Balbiani granules as a model for studying RNA synthesis and processing

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Summary
We investigated, by ultrastructural immunocytochemistry, the localization and molecular composition of Balbiani granules from *Chironomus thummi* polytene chromosomes. The salivary gland cells produce huge amounts of RNA coding for a single protein, hence the mRNAs have all the same length and the resulting granules share identical characteristics. Our results show the localization of cleavage, polyadenylation and splicing factors on the nascent perichromatin fibrils and, more rarely, on the already structured Balbiani granules.

Key words: cell nucleus, RNA transcription, Balbiani granules.

Introduction

RNA synthesis in the cell nucleus gives rise to perichromatin fibrils (PF) and perichromatin granules (PG), which are ribonucleoprotein (RNP) containing structures representing the morphological evidence of transcription at electron microscopy (Bernhard, 1969).

It is known that PF represent the most frequent and mobile complexes among the RNA-containing structures. Incorporation of RNA precursors as well as specific immunolabeling have shown that co-transcriptional splicing and capping occur therein (Fakan, 1994; Fakan and Bernhard, 1971; Fakan *et al.*, 1986).

As for PG, they probably represent a storage form of RNA and likely originate from the folding of PF. According to the most generally accepted theory, PG play a role in the export/accumulation of mRNAs in the nucleus: this is based on the results of different experiments in which drugs and hormones have been used to purposely alter RNA metabolism or transport to the cytoplasm.

In support of this theory it must be underlined that the PG observed in mammals share close similarities in their structure and composition to the Balbiani ring granules (BG), *i.e.* the nuclear mRNP found in the polytene chromosomes of *Drosophila* and *Chironomus* species (Daneholt, 2001). The comparison of the sub-structural features of BG and PG using the same preparation procedures showed that single granules of either type are indistinguishable at electron microscopy as for their fine structure (Vàzquez Nin *et al.*, 1996; 1997). Studies mainly performed by immunoelectron microscopy demonstrated that both the PG and BG contain already spliced and polyadenilated mRNA, thus suggesting that both structures should likely derive from mature PF (Vàzquez Nin *et al.*, 1996).

The aim of this study was to investigate, by ultrastructural immunocytochemistry, the localization and molecular composition of BG from *Chironomus thummi* polytene chromosomes. In fact, these salivary gland cells produce huge amounts of RNA coding for a single protein, hence the mRNAs have all the same length and the resulting granules should have identical characteristics.

Materials and Methods

Samples of salivary glands from *Chironomus thummi* were prepared for the ultrastructural immunocytochemical analyses at the Centre for Electron Microscopy of the University of Lausanne (Switzerland).

Salivary glands isolated from fourth instar larvae under a stereo microscope were fixed in 4% formaldehyde for 2 hr at 4°C; after thorough washing in phosphate buffered saline (PBS), the glands were immersed in 0.5 M NH_4Cl in PBS for 30 min at room temperature (r.t.) to block free aldheyde groups. The samples were then dehydrated in ethanol, embedded in the acrylic resin LR White, and polymerized at 60°C for 24 hr. Thin (60-70 nm) sections were cut with an ultramicrotome and collected onto 200 mesh nickel grids to be treated for immunocytochemical labeling.

Sections were incubated for 3 min with Normal Goat Serum (NGS) diluted 1:100 in PBS, followed by 5 min incubation in a mixture of 0.05% Tween

