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Intronic sequence with both negative and positive effects on the regulation of alternative transcripts of the chicken β tropomyosin transcripts

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ABSTRACT

The chicken β tropomyosin gene generates three major transcripts by alternative splicing. A pair of internal exons are spliced in a mutually exclusive manner and their utilisation is developmentally regulated. Exon 6A and exon 6B are used respectively in myoblasts and myotubes during the process of differentiation of muscle cells. We have previously reported that, in myoblasts, exon 6B is skipped because of a negative regulation which involves intron as well as exon sequences. In this report, we describe a previously uncharacterized intronic element which is involved in the regulation of the splicing of both exons 6A and 6B. This cis-element is localized 37nt downstream of exon 6A and is approximately 30nt long. Its deletion, as well as modification of its sequence, results in the activation of the use of exon 6B and, at the same time, in the inhibition of the use of exon 6A. The mechanisms by which this region could act are further discussed.

INTRODUCTION

Splicing is the general mechanism by which introns are deleted from premRNAs in order to give rise to the mature transcripts. Splicing can be constitutive, in which case all introns are deleted, or it can be regulated so that not all exons will be found in the mature transcripts for review see (1), the choice of exons being determined by a variety of factors such as the state of differentiation or the type of tissue in which the gene is expressed. Understanding the mechanisms which control this choice is becoming of fundamental importance. Both cis-acting elements and trans-acting factors have been proposed as determinants for splice site selection in alternative splicing. Little is known in vertebrates about the trans-acting factors (2, 3, 4) and most studies have dealt with the characterization of the cis-acting elements.

The gene which codes for the chicken β tropomyosin has provided us with a model system to study the regulation of alternative splicing since two of its internal exons, exons 6A and 6B, present a mutually exclusive pattern of splicing which depends on the state of differentiation of muscle cells: exon 6A is included in the major transcripts accumulated by undifferentiated myoblasts while differentiated myotubes accumulate transcripts which now contain essentially exon 6B (5, 6). We have previously shown that the same pattern of splicing can be reproduced by transfecting into muscle cells a minigene construct which contains the two alternative exons flanked by the common exons 5 and 7 (7) (see also fig.1 A). An extensive mutational analysis using this minigene construct has allowed us to define some of the cis acting elements which control this alternative pattern of splicing. It was shown that, in myoblasts, exon 6B is under a negative control which involves exonic as well as intronic sequences. The exonic sequences are located at the 5′ end of exon 6B and are implicated in the formation of a stem and loop structure which is in part responsible for this downregulation of exon 6B in myoblasts (8, 9). The intronic sequences consist of a long polypyrimidine rich sequence (over 100nt) which spans the region from the branch point up to the acceptor splice site of exon 6B (9), such a pyrimidine stretch, which may increase the efficiency of the lariat formation (10), was shown to have also an important role in the regulation of the splicing of the rat homologous gene (11). These sequences negatively control the use of exon 6B presumably through the interaction with factor(s) which have still to be characterized.

In this communication, we describe the characterization of a new and unexpected intron sequence which participates in the negative regulation on splicing of exon 6B. This sequence is 33nt long and is located 37nt downstream of exon 6A. Its deletion or its modification induces the use of exon 6B in myoblasts. We show that partial deletions of this sequence induce the use of exon 6B but to a lesser extent. Converting the sequence to the complementary one induces also the use of exon 6B. Reinsertion of this sequence elsewhere along the intron does not restore the negative control on the use of exon 6B. Lastly, we also show that this same region may be involved in the control of splicing of exon 6A, since deletion of this sequence induces a marked

