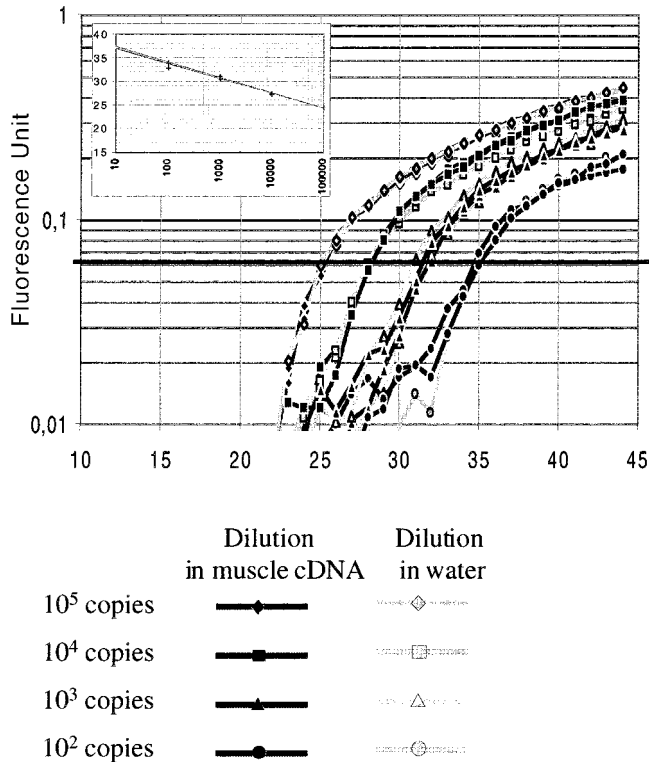


FIG. 3. Quantification of calpain 3 exons 15–16 splicing event using a Scorpion primer. Quantification was assayed on serial dilutions of a plasmid containing calpain 3 isoform lacking exons 15–16. 10-fold dilutions were performed in water (light lines) and in skeletal muscle cDNAs containing the calpain 3 complete isoform in excess (dark lines). Results of quantitative assays are presented as amplification curves for four plasmid dilutions (10^5 , 10^4 , 10^3 , and 10^2 copies) and as their associated standard curves (presented in the inset). The threshold, indicated as a black line, is set at 0.05. Results are shown for an optimized primer and Scorpion probe concentration of 750 nM. Amplification curves are similar between dilutions in water and dilutions in skeletal muscle cDNAs. Standard curves also give similar results, confirming a reduction of the competition process.

As expected, results in muscles from the four transgenic lines tested show a strong increase in global calpain 3 expression, ranging from 5- to 100-fold more than in normal skeletal muscle (Fig. 4A). In the exon 6 minus lines, this represents an elevation of the expression level of the exon 6-spliced isoform of 1000 and 10,000, in comparison with the observed basal level of this isoform in normal muscle (Fig. 4B). The proportions of exon 6-spliced transcripts correspond to 65 and 95% of the calpain 3 global mRNA populations in these mice (Fig. 4C).

DISCUSSION

In this report, we demonstrated, for the first time to our knowledge, the feasibility of using boundary-span-

ning Molecular Beacon and Scorpion probes for quantification of alternative splicing. Using these systems, we were able to, accurately and sensitively, quantify alternative splicing events of the calpain 3 gene.

We first observed the existence of a competition phenomenon preventing accurate quantification of under-represented alternative transcripts. A possible explanation for this inhibition could be a progressive depletion of primers along amplification cycles as the primers can hybridize to the unspliced calpain 3 cDNA present endogenously in the skeletal muscle sample. During the reaction, both isoforms use up primers, but as the complete PCR products predominate, they are preferentially amplified as the primer amounts decrease. Accurate quantification of minor spliced isoforms is then impaired. Depletion of probes is less likely to be responsible for this quantification impairment, as Molecular Beacon and Scorpion do not hybridize to complete transcripts as shown above. Using a boundary-spanning Taqman probe aimed at the quantification of alternative transcripts of the neurofibromatosis type 1 gene, Vandembroucke *et al.* reported a similar competition phenomenon (12). They obtained better results using a boundary-spanning primer in combination with a Taqman probe or with the SYBR green-based assay. In this case, no competition could occur during amplification as the primers cannot hybridize to the full-length cDNA and they were able to detect transcripts at a level of 0.1% of the global transcript population. However, a strong limitation of the boundary-spanning primer strategy is the restricted region in which the primer has to be designed, i.e., the splice junction. Therefore, this strategy may not be adequate in the case of inappropriate nucleotide composition for primer design (loops and GC- or AT-rich regions). In fact, regarding calpain 3, Molecular Beacon and Scorpion boundary-spanning probes were used in a context in which boundary-spanning primers failed to amplify spliced transcripts.

