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Assembly and disassembly of spliceosomes along a specific pre-messenger RNP fiber

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Transcriptionally active Balbiani ring (BR) genes in the salivary glands of the dipteran *Chironomus tentans* were studied by immunoelectron microscopy to establish the distribution of spliceosome components along a specific pre-messenger ribonucleoprotein (pre-mRNP) fiber. The BR genes are 35–40 kb in size with three introns close to the 5′ end and one close to the 3′ end; a very large middle portion lacks introns. As a rule the 5′ introns are spliced concomitant with transcription in the promoter proximal third of the gene, while the 3′ intron is spliced post-transcriptionally. The BR genes with growing pre-mRNPs were visualized *in situ*, while completed and released pre-mRNPs were isolated from the nucleoplasm and studied unfolded on a grid surface. An anti-snRNP antibody (Y12) bound mainly to the promoter proximal third of the BR gene (86%) and only to a minor extent to the middle and distal thirds (7 and 7% respectively). An antibody to an hnRNP protein reacted with the proximal, middle and distal regions to an increasing extent (17, 38 and 45% respectively), reflecting the increase in size of the growing transcription product. In the nucleoplasmic pre-mRNP particle only one end of the RNP fiber was labeled by Y 12, presumably the 3′ end; the anti-hnRNP antibody decorated the entire RNP fiber. Thus, the snRNPs do not associate along the whole pre-mRNP fiber but rather bind to the 5′ and 3′ ends, i.e. the regions containing the introns. The results also imply that the spliceosomes both assemble and disassemble rapidly on the pre-mRNP fiber.

Key words: hnRNP/perichromatin fiber/pre-messenger RNP/snRNP/spliceosomes

Introduction

Splicing of RNA takes place in a specific organelle, the spliceosome (Brody and Abelson, 1985; Maniatis and Reed, 1987). Spliceosomes and the splicing process have been extensively studied *in vitro*: splicing components have been identified, as well as the details of the splicing reaction (Green, 1991; Steitz, 1992; Wassarman and Steitz, 1992; Lamond, 1993). Small nuclear ribonucleoprotein particles have been implicated (Lührmann *et al.*, 1990; Zieve and Sauterer, 1990; Mattaj *et al.*, 1993) and as many as 50–100 protein factors (Reed, 1990; Ruby and Abelson, 1991; Bennet *et al.*, 1992). *In vivo*, the synthesis and assembly of spliceosomes is a complex process and to fully understand splicing it is necessary to know how the spliceosome components are being synthesized, stored and delivered to the sites where splicing takes place (Gall, 1991; Huang and Spector, 1992).

Within the cell nucleus, pre-mRNA (often referred to as hnRNA) appears as perichromatin fibers or granules (Fakan and Puvion, 1980). The fibers (and granules) represent ribonucleoprotein complexes (hnRNPs) (Fakan *et al.*, 1984, 1986) consisting of a complex set of proteins associated with the pre-mRNA (Dreyfuss *et al.*, 1993). It was also established at an early date in biochemical experiments that snRNAs co-sediment with these complexes (Deimel *et al.*, 1977; Howard, 1978). As has been shown more recently by immunoelectron microscopy, snRNP proteins (Fakan *et al.* 1984; Puvion *et al.* 1984) and splicing factors (Spector *et al.*, 1991) are bound to the perichromatin fibers. Putative spliceosomes have been directly visualized on growing RNP fibers on active genes spread according to Miller (Osheim *et al.*, 1985; Beyer and Osheim, 1988). Thus, there is ample evidence that splicesomes are present *in vivo* on the perichromatin fibers containing pre-mRNA.

It is less clear to what extent the assembly process takes place on the pre-mRNA molecule. The synthesis of snRNP particles has been extensively studied: snRNA is synthesized in the nucleus, it is transported to the cytoplasm and it re-enters the nucleus associated with proteins as snRNP particles (Dahlberg and Lundh, 1988; Mattaj 1988; Zieve and Sauterer, 1990). In the nuclei of mammalian cells, the snRNP particles appear not only on the perichromatin fiber, but also in clustered RNP granules, called interchromatin granules (Fakan *et al.* 1984; Puvion *et al.* 1984; Spector *et al.* 1991). A similar situation prevails in amphibian oocytes. The snRNPs are present in the transcription loops of the lampbrush chromosomes, as well as in extrachromosomal RNP-containing bodies, designated snurposomes (Gall and Callan, 1989; Wu *et al.*, 1991). One possibility is that snRNPs are channeled via the interchromatin granules (and snurposomes) to the perichromatin fiber. As the B type snurposomes contain all the snRNPs, as well as splicing factors (Wu *et al.*, 1991), it has even been suggested that spliceosomes could assemble before being delivered to the nascent transcript. Alternatively, the interchromatin granules could represent mainly a storage site for snRNP particles, and as a rule snRNPs reach pre-mRNA directly.

It is not yet clear whether, when the spliceosome components associate with the growing transcripts *in vivo*, they bind along the entire RNP fiber or just at the splice sites. In amphibian lampbrush chromosomes, splicesosome components are present along essentially all loops and the distribution seems even under the fluorescence microscope

(Gall and Callan, 1989; Wu *et al.*, 1991). Furthermore, snRNPs are present in essentially all chromosomal puffs, including the heat-shock puffs (Sass and Pederson, 1984; Vazquez-Nin *et al.*, 1990; Matunis *et al.*, 1993), which has also been taken as evidence for the view that snRNPs are bound to nacent RNPs in a non-discriminant fashion. On the other hand, when active genes from *Drosophila* embryos are spread on a grid surface and studied in the electron microscope, putative spliceosomes are only recorded at the splice sites, indicating a specific assembly there (Osheim *et al.*, 1985; Beyer and Osheim, 1988). It should, however, be noted that all these studies have their limitations. The lampbrush chromosome studies were not performed at high resolution and the intron arrangement along the individual lampbrush loops is not known. Chromosomal puffs are large structures and often comprise several different active genes, making it difficult to draw firm conclusions at the cytological level. In the studies of spread genes, spliceosome components were not directly assayed for and, furthermore, loosely bound spliceosomes/spliceosome components could have been lost during the spreading of the chromosomes at low ionic strength.

In the present study, we have analyzed when and where spliceosomes (or spliceosome components) assemble and disassemble on pre-mRNA in a well-defined transcription unit. We have chosen to study Balbiani rings (BRs), a small set of chromosomal puffs in the salivary glands of the dipteran *Chironomus tentans*. The BRs harbor 35–40 kb long genes encoding secretory proteins (Wieslander, 1994). The genes contain three introns close to the 5′ end and one close to the 3′ end, i.e. the proximal introns are separated from the single distal intron by an exon of the order of 30 kb in size. By analysis at the RNA level it has recently been shown for one of the 5′ introns that it is spliced rapidly in the promoter proximal portion of the gene concomitant with transcription, while the 3′ intron was found to be spliced mainly post-transcriptionally (Baurén and Wieslander, 1994). The active BR genes are well-suited for electron microscopic analysis: the growing RNP fibers can be visualized *in situ* along the gene and the assembly of the fibers can be followed sequentially (Skoglund *et al.*, 1983). Furthermore, due to the exceptional size of the exon distal to the three 5′ end introns, both the assembly and disassembly of spliceosomes at the 5′ end of the transcript can be scrutinized concomitant with transcription. In addition, the completed BR RNP products released into the nucleoplasm can be isolated (Wurtz *et al.*, 1990) and analyzed with regard to the distribution of spliceosome components.