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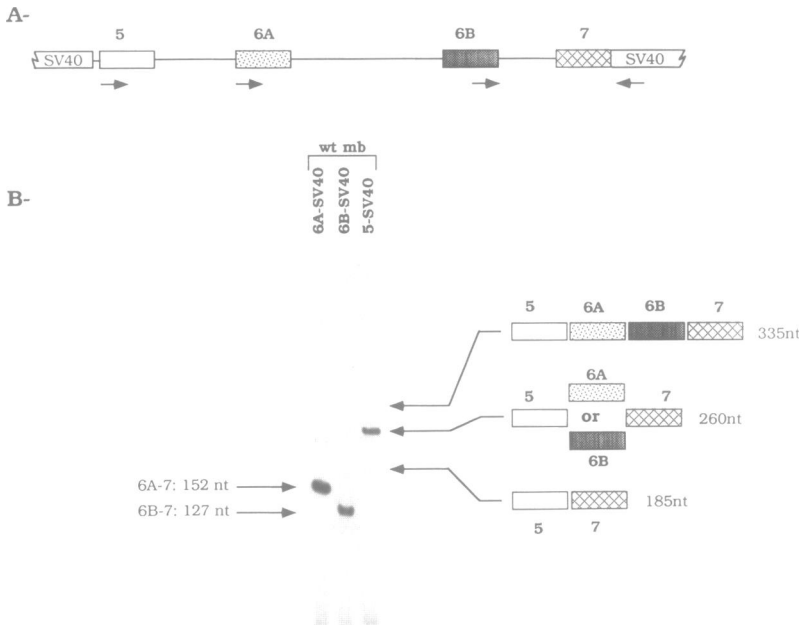

Fig. 1. A. Schematic diagram of the minigene construction. Exon sequences are boxed and the name of each exon is indicated above each box. Under these boxes the approximate localisation of the oligonucleotides used and their sense are represented by an arrow. **B.** Representation of a cDNA-PCR reaction performed between the three sense oligonucleotides used and the antisense SV40 oligonucleotide (SV). The RNA used was extracted from myoblasts transfected transiently by the wild type construct. Each arrow indicates the size, in base pairs, and the localisation on the gel of the amplification products which are obtained from all the possible mRNAs with the amplimers indicated on top of each lane. Some amplification products are not indicated: i. e. lane 6A—SV40 at 227nt a band can be seen, it corresponds to an amplification occuring on the transcript 5-6A−6B-7; lane 6B—SV40 a band is visible at approximately 260nt, it corresponds to the amplification of minor traces of transcripts which still contain the intron between exons 6B and 7 as shown by blotting experiments (data not shown).

decrease in the use of exon 6A. Several hypotheses to explain how this sequence exerts its regulatory functions are further discussed.

MATERIALS AND METHODS

Plasmids

The wild type (wt) minigene construction pBS/SVβalt has been previously described (7). Site directed mutagenesis was performed by the Kunkel method (12, 13) on single stranded construct with a minor modification: the elongation step of the reaction was performed using Taq polymerase (Beckman®) at 37°C. All mutants were sequenced to confirm that unwanted changes were not introduced and were named by the generic prefix pSV followed by the name of the mutation introduced: i.e. pSVΔ4 is the plasmid pBS/SVβalt with the mutation Δ4.

Cells and transfection

RSV transformed quail embryo myoblasts were obtained and transfected using the calcium phosphate method as previously described (7, 14).

cDNA-PCR analysis of the transcripts

RNAs were isolated as described in (15). Specific cDNA of the exogenous transcripts was obtained using 10μg of RNA and oligoSV complementary to SV40 sequences located in the 3′ end of the minigene (see fig.1 A) as a primer for the reverse transcriptase (M-MuLV Reverse transcriptase, Pharmacia®). The reaction was performed in 20μl of 50mM Tris-HCl pH 7,5; 10mM $MgCl_2$; 50mM KCl; 8mM DTT and 1mM dNTPs. Annealing of oligoSV (2.5 picomol) was performed for 5min at 70°C, followed by 5min at 37°C after which time 10 U of M-MuLV reverse transcriptase was added and the reaction was further incubated at 37°C for 60min. 2 to 4μl were then used for the amplification reaction.

