Transcriptional termination in the *Balbiani ring 1* gene is closely coupled to 3'-end formation and excision of the 3'-terminal intron

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We have analyzed transcription termination, 3'-end formation, and excision of the 3'-terminal intron in vivo in the *Balbiani ring 1* (*BRI*) gene and its pre-mRNA. We show that full-length RNA transcripts are evenly spaced on the gene from a position 300 bp upstream to a region 500–700 bp downstream of the polyadenylation sequence. Very few full-length transcripts and no short, cleaved, nascent transcripts could be observed downstream of this region. Pre-mRNA with 10–20 adenylate residues accumulates at the active gene and then rapidly leaves from the gene locus. Only polyadenylated pre-mRNAs could be detected in the nucleoplasm. Our results are consistent with the hypothesis that transcription termination occurs in a narrow region for the majority of transcripts, simultaneous with 3'-end formation. Excision of the 3'-terminal intron occurs before 3'-end formation in about 5% of the nascent transcripts. When transcription terminates, 3' cleavage takes place and 10–20 adenylate residues are added, the 3'-terminal intron is excised from additionally about 75% of the pre-mRNA at the gene locus. Our data support a close temporal and spatial coupling of transcription termination and the cleavage and initial polyadenylation of 3'-end formation. Excision of the 3'-terminal intron is highly stimulated as the cleavage/polyadenylation complex assembles and 3'-end formation is initiated.

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Formation of mRNA in eukaryotic cells requires that the mRNA precursors (called pre-mRNA) are extensively processed in the nucleus and transported to the cytoplasm. At the 5' end of the pre-mRNA, a cap structure is added early in transcription (Shatkin 1976; Salditt-Georgieff et al. 1980; Rasmussen and Lis 1993), and the cap-binding proteins CBP 20 and CBP 80 rapidly become associated with this structure (Izaurralde et al. 1992), apparently cotranscriptionally (Visa et al. 1996). In pre-mRNAs that contain introns, the exons are spliced together in two successive phosphoryl transfer reactions (Staley and Guthrie 1998). At the 3' end, pre-mRNA is cleaved and polyadenylated (Wahle and Keller 1996; Colgan and Manley 1997; Wahle and Kühn 1997).

A common feature of the splicing and 3' processing events is association of multicomponent processing complexes with the pre-mRNA at defined sequences. Splicing requires that >50 different proteins (see Will and Lührmann 1997) and five RNAs come together to form a

functional spliceosome (Krämer 1996; Staley and Guthrie 1998). 3' Processing involves at least 10 different polypeptides that assemble at the site of cleavage and polyadenylation (Wahle and Keller 1996; Colgan and Manley 1997; Wahle and Kühn 1997).

We do not know much about the relationship between the cell biology of the processing machineries (their assembly, function, and disassembly/reuse) and the organization of these machineries on active chromatin. Interactions between different pre-mRNA processing machineries have been observed in vitro and in transfection experiments. The nuclear cap binding complex, CBC, stimulates excision of the most 5'-located intron in vitro (Ohno et al. 1987; Inoue et al. 1989; Izaurralde et al. 1994) and has been shown to facilitate U1 snRNP binding to the cap-proximal 5'-splice site (Lewis et al. 1996). It has also been shown that CBC enhances endonucleolytic cleavage at the polyadenylation site (Flaherty et al. 1997).

Splicing and 3'-end formation can take place independently in vitro (Moore and Sharp 1984, 1985), but it has been suggested that excision of the 3'-terminal intron and 3'-end formation are functionally coupled in vitro

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(Niwa et al. 1990; Niwa and Berget 1991) and in intact cells (Chiou et al. 1991; Liu and Mertz 1993; Nesic et al. 1993; Nesic and Maquat 1994). Components of the spliceosome are not needed for 3'-end formation in vitro (Wahle and Keller 1996), but several observations indicate an involvement of the U1 snRNP in polyadenylation. In SV40 late RNA, U1 snRNP can bind just upstream of the polyadenylation signal (through its U1 RNA; Wassarman and Steitz 1993) and to the upstream polyadenylation efficiency element via its U1 snRNP-A protein (Lutz and Alwine 1994), which results in stimulation of polyadenylation. Polyadenylation is also increased by direct interaction between the U1 snRNP-A protein and the 160-kD subunit of the cleavage and polyadenylation specificity factor, CPSF (Lutz et al. 1996). U1 snRNP can also inhibit polyadenylation at specific sites (Ashe et al. 1997) and the U1 snRNP-A protein inhibits the poly(A) polymerase when bound to the 3' UTR of its own pre-mRNA (Gunderson et al. 1994).

The carboxy-terminal domain (CTD) of the RNA polymerase II large subunit appears to play an important role in synchronizing transcription and the pre-mRNA processing events (for review, see Neugebauer and Roth 1997). The phosphorylated CTD binds the capping enzymes (Cho et al. 1997; McCracken et al. 1997a). CTD overexpression (Du and Warren 1997) and CTD peptide addition to extracts (Yuryev et al. 1996) influence splicing in HeLa cells, which, together with observations that the hyperphosphorylated polymerase II binds snRNPs and SR proteins (Chabot et al. 1995; Mortarillo et al. 1996; Vincent et al. 1996; Kim et al. 1997), indicates that spliceosomal components are also associated with RNA polymerase II.

