Regulation of splicing is responsible for the expression of the muscle-specific 2a isoform of the sarco/endoplasmic-reticulum Ca^{2+} -ATPase

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Tissue-specific alternative processing of sarco/endoplasmic reticulum Ca^{2+} -ATPase 2 (SERCA2) transcripts generates functionally different Ca^{2+} pump isoforms in muscle compared with non-muscle tissues. In non-muscle cells, the SERCA2 pre-mRNA can be polyadenylated at a site located between the donor and acceptor splice site of an intron which is only removed in muscle tissues. To define the *cis*-active elements involved in differential processing, we constructed a minigene (pCM β SERCA2) containing the 3' end of the SERCA2 gene. When stably transfected into a myogenic cell line, minigene transcripts were differentially processed depending on the differentiation state of the cells. This proves that the essential elements required for regulated processing are present in the construct. Furthermore, co-transfection of the pCM β SERCA2 minigene and a myogenin expression

vector in a fibroblast cell line induced muscle-specific splicing of transcripts from pCM β SERCA2. This shows that *trans*-acting factor(s) responsible for muscle-specific processing can be induced by one of the important regulatory genes of muscle differentiation. Inactivation of the non-muscle poly(A) site did not induce splicing in non-muscle cells. This excludes a simple competition model between splicing and polyadenylation, but it is consistent with splicing being very inefficient in non-muscle cells. Moreover, splicing could be induced in non-muscle cells by optimizing the muscle-specific donor splice site and/or by shortening the intron length. We therefore propose that expression of the muscle-specific SERCA2a isoform is the result of activation of an otherwise inefficient splicing process.

INTRODUCTION

Organellar Ca^{2+} -transport ATPases (Ca^{2+} pumps) mediate the uptake of Ca^{2+} into intracellular stores, such as the sarcoplasmic or the endoplasmic reticulum. Three different SERCA genes (1, 2 and 3) have been identified [reviewed in Wuytack et al. (1992)]. In addition, alternative processing of primary gene transcripts leads to a tissue-specific and developmentally regulated pattern of SERCA isoform expression. The SERCA1 gene is exclusively expressed in fast-twitch skeletal muscle. Alternative splicing generates an adult (SERCA1a) and a neonatal (SERCA1b) isoform (Brandl et al., 1986, 1987). The SERCA2 gene is expressed in all other cell types. Tissue-specific differential processing of the 3' end of the SERCA2 mRNA gives rise to two different Ca^{2+} -pump proteins which differ in their C-terminal part (Genteski-Hamblin et al., 1988; Lytton and MacLennan, 1988; de la Bastie et al., 1988; Eggermont et al., 1989). In SERCA2b, the last four amino acids of SERCA2a are replaced by a tail of 49 amino acids. SERCA2b is considered as the 'housekeeping' isoform and is expressed in non-muscle tissues and in smooth muscle. On the other hand, SERCA2a is typical of cardiac and slow-twitch skeletal muscle and is also present in smooth muscle. The expression pattern of the SERCA3 gene is less well documented but has been demonstrated to be expressed in non-muscle tissues (Burk et al., 1989; Wu and Lytton, 1993). Distinct functional characteristics have been reported for the muscle-specific isoforms (SERCA1 and SERCA2a) and their non-muscle (SERCA2b and SERCA3) counterparts (Verboomen et al., 1992; Lytton et al., 1992). The SERCA2 isoforms differ with respect to their Ca^{2+} affinity (SERCA2a < SERCA2b) and their turnover rate for Ca^{2+} transport and ATP hydrolysis (SERCA2a > SERCA2b).

Northern blots and S1 nuclease assays have revealed four different SERCA2 mRNAs (de la Bastie et al., 1988; Eggermont et al., 1990; Plessers et al., 1991). These messengers differ in their 3' end and are expressed in a tissue-specific way. Cardiac, slow-twitch skeletal and smooth-muscle cells contain a SERCA2a-encoding mRNA (class 1), whereas non-muscle cells show class-2 mRNA. A third mRNA (class 3) was detected in muscle and non-muscle cells, and class-4 mRNA is specific to the brain. The class-2, -3 and -4 mRNAs all encode the SERCA2b isoform. The muscle-specific mRNA (class 1) is generated by a unique splicing process which removes an optional intron at the 3' end of the SERCA2 messenger. In contrast, the predominant non-muscle mRNA (class 2) is polyadenylated at a site that lies in the muscle-specific intron. Muscle-type splicing and non-muscle polyadenylation are therefore mutually exclusive.