Amplifications were performed using sense oligonucleotides 6A, 6B and 5 complementary respectively to sequences in exons 6A, 6B and 5, and oligoSV (see the schematic representation of the minigene construct in fig. 1 A for the localisation and the sense of the oligonucleotides used), in a final volume of 50μl containing 10mM Tris-HCl pH 8,3; 50mM KCl; 0,75 to 2mM $MgCl_2$; 0,01‰ gelatin; 200μM each dNTP; 0,5μM of primer and 2,5 U of Taq polymerase (Beckman®). The reaction was performed on a 'Crocodile' Appligene PCR cycler. In order to be able to quantitate the amplification products, 1−2 picomol of 5′ ^{32}P end-labelled oligoSV were also added to the reaction mixture. After a first denaturation step of 3min at 94°C a basic cycle (94°C: 20″; 55°C: 30″; 72°C: 1′) was repeated 10 to 20 times. We have quantified the amount of radioactivity incorporated in the products of amplification following increasing number of cycles and have observed that, under our experimental conditions, amplification is exponential for at least 25 cycles. It is very important to remain under these conditions in order to be able to prevent the formation of heteroduplexes during the last annealing step. The heteroduplex molecules migrate anomalously on non denaturing gels and can produce artifactual bands. The existence of such artifacts has been shown by Zorn (16) for molecules which share common regions (as this is the case here). The expected products of a typical cDNA-PCR experiment done on RNA extracted from myoblasts transfected

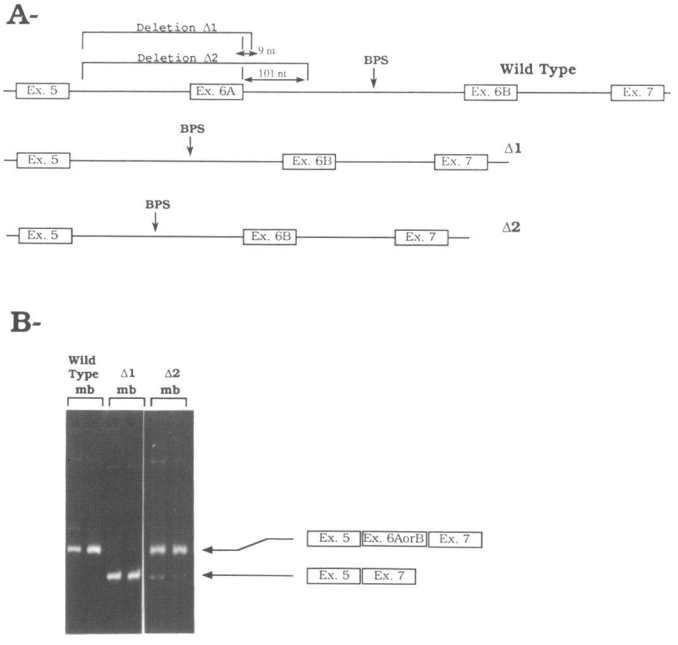

Fig. 2. cDNA-PCR analysis of transcripts derived from transfected myoblasts. **A.** Representation of the position of the deletions Δ1 and Δ2 on the wild type contructs. **B.** Amplifications are performed between amplimers SV and 5 (duplicate experiments are presented). In cells transfected by the wt construction and by Δ2 the major amplification product is at 260nt (5-6A−7 for the wt and 5-6B−7 for Δ2); with Δ1 the major amplification product is at 185nt corresponding to exon 5 spliced directly to exon 7.

