The Xenopus αtrop-tropomyosin gene contains, at its 3'-end, a composite internal/3'-terminal exon (exon 9A9'), which is subjected to three different patterns of splicing according to the cell type. Exon 9A9' is included as a terminal exon in the myotome and as an internal exon in adult striated muscles, whereas it is skipped in nonmuscle cells. We have developed an in vivo model based on transient expression of minigenes encompassing the regulated exon 9A9' in Xenopus oocytes and embryos. We first show that the different α-tropomyosin minigenes recapitulate the splicing pattern of the endogenous gene and constitute valuable tools to seek regulatory sequences involved in exon 9A9' usage. A mutational analysis led to the identification of an intronic element that is involved in the repression of exon 9A9' in nonmuscle cells. This element harbors four polypyrimidine track-binding protein (PTB) binding sites that are essential for the repression of exon 9A9'. We show using UV cross-linking and immunoprecipitation experiments that Xenopus PTB (XPTB) interacts with these PTB binding sites. Finally, we show that depletion of endogenous XPTB in Xenopus embryos using a morpholino-based translational inhibition strategy resulted in exon 9A9' inclusion in embryonic epidermal cells. These results demonstrate that XPTB is required in vivo to repress the terminal exon 9A9' and suggest that PTB could be a major actor in the repression of regulated 3'-terminal exon.

Alternative splicing is a widespread mechanism in metazoans, by which multiple mRNAs can be produced from a single gene. This process is often subjected to tissue or developmental control and allows the production of protein isoforms differing in specific domains.

Over the last few years, some general topics have emerged from the intensive studies undertaken to elucidate the mechanism of alternative splicing. It is thus established that regulated exons are generally flanked by suboptimal splice sites whose recognition is modulated by cis-acting regulatory sequences present within exons or introns. In most cases, alternative exons are subject to positive and negative regulation which insures a tight control on their usage (reviewed in Ref. 1). Numerous trans-acting factors that modulate alternative splicing have also been characterized. These regulatory factors belong to two broad families of RNA-binding proteins, designated hnRNP proteins and RS-domain proteins. The latter includes the SR protein subfamily (2, 3) that is involved in constitutive or alternative exon recognition through exonic sequence called exonic enhancer (4).

Despite this significant breakthrough, the basis of tissue regulation of alternative splicing is still poorly understood in vertebrates. Indeed, contrary to the paradigm described in Drosophila, where many splicing events are controlled by tissue-specific factors (5), to date the implication of such factors appears limited in vertebrates.

To overcome this lack of tissue specific factors it was proposed that ubiquitously expressed splicing factors could determine a cellular code based on variation of their relative levels or activities in various tissues (6). A current model proposes that hnRNP packaging prevents exon use and that their displacement is a preamble to a splicing complex assembly. For example, hnRNP A1 can antagonize the promotion of alternative exon usage that occurs through the binding of SR proteins to purine-rich exonic enhancer (7, 8). The ubiquitously expressed polypyrimidine tract-binding protein (PTB designated also hnRNP I) has been implicated in the repression of several alternative exons including exon 7 of β-tropomyosin (9), exon N of GABAγ2 (10), exon 5 of N-methyl-D-aspartate receptor NR1 (11), exons IIb and IIIc of FGF-R2 (12, 13), exon N1 of c-src (14), exon SM of α-actinin (15), exon IIb of fibronectin (16), exon 3 of α-tropomyosin (17), and exon 9 of caspase-2 (18). PTB binds especially to pyrimidine-rich intronic splicing silencer elements present upstream or downstream of the alternative exon. These pyrimidine-rich elements frequently contain several high affinity binding motifs UCUU and (G/U)UCUCU (10, 19). The physiological relevance of PTB in alternative exon repression was recently demonstrated by RNAi-mediated PTB depletion that leads to an increase in exon IIb of FGF-R2 and exon IIb of fibronectin inclusion (20). From all of these data, it was proposed that PTB has a general role in exon silencing and preventing exon definition (21).

3’-Terminal exons can also be differentially processed (reviewed in Ref. 22). However, selection of alternative 3’-terminal exons differs in many aspects from that of internal exon, since it could potentially be regulated at several levels including transcriptional termination and competition between splice sites and cleavage-polyadenylation signals. Use of a proximal 3’-terminal exon can be simply controlled by the selection of a proximal polyadenylation signal, since cleavage at this site excludes the distal 3’-terminal exon and subsequently any competition with this distal exon. Indeed, to date extensive studies performed with the calatonin/calcium gene-related protein (CT/CGRP) and heavy chain immunoglobulin gene
pre-mRNAs have especially highlighted the importance of the cleavage/polyadenylation reaction in the selection of alternative 3'-terminal exons. Thus, the processing of the CT/CGRP pre-mRNA is regulated at the level of polyadenylation through the action of a complex intronic enhancer localized downstream of the proximal 3'-terminal exon (23). In the same way, it was demonstrated that the differential processing of the heavy chain immunoglobulin mu gene during B-cell maturation did not require Ig gene-specific sequences (24) and was regulated primarily by changes in the activity of the general polyadenylation factor CstF-64 (25, 26). Despite the importance of the polyadenylation signals in the regulation of 3'-terminal exons, it is difficult to explain a complete switch between two 3'-terminal alternative exons only by a change in the efficiency of the cleavage-polyadenylation efficiency. It is of particular interest to understand how a proximal exon is excluded when a distal exon is selected. Active repression of the proximal 3'-terminal exon, as described for the internal alternative exons, could be a mechanism to ensure a complete skipping of this exon.

