Inactive chromatin in polytene chromosomes of *Chironomus tentans* partially shows a non-nucleosomal organization

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**Abstract.** Chromatin from salivary gland cells of *Chironomus tentans* was fractionated into soluble and insoluble fractions by gentle sonication at low ionic strength followed by differential centrifugation. The fractions (18,000 g, 35,000 g and 100,000 g pellets) were examined by combined electron microscopic and biochemical analysis in order to correlate structural organization with content and phosphorylation of histone and non-histone proteins and transcriptional activity. The insoluble chromatin (18,000 g pellet) was dominated by inactive band and interband structures but included transcriptionally active regions as well. The analysis of the inactive portion of insoluble chromatin indicated the absence of typical nucleosomal packaging. The DNA filaments were extended and not wrapped around the globular histone granules. Nonetheless, the presence of a full complement of core histones could be demonstrated by biochemical analysis. The soluble chromatin fraction displayed a nucleosomal configuration: the 35,000 g pellet contained long arrays of nucleosomes and the 100,000 g pellet had mono- and oligonucleosome-like material. Thus, three structurally, chemically and functionally different chromatin types could be distinguished: (1) non-nucleosomal chromatin highly folded in bands and extended in interbands with no detectable transcriptional activity; (2) loops of nucleosomal chromatin fibres with low transcriptional activity and low incorporation of $^{32}$P into prominent non-histone proteins; (3) mono- and oligonucleosomal chromatin with essentially no transcriptional activity and no incorporation of $^{32}$P into non-histone proteins.

Introduction

Elucidation of the structure and chemical composition of chromatin is of crucial importance for the understanding of eukaryotic gene regulation. Partial nuclease digestion of isolated nuclei has identified the nucleosomes as basic structural units of chromatin, assembled into 10 nm diameter filament or thin fibre (Oudet et al. 1975; Olins and Olins 1974; Paulson 1982). This nucleosome filament can be coiled into a solenoid, thereby forming a 20–30 nm diameter filament or thick fibre (Thoma et al. 1979; Finch and Klug 1976; McGhee et al. 1983), which may be further folded into radial loops with the loop bases organized together in the central portion of the chromatid (Benyajati and Worcel 1976; Marsden and Laemmli 1979; Hancock and Boulikas 1982). The thin fibre is believed to represent transcriptionally active and the thick fibre inactive chromatin. The conformation and positioning of nucleosomes in transcriptionally active chromatin is, however, controversial (Igó-Kemenes et al. 1982) and altered nucleosomal packaging in active chromatin has been reported from several laboratories (Wu et al. 1979; Scheer 1978; Davis et al. 1983; Prior et al. 1983). Non-nucleosomal chromatin segments with extended DNA filaments (Labhart and Koller 1982), including spacer regions flanking rRNA transcription units (Scheer 1978; Davis et al. 1983) and other regions unrelated to transcriptional activity (Derenzini et al. 1983), have been observed in nucleoli. These results, as well as the observations of Szent-Györgyi and Isenberg (1983) and Rattner et al. (1982) in yeast chromatin, strongly suggest that chromatin can be structurally non-uniform.

The universal applicability of the nucleosome concept has been based on results obtained by digestion with micrococcal nuclease, an enzyme that preferentially cleaves DNA between the nucleosomes. However, in view of the sequence specificity of micrococcal nuclease (Kornberg 1981), implying that digested naked DNA and chromatin give similar digestion patterns, objections can be raised to this procedure. The basic method used to prepare chromatin includes partial nuclease digestion and subsequent lysis of isolated nuclei. This usually means that only the solubilized fraction of chromatin is studied and does not allow conclusions regarding chromatin as a whole.

In the present investigation, a wider and partly differing approach was used. Chromatin was prepared from isolated nuclei of *Chironomus* salivary gland cells by gentle sonication in low salt solution (without EDTA) and separated into soluble and insoluble fractions by centrifugation at 18,000 g as previously described (Egyházi et al. 1983). The soluble fraction was further subfractionated by differential centrifugation into 35,000 g and 100,000 g pellets and a 100,000 g supernatant. The insoluble fraction (18,000 g pellet) and the soluble fractions – together representing the total chromatin – were then studied electron microscopically and biochemically. The results show that about half of the soluble chromatin can be sedimented at 35,000 g. Electron microscopic examination of the spread 35,000 g chromatin fraction revealed the presence of loose and tight strings of up to several hundred nucleosomes. In contrast, the insoluble chromatin contained the usual nucleosome constituents, i.e. DNA and core histones, but the DNA filaments were extended and not wrapped around the histone granules. These observations support earlier ideas.
concerning the structural heterogeneity of the chromatin and indicate that studies of soluble chromatin may not be representative of chromatin as a whole.

