Volume 17 Number 16 1989 Nucleic Acids Research

A subfragment of the β tropomyosin gene is alternatively spliced when transfected into differentiating muscle cells

Domenico Libri, Joëlle Marie[1], Edward Brody[1] and Marc Y.Fiszman*

Molecular Biology Department, Pasteur Institute, Paris and [1]C.G.M., Bâtiment 24,CNRS, Gif sur Yvette, France

Received June 29, 1989; Accepted July 19, 1989

ABSTRACT

A subgenomic fragment of the chicken β tropomyosin gene which contains two alternative exons flanked by common exons was isolated and placed under the control of the SV 40 early promoter. This construction was subsequently used to transfect quail myoblasts together with a Neomycin resistance gene, and to isolate stable transfectants. mRNAs were isolated before and after differentiation and analyzed using a modification of the primer extension method. We show that myoblasts accumulate transcripts which contain the non muscle specific exon joined to the common exons while myotubes accumulate transcripts containing the muscle specific exon. These results, therefore demonstrate that such a subgenomic fragment contains all the necessary information to direct a correct developmentally regulated mutually exclusive splicing. They also strongly suggest that trans acting factors must be involved in the switch of the splicing pattern which takes place during the transition from myoblasts to myotubes. The same regulation cannot be faithfully reproduced during transient expression, since no difference in the use of exons 6A/6B is observed during differentiation and two aberrant minor splicing products are obtained which contain or lack both exons. We suggest that failure of exon 6A to splice to exon 6B is due to the existence of some structural constraints which lower the efficiency with which the intron between them is excised.

INTRODUCTION

Alternative splicing is a widespread mechanism used to generate multiple mRNA molecules from a single gene by differential processing of a unique primary transcript. It represents a potentially powerful mechanism to regulate gene expression in biological systems in which sequential expression of different isoforms is required during development. It also adds a feature of general biochemical flexibility and reversibility to the terminally differentiated and long-lived cells of muscular and nervous systems in which rapid gene reprogramming may be required due to environmental changes (for a review see [1, 2]). In spite of its widespread importance and of the growing number of examples of alternatively spliced genes, very little is known about the factors which influence splice site selection.

A number of *cis*-acting elements have been proposed which may be involved in splice site selection, such as multiple promoters and/or multiple polyadenylation signals [3], intron spacing [4, 5], exon sequences [6, 7, 8, 9, 10], relative strength of splice sites [6, 7] and branch point position [11, 12, 13]. Secondary structures of the primary transcript have also been postulated to play a role in determining the alternative nature of splice sites [14, 15, 16, 17]. Lastly, trans-acting factors are also believed to be involved in tissue specific splicing regulation [9, 18, 19, 20], though little is known about their role and nature mainly because of the lack of suitable systems for *in vitro* reproduction of splicing regulation.

Tropomyosin is a constituent of the sarcomere which plays a key role in the regulation of contraction. It is present, as a family of related isoforms, in several other cell types

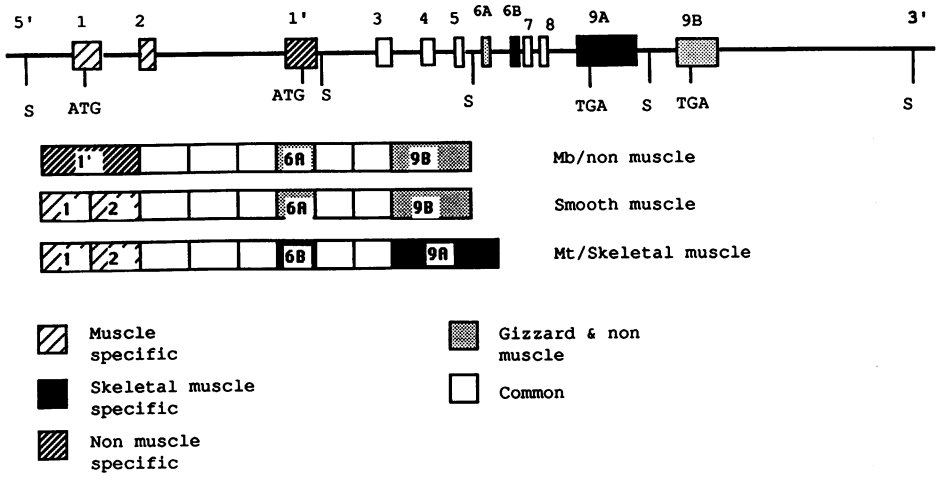


Fig. 1. Schematic representation of the β tropomyosin gene (not drawn to scale) and its mode of transcription. Upper part: genomic fragment containing the 12 exons of the gene.S: SacI sites.
Lower part: different transcripts produced by the tissue specific and developmentally regulated splicing. Mb: myoblasts. Mt: myotubes.

and tissues in which its functions, if any, remain to be elucidated. Two forms of muscular β-tropomyosin are known which are expressed in skeletal muscle (sk βTm) and in smooth muscle (sm βTm). The two proteins are encoded by the same gene by mutually exclusive splicing of two internal exons and by differential 3′ end processing [17]. This gene (fig.1) contains 12 exons of which exons 3 to 5 and 7 and 8 are constitutive; exons 6A and 9B (containing the 3′ end of the molecule) are included only in smooth muscle and in undifferentiated cells transcripts, while exons 6B and 9A (containing the 3′ end of the molecule) are present only in skeletal muscle RNA. Exons 1 and 2 are expressed only in smooth and skeletal muscle RNA while exon 1′(located between exons 2 and 3) is only present in myoblasts and undifferentiated cells mRNA. Exon 1 and 1′ contain the transcription start sites, the 5′ non coding ends, and the 5′ end of the coding parts of the corresponding mRNAs. The myoblast transcript (starting with exon 1′) codes for a shorter non-muscle form of tropomyosin (248 aminoacids versus 284 for muscle forms) (Libri et al., submitted; [21]).