Using a monoclonal anti-snRNP antibody to locate spliceosome components and an anti-hnRNP antibody for comparison, we observed by immunoelectron microscopy that the snRNPs are almost exclusively associated with the transcripts in the proximal third of the active BR gene. In isolated, released nucleoplasmic particles they are confined to only one end of the RNP fiber, presumably the 3′ end. In contrast, hnRNP proteins are distributed along the entire transcripts. We conclude that spliceosome components are only transiently associated with pre-messenger RNP (pre-mRNP) and then selectively in regions with introns. Both the assembly and disassembly of spliceosomes are rapid processes on the pre-mRNP fiber, intimately related to the splicing event.

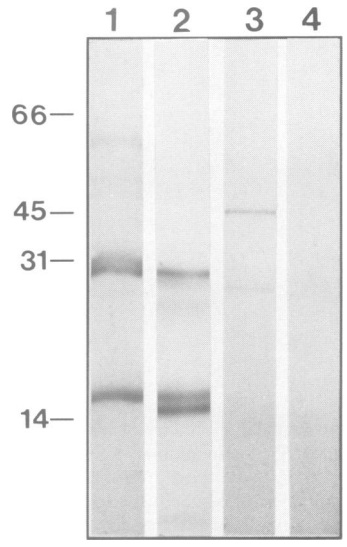

Fig. 1. Western blot analysis of *C.tentans* and HeLa cell proteins using the monoclonal anti-snRNP antibody Y12 and the anti-hnRNP protein antibody 2E4. A HeLa cell extract was analyzed in lane 1 and a *C.tentans* salivary gland extract in lanes 2–4. The Y12 antibody was applied to lanes 1 and 2, the 2E4 antibody to lane 3 and a monoclonal anti-von Willebrand factor (negative control) to lane 4. Size markers (kDa) are shown to the left.

Results

Characterization of the antibodies

To identify the location of spliceosomes we used the monoclonal antibody Y12, which is specific for the Sm epitope present on several polypeptides common to the U1, U2, U4/U6 and U5 snRNPs involved in splicing (Lerner *et al.*, 1981; Pettersson *et al.*, 1984). A monoclonal antibody, 2E4, which recognizes an abundant 45 kDa hnRNP protein in *C.tentans* (T.Wurtz and B.Daneholt, in preparation), served as positive control. As the negative control we used a monoclonal antibody against the human von Willebrand factor. Prior to their application in the immunoelectron microscopy experiments, the antibodies were tested by Western blotting and immunocytology.

The Y12 antibody was first probed against HeLa nuclear proteins. Three main proteins were recorded, with apparent mol. wt of 17, 28 and 30 kDa (lane 1 in Figure1), which represent the D, B and B′ snRNP proteins respectively (Lerner *et al.*, 1981; Pettersson *et al.*, 1984; Paterson *et al.*, 1991). In the *Chironomus* salivary gland sample we also detected three major fractions, corresponding to 15, 17 and 28 kDa (lane 2). This pattern is strikingly similar to that observed in *Drosophila melanogaster* with the Y12 antibody (Paterson *et al.*, 1991; Matunis *et al.*, 1993). Based on the similarity in size and the cross-reactivity, we tentatively identify the *Chironomus* 15 and 17 kDa proteins as counterparts to the human D protein and the 28 kDa protein to the human B protein.

As expected, the 2E4 antibody reacted exclusively with the 45 kDa protein in a *Chironomus* salivary gland sample (lane 3 in Figure 1), while the negative control antibody did not recognize any proteins in the *Chironomus* sample (lane 4).

The three antibodies were also tested in immunocytochemical experiments on *Chironomus* polytene chromosomes. The Y12 and the 2E4 antibodies bound to

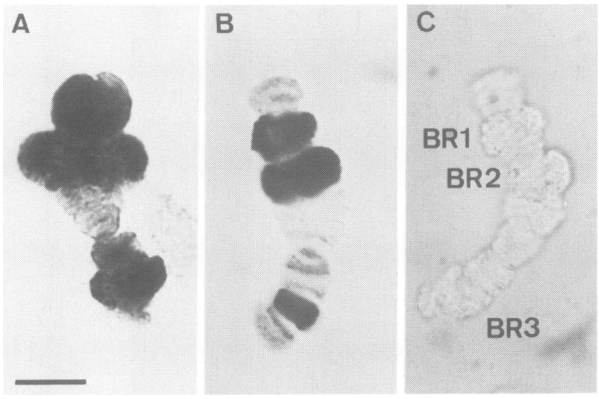

Fig. 2. Immunocytochemical specificity of the anti-snRNP antibody Y12 and the anti-hnRNP protein antibody 2E4. In each experiment isolated chromosomes IV were fixed in formaldehyde and incubated with one of the antibodies. Gold-conjugated secondary antibody was added, the immunoreaction was developed by silver enhancement and the specimen was mounted. Three chromosomes IV are shown as examples: one treated with the anti-snRNP antibody Y12 (**A**), one with the anti-hnRNP protein antibody 2E4 (**B**) and one with a negative control antibody (**C**). Balbiani rings 1–3 are indicated. Bar 10 μm.

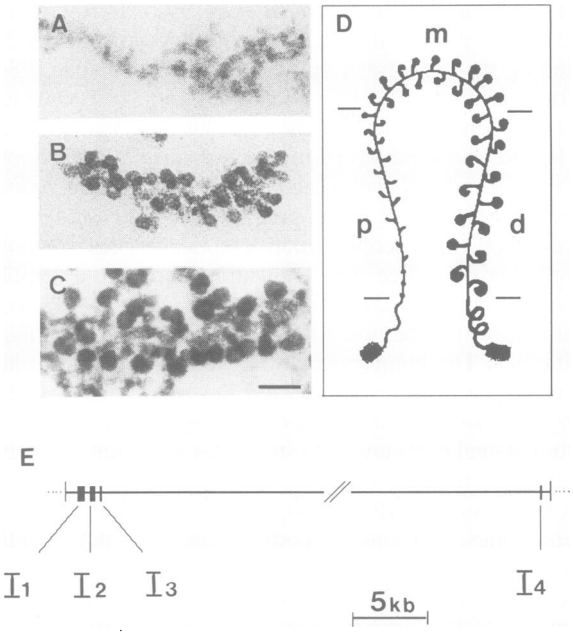

Fig. 3. An active Balbiani ring gene with its growing RNP particles. The promoter proximal, middle and distal thirds of the active gene are presented as electron micrographs (**A**, **B** and **C** respectively) and the active gene as a whole as a schematic drawing (**D**). The organization of the BR gene at the DNA level is outlined below (**E**); the four introns (I 1–4) are indicated. Bar in C 100 nm.

chromosomal puffs, but not to non-puffed regions or nucleoli. Chromosomes IV with the three giant puffs, Balbiani rings 1–3, are displayed in Figure 2. The puffs were heavily decorated by both the Y12 antibody (Figure 2A) and the 2E4 antibody (Figure 2B). When the chromosomes were treated with RNase A prior to the immunoreaction, no antibody binding was recorded (not shown). The negative control antibody did not react with the chromosomes at all (Figure 2C).

