

Demonstration of a Dynamic, Transcription-dependent Organization of Pre-mRNA Splicing Factors in Polytene Nuclei

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Abstract. We describe the dynamic organization of pre-mRNA splicing factors in the intact polytene nuclei of the dipteran *Chironomus tentans*. The snRNPs and an SR non-snRNP splicing factor are present in excess, mainly distributed throughout the interchromatin. Approximately 10% of the U2 snRNP and an SR non-snRNP splicing factor are associated with the chromosomes, highly enriched in active gene loci where they are bound to RNA. We demonstrate that the splicing factors are specifically recruited to a defined gene upon

induction of transcription during physiological conditions. Concomitantly, the splicing factors leave gene loci in which transcription is turned off. We also demonstrated that upon general transcription inhibition, the splicing factors redistribute from active gene loci to the interchromatin. Our findings demonstrate the dynamic intranuclear organization of splicing factors and a tight linkage between transcription and the intranuclear organization of the splicing machinery.

IN the cell nucleus, pre-mRNA is synthesized at gene loci and subsequently transported to the nuclear membrane pores. All pre-mRNAs are processed at their 5' (Shatkin, 1976) and 3' ends (Wahle, 1995) and interact with RNA-binding proteins to form pre-mRNP (Dreyfuss et al., 1993). Pre-mRNP formation is therefore a complex process that requires a coordinated organization of several events and many molecular components. In particular, intron containing pre-mRNAs associate with the five different snRNPs and non-snRNP splicing factors, which form a multicomponent structure, a spliceosome at each intron (e.g., Newman, 1994; Sharp, 1994). After completed splicing, the spliceosomal components stay in the nucleus and are presumably reused. The temporal and spatial organization of the splicing machinery in the intact cell nucleus during this dynamic series of events is not well known.

In interphase diploid nuclei that have a highly complex structural organization, the available information relies, on the one hand, on immunocytochemical localization of spliceosomal components (e.g., Nymann et al., 1986; Spector et al., 1991), and on the other hand, on detection by in situ hybridization of snRNPs (Carmo-Fonseca et al., 1992; Matera and Ward, 1993) and exon or intron sequences in pre-mRNA (Zhang et al., 1994). The snRNPs and non-snRNP

splicing factors are largely concentrated to so-called speckles, forming a network throughout the nucleus, containing interchromatin granules (Spector, 1993), but in addition, exhibit a more diffuse localization surrounding the interchromatin granules, in regions containing perichromatin fibrils and granules (Visa et al., 1993; Fakan, 1994). SnRNPs are also concentrated in coiled bodies (Lamond and Carmo-Fonseca, 1993).

Even though some pre-mRNAs may be spliced at or close to interchromatin granule clusters (Huang and Spector, 1991; Xing et al., 1993), ultrastructural studies (Beyer and Osheim, 1988; Fakan, 1994) and in situ hybridization (Zhang et al., 1994) suggest that it is more likely that spliceosome assembly and splicing occur in the perichromatin regions, at or close to the site of transcription. It therefore appears as if the interchromatin granules (for discussion see Mat-taj, 1994) or coiled bodies (Lamond and Carmo-Fonseca, 1993), despite their high concentrations of splicing factors, are not the actual sites for splicing of pre-mRNAs, and these structures may serve as storage and/or assembly-disassembly sites for components of the spliceosomes. Studies using polytene chromosomes in dipterans, which provide much improved structural resolution, have also shown that hnRNP proteins (Matunis et al., 1993) and spliceosomes (Kiseleva et al., 1994) assemble on nascent pre-mRNA and that splicing can occur cotranscriptionally (Baurén and Wieslander, 1994).

Here, we use the polytene salivary gland cells of the dipteran *Chironomus tentans* to demonstrate a transcription-dependent dynamic organization of the splicing machinery in the nucleus. The polytene cells offer considerable

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experimental advantages for *in situ* studies of intranuclear pre-mRNA processing events. The nuclei are exceptionally large with a diameter of ~ 75 μm , and the individual chromosomes can be seen, well separated from the interchromatin, defined here as the part of the nucleus not occupied by the chromosomes. In particular, the tissue-specific active Balbiani ring (BR)¹ genes can be identified within the intact nucleus.

Our results are compatible with the view that spliceosome components are present in excess in the interchromatin, organized to achieve a close coordination between pre-mRNA synthesis and spliceosome assembly.

Materials and Methods

Animals

C. tentans was cultured as described (Meyer et al., 1983). In the galactose experiments, larvae were kept for 8–10 d in standard cultures supplemented with 0.5 g per 100 ml of galactose in the water. In the DRB (5,6-dichloro-1-b-ribofuranosylbenzimidazole) and actinomycin D experiments, the larvae were kept for 5–6 h in water containing DRB (Sigma Chemical Co., St. Louis, MO) at a concentration of 450 mM or actinomycin D (Calbiochem-Novabiochem Corp., La Jolla, CA) at a concentration of 10 mg/ml (known to inhibit nuclear RNA synthesis by >98%) (Egyházi et al., 1969). The effect of both drugs was checked by observing a complete or almost complete regression of the BR puffs to a condensed chromatin state, which is correlated with inhibition of transcription.

C. tentans embryonic epithelial cells were grown in culture as described by Wyss (1982).

Immunofluorescence

Antibodies. Three mAbs were used in this study: 4G3 (Habets et al., 1989), obtained from Euro-Diagnostica B. V. (Apeldoorn, The Netherlands), 2E4 (Kiseleva et al., 1994; Wurtz et al. in press), a gift from B. Daneholt (Karolinska Institutet, Stockholm, Sweden), and Y12 (Lerner et al., 1981; Pettersson et al., 1984), a gift from Dr I. Pettersson (Karolinska Institutet, Stockholm, Sweden).

Cultured Cells. Cultured *C. tentans* diploid cells were pelleted, resuspended in PBS, and centrifuged onto poly-L-lysine-coated objective glasses using a centrifuge (Cytospin; Shandon Astmoor, Runcorn, UK). The cells were fixed in 4% formaldehyde in PBS for 15 min at room temperature. After washing in PBS, the cells were treated with 0.2% SDS in PBS for 7 min and again washed in PBS. The cells were then kept in 5% milk, 5% BSA in PBS for 1 h. The primary antibody in PBS, 0.5% milk, and 0.5% BSA was applied for 1 h, followed by three washes in PBS. The secondary antibody in PBS, 0.5% milk, and 0.5% BSA was added for 1 h, and the cells were finally washed three times in PBS and mounted in glycerol/PBS (87:13).

Salivary Glands. Salivary glands were dissected from fourth instar larvae and directly fixed in PBS containing 4% formaldehyde on ice for 10–15 min. The glands were washed in PBS three times for 5 min each and incubated in PBS and 0.2% SDS at room temperature for 10 min. After washing three times for 10 min each in PBS, the glands were incubated with PBS containing 5% milk and 5% BSA for 1 h at room temperature, followed by incubation with the primary antibody in PBS, containing 0.5% milk and 0.5% BSA overnight. The glands were then transferred to room temperature for 60 min. Washing in PBS, incubation with the secondary antibody, and mounting was performed as for cultured cells.

Squash Preparations. Squash preparations were prepared essentially as described by Silver et al. (1978). Glands were dissected and kept for 10 min in 25 mM disodium-glycerophosphate, 10 mM KH_2PO_4 , pH 6.8, 30 mM KCl, 10 mM MgCl_2 , 3 mM CaCl_2 , 160 mM sucrose, and 0.5% NP-40 on ice. The glands were fixed in 4% formaldehyde in PBS for 25 min, followed by incubation in 45% acetic acid for 10–20 min. Cells were mechanically separated from the rest of the glands and squashed. The squash preparations were kept in 20 mM Tris-HCl, pH 7.6, 137 mM NaCl (TBS),

first at room temperature for 5 min and then at 4°C for up to 4 h. The preparations were washed in TBS containing 0.05% Tween-20 three times for 5 min each at room temperature and incubated for 1 h at room temperature with the primary antibody, diluted in TBS containing 0.05% Tween-20. The preparations were washed in TBS, 0.05% Tween-20 for three times for 5 min each at 4°C. The secondary rabbit anti-mouse antibody was diluted in TBS, 0.05% Tween-20, and 5% BSA, and incubation was for 60 min at room temperature. After washing in TBS, 0.05% Tween-20 at 4°C, the preparations were mounted in glycerol/PBS.