The switch at the level of the two couples of alternative exons (6A/6B and 9A/9B) is both subjected to tissue and to developmental regulation: undifferentiated myoblasts in culture express exons 6A and 9B while after differentiation, myotubes express exons 6B and 9A.

In an attempt to define the sequences necessary for such a regulation, we have performed transfections of myogenic cells with minigene constructions. In this report, we show that a 0.9 kb fragment of the gene contains enough information to reproduce the developmental regulation for mutually exclusive splicing of exons 6A/6B. *Trans* acting factors are required for such a regulation while a number of *cis* elements like different 5′ ends of the primary transcript and alternative 3′ end processing seem to have little or no importance. On the other hand, only stable transfectants can reproduce such a regulation, which suggests either that such factors are present in limiting amounts or that plasmid integration may be

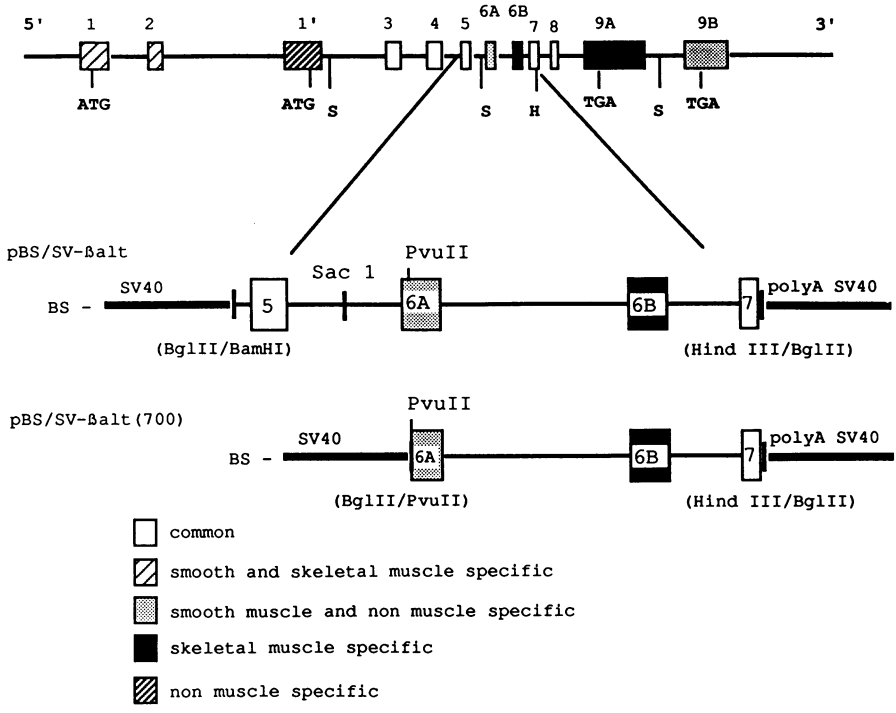


Fig. 2. Minigene constructions. Top line is a schematic illustration of the β tropomyosin gene. The two minigenes (pBS/SV-βalt and pBS/SV-βalt (700)) are shown in the bottom part of the figure. The Bam HI, HindIII, BglII and PvuII sites were lost upon cloning. SV40 sequences are indicated. S: SacI sites. H: HindIII sites. BS: 'Blue scribe' vector. Exon specificity is indicated.

necessary. We present evidence that the intron between the two alternatively spliced exons can be spliced out after transient expression but always with a low efficiency, even in the absence of exon 5 sequences. We suggest the existence of a dual control: an intrinsic low efficiency of excision of the intron between exons 6A and 6B would account for their mutually exclusive character, while trans acting factors would be responsible for the developmental stage specific regulation.

MATERIAL AND METHODS

Minigene construct

pBS/SV-βalt has been obtained by cloning a 0.9kb Bam HI-Hind III fragment of the chicken β tropomyosin gene into a BglII site of plasmid pBS/SV containing the SV 40 early promoter and transcription termination sites (fig.2). This genomic fragment contains all the sequence from part of the intron upstream of exon 5 to part of exon 7 (38 nt). The Bam HI site was introduced by oligonucleotide directed mutagenesis. pBS/SV-βalt(700) was obtained by cloning in the same vector a 0.7kb genomic fragment containing all the sequence from a PvuII site in exon 6A to the HindIII site of exon 7 (fig.2).

Cells and transfection

Quail embryo myoblasts transformed with a temperature sensitive mutant of Fujinami sarcoma virus (FSVtsNY240) were obtained as previously described for Rous sarcoma

virus transformed quail myoblasts [22]. 10 μg of pBS/SV-βalt or pBS/SV-βalt(700) and 1 μg of pBS/LTR-neo [23] were used to transfect 10^6 cells by the calcium phosphate precipitation method [24]. Stable transfectants were selected in the presence of 400 μg/ml of Geneticin (BRL). For transient transfections the plasmid pBS/LTR-neo was omitted and transiently expressed transcripts were harvested after 24 or 48 hours.

Oligonucleotide controlled primer extension

Two methods have been used to analyse the transcripts issued from minigene constructions. The first has been described by Erster et al.[25] in which primer extension is performed on transcripts previously submitted to oligonucleotide directed RNAse H digestion. RNAse H digestion and subsequent manipulations have been performed essentially as described.

In the second method RNAse H digestion is omitted and the reverse transcriptase reaction is simply stopped by the presence of an unlabelled oligonucleotide annealed 3′ to the labelled primer. The occurrence of a specific stop of reverse transcriptase indicates the presence of a sequence complementary to the unlabelled oligonucleotide on the mRNA analysed.