Furthermore, 3'-end formation may influence termination of transcription. An intact polyadenylation sequence is required for transcription termination (Whitelaw and Proudfoot 1986; Logan et al. 1987; Connelly and Manley 1988). Factors involved in 3'-end formation have been implicated in the coupling of transcription termination and 3' processing (Edwards-Gilbert et al. 1993). Mammalian CPSF (Dantonel et al. 1997; McCracken et al. 1997b) and CstF (cleavage stimulation factor; McCracken et al. 1997b) associate with the CTD of RNA polymerase II and mutations in the CTD that prevent this association lead to disturbed transcription termination (McCracken et al. 1997b). In addition, recent mutational studies in yeast have demonstrated that factors involved in pre-mRNA 3'-end cleavage are active in directing termination of transcription (Birse et al. 1998).

To better understand the individual steps of pre-mRNA formation and maturation in vivo and how these events are functionally integrated, quantitative descriptions of pre-mRNA maturation in intact eukaryotic nuclei would be useful as a complement to biochemical and genetic data.

Here, we describe the termination of transcription, excision of the 3'-terminal intron and cleavage/polyadenylation of the *Balbani ring 1* (*BR1*) gene pre-mRNA in the dipteran *Chironomus tentans*. *BR1* gene pre-mRNA

can be isolated as nascent transcripts during transcription, and as released transcripts during transport in the nucleus away from the active gene (Baurén and Wieslander 1994). This experimental system makes it possible to identify and measure the various pre-mRNA processing intermediates in vivo.

Results

Termination of transcription in the BR1 gene

We analyzed the termination of transcription in the *BR1* gene by studying the distribution of nascent pre-mRNA along the 3' end of the gene using two different methods.

RT-PCR The *BR1* gene is ~40 kb long and contains four introns. Exon 4 is unusually long, ~35 kb. It consists of ~150 tandemly arranged repeat units and is probably the result of repeat array expansions (Wieslander 1994). Intron 4 is located ~600 bp upstream of the polyadenylation sequence (Fig. 1A). We used as cDNA/3' PCR primers deoxyoligonucleotides complementary to the pre-mRNA at nine different positions at the 3' end (Fig. 1A). These positions started 310 bases upstream of the cleavage/polyadenylation site (position -310) and ended at position +1180 (we denote the position of the cleavage/polyadenylation site as zero and number the bases upstream -1, etc., and downstream +1, etc.).

One of two 5' PCR primers (A1 at -703 and A2 at -250) was used. The positions of the 5' PCR primers ensured that we did not detect any transcript that had been cleaved at the cleavage/polyadenylation site and subsequently elongated. In parallel, each primer pair was used in RT-PCR reactions on a fixed amount of an in vitro RNA transcript covering the same part of the *BR1* gene. The in vitro RNA transcript thus served as a standard to which each PCR product from the nascent pre-mRNA could be related (Fig. 1B). This enabled us to measure the relative amount of nascent pre-mRNA for each primer pair.

Figure 1C shows that the decrease in the amount of PCR products was almost linear between positions -310 and +450. The amount of PCR product reflects the number of nascent transcripts present from the position of each cDNA/3' PCR primer and downstream of this point, so the linear relationship shows that the RNA transcripts are evenly distributed along the template between positions -310 and +450. Because of the repetitive nature of exon 4 in the *BR1* gene (Wieslander 1994), we could not extend our measurements much further upstream than position -310. Upstream of the polyadenylation site, this linear decrease is expected as it has been observed in the electron microscope that the transcripts are almost evenly spaced along the entire *BR1* gene (Lamb and Daneholt 1979). Downstream of the cleavage/polyadenylation site, the number of full-length transcripts per unit length template is not changed. A line through the experimental points from position -310 to +450 crosses the value of zero transcripts between positions +500 and +700 (Fig. 1C). This region is referred to

Figure 1. Distribution of nascent transcripts on the *BR1* gene analyzed by RT-PCR. (A) Structure of the 3' end and the downstream region of the *BR1* gene shown together with the position of 5' PCR primers (A1 and A2) and 9 different cDNA/3' PCR primers (B–J). (B) Pairwise results of RT-PCR reactions with the cDNA/3' primers B–H (no signal was detected for H–J for the nascent pre-mRNA, only H is shown). In each pair, the left band (IV) represents a fixed amount of an in vitro transcript covering the 3' end and downstream region of the *BR1* gene. The band to the right (BR) represents the nascent *BR1* pre-mRNA. (C) Results quantitated by PhosphorImager analysis. The ratio of the nascent transcript signal to the in vitro transcript signal for each primer pair (A₁–B, A₁–C, A₂–D, A₂–E, etc., in B), as a measure of the number of transcripts, is plotted against the distance from the polyadenylation site. The polyadenylation site is at position 0. Upstream positions have negative values and downstream positions positive values.

as the +600 region. The data show an essentially regular spacing of transcripts up to, but not downstream of, the +600 region. No termination of transcription and no cleavage of transcripts at the polyadenylation site could therefore be detected before the RNA polymerases reach this region.

At positions +700, +813, and +1180 (H–J in Fig. 1A), we detected no signal after 26 PCR cycles. However, we could see small amounts of products after 35 cycles, with the weakest signal seen at position +1180 (data not shown). Therefore, full-length transcripts are also present downstream of the +600 region, but only in very small numbers. We estimate that after 26 PCR cycles, we would have been able to detect 1% of the signal obtained for the in vitro transcript. We detected a signal at the +450 position that is 35% of the in vitro transcript signal (G in Fig. 1A). Compared to the number of full-length transcripts present between position +450 and the +600 region, fewer than 1 of 35 transcripts is therefore present downstream of the +600 region.

