Zfp60, a mouse zinc finger gene expressed transiently during in vitro muscle differentiation

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Abstract The complete cDNA coding sequence of the zinc finger gene Zfp60 is reported. The predicted amino acid sequence of the Zfp60 protein has been found to contain 19 zinc finger motives clustered at the C-terminus. At its N-terminus, Zfp60 shares with other members of the zinc finger gene family two additional conserved amino acid modules named Kruppel Associated Boxes (KRAB). The expression patterns of Zfp60, MyoD and MHC mRNAs have been followed during in vitro myogenic differentiation of C2 cells. We show that the bacterial produced Zfp60 protein binds DNA only in presence of zinc ions. Zfp60 locus has been mapped in chromosome 7, where other Zfp loci are localised.

Key words: cDNA cloning; Zinc finger protein; Myogenic differentiation; Temporal expression; DNA binding; Chromosome mapping

1. Introduction

The formation of mature muscle fibers requires the coordinated expression of many different genes coding for the myotube-specific proteins. These functions are governed by a set of regulatory genes [1]. Myogenic cell lines and myoblast primary cultures can mimic many of the steps characteristic of myogenic differentiation. The possibility to follow and to study the in vitro transition of myoblast cells to myotube provides a useful system to study gene regulation during myogenesis. In the last few years, several muscle regulatory genes have been identified and characterized such as many members of the MyoD gene family. This family of regulatory genes (MyoD, myogenin, Myf5 and MRF4) share the same evolutionarily conserved domains, a basic region involved in DNA binding to the specific target sequence (E box), and a helix loop helix (HLH) implicated in homodimerization and/or heterodimerization with other regulatory protein [2]. The basic idea of this work is the isolation and characterization of other regulatory genes, involved in myogenesis, coding for proteins with the 'zinc finger' motif as a DNA binding domain. A zinc finger motif was first identified in the *Xenopus laevis* RNA polymerase III transcription factor TFIIIA [3,4]. Since then, several zinc finger genes involved in gene regulation have been identified in a variety of animals and plants [5,6].

In a previous work we reported on the characterization of three zinc finger genes, isolated from an adult mouse skeletal muscle cDNA library [7]. In this work we describe one of those genes, termed Mfg3 and presently renamed Zfp60 according to the international convention. We determine the complete cDNA coding sequence and the predicted amino acid sequence of the ZFP60 protein. This protein is characterized by a cluster of 19 zinc finger motifs at the C-terminus and by the two Kruppel associated boxes (KRAB) A and B at the N-terminus. These modules have been shown to define a subfamily of multifinger proteins [8,9] and to be associated with transcriptional repression activity [10–13]. The expression patterns of Zfp60, MyoD and myosin heavy chain (MHC) mRNAs have been followed and compared during myoblast C2 cell line differentiation. We also show that the bacterial expressed Zfp60 protein is able to bind DNA only in presence of zinc ions. Using restriction length polymorphism (RFLP) we assigned the Zfp60 locus to the murine chromosome 7, at about 10 cM from the centromere, where a cluster of Zfp loci maps. This region shows homology with human chromosome 19q13.

2. Materials and methods

2.1. RNA extraction and Northern blotting

Total RNA was extracted from cell lines using the method described by Chomczynski and Sacchi [14]. Total RNA (10 μg) was fractionated by electrophoresis on a 1.2% agarose-formaldehyde gel according to Maniatis and co-workers [15]. The gels were blotted on nylon filters (Hybond N, Amersham) and hybridized according to the manufacturer's instructions. DNA probes were labelled by [α-^{32}P]dATP incorporation using Megaprime Probe Kit (Amersham).

2.2. cDNA library screening

A λ gt11 cDNA library, prepared from RNA of mouse F9 teratocarcinoma cell line differentiated using retinoic acid (0.1 μM) was screened using as a probe the previously isolated cDNA fragment [7], spanning from nt 1447 to nt 1990 (see Fig. 1).