Usually 10 to 30 μg of total RNA and 0.05 to 0.15 picomoles of 5′ labeled primer are annealed in a total volume of 20μl.The ratio of the second oligonucleotide to the labeled primer is about 50:1. Annealing is performed in 50mM Tris-HCl pH 8, 6mM $MgCl_2$, 40mM KCl as follows: the mixture is incubated for 5′ at 65°C and 10′ at 37°C. The reverse transcriptase reaction is performed in 50mM Tris-HCl (pH 8), 6mM $MgCl_2$, 40mM KCl, 0.5mM dNTP and 10U AMV reverse transcriptase (Boehringer), for 45′ at 37°C. The products are analyzed on a 6% polyacrylamide gel.

Seven oligonucleotides were used: oligonucleotide SV is directed against SV40 sequences at the 3′ end of the minigene construction (pBS/SV-βalt) and of pBS/LTR-neo. Oligonucleotides 5,6A and 6B are directed against exons 5,6A and 6B. Oligonucleotides 7 and 2 are directed against sequences of exons 7 and 2 which are not present in the minigene constructions. An additional oligonucleotide (I-6A) is directed against the intron upstream of exon 6B. For all the oligonucleotides employed the presence of a single point of annealing has been verified by RNase H directed cleavage of uniformly labelled T3 transcripts.

All our results were reproduced using both methods.

RESULTS

Correct in vivo splicing of a β tropomyosin minigene in stable transfectants

In order to study the kind of regulation involved in the mutually exclusive splicing of exons 6A and 6B, we decided to investigate the potential influence of the alternative 3′ end processing of the primary transcript on the choice of the internal exons. As already mentioned, undifferentiated muscle cell mRNA contains exon 6A always in conjunction with exon 9B while myotube mRNA contains exon 6B with exon 9A. These terminal exons (9A and 9B) contain the stop codons and untranslated sequences 3′ to the coding region (fig. 1). The choice between exons 9A/9B, which could be made at the level of splicing and/or 3′ end processing, could be epistatic to the choice of exons 6A/6B and might control the latter by a cis-acting mechanism. Another potential cis-element in the developmental regulation is the presence of two transcription start sites, one of which is used in undifferentiated cells (exon 1′) while the other is utilized in myotubes and adult tissues (fig.1). On the other hand, mutually exclusive splicing could be regulated in trans by a set of factors acting independently of the presence and nature of the 5′ and 3′ ends of the primary transcript.

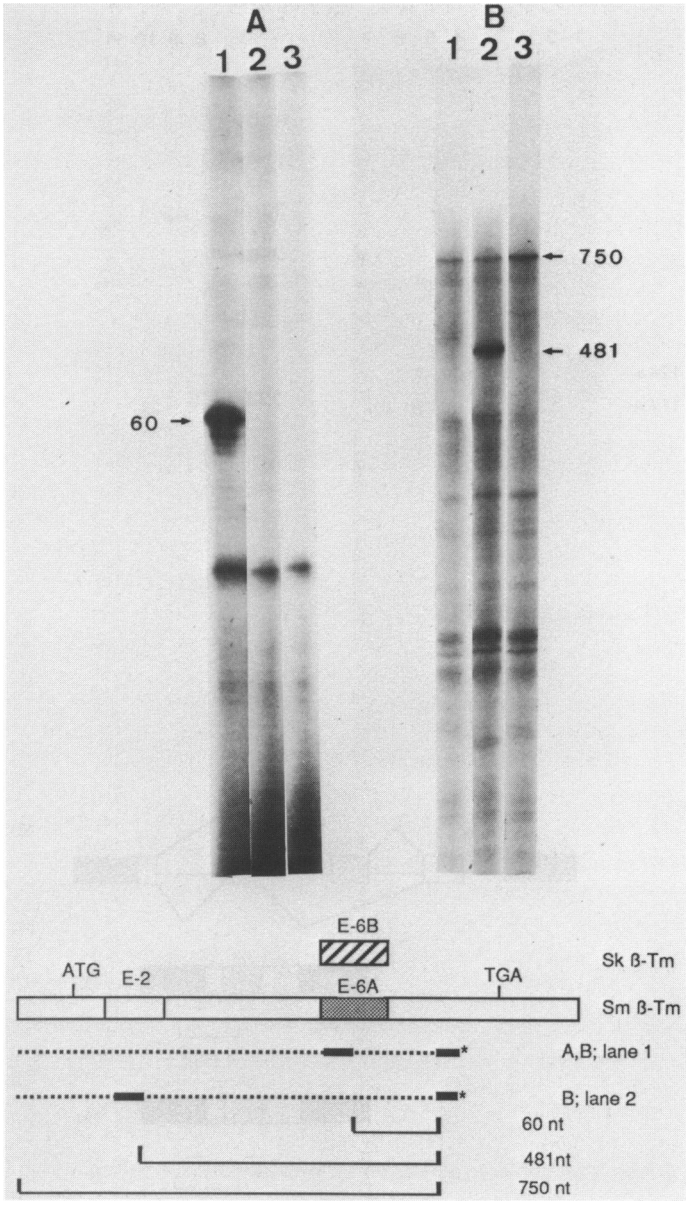
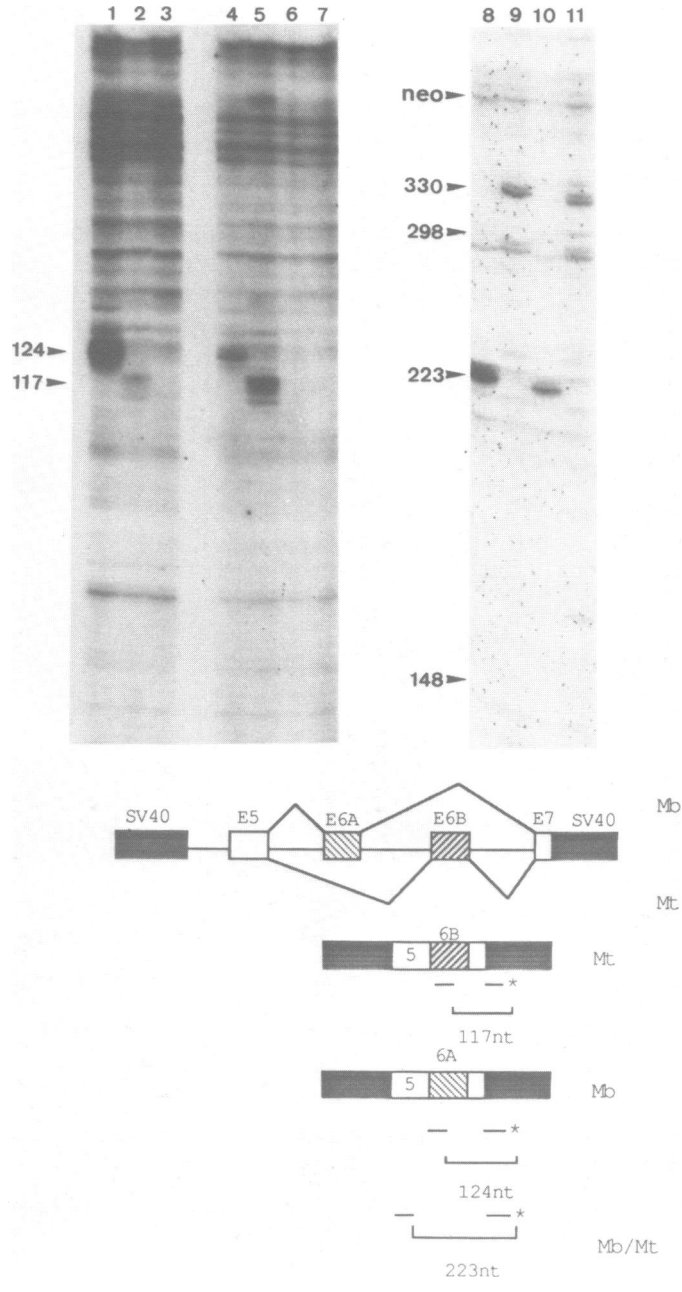