Isolated Chromosomes. Native chromosomes were isolated essentially as described (Kiseleva et al., 1994). Salivary glands were dissected and kept for 30 s in TKM (10 mM triethanolamine-HCl, pH 7.0, 100 mM NaCl, and 1 mM MgCl_2) containing 2% NP-40 and transferred to TKM containing 0.025% NP-40. In this solution, the glands were disrupted by pipetting in siliconized glass pipettes with a diameter of 150–250 μm to release nuclei and individual chromosomes. The chromosomes were transferred with a pipette to a drop of TKM on a poly-L-lysine-coated objective glass, where they stuck to the surface of the glass. The chromosomes were fixed in 4% formaldehyde in TKM for 30 min at room temperature and washed in TKM. After incubation in TKM containing 5% milk and 5% BSA for 30 min at room temperature, the chromosomes were incubated with the primary antibody in TKM containing 0.5% milk and 0.5% BSA for 60 min. After washing in TKM, and 0.05% Tween-20, the secondary antibody was applied in TKM/0.5% milk/0.5% BSA for 60 min. After washing, the chromosomes were mounted in glycerol/PBS.

RNAse treatment of the chromosomes was performed by incubation for 30 min at room temperature in TKM containing 200 mg of RNaseA and 10 U RNaseT1 per ml immediately after isolation. The RNase-treated chromosomes were then washed in TKM, fixed, and incubated with antibodies as described above.

Microscopy

The large salivary gland nuclei with a diameter of ~ 75 μm were imaged in a confocal laser scanning microscope (Sarastro 1000; Molecular Dynamics, Inc., Sunnyvale, CA), as previously described (Wallen, 1991). An immersion oil lens ($\times 40/\text{NA } 1.3$; Nikon Inc., Garden City, NY) was also used.

Nuclei in cultured cells were imaged by a fluorescence digital imaging microscope consisting of an inverted light microscope (Axiovert 35; Zeiss, Oberkochen, Germany) and a cooled charge coupled device camera (Photometrics Ltd., Tucson, AZ) with a 1340/1037 pixel cooled charge coupled device chip (Kodak-Videk). An oil immersion lens and a filter set for rhodamine was used ($\times 100/\text{NA } 1.3$, Plan-Neofluar; Zeiss).

Squash preparations and isolated chromosomes were either photographed in the confocal microscope or in a standard fluorescence microscope (Axioskop; Zeiss).

Microdissection and Western Blotting

Salivary glands were fixed in 70% ethanol for 30 min at 4°C. The glands were stored in glycerol/ethanol (1:1 by vol) and mounted for microdissection on the lower side of a coverglass as described (Lambert and Daneholt, 1975). First, nuclei were isolated mechanically, and subsequently, the chromosomes were separated from the interchromatin material. Proteins were extracted separately from each of the two nuclear compartments and electrophoresed side by side in 12% SDS polyacrylamide gels. The separated proteins were electrophoretically transferred to nylon filters and probed with the 2E4 and 4G3 antibodies. Bound antibodies were detected with HRP-labeled anti-mouse Ig antibodies (ECL; Amersham Intl., Little Chalfont, UK). The staining of the 2E4 and 4G3 protein bands were quantitated by two-dimensional densitometry of the autoradiograms, using a Phoscan system P-1000 (Optronics International, Inc., Chelmsford, MA) and the program Pixie (developed by U. Skoglund and H. Mehlin for use in electron microscope tomography), running on a Silicongraphics Crimson computer. The relative staining of the chromatin or the interchromatin band was, after background subtraction, calculated as percentage of the total staining of the chromatin plus interchromatin bands.

Results

Intranuclear Distribution of Splicing Factors

Throughout this study, three different antibodies were used to analyze the spatial and temporal relationship of the splic-

1. *Abbreviations used in this paper:* BR, Balbiani ring; DRB, 5,6-dichloro-1-b-ribofuranosylbenzimidazole.

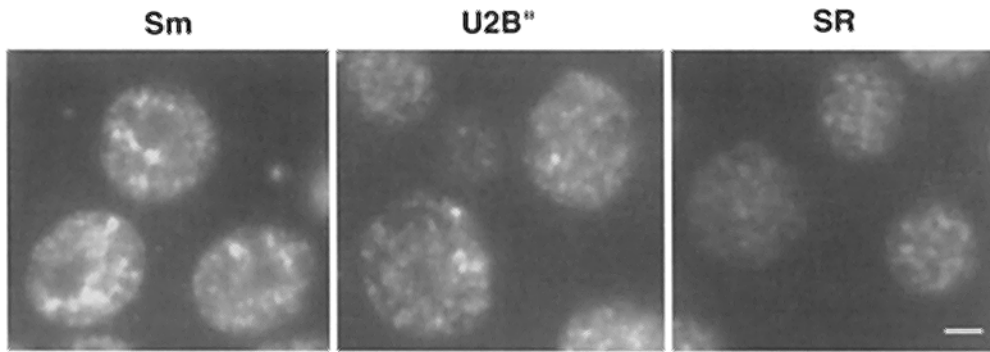


Figure 1. Distribution of splicing factors in diploid *C. tentans* interphase nuclei. Indirect immunofluorescence was performed with the mAbs recognizing the U1, U2, U4/U6, and U5 snRNP Sm proteins (*Sm*), the U2 snRNP B'' protein (*U2B''*), and the SR non-snRNP factor (*SR*), respectively. Bar, 2 μ m.

ing machinery and transcription. The Y12 mAb (Lerner et al., 1981; Pettersson et al., 1984) recognizes the Sm epitope, common to the U1, U2, U4/U6, and U5 snRNPs. The 4G3 mAb detects the U2 snRNP specific B'' protein (Habets et al., 1989). The 2E4 mAb, finally, binds to an SF2/ASF (Krainer et al., 1991; Ge and Manley, 1990) homologue in *C. tentans* (Alzhanova-Ericsson, A., X. Sun, N. Visa, E. Kiseleva, T. Wurtz, and B. Daneholt, manuscript submitted for publication). We therefore detect snRNPs and an SR non-snRNP splicing factor.

The specificity of these three mAbs was assessed as follows: the Y12 antibody immunoprecipitated snRNPs from *C. tentans* cell culture extracts, assayed by PAGE of the precipitated RNA species (data not shown). Y12 also detects three specific *C. tentans* proteins in Western blots, presumably corresponding to the B'/B and D proteins (Kiseleva et al., 1994). The 4G3 antibody was tested in Western blots and was found to specifically and efficiently detect a 31-kD protein (see Fig. 4). The 2E4 antibody specifically recognizes a 45-kD protein, Ct-hrp45, in Western blots (Wurtz et al., 1996; see also Fig. 4) and binds to pre-mRNA in situ (Kiseleva et al., 1994; Alzhanova-Ericsson, A., X. Sun, N. Visa, E. Kiseleva, T. Wurtz, and B. Daneholt, manuscript submitted for publication).

The antibodies were shown to locate snRNPs and the SR non-snRNP splicing factor in *C. tentans* diploid nuclei. For all three antibodies, a speckled nuclear staining pattern superimposed on a more diffuse, even distribution was recorded (Fig. 1). We conclude that the distribution of splicing factors in diploid *C. tentans* nuclei is similar to that described in mammalian diploid interphase nuclei (e.g., Nyman et al., 1986; Spector et al., 1991; Carmo-Fonseca et al., 1992; Huang and Spector, 1992).

