Silver staining of Drosophila polytene chromosomes and the effect of hyaluronidase and lysozyme pretreatment

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Abstract

The Ag-AS technique was used for staining the polytene chromosomes of *D. melanogaster* and *D. lummei*. Bands were stained dark reddish-brown, interbands light yellow. A toromere was heavily stained on the sixth chromosome of *D. lummei*. The staining intensity of nucleoli was lower than that of chromosomes. During a prolonged staining ectopic threads and the nonhomogeneous structure of nucleoli were revealed. Pretreatment with RNase caused slight changes in the silver staining pattern of chromosomes; pretreatment with DNase did not result in any visible changes, while after preincubation with proteolytic enzymes chromosome morphology was destroyed. Hyaluronidase and lysozyme removed the silver-reducing components from chromosomes without destroying the general chromosome structure. Each of these two enzymes acts specifically: hyaluronidase affects the morphology of chromosomes, but not nucleoli and bands at heat shock puffs, whereas the action of lysozyme is probably evenly distributed between chromosomes and nucleoli.

Introduction

The ammoniacal silver staining (Ag-AS) technique has been extensively used for the localization of nucleolar organizer regions (NORs) on mitotic chromosomes in a wide variety of vertebrates (e.g. Goodpasture & Bloom, 1975; Tantravahi *et al.*, 1976; Bloom *et al.*, 1978; Nardi *et al.*, 1978; Schmid, 1978), in the insect *Achaeta domesticus* (Howell, 1977; Czaker, 1978) and in plants (Schubert *et al.*, 1979). It has also been used for the staining of the lampbrush chromosomes of *Triturus cristatus carnifex* (Varley & Morgan, 1978), as well as of synaptonemal complexes (e.g. Dresser & Moses, 1980) or of polytene chromosomes of two Dipterans – *Sciara coprophila* (Black & Ansley, 1964) and *Rhynchosciara hollanderi* (Stocker, 1978; Stocker *et al.*, 1978). Although the mechanism of silver staining is not yet known in detail, it is supposed that silver is intensively absorbed by specific non-histone proteins of NORs which were active in the previous interphase (Howell, 1977, Schwarzacher *et al.*, 1978; Olert *et al.*, 1979).

In this study the Ag-AS technique was used for staining the polytene chromosomes of *Drosophila*. It was shown that the intensively stained component is removed after the chromosomes have been pretreated with hyaluronidase and lysozyme.

Material and methods

Drosophila melanogaster (Oregon R) and *D. lummei* Hackman (Moscow strain) salivary gland chromosome preparations of the late third instar larvae were made using the conventional squashing technique. For most of the experiments larvae of *D. melanogaster* had been prelimarily kept at 37 °C for 30 min, i.e. we used larvae with developed 'heat

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shock' puffs. *D. lummei* larvae were cultured at 14 °C in order to obtain toromeres in the sixth chromosome.

Silver staining was carried out according to modified methods of the Ag-AS technique (Goodpasture & Bloom, 1975). Three drops of a 50% solution of silver nitrate were placed on the surface of each slide and covered with a coverslip. The slides were heated to 65 °C for 2–3 min until silver crystals were formed along the edges of the coverslip. Then the coverslip was removed and the slide rapidly washed and dried. After that 2 drops of an ammoniacal-silver solution (4 g $AgNO_3$ per 5 ml H_2O and 2.5 ml concentrated NH_4OH) and 2 drops of 3% formalin (neutralized with sodium acetate) were placed on the slide surface. The slide was covered with a coverslip and the staining process was monitored under a light microscope.

Giemsa staining: Two times diluted azure-eosine stock solution (according to Romanovsky-Giemsa) for 10 min, followed by 10 sec rinsing in distilled water and air-drying.