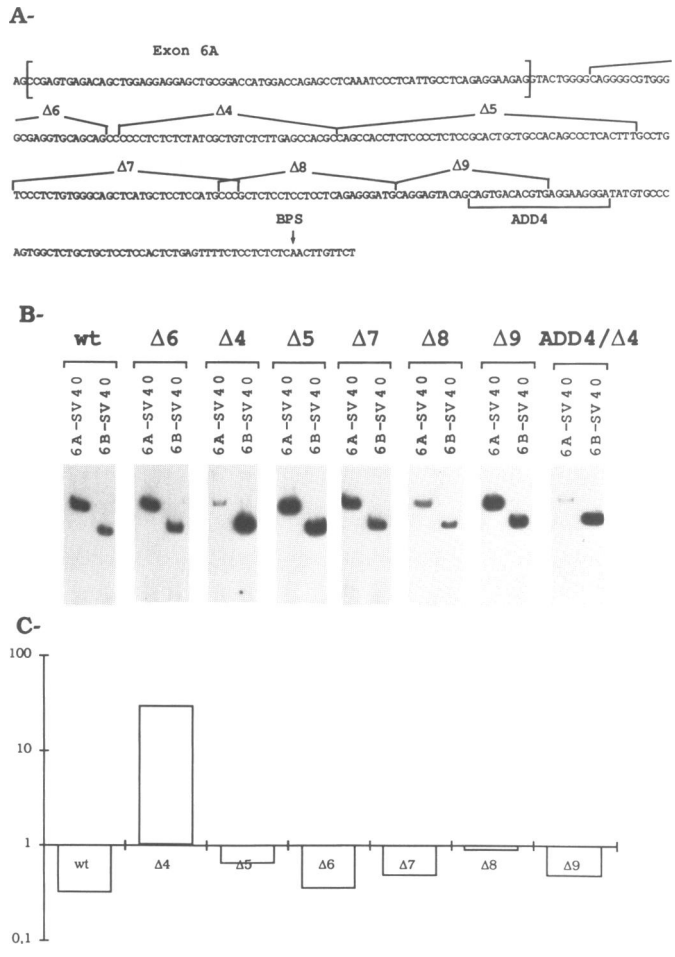

Fig. 3. A. Schematic representation of the deletions introduced on the sequence downstream of exon 6A (from Δ4 to Δ9), in the mutant ADD4/Δ4 the region Δ4 which has been deleted from its original place was inserted in place of the sequence delineated by the brackets ADD4. **B.** Amplifications reactions performed between oligonucleotides 6A, 6B and SV for all the mutants described above. **C.** This histogram represents the ratios 6B/6A for the different deletion mutants described above. The scale for the ordonates is logarithmic. For the wild type construct the ratio 6B/6A fluctuates around 0.3, for the mutant Δ4 it fluctuates around 30, for the other mutants this ratio fluctuates between 0.05 (Δ8) and 0.3 (Δ6).

transiently by the wild type construct is shown on fig. 1 B, the nature of the major and the minor bands is detailed on the legend of fig. 1 B. Each amplification experiment was repeated and some of the transcripts were also analysed by controled primer extension (7, 9) with essentially the same results.

Quantification of the radiolabelled PCR products was done by drying the gels and scanning them on a Molecular Dynamics β scanner apparatus. Each quantification experiment was performed at least three times.

RESULTS

In order to show that in myoblasts exons 6A and 6B are not involved in a process of competition, different minigene constructs have been prepared in which exon 6A was deleted together with the preceding intron. Both constructs have the same 5′ starting position but differ from one another by the location of their 3′ ends which extent by different lengths within the intron which separates exons 6A and 6B (IVSAB). These constructs conserve the exon 5 donor splice site and share the deletion of exon 6A and of the upstream intron, which has been shown to play no role in exon 6B splicing inhibition (7). When these constructs were used to transfect quail myoblasts and QT6 fibroblasts, it was obvious that they could be organized into two groups depending on whether exon 6B was included or not in the transcripts. A representative construct of each group is shown in figure 2. As indicated in figure 2A, mutants MutΔ6A (Δ1) and Δ2 are both deleted of exon 6A and of the upstream intron, they both conserve the donor splice site of exon 5, but while deletion Δ1 ends 9nt downstream of exon 6A, deletion Δ2 ends 101nt downstream of exon 6A. Each mutant was used to transfect quail muscle cells, the transcripts were isolated either before or after differentiation and analyzed by cDNA-PCR as detailed in the Methods section. In myoblasts, the splicing pattern of the wild type generates a major transcript which contains exons 5, 6A and 7 (7) while Δ1 gives a transcript which only contains exons 5 and 7 and Δ2, a transcript which contains exons 5-6B and 7 (Fig. 2B). This result for the wild type and Δ1 is expected (14), but the result for Δ2 is not since it indicates that the region deleted in Δ2 but not in Δ1 can regulate splicing of exon 6B.