We conclude that in *C.tentans* the anti-Sm antibody Y12 specifically recognizes counterparts to the human D and B snRNP proteins. The antibody selectively decorates the transcriptionally active regions on the polytene chromosomes, except for the nucleoli. As the immunoreaction is RNase sensitive, the antigens present on the polytene chromosomes are presumably associated with RNA. Thus, the distribution of the Y12 antibody is likely to reflect the distribution of the snRNP particles along the polytene chromosomes (see also Discussion).

The structure of the transcriptionally active BR genes

The active BR genes can be visualized by electron microscopy *in situ* as transcription loops (Figure 3) (Daneholt, 1992). A schematic presentation of the morphology is given in Figure 3D and the proximal (p), middle (m) and distal (d) thirds of the gene have been demarcated in the figure. In the proximal region, the growing RNPs appear as thick RNP fibers growing longer and longer downstream of the region, while in the middle and distal regions the 5′ end of the RNP fiber is being packed into a dense globular structure, which increases in diameter along the gene. The ultrastructures of the proximal, middle and distal regions are displayed in Figure 3A–C respectively. The border between the proximal region and the rest of the gene is rather distinct, while the transit from the middle to the distal third is gradual (Figure 3D). With experience it is possible to classify the various observed gene segments into the three regions, although a low proportion of segments remains uncertain (less than 10%).

In Figure 3E the structure of the BR gene at the DNA level is outlined with the position of the four introns (I 1–4) indicated (Wieslander, 1994). The entire gene comprises 35–40 kb, i.e. each third of the gene corresponds to 12–13 kb. The three introns in the 5′ end reside in the first third of the proximal region and the fourth intron in the very end of the distal region. Thus, most of the gene does not contain introns. The BR gene is therefore well-suited for an immunoelectron microscopy analysis of the splicing of the first three introns, as the events can be followed downstream of the introns for a long time with no additional introns being included in the transcript.

Immunoelectron microscopy of growing BR RNP particles in situ

The binding pattern of the Y12 antibody to the growing RNP particles along the BR gene can be seen in Figure 4A–C; segments from the proximal (p), middle (m) and distal (d) regions have been indicated. It can be directly observed that most of the gold particles are confined to the proximal segments (5–10 gold particles in each proximal area) and only occasional particles appear in the middle and distal segments. Figure 4D represents the negative control, which shows that there are almost no background gold particles, i.e. essentially all the gold particles in Figure 4A–C are significant. As the three types of segments appear in the sections at about the same frequency, each representing about one third of the gene, the relative number of gold particles in the identified segments directly approximates the relative distribution of gold particles in the corresponding regions along the gene.

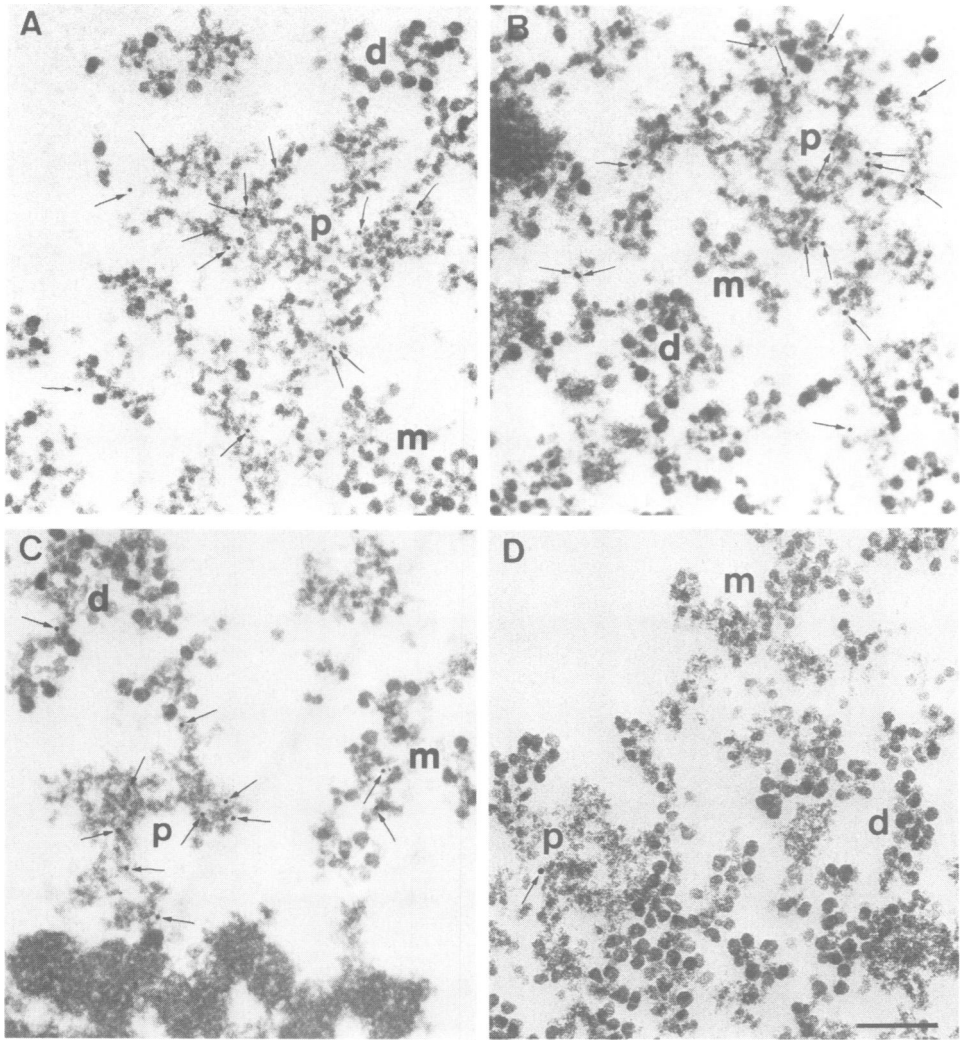

Fig. 4. Immunoelectron microscopy analysis of the distribution of snRNP proteins along transcriptionally active Balbiani ring genes. (**A**–**C**) Experiments with the anti-snRNP antibody Y12. (**D**) Experiments with the negative control antibody. (A–C) Gold particles (thin arrows) are seen over the proximal (p) region of the BR genes, while the middle (m) and distal (d) regions only exhibit a few particles. Bar 250 nm.