In this study, with the aim of investigating the involvement of polyadenylation versus splicing in the selection of 3'-terminal regulated exons, we developed an in vivo splicing model based on the differential processing of the Xenopus a-tropomyosin pre-mRNA. This pre-mRNA possesses three alternative 3'-terminal exons designated exons 9A9', 9B, and 9D, whose differential selection generates three distinct mRNAs (27, 28). In this article, we focus our attention on the regulation of exon 9A9' that is subjected to a complex regulation. Exon 9A9' is skipped in nonmuscle cells, whereas it is used as a terminal or internal exon in somitic and adrenal stripped cells, respectively. For this reason, we designated this exon as a composite intron/3'-terminal exon. Using minigenes whose expression is targeted in embryonic epidermal or somitic cells or oocytes, we show that a genomic fragment encompassing exon 9A9' contains all of the cis sequences necessary for accurate regulation during development. By a mutagenesis approach, we characterize an intronic element lying between the branch site and exon 9A9' involved in the repression of this exon in nonmuscle cells. This element, which is pyrimidine-rich, binds PTB in vitro and contains four high affinity PTB binding sites. We first showed that mutation of these binding sites, which abolishes PTB binding, correlates with the loss of exon 9A9' repression in the oocyte and embryonic epidermal cells. To ascertain a physiological role of XPTB in exon 9A9' repression, we depleted endogenous XPTB in Xenopus embryos using a morpholinobased translation inhibition strategy. XPTB knockdown resulted in a strong inclusion of exon 9A9' in embryonic epidermal cells. This study demonstrates that the composite exon 9A9' is repressed in nonmuscle cells as a 3'-terminal exon by PTB and suggests that PTB could be a major actor in repression of regulated 3'-terminal exon. It also establishes Xenopus oocyte and embryo as valid models to study the tissue-specific regulation of alternative splicing in vivo.

**EXPERIMENTAL PROCEDURES**

**Embryo Culture and Injection**—Xenopus laevis eggs were obtained from laboratory-reared females and fertilized artificially. Embryos were staged according to the table of Nieuwkoop and Faber (29). For DNA injection, 250 pg of supercoiled DNA were injected in one blastomere at the two-cell stage. Injections were carried out in F1 medium (30) containing 5% Ficoll, and the embryos were cultured until Stage 26.

**Oocyte Culture and Injection**—Pieces of X. laevis ovaries were dissected and treated with collagenase, and individual Stage VI oocytes were prepared for microinjection as described in (31). Injection and culture of oocytes were carried out in OR2 medium (32). For nuclear injection, oocytes were previously centrifuged to allow the visualization of the germinal vesicle as described in Ref. 33. For minigene injection, 1 ng of supercoiled DNA was microinjected into the nucleus. For RNA competition experiments, different amounts of in vitro transcribed RNA were microinjected with minigene.

**Plasmid Constructs**—The plasmids pBS-SV40, pBS-actin, and pBS-keratin, which contain the SV40 early promoter, the Xenopus cardiac actin promoter, and the Xenopus keratin promoter, respectively, have been described previously (34). Wild type minigenes were generated by subcloning the BamHI/Sall-digested Xag7–9B fragment from the pGEM-Xag7–9B plasmid into the different expression vectors.

All of the mutated minigenes were produced by primer-directed PCR mutagenesis and cassette substitutions. Two cassettes were used depending on the localization of the mutation. A first cassette localized between Snail and MluI sites present within the 5' region and 3' region, respectively, of intron 7-8 was used to substitute all of the mutations introduced in intron 7-8 upstream of the site. A second cassette localized between the MluI restriction site and a BsaBI restriction site present within the 5' region of intron 9A9'-9B was used to substitute all of the mutations introduced in intron 7-8 downstream of the MluI restriction site or in exon 9A9'. For each mutation, the amplified region was cloned into the pGEM-T vector and sequenced. The mutated fragment was then substituted to the wild type sequence using the SnaB1/MluI or the MluI/BsaBI cassettes.

The region 150PY present within intron 7-8 was PCR-amplified with the following primers: Int7-8 ss2 (5'-TGGCAGAATTCTCAGAACCCT-3') and Int7ssas2 (5'-GTTGCCAACAAAAGAAGGAGG-3'). The resulting fragment was cloned into pGEM-T to generate the plasmid pGEM-150PY. The region 150BS was amplified using the plasmid pBS-AG (Stratagene) as template and the following primers: pBS150as (5'-CCGGCCGCACTATCTCGGTC-3') and pBS150as (5'-GAATCAATGGC-GAATGGGCG-3'). The AG dinucleotides present, within the pBS50 sequence, in positions 30 and 70 were subsequently mutated to TT by primer-directed PCR mutagenesis. The resulting fragment was cloned into pGEM-T to generate the plasmid pGEM-150BS.

For cross-link experiments, the open reading frame of XPTB was amplified by PCR from the hnRNPI cDNA (35) using an upstream primer containing a BamHI restriction site and a downstream primer designed to remove the stop codon and containing a NotI restriction site. The product was cloned as a BamHI/NotI fragment into the plasmid pT7TS-V5. The region 150pBS present within intron 7-8 was amplified using the plasmid pBS(+) (Stratagene) as template and the following primers: pBS150as (5'-CCGGCCGCACTATCTCGGTC-3') and pBS150as (5'-GAATCAATGGC-GAATGGGCG-3'). The PT7TS-V5 vector was constructed by cloning the V5 epitope tag into the BglII/Spel restriction sites of the pT7-TS vector (provided by Paul Krieg, University of Texas).