To test this hypothesis, smaller deletions were introduced downstream of exon 6A, as shown on figure 3A. All mutants were transfected into myoblasts and the transcripts analysed as already described. Figure 3B shows a comparison of the products of amplifications performed between oligonucleotides SV and either oligonucleotides 6A or 6B in the case of the various mutants. All these amplifications were made using a radiolabelled

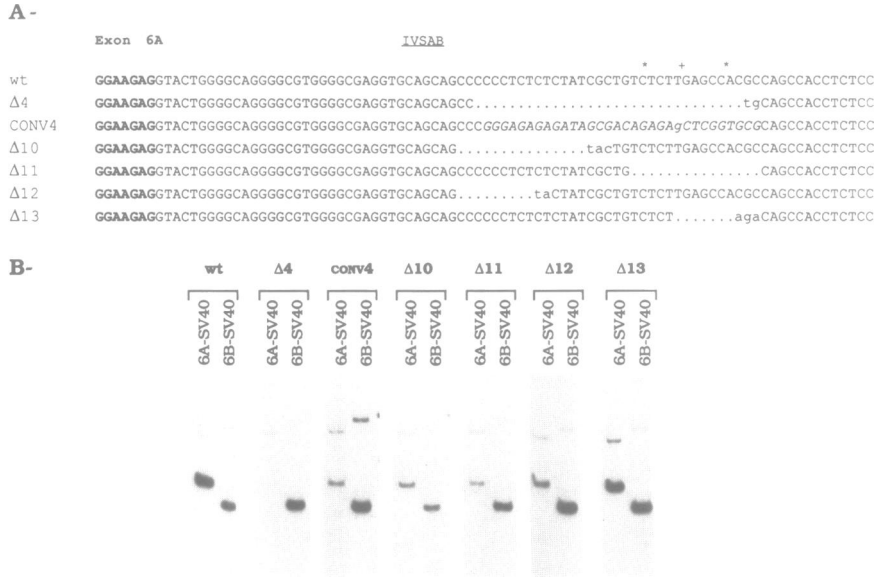

Fig.4. A. Sequences of the minigene constructs corresponding to the partial mutations of region Δ4. Points indicate the nucleotides which are deleted, lower case indicate modification of the wild type sequence and italics represent modifications corresponding to the complementary sequence of the region Δ4. **B.** cDNA PCR analysis of the minigenes described above, amplifications were performed between oligonucleotides 6A, 6B and SV. The ratio 6B/6A fluctuates around 1.5 for Δ10, 5 for Δ11 and Δ12, and 1.2 for Δ13.

oligonucleotide SV which allows us to make quantitative measurements (see M. & M. for the discussion of this point). As can be seen on figure 3C, in the case of the wild type construct, the ratio 6B/6A is less than one (it fluctuates around 0.3). With all the mutants this ratio remains less than one except for mutant Δ4 in which case this ratio is between 20 to 30. The preferential use of exon 6B in myoblasts was confirmed by Northern (data not shown). Thus, we can conclude that the deletion Δ4 drastically affects the balance between the two exclusive exons 6A and 6B by inducing the use of exon 6B in a non muscle environment.

In an attempt to localize more precisely the region implicated in this control of alternative splicing, we have constructed smaller deletions (labelled Δ10, Δ11, Δ12 and Δ13; fig.4 A) which are contained within the original deletion Δ4. Analysis of these additional mutants is shown on figure 4B. As can be seen, all the mutants induce the use of exon 6B in myoblasts, but none of them to the same extent as Δ4 (the ratio 6B/6A is between 1,2 forΔ13 and 5 for Δ11 and 12; fig. 4 B).

