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Transcription and Splicing Patterns of M- and O-Type *P* Elements in *Drosophila bifasciata, D. helvetica,* and *Scaptomyza pallida*

Elisabeth Haring, Sylvia Hagemann, Wilhelm Pinsker

Institut für Medizinische Biologie, AG Allgemeine Genetik, Universität Wien, Währingerstr. 17, A-1090 Vienna, Austria

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Abstract. RT-PCR was applied to analyze the splicing patterns of *P*-element-derived mRNAs in *Drosophila bifasciata, D. helvetica,* and *Scaptomyza pallida. D. melanogaster* was used as a control. The experiments revealed that *P* elements are transcribed in all species investigated. However, there are differences in the splicing patterns of IVS3, which has to be removed in order to produce transposase mRNA instead of repressor mRNA. These differences are observed among species as well as between the *P* element subfamilies, the M and the O type, which coexist in the genomes of *D. bifasciata* and *S. pallida.* In *D. helvetica* M-type transposase mRNA was found in the germline and repressor mRNA in the soma, as has been previously described for the canonical (M-type-related) *P* element of *D. melanogaster*. In contrast, in *S. pallida* only repressor mRNA of M-type elements was detected in all tissues. In *D. bifasciata,* M-type IVS3, although activated both in the soma and the germline, is never completely excised. Instead, two alternative double-spliced variants occur in which two small introns are removed within the IVS3 region. One of these variants codes for a protein 12 aa longer than the regular transposase. Taking these findings together, transposase production and transpositional activity of M-type elements seem to be limited to *D. helvetica* and *D. melanogaster,* whereas M-type elements have become immobile in *D. bifasciata* and *S. pallida.* Unlike the M type, the splicing of O-type transcripts in *D. bifasciata* and *S. pallida* follows the classical rules of tissue-specific *P* element regulation: transposase mRNA is produced exclusively in the germline whereas repressor mRNA is formed in somatic cells. Thus O-type elements are thought to be still transpositionally active in both species. This finding is in accordance with the postulated recent transfer of O-type elements between the gene pools of *D. bifasciata* and *S. pallida.* In addition, we were able to show that the IVS3 double-spliced variants of both *P* element types are produced regularly in all species of the genus *Drosophila* investigated so far, but not in *S. pallida.*

Key words: *Drosophila* — *Scaptomyza* — *P* element — RT-PCR — Expression — Splicing pattern

Introduction

The evolutionary history of *P* elements, a family of DNA transposons widely distributed in the genus *Drosophila,* has been intensively studied over the last two decades (for reviews see: Engels 1992; Kidwell 1994). Two main features of their evolutionary behavior have become apparent: rapid propagation in newly infected gene pools (Kidwell 1983; Anxolabéhère et al. 1988) and the occasional horizontal transmission between genetically isolated species (Daniels et al. 1990; Hagemann et al. 1994, 1996a; Clark et al. 1994). Analyzing *P* element sequences isolated from various members of the *willistoni* and *saltans* species group, Clark et al. (1995) were able to distinguish two different classes of elements. The canonical elements which closely resemble the *P* element

Abbreviations: aa, amino acids; IVS, intervening sequence; nt, nucleotides; RT-PCR, reverse transcription–polymerase chain reaction

Correspondence to: W. Pinsker; *e-mail:* a5171dae@vm.univie.ac.at

of *D. melanogaster* show only a low degree of sequence divergence and are thought to be active transposons and relatively recent additions to the respective genomes. In contrast, the noncanonical *P* elements show considerably more sequence variation. They may represent ancestral elements having been transmitted vertically in the respective lineages for rather long time periods.

In the *obscura* species group the diversification of *P* element sequences appears more pronounced. Three distinct subfamilies differing by about 30% nt divergence from each other have been described so far:

1. T type: *P* elements of this subfamily have been isolated from *D. ambigua*, *D. obscura*, and *D. tristis* (Hagemann et al. 1996b). According to sequence similarities the terminally truncated *P* homologues from *D. subobscura* (Paricio et al. 1991), *D. guanche* (Miller et al. 1992), and *D. madeirensis* (Paricio et al. 1996) have to be considered as immobilized derivatives of T-type transposons. Thus the T type may have been present at the basis of the lineage leading to the Palearctic species of the *obscura* group.
2. O type: Although *D. bifasciata* and *D. imaii* belong to the Palearctic *obscura* clade, T-type elements are missing in these species. Instead, their genomes contain members of two other subfamilies, the O type and the M type. *P* elements of the O-type subfamily seem to be transpositionally active (Haring et al. 1995). The high sequence similarity (>99%) to O-type elements of *Scaptomyza pallida* indicates that the O-type elements have entered the *D. bifasciata/D. imaii* lineage recently.
3. M type: Among the three subfamilies the M type is the closest relative of the canonical *P* element of *D. melanogaster* (O'Hare and Rubin 1983) and the *P* elements described so far in species of the *willistoni* and *saltans* group (Lansman et al. 1987; Daniels et al. 1990; Clark et al. 1995). In the *obscura* group M-type elements are found in three species: *D. bifasciata* (Hagemann et al. 1992), *D. imaii* (Haring et al. 1995), and *D. helvetica* (Haring 1997). At least in *D. bifasciata* and *D. imaii* M-type elements are thought to be transpositionally inactive (Haring et al. 1995), although one complete element with intact reading frames has been isolated (Hagemann et al. 1992). Sequence similarity to the M-type elements of *S. pallida* (Simonelig and Anxolabéhère 1991) is rather high (90%). It is assumed that the M-type elements of *D. bifasciata/D. imaii* have been acquired by horizontal transfer from the genus *Scaptomyza*. This invasion probably predates the more recent invasion of O-type elements (Hagemann et al. 1996a). The potential origin of M-type elements in *D. helvetica*, a species which has split off at the basis of the lineage leading to the other Palearctic *obscura* species (Lakovaara et al. 1976), is discussed elsewhere (Haring 1997).