Similar processing choices, *i.e.* splicing *versus* polyadenylation, have been described for IgM heavy chain gene, for the calcitonin/calcitonin gene-related peptide (CT/CGRP) gene and for *Drosophila doublesex* (*dsx*) [reviewed in Smith et al. (1989) and McKeown (1992)]. In each case there is a choice between polyadenylation at an internal site and splicing to a downstream exon. The processing of IgM heavy-chain mRNA seems to be regulated by an increase in the global cleavage-polyadenylation efficiency during maturation of B-lymphocytes which leads to preferential usage of the internal poly(A) site in plasmacytes (Danner and Leder, 1985; Galli et al., 1988; Peterson et al., 1991). In contrast, CT/CGRP and *dsx* processing are regulated at the level of splicing (Leff et al., 1987; Emeson et al., 1989). For *dsx* the weak female-specific acceptor splice site is skipped in males, but is specifically activated by the Tra and Tra2 proteins in females (Hoshijima et al., 1991; Ryner and Baker, 1991; Tian and Maniatis, 1992).

Abbreviations used: SERCA, sarco/endoplasmic-reticulum Ca^{2+} -ATPase; *dsx*, *doublesex*; CT/CGRP, calcitonin/calcitonin gene-related peptide; 5'D1, 5' donor splice site 1; 5'D2, 5' donor splice site 2; 3'A, 3' acceptor splice site; pA_u, upstream polyadenylation site; pA_d, downstream polyadenylation site; RT, reverse transcriptase; ASF/SF2, alternative splicing factor/splicing factor 2.

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Figure 1 pCM β SERCA2 construction

pCM β SERCA2 was constructed using as starting materials pCM β neo, pUC19, p β 5'SVBgIII, pSERCA2-5 and pER-15 as outlined in the Materials and methods section. Restriction fragments used are indicated with a dashed line. Thin lines are bacterial sequences. The simian virus 40 promoter sequences are represented as black boxes and the cytomegalovirus sequences as boxes with + signs. The Neo gene is hatched and the rabbit β -globin regions are stippled. The SERCA2 sequences are represented as open boxes and the relevant SERCA2 exons are numbered as described by Eggermont et al. (1991). DFR refers to the downstream flanking region of SERCA2. B, *Bam*HI; E, *Eco*RI; H, *Hind*III; P, *Pvu*I; Sf, *Sfa*NI; Sm, *Sma*I; Sp, *Sph*I; Amp, β -lactamase gene.

We have investigated whether processing of SERCA2 mRNA, which is responsible for the specific formation of either the muscle or the non-muscle Ca²⁺-pump isoforms, is regulated at the polyadenylation or the splicing level. In order to do this, we constructed and modified a minigene (pCM β SERCA2) containing the 3' end of the SERCA2 gene. From transfection studies, we conclude that SERCA2 processing is regulated at the splicing step and not at the polyadenylation step. Moreover, we found that the *trans*-acting factor(s) responsible for activation of splicing can be induced by myogenin.

MATERIALS AND METHODS

Cell culture

BC₃H1 mouse myoblasts were cultured as described previously (De Smedt et al., 1991). For differentiation studies, the cells were seeded at a concentration of 5000 cells/cm² in 175 cm² flasks in Dulbecco's modified Eagle's medium supplemented with 20% (v/v) fetal calf serum, 3.5 mM L-glutamine, 0.9% (v/v) non-essential amino acids, 85 μ g of streptomycin/ml and 85 units of penicillin/ml (Flow Laboratories, Irvine, Scotland, U.K.). After 3 days, differentiation was induced by switching the BC₃H1 cells

to medium containing 0.5% (v/v) serum. The cultures were supplied with fresh growth medium at days 2, 4, 7, 10 and 14 after the start of differentiation. C3H/10T1/2 fibroblasts (clone 8) were obtained from ATCC (Rockville, MD, U.S.A.) and were cultured in the same way as the BC₃H1 cells except that 10% fetal calf serum was used in the growth medium.

pCM β SERCA2 construction and modifications

The construction of pCM β SERCA2 is outlined in Figure 1. We started from the pCM β neo vector [kindly provided by Dr. L. Nelles, Center for Thrombosis and Vascular Research, Leuven, Belgium and described by Lijnen et al. (1990)]. This vector contains the human cytomegalovirus immediate-early promoter, the neomycin phosphotransferase gene (Neo) flanked by the simian virus 40 early promoter/enhancer and the rabbit β -globin 3' processing signals. In this pCM β neo vector, a *Pvu*I (partial digest)–*Hind*III restriction fragment containing the rabbit β -globin 3' processing signal was replaced by a *Pvu*I–*Hind*III fragment containing the polycloning site of pUC19 (Gibco-BRL, Gaithersburg, MD, U.S.A.), resulting in pCM19neo. Subsequently, a *Sfa*NI (blunt ended)–*Eco*RI fragment from the