We also established the distribution of the gold particles between the proximal, middle and distal gene regions in an alternative, more quantitative approach. Three BRs from three different animals were studied. For each BR, 10 central sections were scrutinized and all the gold particles were analyzed (~500 particles). Each gold particle was classified according to its regional location and the distribution was expressed as a percentage (left panel in Figure 6). Most of the gold particles are in the proximal region (86%), while considerably fewer reside in the middle (7%) and the distal regions (7%). These results are in good agreement with the direct evaluation mentioned above of identified proximal, middle and distal regions in the electron micrographs (Figure 4).

The preferential binding of the Y12 antibody to the proximal region argues against a non-specific distribution of the snRNP proteins along the growing RNP fibers. If the snRNPs were randomly distributed along the growing RNP fibers, the binding should increase along the gene, as the RNP fibers are growing linearly from the proximal towards the distal end of the gene. To test whether a protein likely to be present along the entire RNP fiber displays a gradual increase in antibody labeling from the proximal towards the distal end of the gene, we studied an antibody to a *Chironomus* hnRNP protein, 2E4. The results with the 2E4 antibody are displayed in Figure 5. Again, the negative control almost lacks gold particles (Figure 5D). In this case it is clear that the label is distributed above all the three gene regions and the relative labeling intensity is increasing along the gene from the proximal to the distal region. This was verified by a quantitative analysis similar to that carried out in the Y12 experiment. In this case, 1500 gold particles were classified in each BR and again three BRs from three different animals were studied. The label is increasing along the gene: 17, 38 and 45% in the proximal, middle and distal regions respectively (right panel in Figure 6). As the theoretical distribution should correspond to the ratio 1:3:5, there is an indication of a decreasing accessibility of the epitope along the gene, but clearly the epitope is also available to a large extent in the densely packed particle. Thus, at least for one anti-hnRNP antibody, 2E4, it is possible to reveal the corresponding epitope in both the stalk and globular portions of the growing RNP

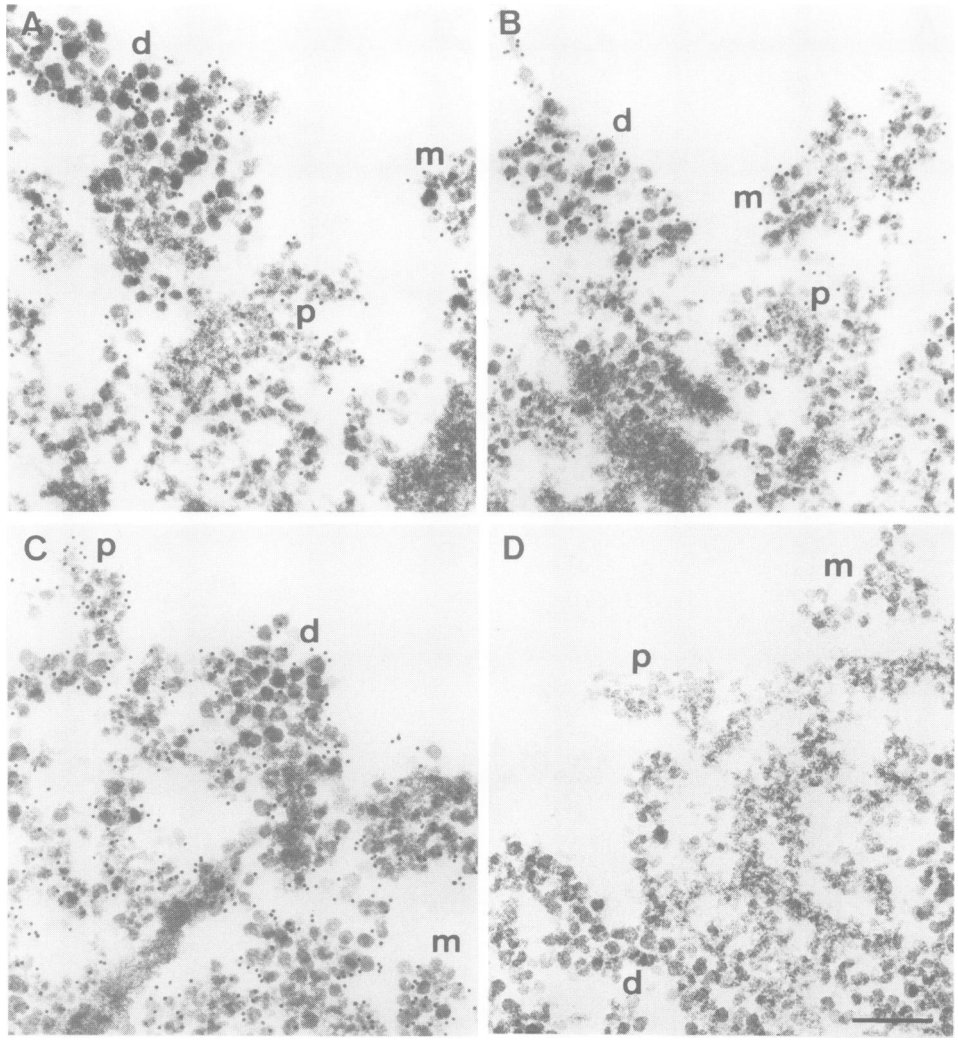

Fig. 5. Immunoelectron microscopy analysis of the distribution of an hnRNP protein along transcriptionally active Balbiani ring genes. (**A**–**C**) Experiments with the anti-hnRNP protein antibody (2E4). (**D**) Experiments with the negative control antibody. (A–C) Gold particles are abundant in the proximal (p), middle (m) and distal (d) regions of the gene. Bar 250 nm.

particles, i.e. irrespective of whether the fiber is loosely or more densely packed in the particle. We conclude that the Y12 distribution is likely to represent the actual location of the snRNP particles along the active gene, i.e. the snRNPs are preferentially located in the proximal region (see also Discussion).

Two additional control experiments were carried out. An antibody (H111) to a U1 RNP-specific protein, the 70 kDa protein, was used to further test whether the Y12 distribution represents that of snRNP. This antibody gave the same predominant promoter proximal distribution as Y12 in immunoelectron microscopy experiments (not shown), which supports the conclusion that the Y12 experiments reveal the location of snRNPs along the gene.

The chromosomes were released in an isotonic solution, but it could still be argued that loosely associated snRNPs were lost during the course of chromosome isolation. Therefore, the immunoelectron microscopy experiment with the Y12 antibodies was also performed on salivary gland cryosections (see Materials and methods). Again, the labeling was mainly confined to the promoter proximal region of the gene (not shown), indicating that this is the true distribution *in vivo*.