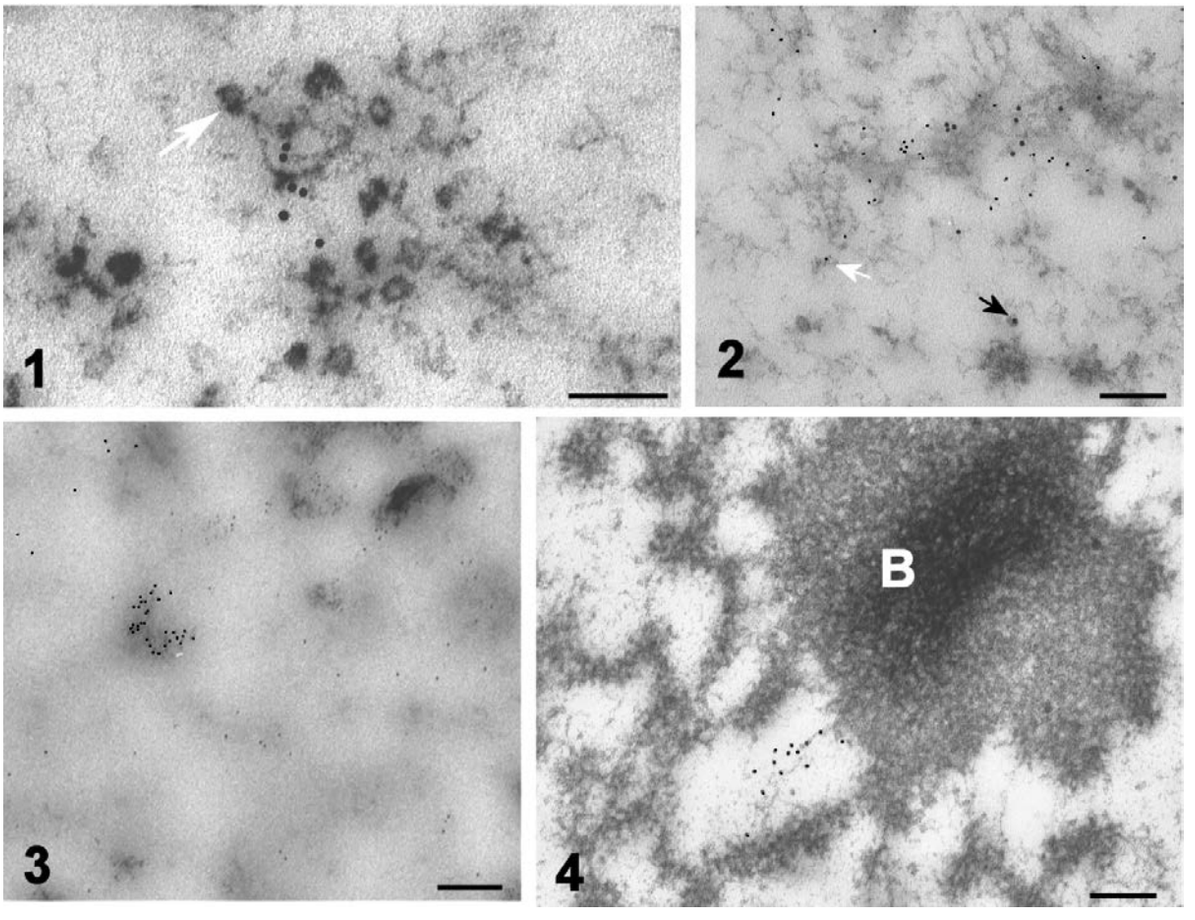

Figure 1. After EDTA regressive staining, Balbiani granules can be seen during the folding of the fibril (arrow); immunolabeling for RNase.
Figure 2. Double immunolabeling for RNase A (large gold grains) and methylcap (small grains). The black arrow indicates a completely formed and coiled granule, while the white one a labeled fibril on which capping is still undergoing.
Figure 3. CPSF 100 is labeled on the fibril by 6 nm gold grains. The sample is stained selectively for RNA with terbium citrate.
Figure 4. After immunolabeling for SC-35 splicing factor: the gold grains are localized on a transcript emerging from the Balbiani ring (B).
Bar = 100 nm

20 plus 0.1% bovine serum albumin (BSA) in PBS (PBS-Tween-BSA); the sections were then incubated for 17-24 hr at 4°C with specific antibodies (see Table 1) diluted in PBS-Tween-BSA, and finally with the appropriate gold-conjugated secondary antibodies diluted 1:20, for 30 min at r.t. In some experiments, double immunolabeling was performed using secondary antibodies conjugated with differently sized gold particles.