Fig. 3. Analysis of chicken gizzard RNA by the C.P.E. method. 30μg of total RNA were analyzed. Labeled primer is directed against exon 7 of the gene. Oligonucleotides directed against exons 6A, 6B and 2 were used to block elongation of the labeled-primer. Lane 1 (A and B): block by oligonucleotide 6A; lane 2 (A): block by oligonucleotide 6B; lane 2 (B): block by oligonucleotide 2 and lane 3 (A and B): free extension. Bands which are common to all the lanes represent unspecific stops of reverse transcriptase.
The lower part is a schematic representation of the experiment.


1 2 3
4 5 6 7
8 9 10 11
neo
330
298
124
117
223
148
SV40
E5
E6A
E6B
E7
SV40
Mb
Mt
6B
5
Mt
117nt
6A
5
Mb
124nt
Mb/Mt
223nt

To solve this problem we tried to reproduce the same developmental regulation of splicing in a molecule of primary transcript lacking such 3′ and 5′ sequences. A hybrid minigene construction containing SV40 sequences responsible for initiation and termination of transcription joined to a β-tropomyosin genomic fragment containing exons 5 (constitutive)−6A (smooth muscle/myoblasts)−6B (skeletal muscle/myotubes) and part of exon 7 (constitutive) (fig. 2), has been used to transfect quail myogenic cells. Stable transformants have been isolated, total mRNAs have been prepared from both myoblasts and myotubes, and further analyzed. To avoid interference in the analysis of exogenous transcripts by the endogenous transcripts (quail and chicken β-tropomyosin transcripts cannot be distinguished by S1 protection experiments or northern blot analysis), we have utilized a modification of the primer extension method (controlled primer extension, C.P.E., see Mat. and Methods). As an application of the method, Fig. 3 shows the analysis of chicken gizzard β tropomyosin mRNA in which extension is performed from a primer complementary to exon 7 and stopped with oligonucleotides complementary to exon 6A, exon 6B or exon 2. Extension of the primer is blocked when gizzard RNA is annealed with oligonucleotide 6A, as demonstrated by the presence of a 60nt fragment (Fig. 3A, lane 1), or in the presence of oligonucleotide 2, in which case a 481nt fragment is detected (Fig. 3B, lane 2). As expected [17], no specific block is detected with oligonucleotide 6B (FIG. 3A, lane 2). When no other oligonucleotide is present but the primer, a 750nt fragment is detected which corresponds to the full length extension product (Fig. 3A and 3B, lanes 3).

This technique was used to analyze RNA isolated from stable transfectants. To prime the exogenous transcript, an oligonucleotide directed against SV40 sequences located at the 3′ end of the minigene construction has been used (oligonucleotide SV). To stop the reverse transcription reaction we used the previously mentioned oligonucleotides 6A and 6B and an oligonucleotide directed against exon 5 (oligonucleotide 5). As can be seen in fig.4 the major splicing product of the exogenous primary transcript in myoblasts contains exon 6A joined to exon 7, as indicated by the specific stop induced by oligonucleotide 6A (124nt.) in the reverse transcription reaction (Fig. 4, lane 1). On the other hand, in myotubes, the major product contains exon 6B and exon 7 (Fig. 4, lane 5). In both undifferentiated and differentiated cells, exon 5 is correctly utilized and spliced either to exon 6A (Fig. 4, lane 8) or to exon 6B (Fig. 4, lane 10), as shown by the unique stop at 223nt. It is interesting to note that there are no detectable bands which could indicate the absence of both exons 6A and 6B (splicing of exon 5 to exon 7) from the final transcript (expected length 148nt) nor bands indicating their simultaneous presence on the same molecule (expected length 298nt).

