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The intranuclear site of excision of each intron in Balbiani ring 3 pre-mRNA is influenced by the time remaining to transcription termination and different excision efficiencies for the various introns

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ABSTRACT

The 10.9-kb Balbiani ring 3 (BR3) gene contains 38 constitutively excised introns. Both nascent and nucleoplasmic, released BR3 gene pre-mRNA can be isolated by microdissection of the polytene salivary gland nuclei in which the gene is transcribed.

Here we analyze the order of intron excision in relation to transcription and to intranuclear transport. We demonstrate that the introns are excised with an overall 5′ to 3′ polarity that is established during transcription and maintained during transport. In contrast, we also show that individual introns are excised at very different rates and that neighboring introns are removed in a preferred order that is not necessarily 5′ to 3′. Splicing factors are, in addition, shown to associate with the nascent BR3 pre-mRNA. Our data argue that functional spliceosomes assemble rapidly as introns appear in the pre-mRNA, but that intron-specific properties influence the kinetics of spliceosome assembly and/or function, resulting in cotranscriptional excision of some introns, preferentially those located in the 5′ part of the pre-mRNA, and posttranscriptional excision of other introns, preferentially those located in the 3′ part of the pre-mRNA.

Keywords: polytene chromosomes; pre-mRNA processing; spliceosome; splicing factors

INTRODUCTION

Pre-mRNA splicing, the excision of introns from mRNA precursors, requires the assembly at each intron of a multicomponent complex, the spliceosome. A spliceosome is built from the five different snRNPs, U1, U2, U4, U5, and U6 (Hermann et al., 1995), and a number of non-snRNP proteins such as U2AF (Zamore & Green, 1992), which binds to the polypyrimidine tract at the 3′ splice site, and the family of SR proteins (Zahler et al., 1992; Blencowe et al., 1995; Screaton et al., 1995), known to play an important role in both constitutive and regulated splicing (Sun et al., 1993; Tian & Maniatis, 1993; Staknis & Reed, 1994).

In vitro studies have shown that the spliceosome forms on the pre-mRNA in several steps, recorded as biochemically separate complexes (Konarska & Sharp, 1986; Bennett et al., 1992). In the first, E complex, the U1 snRNP base pairs to the 5′ splice site (Séraphin & Rosbash, 1989; Michaud & Reed, 1991; Jamison et al., 1992) and the U2AF protein is bound to the 3′ splice site (Zamore & Green, 1992). The 5′ and 3′ splice sites are associated in this complex (Séraphin & Rosbash, 1989; Jamison et al., 1992; Michaud & Reed, 1993). SR proteins are thought to promote this early recognition step and to mediate the association of the 5′ and 3′ sites (Wu & Maniatis, 1993).

The U2 snRNP binds to the branch site, resulting in a prespliceosomal A complex, and the U4, U5, and U6 snRNPs are added to form the mature spliceosome (for a review see Green, 1991).

In the dynamic spliceosome, the RNA components of the snRNPs participate in an ordered series of RNA–RNA interactions between themselves and with the pre-mRNA, presumably to catalyze the two successive transesterification reactions of splicing (e.g., Nilsen, 1994; Sharp, 1994).

Pre-mRNAs may contain from zero introns to more than 50 introns, and the length of both introns and

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exons may vary quite considerably. In addition, the splicing machinery can be influenced to accommodate regulated recognition of splice sites in the many alternatively spliced pre-mRNAs. A number of factors have been described that all influence the recognition of an intron, e.g., the sequence of the 5′ and 3′ splice sites, the lengths of the introns and exons, and the presence of exon-located splicing enhancer sequences (Watakabe et al., 1993; Xu et al., 1993).

Several general models have been proposed to explain the accurate and ordered selection and utilization of splice sites in a multi-intron pre-mRNA. Experimental data are inconsistent with a linear scanning model in which splicing components attach to a specific site in an intron and scan for the 5′ and 3′ splice sites bordering the intron (Kuhne et al., 1983; Konarska et al., 1985; Solnick, 1985). To explain how exon skipping is avoided, and to explain the uniform length of vertebrate exons and the effect of 5′ splice site mutations, an exon definition model has been proposed (Robberson et al., 1990) in which initial interaction between a 3′ splice site and the downstream 5′ splice site across the exon is followed by subsequent rearrangement to form a spliceosomal association between the ends of each intron. This order of events receives support from experimental observations of some introns (Niwa et al., 1992), but not of others (Baurén & Wieslander, 1994; Talerico & Berget, 1994).

In line with observations that spliceosomes assemble (Beyer & Osheim, 1988; Baurén & Wieslander, 1994; Kiseleva et al., 1994) and hnRNPs bind to pre-mRNA (Matunis et al., 1993) cotranscriptionally, it has also been proposed that introns are committed to splicing according to a first come, first served principle (Aebi & Weissmann, 1987). To better understand the mechanisms that are involved in the ordered excision of introns from multi-intron pre-mRNAs, it is important to characterize the splicing of specific pre-mRNAs in vivo.

Here, we have analyzed the excision of introns in the Balbiani ring 3 (BR3) gene pre-mRNA in the dipteran *Chironomus tentans*. The BR3 pre-mRNA offers experimental advantages, firstly, because the polytene nuclei of the salivary gland cells allow the isolation of nascent pre-mRNA as well as the released pre-mRNA in the nucleoplasm and, secondly, because the exon–intron structure of the BR3 gene pre-mRNA allows analysis of the order of intron excision along the pre-mRNA. We demonstrate directly that in vivo the introns are excised in an overall 5′ to 3′ order, which is initiated cotranscriptionally, but that neighboring introns are removed in a preferred order that is not necessarily 5′ to 3′. This temporal order of intron excision is transferred into a spatial polarity of intron excision; 5′-located introns are preferentially excised cotranscriptionally, whereas more 3′-located introns are more likely to be excised posttranscriptionally.

RESULTS

We have aimed at studying the temporal order of intron excision in the 10.9-kb BR3 gene pre-mRNA in vivo and its relation to the functional and spatial organization of the nucleus. Except for the first intron, all 38 introns in the BR3 gene are short (Fig. 1), between 53 bp and 288 bp. The exons are between 17 bp and 678 bp (Paulsson et al., 1990). Together, the 38 introns cover half of the gene and the final mRNA is 5.5 kb. The BR3 gene structure therefore makes the BR3 pre-mRNA highly suitable for studies of intron excision in relation to the position of the introns in the gene.