p β 5'SVBgIII vector containing the rabbit β -globin sequence [kindly provided by Dr. N. J. Proudfoot, Sir William Dunn School of Pathology, Oxford, U.K. and described by Grosveld et al. (1982)] was cloned into *Bam*HI(blunt ended)-*Eco*RI-linearized pCM19neo, resulting in pCM19neoR β G. In the *Eco*RI site of this vector, the 3' end of the SERCA2 gene was cloned. In order to obtain this fragment, a *Sma*I-*Eco*RI fragment of the pig genomic clone pSERCA2-5 (Eggermont et al., 1991) was cloned into *Sma*I-*Eco*RI-linearized pGEM-7Zf(+) (Promega, Madison, WI, U.S.A.). The *Sph*I fragment from this vector replaced the *Sph*I fragment in the pig cDNA clone pER-15 (Eggermont et al., 1990) and the *Eco*RI fragment from the resulting pSERCA2 vector was cloned into the *Eco*RI site of pCM19neoR β G resulting in pCM β SERCA2.

Mutations were created using the Altered Sites *in vitro* Mutagenesis System (Promega). A *Xba*I-*Xba*I and a *Xba*I-*Afl*II restriction fragment was cloned into the pALTER vector in order to modify the optional donor splice site (5'D1) and the upstream polyadenylation site (pA_u) respectively. In Mut1, the 5'D1 site (TG/GTAAAG) was converted at positions +5 and +6 into the consensus donor splice site [TG/GTAAGT (Shapiro and Senapathy, 1987)]. In Mut2, the pA_u was destroyed by two point mutations in the AATAAA consensus sequence resulting in ATGAAA. A large fragment (1924 nt) of exon 23 and 24 was deleted by removing the *Afl*II-*Eco*NI restriction fragment. Klenow fill-in and recircularization of the vector resulted in De11. In Sub1, a 1924 nt-long PCR fragment amplified using λ DNA (molecular-mass marker III; Boehringer-Mannheim, Mannheim, Germany) as starting material, was cloned into the *Bst*EII restriction site of pCM β SERCA2. The substituted DNA sequence corresponded to the 11425–13349 part of the λ DNA as described by Sanger et al. (1982), except that position 11426 was mutated to a T in order to create a *Bst*EII restriction site and one G was deleted at position 11432. All the modifications were confirmed by sequencing. The sequences of the mutated fragments were verified before transferring them from pALTER to the construct in order to exclude unwanted additional modifications.

DNA transfections

BC₃H1 and C3H/10T1/2 cells were stably transfected using the calcium phosphate coprecipitation method. Briefly, 24 h before transfection exponentially growing cells were trypsinized and seeded at a density of 50000 cells/cm² in cell culture flasks (75 cm²). The DNA to be transfected (0.35–1 $\mu\text{g}/\text{cm}^2$) was diluted in HEPES-buffered saline (pH 7.05). CaCl_2 was added to a final concentration of 125 mM and the mixture was kept at room temperature for 30 min. The monolayers were incubated with the calcium phosphate/DNA coprecipitate for 15 min at room temperature and thereafter growth medium was added. After 48 h, the cells were trypsinized and replated at lower cell density in selection medium consisting of growth medium with 400 $\mu\text{g}/\text{ml}$ geneticin (Gibco-BRL). Co-transfection of the C3H/10T1/2 cells with the myogenin expression vector (EMSV-Myo8; kindly provided by E. N. Olson, M. D. Anderson Cancer Center, Houston, TX, U.S.A.) and pCM β SERCA2 was performed using a 50:1 ratio (100 μg of EMSV-Myo8, 2 μg of pCM β SERCA2) in order to ensure that each cell containing pCM β SERCA2 DNA also expresses myogenin.

RNA isolation and reverse transcriptase (RT)-PCR

Total RNA was isolated from transfected or untransfected BC₃H1 and C3H/10T1/2 cells as described by Chirgwin et al.