RNA-ligase mediated RT-PCR (LM-RT-PCR) In the second method of measuring the distribution of transcripts along the 3' end of the *BR1* gene, a deoxyoligonucleotide was ligated to the 3' end of the nascent *BR1* pre-mRNAs to obtain a defined handle at the 3' end of each such nascent pre-mRNA transcript. A second, complementary, deoxyoligonucleotide was then annealed to the ligated deoxyoligonucleotide, serving as a start for cDNA synthesis. Subsequently, the 3' ends of

the transcripts, from a defined upstream position, were amplified by PCR.

Figure 2, lane 1, shows the distribution of the 3' ends of the nascent *BR1* pre-mRNA, measured from position –20. Many different-sized PCR fragments are seen with lengths from below 100 bp (the combined length of the PCR primers is ~70 bp) up to ~700 bp. The PCR products are relatively evenly distributed up to 500–550 bp and then gradually decline. We conclude that the size distribution of PCR products reflects an expected even distribution of the nascent pre-mRNA along the gene.

In these experiments, PCR products were seen only when reverse transcriptase was included in the cDNA reaction (Fig. 2, lane 3). We have shown that no degradation of RNA occurs during the ligation step (data not shown). When the position of the 5' PCR primer was shifted upstream, from position –20 to position –703 with the same cDNA population, the regular distribution of PCR products extended in length approximately a corresponding distance (Fig. 2, lane 2). This result rules out the possibility of extensive RNA degradation during extraction and LM-RT-PCR and shows that transcripts longer than 600 bases were present in the nascent pre-mRNA preparation. It also demonstrates that the drop in the amount of PCR products at a length of 600–700 bp in Figure 2, lane 1, is not attributable to an inability to detect longer fragments in the LM-RT-PCR experiment. We conclude that the pattern seen in the LM-RT-PCR assay agrees with our RT-PCR results above.

The RT-PCR results showed that a small number of

Figure 2. Distribution of nascent transcripts on the *BR1* gene analyzed by LM-RT-PCR. Autoradiographs of the final PCR products, separated on 6% sequencing gels. (M) Size markers. The same markers were used in both panels. The gel on the *right* was run a shorter distance. Sizes of the markers are given in bases (the two lowest bands, 151 and 140 bases respectively, are not seen in the *left* panel). (L) Second set of size markers, consisting of a ladder with a periodicity of 123 bases. (Lane 1) The 5' primer position was at position -20; (lane 2) the primer position was at position -700; (lane 3) no PCR product was obtained if reverse transcriptase was left out of the cDNA reaction; (lanes 4-6) the 5' primers were at positions +276, +496, and +790, respectively.

full-length transcripts are present downstream of the +600 region. In Figure 2, this was also seen as the presence of very small amounts of transcripts extending longer than 700 bases (lane 1).

By shifting the 5' PCR primer closer to the +600 region and increasing the number of PCR cycles to 35 or 40, we could better detect these long transcripts in our LM-RT-PCR assay. In Figure 2, lanes 4, 5, and 6, the 5' PCR primer was shifted to positions +276, +496, and +790, respectively. Then, we detected PCR products that depended on reverse transcriptase. The size distribution was considerably more uneven along the template when the 5' primer was shifted than it was when the 5' primer was at position -20 or -703 (Fig. 2, lanes 1,2). The size distribution was more uneven for locations far downstream (Fig. 2, lanes 5,6). In all cases there was an accumulation of transcripts at specific sites. This is consistent with the hypothesis that RNA polymerases rarely and randomly slip past the +600 region. Further, it is possible that the polymerases in addition are stalled at several preferred regions in the downstream chromatin fiber.

Cleaved nascent transcripts were not detected downstream of the +600 region When the polymerases reach the +600 region, virtually all RNA transcripts (>97%) are either cleaved at the polyadenylation site and/or transcription is terminated. If all the polymerases continue to transcribe after the pre-mRNA has been cleaved, short

nascent RNA transcripts should remain and be present downstream of the +600 region. These transcripts would then extend in the 5' direction, maximally up to the polyadenylation site. To look for such short transcripts, we isolated the nascent *BR1* pre-mRNA population by microdissection. In the subpopulation that had been transcribed past the +600 region (cDNA/3' PCR primer at position +700, Fig. 3A), we compared the number of long pre-mRNAs (pre-mRNAs that were detected by the 5' PCR primer located upstream of the polyadenylation site, A1 in Fig. 3A) with the number of potentially short transcripts (with a 5' PCR primer downstream of the polyadenylation site, A2 in Fig. 3A).

No RNA transcripts were detected in RT-PCR analysis after 26 cycles with either of the two 5' primers. Approximately similar signals with respect to the control in vitro transcript signals were seen after 35 cycles (Fig. 3B). This result is expected if both 5' primers see the same population of transcripts. We can therefore rule out the presence of substantial amounts of short RNA transcripts in the region downstream of the +600 region. If cleavage at the polyadenylation site is followed by a very rapid 5' → 3' degradation of the transcripts, resulting in very short transcripts (shorter than 150 bases), we would not detect them in this experiment.

Transcripts not cleaved at the polyadenylation site could not be detected in the nucleoplasm The PCR experiments could not distinguish between the possibility that the transcripts were released from the template as a result of transcription termination and the possibility that they were released as the result of endonucleo-

Figure 3. Short nascent transcripts are not found downstream of the +600 region in the *BR1* gene. (A) Positions of 5' PCR primers (A1 and A2) and a common cDNA/3' primer. (B) Result of RT-PCR analysis on one and the same population of nascent *BR1* gene pre-mRNA, using the cDNA/3' primer in combination with either the A1 5' primer (A1) or the A2 5' primer (A2). (IV) In vitro transcript. The numbers 26 and 35 indicate the number of PCR cycles.