We have chosen to analyze the excision of three introns at the 5′ end of the gene, introns 4–6; three introns at the middle of the gene, introns 20–22; and three introns at the 3′ end of the gene, introns 35–37 (Fig. 1). The reason for analyzing three introns in the same reaction at each position was to measure an average situation for several introns at each position and to be able to experimentally detect all splicing intermediates. It also allowed us to compare, at each position, the excision of introns with different lengths and different splice site sequences.

The large size of the polytene salivary gland cell nuclei, in which the BR3 gene is specifically expressed, allows isolation by microdissection of the active BR3 gene locus, containing nascent BR3 gene pre-mRNA, as well as the nucleoplasm, containing the released BR3 gene pre-mRNA during transport to the nuclear pores (Fig. 2). This is exploited in the analysis of the spatial relation of splicing, transcription, and intranuclear transport.

The analysis of intron excision was performed by RNA-PCR, using a cDNA and 3′ PCR primer located in exon 6, 22, or 37, (Fig. 1). The ^{32}P-labeled 5′ PCR primer was located in exon 4, 20, or 35. At each position, the nonspliced, the completely spliced, and the six possible splicing intermediates of the pre-mRNA could therefore theoretically be detected. As described in the Materials and methods and in the legend to Table 1, the PCR fragments representing the various detectable splicing intermediates at each position were analyzed on polyacrylamide sequencing gels and quantitated relative to each other by phosphorimage analysis. The identity of the PCR products was indicated by the size of the fragments according to the electrophoretic separation and determined by eluting each fragment from the gel, followed by direct sequencing, thus establishing the presence/absence of the introns in each fragment. In order to test that the PCR products faithfully represented the relative amounts of pre-mRNA splicing intermediates in the original extract, we performed experiments in which the amount of PCR products were recorded at different number of PCR cycles. As shown in Figure 3, the amount of PCR products increased exponentially up to about 28 cycles

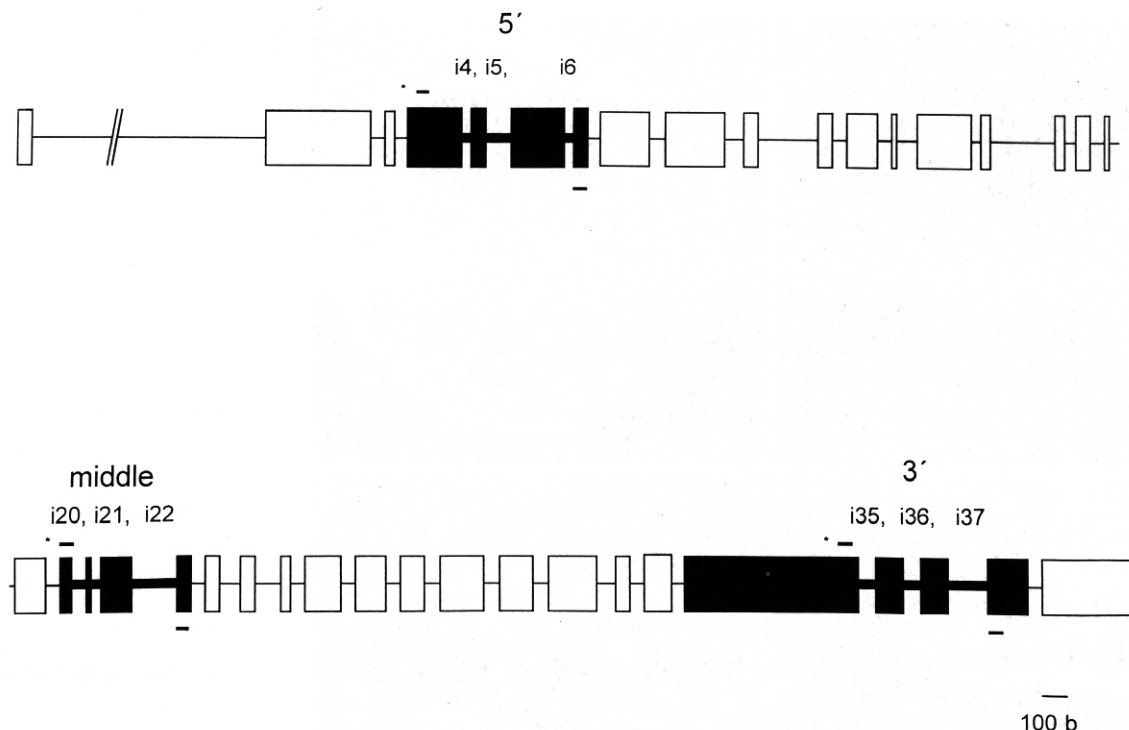

FIGURE 1. Exon–intron structure of the unspliced BR3 gene pre-mRNA. Introns at the three analyzed positions, 5′, middle, and 3′, are indicated. At each position, the analyzed exons (boxes) and introns (thin lines) are shown in black. Positions of the common cDNA and 3′ PCR primers are shown as a single line below the pre-mRNA structure and the labeled 5′ PCR primer positions are shown by a line with a star above the pre-mRNA. The pre-mRNA is drawn to scale and the bar represents 100 bases.

TABLE 1. Quantitation of splicing products in nascent and released BR3 gene pre-mRNA.[a]

	BR3 locus									Nucleoplasm								
	5′			Middle			3′			5′			Middle			3′		
Intron(s) excised	i4	i5	i6	i20	i21	i22	i35	i36	i37	i4	i5	i6	i20	i21	i22	i35	i36	i37
None	60.1		68.1	86.5		89.4	93.7		94.2	Not detect.			1.6		3.4	2.3		3.1
			45.8			75.0			92.1						1.0			1.8
i4; i20; i35	2.9		7.4				0.8		1.3	Not detect.						1.7		2.3
			0.1	0.2*		0.6			0.1				Detect.*					1.3
i5; i21; i36	8.3		13.3			Detect.	Not detect.			Not detect.						Not detect.		
			3.1															
i6; i22; i37	Not detect.			1.2		13.2	Not detect.			Not detect.			2.0		4.2	Not detect.		
						0.7									1.6			
i4, i5; i20, i21; i35, i36	6.2		10.3	Detect.			0.5		1.0	1.1		1.8	0.1		0.1	4.5		6.4
			3.1						Detect.			Detect.			Detect.			3.0
i5, i6; i21, i22; i36, i37	5.4		8.7	0.4		0.9	Not detect.			0.7		1.7	0.9		1.9	Not detect.		
			3.1			0.2						Detect.			0.9			
i4, i6; i20, i22; i35, i37	Detect.			0.1		1.1	Detect.			Not detect.			0.4		1.0	0.1		0.5
						0.1									0.3			Detect.
All	17.3		22.5	10.5		12.9	6.1		6.6	98.2		100.0	94.9		96.1	91.6		93.7
			13.1			4.9			4.9			96.7			89.2			88.2