**Isolation of RNA and RT-PCR Analysis of the Exogenous Transcript**—Total RNA was isolated from oocytes and embryos by Harland and Misher's proteinase K/LiCl method (36). When RNA preparations were used to assay exogenous transcripts, a RQ1 DNase treatment was added in order to remove any residual plasmid DNA template. For RT-PCR, cDNAs were obtained using 10 µg of RNA and 1 µg of an oligo(dT) anchor primer (5'-TTTTTTTTTTTTTTTTCAGTGAGCAC-3') followed by 5 min at 37 °C, followed by 25 µl of 50 mM Tris-HCl, pH 8.3, 75 mM KCl, 3 mM MgCl₂, 10 mM dithiothreitol, and 1 mM dNTPs. Annealing of the oligo(dT) anchor primer was performed for 3 min at 85 °C, followed by 5 min at 37 °C, after which 100 units of Moloney murine leukemia virus reverse transcriptase (Promega) was added, and the reaction was further incubated at 37 °C for 30 min. Samples of 5 µl were then used for the PCR amplification that was performed with 32P-end-labeled sense primers specific of each minigene and an antisense PCR-anchor primer (5'-CAGCCTGTAGCTCTCCGGC-CCAGC-3'). Sense primers were either actin ex7s oligonucleotide (5'-CACAGCCAGGGATCAGTC-3') or keratin ex7s oligonucleotide (5'-GTCGGAGATTCAGCTCCGCAG-3') or sv40ex7s oligonucleotide (5'-CTGGCAAGATCCGCTGCGA-3'). Following an initial denaturation at 94 °C for 3 min, the reaction was carried out for 22 cycles at 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 3 s. Labeled PCR products were analyzed on a 4% non-denaturing polyacrylamide gel. Quantification of radioactive PCR products was performed on a “Storm” Amersham Biosciences PhosphorImager using ImageQuant software. In all cases, at least three independent microinjection experiments were carried out and analyzed. For the analysis of mutated minigenes, the wild type minigene was always microinjected in parallel as a control.

**In Vitro Transcription**—150PY and 150BS competitor RNAs were transcribed from pGEM vectors, linearized at a SalI site, using the T7 transcription kit. The riboprobe was then analyzed on a 4% nondenaturating polyacrylamide gel. Quantification of radioactive probe was performed on a “Storm” Amersham Biosciences PhosphorImager using ImageQuant software. In all cases, at least three independent microinjection experiments were carried out and analyzed. For the analysis of mutated minigenes, the wild type minigene was always microinjected in parallel as a control.

1 The abbreviations used are: RT, reverse transcriptase; PTB, poly(pyrimidine tract-binding protein); XPTB, Xenopus poly(pyrimidine tract-binding protein); hnRNP, heterogeneous nuclear ribonucleoprotein; MO, morpholinol oligomer; PCNA, proliferating cell nuclear antigen; nt, nucleotide(s).
mMessage mMachine T7 kit (Ambion). Recombinant V5-tagged XPTB-capped mRNA was transcribed from pT7TS-V5 vector, linearized at BamHI, with the mMessage mMachine T7 kit (Ambion).

Full-length recombinant V5-tagged XPTB was produced in nuclease-treated rabbit reticulocyte lysate according to instructions (Promega).

Antibodies and Western Blot Analysis—XPTB antibodies were raised in rabbits against two separate synthetic peptides encompassing residues 40–55 (peptide 1, YGSNQNSKDFKPKGDGR) and 438–453 (peptide 2, PREGQEDQQLKDYS) of XPTB by Eurogentec (Seraing, Belgium). XPTB antibodies used in this study were then purified by immunofinity chromatography against the second synthetic peptide. Anti-V5 monoclonal antibody was purchased from Invitrogen. PCNA and β-tubulin monoclonal antibodies were purchased from Sigma.

For Western blot analysis, proteins were loaded onto 10% SDS-PAGE minigels and electrophoresed. Gel-separated proteins were then electroblotted onto nitrocellulose membrane (Schleicher and Schuell, mini-gels and electrophoresed. Gel-separated proteins were then electroblotted onto nitrocellulose membrane (Schleicher and Schuell), followed by a blocking of the membrane in a blocking buffer (25 mM Tris-HCl, 150 mM NaCl, 0.2% Tween 20, 5% nonfat dry milk, pH 8). The membranes were then sequentially incubated in wash buffer and alkaline buffer (100 mM Tris-HCl, pH 9), and proteins were revealed with a nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate kit (Sigma). For XPTB quantification, the protein was detected with the enhanced chemifluorescence technique according to the manufacturer's instructions (Amersham Biosciences) and quantified by PhosphorImager (Amersham Biosciences).