In spite of the 30% divergence at the sequence level, the basic molecular structure of full-sized elements is the same in all three types. The termini are formed by inverted repeats and the coding region comprises four exons (numbered from 0 to 3). In *D. melanogaster* two different proteins can be produced from the primary transcript. The transposase, coded by exons 0–3, is made only in germline cells. In somatic tissue the third intron (IVS3) is not removed and therefore the mature mRNA codes for a shorter protein, coded by exons 0–2, which acts as a repressor of transposition (Rio et al. 1986; Rio 1990). The mechanism of differential splicing restricts transpositional activity to the germline, thus minimizing deleterious effects on the host organism by insertional mutations (Laski et al. 1986; Laski and Rubin 1989). Somatic inhibition of IVS3 splicing involves the assembly of a multiprotein complex on a regulatory sequence in the IVS3 5′ exon (Siebel and Rio 1990; Siebel et al. 1992). One of these host-encoded proteins is the *P* element somatic inhibitor (PSI) that acts as a tissue-specific regulator of IVS3 splicing in vivo and is required for inhibition of IVS3 splicing in vitro (Siebel et al. 1994, 1995; Adams et al. 1997).

Up to now, investigations of expression and regulation have been exclusively carried out with the *D. melanogaster P* element. In the present study we analyze the expression patterns of M- and O-type elements in different species at the mRNA level. In *D. bifasciata* and *S. pallida* full-sized *P* elements of two distinct subfamilies coexist in the same genome (Hagemann et al. 1994, 1996a). It is not known, however, if both types of elements are transcribed and if the mature mRNA has the coding capacity for functional proteins (repressor and transposase). Furthermore, we wanted to see whether the tissue-specific splicing pattern described for the *D. melanogaster P* element is the same in all species. Finally, it was our aim to elucidate the differences between transpositionally active (O type?) and inactive (M type?) subfamilies and how their mobility is regulated. For this purpose we used RT-PCR to investigate *P*-element-derived mRNAs in *D. bifasciata*, *S. pallida*, *D. helvetica*, and, as a control, *D. melanogaster*.

Materials and Methods

Fly Stocks. D. melanogaster (Harwich, USA; standard P-strain), *D. bifasciata* (Pavia, Italy), *D. helvetica* (Zürich, Switzerland), *S. pallida* (laboratory strain obtained from P. Capy, Gif-sur-Yvette, France).

Amplification of mRNA Sequences (RT-PCR). The lysate mRNA capture kit (Amersham) was used for specific amplification of *P* element mRNAs according to manufacturer's instructions with the following modifications: Preparations of heads (from five flies), testes (20 flies), or ovaries (15 flies) were homogenized in 100-μl lysis solution. The mRNA was bound selectively to poly-T membranes according to manufacturer's instructions. Elution of mRNA was done in 20-μl reaction buffer at 90°C for 3 min. Reverse transcription was carried out

Table 1. List of PCR primers used in this study[a]

Primer	Sequence	Position	Reference sequence
Me0+	CCAGGCAAATAAAAGAAGGCGA	337–358	a
Me3.1−	CGATGTCTGCAGGTATGTTGTT	2332–2311	a
Me2+	GCCGTCTTAACCAAGACATAGT	1851–1872	a
Me3.2−	TCGTCATCTTCAGGCTCAACAT	2276–2297	a
Mil+	TACGCAAACAGAGTGCTGTT	436–447, 512–519	a
Mi2+	GTGTATCAGAATCAAAAACCTG	1168–1174, 1242–1251	a
Mi3−	TTCTGTATTCTTGGCTATTAAA	1960–1967, 2159–2172	a
Mi3b−	TCTGTATTCTTGGATTCTCG	2062–2068, 2159–2171	a
Mi3b′−	CTGTATTCTTGGCTACATTCTC	2063–2072, 2159–2170	a
Oe0+	GAAAGAGGATGAAAGTGTTA	400–419	b
Oe3.1−	TAAATCCAGATTGCGTTGAT	2463–2482	b
Oe3.2−	CATAGCGTCCTCCGTTAGTT	2397–2416	b
Oi1+	TACGGAAACTGAGGACACAG	443–454, 543–550	b
Oi2+	TATCTGAAGAAAAAACCTGG	1212–1222, 1289–1299	b
Oi3−	CTCTGTATTCTTGGCTACAAT	2008–2212	b
Oi3b−	CTGTATTCTTGGATTCACAA	2112–2119, 2199–2210	b
Oi3b′−	CTGTATTCTTGGCAACATTCAC	2114–2123, 2199–2210	b
mel-i1+	TACACAAACAGAGTCCTGTT	431–442, 501–508	c
mel-i2+	= Mi2−	1157–1168, 1222–1231	c
mel-i3−	CATTTCTGTATTCCTGGCTATT	1943–1947, 2138–2154	c
mel-e3−	TGTCCTCTTCGATATTTCCC	2166–2185	c
mel-i3b−	CTGTATTCCTGGATTCTCAA	2041–2048, 2138–2149	c
mel-i3b′−	= Mi3b′−	2043–2052, 2138–2149	c

[a] Primer sequences are given left to right from 5′ to 3′ ends. Primer designation: M, O, mel stand for the respective *P* element type; the letters e and i denote the binding region (e = primer binds within exon, i = intron-spanning primer); the number refers to the exon or intron number; + and − indicate the orientation with respect to the *P* element. Positions refer to the reference sequences: a = *IbifM*-3 (M type of *D. bifasciata*), b = *IbifO*-1 (O type of *D. bifasciata*), c = pπ25.1 (*P* element of *D. melanogaster*)

according to the manufacturer's protocol using 20-μl mRNA solution for each reaction. Reverse transcriptase was then inactivated by phenol extraction followed by chloroform extraction. mRNA was precipitated with 1/10 vol 3 M NaAc and 2 vol EtOH (100%).