[a] PCR products representing unspliced, completely spliced, and all detected splicing intermediates at each position (5′, middle, and 3′) of the pre-mRNA were quantitated by phosphorimage analysis. Each PCR product (see Fig. 5) was expressed as the proportion (in percent) of the sum of all PCR products in that experiment. In the table, the median values and the maximum and minimum values for each product are given. Using the Kruskal–Wallis one-way analysis of variance by ranks (Siegel & Castellan, 1988), it was found that the values for completely spliced products at the 5′, middle, and 3′ positions were significantly different ($P = 0.001$). Comparing the positions pairwise using the Mann–Whitney test (Altman, 1991) showed that the 5′ and middle position values were significantly different ($P = 0.002$) and that the middle and 3′ position values also were different ($P = 0.055$). At each position, the relative proportions of the various splicing intermediates were significantly different according to the Kruskal–Wallis analysis: 5′ BR3 ($P = 0.001$), middle BR3 ($P = 0.0026$), middle nucleoplasm ($P = 0.006$), 3′ BR3 ($P = 0.008$), 3′ nucleoplasm ($P = 0.001$). A * means that it could not be determined to what extent the recorded PCR product represented an intermediate lacking intron 20 or 21. Detect., PCR product was observable but represented considerably less than 0.1%.

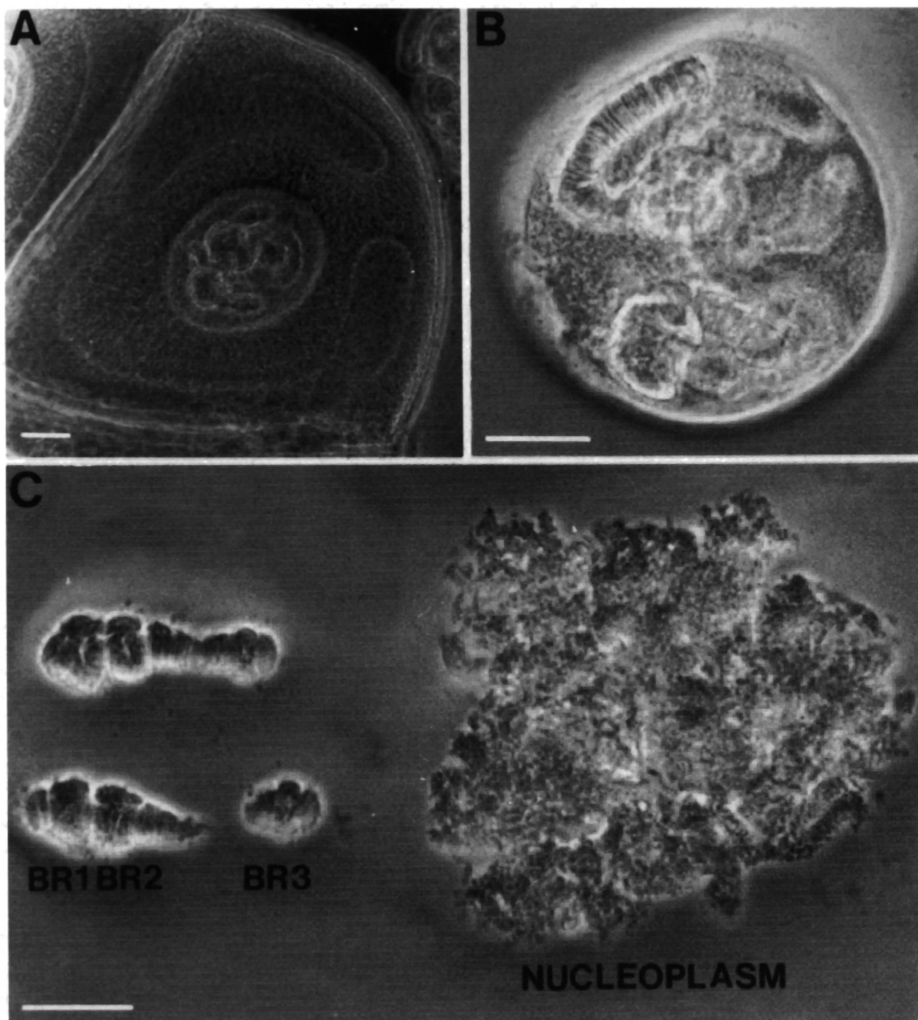

FIGURE 2. Isolation of the BR3 gene locus and nucleoplasm by microdissection. **A:** Salivary gland cell is shown before microdissection. **B:** Single polytene nucleus has been isolated by microdissection. **C:** Chromosome IV and the nucleoplasm have been microdissected from one nucleus. In a separate chromosome IV, the BR3 gene locus has been dissected from the rest of chromosome IV. The BR1, BR2, and BR3 loci are indicated. The bar, in all instances, represents 20 μm.

for the completely spliced band, which was the most abundant, and the same was true for a splicing intermediate present in the lowest amount, which was consistently two orders of magnitude below the completely spliced product. In our measurements, we used 26 PCR cycles to still be within the exponential phase of the PCR and yet to reach sufficient sensitivity to detect the less abundant intermediates.

Because the various splicing intermediates were present in very different amounts (see Table 1), we were also concerned that the different internal structure of the intermediates (lacking zero, one, two, or three introns) could influence the cDNA synthesis and/or PCR. To check this possibility, we produced in vitro RNA transcripts representing all eight variants of the pre-mRNA at the 5′ position. By mixing equimolar amounts of the in vitro transcripts, we have shown that they were all equally good substrates for both cDNA synthesis and PCR (data not shown). We therefore conclude that the observed and measured PCR fragments reflect the true relative amounts of pre-mRNA splicing products.