Materials and methods

Material. The method of culturing Chironomus tentans has been described previously (Beermann 1952; Lambert and Daneholt 1975). Salivary glands were dissected from 6- to 8-week-old fourth instar larvae.

Labelling conditions. To study post-synthetic phosphorylation of nuclear proteins, salivary glands (50-100 in each experiment) were excised into phosphate-free Cannon medium (Ringborg and Rydlander 1971) and incubated for 25 min at 20°C. The glands were then transferred into 100-200 µl fresh phosphate-free medium containing 20 mCi/ml of carrier-free 32P-orthophosphate (The Radiochemical Centre, Amersham, England) and incubated for 10 min (Egházi et al. 1983). For labelling of RNA, 32P-orthophosphate was replaced by 1 mCi/ml of γ-32P-ATP (400 Ci/mmol; The Radiochemical Centre) in regular Cannon medium and the glands were incubated for 5 min.

Fractionation of chromatin and extraction of proteins and RNA. Preparation of nuclei from salivary glands was carried out as described by Sass (1980a). Nuclear isolation medium contained 90 mM KCl, 8 mM NaCl, 0.5 mM MgCl2, 0.5 mM CaCl2, 15 mM Na-K phosphate, pH 6.0, 0.02% Nonidet P40, 1 mM NaF and 0.1 mM phenylmethylsulphonylfluoride (PMSF). Insoluble chromatin was fractionated according to Egyházi et al. (1983). The nuclei were sonicated in a Branson Sonifier Model B-30 at a setting of 2 at 15 s intervals for a total time of 1 min at 0°C in a solution containing 1.5 mM KCl, 1 mM NaF and 1 mM PMSF, pH 6.0, following which the homogenate was centrifuged at 18,000 g (Sorval RC-5B) for 10 min at 4°C. The supernatant was decanted and recentrifuged at 35,000 g (Beckman L-5-50, SW 50-1 rotor) for 15 min. The resulting supernatant was also decanted and recentrifuged at 100,000 g for 120 min. The resulting supernatant was decanted, layered on 10 ml of 80% sucrose solution and spun at 100,000 g for 120 min (the supernatant discarded). The 18,000 g pellet (insoluble fraction) was also resuspended in 100 µl of tenfold diluted sonication medium, layered on 10 µl of 80% sucrose solution and spun at 100,000 g for 120 min. The three pellets were then examined by electron microscopy.

Electron microscopy. Dispersed chromatin pellets were placed on copper grids coated with a carbon-stabilized Formvar film. After 3-12 min of attachment, excess liquid was removed with filter paper. The specimens were then stained for 1 min with 0.3% uranyl acetate or 1% phosphotungstic acid (adjusted to pH 7.0 with NaOH). Uranyl acetate staining was in some cases followed by rotary shadowing with platinum (10°, 12 cm), using an Edwards vacuum-coating unit. The grids were examined at 80 kV in a Philips EM 300 electron microscope equipped with an anticontamination device cooled with liquid N2. The magnifications were calibrated with a carbon replica of a ruled diffraction grating with 2,160 lines/mm (Ladd).

Results

Fractionation of total chromatin into insoluble and soluble fractions

Chromatin from Chironomus salivary gland cells was isolated by sonication of isolated nuclei at low ionic strength followed by differential centrifugation (Egházi et al. 1983). In this way, the material was divided into an insoluble (18,000 g pellet) and a soluble (18,000 g supernatant) fraction. Electrophoretic separation in 12% SDS-polyacrylamide gels of proteins released from the soluble and insoluble chromatin fractions is shown in Figure 1. The staining profile of the soluble chromatin fraction (lane A) includes one-third of the core histones and non-histone proteins. Most of the non-histone bands in the soluble fraction are also recognized in the insoluble fraction (lane B), although the intensity of staining in the two lanes partly differs. The distribution of DNA between the two fractions parallels that of core histones (Table 1). In contrast, on the basis of DNA and core-histone content (Egházi et al. 1983), the soluble chromatin fraction contains less of prominent, rapidly phosphorylated non-histone proteins than the insoluble chromatin fraction. The ionic conditions used here proved optimal for separation of chemically and structurally distinct chromatin samples.

Fractionation and biochemical analysis of soluble chromatin

The soluble chromatin was further fractionated into three subfractions by differential centrifugation: material pelleting at 35,000 and 100,000 g, and the remaining supernatant fraction. Before preparation of chromatin, salivary glands were incubated with 32P, for 10 min to label nuclear pro-