To test if the effect of Δ4 was due to a physical mechanism, for example by bringing close to the 5′ end of IVSAB regions which are normally more distant from it, we converted the region corresponding to the deletion Δ4 into its complementary sequence (fig. 4 A: CONV4). This construct induces the use of exon 6B to the same extent as deletions Δ10, Δ11, Δ12 and Δ13 but not to the extent of Δ4 (fig. 4 B). All these results point to the fact that both the size and the sequence of the region Δ4 is important. As a last attempt to characterize Δ4 and its mode of action, we decided to test whether this region could be moved along IVSAB. In one case, the region Δ4 was first deleted and then reinserted approximately 100nt further downstream (Fig. 3, Mutant ADD4). Among the various clones which were isolated, none showed the exact sequence for the reinserted Δ4 region. However, two clones had almost the exact sequence: clone 23 has a substitution T→G on the T marked by a + (fig 4 A wt) and clone 105 has a substitution C→A on the first C marked by a * and lacks the second C marked by a *. Both clones (23 and 105), when transfected into myoblasts present the same pattern of splicing. In all cases, exon 6B is used preferentially to exon 6A and the ratio 6B/6A is the same as the one obtained with mutant Δ4 (fig. 3 B cl.23 represented). Thus, once deleted, the sequence Δ4 cannot be reinserted and restore the wild type phenotype.

Deletion Δ4 has a dual effect on the regulation of the splicing of exon 6A and of exon 6B

During the course of the analysis of mutant Δ4, we became aware of the fact that this mutant, besides its activation of exon 6B could also have an inhibitory effect on the use of exon 6A. When amplification is performed between exon 5 and exon 7, in the case of the wt construct the major product is 260nt long and corresponds to transcripts containing exons 5−6A−7 while in the case of mutant Δ4, two products are detected, one at 260nt and the other at 185nt which corresponds to transcripts containing exons 5−7. This second type of transcript can represent up to 35% of the normal transcript and has been found with all the constructs in which the region Δ4 is involved but not with any of the other deletions (data not shown).

To test the possibility that splicing of exon 6A is affected by the mutation Δ4, four mutant minigenes were constructed: mutant K6A/wt where both splice sites of exon 6A are destroyed, mutant MutK6B (K6B/wt) where both splice sites of exon 6B are destroyed (fig. 5 A) and mutants K6A/Δ4 and K6B/Δ4 for which the deletion Δ4 has been introduced together with the mutations affecting the splice sites of exons 6A or 6B respectively (fig. 5 B). All these constructs were transfected into quail myoblasts and the transcripts were analyzed by cDNA-PCR using amplification between exon 5 and exon 7. When K6B/wt and