Transcription, 3' processing, and intron excision

lytic cleavage at the 3' polyadenylation site. In an attempt to clarify this question, we investigated whether transcripts that had not been cleaved at the polyadenylation site could be detected in the nucleoplasm. If such transcripts were present it would suggest that transcripts are released from the template before 3'-end formation. Figure 4 shows the results obtained from RNA extracted from isolated nucleoplasm and analyzed for the presence of uncleaved *BR1* transcripts by RT-PCR. Lane 1 clearly shows transcripts with a 3' cDNA/PCR primer positioned upstream of the polyadenylation site (position -20). The 5' PCR primer was located upstream of intron 4, which explains why we recorded both spliced and unspliced transcripts. The spliced transcripts account for ~95% and the unspliced transcript for 5%, in agreement with previous results (Baurén and Wieslander 1994). In lane 2 of Figure 4, we used the same 5' PCR primer but shifted the position of the cDNA/3' PCR primer downstream of the polyadenylation site to position +105. Then, we were unable to detect any signal at all. We conclude that transcripts that have not been cleaved at the polyadenylation site can only be detected in the active *BR1* gene locus and not in the nucleoplasm.

Polyadenylation of the *BR1* pre-mRNA

The addition of a poly(A) tail to the *BR1* pre-mRNA was monitored by measurement of the length of the poly(A) tail in pre-mRNA isolated by microdissection from the *BR1* gene locus, the nucleoplasm, and the cytoplasm. Only picogram quantities of pre-mRNA could be obtained from the gene locus and the nucleoplasm, so we used the method of LM-RT-PCR.

Figure 5 shows the length distribution of the PCR fragments obtained after LM-RT-PCR. The size distribution for the cytoplasmic *BR1* mRNA centered around 60 adenylate residues, with a range from 0 to 150, on the basis of PhosphorImager analysis (Fig. 5, lane 2). The PCR

Figure 4. Uncleaved transcripts cannot be detected in the nucleoplasm. RNA was extracted from microdissected nucleoplasm and *BR1* pre-mRNA was analyzed by RT-PCR. In the -20 lane, the cDNA/3' PCR primer was located at position -20 (i.e., upstream of the polyadenylation site, D in Fig. 1A) and the 5' PCR primer at position -703, just upstream of intron 4 (A1 in Fig. 1A). The lower, thicker band is spliced transcripts and the upper, weaker band unspliced transcripts. The length of the intron is 55 bases. In the +105 lane, the 5' PCR primer was the same as for the -20 lane, but the position of the cDNA/3' PCR primer was at +105 (i.e., downstream of the polyadenylation site, E in Fig. 1A). The number of PCR cycles was 35.

Figure 5. Length distribution of poly(A) tails. The length of the poly(A) tail was analyzed by LM-RT-PCR. The PCR products were sized on a 6% sequencing gel. The 5' primer was at position -151 and the 3' PCR primer was 32 bases long. (Lanes 1,3) Result when reverse transcriptase was excluded from the cDNA reaction; (lane 2) cytoplasmic *BR1* mRNA; (lane 4) nucleoplasmic *BR1* pre-mRNA. Size markers are given in bases (M).

products from the center of this distribution were eluted and sequenced. The sequence showed that the PCR products were correctly polyadenylated pre-mRNAs and that the poly(A) tail was around 65 adenylate residues long (data not shown). This agrees with the length measured according to Parker and Decker (1993). We conclude that *BR1* mRNA has a relatively short poly(A) tail in the cytoplasm, with an average length of 60 adenylate residues.

The length distribution of the poly(A) tail of *BR1* pre-mRNA in the nucleoplasm was broader than that of the cytoplasmic mRNA, ranging from 0 to 300, on the basis of PhosphorImager analysis, and that the average length was greater, ~100 adenylate residues (Fig. 5, lane 4).

We could also detect polyadenylated *BR1* pre-mRNA associated with the microdissected *BR1* gene locus. This polyadenylated pre-mRNA made up a very small amount (<0.2%) of the total *BR1* pre-mRNA molecules present at the gene locus at any time (see below). Therefore, we found that we had to enrich the population to measure the length of the poly(A) tail. First, we converted the total pre-mRNA present in the *BR1* gene locus into PCR fragments, using the LM-RT-PCR method. The 5' primer was located at position -151. The PCR fragments that were obtained were digested with *DraI*, as the *BR1* gene has a cleavage site for this enzyme 23 bp downstream of the poly(A) addition site. PCR fragments corresponding to nascent transcripts downstream of this position should then have been cleaved, but PCR fragments corresponding to polyadenylated pre-mRNA should not. Subsequently, we reamplified the *DraI*-digested PCR fragments using the same 5' primer and a modified 3' primer. This 3' PCR primer had two thymidine residues added to its 3' end to favor amplification in the LM-RT-PCR step of sequences having poly(A) tails juxtaposed to the ligated oligonucleotide.