(1979). Oligo(dT)-primed first-strand cDNA was synthesized typically in a 20 μl volume containing 1 μg of total RNA, 200 units of M-MLV-RT (Gibco-BRL), 1 $\times$ M-MLV-RT buffer (Gibco-BRL), 1 mM dATP, dCTP, dGTP and dTTP (Boehringer-Mannheim), 20 units of RNAase inhibitor (rRNasin; Promega), 200 ng of oligo(dT)₁₅ (Boehringer-Mannheim), and incubated at 37 °C for 1 h. Portions of the first-strand cDNA mix were then PCR amplified using Hot Tub DNA polymerase (Amersham International, Amersham, Bucks., U.K.). The PCR mixture (50 μl) contained 1 $\times$ Tub buffer (low Mg^{2+} ; Amersham International), 0.2 mM each dNTP, 25 pmol of each forward and reverse primer(s) and 2.5 units of Hot Tub DNA polymerase. When homologous primers were used, each cycle consisted of 1 min denaturation at 95 °C, 1 min annealing at 55 °C and 1 min 30 s extension at 72 °C. When heterologous primers were used, annealing was performed at 45 °C during the first two cycles. The amplification products (20 μl of the final PCR mixture) were separated on a 1% agarose or a 6% polyacrylamide gel and DNA was stained with ethidium bromide. The exact nature of the relevant PCR fragments was determined by sequencing and/or restriction-enzyme digestion. Cloning of PCR fragments was performed as described previously (De Jaegere et al., 1993). In order to ensure that there was no contaminating genomic DNA present in the cDNA samples, we performed PCR amplifications in parallel starting from RNA samples. These controls were always negative. Essentially the same results were obtained using transiently transfected cells from which RNA was isolated 48 h after transfection. The results were also not affected when polyadenylated RNA was used instead of total RNA (results not shown).

The SERCA2 primer sequences were derived from the pig genomic SERCA2 sequence (Eggermont et al., 1991) and correspond to the following sequences: Uf = 5'-CTCCATCTGC-CTGTCCAT-3' (nt 1878–1895, exon 20), C1 = 5'-GCGGTTAC-TCCAGTATTG-3' (nt 5527–5544, exon 25), C2 = 5'-AGACCA-GAACATATCACT-3' (nt 2267–2284, exon 22) and P1 = 5'-GGCTGATGGCTTCTAAGT-3' (nt 5843–5860, exon 25). The U1 primer (5'-GAGGTTCTTCGAGTCCTT-3') corresponds to nt 299–316 (exon 2) of the rabbit β -globin sequence as described by Van Ooyen et al. (1979). When two different antisense primers were used simultaneously in combination with the same sense primer, it was verified that the result was the same as when the antisense primers were used separately.

RESULTS

Comparison of pCM β SERCA2 RNA and endogenous SERCA2 mRNA processing

To study the *cis*-active elements involved in the regulation of SERCA2 transcript processing, we constructed a minigene, pCM β SERCA2, containing the 3' end of the SERCA2 gene. The processing pathways of SERCA2 and pCM β SERCA2 transcripts are shown schematically in Figure 2. Class-1 RNA is formed after splicing of an upstream donor splice site (5'D1) to a downstream acceptor (3'A) and polyadenylation at the downstream poly(A) signal (pA_d). Endogenous class-1 mRNA is muscle-specific and is translated into the SERCA2a isoform. Polyadenylation at the upstream poly(A) signal (pA_u), located between the donor and acceptor splice site, prevents splicing and generates the class-2 RNA. Endogenous class-2 mRNA is found in non-muscle cells and is translated into the SERCA2b isoform. Two other RNA species can be formed which differ from class 2 in their 3' untranslated region. An unspliced class-3 RNA can be formed when polyadenylation occurs at the downstream poly(A) site (pA_d). Splicing from a second donor (5'D2) to the same

Figure 2 Schematic representation of the alternative 3' end processing of SERCA2

The 3' end of the SERCA2 gene (a) and the context of the SERCA2 part in pCMβSERCA2 (b) are shown schematically. The processing signals present in SERCA2 are indicated as follows: 5'D1 and 5'D2, 5' donor splice sites 1 and 2 respectively; 3'A, 3' acceptor splice site; pA_u and pA_d, upstream (u) and downstream (d) polyadenylation signal. The encircled numbers refer to the alternative processing patterns of the 3' end of SERCA2 giving rise to different mRNA classes. The relative position of the sense and antisense PCR primers used to amplify the different RNA classes are depicted as arrows beneath the sequence. The β-globin regions are stippled whereas the SERCA2 sequences are open boxes. Coding and non-coding sequences are represented by wide and narrow boxes respectively. Partial exon sequences are in parentheses. DFR, downstream flanking region of SERCA2; CMV-IE, cytomegalovirus-immediate early promoter.