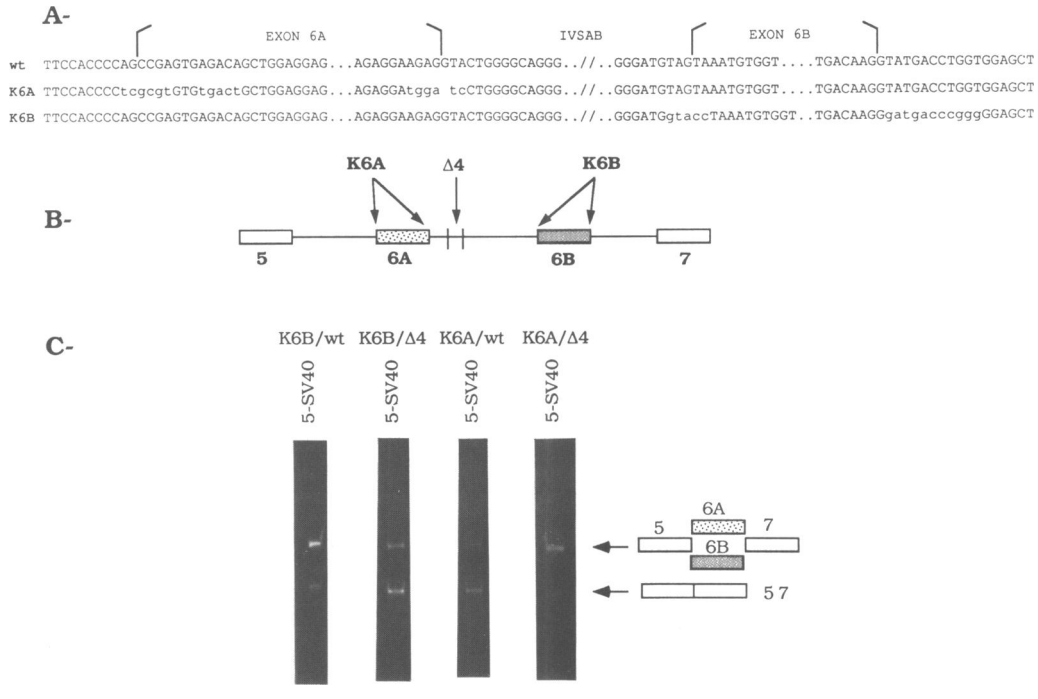

Fig.5. A. Sequences of the minigenes K6A and K6B are shown. As can be seen the modifications on K6A concern the acceptor, and putative downstream cryptic acceptors, and the donor splice site of exon 6A. For K6B the modifications are similar but concern exon 6B. **B.** Schematic diagram of the minigene with the relative position of the different mutations. **C.** cDNA-PCR analysis of transcripts derived from transfected myoblasts. Amplification were performed between amplimers SV and 5. Two products are obtained and the major product whether includes an alternative exon (first and last lanes) or excludes it (second and third lanes).

K6B/Δ4 are compared, the major amplification product is of the type 5-6-7 for K6B/wt and 5-7 for K6BΔ4 (fig. 5 C). This result clearly indicates that when exon 6A is the only available exon, in the presence of the deletion Δ4, this exon is preferentially skipped, and is in agreement with the analysis of mutant Δ4 in vitro with HeLa cell extracts (M. Gallego and al. in preparation). Comparison of the two other mutants, K6A/wt and K6A/Δ4, gives the opposite result. In the case of K6A/wt, the major product of amplification is of the type 5-7 while in the case of K6A/Δ4, it is of the type 5-6-7 (fig. 5 C). This result confirms that when exon 6B is the only choice, it is not used in myoblasts (14) unless it is activated by deletion of the region Δ4.

DISCUSSION

In this communication, we describe the characterization of a new cis acting element which is involved in the regulation of alternative splicing of the chicken β tropomyosin transcript. This element, which we have called Δ4, is a sequence of 33nt which is located 37nt downstream of the donor splice site of exon 6A in IVSAB. Its deletion or modifications seems to have two effects, the most noticeable is the almost exclusive use of exon 6B in undifferentiated myoblasts and a second, which appears to be more subtle, is a down regulation on the use of exon 6A. This latter point is best observed in a double mutant in which Δ4 is deleted at the same time as the use of exon 6B is blocked by mutating the two splice sites of exon 6B (MutK6B/Δ4). When such a mutant is transfected into muscle cells, the main transcript which accumulates contains exon 5 spliced directly to exon 7 while the same cells transfected with the single mutant MutK6B/wt accumulates a transcript which contains the three exons 5, 6A and 7 (fig. 5 C). Finally, the entire element seems to be required for its full expression since partial deletions have only a partial effect and furthermore, its location seems to be important since moving the region Δ4 in another part of IVSAB does not restore a wild type phenotype of alternative splicing.