The PCR products were sized on a sequencing gel (Fig. 6, lane 1) and compared to a non-*DraI* cleaved sample

Figure 6. Length distribution of poly(A) tails in *BR1* pre-mRNA isolated from the *BR1* gene locus. The pre-mRNA was analyzed by LM-RT-PCR, and the PCR products were separated on a 6% sequencing gel. (M) Size markers given in bases. (Lane 1) *DraI* cleaved; (lane 2) *DraI* cleaved -RT control; (lane 3) non-*DraI*-cleaved -RT control; (lane 4) non-*DraI* cleaved. The positions of the bands at 190 and 200 bases are marked.

that had otherwise been treated identically (Fig. 6, lane 4). The distance from the 5' primer to the polyadenylation site is 151 bases, and the 3' PCR primer is 32 bases long. Thus, all PCR fragments shorter than 183 bases are nascent transcripts. Apart from such short fragments, two rather narrow bands are seen in both lane 1 and lane 4, centered around 190 and 200 bases in length. The top band was more accentuated in the *DraI* cleaved sample (lane 1), being more clean at its upper side. As discussed above, 183 bases are accounted for by the length between the 5' primer and the polyadenylation site. Taking into account the width of the bands, the poly(A) tail lengths in the two bands is about 10 (± 5) and 20 (± 3) respectively. The band corresponding to 10 adenylate residues was stronger. Both bands from lanes 1 and 4 were eluted and sequenced (data not shown), and were indeed correctly polyadenylated *BR1* pre-mRNA with the calculated number of adenylate residues. In addition, the eluted fragments were cloned into a plasmid vector and a small number of clones were sequenced (data not shown), confirming the results of the direct sequencing.

In conclusion, *BR1* pre-mRNA polyadenylation is initiated at the site of the active gene. Polyadenylation proceeds during transport through the nucleoplasm and reaches an average length of 100 adenylate residues. In the cytoplasm, the poly(A) tails are shortened, resulting in an average length of about 60 adenylate residues.

Excision of the 3'-terminal intron

We wanted to study the relationship between excision of the 3'-terminal intron, intron 4, transcription termination and cleavage/polyadenylation. Therefore, we analyzed *BR1* pre-mRNA populations of defined processing intermediates and measured the fraction of pre-mRNA in which intron 4 had been excised in each population.

Intron excision in nascent *BR1* transcripts We initially measured the proportion of spliced pre-mRNA in the na-

scent population that was located downstream of the polyadenylation site. The cDNA/3' PCR primer was at position +125 and the 5' PCR primer at position -703, upstream of intron 4. The spliced nascent transcripts comprised 5%–6% of the total (data not shown), meaning that ~5%–6% of all nascent transcripts present from this point downstream had lost intron 4. Most of these spliced transcripts were present between position 125 and the +600 region as could be seen from the distribution of transcripts (Fig. 1C).

It has been suggested that removal of the 3'-terminal intron inhibits 3' cleavage and polyadenylation of pre-mRNA (Liu and Mertz 1993). To investigate this possibility, we compared the proportion of nascent transcripts that had excised intron 4 from two subpopulations, one consisting of all transcripts from position +125 and downstream and one consisting of the transcripts present from position +700 and downstream (Fig. 7A,B). In this experiment, we had to increase the number of PCR cycles to 35 to detect a significant signal. This means that the relative proportion of spliced transcripts could not be accurately quantitated. An increase in the number

Figure 7. Excision of intron 4 in nascent and polyadenylated *BR1* pre-mRNA. (A) The structure of the 3' end and downstream region of the *BR1* gene together with the position of the labeled 5' PCR primer and two unlabeled alternative cDNA/3' PCR primers (A and B). The position of an *EcoRV* cleavage site used to cleave the PCR products prior to electrophoretic separation is indicated. (B) RT-PCR analysis of the same population of nascent *BR1* gene pre-mRNA with the two alternative primer pairs is shown. (A) The 5' primer was combined with the A primer; (B) the 5' primer was combined with the B primer. The top band represents the unspliced pre-mRNA and the lower band represents the spliced pre-mRNA. (C) RT-PCR analysis of polyadenylated pre-mRNA at the *BR1* gene locus and in the nucleoplasm. RNA was isolated from the microdissected *BR1* gene locus (*BR1*) and nucleoplasm (NP) and the proportion of pre-mRNA with (unspliced) or without (spliced) intron 4, was determined.

of cycles gave an overestimation of the smaller of the two populations, in this case, the spliced molecules. Our results show that 5%–6% for the +125 primer population (Fig. 7B, lane A) and 7%–10% for the +700 primer population (Fig. 7B, lane B) were spliced, indicating that a somewhat greater proportion of transcripts lose intron 4 downstream of the +600 region, but that there was no substantial overrepresentation of spliced transcripts. This result also indicates that the 3'-terminal intron can be excised without interference from transcription termination and 3'-end formation.

Intron excision in polyadenylated *BR1* pre-mRNA at the active gene locus and in the nucleoplasm Using an oligo (dT) primer for cDNA synthesis with a specific sequence extension at its 5' end, a 3' PCR primer complementary to the extension sequence and a 5' PCR primer located upstream of intron 4 (position –703), we measured the fraction of spliced pre-mRNA in the population of polyadenylated pre-mRNA associated with the *BR1* gene locus. We found that a vast majority of these pre-mRNAs with short poly(A) tails have lost intron 4 (Fig. 7C, *BR1* lane). The proportion of spliced pre-mRNAs was between 76% and 83%, with an average of 80%, as measured by PhosphorImager analysis in four independent experiments.

Figure 7C also shows that in pre-mRNA that has been transported through the nucleoplasm away from the *BR1* gene locus, the proportion of spliced molecules has increased to 95% (NP lane; the range from four independent experiments was 94%–96%, with an average of 95%). In the cytoplasm no unspliced mRNAs could be seen (data not shown).