Immunoelectron microscopy of unfolded RNP in isolated BR particles

Immunoelectron microscopy has also been carried out on isolated BR particles to decide whether or not snRNP particles could be detected in completed and released BR RNPs. The experiments were designed to allow an analysis of the distribution of snRNPs along the RNP fiber. Nucleoplasmic BR RNP particles were isolated as 300S particles as described earlier (Wurtz *et al.*, 1990) and the BR RNP fiber was unfolded in low ionic strength medium and spread on a grid surface. The distribution of snRNP proteins along the fiber was determined by immunoelectron microscopy using the Y12 antibody. Again, the anti-hnRNP antibody was used as the positive and the anti-von Willebrand factor antibody as the negative control.

The results of the immunoelectron microscopy experiments with the unfolded BR RNP fiber are shown in Figure 7. A non-treated BR particle is presented for comparison in Figure 7N. About 30% of the particles appeared as RNP ribbons (~0.5 μm in length, 20–25 nm width), each correponding to the bent ribbon forming the ring-like structure of the compact particle (Skoglund *et al.*, 1986). The two ends of the ribbon are likely to correspond

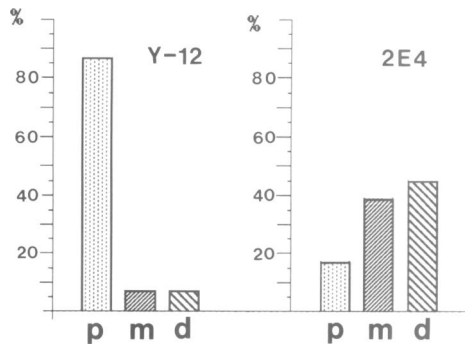

Fig. 6. The distribution of snRNP proteins (left) and an hnRNP protein (right) along a transcriptionally active Balbiani ring gene as determined by immunoelectron microscopy. The quantitative analysis was based on three snRNP experiments with the Y12 antibody (cf. Figure 4) and three hnRNP protein experiments with the 2E4 antibody (cf. Figure 5). Each individual gold particle was classified as to gene region: proximal (p), middle (m) or distal (d). The number of gold particles in each region is expressed as a percentage of the total number of gold particles along the entire gene (for further details see Materials and methods). The snRNP proteins are mainly distributed in the proximal region of the BR gene, while the hnRNP protein increases in amount from the proximal towards the distal region.

to the 5′ and 3′ ends of the transcript. Extended ribbons of this kind have been chosen to analyze the distribution of antibody binding along their length. In the Y12 experiment, only 3–5% of all extended RNP ribbons exhibited gold particles, which corresponds well to the low fraction of intron-containing RNA in nucleoplasmic RNA (Baurén and Wieslander, 1994). Whenever label appeared, only one end of the ribbon was labeled (Figure 7A–E); there was no evidence for a distribution along the entire BR ribbon. The anti-hnRNP antibody on the other hand always decorated the entire ribbon, indicating an even distribution along the fiber (Figure 7F–I). The negative control showed essentially no immunoreaction (Figure 7K–M). It is concluded that the snRNP proteins are non-randomly distributed along the RNP fiber, in this case at one end of the completed transcription product, presumably the 3′ end (see also Discussion).

We conclude from the Y12 experiments with growing BR RNP fibers *in situ* and with unfolded completed RNP fibers of isolated BR particles that the snRNP proteins occupy specific regions along the RNP fiber. This pattern is quite different from the even distribution of an hnRNP protein studied in parallel. The snRNPs are recorded in the proximal, and presumably also in the most distal, part of the RNP fiber, i.e. their distribution corresponds to the location of the introns. This would suggest that the assembly of the spliceosomes occurs selectively in the vicinity of the introns and that both the assembly and disassembly are rapid processes taking place concomitant with or shortly after the completion of transcription.

Discussion

Demonstration of snRNPs associated with nascent RNP

In the present study the appearance of spliceosomes on growing RNP particles has been demonstrated by immunoelectron microscopy using the Y12 antibody. This antibody is known to recognize the Sm epitope on several snRNP polypeptides (Lerner *et al.*, 1981; Pettersson *et al.*, 1984; Paterson *et al.*, 1991) and the epitope shows a remarkable evolutionary stability (Lührmann, 1988). In *Chironomus*, the three main bands that react with the Y12 antibody in Western blots are likely to represent equivalents to the predominant human B and D snRNP proteins. That Y12 does react with snRNP particles in *Chironomus* has been directly demonstrated in immunoprecipitation experiments, in which the snRNA species were recorded (Sass and Pederson, 1984). In immunocytology experiments, Y12 decorates the RNA-containing puffs, but not other regions of the polytene chromosomes (Figure 2; see also Sass and Pederson, 1984; Vazquez-Nin *et al.*, 1990). Furthermore, the reactivity to the puffed areas is RNase-sensitive, i.e. the proteins are likely to be part of RNA-containing complexes. We conclude that the Y12 antibody is a suitable probe for snRNP proteins in *Chironomus*.

The nature of the Y12 association with RNP particles was further investigated at the ultrastructural level in the Balbiani rings. Essentially all the gold particles in the immunoelectron microscopy experiments were bound to nascent RNP particles connected to the BR genes. Completed BR granules are only occasionally seen within the BRs, while interchromatin granules were not recorded in the isolated polytene chromosomes. We conclude that the snRNP proteins were associated with growing pre-mRNP particles in the BRs, which confirms a study by Vazquez-Nin *et al.* (1990). The snRNPs are probably present as components of spliceosomes, which is supported by electron microscopic analysis of spread BR genes showing that spliceosome-sized granules reside in the 5′ end region of the growing RNP transcripts (H.Trepte, personal communication; see also, for example, Beyer and Osheim, 1988).