The conditions of pretreatment with enzymes and NaOH were as follows (Khachaturov *et al.*, 1975; Howell, 1977; Byarugaba, 1978; Varley & Morgan, 1978):

(a) RNase purified by preparative polyacrylamide gel electrophoresis, 100 μg/ml in 2×SSC, 37 °C, 1 hour;

(b) Pancreatic DNase I (Serva), 100 μg/ml in 0.01 M $MgCl_2$ 37 °C, 1 hour;

(c) Trypsin, 100 μg/ml in 2×SSC, room temperature, 5 minutes;

(d) Pepsin (Schuchardt), 10 mg/ml in 0.9 M NaCl, pH 1.6, 37°C, 1 hour;

(e) Pronase E (Serva), 100 μg/ml, water solution, 37 °C, 1 hour;

(f) Testicular hyaluronidase (Calbiochem), 100 μg/ml in 0.1 M phosphate buffer, pH 6.0, 37 °C, 1 hour;

(g) Hen's egg-white lysozyme (Serva), 100 μg/ml in 0.1 M phosphate buffer, pH 7.2, 37 °C, 1 hour;

(h) 0.1 N NaOH, room temperature, 20 minutes.

After each treatment slides were washed in two changes of water, 70% and 95% ethanol, and air-dried.

The preparations were analyzed with a Zeiss NU-2 microscope (K. Zeiss, Jena) with a 100× oil immersion objective.

Results

Staining of non-treated preparations

The following changes were observed during the staining of polytene chromosomes. After the first stage ('the impregnation') chromosomes turned light yellow. During the very first moments of the second stage ('the development') the yellow colour grew more intense, the chromosome bands then turned reddish brown, while interbands remained yellow. Simultaneously nucleoli acquired a reddish tinge, but they were stained more lightly than the bands and their structural inhomogeneity was clearly visible. The protoplasm around chromosomes also turned yellow, but was paler than observed using usual methods of staining of polytene chromosomes (Fig. 1), although some

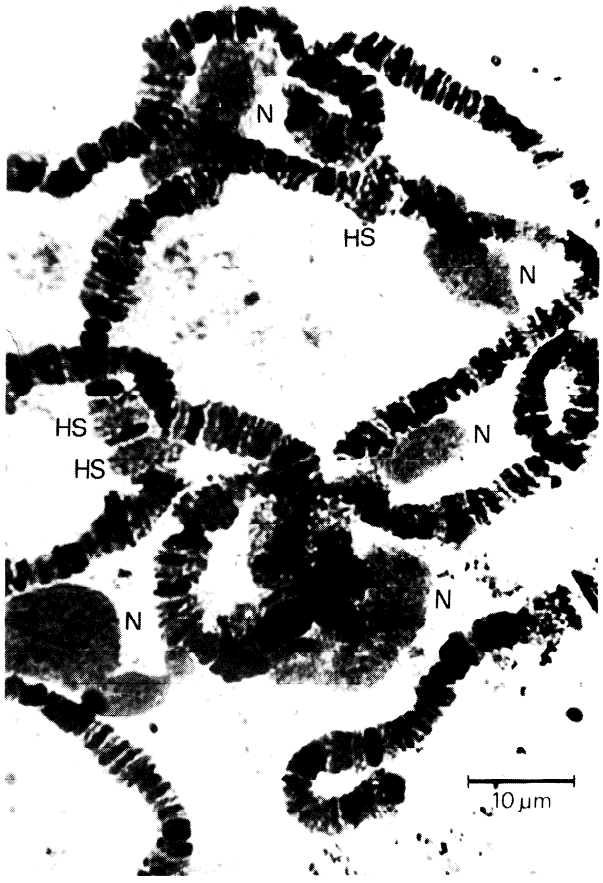

Fig. 1. Silver-stained chromosomes of *D. melanogaster*. Five nucleoli (N) and heat shock puffs (HS) can be seen.

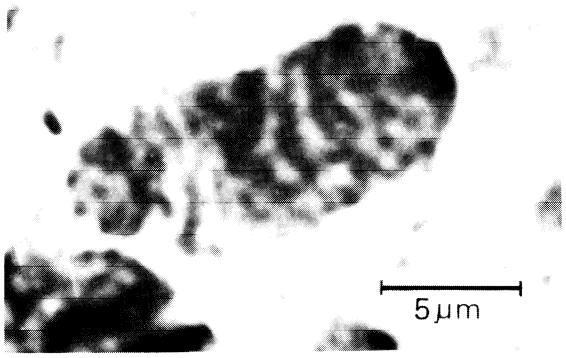

Fig. 2. The sixth chromosome of *D. lummei.* Noted dark toromere at the end of the chromosome.

regions were heavier stained with silver than one could expect on the basis of the result of Giemsa staining.