UV Cross-linking and Immunoprecipitation—The 150PY and 150PY mut PTB 1–4 RNAs were synthesized directly from DNA templates amplified by PCR. pGEM-T 150PY was used as the PCR template with the T7150PY primer that comprises the sequence of the T7 promoter (T7150PYwt (5′-AAATTAATACGACTCACTATAGG-3′) and the In7-8 as 2 primer (5′-GGATCCAAAGGGGGGAGGG-3′)). pGEM-T 150PY mut PTB 1–4 was used as the PCR template with the T7150PY primer that comprises the T7 promoter (5′-AAATTAATACGACTCACTATAGGTTGTCCTGCAATGTCTC-3′) and the In7-8 mut PTB as primer (5′-GGCAAGGGGGGAGGGTAGTAGGGGC-3′). 5 pmol of PCR product were used for transcription in a 30-μl reaction mixture containing 20 mM MgCl2, 0.2 mM EDTA, 0.5 mM dithiothreitol, 16% glycerol, 1 mg/ml RNA (3.5 pmol, about 200,000 cpm) was incubated for 15 min at 4 °C. RNA components were digested by the addition of 100 units of RNase A and 100 units of RNasin (Promega); 3 mM each rATP, rCTP, and rGTP; 0.3 mM MgCl2, 100 mM NaCl, 40 mM Tris-HCl, pH 7.9; 10 mM dithiothreitol; 10 μg/ml yeast tRNA. The reaction mixtures were then irradiated for 10 min at 4 °C with a 254-nm UV light at a distance of 10 cm from the source. RNA components were digested by the addition of 100 units of RNase T1 for 1 h at 37 °C. The labeled cross-linked proteins were separated on 10% SDS-PAGE and visualized on a PhosphorImager or by autoradiography.

In vitro translated XPTB was immunoprecipitated using Protein G-Sepharose beads coated with monoclonal anti-V5 antibody (Invitrogen). Endogenous XPTB from germinal vesicle extracts was selected using protein A-Sepharose beads coated with our anti-XPTB serum. Immunoselection was performed at 4 °C in IP buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% Igepal). The beads were subsequently washed three times with IP buffer containing 0.25% Igepal. Immunoselected proteins were eluted with 20 μl of SDS-PAGE loading buffer and fractioned on a 10% SDS-PAGE. Cross-linked proteins were visualized on a PhosphorImager or transferred to nitrocellulose membrane for Western blot analysis.

Morpholino Oligonucleotide Injection and XPTB Rescue—Morpholino oligonucleotides (MO) were obtained from Gene Tools, LLC. The XPTB-MO sequence is 5′-AAACATCTCTCCGACTGACCTAA-3′, and the control MO sequence is 5′-CCCTTATCCCTCTAACTGAGAATTAT-3′. Oligonucleotides were resuspended in distilled H2O to a stock concentration of 10 μg/ml, and 20 ng/blastomere was injected into both blastomeres at the two-cell stage. For XPTB rescue, a V5-tagged XPTB mRNA that had third base modification at the 5′-end to prevent its hybridization with XPTB-MO was prepared. The sequence of the 5′-end modification is 5′-GGGATCCACCATGGAGGGCATAGCTCAAGATATAACGACTCACTATAGG-3′. The start codon and modified nucleotides are underlined. 100 pg of V5-tagged XPTB mRNA was microinjected together with 40 ng of XPTB-MO.

RESULTS

Minigenes Driven by Tissue-specific Promoters Recapitulate the Specific Use of Exon 9A9 in Muscle Tissue and Its Repression in Nonmuscle Tissues.—The differential processing of the 3′-terminal region of the Xenopus α-fast tropomyosin pre-mRNA generates three distinct mRNAs (Fig. 1A). In somites, exon 9A9 is used as a terminal exon with the selection of a polyadenylation signal present within exons 9A9, 9B, and 9D, respectively. A, schematic diagram of the minigene constructs. The boxes represent exons, and horizontal lines represent introns. The boxes represent exons, and horizontal lines represent introns. Positions of the anchor primer and the 5′-end–radiolabeled primers that hybridize at the junction of the promoter and exon 7 are indicated by the arrows. C, the structure and size of all splicing events are given. An asterisk marks the end of the PCR product that is radiolabeled.
present within exon 9B and designated αα (where “α” represents adult) is used. Therefore, exon 9A9α behaves like a composite internal/terminal exon, depending on the myogenesis stage at which the α-tropomyosin gene is expressed. Finally, in nonmuscle cells, the processing reaction skips over exons 9A9α and 9B and splices exon 8 directly to the terminal exon 9D to produce XTM05 RNA (27).

To study the mechanisms involved in the regulation of the composite exon 9A9α during development, the genomic region encompassing exon 7 to exon 9B was cloned by PCR and further analyzed (the sequence and its analysis are available as supplementary Fig. 1). The genomic fragment, designated Xag7–9B, was then cloned downstream of tissue-specific promoters in order to study the differential use of the terminal exons in muscle and nonmuscle tissues during early development. Three promoters were used: the Xenopus cardiac actin promoter that is activated, around Stage 12, specifically in somites and embryonic heart (37); the Xenopus larval keratin promoter that is specifically activated in epidermis (38); and the SV40 early promoter that is functional in Xenopus oocyte (39). The cardiac actin and keratin-driven minigenes were injected into two-cell stage embryos, which were then cultured until tail bud stage (Stage 26). The mRNA was assayed by labeled 3’-rapid amplification of cDNA ends in samples representing equal numbers of whole embryos (Fig. 1B). The size and the composition of each expected PCR product are indicated in Fig. 1C.