2.3. Cloning the cDNA 5′ region

In order to isolate the 5′ region of Zfp60 mRNA we used the reverse PCR technique first described by Uematsu [16], and modified as previously described [17]. Briefly, three oligonucleotides were synthesized in the known extreme of the 5′ end of Zfp60 cDNA: antisense oligonucleotide (A) from nt 551 to nt 573, sense oligonucleotide (B) from nt 524 to nt 547, antisense oligonucleotide (C) from nt 330 to nt 353. For cDNA synthesis, 1 μg of total RNA prepared from mouse F9 teratocarcinoma cell line induced to differentiate with retinoic acid [18] was used. RNA was primed using 10 ng of oligonucleotide A. First strand and second strand cDNA synthesis was performed using the Promega RiboClone cDNA System kit according to the manufacturer's instructions. The double strand cDNA in a total volume of 7 μl of TE buffer (10 mM Tris-HCl pH 8, 1 mM EDTA)

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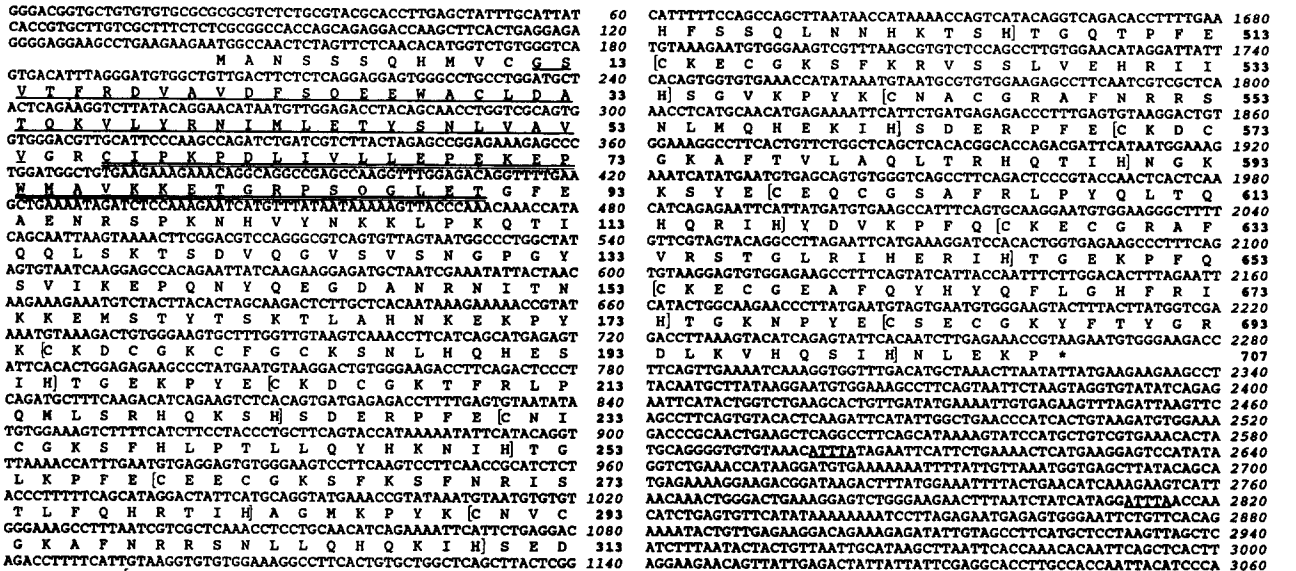

Fig. 1. Nucleotide sequence of Zfp60 cDNA and its deduced amino acid sequence. Nucleotides are numbered on the right, amino acid sequence is numbered in bold characters. The amino terminal KRAB boxes A and B are underlined and double underlined respectively. Individual zinc finger domains are shown between square brackets. The polyadenylation signals are in bold characters; the 'ATTTA' sequences, potentially involved in mRNA selective degradation, are underlined. The Zfp60 nucleotide sequence has been deposited at GenBank, with accession number U48721.

was circularized according to Uematsu [16]. The amplification of the circularized double strand cDNA was accomplished using the Zfp60 oligonucleotides B and C and the GeneAmp PCR Core Reagents Kit (Perkin Elmer Cetus). The amplification cycles consisted of denaturation for 30 s at 94°C, annealing for 30 s at 66°C, and extension for 1 min at 72°C. 10 μl of the PCR products were analyzed by 1.6% agarose gel electrophoresis and transferred to Amersham Hybond N nylon membrane. Specific products for Zfp60 sequences were detected by hybridization using [γ-^{32}P]ATP end labelled oligonucleotide A as a probe. The PCR products were then directly subcloned using the TA Cloning Kit (Invitrogen).

2.4. DNA sequencing

DNA fragments of cDNA clones were subcloned either in pCR vector (Invitrogen) or pBluescript (Stratagene) and both strands were sequenced using the Sequenase kit according to the manufacturer's instructions (United States Biochemical).