In contrast to the low resolution obtained in the diploid nuclei, the distribution of snRNPs and the non-snRNP splicing factor in *C. tentans* polytene nuclei is depicted in Fig. 2. A clear distinction between splicing factors associated with chromatin and with interchromatin can be seen.

In chromatin, the highest local concentrations of both kinds of splicing factors are at specific chromosomal loci. Among these are the tissue-specific active BR1, BR2, and BR3 loci, which harbor the highly transcribed BR1, BR2.1 plus BR2.2, and BR3 genes, respectively. The first three of these genes each contains four introns (Wieslander, 1994), and the BR3 gene has 38 introns (Paulsson et al., 1990). At each of the three loci, 5–10 pg of nascent pre-mRNA is present (Edström et al., 1978). For each individual puff,

the intensity of staining is approximately proportional to the size of the puff, which is known to be related to the amount of nascent RNA present. It was also noted that the BR3 locus often was more intensely stained than the BR1 and BR2 loci, presumably reflecting the fact that the BR3 gene contains nine times more introns than the BR1 and the two BR2 genes.

A large number of additional chromosomal loci were labeled. In the intact nuclei the most intensely stained loci can be seen (Fig. 2). In isolated chromosomes or squash preparations, allowing more detailed analysis of individual chromosomes, more weakly stained loci are also observed (see Fig. 5), and the total number of loci is large. The strongly labeled loci were essentially the same for all three epitopes, and some loci correspond to the position of known intron-containing, tissue-specific, transcribed genes (Wieslander, 1994).

The chromosomal labeling with the SR non-snRNP antibody is sensitive to RNase treatment, as shown using isolated native chromosomes (Fig. 3). The same result was obtained with the anti-Sm protein antibody (not shown). We therefore conclude that the splicing factors that are located in chromosomal loci are associated with RNA.

In the interchromatin, staining is relatively homogeneous all over the nucleus. In detail, the staining has a reticular appearance, which may be more clearly seen in some areas free of chromosomes (see Fig. 1) and in partially squashed nuclei, where the chromosomes have been dislocated in relation to the rest of the nucleus (Fig. 2B). Apart from the reticular pattern, no obvious intensely stained local regions could be detected in the interchromatin space in such preparations.

Quantitation of Splicing Factors in Chromatin and Interchromatin

The large polytene nuclei allow separation of the interchromatin from the chromosomes by microdissection (Fig. 4, A–C). In Western blot analysis of the separated nuclear compartments (Fig. 4D), the proportion of the SR non-snRNP splicing factor associated with the chromosomes was 10% and with the interchromatin 90%. For the U2 snRNP-specific B'' protein, the distribution was 20% associated with the chromosomes and 80% with the interchromatin. In spite of the intense staining of many chromosomal loci (Fig. 1), the majority of the spliceosomal components are therefore at a given moment present in the interchromatin.

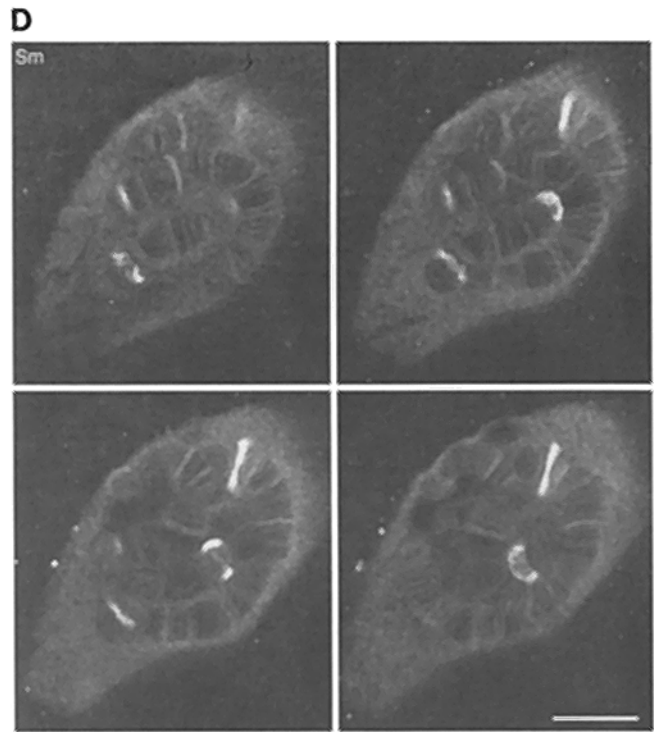
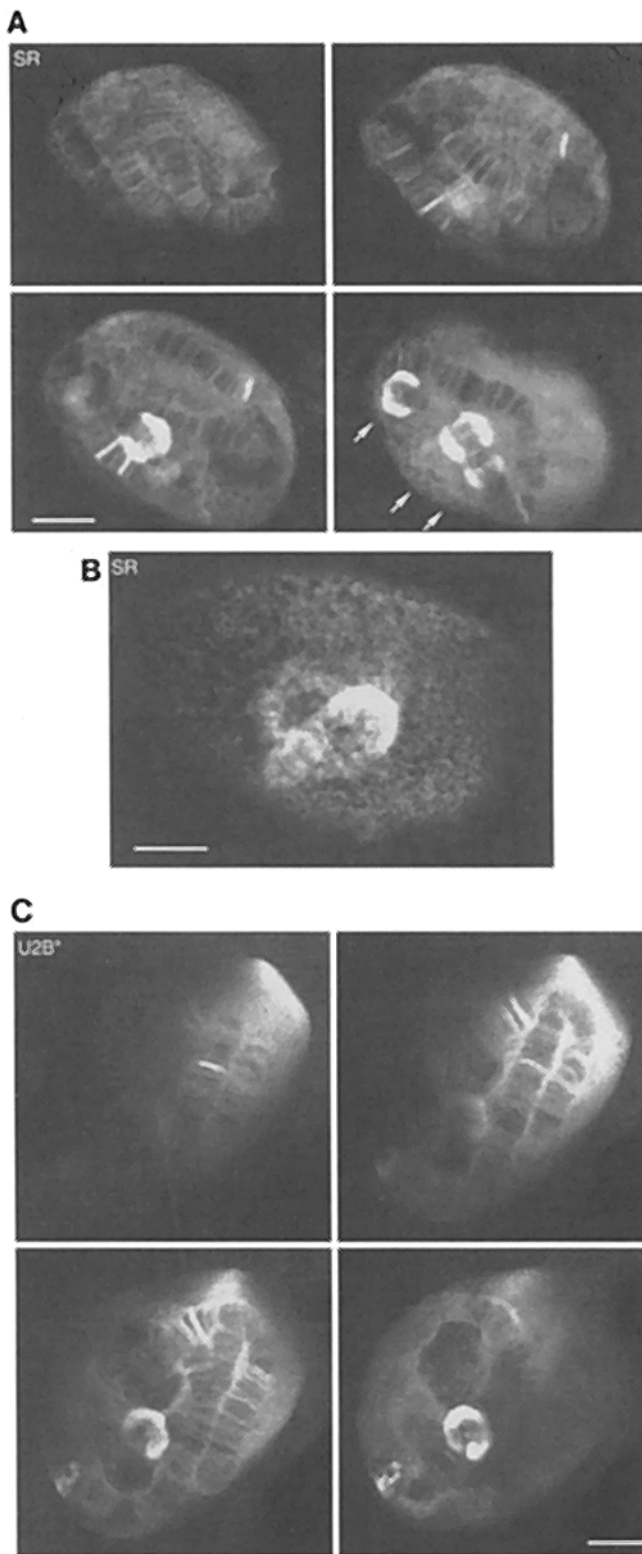


Figure 2. Distribution of splicing factors in polytene salivary gland cell nuclei of *C. tentans*. Indirect immunofluorescence was performed on intact salivary gland cells with mAbs detecting SR non-snRNP factor (SR) (A and B), U2 snRNP B'' protein (U2B'') (C), and U1, U2, U4/U6, U5 snRNP Sm proteins (Sm) (D). Stained chromosomal loci are clearly seen; among these are the intensely stained BR gene loci that are most obvious in the last section in A (arrows). In B, the nucleus has been squashed gently, resulting in dislocation of the polytene chromosomes. This allows inspection of a substantial part of the interchromatin space in the nucleus. The distance between each optical section is 5 μm in A and 3 μm in C and D. Bars, 20 μm .