The results are presented in Fig. 2A. A major product of 352 nt, containing exon 9A9α (α7-type RNA) was present with the cardiac actin promoter. A faint product of 468 nt, containing exons 9A and 9B (α2-type RNA) was also present. A minor splicing product of 389 nt was also amplified. Cloning and sequencing of the corresponding fragment showed that it corresponds to a maturation product in which exon 8 was spliced directly to exon 9B, and it was designated αα1αA9α. Quantification of the different amplified fragments showed that α7-type RNA represents about 87% of the matured transcripts, whereas the minor products α2-type and αα2αA9α-type RNAs represent altogether <12% of the mature RNA. On the other hand, with the keratin-driven minigene, >70% of the mature transcripts correspond to αα2αA9α-type RNA. In non-muscle tissues, the endogenous α-tropomyosin gene produces RNA containing exon 8 and exon 9D but no αα2αA9α-type RNA. Therefore, our results suggest that in the context of the minigene, in which exon 9D is not present, the specific repression of exon 9A9α in nonmuscle cells still occurs and that exon 8 is spliced to exon 9B as a default choice.

Splicing of the endogenous α-tropomyosin pre-mRNA is also strictly regulated in the oocyte, since only one α-tropomyosin-derived mRNA (XTM05) is present in such cells (27). Therefore, oocytes could be envisioned as an attractive model to study the α-tropomyosin pre-mRNA splicing regulation. Accordingly, we tested the behavior of a minigene in such a context. An SV40-driven minigene was injected in the germline vesicles of Stage VI oocytes, followed by a 6-h incubation. The mRNA was assayed by 3’-rapid amplification of cDNA ends. As described in embryonic epidermal cells, >80% of αα2αA9α-type RNA was produced in oocytes (Fig. 2B). Two bands corresponding to unspliced or partially spliced products primed from a poly(A) stretch present within intron 8-9A were also amplified. Their prevalence varies between experiments (data not shown), which could be related to a variation in the transcription efficiency according to the oocyte batch. The presence of these unspliced or partially spliced products suggests that splicing of 7–9B pre-mRNAs was rate-limiting compared with transcription in oocytes.

Altogether, the results observed in the embryo are in accord-
poorly defined exon whose recognition, in muscle and nonmuscle cells, is modulated by cis acting regulatory sequences present within exons or introns. Since mutations in exon 9A9 have not revealed any exonic enhancer (data not shown), we focused our attention to the intron upstream of exon 9A9.

The Major Branch Site Used in the Splicing of Exon 8 to Exon 9A9 Is Localized 274 nt Upstream of the 3' Splice Site of Exon 9A9—The analysis of the intronic region upstream of exon 9A9 showed many potential branch sites lying in a region between –130 and –274 nt from the 3' splice site (see supplementary Fig. 1). To assign experimentally the major branch site used under the control of the cardiac actin promoter was injected into Xenopus embryos. RNA was then subjected to RT-PCR and analyzed as described in the legend to Fig. 2. Quantification was performed on a PhosphorImager to yield the proportion of each product.

To determine whether sequences lying between the branch site and the 3' splice site of exon 9A9' are important in the regulation of this exon, we constructed three deletion mutants designated Δ1, Δ2, and Δ3 in which 80, 150, and 230 nt, respectively, were removed upstream of the 3' splice site. To avoid any direct effect upon the 3' splice site, the deletions maintained the last 14 nt of the intron upstream of exon 9A (Fig. 4A). In nonmuscle cells, the mutant Δ1 resulted in partial activation of exon 9A9' (Fig. 4B, lane 2). The effect was reinforced with the mutants Δ2 and Δ3 in which 100% of mature RNA contained exon 9A9' (lanes 3 and 4). These results suggest that repressor elements lie in the region deleted in mutant Δ2. To ensure that the effect of the mutation Δ2 was not secondary to a shortening of the intron or to a bringing closer of the branch point and the 3' splice site, the region was replaced by a 150-nt sequence from pBluescribe (150pBS). This substitution resulted in 77% of exon 9A9' usage compared with 100% usage when the element was deleted (lane 5). This result suggests that although the long distance between the branch site and the 3' splice site may contribute to the weakness of the 3' splice site, specific sequences lying between –165 and –15 from the AG border are probably involved in exon 9A9' repression in nonmuscle cells. In muscle cells, in which exon 9A9' is already used very efficiently, the different deletions did not change the splicing pattern (Fig. 4C, lanes 2–4).

The 150-nt silencer element was notable for its pyrimidine-rich composition with four UCUU motifs corresponding to high affinity PTB binding sites (10, 19). Since PTB has been described to repress the splicing of some alternative exons, we sought to determine whether these PTB binding sites designated PTB1–PTB4 (Fig. 4A) are involved in the repression of exon 9A9' and whether PTB binds to the repressor element. For each PTB site, uridines were mutated to cytidines in order to maintain the overall pyrimidine composition. Each mutant was placed under the control of the keratin promoter and analyzed independently and in combination. Individual mutations and double mutations had no effect (data not shown), whereas mutation of all four PTB binding sites present within the 150-nt silencer element was as effective as the 150pBS substitution (Fig. 4D, lane 2; compare with Fig. 4B, lane 5). This suggests that it is not only the overall pyrimidine composition of the 150PY element that is involved in the repression of exon 9A9' but that PTB-specific binding sites are required for the repression of exon 9A9' in epidermal cells.