PCR was done in a volume of 100 μl with 2 units Dynazyme DNA polymerase in polymerase buffer (Finnzymes OY), 0.5 μM of each primer, and 0.2 mM of each dNTP. The solutions were heated to 95°C for 5 min and then put through 30 reaction cycles: 95°C for 30 s, primer-specific annealing temperature for 30 s, 72°C for 2.5 min, followed by a final extension at 72°C for 10 min. Annealing temperature: T_m − 4°C; if the T_m values of the two primers differed, the annealing temperature was set as T_m − 2°C of the primer with the lower T_m. The primers used in this study are listed in Table 1.

Elution and Cloning. PCR products were separated on 1% agarose gels. After elution by means of the Sephaglas BandPrep Kit (Pharmacia) fragments were cloned into the pT7Blue T-Vector (Novagen). Sequencing was accomplished by the dideoxy chain termination technique using the Sequenase ImagesTM Non-Isotopic DNA Sequencing System (USB). Nomenclature of clones follows the designation system introduced by Hagemann et al. (1996b). The first letter stands for the geographic origin followed by an abbreviation of the species name, the subfamily type, and the clone number. The letter c signifies that the clone is derived from cDNA.

Sequences. Positional information for the various *P* element types refers to the following reference sequences: M type, *D. bifasciata: IbifM*-3 (Hagemann et al. 1992; GenBank accession number X60990), O type, *D. bifasciata: IbifO*-1 (Hagemann et al. 1994; X71634), *D. melanogaster*: pπ25.1 (O'Hare and Rubin 1983; X06779). The clone *PS18* was used as a representative of M-type elements of *S. pallida* (Simonelig and Anxolabéhère 1991; M63342).

Results

Experimental Strategy

The aim of the present study was to investigate whether and how the two *P* element types (M and O) are expressed in *D. bifasciata* and *S. pallida.* We especially wanted to know if different types of mature mRNAs are produced through tissue-specific splicing, as is the case in *D. melanogaster.* In addition, we tested the expression of recently discovered M-type elements in *D. helvetica* (Haring 1997), which is, like *D. bifasciata*, a member of the *obscura* group. For this purpose RT-PCR (reverse transcription followed by PCR amplification) was carried out with RNA samples prepared from both somatic and germline tissues. According to the splicing pattern found in *D. melanogaster*, transposase mRNA (IVS3 excised) was expected to occur in ovaries and testes only. As the gonads are composed of both germline and somatic cells, repressor mRNA (containing IVS3) also was expected in ovaries and testes. On the other hand, somatic cell lysates obtained from heads should contain repressor mRNA only.

Figure 1 depicts the primers applied and their respective binding sites within the M- and O-type *P* elements of *D. bifasciata* (*IbifM*-3, *IbifO*-1) and *D. melanogaster* (pπ25.1), respectively. Reverse transcription was carried

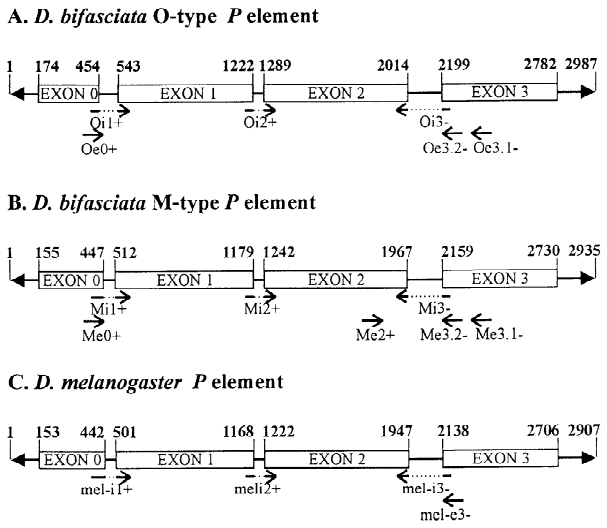

Fig. 1. Primer binding sites in the different *P* elements: **A** *D. bifasciata* O type (*IbifO*-1), **B** *D. bifasciata* M type (*IbifM*-3), and **C** *D. melanogaster* (*pπ25.1*).

out with an antisense primer binding within exon 3. Then, in a first round of PCR amplification, this exon-3–specific primer was combined with a primer binding in exon 0, generating a PCR product which comprises all three splicing junctions (of IVS1, IVS2, IVS3) known from the *D. melanogaster P* element. Since the transcription rate of *P* elements is rather low, another round of PCR amplification with internal primers was necessary to obtain enough product to be visualized in agarose gels. These reamplifications were accomplished with intron-spanning primers on the 5′ side (spanning either IVS1 or IVS2) combined with primers binding to the interior section of exon 3 at the 3′ side. Since intron-spanning primers are complementary to the 5′ and 3′ adjacent sequences of the respective intron, they bind to processed mRNA only but not to contaminating genomic DNA or pre-mRNA. The PCR products obtained in these reamplifications include the entire IVS3 region, allowing one to differentiate between alternative splicing variants. In addition, IVS3-spanning primers (Oi3− and Mi3−, respectively) were designed for the specific detection of transposase mRNA and for control experiments with PCR-derived clones. Tests with genomic DNA revealed that none of the intron-spanning primers bind nonspecifically to DNA (data not shown).