2.5. Cell cultures

The mouse myoblast C2 cell line [19] clone 7 was obtained from Dr. M. Buckingham (Institut Pasteur, Paris, France). Cells were maintained as undifferentiated myoblasts in Dulbecco's modified Eagle medium (DMEM) supplemented with 20% fetal calf serum (FCS) in a 6.5% carbon dioxide atmosphere. To induce cell differentiation, myoblasts were grown to confluence and shifted to DMEM supplemented with 2% FCS.

2.6. Expression of recombinant fusion protein

The entire coding region of Zfp60 cDNA was cloned in pQE-30 vector (Qiagen). Zfp60 bacterial fusion protein was expressed and purified according to the manufacturer's instructions.

2.7. Gel electrophoresis and South-Western blotting

Purified protein obtained in bacteria (0.5 μg per lane) was separated on 9% SDS-polyacrylamide gel, blotted to nitrocellulose paper (Hybond C, Amersham) as described by Harlow and Lane [20]. South-Western blotting was performed according to Sukegawa and Blobel [21]. In particular two identical filter blots (A and B) were incubated for 12 h in renaturation buffer A (50 mM Tris-HCl pH 7.5, 100 mM KCl, 1% Triton X-100, 10% glycerol, and 0.1 mM $ZnCl_2$) and in renaturing buffer B (50 mM Tris-HCl pH 7.5, 100 mM KCl, 1% Triton X-100, 10% glycerol, 10 mM EDTA, and 2 mM DTT) respectively. Total mouse genomic DNA was sonicated to a final size of about 1 kb, labelled with [α-^{32}P]dATP and random hexanucleotide primers. 5×10^4 cpm per ml were added and incubated for 3 h to both filters. The filters were washed five times, for 10 s, in the respective buffers A and B, and autoradiographed. All incubations were performed at 4°C. In order to normalize the amount of protein loaded in each lane, the same filters were then incubated with Zfp60 mouse polyclonal antibody. Proteins were then visualized using an alkaline phosphatase-conjugated goat anti-mouse.

2.8. Genetic mapping of Zfp60

Details on the interspecific test cross population of 106 male (C3H/He×*M. spretus*)×C57BL/6J, designed HSB, have already been reported [22]. The marker locus Lig1 was identified by segregation analysis of the three *M. spretus* polymorphic bands, detected by the hybridization of HSB TaqI-digested genomic DNAs with the Lig1 cDNA [23]. A genetic linkage map was constructed by multipoint analysis of the data, with the MAPMARKER/EXP program [24,25]. Genetic distances were computed by use of Haldane's mapping function. Linkage between markers was considered significant if the LOD score was >3. Zfp60 locus was mapped by restriction length polymorphism (RFPL) analysis of a 813-bp fragment from 3′-UTR

region of the murine Zfp60 gene. The fragment was obtained by PCR amplification of 75 ng of genomic DNA. 5 pmol of specific primers: from nt 3042 to nt 3072 (5'-GCCACCAATTACATCCCATGC-CTCCTGTGC-3') and from nt 3856 to nt 3827 (5'-GGGATCCATA-TACACTCACAAGCATTTAC-3') were designed from Zfp60 cDNA. Samples were subjected to 40 PCR cycles consisting of denaturation at 95°C for 20 s, annealing at 52°C for 30 s and extension at 72°C for 30 s. PCR samples were digested using the restriction enzyme *Hin*fI. We detected a RFLP between *Hin*fI-digested 3'-UTR fragments of C3H/He (H) and C57BL/6J (B) mouse strains and *M. spretus* (S) strain. A *Hin*fI site, present in H and B strains but absent in the S strain, gave rise to a RFLP. We analyzed the segregation of this band in the HBS population. The segregation showed the expected 1:1 pattern.