Figure 3 Comparison of the processing of RNA of pCMβSERCA2 and endogenous SERCA2 mRNA in transfected BC₃H1 cells

BC₃H1 cells were stably transfected with pCMβSERCA2 and total RNA extracts were prepared at 0, 2, 4, 7, 10 and 14 days after the onset of differentiation. Oligo(dT)-primed cDNA was amplified by PCR using a mixture of class-1 and class-2/3-specific primers. The cDNA from pCMβSERCA2 (a) was amplified by 25 cycles with U1, P1 and C2 as primers and starting from 5% of the cDNA first-strand mixture. The endogenous SERCA2 cDNA (b) was amplified by 20 cycles including two cycles at a lower annealing temperature using Uf, C1 and C2 starting with 1% of first-strand cDNA mixture. The samples were run on a 1% agarose gel for 120 min at 5 V/cm. In the control lane (C) the cDNA samples were replaced by water. The molecular-mass marker VI (Boehringer-Mannheim) was used as standard. The amplified fragment as well as the primers are shown on the left of the gels.

Figure 4 Myogenin expression induces processing of pCMβSERCA2 RNA in transfected C3H/10T1/2 cells

C3H/10T1/2 cells were stably transfected with pCMβSERCA2, with (+) or without (−) the myogenin expression vector EMSV-Myo8. Oligo(dT)-primed cDNA was prepared from total RNA extracts of undifferentiated (U) and differentiated (D) cells that were cultured for 8 days in medium with a high or low serum concentration respectively. PCR amplification (30 cycles) was performed starting from 50% of the cDNA first-strand mixture using U1, P1 and C2 as primers. In the control lane (C) water replaces the cDNA samples. The molecular-mass marker VI (Boehringer-Mannheim) was used as a standard. The samples were run on a 1% agarose gel for 120 min at 5 V/cm. The amplified fragments as well as the primers are shown on the left of the gel.

acceptor as used in class 1 gives rise to class-4 RNA. Endogenous class-3 mRNA is present in muscle and non-muscle tissues, and class-4 mRNA is specific to the brain. Class-4-type splicing of pCMβSERCA2 transcripts was not detected under experimental conditions used (results not shown).

BC₃H1 is a myogenic cell line which is well characterized with respect to muscle-cell differentiation (Edmondson and Olson, 1989; Brunetti and Goldfine, 1990). A differentiation-dependent change in alternative RNA processing was reported for the endogenous SERCA2 transcripts (De Smedt et al., 1991). In order to find out whether the pCMβSERCA2 transcripts were also processed in a differentiation-dependent way, BC₃H1 cells were stably transfected with pCMβSERCA2 and allowed to differentiate for 14 days. Oligo(dT)-primed first-strand cDNA was prepared and fragments of the transcripts were PCR amplified. Figure 2 shows the relative positions of the PCR primers used to discriminate between class-1 and class-2/3 RNA. Muscle-specific class-1 transcripts from pCMβSERCA2 were amplified using the U1 sense oligonucleotide located in exon 2 of β-globin and the P1 antisense oligonucleotide located in exon 25 of SERCA2. PCR amplification of class-2/3 RNA was carried out using the same U1 sense primer combined with the C2 antisense primer in exon 22 of SERCA2. By choosing one primer in the β-globin and the other in the SERCA2 part of the construct, we were able to amplify specific pCMβSERCA2 transcripts, distinguishing them from endogenous SERCA2 transcripts. By combining these three primers, three different fragments of 414, 602 and 987 bp could be amplified (Figure 3a). The smallest fragment (414 bp) corresponds to class-2/3 RNA. The 987 bp fragment is amplified from a precursor of class-2/3 RNA that still contains the second β-globin intron between exon 2 and 3. This fragment is not due to contamination with genomic DNA as PCR without RT yielded no band (results not shown). The 602 bp fragment corresponds to class-1 RNA. Figure 3(a) shows that the two class-2/3 fragments were amplified at all stages of differentiation, although the amount of precursor class-2/3 RNA decreased drastically during differentiation. However, the class-1 specific fragment (602 bp) first appeared after 4 days of muscle-specific differentiation (Figure 3, lane 3). This indicates that minigene transcripts can be processed in a muscle-specific

Figure 5 Effect of modifications of the pCM β SERCA2 construct on RNA processing in transfected C3H/10T1/2 cells