In Figure 4, the exon–intron structure of the eight possible PCR fragments obtainable from analysis of introns 4–6 at the 5′ position of the BR3 pre-mRNA is depicted as one example. In Figure 5, autoradiograms of the PCR fragments obtained at the 5′, middle, and 3′ positions of the BR3 pre-mRNA are shown, both for the nascent pre-mRNA and for the pre-mRNA isolated from the nucleoplasm. It is evident that most but not all possible splicing intermediates are detected. The relative proportion of each detectable intermediate at the 5′, middle, and 3′ positions of the pre-mRNA is given in Table 1 (average of 4–8 individual experiments). From these results, several conclusions can be drawn. Firstly, in the nascent BR3 pre-mRNA population, the 5′-located introns are excised before more 3′-located introns. This overall polarity of splicing can be seen when the percentage of totally spliced pre-mRNA is plotted against the position of the introns in the pre-mRNA (Fig. 6). The percentage of totally spliced product decreases in an essentially linear manner, implying that there is, on average, a direct relationship between the probability of excision of an intron and the remain-

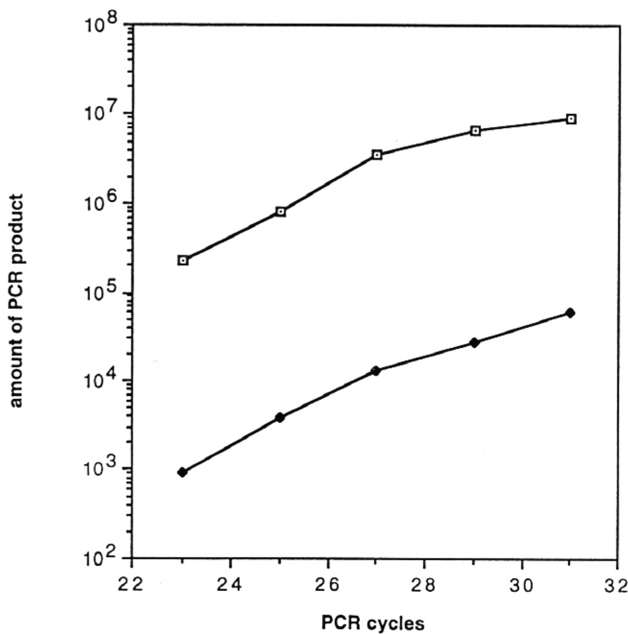

FIGURE 3. Relationship between the amount of PCR products and the number of PCR cycles. RNA extracted from 30 BR3 gene loci was transcribed into cDNA using a primer in exon 38. The cDNA was amplified with PCR for 23, 25, 27, 29, or 31 cycles. Products were separated on a sequencing gel and quantitated by phosphorimage analysis. The amount of PCR product is plotted against the number of PCR cycles for the completely spliced pre-mRNA (open squares) and for the splicing intermediate retaining only intron 36 (filled squares), which is present in approximately 1% of the amount of the completely spliced product.

ing distance of transcription. If instead the percentage of all pre-mRNA in which at least one intron is excised is analyzed (Table 1: 5′, 39.9%; middle, 13.5%; 3′, 6.3%), the overall 5′ to 3′ polarity can still be seen, but the decrease is no longer linear. There is a considerable drop between the 5′ and middle positions and a slower decline between the middle and 3′ positions. This reflects that, at the 5′ position, excision of intron 5 is exceptionally efficient. We have measured the excision of intron 5 separately and shown that 35% of the nascent transcripts lack intron 5 (data not shown), a value that agrees well with sum of all intermediate splice products in which intron 5 is excised (37.2%, Table 1).

Secondly, it can be noted that more than 90% of all introns are removed from the nucleoplasmic, released pre-mRNA at a given moment. Even so, the 5′-located introns are essentially completely removed, whereas this is not the case for the more 3′-located introns, i.e., the 5′ to 3′ polarity is still observed in the nucleoplasmic pre-mRNA.

We conclude that excision of the 5′-located introns is both initiated and completed earlier than for the more 3′-located introns, and that there is an overall polarity that is proportional to the distance remaining to the 3′ end of the pre-mRNA.

Based on electron microscope observations of the transcribing BR3 gene in situ (I. Wetterberg, L. Wieslander, and U. Skoglund, unpubl.), and on analogy

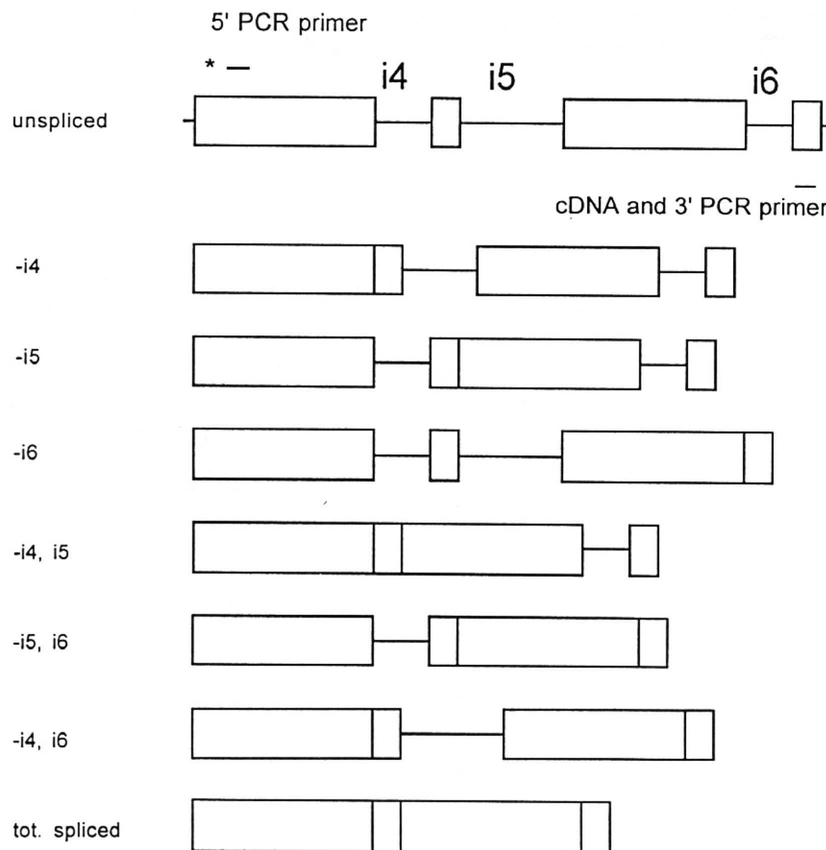

FIGURE 4. Structure of splicing intermediates and the completely spliced product at the 5′ position. The 5′ position of the unspliced pre-mRNA is shown on top with the positions of the cDNA/3′ PCR primer and the labeled 5′ PCR primer. Below, the six possible splicing intermediates and the completely spliced product are depicted schematically.