3. Results

3.1. Structural organization

To isolate the Zfp60 complete cDNA we screened a λ cDNA library prepared from the mouse F9 teratocarcinoma cell line differentiated using retinoic acid [18]. We used as a probe the previously isolated cDNA fragment, corresponding to a portion of the zinc finger coding region [7]. Several Zfp60 positive λ cDNA clones were isolated. The longest cDNA clone comprised both the 3' untranslated region and the complete zinc finger coding portion of the Zfp60 mRNA, but it was missing the 5' region of the transcript. To isolate this portion of the Zfp60 mRNA, we used the inverse circular PCR technique [16,17]. The longest Zfp60 cDNA sequence obtained using different cloning strategies was 3923 bp long (GenBank accession number U48721). An AUG was identified at nt 142–144, located in an appropriate context for an initiation codon [26]. This AUG was preceded by an in-frame stop codon: nt 133–135. As shown in Fig. 1, the AUG codon opens an uninterrupted reading frame encoding a 707 aa long polypeptide. At the C-terminus, 19 zinc finger motifs are clustered, classifying Zfp60 as a multifingered protein. The N-terminal portion of the protein contains two conserved peptide modules termed KRAB, box A and box B, which have been found to occur in several members of the zinc finger multigene family [8,9]. A 1660 nt long untranslated region is present at the 3' end of Zfp60 mRNA. In this region several 'AUUUA' sequences are present (see Fig. 1), frequently associated with transcripts having an elevated turn-over [27]. Two canonical polyadenylation consensus sequences are arranged in tandem at nt 3857–3862 and nt 3886–3891, a poly A tail is present at the end of the cDNA.

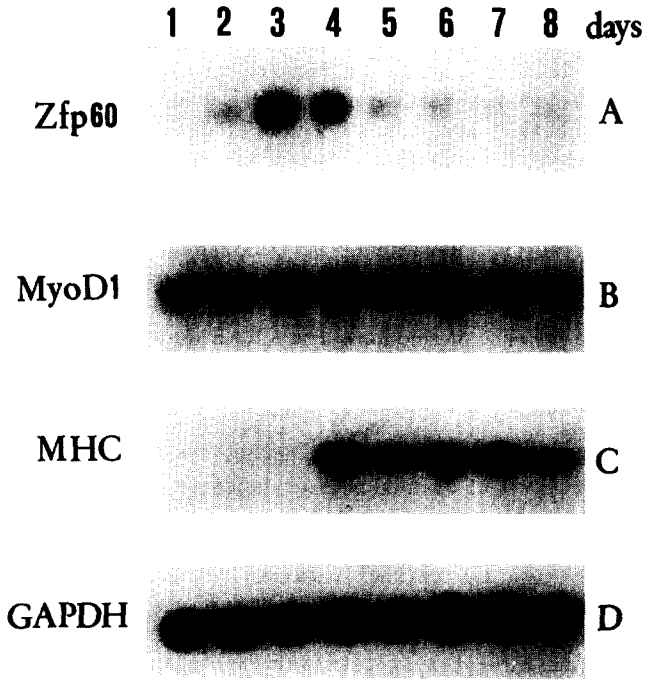

Fig. 2. Northern blot analysis of total RNA isolated from the mouse myoblast C2 cell line on different days during differentiation. On day 2 cells are shifted to the differentiation medium. The same filter was hybridized with the probes indicated on the left. Panel A: Zfp60 cDNA, panel B: MyoD1 cDNA, panel C: myosin heavy chain cDNA, and panel D: glyceraldehyde-3-phosphate-dehydrogenase cDNA.

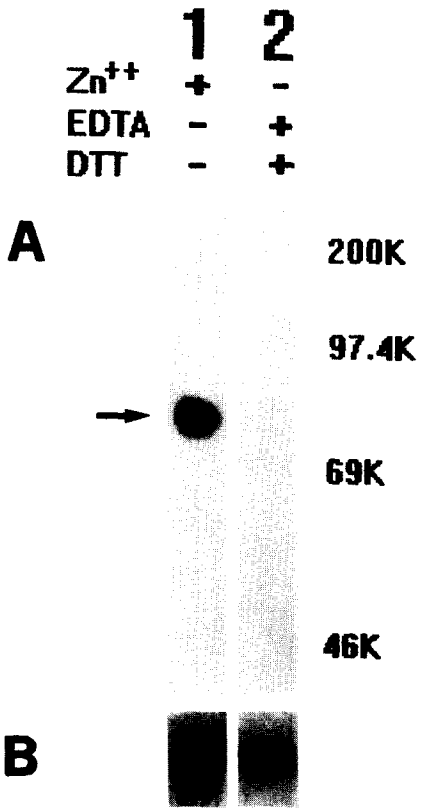

Fig. 3. South-Western blot analysis of a bacterially expressed and purified Zfp60 fusion protein. The proteins were separated on a 9% SDS-polyacrylamide gel and blotted to nitrocellulose filter. Blots were probed with [α-^{32}P]dATP labelled mouse genomic DNA either in the presence of zinc ions (lane 1) or in the presence of EDTA and DTT (lane 2). Panel A: The fusion protein Zfp60 is marked by an arrow. Positions of the molecular mass standard proteins are shown. Panel B: The same filters were incubated with a Zfp60 mouse polyclonal antibody in order to normalize the amount of protein loaded in each lane. Proteins were visualized using an alkaline phosphatase-conjugated goat anti-mouse.