O-Type Transcription in D. bifasciata

RNA samples from heads, ovaries, and testes were prepared. After reverse transcription with the primer Oe3.1−, the first PCR amplification was carried out with the primer pair Oe0+/Oe3.1−. Reamplification was done with several primer sets. Table 2 shows the PCR products obtained after reamplification. Fragments of the size expected from repressor mRNA were found in all tissues. The band derived from repressor mRNA was more intensive in ovaries than in testes, where it was even found to be absent in some PCR experiments. This might be due to the higher proportion of somatic tissue in ovaries compared to the testes. On the other hand, the fragments corresponding to transposase mRNA were obtained only from ovaries and testes but never from heads. With primer Oi1+ additional shorter fragments were produced sporadically. These fragments probably stemmed from transcripts of internally deleted O-type elements. In order to confirm that the supposed transposase-derived fragments of 1,636 bp (Oi1+ / Oe3.2−) and 955 bp (Oi2+ / Oe3.2−) are not amplification products from deleted elements, the specific IVS3-spanning primer Oi3− was employed. With this primer, products can be obtained only from mRNA lacking IVS3. As with primer Oe3.2−, fragments matching the sizes expected for transposase mRNA were exclusively obtained from ovaries and testes, whereas no products were detected in RNA samples from heads.

Table 2. PCR products from O-type repressor and transposase mRNAs[a]

Primer	Fragment size expected (bp)	Fragments obtained (bp)		
		Heads	Ovaries	Testes
D. bifasciata				
Oi1+ / Oe3.2−	R: 1820	Yes	Yes	Yes
	T: 1636	—	Yes	Yes
	D: —	1,200	950, 1,200	950, 1,200
Oi2+ / Oe3.2−	R: 1137	Yes	Yes	Yes
	T: 955	—	Yes	Yes
Oi1+ / Oi3−	R: —	—	—	—
	T: 1432	—	Yes	Yes
	D: —	—	800	550, 800
Oi2+ / Oi3−	R: —	—	—	—
	T: 749	—	Yes	Yes
S. pallida				
Oi1+ / Oe3.2−	R: 1820	Yes	Yes	Yes
	T: 1636	—	Yes	Yes
Oi2+ / Oe3.2−	R: 1137	Yes	Yes	Yes
	T: 955	—	Yes	Yes
Oi1+ / Oi3−	R: —	—	—	—
	T: 1432	—	Yes	Yes
Oi2+ / Oi3−	R: —	—	—	—
	T: 749	—	Yes	Yes

[a] ''Yes'' denotes that the expected fragment was observed. R = repressor mRNA, T = transposase mRNA, D = fragments derived from transcribed elements with internal deletions

All fragments from presumptive transposase and repressor mRNAs listed in Table 2 were cloned and analyzed by PCR experiments. Sequence analyses were carried out with three cDNA clones derived from reamplification with primers Oi1+ / Oe3.2−. These clones contain the complete IVS3 region. Two clones represent repressor mRNA (*IbifcO*-9, *IbifcO*-31) and one clone transposase mRNA (*IbifcO*-28). Analysis of the splicing sites of IVS2 and IVS3 (the splice site of IVS1 is covered by primer Oi1+) revealed that the three splicing junctions are located at positions homologous to

Table 3. PCR products from M-type repressor and transposase mRNAs[a]

Primer	Fragment size expected (bp)	Fragments obtained (bp)		
		Heads	Ovaries	Testes
D. bifasciata				
Mi1+ / Me3.2−	R: 1,736	Yes	Yes	Yes
	T: 1,545	>1,545	>1,545	>1,545
Mi2+ / Me3.2−	R: 1,068	Yes	Yes	Yes
	T: 877	>877	>877	>877
Mi1+ / Mi3−	R: —	—	—	—
	T: 1,420	—	—	—
Mi2+ / Mi3−	R: —	—	—	—
	T: 752	—	—	—
S. pallida				
Mi1+ / Me3.2−	R: 1,721–1,766	Yes	Yes	Yes
	T: 1,545–1,590	—	—	—
Mi2+ / Me3.2−	R: 1,053–1,098	Yes	Yes	Yes
	T: 877–922	—	—	—
Mi1+ / Mi3−	R: —	—	—	—
	T: 1,420	—	—	—
Mi2+ / Mi3−	R: —	—	—	—
	T: 752	—	—	—

[a] ''Yes'' denotes that the expected fragment was observed. In *PS18* of *S. pallida* primer Me3.2− binds to a tandem repetitive region with up to five repeats of a 15-bp unit. Therefore different fragments could be generated from a single type of mRNA. The size range of the expected fragments is indicated. R = repressor mRNA, T = transposase mRNA

those described for the *D. melanogaster P* element (Fig. 1A). Moreover, the tissue-specific differential splicing of O-type transcripts also resembles the processes in *D. melanogaster*. IVS3 splicing occurs exclusively in the germline.

M-Type Transcription in D. bifasciata

The same RNA samples (from heads, ovaries, and testes) that had been analyzed with the O-type primers were employed for investigation of M-type mRNAs. Primer Me3.1− was used for reverse transcription; the first PCR amplification was done with the primer pair Me0+ / Me3.1−. The PCR products obtained after reamplification with different primer pairs are listed in Table 3. With the primer pair Mi1+ / Me3.2− the fragment sizes expected from repressor and transposase mRNAs are 1,736 bp and 1,545 bp, respectively. With primer pair Mi2+ / Me3.2− the corresponding fragments are 1,068 bp and 877 bp. At first sight the sizes of the PCR products in both experiments matched the expectations (Fig. 2). Yet, surprisingly, both presumed mRNAs, repressor and transposase, were found in somatic and germline tissue as well. In the specific tests for detection of transposase mRNA using the IVS3-spanning primer Mi3− for reamplification, no products were obtained. This finding indicates that in *D. bifasciata* IVS3 of M-type transcripts is not spliced, at least not in the way described for transposase mRNA of *D. melanogaster*.