In conclusion, intron 4 is excised from 5% of the transcripts within 1.2 kb of transcription after the intron appears in the pre-mRNA (the distance from intron 4 to the +600 region). Closely linked in time to cleavage and addition of the first 10–20 adenylate residues and to termination of transcription, intron excision then proceeds very rapidly. Intron excision is completed during transport away from the gene locus.

Discussion

Termination of transcription

We have demonstrated that in the active *BR1* gene, all RNA polymerases continue transcription to a region 500–700 bp downstream of the polyadenylation sequence. Downstream of this region, very few RNA transcripts are present. Our experimental data do not allow the conclusion that a defined sequence for termination of transcription is present in the *BR1* gene. Two observations are, however, consistent with >95% of the polymerases leaving the DNA template in a restricted region. First, no short nascent transcripts were detected upstream or downstream of the +600 region. This argues against a model in which pre-mRNA cleavage at the polyadenylation site precedes termination of transcription (Proudfoot 1989). Second, analysis of the chromatin

upstream and downstream of the +600 region in the *BR1* gene suggests differences in organization. In attempts to study the chromatin organization (data not shown), we have experimentally increased the density of polymerases on the active gene to approximately one polymerase per 100 bp. This leads to disruption of the regular micrococcal nuclease digestion pattern (Belikov et al. 1998). The nucleosomal pattern is disturbed at the oligonucleosomal level upstream of the +600 region, while the region immediately downstream is not. This observation supports the interpretation that most RNA polymerases leave the DNA template at the +600 region.

According to run-on experiments, transcription in a number of genes continues for a variable distance, 0.3–4 kb, downstream of the polyadenylation sequence and the density of polymerases gradually decreases (for references, see Proudfoot 1989). A few studies, however, have identified defined termination sequences at which efficient transcription termination occurs (Enriquez-Harris et al. 1991; Owczarek et al. 1992; Tantravahi et al. 1993). These sequences are different in different genes, but a common feature is an A-rich region. In the *BR1* gene termination region the sequence AGAAAAAAATG is found at position +690.

In chromatin spreads of *BR* genes, the nascent transcripts grow gradually longer and are evenly spaced until they abruptly disappear (Lamb and Daneholt 1979). The final transcript is followed by an array of nucleosomes in which the first nucleosome appears to be disrupted. Our molecular data agree with the morphological observation. It is also obvious from our data that transcription is not fully terminated in this region. Rarely, unprocessed transcripts are present downstream of the +600 region in the *BR1* gene. These rare transcripts are presumably difficult to detect morphologically.

*Polyadenylation of the *BR1* pre-mRNA*

The *BR1* mRNA is very abundant in the cytoplasm and has a half-life of about 20 hr (Edström et al. 1978). In the cytoplasm, it has a short poly(A) tail that, on average, is about half the length of the poly(A) tail of the nucleoplasmic precursor. This observation agrees with previous findings of the fate of isotopically labeled poly(A) in *BR* gene mRNA (Egyházi et al. 1979). We also analyzed the polyadenylation events within the nucleus. All *BR1* pre-mRNA in the nucleoplasm had been cleaved at the polyadenylation site (Fig. 4), indicating that cleavage/polyadenylation is closely linked to the release of the transcripts from the gene template. We also found that transcription proceeds for about 600 bp after the AAUAAA sequence has appeared in the transcript. It is a general finding that termination of transcription occurs at a distance from the poly(A) sequence (Proudfoot 1989; Tantravahi et al. 1993). This finding indicates that the AAUAAA sequence is not immediately available for the CPSF and CstF, which are associated with the RNA polymerase II CTD. It also suggests that the assembly of all components of the 3' cleavage complex requires some time. The transcription elongation rate in the *BR1* gene

is 30–35 bases/sec, which means that ~20 sec elapse after the appearance of the AAUAAA sequence before cleavage takes place at the polyadenylation site.

The poly(A) tails of the *BR1* pre-mRNA that was still associated with the active gene locus were preferentially of two different lengths, 10 or 20 adenylate residues. Distinct changes in the polyadenylation complex, both after addition of 10 adenylate residues and after addition of 30 adenylate residues have been observed in vitro (Bardwell and Wickens 1990). It is well established that in vitro the poly(A) further polymerase requires the stimulation of both CPSF and poly(A)-binding protein II (PAB II) to switch from a distributive to a processive elongation mode and that PAB II needs at least 10 adenylate residues for binding (Wahle and Kühn 1997). Our data from in vivo experiments also suggest that the early phase of polyadenylation differs from the later phase and that transitions in the polyadenylation complex take place during the early phase. It is quite possible that the early phase occurs while the transcript still remains associated with the active gene, perhaps in a cleavage complex (Wahle and Kühn 1997) whereas the processive elongation mode takes place in the *BR1* pre-mRNP during transport through the nucleoplasm.

Coupling between termination of transcription, cleavage/polyadenylation, and intron excision

We have analyzed only that part of the pre-mRNA population present at the *BR1* gene locus that contains sequences complementary to the cDNA and 3' PCR primers. This population contains several subpopulations: (1) nascent transcripts on the final 1.2 kb of the gene (these transcripts comprise $\sim 1/35$ of the whole nascent pre-mRNA spectrum on the *BR1* gene); (2) pre-mRNAs that have been cleaved at the polyadenylation site and on which polyadenylation has been initiated; and possibly (3) cleaved but not yet polyadenylated pre-mRNA. Data from in vitro experiments suggest a very tight coupling between cleavage and polyadenylation (see Wahle and Keller 1996; Wahle and Kühn 1997). If this is also true in vivo, the latter of the three subpopulations cannot be formed.