(a) Schematic representation of modifications made to pCM β SERCA2. In Mut1 the muscle-specific donor splice site was optimized, and in Mut2 the upstream polyadenylation signal was destroyed. In Del1 an A/II–EcoNI fragment of 1924 nt was deleted, and Sub1 contained a λ DNA fragment of the same length cloned in the BstEII restriction site. The λ DNA fragment is shown as a hatched box. A, A/II; B, BstEII; EN, EcoNI; X, XbaI. In (b) the effect of the mutations and deletion on the processing of the different transcripts is shown. C3H/10T1/2 cells were stably transfected with pCM β SERCA2 and pCM β SERCA2 containing the modifications indicated at the top of each lane. Oligo(dT)-primed cDNA was prepared from total RNA of cells cultured for 8 days in growth medium. PCR amplification (35 cycles) was performed starting from 25% of cDNA first-strand mixture and with U1, P1 and C2 as primers. The samples were run on a 6% polyacrylamide gel for 90 min at 12 V/cm. Molecular-mass marker III (Boehringer-Mannheim) was used as standard. Representations of the amplified fragments and the primers are shown on the left of the gel.

way. The *cis*-active elements required for muscle-specific processing are therefore present in pCM β SERCA2.

We then compared the processing switch for the pCM β SERCA2 transcripts with that of the endogenous SERCA2 transcripts. In order to amplify the different endogenous SERCA2 RNAs present in BC₃H1 cells, we used sense primer Uf corresponding to a sequence in exon 20 of SERCA2 in combination with C1 (exon 25) and C2 (exon 22) as antisense primers. As shown in Figure 3(b), class-1 RNA appears 4 days after the BC₃H1 cells had been switched to differentiation medium (lane 3), whereas class-2/3 RNA is present in undifferentiated as well as in differentiated cells. These results demonstrate that

muscle-specific processing of endogenous SERCA2 and pCM β SERCA2 transcripts is activated at the same time.

Effect of myogenin on the processing of pCM β SERCA2 transcripts

The myogenic differentiation of BC₃H1 cells was shown to be tightly coupled to the expression of myogenin, a transcription factor of the helix–loop–helix family (Edmondson and Olson, 1989; Brunetti and Goldfine, 1990). In order to discover whether SERCA2 processing is related to expression of myogenin, we examined whether the muscle-specific splicing of RNA transcribed from pCM β SERCA2 could be induced by

myogenin. The myogenin expression vector, EMSV-Myo8, and pCM β SERCA2 were stably co-transfected in the fibroblast cell line C3H/10T1/2. As can be expected for a non-muscle cell line, C3H/10T1/2 cells transfected with pCM β SERCA2 alone, do not splice the pCM β SERCA2 transcripts and consequently only contain class-2/3 RNA (Figure 4, lanes 4 and 5). After transfection of the myogenin expression vector and deprivation of the cells of hormones and growth factors, the appearance of the cells drastically changed as they fused and formed myotubes. Under these conditions, myogenin mRNA could easily be detected using the method described by De Jaegere et al. (1993) (results not shown). Moreover, we found that, in these conditions, besides the band representing the class-2/3 RNA (414 bp), a second 602 bp fragment was amplified (Figure 4, lane 2). The latter fragment corresponds to the muscle-specific class-1 RNA. This result indicates that myogenin can induce the *trans*-acting factor(s) responsible for muscle-specific splicing.

Effect of pCM β SERCA2 modifications on muscle-specific splicing in non-muscle cells

In order to explore how the *trans*-acting factors regulate SERCA2 processing, we modified pCM β SERCA2 as schematically represented in Figure 5(a). We based these modifications on the competition model proposed by Peterson and Perry (1989) to explain the regulation of the Ig μ 3' end processing. In this model, splicing and polyadenylation compete with each other and the outcome of this competition is determined by the balance between splicing and polyadenylation efficiency. Processing of the SERCA2 transcript could be due to competition between polyadenylation at the pA_u site and splicing from 5'D1 to 3'A. If this working hypothesis is correct, alterations in the strength of one pathway should switch the processing pattern to the other pathway and *vice versa*. We therefore investigated whether it was possible to enforce the muscle-type splicing in non-muscle cells, either by increasing the likelihood of splicing or conversely by preventing polyadenylation at the pA_u site. In a first approach, we increased the strength of the 5'D1 splicing site by mutating the intron part of this non-consensus site to the consensus sequence (Mut1). Secondly, a deletion of a 1924 nt fragment between the donor and acceptor site (Del1) was intended to increase the probability of splicing relative to polyadenylation. Thirdly, polyadenylation at the pA_u site was prevented by mutating the AATAAA consensus sequence for polyadenylation (Mut2). Each of the modifications was introduced into the pCM β SERCA2 construct either alone or in combination. These constructs were then stably transfected in C3H/10T1/2 cells and their effects on the RNA-processing pattern were evaluated using RT-PCR (Figure 5b). We have also included the primers to amplify class-2/3 RNA. No change in the level of the latter RNA species could be detected (Figure 5b).