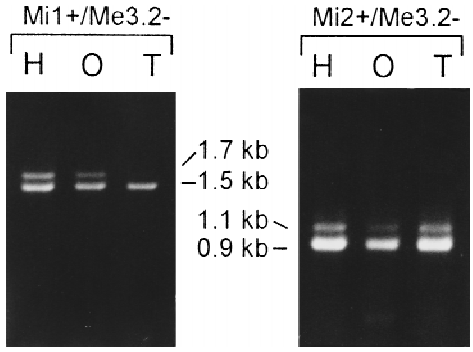

Fig. 2. RT-PCR fragments obtained from heads (*H*), ovaries (*O*), and testes (*T*) of *D. bifasciata* using two different M-type specific primer pairs for reamplification. Fragments approximately match the sizes expected for repressor (1.7 and 1.1 kb) and transposase (1.5 and 0.9 kb) mRNAs.

Both fragments obtained in the reamplification with Mi1+ / Me3.2− were cloned and analyzed by PCR experiments. Two clones were selected for sequence analysis: *IbifcM*-9 (larger fragment, obtained from heads) and *IbifcM*-15 (shorter fragment, obtained from testes). *IbifcM*-9 was sequenced completely. It contains IVS3, whereas IVS2 is spliced as expected (splicing junctions of IVS1 are covered by the intron-spanning primer Mi1+). Thus the 1,736-bp fragment comes from a repressor mRNA with excised IVS1 and IVS2.

For *IbifcM*-15 only the IVS3 region was analyzed (PCR control with primer Mi2+ had shown that IVS2 was excised as expected). The sequence revealed a novel splicing pattern: A double splice that leads to the removal of two introns within the boundaries of IVS3, leaving behind a rather short exon of 36 bp. Activation of splicing occurs at the expected 5′ splice site of IVS3 at pos. 1968, but instead of the expected 3′ splice site at pos. 2158 another junction is used, leading to the removal of a 69-bp intron. Activation of another donor site (37 bp downstream) in combination with an acceptor site homologous to that of IVS3 leads to the removal of a 86-bp intron.

The reading frame of this novel type of mRNA is intact and codes for a protein 12 aa longer than the predicted transposase. A similar mRNA was found in *D. melanogaster* (Chain et al. 1991). However, in that case the second intron within the IVS3 region had a different 5′ site. Splicing at this position leads to a frame shift and thus to a truncated protein. Chain et al. (1991) assumed that this protein may serve as a repressor contributing to the P cytotype in the germline of *D. melanogaster*. The 5′ site of this second intron is located 4 bp upstream with respect to that found in *D. bifasciata*. Although it seemed that the two different modes of double splicing occur in a species-specific manner, the sequence analysis revealed that the two alternative splice sites are present in both species. To find out whether the *D. melanogaster* splicing variant is also made in *D. bifasciata*, a new series of RT-PCR experiments from total fly homog-

Fig. 3. Alignment of the IVS3 region in the *P* elements of *D. melanogaster* (*mel*), *S. pallida* (*palM*), *D. helvetica* (*hel*), *D. bifasciata* M type (*bifM*) and O type (*bifO*). The alternative splicing sites 3a, 3b, and 3b′ are conserved in all sequences. Splice junctions (*GT, AG*) are *boxed.* The most likely branch points (within the section −21 to −38 from the 3′ splice junction) are marked by *solid boxes;* an alternative branch point (closer to the 3′ splice junction) is indicated by a *dotted box.* The stop codons in the respective exon 3a sequences of *bifO* and *hel* are *double underlined.*

enates was performed. Reamplification was done with the primer pair Me2+ / Me3.2− which flanks the IVS3 region. The three clones sequenced (*IbifcM*-1, *IbifcM*-2, *IbifcM*-6) proved to contain the splicing variant found in *D. melanogaster*. Thus, within *D. bifasciata* three different M-type mRNAs are made: beside the repressor mRNA, two double-spliced variants are produced, one of them retaining an intact reading frame. The classical transposase mRNA with complete removal of IVS3 is not found.

With respect to the established designation of the *D. melanogaster* introns and exons, we modified the nomenclature in the following way (with the sections downstream of exon 2 in parentheses): (1) repressor mRNA (IVS3 + exon 3); (2) transposase mRNA (exon 3; excised: IVS3); (3) double-spliced variant with frame shift (exon 3a + exon 3; excised: IVS3a + IVS3b); (4) double-spliced variant with intact reading frame (exon 3a′ + exon 3; excised: IVS3a + IVS3b′). The two double-spliced variants were named IVS3a / IVS3b splicing and IVS3a / IVS3b′ splicing, respectively. Figure 3 shows the alignment of the IVS3 regions of five *P* elements. The various splicing sites are perfectly conserved and possible branchpoint sequences are found within the expected distances from the splice sites (minimum: 38 nt from the 5′ splice site and 15 nt from the 3′ splice site) according to Mount et al. (1992).

Double Splicing of IVS3

The observation of double splicing raises several questions: First, is there any tissue specificity concerning the double-spliced variants? Since no M-type transposase mRNA was detected in *D. bifasciata*, the variant with the intact reading frame could perhaps encode a transposase-like protein which is expressed in the germline only. Second, is the double-spliced variant with intact reading frame also produced in *D. melanogaster?* Third, does the alternative splicing mode of IVS3 also exist for O-type elements?