To detect RNA, the immunolabeled sections were stained with 0.2M terbium citrate pH 8.5 for 45 min at r.t. and briefly washed with water (Biggiogera and Fakan, 1998): terbium ions bind guanosine monophosphate in the RNA molecules (Ringer *et al.*, 1980) and the electron dense product allows an effective visualization at high resolution.

To label RNPs, the immunolabeled sections were submitted to the EDTA regressive procedure (Bernhard, 1969): briefly, the sections were stained for 3 min with 7% uranyl acetate in water, washed and dried; they were then incubated for 3-5 min with 0.2M EDTA, pH 7.0, washed and dried, to be finally stained with lead citrate for 1 min. Uranyl acetate binds both RNPs and DNA in the nucleus, while EDTA is able to remove uranyl from DNA thus bleaching chromatin; on the contrary, a higher contrast will be acquired by the RNP containing structures.

Results

At the electron microscope, the EDTA regressive staining is very useful to interpret the structure and the nature of the polytene chromosome in *Chironomus*. In Figure 1, the BG appear darker than the nucleoplasmic background and are easily identified.

We utilized an anti-RNase A antibody specifically labeling the enzyme, both in the active and inactive form; in the same experiment, an additional labeling with the H20 antibody was used to recognize the 2,3,3-methylguanosine present at the 5' site of the transcript. An elongated fibril containing nascent pre-mRNA can be seen labeled by the H20 probe in Figure 2: in the same micrograph it is worth noting that several fibrils were labeled by the anti-RNase antibodies. It must be underlined here that double-labeled fibrils were never found.

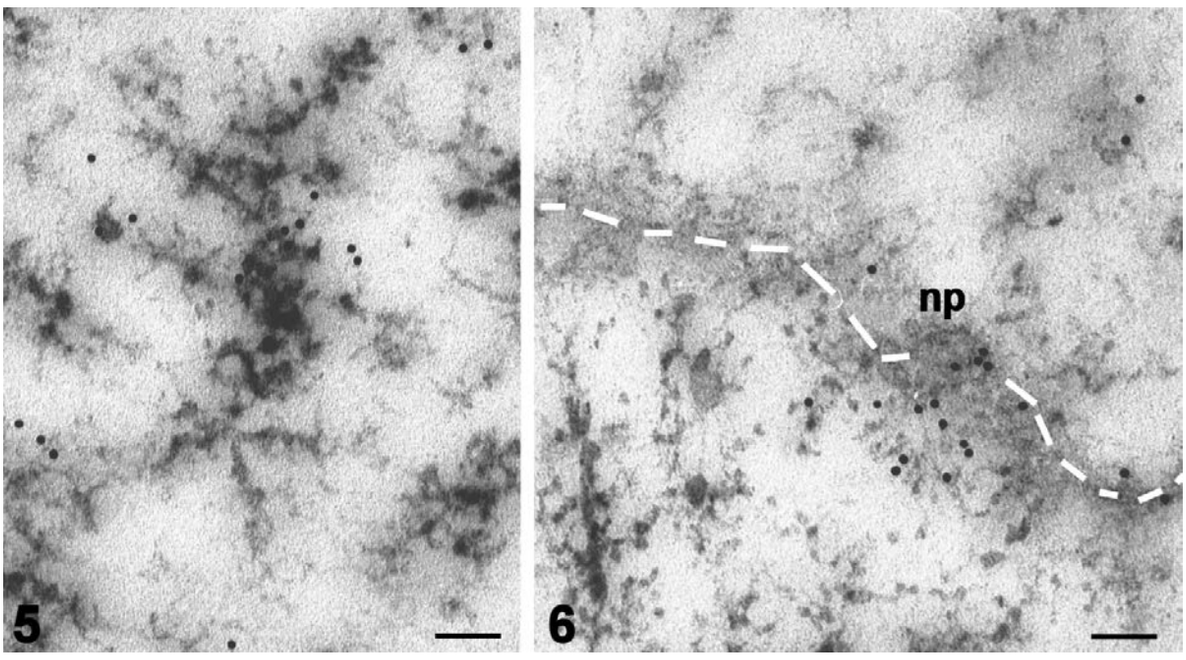

Figure 5. Labeling for SC-35 is present both on the fibril and on the completed granule.
Figure 6. Passage of the granules from the nucleus to the cytoplasm. The nuclear envelope is underlined in white. At the pore complex (np) SC-35 is still associated with the BG.
Bar = 100 nm

Table 1. Antibodies used for the immunocytochemical analyses.