Fig. 4. C.P.E. analysis of transcripts originating from pBS/SV-βalt minigene construction after stable tranfection. Labeled primer is directed against SV40 sequences present in both pBS/SV-βalt and pBS/LTR-neo. Lanes 1−3 and 8−9: RNA isolated from myoblasts; lanes 4−7 and 10−11: RNA isolated from myotubes. Extension is blocked by oligonucleotide 6A (lanes 1 and 4); oligonucleotide 6B (lanes 2 and 5); oligonucleotide I-6A (lane 6) and oligonucleotide 5 (lanes 8 and 10). Lanes 3, 7, 9 and 11 represent free extension. Transcripts have been digested with RNase H prior to primer extension in lanes 8 to 11. The size of the final product transcribed from pBS/SV-βalt is 330nt while the product transcribed from pBS/LTR-neo is larger and labeled 'neo'. 148 and 298 indicate the expected length of the product if exons 6A and 6B were respectively both skipped or included. The lower part is a schematic representation of the experiment.

In both myoblasts and myotubes minor reverse transcriptase stops can be obtained respectively with oligonucleotide 6B (Fig. 4,lane 2) and oligonucleotide 6A (Fig. 4,lane 4). These stops may be due to the presence of differentiated cells among the myoblasts and to undifferentiated cells among the myotubes. This possibility is confirmed by the morphology of the cultures and by the analysis of endogenous transcripts by S1 protection (data not shown).

Our results demonstrate that alternative splicing of exons 6A/6B is not regulated in cis by the choice of exons 9A/9B at the 3′ end of the mRNA nor by the choice of the two different starts of transcription (exon 1 or exon 1′). This last result was somewhat expected since adult smooth and skeletal muscles, which employ different patterns of splicing, utilize the same transcription initiation site [17]. Furthermore, since the minigene primary transcripts are identical in myoblasts and myotubes, trans-acting factors have to be postulated in order to allow a specific splicing of both exons to occur depending on the state of differentiation of the cell. This also implies that such trans-acting factors can distinguish an 'alternative' exon from a constitutive one by using information which is contained in the 0.9Kb genomic fragment.

Splicing of the β tropomyosin minigene in transient transfectants

To investigate the effects of stable integration of pBS/SV-βalt on alternative splicing, we have harvested transiently expressed RNAs from transfected myoblasts and myotubes. The analysis of transcripts by controlled primer extension is shown in fig. 5 A and B. As can be clearly seen in lanes labelled 6A and 6B in myoblasts (Mb) and myotubes (Mt), the relative amounts of RNAs containing exon 6A or exon 6B does not significantly change following differentiation. These results have been reproduced many times using different differentiating cultures in order to overcome some experimental variability due to incomplete differentiation of myoblasts. Though slight variations in the ratio of the two transcripts were observed, mRNA containing exon 6B was never the main product of splicing in myotubes while a transcript containing exon 6A was always present in higher amounts. In no case were changes in the ratio of mRNA species containing exons 6A or 6B observed after differentiation. These experiments show that the tissue specific character of mutually exclusive splicing of exons 6A and 6B is lost in transient expression of the minigene and that regulation by trans acting factors is greatly reduced if not absent.

Moreover, a number of additional minor splicing products are also observed. As can be seen in the overexposed myoblast section of fig. 5A, three additional bands showing specific reverse transcriptase stops are present in lanes 6A and 5. Two of these stops (bands at 199nt, lane 6A, and at 298nt, lane 5) are best explained if exons 6A and 6B are spliced together in a transcript containing also exon 5 and 7. On the other hand, the band at 148nt (lane 5) indicates the presence of an mRNA species in which exon 5 is joined directly to exon 7 and both exons 6A and 6B are skipped (see also fig. 5B). The same aberrant splicing products can be observed in the myotubes section of fig.5A although they are barely visible due to the shorter exposure of the autoradiogramm and of the quasi coincidence of the band at 298nt (lane 5) with a non specific stop of reverse transcriptase. As expected we see no modulation in the expression of such species during differentiation (compare myoblasts and myotubes sections of fig. 5A, and data not shown) although some variations in their ratios can be observed among different transfection assays. These observations show that the intron between exons 6A and 6B, which is never excised from the endogenous primary transcript, is a functional intron and can be spliced *in vivo*, albeit with a very low efficiency.