We could measure the proportion of pre-mRNA from which intron 4 had been excised in the combined population (~10%, data not shown; see also Baurén and Wieslander 1994), and in the two first subpopulations separately (~5% and 80% respectively). This allowed us calculate the relative proportion of the pre-mRNA processing intermediates present at the active gene locus. We found that the nascent transcripts account for ~93% of the analyzed population. More than 97% of these nascent transcripts are present between position -600 and the +600 region, while <3% are present downstream of this region. Our data show that nascent transcripts do not accumulate during transcription termination (Figs. 1 and 2). The polyadenylated pre-mRNAs make up ~7% of the analyzed population, and we conclude that polyadenylated pre-mRNAs are present at the active gene locus transiently. The polyadenylated pre-mRNAs make up

<0.2% of the full spectrum of nascent pre-mRNA on the entire *BR1* gene.

We have not been able to detect *BR1* pre-mRNA that has been released from the gene before 3'-end formation (Fig. 4), nor have we detected short transcripts at the gene locus, indicative of cleavage at the polyadenylation site prior to transcription termination (Figs. 1 and 3). We have, however, demonstrated that 3'-end formation of *BR1* pre-mRNA is initiated at the active gene locus (Fig. 6). Our estimations of the proportions of the different types of pre-mRNA at the end of the *BR1* gene (see above) allow us to conclude that the vast majority of the transcripts leave the gene locus as they reach the +600 region. The transcription rate in the *BR1* gene is ~30 bases/sec. All nascent transcripts present at a given time between -600 and the +600 region will reach the +600 region within 40 sec. Less than 3% will continue to be transcribed past this region and at a given moment only 7% remain at the gene locus as polyadenylated transcripts. Unless considerable degradation of pre-mRNA occurs, almost 90% of the transcripts must therefore leave the gene template, initiate 3'-end formation and move away from the gene locus in a short time period. The ratio between nascent (93%) and polyadenylated (7%) transcripts suggests that this time period is of the order of a few seconds. We suggest that there is a spatial and temporal coupling between transcription termination and 3'-end formation. Such an interpretation agrees with previous observations that transcription termination depends on a polyadenylation sequence (Whitelaw and Proudfoot 1986; Logan et al. 1987; Connelly and Manley 1988) and with recent genetic evidence that factors involved in the endonucleolytic cleavage part of 3'-end formation also direct termination of transcription (Birse et al. 1998).

The excision of the 3'-terminal intron, intron 4, commences rapidly and at the same time as transcription. This is as expected, since the spliceosome components assemble as soon as the intron appears in the nascent pre-mRNA (Wetterberg et al. 1996). Five percent of the nascent transcripts have excised the intron within 1.2 kb of its appearance in the growing transcript and before 3'-end formation. The proportion of polyadenylated *BR1* gene pre-mRNA that lacks intron 4 increases from 80% to 95% as it moves from the active gene locus to the nucleoplasm. We conclude that excision of the 3'-terminal intron and 3'-end formation can occur in any order.

It has been suggested that selection of the cleavage/polyadenylation site must precede the excision of the 3'-terminal intron (Liu and Mertz 1993). Our data do not rule out such a connection. We do, however find that both spliced and unspliced nascent transcripts slip by the +600 region without being 3' processed and no accumulation of spliced pre-mRNA is evident. All the *BR1* pre-mRNA that we could detect in the nucleoplasm was 3' processed. This means that the few nascent transcripts downstream of the +600 region are either substrates for 3' processing or that they are degraded. At the same time, the most significant result is that excision of the 3'-terminal intron is drastically increased when cleavage

at the polyadenylation site and polyadenylation occurs. The fraction of transcripts in which intron 4 has been excised, increases from 5% to 80%, while the pre-mRNA is still associated with the active gene locus, but before 10–20 adenylate residues are added to the 3' end. We estimate that the degree of stimulation of splicing is between 15- and 20-fold. As the export of polyadenylated transcripts is rapid as soon as 10–20 adenylate residues have been added, it is possible that the degree of stimulation of splicing is even higher. We can compare the excision of intron 4 with the rate of cotranscriptional excision of intron 3 in the same gene (Baurén and Wieslander 1994). Intron 3 is excised from 50% of the nascent transcripts after 2.5 min (corresponding to 5 kb of transcription) and from about 80% of the transcripts within 10 min (corresponding to 20 kb of transcription). With these values in mind, a stimulation of excision of intron 4 coupled to 3'-end formation of 15- to 20-fold is a reasonable figure, although it should be remembered that different introns can be excised at different efficiencies (Wetterberg et al. 1996). Therefore, our data are consistent with the hypothesis that initiation of intron excision can occur without interaction between splicing and 3' processing components. The acceleration of intron excision as cleavage and polyadenylation occur, however, suggests that such interactions take place in the *BR1* pre-mRNA in vivo at the time when the 3' processing factors assemble.

Materials and methods

Animals

C. tentans was cultured as described (Meyer et al. 1983). Salivary glands from fourth instar larvae were used in all experiments.

Isolation of nascent and nucleoplasmic pre-mRNA and cytoplasmic mRNA

Salivary glands were fixed in ethanol/acetic acid (3:1) for 30 min on ice. The glands were then rinsed three times in 70% ethanol for 10 min each time on ice and stored in ethanol/glycerol (1:1, by vol) at -200°C . The glands were placed on the lower side of a cover glass in a small drop of ethanol:glycerol and surrounded by paraffin oil. Nuclei, individual chromosomes, *BR1* gene loci, nucleoplasm or cytoplasm were dissected with glass needles, using a de Fonbrune micromanipulator (Lambert and Daneholt 1975). During dissection, the nuclei were viewed in phase contrast at a magnification of 100–400 $\times$ in a Zeiss microscope.