We were able to circumvent the competition problem by increasing the amounts of primers and probes used in the quantification assays and we demonstrated that quantification systems based on boundary-spanning Molecular Beacon and Scorpion probes are efficient, even with minor spliced variants. It should be noted that less primer is necessary to restore quantification efficiency with Scorpion probes than with Molecular Beacon. In the Scorpion primer, the probe is attached to the primer, giving a higher probability that the probe will find its specific target due to spatial proximity compared to a separated probe. However, it should be kept in mind that the aim of this study was not to compare Molecular Beacon and Scorpion probe systems and that these systems quantify two different spliced variants, making them not directly comparable.

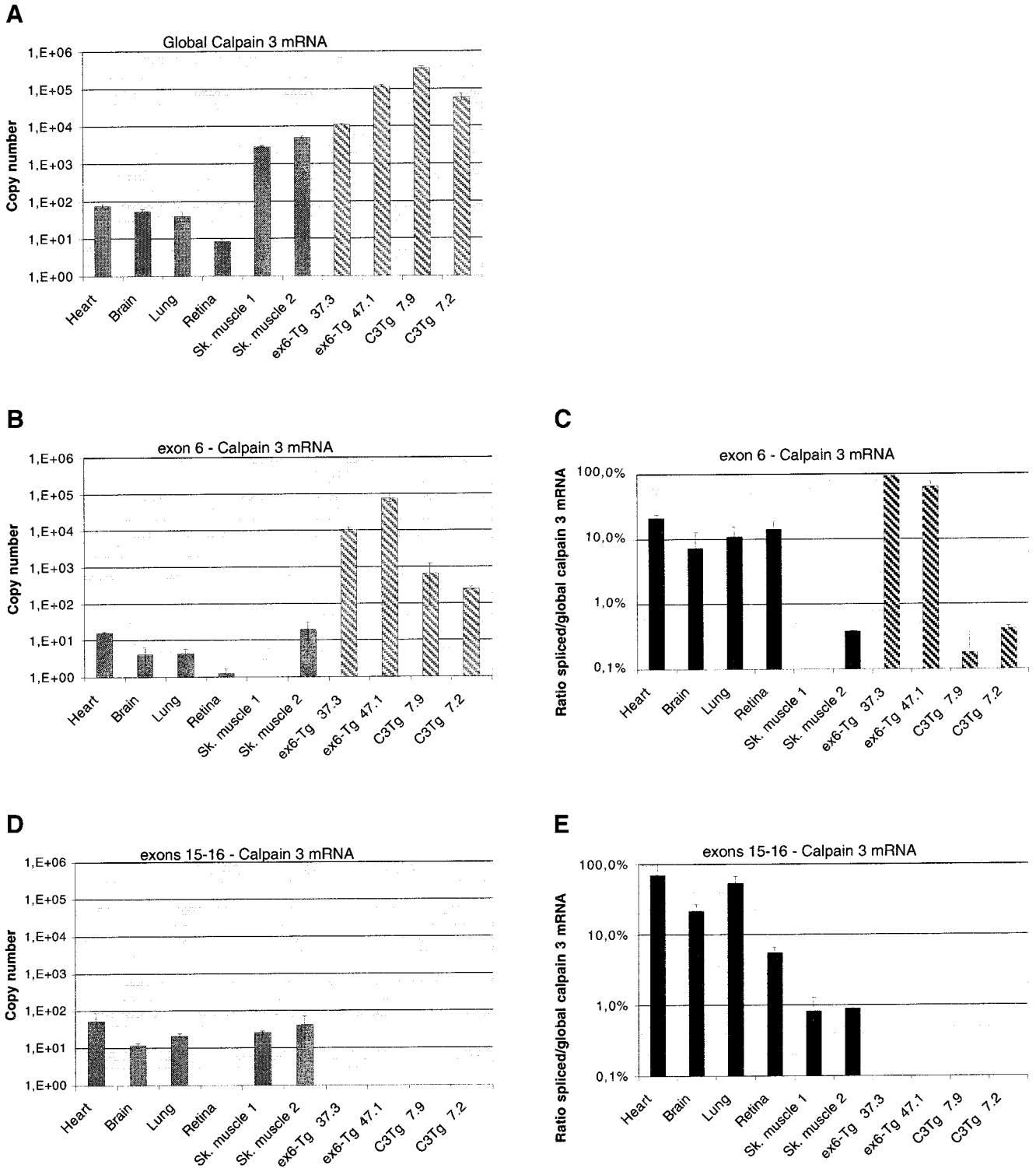


FIG. 4. Quantification of calpain 3 splicing events in biological samples. The biological samples correspond to heart, brain, lung, retina, and skeletal (Sk.) muscle from normal mice. Ex6-Tg37.3, ex6-Tg47.1, C3Tg 7.9, and C3Tg 7.2 correspond to skeletal muscle cDNA from transgenic mice and are indicated as hatched bars. Ex6-Tg37.3 and ex6-Tg47.1 are transgenic for a calpain 3 cDNA without exon 6 and C3Tg 7.9 and C3Tg 7.2 for a complete calpain 3 cDNA. (A) Quantification of global calpain 3 mRNA population, (B) quantification of exon 6 minus splicing event, and (D) quantification of exons 15–16 splicing event. For these three graphs, data are presented in copy number. (C and E) The ratio between transcripts lacking exon 6 or exons 15–16 and calpain 3 global mRNA population present in the samples is graphed.

Although we have not tested it, an identical strategy of increased amount of primers should also work with Taqman probes. In addition, the Molecular Beacon and Scorpion primers are systems that could be used with boundary primers. These methods can also be adapted to multiplex PCR with different fluorophores for each probe and can be used to quantify simultaneously different splicing events. Therefore, several possibilities exist to quantify splicing variants specifically. We suggest that there is no absolute solution and that the choice of a quantification system should depend in part on structure and base composition of the gene and may have to be determined experimentally. The validation steps of a system should comprise the demonstration of a specific amplification with efficiency close to 100% and the possibility to detect linearly the spliced sequence of the unspliced sequence with ratio down to 1%.