3.2. Expression analysis

We have previously reported that Zfp60 gene expression is strongly induced upon differentiation both in the F9 teratocarcinoma cell line and in the C2 myogenic cell line [7]. Here we show a detailed analysis of the expression pattern of Zfp60 in total RNA prepared at different times following the onset of differentiation of the myogenic C2 cell line. As shown in Fig. 2A a specific Zfp60 hybridization signal is present at low level in C2 cycling cells on days 1 and 2, it peaks on day 3,

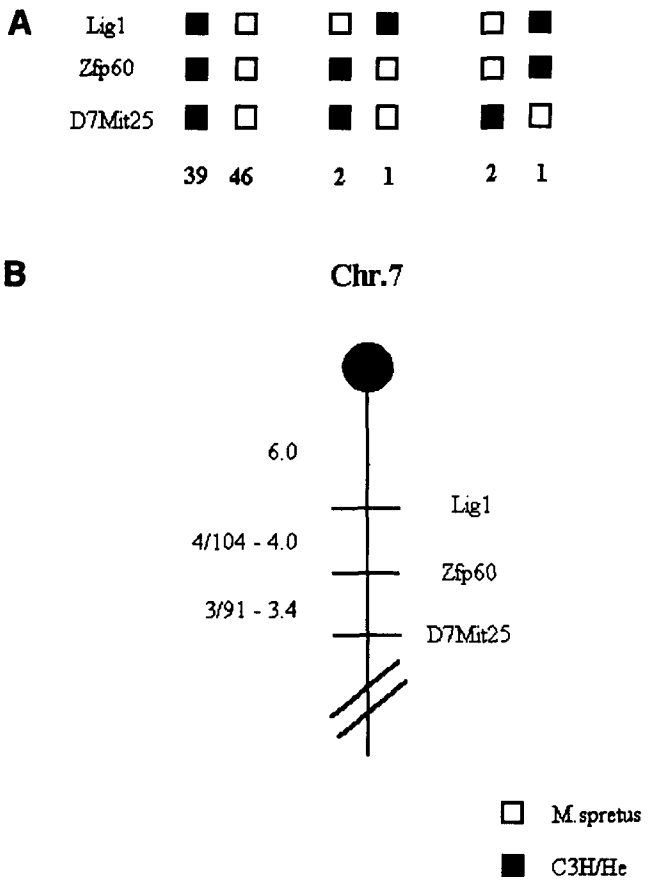

Fig. 4. Haplotype data of completely typed HSB mice. Panel A: Each column represents the type of chromosome identified in the progeny. The number of progeny exhibiting each type of chromosome is listed at the bottom. The open squares represent the *M. spretus* allele; the black squares stand for the C3H/HeJ allele. Panel B: The partial genetic linkage map of the mouse chromosome 7 showing the location of the Zfp60 gene. The recombination frequencies are shown on the left and are calculated by multipoint analysis of recombinants over the number of animals typed for each pair of loci, and expressed as genetic distances in cM (computed using Haldane's function). Distance of the Lig1 marker from the centromere is also indicated.

concomitantly with the differentiation commitment, and then it slowly declines from day 4 on. Fig. 2B shows the expression pattern of MyoD, one of the muscle-specific determining/transcription factors [1,2]; its hybridization signal remains nearly constant during differentiation progression. In fact, in the C2 cell line, the MyoD gene product is present in an inactive form in cycling conditions. In order to follow the differentiation progression the MHC gene transcript has been visualized (Fig. 2C). It appears on day 4 and then remains constant during the terminal differentiation. The amount of RNA loaded in each lane has been normalized using as a probe GAPDH cDNA (Fig. 2D). These results suggest that Zfp60 gene has a refined pattern of expression correlated to the onset of differentiation, but not to the maintenance of this stage.