Recruitment of Splicing Factors to Transcriptionally Activated Genes and Displacement from Inactivated Genes

Environmental changes can drastically change the transcription of the genes in the BR gene family. The BR6 gene, which under standard culturing conditions is not transcribed or transcribed at a very low level, is induced in the

presence of galactose (Beermann, 1973; Lendahl and Wieslander, 1984). At the same time, the two BR2 genes, BR2.1 and BR2.2, are turned off at the transcriptional level (Nelson and Daneholt, 1981). Transcription of the BR1 and BR3 genes is essentially not influenced. The *C. tentans* larvae grow and develop normally in the presence of galactose, and the described transcriptional changes are be-

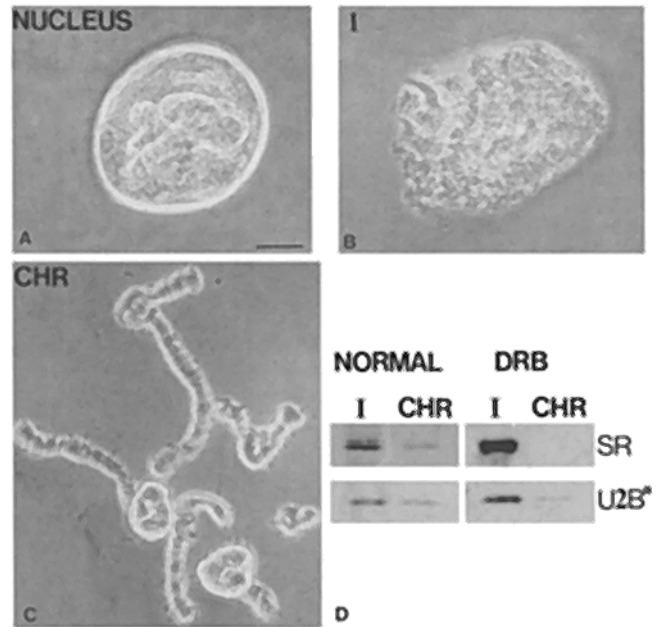
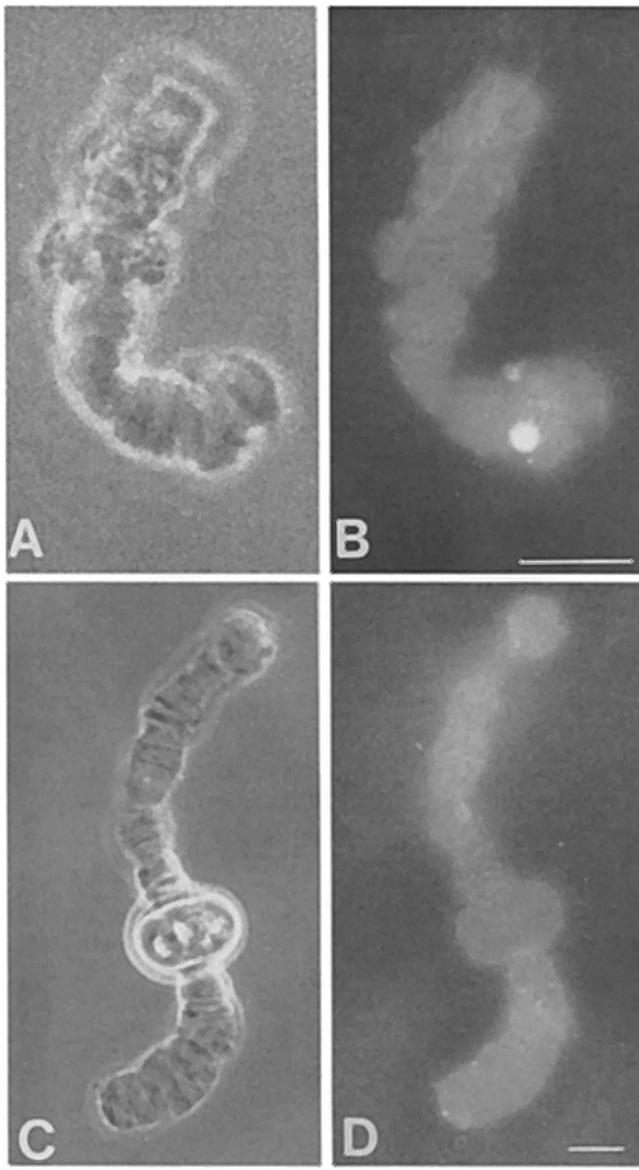


Figure 4. Quantitation of splicing factors in chromatin and interchromatin. Salivary gland cells from normal larvae and larvae treated with DRB were fixed and prepared for microdissection. First, nuclei were microdissected (A), and subsequently, the nuclei were separated into interchromatin (B) and chromosomes (C). Proteins present in the interchromatin and in the chromosomes from 180 nuclei were extracted and subjected to Western blot analysis. The filters were probed with antibodies detecting the 45-kD SR non-snRNP factor (SR) and the 31-kD U2 snRNP B'' protein (U2B''). In D, material from normal larvae and material from DRB-treated larvae is shown. I, interchromatin; CHR, chromosomes. Bar in A, 20 μ m. The magnification is the same in A, B, and C.

lied to be gene specific and represent a physiological response to environmental changes (Lendahl and Wieslander, 1984). We used this situation to analyze the distribution of splicing factors in relation to specific changes in gene activity under normal physiological conditions.

In Fig. 5, it is demonstrated that none of three antibodies stains the BR6 gene locus before galactose induction, and that after induction, the BR6 gene locus is heavily stained. In Fig. 6, it is also demonstrated that the result is precisely the opposite for the BR2 locus; before induction the BR2 locus is stained, and after induction, it is not

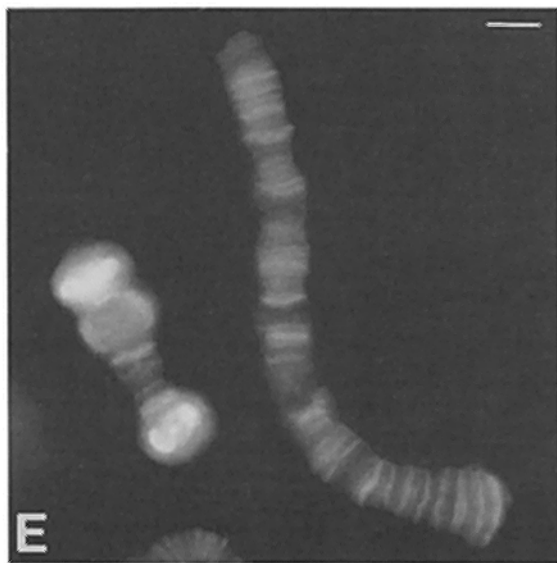


Figure 3. The association of splicing factors with gene loci is sensitive to RNase. Native chromosomes were isolated and treated with RNase before fixation and subsequent immunofluorescence. (A) Isolated RNase-treated and fixed chromosome IV stained with the anti-SR non-snRNP splicing factor antibody and viewed in phase contrast. (B) The same chromosome IV seen in the fluorescence microscope. (C) Isolated RNase-treated and fixed chromosome III stained with the anti-SR non-snRNP splicing factor antibody and viewed in phase contrast. (D) The same chromosome III in the fluorescence microscope. (E) Chromosome IV and chromosome III, which have not been RNase treated, stained with the same antibody as in A–D. Bars, 10 μ m. The magnification is the same in A and B, as well as in C and D.