To answer these questions, type-specific primer sets were designed: one for M-type transcripts, one for O-type transcripts, and one for the *D. melanogaster P* element. For each *P* element type two primers were made that recognize specifically one of the two splicing variants by spanning IVS3b and IVS3b′, respectively. Furthermore, intron-spanning primers for *D. melanogaster* were made to detect the conventional repressor and transposase mRNAs (Fig. 1).

To investigate tissue specificity of the double-splice M-type variants in *D. bifasciata*, the primer pairs Mi1+ / Mi3b− and Mi2+ / Mi3b− were used to detect the splice variant with interrupted reading frame. For the other variant Mi1+ / Mi3b′− and Mi2+ / Mi3b′− were employed. All four expected fragments could be amplified from heads, ovaries, and testes, indicating that donor site selection of IVS3b vs. IVS3b′ is not tissue specific.

Reverse transcription of mRNA from *D. melanogaster* heads, ovaries, and testes was done with primer me1-e3− followed by PCR amplification with me1−i1+ / me1-e3−. In the reamplifications primer me1-i2+ was combined with me1-e3− for detection of transposase and repressor mRNAs, or me1-i3− for specific detection of transposase mRNA. In this control experiment the fragment expected from repressor mRNA was obtained from all tissues, whereas the PCR products derived from transposase mRNA were only found in ovaries and testes. To identify the two alternative splicing variants in *D. melanogaster*, me1-i2+ was combined with me1-i3b− and me1-i3b′−, respectively. With both primer pairs PCR products were exclusively obtained from ovaries and testes. These data suggest that the double splicing of IVS3 occurs regularly in *D. melanogaster* germline and that both donor sites of IVS3b are used.

For the O type the following four primer pairs were used: Oi1+ / Oi3b−, Oi2+ / Oi3b−, Oi1+ / Oi3b′−, and Oi2+ / Oi3b′−. The fragments corresponding to the two

splicing variants were detected in testes only, whereas in heads and ovaries no PCR products were obtained. Interestingly, both splicing variants originating from O-type elements have interrupted reading frames.

M-type Transcription in D. helvetica

The *P* element of *D. helvetica* clearly belongs to the M-type subfamily. No other *P* element types are found in this species. A full-length *P* element copy has been already cloned and sequenced (Haring 1997). Therefore the binding properties of the various primers could be deduced from the sequence of that copy: Primers Mi1+, Me3.1−, and Mi3− bind without mismatch, Mi2+, Me3.2−, Mi3b−, and Mi3b′− with one, and Me0+ with two mismatches. Reverse transcription, first PCR amplification, and reamplifications were done in the same manner as with *D. bifasciata.* The results indicate that the splicing patterns in *D. helvetica* are identical to that of *D. melanogaster*, where IVS3 activation is restricted to the germline. Repressor mRNA was found to be present in heads, ovaries, and testes, whereas transposase mRNA with spliced IVS3 was detected exclusively in germline tissue. Reamplification with the two primers specific for the double-spliced mRNAs revealed that both variants are made in germline cells of *D. helvetica* but not in somatic tissue. Both double-spliced variants have interrupted reading frames, as is the case with the O-type elements (Fig. 3).

P *element Transcription in* S. pallida

The O-type elements of *S. pallida* were found to be nearly identical to those of *D. bifasciata* (Hagemann et al. 1996a). Therefore, the same primer sets could be used for both species. The results obtained in all reamplifications were the same as in *D. bifasciata* (Table 2) except that no transcripts from internally deleted elements were found. PCR products from transposase mRNA (either with primer Oi3− or Oi3.2−) were obtained from germline tissue only, whereas fragments derived from repressor mRNA were found in heads, and sometimes in ovaries and testes, too (Fig. 4). In *D. bifasciata* the presence of additional smaller fragments was ascribed to transcription of internally deleted elements. The absence of shorter fragments in *S. pallida* confirms this assumption and excludes the alternative possibility that the short fragments in *D. bifasciata* might represent PCR artifacts derived from larger transcripts. It has been shown previously (Hagemann et al. 1996a) that our laboratory strain of *S. pallida* harbors only one size class of small internally deleted O-type elements, whereas the strain of *D. bifasciata* contains several truncated O-type copies with smaller deletions. The IVS3 regions of the following clones were sequenced: *FpalcO*-1, *FpalcO*-4 (from heads, primer: Oi1+ / Oe3.2−), *FpalcO*-2 (from ovaries, primer: Oi2+ / Oe3.2−), and *FpalcO*-8 (from testes, primer: Oi2+ / Oe3.2−). As indicated by the presence of IVS3, *FpalcO*-1, *FpalcO*-2, and *FpalcO*-4 are derived from repressor mRNA. In contrast, clone *FpalcO*-8 lacks IVS3 and thus has to be considered as an amplification product from transposase mRNA. In summary, O-type transcription and splicing in *S. pallida* meets the basic model of *P* element expression in *D. melanogaster* given in most textbooks: repressor in somatic cells and transposase in the germline.

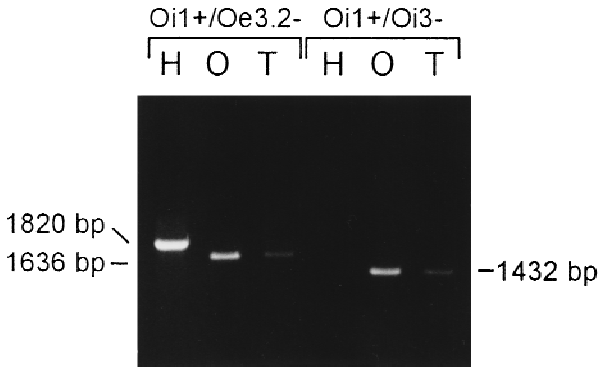

Fig. 4. RT-PCR fragments obtained from heads (*H*), ovaries (*O*), and testes (*T*) of *D. bifasciata* using two different O-type-specific primer pairs for reamplification. The fragments correspond to repressor (1,820 bp) and transposase (1,636 and 1,432 kb) mRNAs. Transposase-derived fragments are found only in PCR amplifications from ovaries and testes.