Primary antibody	Source	Antigen	Dilution	References
Anti-RNAse A	Rabbit	RNase from bovine pancreas	1:100	Painter *et al.,* 1973
H20	Mouse	2,2,3-trimethylguanosine cap at the the 5' terminal of the UsnRNP	1:50	Luhrmann *et al.,* 1982
CPSF 100	Rabbit	100 kDa subunit of CPSF	1:20	Jenny *et al.,* 1994
CPSF 160	Mouse	160 kDa subunit of CPSF	1:50	Jenny *et al.,* 1994
Anti-SC-35	Mouse	SC-35 Splicing factor	undiluted	Fu and Maniatis, 1990

The fibrils may give rise to BG by folding, and in some cases (Figure 2, arrows) the labeling can be observed also on these structures. Immunolabeling for the methyl-cap was, however, quite rare: likely, as the folding of the fibril starts at the 5' end the epitope would precociously become hidden to the antibody.

Termination of the RNA synthesis is linked to polyadenylation, and the cleavage and polyadenylation specificity factor (CPSF) is involved with its four subunits of 160, 100, 73 and 30 kDa (Schul *et al.*, 1996); in its action, this factor moves together with RNA polymerase along the DNA molecule and may be detected by anti-CPSF100 and anti-CPSF160 kDa antibodies. In Figure 3 the labeling for CPSF100 is shown: interestingly, the signal is rather abundant, indicating that at this step of BG formation the epitopes are still openly available for antibody binding. One of the key steps in mRNA maturation is splicing, *i.e.* the removal of introns which are four in the case of the gene expressed in salivary glands. The SC-35 splicing factor is an essential non-snRNP factor which locates on the BG at different levels of granule formation. SC-35 was detected on the puffs of polytene chromosomes, where chromatin decondenses during transcription (Figure 4). Moreover, this factor can also be seen in the nucleoplasm, in correspondence of the fibrils on the way to coiling and form BG (Figure 5). Finally, the labelling for SC-35 was also found close to the nuclear envelope, which is well visible in Figure 6, where BG start to unravel to cross the nuclear pore.

Conclusions

RNA maturation is a complex process involving several factors which mostly act co-transcriptionally. Investigation over the last twenty years allowed to unravel this mechanism as far as PF are concerned (Cmarko *et al.*, 1999); on the contrary, the study of the role and dynamics of PG proved to be much more difficult. So far, the folding of the fibril was only observed in BG, thanks to the large number of these structures which can easily be examined at electron microscopy and isolated for biochemical analyses (Daneholt, 1975, 1997, 2001). We have shown here that salivary glands represent a model of choice to detect the association of maturation factors to pre-mRNA, since these cells synthesize one mRNA only, thus producing a homogeneous pool of pre-mRNA molecules.

Our results show the localization of cleavage, polyadenylation and splicing factors on the nascent PF and, more rarely, on the already structured BG. Interestingly, the anti-methylcap is present almost exclusively on the fibrils, since the epitope is hidden during the folding. On the contrary, SC-35 can be detected on both structures and is still present nearby the nuclear pore and in the cytoplasm. On some fibrils, we found immunolabeling for RNase A but not for methyl-cap, and *vice versa*: this could indicate that the fibrils labeled for RNase may undergo degradation even before capping or, alternatively, that the methyl-cap may be degraded first with the resulting loss of the target epitope.

These present findings confirm that BG in polytene chromosomes are an especially convenient natural model for investigating *in situ* the molecular interaction of specific factors involved in RNA synthesis and maturation.

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