To analyze whether the Xenopus PTB (XPTB) binds to the 150-nt pyrimidine-rich silencer element, a recombinant XPTB protein tagged with a V5 epitope was synthesized in a nuclease-treated rabbit reticulocyte lysate. XPTB programmed lyase and nonprogrammed lysate were then tested by UV cross-linking using probes corresponding to wild type 150PY RNA or mutated 150PY RNA in which the four PTB binding sites were mutated. After immunoprecipitation of the cross-link products with a V5 antibody, a strong signal migrating at about 60 kDa, the expected mobility for XPTB, was detected in the XPTB protein tagged with a V5 epitope.
the mutated 150PY RNA (lane 3), although equal amounts of the recombinant protein XPTB-V5 had been immunoprecipitated (Fig. 5, bottom panel, compare lanes 1 and 3). Finally, this signal was specific for XPTB-V5 (compare with lanes 2 and 4). Therefore, XPTB can specifically interact with the four high affinity UCUU motifs present within the 150PY element. These data suggest that PTB is a strong candidate for the activity that represses exon 9A9' in embryonic epidermal cells.

**PTB Represses Exon 9A9' Usage in Oocyte through Binding to Four High Affinity PTB Binding Sites**—The Xenopus oocyte offers a unique possibility to study alternative splicing regulation. We therefore wished to study whether repression of exon 9A9' in this nonmuscle cell was controlled in a similar way as in embryonic epidermal cells. For this purpose, the mutants Δ2 and mutPTB 1–4 placed under the control of the SV40 promoter were tested in the oocyte. In agreement with its behavior in embryonic epidermal cells, the mutation Δ2 gave rise to an almost complete derepression of exon 9A9' in oocyte (Fig. 6A, lane 2). Similarly, the mutation mutPTB 1–4 resulted in a strong activation of exon 9A9' usage (lane 3), indicating that mutation of the four high affinity PTB binding sites was also sufficient to derepress exon 9A9' in the oocyte. These results suggested that PTB was involved in the repression of exon 9A9' in the oocyte through binding to the four high affinity binding sites. To confirm this assertion, we tested whether competitor RNA corresponding to the sequence that had been removed in mutant Δ2 150pBS. The four UCUU PTB motifs designated PTB1–PTB4 are framed. B and C, the mutated minigenes comprising the different deletions and under the control of the keratin (B) or cardiac actin (C) promoters were injected into Xenopus embryos. D, a mutant minigene, driven by the keratin promoter, in which the four PTB UCUU motifs were mutated to CCCC, was microinjected into Xenopus embryos. B–D, RNA was then subjected to RT-PCR and analyzed as described in the legend to Fig. 2. Quantification was performed on a PhosphorImager to yield the proportion of each product.

**Fig. 4.** PTB motifs within an intronic silencer element present upstream of exon 9A9' mediates its silencing in embryonic epidermal cells. A, nucleotide sequence of deletion mutants in intron 8–9A. The partial nucleotide sequence of intron 8–9A is presented. The major adenosine branch site is shown in **boldface capital letters**, whereas the putative adenosine branch sites are indicated in **boldface lowercase letters**. The deletions Δ1, Δ2, and Δ3 extended from **vertical mark I** to **vertical marks II, III, and IV**, respectively. They maintained the 14 last nucleotides of intron 8–9A and extended over 80, 150, and 220 nt, respectively. A 150-nt sequence derived from pBS was substituted to the sequence deleted in the mutant Δ2 to produce the mutant Δ2 150pBS. The four UCUU PTB motifs designated PTB1–PTB4 are framed. B and C, the mutated minigenes comprising the different deletions and under the control of the keratin (B) or cardiac actin (C) promoters were injected into Xenopus embryos. D, a mutant minigene, driven by the keratin promoter, in which the four PTB UCUU motifs were mutated to CCCC, was microinjected into Xenopus embryos. B–D, RNA was then subjected to RT-PCR and analyzed as described in the legend to Fig. 2. Quantification was performed on a PhosphorImager to yield the proportion of each product.

**PTB Represses in Vivo a Regulated 3′-Terminal Exon 22171**
to the 150pBS sequence that is unable to restore exon 9A9′ splicing was ineffective in activating exon 9A9′ splicing (Fig. 6B, lanes 5–7).

The efficient binding of the splicing repressor XPTB to the 150PY RNA is consistent with the hypothesis that the 150PY competitor RNA traps XPTB, which is required for exon 9A9′ repression. To assess the ability of XPTB to repress exon 9A9′ splicing in the oocyte, we first studied its expression in this cell. Western blot analysis was performed on oocytes with affinity-purified polyclonal antibodies raised against polypeptides. Stage VI oocyte extracts as well as purified nuclear and cytoplasmic fractions (Fig. 7A, lanes 1–4) were analyzed. The membrane was also probed with PCNA and β-tubulin antibodies to check the purity of the cytoplasmic and nuclear fractions. A protein with an apparent molecular mass of 60 kDa is detected in both cytoplasmic and nuclear fractions (lanes 2–4). This protein is not detected with the preimmune serum (data not shown). We conclude therefore that XPTB is present in the nucleus and cytoplasm of Stage VI oocyte.

To directly test whether XPTB present in oocyte nuclei can bind to the 150PY RNA, UV cross-linking of oocyte nuclei, UV cross-linking of oocyte nuclei was performed with the wild type 150PY RNA and the mutated 150PY RNA. In vitro translated radiolabeled XPTB protein was run in parallel for comparison. A band at ~60 kDa, the expected mobility for XPTB, was present for the wild type RNA (Fig. 7B, lane 3). This band co-migrates with the in vitro translated XPTB (compare lanes 3 and 5). A faint signal was also detected for the mutated RNA (lane 1), suggesting that in the context of a nuclear extract, a low level of XPTB binding to the 150PY RNA occurs outside of the four high affinity binding sites. To confirm that the cross-linked band was XPTB, an immunoprecipitation was then performed. The 60-kDa band was immunoprecipitated specifically by anti-XPTB serum (Fig. 7C, lane 2) but not by preimmune serum (lane 1), confirming that nuclear XPTB can bind to the 150PY repressor element. Our data strongly suggest that PTB represses exon 9A9′ usage in the oocyte through binding to PTB 1–4 high affinity binding sites.