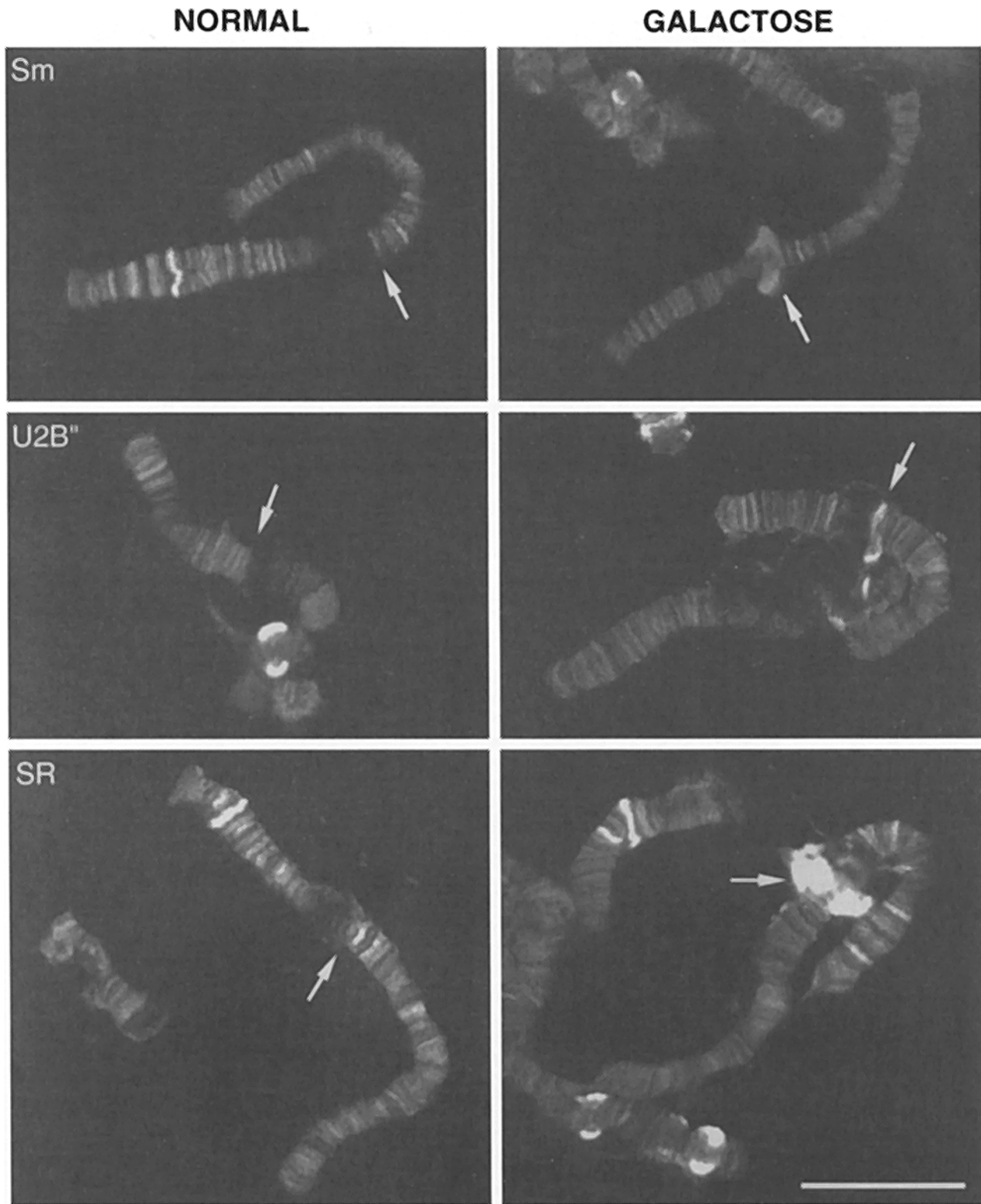
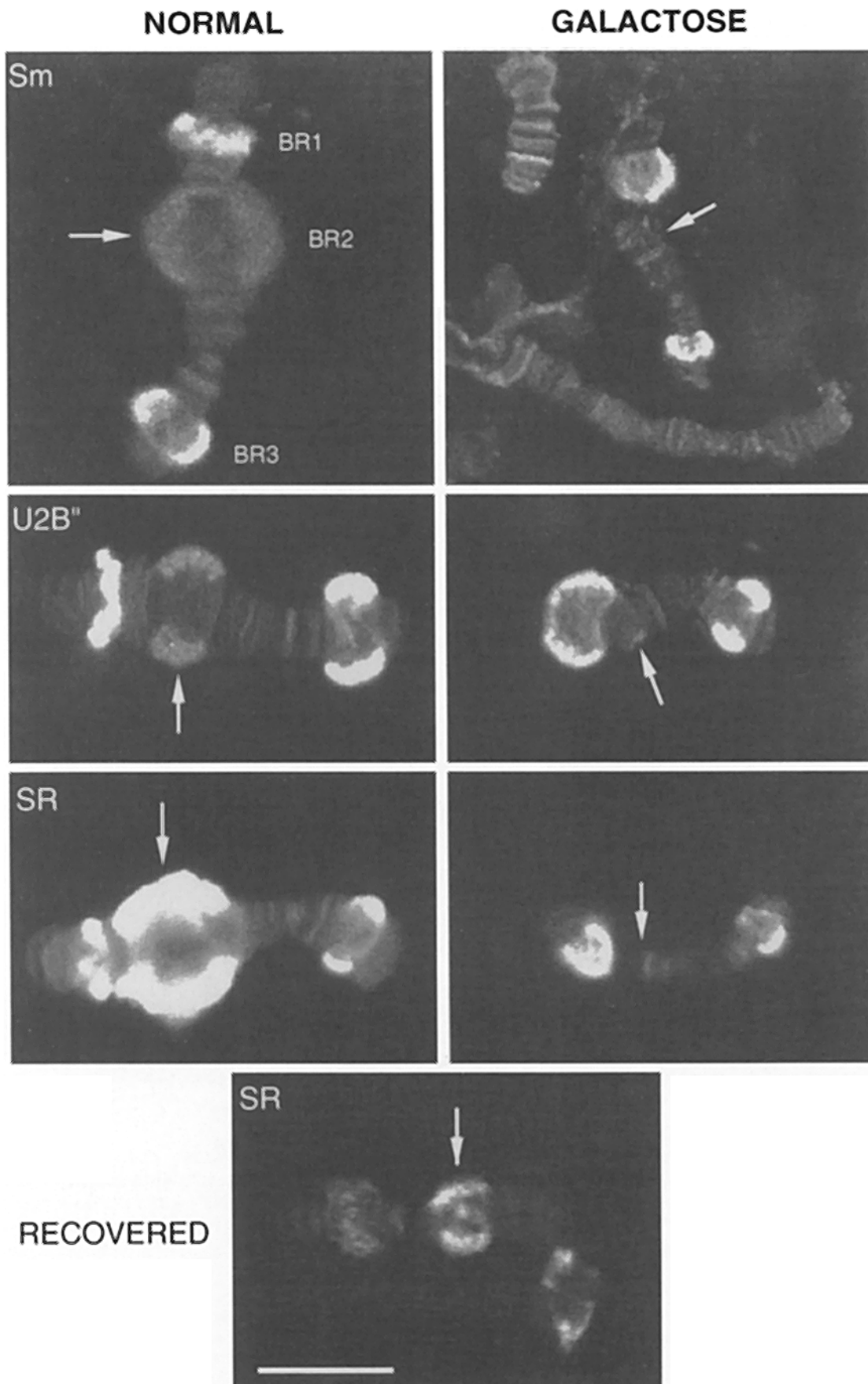


Figure 5. Recruitment of splicing factors to the activated BR 6 gene. Squash preparations of salivary glands of larvae from normal cultures and from larvae treated with galactose were stained with the antibodies detecting the Sm snRNP proteins (*Sm*), U2 snRNP B'' protein (*U2B''*), or the SR non-snRNP splicing factor (*SR*). The BR6 locus on chromosome III, close to a nucleolar organizer region, is indicated with an arrow. Bar, 40 μ m.