The M-type elements of *D. bifasciata* and *S. pallida* are closely related (90% identity). Thus several primers designed for *D. bifasciata* can also be utilized for *S. pallida.* Me3.1−, Mi1+, and Mi3− bind to the *S. pallida* M-type element *PS18* without mismatch. Me0+, Mi2+, and Me3.2− bind with one or two mismatches only and were supposed to recognize the *S. pallida* M-type elements, too.

For reverse transcription Me3.1− was used. The first PCR amplification was done with the primer pair Me0+ / Me3.1−. For reamplification the following primer pairs were employed: Mi1+ / Me3.2−, Mi1+ / Mi3−, Mi2+ / Me3.2−, and Mi2+ / Mi3−. Whenever the primer Mi3− was used, no PCR products were obtained, indicating that IVS3 splicing of M-type elements does not occur in *S. pallida.* Reamplification with Me3.2− produced single fragments in the sizes expected from repressor mRNA. Sequence analyses of several clones confirmed the presence of IVS3: *FpalcM*-9 (from heads, primer Mi1+ / Me3.2−), *FpalcM*-5 (from testes, primer Mi2+ / Me3.2−), *SpalcM*-5, and *SpalcM*-8 (from ovaries, primer Mi2+ / Me3.2−).

Although in *S. pallida* no additional fragments (beside those attributed either to repressor or transposase mRNA) were detected in the PCR experiments described above, a specific search for double-spliced variants was carried out. The IVS3b and IVS3b′ spanning primers designed for the M-type sequence of *D. bifasciata* bind

Table 4. Proteins and IVS3 splicing in different tissues and species[a]

	D. bifasciata		*S. pallida*		*D. helvetica*		*D. melanogaster*	
M type								
Soma	R	No	R	No	R	No	R	No
	R-like	3a + 3b	—	—	—	—	—	—
	T-like	3a + 3b′	—	—	—	—	—	—
Germline	R	No	R	No	R	No	R	No
	—	—	—	—	T	3	T	3
	R-like	3a + 3b	—	—	R-like	3a + 3b	R-like	3a + 3b
	T-like	3a + 3b′	—	—	R-like	3a + 3b′	T-like	3a + 3b′
O type								
Soma	R	No	R	No				
Germline	R	No	R	No				
	T	3	T	3				
	R-like	3a + 3b	—	—				
	R-like	3a + 3b′	—	—				

[a] Presumed proteins: R = repressor; T = transposase; R-like = splicing variant with frame shift in exon 3; T-like = splicing variant with intact reading frame in exon 3. IVS3 splicing: No = no splicing of IVS3; 3, 3a + 3b, 3a + 3b′ = introns removed

to the *S. pallida* M-type element *PS18* and *PS2* without mismatch. In order to test whether the *D. bifasciata* primers Oi3b− and Oi3b′− could be also used for *S. pallida*, the clone *FpalcO*-1 derived from repressor mRNA was sequenced. The analysis showed that the *D. bifasciata* primers Oi3b− and Oi3b′− bind perfectly to the *S. pallida* O-type element. These intron-spanning primers were used in combination with Mi1+, Mi2+, Oi1+, and Oi2+. In contrast to the other species tested, no PCR products were obtained. Thus double splicing within IVS3 obviously does not occur in *S. pallida*, neither in M-type nor in O-type transcripts.

Discussion

The RT-PCR experiments clearly show that in *D. bifasciata* and *S. pallida* both O-type and M-type elements are transcribed. The M-type elements of *D. helvetica* are transcribed, too (Table 4).

O-type transcripts are spliced differentially, in the same manner as the *D. melanogaster P* element: In *D. bifasciata* as well as in *S. pallida*, repressor mRNA is found in somatic tissue, whereas transposase mRNA is made only in germline cells. M-type repressor mRNA containing the complete IVS3 is found in somatic tissue (including the somatic parts of the gonads) of *D. bifasciata, D. helvetica, D. melanogaster*, and *S. pallida*. M-type transposase mRNA with completely excised IVS3 is produced only in the germline of *D. helvetica* and *D. melanogaster*. In *S. pallida* no activation of M-type IVS3 was observed at all, indicating that in this species only repressor proteins are produced. In contrast, an alternative splicing pattern was found in *D. bifasciata:* Two introns are removed within the boundaries of IVS3, whereby for the second one the usage of two alternative 5′ sites results in two double-spliced mRNA variants. One of them retains an open reading frame coding for a protein 12 aa longer than the predicted transposase. This mode of IVS3 double splicing is found in *D. bifasciata, D. helvetica*, and *D. melanogaster*, but not in *S. pallida*. In *D. helvetica* and *D. melanogaster* double splicing is germline specific, like the complete excision of IVS3. In *D. bifasciata* germline-specific double splicing is observed only with O-type transcripts, whereas double-spliced M-type variants are found in all tissues. Theoretically, one could imagine splicing variants where only one of the internal introns of IVS3 is removed. Yet, none of these two possible splicing products (neither with O nor with M type) was observed. Thus it can be assumed that IVS3a splicing is an obligatory step in the double splice reaction. Another interesting question is how the splicing of IVS3b (IVS3b′) is repressed in the soma. One possibility would be that the 5′ splice sites of both intron 3a and 3b (3b′) are inhibited by the multiprotein complex binding to the pseudo-5′ splice sites in the 5′ exon region of IVS3 (Laski and Rubin 1989; Siebel et al. 1992). Alternatively, an additional pseudo-5′ splice site (AAAGTGTGG) located in the miniexon 3a at the appropriate distance from the IVS3b (3b′) 5′ splice sites could be responsible for splicing repression of IVS3b (3b′).