**XPTB Is Required to Repress Exon 9A9′ in Embryonic Epi-**

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**FIG. 5.** The silencer element binds PTB through the four UCUU motifs. Wild type 150PY RNA (WT) and 150PY RNA mutated at the four high affinity PTB binding sites (M) were incubated in rabbit reticulocyte lysates that had been programmed with XPTB-V5 mRNA (Lys-XPTB) or not (Lys-O). Samples were UV-irradiated and treated with RNase T1, and cross-linking products were then immunoprecipitated with anti-V5 antibodies. The immunoprecipitated proteins were resolved onto a 10% polyacrylamide gel in the presence of SDS and visualized by autoradiography (top panel) or Western blotting with anti-V5 antibodies (bottom panel). The 60-kDa cross-linked XPTB protein is indicated by the arrow. The protein size markers are indicated to the right.

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**FIG. 6.** Derepression of exon 9A9′ by 150PY RNA competitors. A, the Δ2 and mutPTB 1–4 mutants driven by the SV40 promoter were microinjected into the oocyte nucleus. B, the wild type minigene was injected, alone or with increasing amounts (13, 65, and 325 fmol) of in vitro transcribed 150PY and 150pBS RNAs, into the oocyte nucleus. A and B, RNA was subjected to RT-PCR and analyzed as described in the legend to Fig. 2. Quantification was performed on a PhosphorImager to yield the proportion of each product.

dermal Cells—Morpholino-based translational inhibition is a powerful tool to specifically knock down the level of an endogenous protein during *Xenopus* development (40, 41). An MO directed against the 5′ region of XPTB mRNA or a control nonspecific morpholino oligomer was microinjected into both blastomeres of *Xenopus* embryos at the two-cell stage. Microinjection of embryos with the XPTB-MO but not with the nonspecific MO resulted in a strong inhibition of the translation of XPTB. Indeed, 100% of Stage 26 embryos (n = 30) injected with MO-XPTB showed a 7-fold reduction of endogenous XPTB as assayed by Western blot quantification (Fig. 8A, lanes 5–9; compare with lanes 1 and 2). To further address the effect of XPTB depletion on the inclusion of exon 9A9′ in nonmuscle cells, XPTB-MO were microinjected along with the minigene encompassing exons 7–9B and driven by the *Xenopus* keratin promoter that targets expression to epidermis. Embryos were fixed at Stage 26, and extracted RNA and proteins were analyzed by RT-PCR analysis and Western blot, respectively. As previously described, a strong skipping of exon 9A9′ was observed in the morpholino-uninjected control embryos (Fig. 8B, lane 1). XPTB-MO-mediated knockdown of XPTB resulted in an almost complete inclusion of exon 9A9′ (lanes 3 and 4), whereas the MO control did not change the processing pattern (lane 2). To confirm that the XPTB-MO-induced phenotype was generated by a specific knockdown of XPTB, in vitro transcribed V5-tagged XPTB...
mRNAs that have a third-base modification in the sequence spanning the translation start site were injected together with XPTB-MO. Rescued expression of V5-tagged XPTB in endogenous XPTB-depleted embryos restored exon 9A9 skipping (lanes 5 and 6), demonstrating that XPTB can directly repress exon 9A9. These results demonstrate that endogenous XPTB is required in vivo to repress exon 9A9 in embryonic epidermal cells in the context of a minigene construct.

DISCUSSION

In the present study, we have analyzed the differential usage of the composite internal/terminal exon 9A9/H11032 of the Xenopus o-fast tropomyosin pre-mRNA. This exon was chosen because it appears to be a good model to study the contribution of splicing and polyadenylation in the regulation of 3'-terminal exons.

To study the mechanisms involved in the regulation of exon 9A9', we first cloned the genomic region encompassing exons 7-9B and constructed minigenes driven by tissue-specific promoters. Our results show that the pre-mRNAs derived from these minigenes are spliced according to the tissue-specific promoters. In muscle cells, exon 9A9' is used as a terminal exon to produce 9A7-type RNA, whereas it is skipped in nonmuscle cells to produce α2Δ9A-type RNA. Although no endogenous isoform corresponding to a direct splicing of exon 8 to exon 9B was reported in Xenopus, the generation of α2Δ9A-type RNA from our minigenes indicates that there is no intrinsic blockage of the splicing of both exons. Indeed, in rat, a brain-specific isoform designated TMBr2 is synthesized by direct splicing of exon 8 to exon 9B, resulting in the inclusion of a specific 30-amino acid carboxyl sequence (42). This suggests that re-
pression of exon 9A in nonmuscle cells can be controlled independently of exon 9B selection.