The preferential location of snRNPs in the proximal region of the BR genes

The Y12 antibody bound almost exclusively to the growing RNP fibers in the proximal region of the BR genes. This result suggests that the snRNP proteins are preferentially present in the proximal portion of the gene. Alternatively, it could be argued that the snRNP epitopes are not accessible in the more densely packed RNP particle in the middle and distal portions of the gene. Two sets of data make this latter interpretation less likely. First, the positive control experiment with the anti-hnRNP antibody shows that the densely packed RNP particle can also be accessible for antibody binding, although this could differ between different epitopes. Second, the stalk portion of the RNP particles in the middle and distal regions did not react with the Y12 antibody. This is important, as a stalk portion of more distal RNP granules should be equivalent to a growing RNP fiber in the proximal portion of the gene: both the RNP fiber in a stalk and a growing RNP fiber in the proximal portion of the gene are loosely coiled (both correspond to recently transcribed RNA). Therefore, even if the compacted globular portion of the downstream growing RNP particles were not accessible to the Y12 antibody, the stalk portion of each growing RNP particle ought to be, which should then result in an approximately even distribution of Y12 labeling along the gene (cf. Figure 3). As the proximal region is by far the most

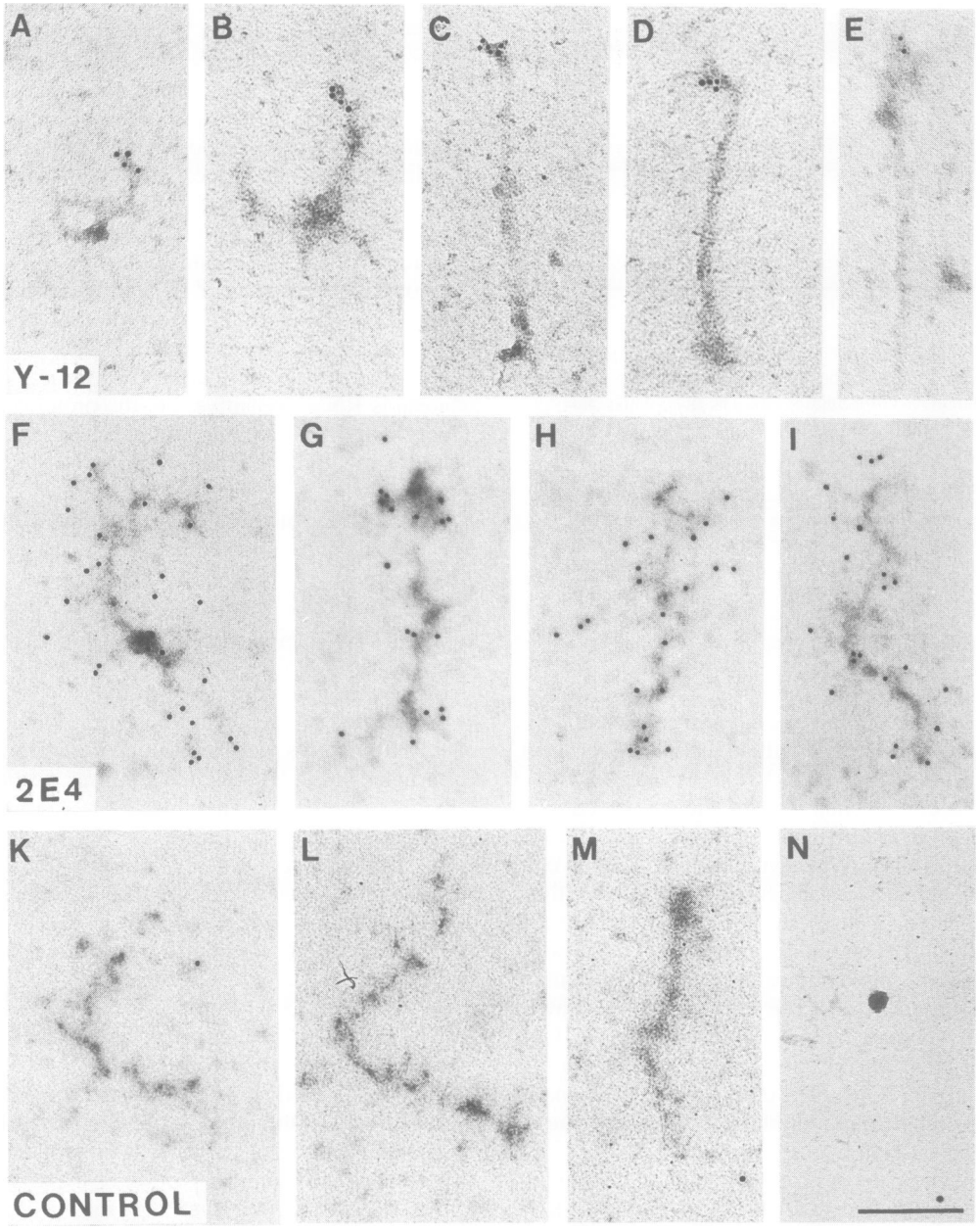

Fig. 7. Immunoelectron microscopy analysis of unfolded BR RNP particles using the anti-snRNP antibody Y12 and the anti-hnRNP protein antibody 2E4. The BR RNP particles were isolated, treated in low ionic strength medium and adsorbed onto an electron microscope grid. The immunoreactions were carried out as described in Materials and methods. Only unfolded particles showing the RNP ribbon extended were studied (~30%) (A–M). A non-treated native BR particle is shown for comparison (**N**). (**A**–**E**) Y12; (**F**–**I**) 2E4; (**K**–**M**) the negative control antibody. The Y12 antibody is characteristically bound only to one end of the RNP ribbon, while the 2E4 antibody is distributed along the entire ribbon; the control lacks signal. Bar 250 nm.

immunoreactive, it is concluded that this decoration is due to the presence of more snRNP proteins in the proximal region of the gene, rather than to inaccessibility of putative snRNP epitopes in the two more distal regions. Thus, the snRNPs are likely to be preferentially located in the promoter proximal portion of the BR genes.

Assembly and disassembly of spliceosomes

As a rule, the growing RNP transcripts are about evenly distributed along the active BR genes (Figure 3) (Lamb and Daneholt, 1979). This implies that the initiation of transcription takes place at a more or less constant rate and that there is no or very limited premature termination; the RNA polymerases sweep along the gene from the initiation site to the regular termination site. The observation that the growing RNP particles in the promoter proximal region harbor snRNPs, while only a minor proportion of those present in the middle and distal regions do, therefore suggests that the spliceosome components are transient structures on the growing RNP fiber. Thus, it seems likely that spliceosomes are formed immediately upon synthesis of the 5′ end intron sequences and they usually vanish before the polymerases enter the middle segment of the gene. This result on the assembly and disassembly of spliceosomes is in good agreement with the recent study of Baurén and Wieslander (1994), who

directly demonstrated that one of the 5′ introns is removed from most of the growing transcripts while these still reside in the proximal region of the gene.

As we can observe the formation and disappearance of the 5′ spliceosomes on the growing transcripts and we know the transcription parameters, it is possible to estimate the kinetics of the process. The transcription time for the entire gene is of the order of 20 min (Egyhazi, 1975; Daneholt, 1982), i.e. any given polymerase will not spend more than 7 min in the proximal third of the gene. Furthermore, as it can be assumed that the spliceosomes are formed subsequent to the synthesis of the corresponding introns, it can be estimated that both the assembly and disassembly process ususally takes place within less than 5 min. This is still a maximum value for splicing of single introns *in vivo*, as there are three closely spaced 5′ introns in each transcript and the corresponding splicing reactions do not necessarily occur simultaneously. Based on their investigation on the RNA level of intron 3 in the 5′ end, Baurén and Wieslander (1994) concluded that 50% of the intron sequences had disappeared 2.5 min after having been transcribed. These results on BR genes are in good agreement with the detailed kinetic analysis performed by Beyer and Osheim (1988) on transcription units in *Drosophila* embryos. They estimated that splicing, including the removal of the bulk of the spliceosome from the nascent transcript, was completed within 3 min.