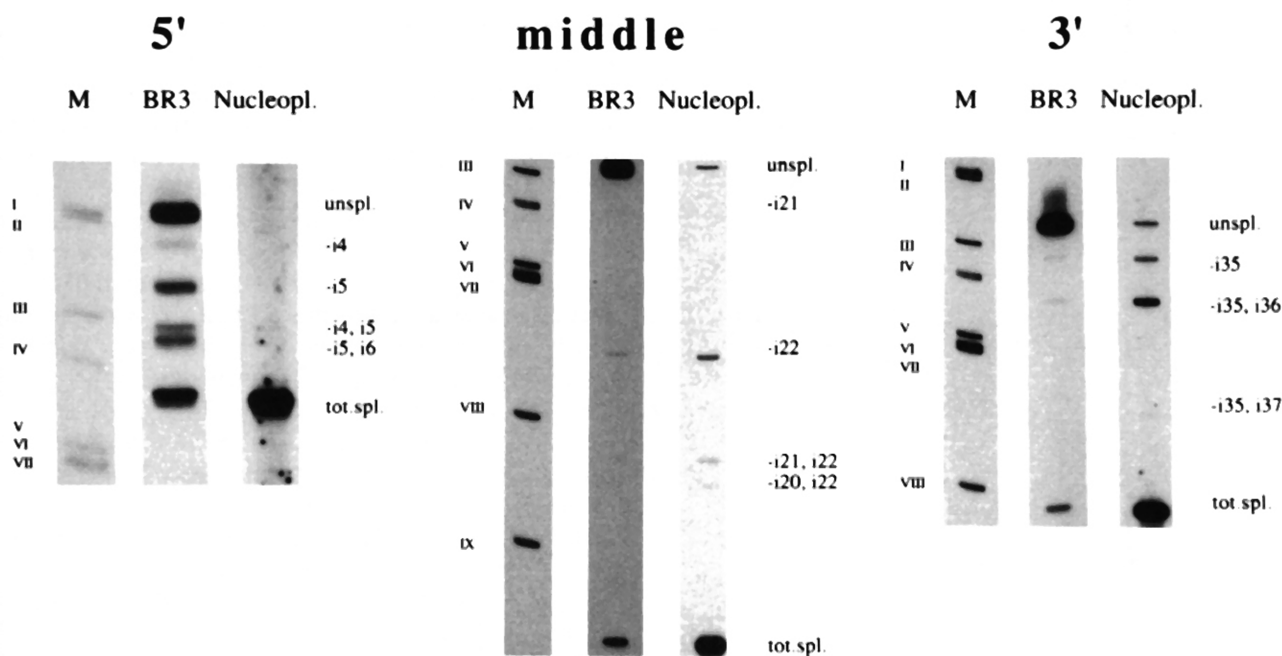

FIGURE 5. Detection and quantitation of splicing products. Splicing products at the 5′, middle, and 3′ positions, which are present in the population of pre-mRNA in the BR3 gene locus (BR3) as well as in the population of nucleoplasmic BR3 pre-mRNA (Nucleoplasm), are shown. PCR fragments corresponding to the splicing products were separated on sequencing gels and visualized by autoradiography. To the left are size markers (M), marked I–IX, with sizes from top to bottom of 786, 713, 553, 500, 427, 417, 413, 311, and 249 bases. To the right, the position of the unspliced, the totally spliced fragments, and the splicing intermediates are indicated. Splicing intermediates are identified according to which introns have been excised.

with the situation in the related BR1 and BR2 genes (Daneholt, 1982; Skoglund et al., 1983), the pre-mRNA extracted from the microdissected BR3 gene locus represents the nascent pre-mRNA population along the 10.9-kb gene. To substantiate this argument, we have shown that pre-mRNA is present at the BR3 gene locus, which is uncleaved at the poly(A) site and which has excised the most 3′-located intron, intron 38. In this experiment, the cDNA and 3′ PCR primer was placed downstream of the cleavage site to ensure that only noncleaved pre-mRNA was analyzed. We then detected a small proportion of pre-mRNA (approximately 1–2%), in which intron 38 had been removed (data not shown). Because we have demonstrated above that 5′-located introns are removed before 3′-located introns, we conclude from this experiment that the established splicing events observed in BR3 pre-mRNA, isolated from the BR3 locus, take place cotranscriptionally. This is in agreement with our previous study of the excision of introns in the BR1 gene (Baurén & Wieslander, 1994).

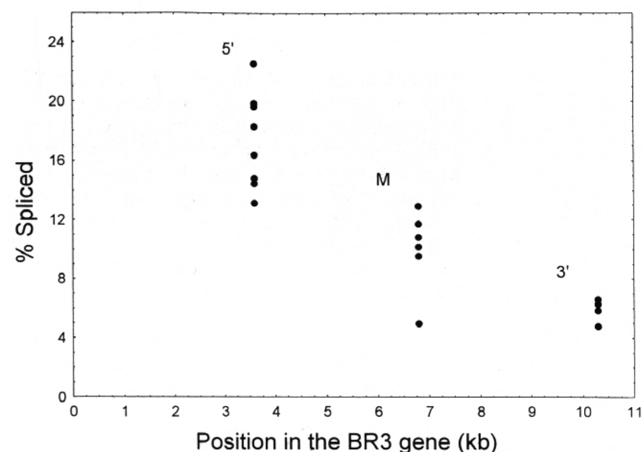

FIGURE 6. Overall 5′ to 3′ polarity of intron excision in the nascent BR3 gene pre-mRNA. The proportion (in percent) of completely spliced pre-mRNA in the nascent BR3 gene pre-mRNA at the 5′, middle, and 3′ positions (5′, M, 3′) is plotted against the locations of the introns in the unspliced 10.9-kb BR3 gene pre-mRNA. Each point represents one independent experiment.