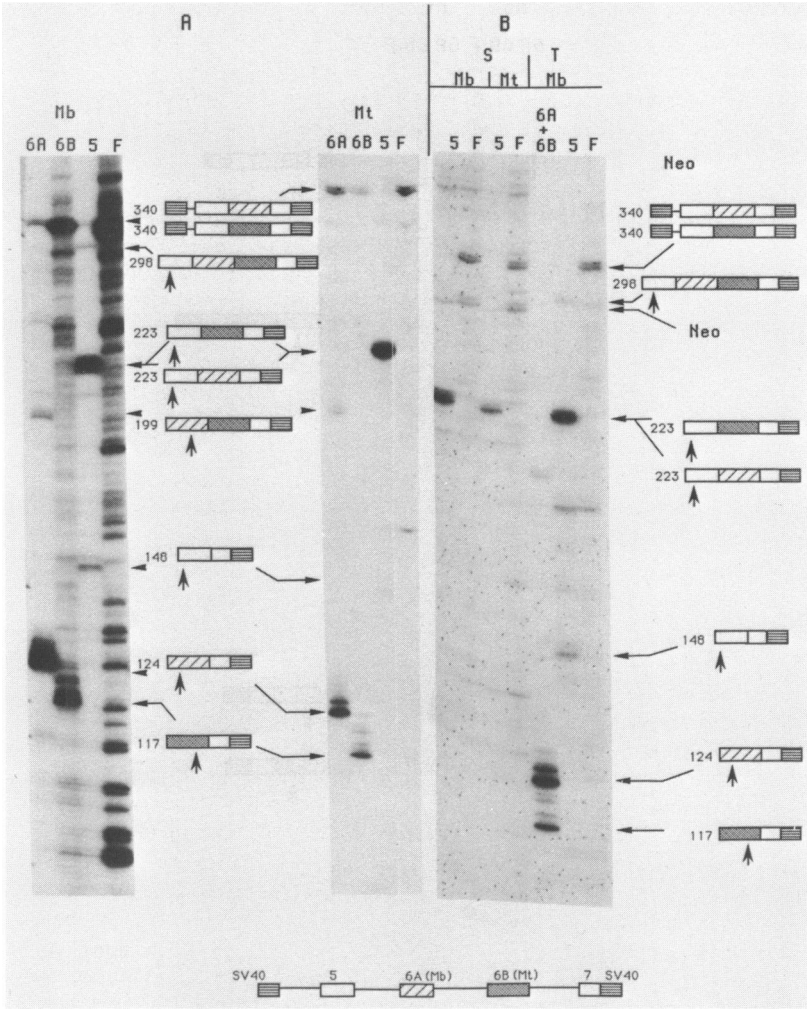


Fig. 5. C.P.E. analysis of transcripts after transient expression of the minigene constructions. Transcripts have been cleaved with RNase H prior to primer extension. Part A: transcripts from pBS/SV-βalt spliced either in myoblasts or in myotubes. Mb: myoblasts; Mt: myotubes. Lanes labelled 6A,6B and 5: mRNA digested with RNAse H after hybridization with respectively oligonucleotides 6A, 6B and 5. Lane labelled 6A+6B: mRNA digested with RNAse H after annealing with a mixture of oligonucleotides 6A and 6B. Lanes labelled F: free reverse transcriptase extension. Part B is a direct comparison between stable (section S, Mb and Mt) and transient transfectants (section T, Mb). A schematic representation of each splicing product is indicated.together with their size in nucleotide. 'Neo' indicates the primer extension product of transcripts derived from pBS/LTR-neo (present only in stable transfectants). Arrows indicate the point of RNAse H cleavage. Note the quasi coincidence of a non specific stop of reverse transcriptase with the band at 298nt (compare lanes 5 and F in section T of part B). Such non specific stop is absent in primer extension of previously cleaved transcripts (compare all lanes 5 with lanes F). An additional band migrating slightly faster is visible in section S (stable transfectants) of part B, which is due to primer extension of transcripts derived from pBS/LTR-neo and which is not present in primer extension analysis of transcripts derived from transient transfectants.

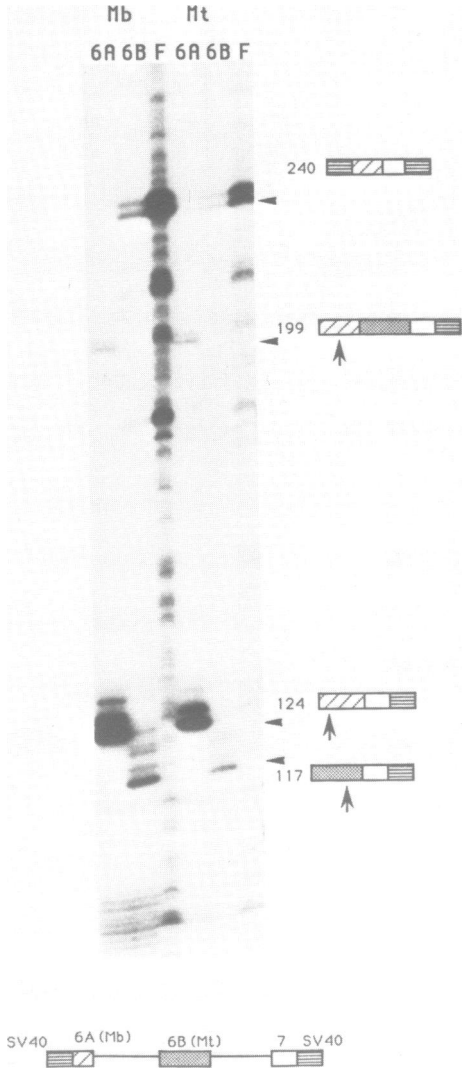


Fig. 6. C.P.E. analysis of transcripts derived from pBS/SV-βalt(700) after transient expression of the minigene construction in myoblasts or in myotubes. Transcripts have been cleaved with RNase H prior to primer extension. Mb: myoblasts; Mt: myotubes. Lanes labelled 6A and 6B: mRNA digested with RNAse H prior annealing with respectively oligonucleotides 6A or 6B. Lanes labelled F: free reverse transcriptase extension. Arrows indicate the point of RNAse H cleavage. Schematic representation of both minigenes and of each splicing product is indicated.

Skipping of both alternative exons clearly indicates that inclusion of exon 6A is not an automatic process whenever exon 6B is excluded by the splicing apparatus. Lastly both observations (simultaneous inclusion or exclusion of exons 6A and 6B) show that the mutually exclusive character of this couple of exons can be partially lost in transient transfection assays.

Transient expression of a truncated minigene lacking exon 5 sequences.
A genomic fragment containing two alternatively spliced exons and two flanking common exons represents a minimal substrate to reproduce both the mutually exclusive character and the tissue specificity of this kind of alternative splicing. We have shown that in transient expression assays, only the mutually exclusive character of the couple of exons 6A/6B is partially retained. To investigate whether an entire exon 6A and upstream sequences were required to prevent splicing of exons 6A to exon 6B, we have performed transient transfections of myogenic cells using a truncated minigene construct in which SV40 promoter sequences are joined directly to a PvuII site in exon 6A (see fig.2 and Material & Methods). In this construct only the donor site of exon 6A is present and it can be spliced either to the acceptor site of exon 6B or to the acceptor site of exon 7. As shown in fig. 6 (lanes 6A in myoblasts and myotubes sections), exon 6A donor site splices preferentially to exon 7 acceptor site and with a low efficiency to exon 6B acceptor site as indicated by the weak signal at 117nt (lane 6B) and at 199nt (lane 6A). As expected, no or little control *in trans* is observed during the transition myoblasts-myotubes (compare ratios of 6A-7 to 6A-6B-7 products in myoblasts and myotubes). This result indicates that sequences upstream of the PvuII site in exon 6A have no regulatory function with respect to splicing of exon 6A to exon 6B.