The Ag-AS method enabled us to reveal some chromosomal details clearly. Thus, the regions of heat shock puffs did not stain as intensively as bands (Fig. 1) and seemed to have a granular structures. The *D. lummei* toromeres in the sixth chromosomes were almost black (Fig. 2).

If the 'development' was continued for a longer time, all the bands became black, many threads between chromosomes or chromosomes and nucleoli were revealed (Fig. 3).

Staining after pretreatment

(a) *RNase treatment:* RNase pretreatment did not change the chromosome silver staining pattern (Fig. 4). Thick bands became more clearly visible because thin bands were not seen at all. Nucleoli were stained as intensively as chromosomes.

After Giemsa staining of RNase pretreated preparations nucleoli and cytoplasm were not stained. The general colour of chromosomes was reddish, not blue-purple as in the case of untreated preparations.

(b) *DNase treatment:* After DNase pretreatment the morphology of silver stained chromosomes seemed to be changed only slightly. The difference in staining intensity between bands and interbands was not as clear as in untreated preparations.

Fig. 3. D. melanogaster chromosomes stained for a long time. The nucleolus has a nonhomogeneous structure; ectopic threads and threads connecting the nucleolus to chromosomes are visible.

Nucleoli seemed to be unaffected. The colour of chromosomes and nucleoli was reddish-brown.

(c) *Treatment with proteolytic enzymes:* Pretreatments with all three proteolytic enzymes used (pepsin, pronase, trypsin) resulted in changes in polytene chromosome morphology. The band structure disappeared, chromosomes became almost unstainable with silver, the colour was pale yellow. Nucleoli were destroyed to a lesser degree than chromosomes; they were stained more intensively than chromosomes, turning russet.

The change in chromosomal morphology was also confirmed by Giemsa staining: chromosome structure was somehow affected and they were stained weakly.

(d) *Treatment with lysozyme and hyaluronidase:* The hyaluronidase-treated polytene chromosomes were stained with silver very weakly, but some bands remained red-brown (Fig. 5). In particular, heat shock puffs were seen in the middle of heavily

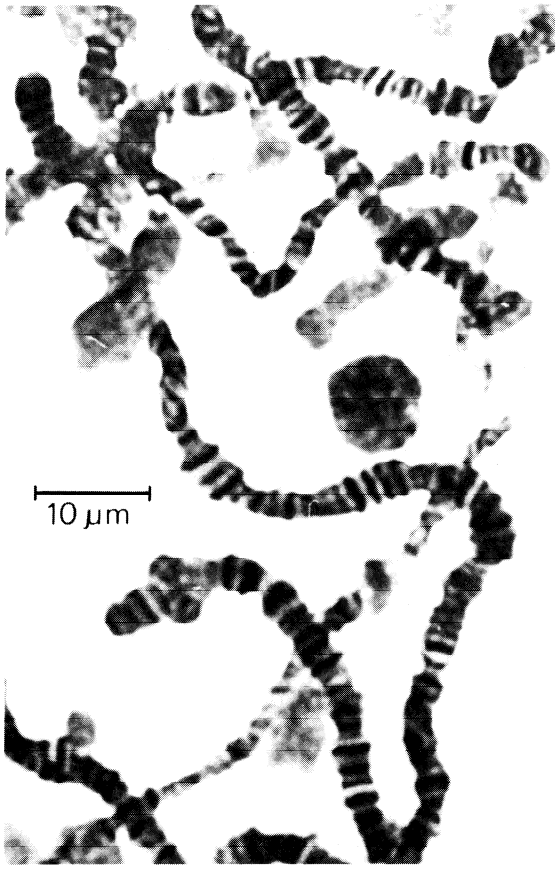

Fig. 4. Silver-stained chromosomes of *D. melanogaster*, pretreated with RNase. Bands have clear boundaries; the intensity of coloration of nucleoli and chromosomes are almost the same; specific pattern of X2B puff structure is seen.