It is interesting to note that it is the first time that a sequence located upstream of the branch point is shown to influence the regulation of alternative splicing. Using the rat homologous gene, Helfman and his colleagues have shown that the region of IVSAB located upstream of the branch point can be deleted from the minigene without noticeably affecting the pattern of splicing (11). Part of the discrepancy between their result and ours could be that their experiments were performed in vitro while ours have been obtained in vivo. In agreement with this conclusion is the report by M. Gallego who, using in vitro splicing, have been unable to obtain any activation of exon 6B with mutant Δ4 (M.Gallego unpublished result).

Analysis of the sequence of the region Δ4 may provide us with some indications on the mechanism by which this element could act. A first hypothesis is that region Δ4 is involved in the formation of a secondary structure which regulates the use of exon 6B and/or exon 6A. Indeed, analysis of the sequence of the 5′ region of IVSAB using the Zucker algorithm (17) indicates that region Δ4 can form a small stem and loop structure with the nucleotides located upstream of it. This region corresponds to the region called Δ6. However, that this structure plays any regulatory role is ruled out by the observation that mutant Δ6, which disrupts the structure, is devoid of any effect either on activation of exon 6B or inhibition of exon 6A.

A more likely explanation is that region Δ4 may be recognized by some important factor. Examination of Δ4 sequence shows that it is part of a polypyrimidine rich region which extend from position +35 in IVSAB over more than 90nt (see fig. 3 A). A second region which is also very rich in polypyrimidine is located between the branch point and the 3′ end of IVSAB. It is interesting to note here that the presence of two large pyrimidine rich domains is a characteristic feature of introns located between two alternative, mutually exclusive exons in tropomyosin genes. Indeed, this situation is observed for the chicken β gene IVSAB (7), the Xenopus laevis β gene IVS6A6B (A. Piseri and D. Libri unpublished observation), the rat β gene IVS6-7 (18, 19), the chicken α gene (20) and the rat homologous α gene IVS2-3 (21) (although we must note that in this intron the BPS is located upstream from the two pyrimidine rich domains). The fact that an important regulating element, i.e. the region of the deletion Δ4, is located in the first pyrimidine rich domain of IVSAB could indicate that the existence of two polypyrimidine regions in introns located between two alternative exons may have a functional signification. However, this conclusion is partially contradicted by the fact that the adjacent downstream region of the deletion Δ4 (region Δ5), also located in this domain, was shown to have no effects.

Recently, protein factors with affinity for polypyrimidine rich sequences have been described and it has been suggested that they could play a role in the regulation of alternative splicing (18, 22, 23, 24). The composition of Δ4 probably allows it to bind some of the polypyrimidine binding factors such as PTB and perhaps hnRNPs (25). It is however difficult to propose that region Δ4 regulates alternative splicing of the β tropomyosin transcript by interacting with a factor which simply recognizes polypyrimidine streches since regions such as Δ5 or Δ7, which have the same polypyrimidine content as Δ4 (78% for Δ4, 75% for Δ5 and 71% for Δ7), and almost the same C/T ratio (see fig. 3 A), have no effect on the use of either exon 6A or exon 6B. One would therefore have to postulate that region Δ4 interacts with a sequence-specific factor. Moreover, the fact that sequence Δ4 cannot be move around within IVSAB (no restoration of the wild type phenotype was observed after a transfer of this sequence 99nt downstream: mut. ADD4/Δ4 fig. 3) seems to indicate that its location is important.

Lastly, although we have no experimental evidence to suggest that the effects of region Δ4 on exon 6A and exon 6B involve the same mechanism of regulation, the possibility could exist that these two effects are linked. For example, Δ4 could act as a trap and locally deplete or enrich the splicing machinery in some factors acting or not in a dose dependent manner, such as PTB or U2AF and would prevent, by this way, the assembly of the splicing components on IVSAB branch site or on IVSAB acceptor splicing site, and would favour their assembly around IVSAB donor site. By this way it would be possible to explain the effects of region Δ4 on both 5′ and 3′ splice sites. According to this hypothesis, region Δ4 would be implicated in the process of non recognition of IVSAB as an intron sequence in myoblasts.

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