Unfortunately we failed to obtain stable transfectants from this minigene construction.

DISCUSSION

Alternative splicing is a term which is used to designate a number of complex processes which take place during the maturation of primary transcripts and by which multiple mature mRNAs are generated from single genes. A review of the various patterns of alternative splicing has been made recently by Breitbart et al.[2] and Andreadis et al.[1]. It includes exemples of intron retention, presence of internal acceptor or donor sites, alternative promoters or 3′ terminal exons, inclusion/exclusion of 'cassette' type exons and mutually exclusive splicing.

This latter can be seen as a combination of two 'cassette' type exons whose inclusion/exclusion in the mature transcript is co-ordinated in such a way that only one exon is present at a time. When mutually exclusive splicing is regulated in a tissue or development specific way, the choice of the exon to be included is determined by the specific environment in which splicing takes place. Thus, two events occur at the same time in regulated mutually exclusive splicing: the two exons should never be spliced together and a choice must be made to assure the presence of the right exon in a given cell type.

Tissue-specific or developmentally regulated splicing has been successfully reproduced on transcripts issued from minigene constructions. Control of expression of 3′ terminal exons has been obtained in a construct containing a fragment of the Calcitonin/CGRP gene [18] while regulation of inclusion/exclusion of exons of the 'cassette' type has been achieved in a number of genes such as skeletal troponin T [19], leucocyte common antigen (LCA) [9] and fibronectin [20]. These results have also shown that trans acting factors must be postulated to assure such a control.

Cis elements also have been shown to be implicated in the regulation of alternative splicing. A role for exon sequences has been proposed by Mardon et al.[8] for the fibronectin gene and more recently by Streuli et al. [9] and Hampson et al.[10] respectively for a cassette type exon in the LCA gene and for a retained intron in the bovine growth hormone gene. Smith et al.[13] have recently shown that the abnormal proximity of the branch point

to the donor site is responsible for the block of splicing of a couple of mutually exclusive exons in the α-tropomyosin gene, while Helfman and his colleagues [26] have shown that a critical event in splicing of mutually exclusive exons 6 and 7 of the β-tropomyosin gene in the rat (corresponding to exons 6A and 6B of the chicken gene) is the removal of the intron following the skeletal muscle specific exon (exon 7). Lastly, we and others [14, 15, 16, 17] have proposed a role for secondary structures of the primary transcript in splice site selection.

In the present report we show that the primary transcript issued from a minigene contruction containing a O.9Kb genomic fragment can undergo faithfully regulated mutually exclusive splicing after transfection in myogenic cells. Both the mutually exclusive character of the couple of exons 6A/6B of the β-tropomyosin gene and the developmental regulation concerning the choice of the right exon have been successfully reproduced in a differentiating myogenic system after isolation of stable transfectants. This result indicates that all the information required *in cis* for the developmental specificity of splicing and the mutually exclusive character of the couple of exons is inherent to the sequence between exon 5 and exon 7. Other cis elements like differential 3′ end processing or different transcription initiation sites have little or no influence on internal alternative processing. In addition, the existence of trans acting factors must be postulated to explain a different processing of the same primary transcript in myoblasts and myotubes.

On the other hand, during transient expression, analysis of the splicing products derived from the minigene construction shows that the developmental regulation is lost while the mutually exclusive character of exons 6A/6B is partially retained. Indeed, no difference is observed between the splicing products accumulated in myoblasts or myotubes, and two minor aberrant mature transcripts are also produced in which both exons are either included or skipped at the same time. Both the necessity for plasmid integration as well as the higher amount of transcripts in transient transfectants could account for this loss of regulation. As a matter of fact, we have always observed that transient transfectants accumulate higher amounts of exogenous transcripts per μg of total RNA when compared to stable transfectants (the ratio varies from 2:1 up to approximately 100:1). Furthermore, it must be considered that after stable transfection almost all the cells express the exogenous minigene, while during transient transfection only few transfected cells contain and transcribe the exogenous construct. We have estimated the efficiency of transfection in our system to be in the order of 10^{-3} (our unpublished observations). This would mean that each transiently transfected cell expresses 10^3 to 10^5 times more exogenous transcript than a stably transfected cell. This difference could reflect a difference in copy number of the plasmid containing the minigene construction per cell as well as (probably to a lesser extent) a difference in activity of the SV40 promoter before and after integration in the cell genome. Thus, loss of tissue regulation of mutually exclusive splicing and/or aberrant splicing may be correlated with such high amounts of primary transcript, possibly by the titration of specific factors.

It is interesting to note that whichever may be the cause for such deregulation, it does not seem to affect to the same extent the two aspects of alternative splicing which have been previously mentionned: the tissue specificity and the mutually exclusive character of the couple of exons. Developmental specificity of splicing is no longer observed, while the mutually exclusive character of exons 6A and 6B is partially retained since both splicing of exon 6A to exon 6B as well as skipping of the two exons can occur but with a low frequency. Moreover, we observed that at least the aberrant product of splicing in which exon 5 is joined directly to exon 7 disappears very early during the establishment of stable

transfection after no more than 4 cell divisions, while it takes more than 10 days for the tissue specificity to be restored (data not shown).