Even if most spliceosomes have disappeared prior to the entry of the RNA polymerase into the second third of the gene, there are still some left in the middle and perhaps even in the distal part of the gene. According to the immunoelectron microscopy data, the snRNP concentration is less than 10% in the distal two thirds of the gene compared with that in the proximal third. Also, Baurén and Wieslander (1994) noted that the 5′ end intron sequences continue to be removed along the entire BR gene, probably reflecting the stochastic nature of the process.

The occurrence of snRNPs in the distal segment could correspond to some remaining 5′ spliceosomes, as well as newly formed 3′ spliceosomes. The single 3′ intron is likely to be close to the 3′ end of the transcript [~600 bases from the poly(A) addition site], although the precise termination site has yet to be determined. Baurén and Wieslander (1994) showed in their RNA analysis that ~5–10% of the 3′ introns were removed from the nascent BR transcripts, suggesting that the distal spliceosomes that we observe should include 3′ end spliceosomes. Most of the 3′ introns were excised outside the BRs in the nucleoplasm and this process took place rapidly; most by far of the BR transcripts in the nucleoplasm were completely spliced. Our own observation that only 3–5% of the unfolded BR particles carry snRNPs, and then exclusively on one end, is in good agreement with these data. Thus, the 3′ spliceosomes are also assembled and disassembled very rapidly. A precise estimate of the time required is difficult to make, as the turnover time of BR transcripts in nucleoplasm is unknown.

Assembly of the BR pre-mRNP particle and its relation to splicing

The basic element in the BR particle has the same general properties as the perichromatin fibers observed *in situ* in eukaryotic nuclei (Puvion-Dutilleul, 1983) and the unfolded thin nascent RNP fibers revealed in different tissues and species with the Miller chromatin spreading technique (Beyer and Osheim, 1990). The thin smooth fiber is likely to consist of a pre-mRNA molecule associated with abundant hnRNP proteins, the core proteins, along the entire transcript.

The present study shows how one hnRNP protein, the 45 kDa protein recognized by the 2E4 antibody, is being added to the growing pre-mRNA molecule. As the density of the immunolabeling increases along the gene, it seems likely that the protein is added to the RNA molecule in parallel with the synthesis of the RNA. The 45 kDa protein is, therefore, likely to be an hnRNP core protein and one of the main constituents of the thin RNP fiber.

The assembly and disassembly of spliceosomes are probably closely related to the formation of the pre-mRNP fiber. In the case of lampbrush chromosomes, snRNPs and splicing factors appear along the chromosomal loops and essentially along all loops (Gall and Callan, 1989; Wu *et al.*, 1991). It has been assumed that the splicing components associate with the RNP fiber along its entire length, i.e. they should be bound to intron as well as exon sequences. It has even been proposed that unitary hnRNP/snRNP particles, containing all splicing components, are pre-assembled in the B snurposomes and transported to the lampbrush chromosomes to be associated with newly transcribed RNA (Wu *et al.*, 1991). Likewise, Sass and Pederson (1984) concluded from their studies of snRNPs in Balbiani rings and heat-shock puffs that snRNPs could be 'a constitutive element of transcript packaging, irrespective of the presence or absence of introns'. It should, however, be recalled that in neither the lampbrush chromosome experiments nor in the puff studies could the distribution of snRNPs be directly related to the intron–exon structure of the genes; the intron–exon arrangement was not known or the individual genes were not visualized and properly defined.

Our data on the appearance of snRNPs along the BR genes do not support the concept of random distribution. On the contrary, the snRNPs are located more or less exclusively to those areas containing introns, i.e. in the 5′ and 3′ end regions of the transcripts. The large middle portion of the transcript seems to be devoid of snRNPs. This is most convincingly shown by the absence of Y12 antibody binding to the stalk portions of the growing RNP particles in the middle region of the gene *in situ* and to the RNP fiber of the unfolded RNP particles. The observed non-random distribution of snRNPs along the BR transcripts is in very good agreement with the studies performed by Beyer and co-workers on spread active genes from *Drosophila* embryos (Osheim *et al.*, 1985; Beyer and Osheim, 1988). Here, putative spliceosomes (or spliceosome intermediates) were only recorded at the splice sites. Furthermore, Amero *et al.* (1992) could show that snRNPs and hnRNP proteins are not present in the same stoichiometric amounts in all chromosomal puffs in *Drosophila*. They concluded that presumably the abundance of hnRNP proteins is related to the amount of RNA at an active locus, while that of snRNPs reflects, rather, the number of introns. Thus, at least in somatic cells, the binding of snRNPs seems to be closely linked to the

formation of spliceosomes, rather than to the overall assembly of the pre-mRNP fiber.

Our data very much favor a dynamic situation on the active gene, with spliceosome components transiently bound to the growing RNP. It is striking that spliceosome assembly is initiated in close relation to the transcription process and the formation of the pre-mRNP particle. It is even possible that snRNP binding and the commitment to splicing is accomplished at the very assembly of the pre-mRNP (Aebi and Weissmann, 1987). The fact that one of the core proteins of the RNP particle, A1, can affect splice site selection (Mayeda and Krainer, 1992) could argue for such a concept linking transcription, assembly of the pre-mRNP fiber and assembly of the spliceosome closely together.

Materials and methods

Material and drug treatment

Chironomus tentans was cultivated as described by Lezzi *et al.* (1981). Prior to analysis, fourth instar larvae were kept for 3–5 h at 22°C in cultivation water provided with pilocarpine nitrate (0.1 mg/ml) (Mähr *et al.*, 1980).

Antibodies

The mouse monoclonal anti-Sm antibody Y12 was a gift from Dr J.A.Steitz and the mouse monoclonal anti-U1 70 kDa protein antibody (H111) a gift from Dr R.Lührmann. The mouse monoclonal anti-*Chironomus* hnRNP antibody 2E4 was prepared in our laboratory (T.Wurtz and B.Daneholt, in preparation). The mouse monoclonal anti-human von Willebrand factor antibody was purchased from DAKO.

Western blot analysis

Sample preparation. For extraction of proteins, salivary glands were dissected manually from two fourth instar *C.tentans* larvae and fixed in 70% ethanol for 30 min at 4°C. The cells were separated from the fixed glands with dissection needles and collected in glycerol:ethanol (1:1) at 4°C. The proteins were dissolved in SDS sample buffer (Laemmli, 1970) and boiled for 10 min. HeLa nuclear extracts active in *in vitro* pre-mRNA splicing assays were prepared from cells grown in suspension as described by Dignam *et al.* (1983). Low molecular weight protein markers from BioRad were also dissolved in sample buffer and briefly boiled.