In accordance with the competition model, muscle-specific class-1 splicing, resulting in a PCR fragment of 602 bp, could be detected in non-muscle cells when the strength of the donor splice site was increased (Figure 5b, lanes 3, 4, 7 and 8) and/or when a 1924 nt intron fragment was removed (Figure 5b, lanes 2, 4, 6 and 8). From these semiquantitative analyses it also appeared that the effect of Mut1 and Del1 on splicing is additive. However, destroying the upstream polyadenylation site did not induce splicing (Figure 5b, lane 5), i.e. splicing did not occur even when it was the only possible option. To verify the latter result we constructed another polyadenylation-deficient construct by deleting a fragment (nt 2998–3190; Eggermont et al., 1991) containing the complete pA_u site. Again, we could not detect muscle-specific splicing in non-muscle cells (results not shown).

Figure 6 Effect of deletion and intron substitution on RNA processing in stably transfected C3H/10T1/2 cells

C3H/10T1/2 cells were stably transfected with pCM β SERCA2, pCM β SERCA2(Del1) and pCM β SERCA2(Del1,Sub1). These modifications are shown schematically in Figure 5(a). Oligo(dT)-primed cDNA (25% of the first-strand mixture) prepared from total RNA of 8-day-old cells was PCR amplified. In (a) U1 and P1 were used as primers in order to amplify (50 cycles) the class-1 fragment. (b) PCR amplification (35 cycles) of fragments using U1 and C2 as primers. Samples were separated on a 6% polyacrylamide gel (90 min, 12 V/cm) and molecular-mass marker III (Boehringer-Mannheim) was used as standard. A schematic representation of the amplified fragments and the primers are shown on the left.

Similar results were obtained when the polyadenylation-deficient constructs were expressed in undifferentiated BC₃H1 cells (results not shown). Taken together, these results indicate that regulation of the muscle-specific splicing is independent of internal polyadenylation.

Effect of intron size on splicing

The effect of the deletion in pCM β SERCA2(Del1) on the promotion of splicing could be explained in two ways. Either the deletion removes a specific regulatory sequence which is responsible for inhibition of splicing in non-muscle cells, or the effect of the deletion may simply be due to a decreased distance between the 5'D1 and 3'A, thus increasing the probability of splicing. In order to discriminate between the two possibilities, the deleted fragment was replaced by an unrelated DNA sequence derived from λ phage DNA and the effect on splicing was evaluated (Figure 6a). Lane 2 shows the amplification of the class-1 fragment after transfection of the C3H/10T1/2 cells with

the deleted construct, pCM β SERCA2(Del1). The class-1 fragment was absent after transfection of both the wild-type construct (Figure 6a, lane 1) and the substituted construct, pCM β SERCA2(Del1,Sub1) (Figure 6a, lane 3). The class-2/3 RNA was present in the three transfected cell lines indicating that the minigene is expressed to comparable levels (Figure 6b). The wild-type pattern can thus effectively be restored by non-specific sequence substitution indicating that spacer effects rather than specific sequence effects modulate splicing efficiency.

DISCUSSION

The primary transcripts from pCM β SERCA2, containing the 3' end of SERCA2, are processed in a similar way to the endogenous SERCA2 transcripts. The muscle-specific splicing of pCM β SERCA2 transcripts and the endogenous SERCA2 RNA is switched on at the same moment during differentiation of BC₃H1 cells. Moreover, the transcripts from the minigene can be spliced in the muscle-specific way in fibroblasts co-transfected with myogenin and pCM β SERCA2. These results demonstrate that all the essential *cis*-active elements required to regulate processing are present in the SERCA2 part present in pCM β SERCA2.

As expression of myogenin is a sufficient condition to induce muscle-specific splicing of the transcripts from pCM β SERCA2 in a fibroblast cell line, we conclude that this transcription factor can induce the *trans*-acting factor(s) responsible for the muscle-specific splicing. Besides the conversion of C3H/10T1/2 fibroblasts into myoblasts, myogenin can convert many other cell types into muscle cells and a strong correlation between myogenin expression and myoblast differentiation has been reported [reviewed in Olson (1990) and Edmondson and Olson (1993)]. Moreover, myogenin is absolutely required for the induction of functional skeletal muscle as can be concluded from myogenin-gene-disruption experiments in mouse (Hasty et al., 1993; Nabeshima et al., 1993). Besides the induction of gene transcription of typical sarcoplasmic-reticulum proteins and muscle-type contractile proteins (Arai et al., 1992), induction by myogenin of muscle-specific alternative processing was reported for α - and β -tropomyosin (Saitoh et al., 1990). The pathway downstream of myogenin, leading to muscle-specific processing, seems to differ for SERCA2 and tropomyosin. This can be concluded from the fact that in contrast with the regulation of tropomyosin processing (Saitoh et al., 1990), the muscle-specific RNA processing of SERCA2 was strongly dependent on ongoing protein synthesis (De Jaegere et al., 1993). The expression of myogenin is, however, not a necessary condition, as the muscle-specific splicing of SERCA2 also occurs in cardiac muscle (Brandl et al., 1987; Lompré et al., 1989) which lacks myogenin or any other known member of the MyoD family (Edmondson and Olson, 1993). The *trans*-acting factor(s) responsible for muscle-specific processing of SERCA2 can therefore also be induced by a factor different from myogenin.