In Figure 7, we also demonstrate that splicing factors are highly concentrated at the transcribing chromatin loops of the BR3 gene, using antibodies against the U2 snRNP-specific U2B″ protein and a SR non-snRNP splicing factor. Binding of the antibodies is sensitive to RNase treatment prior to the antibody reaction (Baurén et al., 1996), showing that the splicing factors bind to BR3 gene pre-mRNA at the site of transcription.

Based on these data, we conclude that splicing is initiated and to a considerable extent completed cotranscriptionally. Hence, the overall polarity of splicing is established cotranscriptionally and this polarity is then maintained during transcription as well as during transport away from the gene locus through the nucleoplasm.

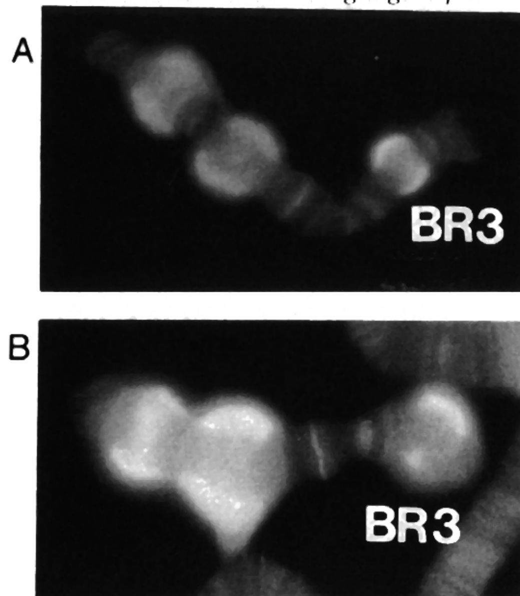

FIGURE 7. Association of splicing factors with pre-mRNA at the site of transcription in the BR3 gene locus. Squash preparations of salivary gland cells were stained with antibodies detecting (**A**) the U2 snRNP B″ protein and (**B**) an SR non-snRNP splicing factor. The BR3 gene is located on chromosome IV. Two additional highly transcribed genes, the BR1 and BR2 genes, are located on the same chromosome and are also intensely stained with the antibodies. Chromosome IV is approximately 50 μm in length.

In Table 1, it can also be seen that, even though there is an overall 5′ to 3′ polarity of intron excision, this polarity is not necessarily observed locally in the pre-mRNA, i.e., for the three neighboring introns at each of the three positions investigated. The fact that, at each position, we analyze three introns at the same time makes it possible to record sequential intron excisions. We could then detect a three-step process in which one, two, or all three introns were removed and we could also observe that there were several different pathways to the completely spliced end product. At each position, the relative proportions of the splicing intermediates were statistically significantly different as tested in a nonparametric test (see Table 1). From these proportions, it is evident that at, e.g., the 5′ position, intron 4 or intron 5 can be excised first, but the intermediate lacking only intron 5 is the most abundant (see Table 1). Among the intermediates lacking two introns, almost all lack intron 5, in combination with the lack of either intron 4 or intron 6, and only very few intermediates retain intron 5. In addition, it can be seen from Table 1 that, in the entire nascent population, 37.2% of all transcripts lack intron 5, 26.4% lack intron 4, and 22.7% lack intron 6. These data show that different introns are excised with different efficiencies and that, in a majority of the transcripts, intron 5 is excised, whereas introns 4 and 6 are still present. If we assume that these efficiencies are mainly due to the structure of each intron and its immediate surroundings and do not change depending on the presence or absence of a neighboring intron, which is consistent with our recorded relative proportions (see the Discussion), then the relative proportions reflect the order of intron excision. Based on these data, we conclude that the preferred order of intron excision is 5, 4, 6. The same analysis for the middle position (including analysis of the situation in the nucleoplasm) suggests that the preferred order is 22, 21, 20. However, here it is not obvious that introns 20 and 21 are removed in any preferred order. For the 3′ position, the deduced order is 35, 36, 37.

We conclude that, at a specific position in the BR3 pre-mRNA, the introns are excised with different efficiencies and that, consequently, neighboring introns are excised in a preferred order that is not necessarily 5′ to 3′.

DISCUSSION

Introns are committed to excision cotranscriptionally

Our data suggest that, for nascent BR3 gene pre-mRNA, there is a linear relationship in vivo of intron excision and the distance remaining to the transcription termination site (Fig. 6). This relationship is in agreement with our previous study of the BR1 gene pre-mRNA (Baurén & Wieslander, 1994) and, if data from the two genes are combined, the linear relationship holds from about 1 kb to 35 kb from the site of transcription termination. This relationship was recorded for nascent pre-mRNA, which means that distance is equivalent to time, i.e., remaining time of transcription.

The simplest explanation for this relationship to time is that introns are recognized and the splicing process initiated in close connection to the transcription process. In vitro it has been shown that a prespliceosomal complex forms very rapidly and without requirement for ATP, containing U1 snRNP, U2AF, and SR proteins (Bennett et al., 1992; Fu, 1993; Staknis & Reed, 1994). It is therefore possible that introns are committed to splicing by association with these factors very rapidly also in the intact cell nucleus. Our experimental in vivo data show that splicing factors associate with the nascent pre-mRNP and that intron excision is initiated during transcription, supporting the proposition of a cotranscriptional first come, first served model (Aebi & Weissmann, 1987). The order of intron excision has been analyzed in a number of other pre-mRNAs (e.g., Lang & Spritz, 1987; Gudas et al., 1990; Weil et al., 1990; Kessler et al., 1993). In some but not all instances, a 5′ to 3′ polarity has been observed. The discrepancy between some of these results and ours, we believe, lies in the fact that most of the studies concern neighboring introns where the polarity effect is not obvious and overruled by other factors, see below.