Gel electrophoresis and Western blotting. The samples were electrophoresed in a 12.5% polyacrylamide gel according to Laemmli (1970). After completion of the run, the proteins were electrophoretically transferred onto an Immobilon-PVDF Membrane (Millipore) in 0.2% SDS and 20 mM Tris–glycine electrode buffer (pH 8.3) for 2 h at 80 V. The membrane was blocked for 2 h with 5% bovine serum albumin (BSA) and 1% non-fat dry milk in phosphate-buffered saline, pH 7.0 (PBS), and immunoassayed for 1 h with the Y12, 2E4 or anti-von Willebrand factor antibody diluted in PBS (final concentration 20 μg/ml). The membrane was washed twice in 0.1% Tween 20 and PBS for a total of 30 min. The antibodies were detected with anti-mouse antibody–alkaline phosphatase conjugates (Promega; 0.3 mg/ml) diluted 1:1000 in PBS containing 0.5% BSA. The phosphatase reaction was developed by incubation of the membrane for 10–20 min in a solution of 0.33 mg/ml NBT and 0.165 mg/ml BCIP color development substrate (Promega) in 10 ml of 0.1 M NaCl, 0.005 M $MgCl_2$ and 0.1 M Tris–HCl (pH 9.5). The reaction was stopped by washing the membrane in distilled water.

Immunocytology

Chromosome isolation. Larvae were decapitated and the salivary glands were isolated. The glands were placed for 30 s in 2% Nonidet P-40 (NP-40) in TKM (100 mM KCl, 1 mM $MgCl_2$ and 10 mM triethanolamine–HCl, pH 7.0), transferred to 0.025% NP-40 in TKM and disrupted by a pipetting technique to release polytene chromosomes. Single chromosomes IV were identified and transferred to a siliconized glass depression slide to which they attached (for details see Björkroth *et al.*, 1988). All manipulations were performed on a cold stage at close to 0°C. The isolated chromosomes were fixed with 3.7% formaldehyde in TKM for 30 min at room temperature and washed with three changes of TKM.

Immunolabeling. The chromosomes were incubated for 30 min at room temperature in a humid chamber on a rocking platform with 40 μl of 1% BSA in TKM. They were then incubated for 90 min with 40 μl of the primary antibody diluted 1:100 for Y12, H 111 and 2E4 and 1:20 for the anti-von Willebrand factor antibody in TKM (final concentrations 20 μg/ml). The slides were washed for 3×5 min in 0.1% Tween 20 in TKM and then incubated for 90 min with the secondary antibody, the goat anti-mouse antibody–gold (10 nm in diameter) conjugate (Amersham) diluted 1:50 in 0.5% BSA and TKM. The slides were washed as above and rinsed in distilled water. An immunogold silver enhancement solution (IntenSEM, Amersham) was applied according to manufacturer's instructions for 5–15 min at room temperature. Subsequently, the samples were rinsed in distilled water and mounted in 30% glycerol. Photographs were taken with Agfapan 25 film in a Zeiss photomicroscope.

Immunoelectron microscopy of isolated chromosomes

Chromosome isolation and immunolabeling. Chromosomes IV were isolated and the immuno reactions were carried out as described above under Immunocytology.

Electron microscopy. After the incubation with the secondary antibody–gold conjugate and washing for 3×5 min in TKM, the specimens were post-fixed in 2% glutaraldehyde in TKM. They were then again rinsed in TKM for 3×5 min, dehydrated in 90% ethanol and embedded in Agar 100 resin. Chromosomes IV with well-developed Balbiani rings were sectioned and stained as previously described (Björkroth *et al.*, 1988). The specimens were examined and photographed in a Jeol TEM-SCAN 100 CX microscope at 60 kV.

Quantitative analysis. Three chromosomes IV from three different larvae were chosen for a detailed investigation of the binding of the antibody Y12 along the active BR gene. For each chromosome, 10 central sections through a BR were scrutinized and each gold particle was classified as to its location in a proximal, middle or distal region. In each experiment, the number of gold particles in each region was expressed as a percentage of the gold particles studied. Finally, an average value for each segment was calculated from the results in the three experiments. The binding of the 2E4 antibody was analyzed in the same way.

Immunoelectron microscopy on ultrathin cryosections

This was essentially performed according to the procedure described by Tokuyasu (1980). Salivary glands were fixed in a fresh solution of 4% paraformaldehyde and 0.1% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.2, for 20–25 min and cryoprotected in 2.3 M sucrose. Ultrathin cryosections were picked up on drops of 2.3 M sucrose and deposited on nickel grids coated with formvar and carbon. Grids were blocked with 10% calf serum and processed for immunocytochemistry using either the Y12 or the anti-von Willebrand factor (negative control) antibodies at ~20 μg/ml. A secondary antibody conjugated to 5 nm gold particles (Amersham) was used in the second step of the immunolabeling procedure. The sections were finally stained with 2% aqueous uranyl acetate for 5–7 min and embedded in polyvinyl alcohol (9–10 kDa; Aldrich) containing 0.4% uranyl acetate. The specimens were examined in a Jeol TEM-SCAN 100 CX microscope at 80 kV.

Immunoelectron microscopy of isolated and unfolded BR RNP particles

Isolation of BR RNP particles. BR RNP particles were extracted from salivary glands and centrifuged in a sucrose gradient as previously described (Lönnroth *et al.*, 1992). A 300S fraction contained the BR RNP particles.

Low salt treatment and electron microscopy of BR RNP particles. BR particles were obtained in TKE (0.1 M KCl, 10 mM EDTA and 10 mM triethanolamine–HCl, pH 7.0), were diluted 10-fold with distilled water and incubated for 15 min at 5°C. The sample was fixed in 2% glutaraldehyde and placed in microchambers provided with carbon-coated grids; prior to use, the grids had been glow-discharged for 5 min, rendering the carbon film hydrophilic. The particles were centrifuged at 4°C for 15 min at 20 000 *g*. After centrifugation the grids were washed in 0.1× TKE, incubated for 20 min at room temperature in a humid chamber on top of a drop of 20 μl of 1% BSA in 0.1× TKE. The specimens were incubated for 40 min on top of a drop of 20 μl of the solution containing the Y12 antibody, the 2E4 antibody or the anti-von Willebrand factor antibody (final concentrations ~20 μg/ml). The grids

were washed for 3×2 min on top of drops of 0.01% Tween 20 in 0.1× TKE and incubated for 40 min with the secondary antibody–gold conjugate (diluted 1:50) in 0.5% BSA in 0.1× TKE. The specimens were briefly rinsed in 0.1 x TKE and subsequently in distilled water and then stained in 1% uranyl acetate for 10 min, washed in distilled water and air dried. The specimens were examined and photographed in a JEOL TEM-SCAN 100 CX microscope at 60 or 80 kV.

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