Isolated nuclear components or cytoplasm were transferred to a microcentrifuge tube, and RNA was extracted as described previously (Baurén and Wieslander 1994). Total salivary gland RNA was extracted from whole salivary glands as described by Edström et al. (1982).

RT-PCR

Nascent pre-mRNA to be used in RT-PCR was purified by hybridization to 5'-biotinylated deoxyoligonucleotides complementary to specific positions at the 3' end of the *BR1* pre-mRNA, followed by binding to magnetic Dynabeads M280 coated with streptavidin as described by the manufacturer (Dy-

nal). Nucleoplasmic pre-mRNA was used without this purification step. The cDNA synthesis and subsequent PCR were performed as described previously (Baurén and Wieslander 1994).

In all PCR analyses, two controls were included. One contained the negative control from the cDNA reaction in which no reverse transcriptase was included, and the other control contained all the PCR components but no cDNA. Tests were performed to ensure that the amplification was in the exponential phase and that the PCR products reflected the amount of initial RNA.

The PCR products were extracted with phenol/chloroform (1:1 by volume), ethanol precipitated, and analyzed on 4%–6% polyacrylamide sequencing gels. The results were analyzed with a PhosphorImager (Molecular Dynamics) and the software ImageQuant (Molecular Dynamics).

For analysis of the proportion of spliced and unspliced pre-mRNA, the PCR fragments were ethanol precipitated, dissolved in the recommended *EcoRV* buffer, and digested with *EcoRV* (New England Biolabs) overnight before loading on to the sequencing gels.

A 3.5-kb *EcoRI* fragment of a *BR1* gene genomic clone was subcloned into Bluescript (Stratagene) and transcribed in vitro by T7 RNA polymerase (Megascript, Ambion). The in vitro transcript was purified by agarose electrophoresis. After elution from the gel, the concentration was measured by absorption at 260 nm, and a fixed amount of the transcript was reverse transcribed and amplified by PCR as described above.

LM-RT-PCR

BR1 pre-mRNA was extracted from 100 microdissected *BR1* gene loci (–500–1000 pg of nascent pre-mRNA) as described above. A total of 6 pmoles of a 3'-NH₂-deoxyoligonucleotide was ligated to the 3' ends of the RNA in 50 mM Tris-HCl (pH 8.0), 10 mM Mg₂Cl₂, 10 $\mu\text{g}/\text{ml}$ BSA, 1 mM hexamine cobalt chloride, 25% PEG, 20 mM ATP, 2 mM DTT, 30 units of RNasin, and 15 units of T4 RNA ligase in a total volume of 15 μl for 6 hr at 18°C . Conditions for ligation of deoxyoligonucleotides to single-stranded DNA have been reported by Tessier et al. (1986), and components used were partially obtained from Clontech. A total of 8 μl of the ligation mixture was mixed with 20 pmoles of an deoxyoligonucleotide complementary to the ligated NH₂-deoxyoligonucleotide and heated at 65°C for 5 min. cDNA synthesis was performed at 45°C for 45 min in a total volume of 30 μl . The reaction mixture contained 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM Mg₂Cl₂, 10 mM DTT, 1 mM dNTPs, 50 units of RNasin, and 10 units of AMV reverse transcriptase (Clontech) or M-MLV reverse transcriptase (SuperScript, GIBCO BRL).

The reaction was stopped by addition of 1 μl 0.5 M EDTA and the RNA was hydrolyzed at 65°C for 30 min after addition of 6 M NaOH. Acetic acid (6 M) was added to neutralize the pH before the cDNA was purified on a glass matrix support (Geno-Bind, Clontech) as described by the manufacturer and eluted in 50 μl of water. A total of 5–20 μl was used for PCR (hotstart, 26 cycles of 30 sec at 94°C , 30 sec at 50°C and 60 sec at 72°C). The 5' primer, complementary to an internal sequence, was ³²P-labeled by T4 polynucleotide kinase and the 3' primer was identical to the cDNA primer. The PCR products were separated on 5%–8% polyacrylamide sequencing gels. The results were analyzed by use of a PhosphorImager (Molecular Dynamics) and the software ImageQuant (Molecular Dynamics).

Measurement of the length of poly(A) tails

Pre-mRNA and mRNA used for poly(A) tail measurements were ligated to the 3' NH₂-deoxyoligonucleotide and RT-PCR was

carried out as described above. The length of the poly(A) tail on cytoplasmic *BR1* gene mRNA was also measured by deoxyoligonucleotide-directed RNaseH cleavage of *BR1* gene mRNA and Northern blot analysis, essentially as described by Decker and Parker (1993).

Sequence analysis of PCR products

The PCR products that were of the correct size to be splicing intermediates were located by autoradiography, cut out from the dried polyacrylamide gel and eluted in 200 μ l of water. After boiling for 15 min and a short centrifugation, 180 μ l of clear liquid was transferred to a second tube and precipitated with 3 vol of ethanol after the addition of 1/10 vol of 3 M sodium acetate, with 40 μ g of glycogen as carrier. The pellet was dissolved in 20 μ l of water, and 10 μ l was used for reamplification by PCR. The 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 sequences were analyzed by use of the computer program GAP from GCGs Wisconsin package 8.0 (Devereaux et al. 1984).

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Transcriptional termination in the *Balbani ring 1* gene is closely coupled to 3'-end formation and excision of the 3'-terminal intron

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