3.3. DNA binding activity

A fusion protein coding for the complete Zfp60 was expressed and purified in *E. coli*. Purified protein was separated by SDS-PAGE, blotted to nitrocellulose, and then probed with sonicated and [α-^{32}P]dATP labelled mouse genomic DNA. The probing was performed either in solutions containing $ZnCl_2$ or in solutions containing EDTA and DTT [21]. As shown in Fig. 3 the mouse DNA binds Zfp60 protein in the presence of zinc ions, whereas no DNA binding was observed in the presence of EDTA and DTT. These data demonstrate that Zfp60 protein is capable of binding DNA in a zinc-dependent fashion.

3.4. Genetic mapping of Zfp60

RFLP analysis was used to identify restriction polymorphisms in a PCR amplified fragment (813 bp) from the 3′-UTR region of the murine Zfp60 gene. The fragment was obtained by PCR amplification of mouse genomic DNA using specific primers of Zfp60 cDNA (see Section 2). We detected a RFLP between the *Hin*fI-digested 3′-UTR fragment of C3H/He (H) and C57BL/6J (B) mouse strains and *M. spretus* (S) strain. A *Hin*fI site, present in strains H and B, but absent in the S strain gave rise to a RFLP. Its segregation was analyzed in the HBS population showing the expected 1:1 pattern (Fig. 4A). Therefore, we could assign the Zfp60 locus to the murine chromosome 7, at about 10 cM from the centromere (Fig. 4B). Other zinc finger coding genes have been mapped in this chromosome region, such as Zfp29, Zfp30, Zfp36, Zfp56 [28–30] and Zfp59 (our unpublished data). This region shows homology with human chromosome 19q13, where the human ZNF36 gene maps, the homologue of the murine Zfp36 [30].

4. Discussion

The differentiation processes require the involvement of a cascade of signals and regulatory molecules in order to govern the expression of specific structural genes. The search for regulatory genes appears to be fundamental for the understanding of these sophisticated processes. Here we report on Zfp60, a mouse zinc finger coding gene, which is transiently expressed during in vitro muscle differentiation. To date, only one other member of the multifinger gene family, named ZT3, has been reported to be expressed during skeletal muscle differentiation [31]. The Zfp60 protein has a cluster of 19 zinc fingers and belongs to the KRAB multifinger gene family. The data presented here clearly demonstrate that the bacterially expressed Zfp60 protein binds DNA and that the binding is dependent on the presence of zinc ions. The KRAB domain, located in the N-terminal portion of Zfp60, has been shown to be a potent transcriptional repressor element [10–13]. Its predicted α-helical structure has been proposed to mediate protein-protein interaction. This hypothesis has been recently supported by the report of co-immunoprecipitation of a protein likely interacting with the KRAB domain [32]. In the present study we follow the expression of Zfp60 during the differentiation of the myogenic C2 cell line. A specific hybridization signal is present at a very low level in C2 cycling cells and increases when the cells are switched to the differentiation medium. Its maximum expression coincides with the massive entry of cells in G0 phase and then declines concomitantly with myoblast cells fusion. This transcription pattern has been confirmed in experiments performed on mouse embryonic primary myogenic cells (data not shown). Moreover, the presence in the 3′ untranslated region of Zfp60 mRNA of several destabilizing 'AUUUA' sequences is coherent with the presence of Zfp60 transcript in a very narrow window during myogenic

differentiation. This peculiar expression pattern suggests that Zfp60 protein is transiently required during myogenic differentiation. Zfp60 mRNA peaks concomitantly with the cell entry to the myogenic terminal differentiation program. During this process myogenic cells undergo profound structural and functional changes, including cell growth arrest, cell alignment and fusion to form multinucleate myotubes. Zfp60 with its large number of zinc fingers and the KRAB regulatory element could effect multiple contacts with DNA and play a role in the change of chromatin structure, which accompanies the transition of a cycling myoblast to mature multinucleate myotubes. The fact that several ZFPs have been indicated to participate in the formation of chromatin structure [17,21,33–35] supports this hypothesis. Moreover, a gene that shares a high degree of homology with Zfp60, namely Zfp59, has been shown to be tightly associated with nuclear scaffold structure during murine male germ line differentiation [17]. In particular it is interesting to note that the Zfp60 and Zfp59 genes have been assigned to the murine chromosome 7, in a region where a cluster of other Zfp loci maps. Taken all together these data suggest that Zfp60, as well as Zfp59, may belong to a cluster of structurally and functionally correlated genes.

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