An overall 5′ to 3′ polarity of intron excision does not necessarily have to be explained by a close coupling

between the transcription process and formation of a prespliceosomal complex. It is known that the 5′ end of all pre-mRNA is chemically modified into the cap structure, $m^7G(5')ppp(5')N$ (Shatkin, 1976). Capping occurs cotranscriptionally (Salditt-Georgieff et al., 1980), and specific proteins are bound to the cap structure (Patzelt et al., 1983; Rozen & Sonenberg, 1987). Initial studies showed that the cap structure is needed for efficient splicing (Konarska et al., 1984; Edery & Sonenberg, 1985). It has also been shown that an initial step in splicing is influenced by the cap binding protein complex (Izaurralde et al., 1994), and that the cap structure stimulates excision of an upstream intron more than a downstream intron (Ohno et al., 1987; Inoue et al., 1989). It is therefore possible that the cap structure with its associated proteins interacts with the splicing machinery, resulting in a 5′ to 3′ polarity effect of intron excision. Another theoretical possibility is that the overall structure of the pre-mRNP influences the splicing machinery in a directional manner. Our data do not discriminate between these possibilities, but, because of the long-range effect, up to 35 kb from the 5′ end of the pre-mRNA, and the linearity (see Fig. 6), we favor the interpretation of a cotranscriptional commitment to splicing, presumably through the assembly of a prespliceosomal complex at each intron, rather than a directional influence of the cap structure or the overall pre-mRNP structure. Our data also underline that, whatever the reason for the polarity effect, it must be established cotranscriptionally and continue to act posttranscriptionally because we record the polarity in the many introns excised after completed transcription.

Cotranscriptional versus posttranscriptional splicing

Originally it was assumed that splicing was strictly a posttranscriptional process, based on several findings that polyadenylated but unspliced pre-mRNA were observed (see Nevins, 1983). Studies of the adenovirus late transcription unit (Keohavong et al., 1982; Mariman et al., 1983), electron microscopy analysis of transcribing *Drosophila* genes (Osheim et al., 1985; Beyer & Osheim, 1988), analysis of the 60-kb inducible E74A gene in *Drosophila* (LeMaire & Thummel, 1990), and direct isolation of nascent, spliced pre-mRNA (Baurén & Wieslander, 1994; Wuarin & Schibler, 1994) have established that splicing may also be a cotranscriptional process. As pointed out, our data support the view that introns are committed to be excised cotranscriptionally, but that the very splicing reaction requires some time to perform. In the present study, we therefore demonstrate that splicing of a defined pre-mRNA may be both cotranscriptional and posttranscriptional, depending on the location of the intron in relation to the 3′ end of the pre-mRNA. In addition, intron-specific properties, such as the sequence of 5′ and 3′ splice sites and the pre-mRNA secondary structure (Balvay et al., 1993), will influence the kinetics of splicing (see below). We would therefore expect that the intranuclear location of splicing will be different for different pre-mRNAs and for different introns within one and the same pre-mRNA.

We have also noted that, even when posttranscriptional, intron excision can be almost completed very close to the gene locus (G. Baurén & L. Wieslander, in prep.). Other introns may presumably be excised later during intranuclear transport and this type of variation will also contribute to differences in intranuclear location of splicing.

Intron-specific properties determine the local order of intron excision

At each of the three positions in the BR3 gene, we could detect all or almost all six splicing intermediates. As observed in other genes (e.g., Gudas et al., 1990; Kessler et al., 1993), there was a preferred, nonrandom but not obligatory order of intron excision. Moreover, the order was not 5′ to 3′ at each position, showing that the mechanism resulting in the overall polarity of intron excision in pre-mRNA discussed above is not determining the local order of intron excision. Each intron has its specific properties (see Aebi & Weissmann, 1987), such as the sequence of the 5′ and 3′ splice sites and the branch point, the length, and interaction with possible enhancer sequences in the neighboring exon (Watakabe et al., 1993; Xu et al., 1993).

In our experiments, we could measure the proportion of splicing intermediates lacking one, two, or all three introns, i.e., we could follow the result of a three-step pathway leading to the completely spliced end product. At each step, any of the remaining introns could be excised first, but the probabilities varied considerably for each intron. We found that we could simulate the experimentally observed proportions of all splicing intermediates in the three-step pathway, if we assumed that each intron had a fixed probability of excision, regardless of the presence or absence of any of the other two introns. It therefore appears as if each intron had defined properties that did not change depending on the presence or absence of the neighboring intron or the exact structure of the surrounding exons. We therefore conclude that intron-specific properties have a strong influence on the splicing reaction. We could not correlate the efficiency of excision to any single property, such as the similarity of 5′ or 3′ splice sites to consensus sequences for these sites in *Drosophila* or *C. tentans*, or to the length of the introns. It is therefore likely that the particular nucleotide sequence at many positions within and immediately surrounding each intron together contribute to an efficiency of excision, which is specific for every intron.

MATERIALS AND METHODS

Animals

C. tentans was cultured as described (Meyer et al., 1983). Fourth instar larvae were used throughout the experiments.

Isolation of nascent and nucleoplasmic BR3 pre-mRNA

Salivary glands were dissected and fixed in ethanol:acetic acid (3:1) on ice for 30 min, washed with 70% ethanol for 30 min on ice, and stored in ethanol:glycerol (1:1) at −20 °C. The fixed glands were attached to a cover glass in a small drop of ethanol:glycerol and surrounded by paraffin oil as described by Lambert and Daneholt (1975). Microdissection was carried out using a de Fonbrune micromanipulator equipped with two glass needles. The needles and the glands were observed through a phase contrast microscope (see Fig. 2), at a magnification of 400 or 900 times.

For each experiment, BR3 gene loci from 30–50 nuclei or nucleoplasm from 1–2 nuclei were isolated by microdissection and RNA was extracted for 30 min at room temperature in 100 μL extraction buffer (20 mM Tris-HCl, pH 7.4, 1 mM EDTA, 0.5% SDS, 100 μg/mL Proteinase K, and 2.5 μg yeast RNA). After extraction with phenol/chloroform and chloroform, the RNA was ethanol precipitated. RNA isolated from the BR3 loci was then treated with 5 U of RNase free DNase I (Promega) for 25 min at 37 °C in 50 μL of 10 mM Tris-HCl, pH 7.4, 10 mM $MgCl_2$, 10 mM dithiothreitol in the presence of 40 U RNasin (Promega). The RNA was thereafter extracted with phenol/chloroform and chloroform, precipitated with ethanol, and dissolved in water.

cDNA synthesis and PCR

Primers for cDNA synthesis and PCR were chosen from the known sequence of the BR3 gene (Paulsson et al., 1990). For the 5′ position, the two primers correspond to parts of exon 4 and exon 7, respectively; for the middle position, to sequences in exon 20 and 23; and for the 3′ position, to sequences in exons 35 and 38 (see Fig. 1).