Figure 6. Displacement of splicing factors from the transcriptionally shut-down BR2 locus. Squash preparations of salivary gland cells from normal and from galactose-treated larvae were probed with the anti Sm protein (*Sm*), anti U2 snRNP B'' protein (*U2B''*), or anti-SR non-snRNP splicing factor (*SR*) antibodies. The BR1 and BR3 loci are not affected by the galactose treatment (intensely stained at each



end of the chromosome IV), but transcription of the two BR2 genes, both present in the BR2 locus (*arrow*), is shut down, and the splicing factors are displaced. In the last figure (*RECOVERED*), the larvae were first treated with galactose as described above and subsequently returned to normal culture conditions without galactose. It is shown that the splicing factor is again recruited to the reactivated BR2 genes. Bar, 20 μ m.

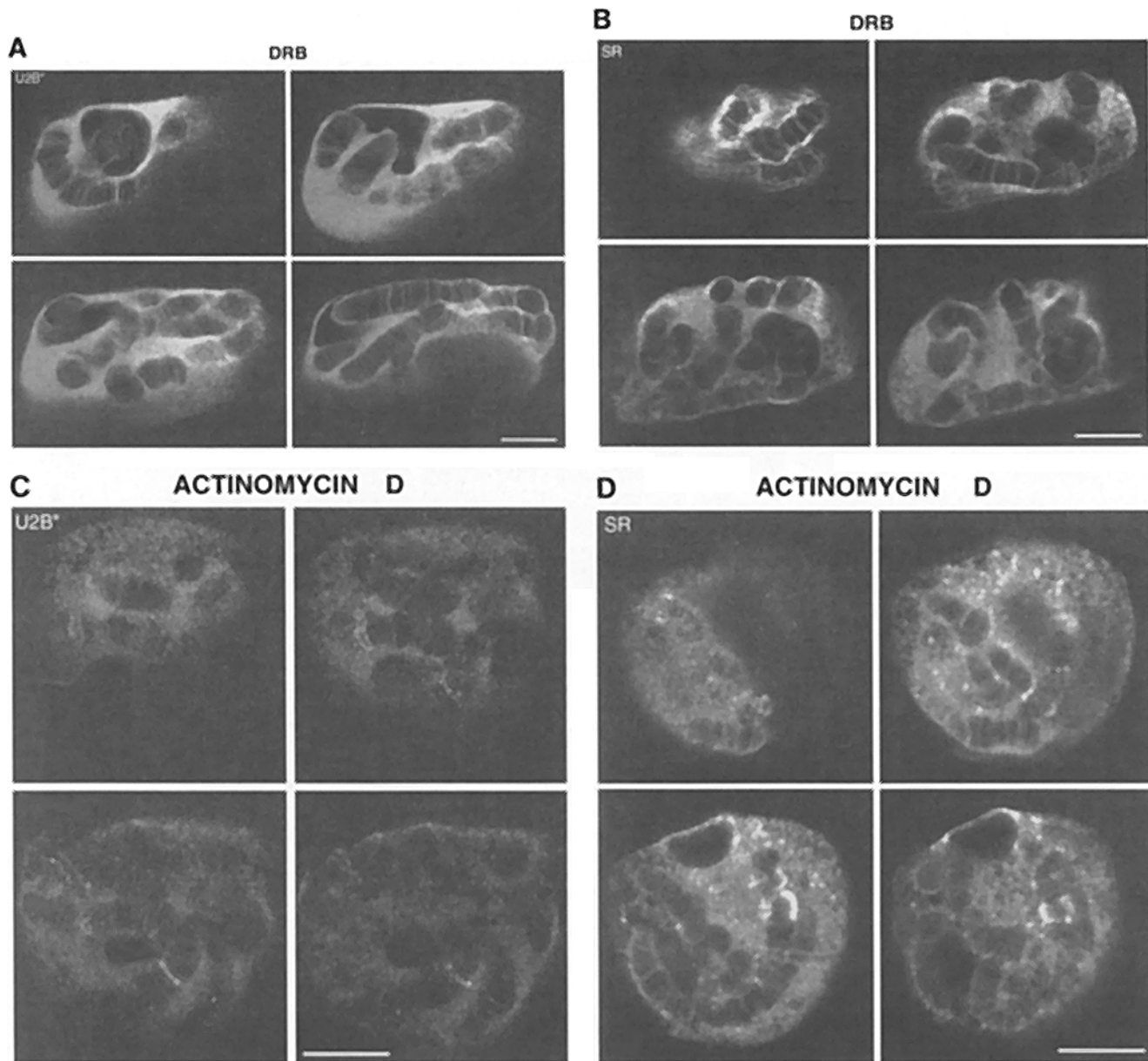
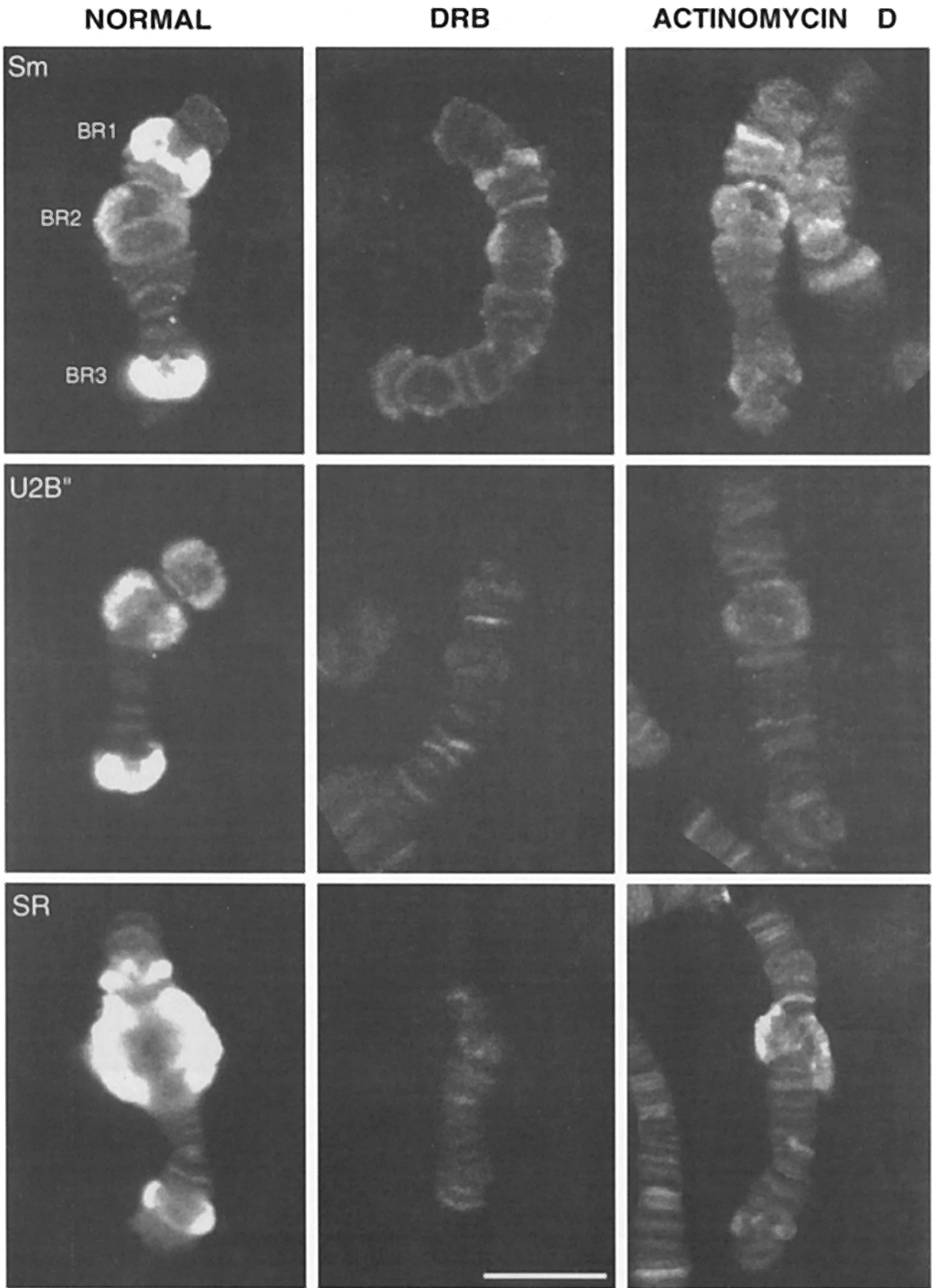