Fig. 5. Silver-stained chromosomes of *D. melanogaster*, pretreated with hyaluronidase. Nucleolus and a few bands are heavily stained. Stained bands (A) at heat shock puffs are seen.

stained bands. Nucleoli were stained more intensively than the majority of chromosome regions; they were revealed as nonhomogeneously stained brown masses. Cytoplasm was hardly stained at all. After prolonged staining yellow threads connecting the chromosomes were seen.

The silver-staining pattern of polytene chromosomes after lysozyme pretreatment also differed from the staining pattern of untreated preparations (Fig. 6). The band structure seemed to be affected, the colour of chromosomes was yellow, but many darker bands were seen and the difference in the intensity of staining of different bands was not as distinct as in the case of hyaluronidase-pretreated chromosomes. Nucleoli were light brown and stained more weakly than chromosomes, their structure and staining patterns were similar to those of the non-treated preparations. Cytoplasm was not stained at all.

The effect of hyaluronidase and lysozyme pretreatment was also seen after Giemsa staining. In both cases bands appeared somewhat changed, only central regions in bands were stained. The intensity of Giemsa staining after lysozyme treatment was very weak, nucleoli were almost unstained, but cytoplasm was rather heavily stained blue. After hyaluronidase pretreatment cytoplasm was not stained by Giemsa.

Successive treatment of preparations with two enzymes (lysozyme, hyaluronidase; lysozyme, RNase; hyaluronidase, RNase) did not reveal any noticeable modifications in the silver-staining pattern as compared with pretreatment with hyaluronidase or lysozyme; RNase abolished silver

Fig. 6. Silver-stained chromosomes of *D. melanogaster*, pretreatment with lysozyme. Many (not all) bands are stained; the nucleolus is stained less intensively than the stained bands.

staining of nucleoli. After the treatment with all the three enzymes the intensity of the staining of chromosomes was very weak even after prolonged staining. Nucleoli were revealed only after prolonged staining and were stained more weakly than chromosomes.

Giemsa staining of preparations successively treated with two or three enzymes indicated a removal of some stainable material from bands. Nucleoli were practically invisible and the cytoplasm was not stained.

(f) *Treatment with NaOH:* Pretreatment of the polytene chromosomes with NaOH solution also affected the silver staining chromosome pattern. Chromosomes were light yellow, their form and band structure seemed to be destroyed. Nucleoli were heavily stained brown; cytoplasm stained yellow.

Discussion

The Ag-AS technique can be used for the staining of polytene chromosomes of *Drosophila*. The staining reveals the band structure of chromosomes, and such details as toromeres, ectopic threads, heat shock puffs, and nucleoli.

D. melanogaster nucleoli are always stained more weakly than polytene chromosomes. Similar results have been obtained for *Rhynchosciara hollaenderi* (Stocker, 1978). By contrast in mitotic chromosomes and diploid cells it is NORs and nucleoli correspondingly which are most heavily stained (e.g. Goodpasture & Bloom, 1975; Schmid, 1978; Schubert *et al.*, 1979; Martin-DeLeon *et al.*, 1978; Pellicia *et al.*, 1978). Nucleoli are also intensively stained in the oocyte chromosome preparations of the newt *Triturus cristatus carnifex* (Varley & Morgan, 1978).

Since the mechanism of silver staining is not yet quite known, the above mentioned difference in intensity between nucleoli and chromosomes is not easy to explain. However, it can be supposed that in the case of interphase nuclei the argentophile substrate is concentrated in nucleoli and NORs, whereas in the case of polytene nuclei it is distributed over a larger area within nucleoli.