Using Molecular Beacon and Scorpion probe-based quantification systems, we evaluated expression levels of alternatively spliced transcripts on samples from different normal tissues and on skeletal muscles of calpain 3 transgenic mice. The aim of this study was to challenge our quantification systems on biological samples known to contain a large range of calpain 3 complete and spliced isoform amounts. The results obtained are in agreement with previously described observations obtained by the RT-PCR method (10) or by Western blot analyses (M. Spencer *et al.*, submitted for publication) and confirmed normal skeletal muscle as the main site of expression of calpain 3. In non-muscle tissues, the global level of calpain 3 is quite low. As a consequence even when the proportion of spliced forms compared to complete forms is high, expression levels of spliced transcripts are always very low. Transgenic samples enabled us to quantify transcript in contexts in which calpain 3 is highly expressed and exon 6 splicing is the major event. As expected, (i) the estimated ratio "spliced/global transcripts" is decreased in mice transgenic for the complete isoform compared to normal muscle, reflecting the existence of a high copy number of the transgene, and (ii) muscles of mice transgenic for the exon 6-spliced isoform demonstrated a high and predominant expression level of this variant. Overall, these normal and transgenic contexts represent a wide range of situations and validate our system using Molecular Beacon as an adequate quantification tool.

Regarding the results provided by calpain 3 quantification in biological samples, a point of discussion would be the functional relevance of the very low expression levels we obtained in the nonskeletal muscle tissues. In the face of such low expression levels, one can wonder whether this observation would not corre-

spond to an illegitimate transcription without biological relevance (13). It is clear that diversity in the transcriptome driven by alternative splicing could serve for the spatial and temporal fine tuning of biological processes. A transcript with usual low abundance could be up-regulated at key moments of cell life in order to address special functions and may represent therefore molecules of biological importance. Moreover, a low level could correspond to only a subpopulation of remarkable or particular cells in the sample (i.e., stem cells).

The quantitative RT-PCR technology, suitable for high throughput, allows the extensive exploration of the expression profiles of alternative transcripts and, therefore, could be very helpful in the exploration of a gene's biology. The demonstration of the usefulness of Molecular Beacon and Scorpion to quantify alternative transcripts extends the possibilities to perform such studies.

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