As already described for several mutually exclusive exons, the 3’ splice region of exon 9A9’ is characterized by a distant branch site. Thus, five potential branch sites are present 149–274 nt upstream of the 3’ splice site of exon 9A9’. Although, we have not experimentally mapped the branch point by an in vitro assay, the dramatic effect of the mutations to the 274 nt branch point or to the polypyrimidine-associated sequence strongly suggests that 274 is the major branch point used in embryonic muscle cells. Whereas a mutual attenuation is often inappropriate to determine a branch point in vitro because of the activation of cryptic sites, this result suggests that the usage of the −274 nt branch point could be highly regulated in vivo. Indeed, the increase in o2 RNA production with the −274 mutation indicates that branch sites other than −274 are functional. Our current explanation is that the other functional branch sites are unavailable in embryonic muscle cells. In this respect, it is interesting to note that we have identified an enhancer element necessary for exon 9A9’ usage in embryonic muscle cells that spans the −196, −158, and −149 potential branch sites.2

Whereas the long distance between the branch site and the 3’ splice site is a prerequisite for exon 9A9’ repression in nonmuscle cells, splicing also could be activated by the removal of specific sequences between the branch site and the 3’ splice site. Competitor RNAs corresponding to these specific sequences activated splicing in the oocyte, which suggests that they bind and sequester factors that normally repress exon 9A9’. The intronic sequence and the corresponding competitor RNA contain four high affinity PTB binding sites. UV crosslinking experiments and immunoprecipitation demonstrated that PTB binds to the competitor RNAs. Involvement of PTB as a repressor was strengthened by the observation that specific mutation of the four high affinity PTB binding sites that strongly reduced PTB binding also reduced exon 9A9’ repression in epidermal cells and the oocyte. To give definitive evidence for XPTB involvement in exon 9A9’ repression we specifically depleted XPTB in embryos using a morpholino-based translation inhibition strategy. As expected, the knockdown of XPTB caused a strong derepression of exon 9A9’ in embryonic epidermal cells. These data demonstrate not only that XPTB is required for exon 9A9’ exclusion in nonmuscle cells but that this exclusion is based mainly or exclusively on XPTB-mediated repression. Importantly, knockdown of XPTB resulted in a stronger increase of exon 9A9’ inclusion than the mutation of the four high affinity PTB binding sites, suggesting that additional PTB binding sites are involved in exon 9A9’ repression.

PTB has been implicated in the repression of alternative exons of many genes including α-fast troponymosin, α-actinin, β-tropomyosin, and c-src (9, 14, 15, 17). In most cases, PTB seems to reinforce the default splicing pattern through the repression of the tissue-specific exon inclusion. Several nonexclusive models have been proposed for PTB action. In some cases, PTB is able to compete the binding of U2AF65 to the polypyrimidine tract (43, 44). Interestingly, an additional high affinity binding site is present within the polypyrimidine tract associated with the −274 branch point. However, it is difficult to test in vivo the relevance of this site by a mutational approach, since its destruction weakens the polypyrimidine tract and therefore prevents exon 9A9’ splicing.2 Numerous exons repressed by PTB also have PTB binding sites upstream and downstream of the exon. Given that PTB can multimerize, it was proposed that PTB can interact across the exon and sequester it (45). Such a model could also be proposed for the repression of exon 9A9’, since in addition to the four high affinity binding sites characterized in this study and present upstream of exon 9A9’, two UCUU motifs are present downstream of exon 9A9’. Interestingly, one of these downstream sites comprises the uridine-rich element of the o2 poly(A) site, and its mutation abolished o2 RNA production in somatic cells.3 This feature does not allow testing by a mutational approach to know whether this downstream site is implicated in the repression of exon 9A9’, but it suggests a new model of regulation in which binding of PTB to the UCUU motif present within the uridine-rich element region could preclude CstF-64 binding. This observation raises the possibility that PTB in addition to repressing splicing could also repress polyadenylation. This activity could be the basis for a more general role of XPTB in repression of regulated proximal 3’-terminal exon. Finally, it was also shown that PTB binding sites can overlap with enhancer elements. In this case, PTB can compete the binding of activator factors, preventing the formation of an activator complex (46, 47). In accord with this model, we identified a complex intronic enhancer element that overlaps with the repressor region.2 We therefore predict a model where the different interspersed binding sites for PTB are involved in the formation of a strong complex repressor in which the branch site −274 and the o2 poly(A) site are inaccessible to the splicing and polyadenylation machineries. In this context, the four high affinity binding sites present within the repressor region and overlapping with the activator sequences could serve as a core element for the binding of PTB that could then bind synergistically to the additional motifs through multimerization leading to a complete sequestration of exon 9A9’.

In contrast to this general repressor function, PTB has also been implicated in the activation of 3’-terminal exons. In vitro experiments demonstrated that PTB could activate the poly(A) signal of the C2 complement gene by binding to a U-rich sequence (designated USE) immediately upstream to the hexanucleotide AAUAAA (48). The mechanical basis of such a stimulatory effect is unknown and remains to be established. In vivo, PTB has also been shown to activate the proximal terminal exon 4 of the calatonin/calcium gene-related protein pre-mRNA by binding to a downstream intronic enhancer (49). It was proposed that this effect was mediated by interfering with the poly(A) binding that is inhibitory for the enhancer activity. Surprisingly, it was not studied whether this effect was partially mediated by exon 5 silencing. Indeed, two PTB high affinity motifs are present within the polypyrimidine tract upstream of exon 5.

Our results establish the Xenopus embryo and oocyte as an attractive model to study alternative splicing regulation in vivo. Our data provide further insights into PTB function as a splicing repressor and support a model in which PTB could prevent the definition of a regulated proximal 3’-terminal exon. The mechanistic explanation of how PTB inhibits such a definition is under investigation using our model system. It should provide new insights into the relationship between polyadenylation and splicing.

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