It is generally assumed that the Ag-staining material is represented mostly by non-histone proteins (Howell, 1977; Schwarzacher *et al.*, 1978; Olert *et al.*, 1979; Bourgeois *et al.*, 1979). These specific proteins seem to be rich in sulfhydryls (Buys & Osinga, 1980). It should be mentioned that in nucleoli and NORs of mitotic chromosomes specific Giemsa-staining acid-insoluble proteins have been found (Matsui *et al.*, 1978).

DNase pretreatment did not affect the staining pattern of polytene chromosomes. Similarly, it did not change the staining patterns of lampbrush (Varley & Morgan, 1978) or mitotic (Buys & Osinga, 1980) chromosomes. Thus, DNase does not remove the main stainable material. However, it should be borne in mind that the heavily silver-stained toromeres of the sixth chromosome of *D. lummei* have an increased DNA content; probably, it consists mainly of endoreplicated satellite DNA (Evgen'ev *et al.*, in press).

RNase pretreatment resulted in a very slight change in silver-staining patterns of chromosomes and nucleoli. The same was shown in the cases of

mitotic or meiotic chromosomes of different animals (Howell, 1977; Schwarzacher *et al.*, 1977; Varley & Morgan, 1978; Buys & Osinga, 1980). Thus it seems possible that RNA itself does not reduce silver, but that treatment with RNase after hyaluronidase or lysozyme treatments abolished silver staining of regions of intensive RNA synthesis – namely, nucleoli, heat shock puffs and some bands. This is in good agreement with the data obtained on nucleoli of mammalian cells which showed that silver is reduced by some components of newly-synthesised RNP (Hausmann *et al.*, 1978; Hofgartner *et al.*, 1979; Hennen, 1979; Bourgeois *et al.*, 1979).

Proteolytic enzymes cause a change in polytene chromosome morphology, they remove a great portion of the silver-staining material, though some stainable substrate remains. The silver staining pattern of mitotic and meiotic chromosomes was also affected after chromosomes had been pretreated with trypsin (Howell, 1977; Schwarzacher *et al.*, 1978; Varley & Morgan, 1978; Buys & Osinga, 1980).

The results show that the glycosidases used remove the main part of the Ag-stainable material from polytene chromosomes. Since silver is absorbed mostly by tissue carbohydrates (Puchtler Waldrop, 1978), one may assume that the material removed is some kind of carbohydrate. It is difficult to say whether those components are glycoproteins or mucopolysaccharides. Glycoproteins have been found in mammalian chromatin (Rizzo & Bustin, 1977; Miki *et al.*, 1978) and even in mitotic chromosomes (Hozier *et al.*, 1979). Moreover, one of the glycosidases used – lysozyme – has been found in histone fractions of chick oviduct chromatin (Conn & O'Malley, 1975). Thus it seems possible that polysaccharides and glycoproteins take part in polytene chromosome organization.

An examination of Giemsa-stained preparations supports the hypothesis that hyaluronidase and lysozyme remove chromosomal components. It has been shown that lysozyme removed some Giemsa-stainable material (mucopolysaccharides?) from mammalian chromosomes (Khachaturov *et al.*, 1975). After staining hyaluronidase-pretreated mouse chromosomes with acridine orange the characteristic pattern of Q-bands was seen (Moutschen, 1976).

Probably, during NaOH treatment some kind of the same carbohydrates are removed from the polytene chromosomes. It is possible that treatment of the oocyte chromosomes of *Achaeta domesticus* (Howell, 1977) or of *Triturus cristatus carnifex* (Varley & Morgan, 1978) with NaOH, and of human mitotic chromosomes with alkaline solution (Howell & Denton, 1976), as well as the salt treatment of the polytene chromosomes of *Chironomus tentans* (Plagens, 1978) results in the removal of similar components. One may suppose that the core of mitotic chromosomes contains polysaccharides similar to those present in polytene chromosomes, as it has been shown that the core can be revealed through silver staining (Howell & Hsu, 1979).

All the data discussed enable us to consider that many chromosome components, including non-histone proteins, are stained by silver, but the main Ag-stainable material of polytene chromosomes consists of polysaccharides, possibly components of glycoproteins.

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