A model in which all the regulation of mutually exclusive developmentally specific splicing is provided by trans acting factors is in theory possible. At least two factors would be required which would in turn either prevent or allow the utilization of one or the other exon. Their expression should be co-ordinated so that only one of them would be present at a time in a given cell type. This would provide both the tissue specificity and the mutually exclusive feature of splicing. Our results do not support such a model since it does not explain how developmental specificity can be lost without the concomitant loss of the mutually exclusive character of the exons. As an alternative model, we suggest the existence of two levels of control which are at least partially independent and which are differently affected by an overproduction of primary transcript or by the necessity for plasmid integration. The first level concerns the mutually exclusive character of the two exons. This could be determined in cis by some intrinsic low efficiency of excision of the intron between the two alternative exons and by the low probability that the splicing apparatus ignores both exons. Smith et al. [13] have recently shown that the intron between two mutually exclusive exons in the rat α-tropomyosin gene cannot be excised in vitro even in the absence of the flanking exons and that this low efficiency of splicing is related to the abnormal proximity of the 5′ splice site to the branch point. Though this cannot be the case for the β tropomyosin gene (the branch point is located about 300nt from the 5′ splice site (Goux-Pelletan et al., submitted) versus only 42nt in the α-tropomyosin gene) other structural constraints can be imagined which would prevent splicing of exon 6A to exon 6B. Secondary structures of the primary transcript could also play an important role. As we have recently proposed [17], every molecule of the primary transcript could exist in one of two alternative states: one in which the exon 6A is accessible and the other in which it is exon 6B. The position of the equilibrium between the two states would determine the relative abundance of the two final splicing products in the absence of specific trans acting factors. A high production of primary transcript could only partially overcome this control.

Trans acting factors would provide the second level of control; their role would be to displace the equilibrium towards one or the other of the two alternative states depending on the cell differentiation stage. This level would be completely abolished in transient transfection assays by titration of the factors and/or by strict requirements for integration of the exogenous DNA.

ACKNOWLEDGEMENTS

The technical assistance of D. Gros is kindly acknowledged. We also wish to thank Dr M.Rosbash for his constant interest and fruitfull discussions. D. Libri is supported by a fellowship from the Association des Myopathes de France. This work was supported by grants from the CNRS, the INSERM, the CEA, the Ministère de l'Industrie et de la Recherche, the Fondation pour la Recherche Médicale Française, the ARC, the Ligue Française contre le Cancer and the Association des Myopathes de France.

*To whom correspondence should be addressed

REFERENCES

1. ANDREADIS, A., M. E. GALLEGO and B. NADAL-GINARD. (1987). Ann. Rev. Cell. Biol. **3**: 207–242,
2. BREITBART, R., A. ANDREADIS and B. NADAL-GINARD. (1987). Ann.Rev.Biochem. **56**: 467–495,

3. GALLEGO, M. and B. NADAL-GINARD. (1988).C.S.H. Meeting on RNA Processing. p. 61. (Cold Spring Harbor Laboratory. Cold Spring Harbor ,New York).
4. FU, X. Y. and J. L. MANLEY. (1987). Mol.Cell.Biol. **7**: 738–748,
5. WIERINGA, B., E. HOFER and C. WEISSMANN. (1984). Cell. **37**: 915–925,
6. REED, R. and T. MANIATIS. (1986). Cell. **46**: 681–?,
7. EPERON, L. P., J. P. ESTIBEIRO and I. C. EPERON. (1986). Nature. **324**: 280–282,
8. MARDON, H. J., G. SEBASTIO and F. E. BARALLE. (1987). Nucleic Acids Res. **15**: 7725–7733,
9. STREULI, M. and H. SAITO. (1989). EMBO J. **8**: 787–796,
10. HAMPSON, R. K., L. LA FOLLETTE and F. M. ROTTMAN. (1989). Mol.Cell.Biol. **9**: 1604–1610,
11. NOBLE, J. C. S., C. PRIVES and J. L. MANLEY. (1988). Genes & Dev. **2**: 1460–1475,
12. REED, R. and T. MANIATIS. (1988). Genes & Dev. **2**: 1268–1276,
13. SMITH, C. W. J. and B. NADAL-GINARD. (1989). Cell. **56**: 749–758,
14. SOLNICK, D. and S. I. LEE. (1987). Mol. Cell. Biol. **7**: 3194–3198,
15. BREITBART, R. E., M. T. NGUYEN, R. M. MEDFORD, A. T. DESTREE, V. MAHDAVI and B. NADAL-GINARD. (1985). Cell. **41**: 67–82.,
16. RUIZ-OPAZO, N. and B. NADAL-GINARD. (1987). J. Biol. Chem. **262**: 4755–4765,
17. LIBRI, D., M. LEMONNIER, T. MEINNEL and M. Y. FISZMAN. (1989). J.Biol.Chem. **264**: 2935,
18. LEFF, S. E., R. M. EVANS and M. G. ROSENFELD. (1987). Cell. **48**: 517–524,
19. BREITBART, R. E. and B. NADAL-GINARD. (1987). Cell. **49**: 793–803,
20. BARONE, M. V., C. HENCHCLIFFE, F. BARALLE and G. PAOLELLA. (1989). EMBO J. **8**: 1079–1085,
21. BRADAC, J. A., C. E. GRUBER, S. FORRY-SCHAUDIES and S. H. HUGHES. (1989). Mol.Cell.Biol. **9**: 185–192,
22. MONTARRAS, D. and M.Y.FISZMAN. (1983). J. Biol. Chem. **258**: 3883–3888,
23. CRIBBS, L. L., N. SHIMIZU, G. E. BUGAISKY, M. Y. FISZMAN, S. JACOVIC and P. K. UMEDA. (1987). Advances in myochemistry. **1**: 173–184,
24. GRAHAM, F. L. and A. J. van der EB. (1973). Virology. **52**: 456–467,
25. ERSTER, S. H., L. A. FINN, D. A. FRENDEWEY and D. M. HELFMAN. (1988). Nucleic Acids Res. **16**: 5999–6014,
26. HELFMAN, D. M., W. M. RICCI and L. A. FINN. (1988). Genes & Dev. **2**: 1627–1638,

This article, submitted on disc, has been automatically converted into this typeset format by the publisher.