For cDNA synthesis, RNA in 16 μL of water was mixed with 4 μL 3′ primer (0.0004 pmol) and 2.5 μg yeast RNA and incubated at 70 °C for 10 min. The sample was then divided into two equal parts, one of which served as a negative control. Four microliters 5× buffer (125 mM Tris-HCl, pH 8.3, 375 mM KCl, 15 mM $MgCl_2$), 2 μL 5 mM dNTPs, 2 μL 0.1 M DTT, and 40 U RNasin were added to each tube. After 2 min at 37 °C, 200 U of reverse transcriptase (SuperScript, GIBCO BRL) was added to one of the tubes and both tubes were left at 37 °C for 1 h. After boiling for 5 min, all of the cDNA reaction was used for the subsequent PCR.

The 5′ primers used for PCR were ^{32}P-labeled by T4 polynucleotide kinase. One microliter of 20 μM primer was mixed with 10 U T4 polynucleotide kinase (New England Biolabs), 50 μCi ^{32}P γ-ATP (New England Nuclear) in 50 mM Tris-HCl, pH 7.6, 10 mM $MgCl_2$, 5 mM dithiothreitol, 0.1 mM spermidine, 0.1 mM EDTA, and incubated for 45 min at 37 °C. The enzyme was inactivated at 65 °C for 5 min and the reaction diluted with water to 300 μL.

PCR was performed as described (Saiki et al., 1988), using hot start and 26 cycles.

In all PCR analyses, two controls were included: one containing the negative control from the cDNA reaction in which no reverse transcriptase was included, the other containing all the PCR components, but no cDNA.

The PCR products were extracted with phenol/chloroform, ethanol precipitated, and dissolved in 8 M urea, 8 mM EDTA, pH 8.3, containing bromophenol blue and xylene cyanol. The samples were run in 4–6% sequencing polyacrylamide gels containing 7 M urea. Kinased *Hinf* I restriction fragments of ϕX174 DNA (Promega) were run in parallel as size markers. The gels were dried and exposed on phosphorimage screens. The results were analyzed using a PhosphorImager (Molecular Dynamics) and the software ImageQuant (Molecular Dynamics).

To assure that the PCRs were in the exponential phase during the conditions used, we performed the above RNA-PCR experiment using 23, 25, 27, 29, and 31 cycles, and the amount of cDNA obtained from one typical cDNA reaction. At each of these points, the PCR products were analyzed on sequencing gels and quantitated by phosphorimage analysis. The relative amounts of PCR products for each splicing intermediate was plotted against the number of cycles. For all detectable splicing intermediates as well as the totally spliced end product, the PCR was in the exponential phase up to about 28 cycles (see Fig. 2).

Additionally, we tested if the various splicing intermediates at one position were equally good substrates in the cDNA and PCR analysis. For the 5′ position, eight in vitro RNA transcripts corresponding to all six splicing intermediates and the unspliced and completely spliced pre-mRNA were produced. This was done for seven of the fragments by reamplifying the purified and sequenced PCR fragments, using a 5′-primer containing the T7 promoter sequence, followed by in vitro transcription (MEGAscript, Ambion). The intermediate lacking only intron 6, which is not detectable in vivo, was obtained by amplifying the corresponding genomic region. To delete intron 6, a 3′ primer complementary to the end of exon 6 and beginning of exon 7 was used together with the same 5′ primer as above. The structure of the PCR product was verified by restriction enzyme analysis.

All eight in vitro transcripts were mixed in equimolar amounts with a total RNA amount of approximately 100 pg, followed by RNA-PCR precisely as described above. The products were separated on a 4% sequencing gel and quantitated by phosphorimage analysis. The relative amounts of the eight PCR products varied between 1 and 2.4 in six separate experiments in a nonsystematic manner.

Sequencing of PCR products

PCR products that, according to their sizes, represented splicing intermediates, were located by autoradiography, cut out from the dried polyacrylamide gel, and eluted in 200 μL water. After boiling for 15 min and a short centrifugation, 180 μL of clear liquid was transferred to a second tube and precipitated with 3 volumes of ethanol after addition of 1/10 volume of 3 M NaAcetate, using 40 μg of glycogen as carrier. The pellet was dissolved in 20 μL water and 10 μL was used for reamplification by PCR. The obtained PCR product was purified by

electrophoresis in a 0.8% low melting agarose gel (FMC Bioproducts) and eluted with GELase (Epicentre Technologies) according to the protocol of the manufacturer.

Approximately 60 ng of the PCR product was used for sequence determination in the dye terminator sequencing reaction (Applied Biosystems, Inc.) and analyzed on an automated ABI model 373A DNA sequencer. The determined sequences were analyzed using the computer program GAP from GCG's Wisconsin package 8.0 (Devereaux et al., 1984).

Immunofluorescence

Two monoclonal antibodies were used, 4G3 (Habets et al., 1989), specifically detecting the U2 snRNP B″ protein (obtained from Euro-Diagnostica B.V.), and 2E4 (Kiseleva et al., 1994; Wurtz et al., 1996), specifically detecting the Ct-hrp45 protein, which is an SF2/ASF (Ge & Manley, 1991; Krainer et al., 1991) homologue in *C. tentans* (A.T. Alzhanova-Ericsson, X. Sun, N. Visa, E. Kiseleva, T. Wurtz, and B. Daneholt, in prep.). Squash preparations were prepared essentially as described by Silver et al. (1978). Salivary glands were dissected and kept for 10 min in 25 mM di Na-glycerophosphate, 10 mM K_2HPO_4, pH 6.8, 30 mM KCl, 10 mM $MgCl_2$, 3 mM $CaCl_2$, 160 mM sucrose, 0.5% Nonidet P-40 on ice. The glands were fixed in 4% formaldehyde in PBS for 25 min, followed by incubation in 45% acetic acid for 10–20 min. Cells were separated mechanically from the rest of the gland and squashed. The squash preparations were kept in 20 mM Tris-HCl, pH 7.6, 137 mM NaCl (TBS), and washed in TBS containing 0.05% Tween-20 for 3 × 5 min at room temperature. The primary antibody was added for 1 h at room temperature, diluted in TBS containing 0.05% Tween-20. The preparations were washed in TBS, 0.05% Tween-20 for 3 × 5 min at 4 °C. The secondary rabbit anti-mouse antibody was diluted in TBS, 0.05% Tween-20, 5% BSA, and incubation was for 1 h at room temperature. After washing in TBS, 0.05% Tween-20 at 4 °C, the preparations were mounted in glycerol/PBS (87/13). The chromosomes were photographed in a standard fluorescence microscope (Axioskop, Zeiss).

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