The relative position of polyadenylation and splice sites at the 3' end of SERCA2 is similar to that in the IgM heavy chain gene. mRNAs coding for the secreted Ig μ protein are formed after polyadenylation at an internal poly(A) site located between the donor and acceptor splice sites of an optional intron. Removal of this intron generates mRNAs encoding the membrane-bound Ig μ protein. Splicing of Ig μ mRNAs is predominant in early-stage B-lymphocytes whereas utilization of the upstream poly(A) site is favoured after maturation into plasmocytes (Alt et al., 1980). Regulation of Ig μ processing depends on the competition between the two mutually exclusive processing reactions: poly-

adenylation at the upstream poly(A) site and splicing (Peterson and Perry, 1986; Peterson and Perry, 1989). The shift from membrane to secreted Ig μ mRNA formation during development is caused by an increase in the polyadenylation efficiency, while the splicing efficiency remains the same (Galli et al., 1988; Peterson et al., 1991). In spite of the similar gene architecture, such a competition model cannot account for the regulated expression of SERCA2 mRNAs. This can be concluded from experiments in which we prevented polyadenylation at the upstream polyadenylation site, either by mutating this site to a non-functional poly(A) site or by deleting the poly(A) signal entirely. This indicates that splicing and polyadenylation do not compete with each other in non-muscle cells and also that splicing is a very inefficient process in these cells. Consequently, regulation of polyadenylation efficiency cannot play a crucial role in the regulation of SERCA2 expression. The appearance of spliced transcripts in BC₃H1 cells or in myogenic C3H/10T1/2 cells rather suggests that the splicing process is activated during myogenic differentiation.

This study also provides some insight in to why non-muscle cells cannot splice SERCA2 transcripts from the 5'D1 to the 3'A splice sites. Firstly, the 5' donor splice site is a weak processing site as it deviates from the consensus sequence in three positions. Splicing could indeed be activated by optimizing the donor splice site. Secondly, the length of the optional intron seems to down-regulate splice efficiency as well. Indeed, by decreasing the length of the intron between 5'D1 and 3'A, it was possible to induce muscle-specific splicing in non-muscle cells. This effect was the result of altered spacing between the donor and acceptor splice site, as replacement of the deleted intron fragment by an unrelated DNA sequence of the same length also inhibited splicing. This therefore excludes the presence of a negatively acting sequence element in the intron. A similar relation between intron length and splicing was also observed for Ig μ (Peterson and Perry, 1986; Tsurushita and Korn, 1987). It was suggested that decreasing the distance between the splice sites facilitates the formation of the spliceosome complex (Tsurushita and Korn, 1987). Finally, the fact that splicing can be activated by various manipulations argues against the presence of a specific splice repressor in non-muscle cells.

These observations suggest that *trans*-acting factor(s) that increase the efficiency of splicing appear during myogenic differentiation. This is in accordance with the observation that ongoing protein synthesis is essential to maintain muscle-specific SERCA2 splicing in BC₃H1 cells (De Jaegere et al., 1993). Potential candidates for *trans*-acting regulatory factors are members of the SR protein family. These proteins are general splice factors, but they also play a role in regulating splicing (Zahler et al., 1992; Mayeda and Krainer, 1992). Moreover, these proteins are expressed in a tissue-dependent way (Zahler et al., 1993). One interesting example of splice regulation by SR proteins is the proposed role of alternative splicing factor/splicing factor 2 (ASF/SF2). It has been shown that this factor can modify donor splice site selection by increasing the strength of U1snRNP binding to donor splice sites (Eperon et al., 1993). Consequently, weak donor splice sites, comparable with the 5'D1 splice site in SERCA2, could be activated in the presence of a higher ASF/SF2 concentration. However, there is no evidence of a change in ASF/SF2 concentration or activity during BC₃H1 differentiation, and the effect of myogenin on the pattern of expression of ASF/SF2 in C3H/10T1/2 cells has not been investigated.

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