The splicing patterns differ not only between *P* element types but also between hosts, e.g., *Drosophila* and *Scaptomyza:* First, M-type mRNAs of *S. pallida* always contain IVS3, indicating that the 5′ splice site of this intron is not used at all. We cannot say whether this is due to IVS3 inhibition in all tissues or to the lack of some unknown activating factors. Second, double splicing of IVS3 is not found in *S. pallida*, neither in O- nor in M-type mRNAs. For the M type this is in accordance with the lack of IVS3 activation: If no cleavage occurs at the IVS3 donor site at all, we cannot expect any of the double-spliced variants. The fact that IVS3 is never removed in *S. pallida* M-type transcripts suggests that this subfamily is transpositionally inactive. Nevertheless, at

least the copy *PS18* was found to transpose when transferred into a *P* element-free strain of *D. melanogaster* (Simonelig and Anxolabéhère 1991). Therefore we have to assume a host-specific, tissue-independent IVS3 inhibiting factor in *S. pallida*. However, this factor exerts its effects only on M-type elements since O-type IVS3 shows somatic inhibition but is activated in germline cells. The absence of IVS3 double splicing in *S. pallida* may be due to another host-specific repressing mechanism, e.g., some factor that binds to the branch site of IVS3a, thus inhibiting its usage.

In *Drosophila* the splicing pattern seems to be uniform in those species *(D. helvetica* and *D. melanogaster)* harboring only one *P* element type. In contrast, within *D. bifasciata* M- and O-type transcripts are spliced in distinct ways. Whereas activation of O-type IVS3 is restricted to germline cells, the donor site of M-type IVS3 is used in soma and germline as well. In *D. melanogaster,* Laski and Rubin (1989) identified the *cis*-acting regulatory sequence for germline specificity which includes a region upstream from the 5′ splice junction of IVS3. Later it was shown that regulation of IVS3 splicing is the result of somatic repression and that pseudo-5′ splice sites in the 5′ exon region of IVS3 are involved (Siebel and Rio 1990; Chain et al. 1991; Tseng et al. 1991; Siebel et al. 1992). Specific RNA binding factors such as PSI (*P* element somatic inhibitor) interact with these regulatory elements and thereby prevent binding of U1 snRNP to the accurate splice site (Siebel et al. 1994, 1995). If the pseudo-5′ splice sites in the 5′ exon region of IVS3 were responsible for inhibition of IVS3 splicing, we would expect to find sequence differences between the two *P* element types in this section. However, among all *P* element types, no matter from which species, the entire region is highly conserved. The only substitution is found in the O-type sequence, destroying one of the two pseudo-5′ splice sites. Interestingly, just the splicing pattern of O-type elements matches exactly that of the *D. melanogaster P* element. So the question remains: If germline specificity of O-type IVS3 splicing is due to somatic inhibition (as in *D. melanogaster*), why has this mechanism no effect on M-type elements? Sequence differences between the two *P* element types cannot be responsible for this phenomenon: Germline specificity has been found for O-type elements of *D. bifasciata* and *S. pallida* as well as for M-type elements of *D. helvetica* and *D. melanogaster*. In fact, it seems to occur irrespective of *P* element type and host species. The M-type elements of *D. bifasciata* are the only exception. One explanation would be an IVS3 activating host-specific factor that specifically acts on the M-type elements of *D. bifasciata.* Alternatively, we could postulate an influence of O-type elements on the splicing of M-type transcripts. Even the different splicing patterns found within *S. pallida* could be ascribed to some interactions between the two *P* element types.

Finally, we have to consider how the proteins encoded by the double-spliced mRNAs may function. First, the variants with interrupted reading frames (both O-type variants, the IVS3a / IVS3b variant of the *D. bifasciata* M type, and both double-splice variants of the *D. helvetica* M type) could encode a repressor-like protein contributing to the P cytotype in the germline as proposed by Chain et al. (1991). Second, the IVS3a / IVS3b′ variants coding for transposase-like proteins that are produced in the germline (M type of *D. bifasciata* and *D. melanogaster*) could function either as a transposase equivalent or (e.g., because of different folding or dimerization properties) as an additional repressor involved in the fine tuning of germline specific regulation. In *D. bifasciata* the presence of a transposase-like protein with transposase function in somatic tissue is difficult to imagine. One possibility is regulation at the translational level preventing the production of a transposase-like protein in the soma.

If we try to interpret the results from an evolutionary point of view, including the effects of host-transposon interactions on the life cycle of *P* elements, we come to two conclusions: First, the principles of *P* element regulation (alternative splicing, germline specificity) hold true irrespective of the *P* element subfamily or host species. For example, sequence divergence between O- and M-type elements is high (30%). Nevertheless, we have shown that O-type elements behave in the classical manner in members of two genera. It seems to be an important property of *P* elements to be able to retain their mobility and regulation patterns after horizontal transmission into a new host species. Second, the presence of two *P* element types within one species seems to result in the inactivation of one type. For the M-type elements of *S. pallida* which are thought to be old components of the genome of this species (Simonelig and Anxolabéhère 1994) it has been shown that they are able to transpose in *D. melanogaster*. Yet, in *S. pallida*, their activity obviously is shut off. For the M type of *D. bifasciata* we still have only indirect hints that it is down-regulated. Nevertheless, the splicing mode of M-type transcripts in this species is exceptional. Further investigations including transformation experiments of *D. melanogaster* with *D. bifasciata* M-type elements will show how they act in a *P*-element-free host species.

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