Figure 7. Displacement of splicing factors from the chromosomal loci into the interchromatin upon general transcription shutdown after DRB and actinomycin D treatment. The distribution is shown of the U2 snRNP B'' protein (U2B'', A and C) and the SR non-snRNP splicing factor (SR; B and D), after treatment with DRB (A and B) or actinomycin D (C and D). The staining is concentrated to the interchromatin of the nuclei (compare the untreated nuclei in Fig. 2). The chromosomes appear as dark regions but retain a decreased number of stained loci. The four pictures in each set represent consecutive optical sections with a distance between each section of 5 μm in A and B and 3 μm in C and D. Bars, 20 μm .

stained with any of the three antibodies. It can also be seen that the BR1 gene locus is essentially unaffected by the galactose induction. We could not detect any change in staining of any other chromosomal loci nor any obvious change in the relative staining of chromosomal loci and in-

terchromatin (data not shown). We also checked that the galactose effect is reversible. After 2 d in normal culture water lacking galactose, the splicing factors are no longer present in the BR6 locus but now reappear in the BR2 locus (Fig. 6).

Figure 8. Splicing factors are displaced from most, but not all, gene loci upon general transcription shutdown by DRB and actinomycin D. Squash preparations of salivary gland cells from normal larvae and from larvae treated with DRB or actinomycin D were stained with antibodies detecting the Sm proteins (*Sm*), the U2 snRNP B'' protein (U2B''), or SR non-snRNP splicing factor (SR). In the normal situation, all three active BR loci are intensely stained. After DRB treatment, the BR loci are inactivated and almost unstained, while other loci are relatively resistant to the treatment. After actinomycin D treatment, the staining is very much reduced but not to the same extent as after DRB treatment, especially for the anti-SR non-snRNP antibody. Bar, 20 μm .



Intranuclear Redistribution upon Experimentally Induced General Transcription Shutdown

Transcription can be blocked by treatment with DRB and actinomycin D. The effect of experimental inhibition of transcription on the intranuclear distribution of the splicing factors was assayed at three different levels: in intact nuclei, on individual chromosomes, and by quantitation of the splicing factors in microdissected chromatin and interchromatin preparations.

In intact nuclei, all three antibodies stained the interchromatin heavily, and the chromosomes were relatively unstained, appearing as darker regions within the nuclei (Fig. 7). Upon close inspection, a few chromosomal loci could be seen to be labeled. The BR loci were, as a rule, not stained. From these data, it appears as if the majority of splicing factors leave the chromatin upon transcriptional shutdown. In the case of DRB-treated animals, this effect is reversible. After bringing the larvae back to water lacking DRB for a few hours, the strong staining of BR loci and other chromosomal loci returns (data not shown).

In Fig. 8, the effect of DRB and actinomycin D treatment can be seen on the antibody staining of chromosome IV. Before treatment, the BR loci are strongly stained and after treatment, the staining is very low or totally absent. Morphologically, this is accompanied by condensation of the transcribing chromatin loops into more compact chromosome loci. The reduced staining is more pronounced for DRB-treated animals than for actinomycin D-treated animals. This is compatible with the known effects that DRB blocks transcription of hnRNA at or close to the site of initiation (Egyházi, 1975, 1976; Sehgal et al., 1976), while actinomycin D binds to DNA and blocks elongation of nascent RNA.

It is also evident that while the effect is clear for the BR loci, it is not as clear for other chromosomal loci on the same chromosome. It is a consistent finding that a small number of chromosomal loci all over the chromosomes are less sensitive to the drug treatment. The staining of these loci is sensitive to RNase treatment before antibody staining (data not shown), indicating that the splicing factors are associated with RNA. It has been reported earlier that after DRB treatment, hnRNA remain on the chromosomes for long periods (Egyházi, 1976). The nature of these long-lived and/or non-DRB-sensitive chromatin-associated RNAs is not known.

Chromosomes and interchromatin were, in addition, separated by microdissection from animals treated with DRB (Fig. 4). As described, the two nuclear fractions were extracted, electrophoretically separated, and probed with the anti-U2 snRNP B'' and anti-SR non-snRNP antibodies. The chromosome-associated staining was now reduced to 6%, as compared with 20% in nontreated animals for the U2 snRNP B'' antibody. In the case of the SR non-snRNP factor, a strong signal was obtained in the interchromatin fraction, but no signal above background could be recorded in the chromosomal fraction.

We conclude from these analyses that upon transcriptional shutdown with DRB and actinomycin D, the splicing factors dissociate from most gene loci and accumulate in the interchromatin. If the drug effect is reversible as for DRB, the splicing factors again appear at the actively transcribing genes.

Discussion

Spliceosome Factors Are Recruited to Nascent Pre-mRNA

In this study we show that in the intact nucleus, splicing factors are highly enriched in actively transcribing gene loci, where they are associated with pre-mRNA. More significantly, we also demonstrate that the splicing factors are recruited to actively transcribing genes and leave genes in which transcription is turned off.

Previous studies have demonstrated an overall dynamic organization of snRNPs in the nucleus in relation to cell type (Spector et al., 1992; Matera and Ward, 1993), terminal erythroid differentiation (Antonioni et al., 1993), RNA synthesis inhibitors (Carmo-Fonseca et al., 1992, 1993), and heat shock (Spector et al., 1991).

The spatial relation of spliceosome assembly, splicing, and transcription has been unclear, mainly because diploid nuclei do not easily lend themselves to studies in which it is possible to demonstrate association of splicing factors and splicing with individual genes (for discussion see Matraj, 1994). Such an association has been indicated from colocalization of antibody and oligonucleotide probes (Zhang et al., 1994). Studies of adenovirus-infected cells have also suggested recruitment of splicing components to the site of adenovirus gene transcription (Jiménez-García and Spector, 1993; Puvion-Dutilleul et al., 1994; Bridge et al., 1995), but this association is not clear (Zhang et al., 1994), and in addition, adenovirus infection leads to extensive morphological rearrangements in the nucleus that complicate interpretation of relocalization as a cause of changed gene activity (Bridge et al., 1993).

The BR gene family, in which the individual genes have evolved slightly different transcriptional regulation (Beer-mann, 1973; Lendahl and Wieslander, 1987), has allowed us to experimentally turn on and off specific BR genes that at the same time can be morphologically identified. Under these conditions, the *C. tentans* larvae live and develop normally, and the activation/repression of the BR6 and BR2 genes reflects a physiological response to environmental changes (Lendahl and Wieslander, 1987; Galler et al., 1984). We therefore directly demonstrate a dynamic reorganization of splicing factors in relation to gene-specific transcription under physiological conditions.

Our results are pertinent to models for how the splicing machinery operates in the intact cell nucleus, arguing that the spliceosome machinery must be organized to allow immediate recognition and binding to nascent pre-mRNA cotranscriptionally. Our results are in agreement with previous studies that have shown that hnRNP proteins are associated with sites of pre-mRNA synthesis (Matunis et al., 1993) and that spliceosome factors are associated with nascent pre-mRNA on polytene chromosomes (Sass and Pederson, 1984; Matunis et al., 1993; Kiseleva et al., 1994), on perichromatin fibrils (Fakan et al., 1984), and in spreads of active genes (Fakan et al., 1986).

The efficient association of splicing factors with nascent pre-mRNA implies that either a large excess of factors exists in the nucleus, allowing rapid binding to the emerging pre-mRNA during transcription (see below), and/or a specific coordinated organization of active chromatin, the transcription machinery, and the splicing machinery. The

latter alternative is attractive in the light of recent observations of association of splicing factors and wt1, a protein implicated in transcriptional control (Larsson et al., 1995).

Site of Splicing and Chromatin-Interchromatin Distribution of Splicing Factors

Our data emphasize that splicing factors associate with pre-mRNA at a considerable number of gene loci, suggesting that the majority, if not all, of intron-containing pre-mRNAs associate with splicing factors cotranscriptionally. Here, it should be commented that the SR protein Ct-hrp45 is homologous to the *Drosophila* protein B52 (Champlin et al., 1991). It has been reported that an anti-B52 antibody recognizes a 45–46-kD *C. tentans* protein, presumably Ct-hrp45, and that this antibody decorates active BR genes (Champlin and Lis, 1994). In that study, it was concluded that the B52 homologue in *C. tentans* is bound to chromatin, flanking unfolded transcriptionally active chromatin, and not to pre-mRNA. Based on our RNase experiments (compare BR1, BR2, and BR3 in Fig. 3, B and E) and the previous demonstration of the presence of Ct-hrp45 in BR pre-mRNP (Kiseleva et al., 1994; Wurtz et al., 1996), we conclude that the overwhelming majority of the Ct-hrp45 protein is associated with nascent pre-mRNP in the BR gene loci and in other chromosomal loci (compare chromosome III in Fig. 3, D and E).

EM (Beyer and Osheim, 1988), biochemical isolation (Wuarin and Schibler, 1994), in situ hybridization (Zhang et al., 1994), and direct isolation of gene-specific nascent pre-mRNA (Baurén and Wieslander, 1994) have shown that splicing may be a cotranscriptional process. Some, but not all, of these splicing factors therefore carry out their function in the splicing reaction cotranscriptionally and leave the pre-mRNA at the gene loci.

At the same time, not all pre-mRNA are spliced cotranscriptionally. Polyadenylated pre-mRNA-containing introns have been observed in many cases (e.g., see Nevins, 1983). In *C. tentans* polytene nuclei we have demonstrated that the introns in the BR1 gene pre-mRNA are excised both co- and posttranscriptionally (Baurén and Wieslander, 1994). A similar situation is true for the BR3 gene pre-mRNA, where introns are excised in an overall 5' to 3' direction, and posttranscriptional splicing is extensive (Wetterberg, I., G. Baurén, and L. Wieslander, manuscript in preparation). It is therefore evident that spliceosomes are part of many pre-mRNPs, as these are transported from the gene loci out into the interchromatin.

Nascent pre-mRNA in the *C. tentans* polytene nucleus accounts for ~10% of the total nuclear pre-mRNA content; i.e., 90% of the pre-mRNA is in the interchromatin (Edström et al., 1978). This is almost precisely the same distribution that we have measured for the U2 snRNP, as indicated by the B'' protein and the SR non-snRNP splicing factor. We know that spliceosomes assemble on the nascent pre-mRNA. 10% of the splicing factors is therefore a measure of the amount of splicing factors needed to handle the spliceosome assembly on 10% of the nuclear pre-mRNA. If we would assume that all splicing reactions occur close to the nuclear pore complexes, we would expect the correspondence in distribution between pre-mRNA and splicing factors to be observed. However, we know that considerable cotranscriptional splicing does occur,

and that in the interchromatin, >90% of the pre-mRNA has already completed splicing (Baurén and Wieslander, 1994; Wetterberg, I., G. Baurén, and L. Wieslander, manuscript in preparation). We therefore conclude that there is an excess of splicing factors relative to the number of intron-containing pre-mRNAs and that a substantial part of the splicing factors in the interchromatin is not engaged in splicing. This is in agreement with the large number of snRNPs present in a cell (Green, 1986; Kiledjian et al., 1994) and the apparent functional excess of U1, U2, and U5 snRNAs in yeast (Séraphin and Rosbash, 1989; Rymond and Rosbash, 1992). It is also in agreement with the abundance of splicing factors in mammalian nuclear regions, in which splicing does not appear to occur (for discussion and references see Mattaj, 1994), and in interchromatin in *Drosophila* (Zachar et al., 1993).

Interchromatin-located Splicing Factors

In mammalian diploid nuclei, spliceosome components are, apart from being diffusely distributed in the interchromatin, found to a large extent in aggregates of interchromatin granules. This is a widespread observation in eucaryotic cells but not universal. In insect cells, aggregates of interchromatin granules are not always found, and interchromatin granules containing snRNPs have not been demonstrated in *C. tentans* salivary gland cell nuclei (Vásquez-Nin et al., 1990, 1993).

In this study, we have not detected patterns of staining that could be attributed to aggregates of interchromatin granules. In nuclei of DRB- and actinomycin D-treated cells, no such obvious organization could be detected either. The rather homogeneous reticular localization throughout the interchromatin space is compatible with the presence of snRNP-containing fibrillar aggregates previously observed in the EM (Vásquez-Nin et al., 1990). As all introns are not excised cotranscriptionally (Baurén and Wieslander, 1994; Wetterberg, I., G. Baurén, and L. Wieslander, unpublished data), it is also compatible with the association of splicing factors with not yet spliced pre-mRNAs, which are transported from a large number of gene loci. Some non-snRNP splicing factors may also be associated with pre-mRNA not only at the site of introns and remain so after completed splicing (Kiseleva et al., 1994; Alzhonova-Ericsson, A., X. Sun, N. Visa, E. Kiseleva, T. Wurtz, and B. Daneholt, manuscript submitted for publication). There is no indication that pre-mRNP to be spliced are collected in specific loci in the interchromatin, although we cannot rule out such a possibility. In addition, we do not have the possibility to detect splicing components released after splicing, e.g., associated with the released introns.

In summary, we demonstrate that spliceosomal components are highly dynamic in the nucleus. They associate with pre-mRNA cotranscriptionally. They may then carry out the splicing reaction cotranscriptionally and leave the pre-mRNA, or they may stay associated with the pre-mRNA as spliceosomes at least during the early phase of transport through the interchromatin. Our data (Baurén, G., and L. Wieslander, unpublished data; Wetterberg, I., G. Baurén, and L. Wieslander, unpublished data) argue that most of the splicing reactions then are carried out close to the gene locus. In the interchromatin, splicing fac-

tors are therefore present as part of spliceosomes or possibly as postsplicing spliceosomal components to be reused. In addition, spliceosome factors may be stored, perhaps as partially preassembled spliceosomes (see Mattaj, 1994). The proportion of these various states of the spliceosomal factors should be largely dependent on transcription, i.e., synthesis of intron-containing pre-mRNAs and their transport to the nuclear pore complexes.

To understand this dynamic life, the crucial points to uncover are how spliceosomal components are assembled in relation to active genes, how the spliceosomal machinery is organized relative to the transcription machinery, and how the spliceosomal factors are degraded or recovered for another round of function.

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