

Nucleosome Repeat Structure Is Present in Native Salivary Chromosomes of *Drosophila melanogaster*

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ABSTRACT The regularly repeating periodic nucleosome organization is clearly resolved in the chromatin of the isolated salivary chromosomes of *Drosophila melanogaster*. A new microsurgical procedure of isolation in buffer A of Hewish and Burgoyne (1973, *Biochem. Biophys. Res. Commun.*, 52:504–510) yielded native *Drosophila* salivary chromosomes. These chromosomes were then swollen and spread by a modified Miller procedure, stained or shadowed, and examined in the electron microscope. Individual nucleoprotein fibers were resolved with regularly repeated nucleosomes of ~10 nm diameter. Micrococcal nuclease digestion of isolated salivary nuclei gave a family of DNA fragments characteristic of nucleosomes for total chromatin, 5S gene, and simple satellite ($\rho = 1.688 \text{ g/cm}^3$) sequences.

The repeating periodic nucleosome structure has now been clearly demonstrated in the chromatin of a wide variety of organisms ranging from viruses, yeast, protozoa, and insects to vertebrates (1–3). These demonstrations cover interphase nuclei of tissues varying through early embryonic, liver, thymus, kidney, erythrocytes, ovaries, and testes (4–6), as well as mitotic chromosomes (7). However, there has been a paucity of demonstrations of nucleosomes in what are probably the most extensively studied of all animal chromosomes, viz., the salivary chromosomes of *Drosophila melanogaster*. The only published micrographs employed nuclei rather than cytogenetically mappable spread chromosomes and a swelling procedure which included 2 M urea in conjunction with the detergent Joy (3). There are a number of reasons for this. First, the Miller technique usually takes as its starting material whole nuclei in free suspension rather than spread chromosomes on a surface. Secondly, classically spread salivary chromosomes have been acid-fixed, which very likely induces transitions in ultrastructure. Thirdly, the nuclear membrane of salivary gland cells is particularly difficult to lyse without damage to the chromosomes. We can now readily resolve the nucleosome repeat unit in spread *Drosophila* salivary chromosomes. Our demonstration was made possible by a new isolation procedure yielding native *Drosophila* salivary chromosomes which has been developed in our laboratory (8).

Over the past years we have been developing a microsurgical procedure for isolation of these structures simply in the physiological saline of D'Angelo (9). Physical stabilization of the

structures was achieved by the inclusion of 0.05% formaldehyde (8). Very recently, it has become possible to isolate them in the buffer A of Hewish and Burgoyne (10), the solvent in which early nuclease digestion experiments gave the first evidence of a 200 base pair (bp) repeating module in chromatin. In this solvent the physical stability of chromatin is enhanced by the polycations, spermine, and spermidine, which give rise to physical but not covalent cross-linking.

We have also investigated the organization of *Drosophila* salivary polytene chromatin by micrococcal nuclease digestion. The resulting DNA fragments have been observed *in toto* and also by probing specifically with simple satellite ($\rho = 1.688 \text{ gm/cm}^3$) and 5S gene sequences.

MATERIALS AND METHODS

Isolation of Chromosomes

Drosophila salivary gland chromosomes were isolated in three different solvents, by the following methods. (a) The classical 45% acetic acid squashing procedure. These chromosomes were stained with aceto orcein and photographed using bright-field illumination and a green filter. (b) The micromanipulative procedure of Hill and Watt (8) using the saline of d'Angelo (9) containing 0.05% formaldehyde. (c) The same micromanipulative procedure using buffer A of Hewish and Burgoyne (10). The chromosomes in b and c were unstained and photographed using phase-contrast optics.

Demonstration of Nucleosomes by Electron Microscopy

A gold electron microscope grid with a film of parlodion and evaporated carbon was freshly glow discharged to render it hydrophilic. The grid was

immersed in buffer A, 5 mM mercaptoethanol in the well of a dissection slide (11). A polytene nucleus, isolated by microdissection, was placed in the well close to the grid. Chromosomal material, as in method *c* above, was removed through an incision in the nuclear membrane and placed gently onto the film on the grid. The chromosomes were then swollen and spread at low ionic strength by a modified Miller technique (12). The solvent was quickly changed to distilled water, adjusted to pH 9.0 with Mallinckrodt standard pH 10 buffer (Mallinckrodt Inc., Science Products Div., St. Louis, MO). This was immediately changed to distilled water containing 200 μg *Escherichia coli* transfer RNA/ml (Boehringer Mannheim Biochemicals, Indianapolis, IN; MRE600) adjusted to pH 9.0. The chromosomes were allowed to swell *in situ* for 45 min at room temperature, and the solvent immersing the grid was then changed quickly to 0.1 M sucrose, 10% formaldehyde, pH 8.5. The well in the slide was covered by a round cover slip and the preparation centrifuged at 2,000 g for 3–5 min. The grid was subsequently immersed for 10 s in 0.4% Photoflo freshly adjusted to pH 8.5. It was then touched to a Ross lens tissue and air-dried. The preparation was stained for 40 s in 1% phosphotungstic acid in 70% ethanol. The grid was rinsed 10 s in 95% ethanol and air-dried. Alternatively, the material after drying from Photoflo was rotary shadowed with platinum at 7°. The grids were examined at 60 kV in a JEM100 CX electron microscope.

Preparation of Salivary Gland Polytene Nuclei for Nuclease Digestion

D. melanogaster (Oregon R) were grown at 25°C until they reached midthird instar, then dissected as follows: the larvae were washed with 0.7% NaCl, 0.1% Triton X-100 (wt/vol) solution in a 24 mM Swinnex filter apparatus (Millipore Corp., Bedford, MA) attached to a syringe. The larvae were rinsed with distilled water and placed in *Drosophila* Ringer's solution (0.75% NaCl, 0.035% KCl, 0.021% $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$). The salivary glands were removed by hand dissection and placed in cold (4°C) Ringer's solution in a plastic tube. Because of problems of

stickiness, the glands and isolated nuclei were manipulated in plastic tubes only. Approximately 350 glands (~6 h of dissection) were used per experiment.

The Ringer's solution in the tube of glands was carefully removed and replaced with fresh, cold, Ringer's (1 ml total volume). To this was added sodium deoxycholate (final concentration of 0.2% wt/vol) and Triton X-100 (final concentration at 0.5% vol/vol). This was kept at room temperature for 5 min, with gentle shaking sufficient to keep the glands suspended. They were then disrupted by pipetting approximately 8 to 10 times using a 1 ml plastic tip and automatic pipetter. This suspension was quickly diluted into 5 vol of cold Ringer's solution and filtered through nitex monofilament bolting cloth (120 μm diameter) in a 12 mM Swinnex filter unit (Millipore Corp.). An additional 5 vol of cold Ringer's was used to rinse the tubes and filter, and combined with the previous filtrate. The filtrates were spun for 1.5 min in a clinical centrifuge at full speed. The supernatant was carefully removed and the pellet resuspended in nuclease digestion buffer (see below). The nuclei were kept on ice until digested. The chromosomes of these nuclei were intact as assessed by standard acetic acid squashing procedures followed by phase-contrast microscopy.

Micrococcal Nuclease Digestion of Nuclei

The buffers and procedure followed were those of Wu et al. (13). The yield of nuclei from the above procedure was sufficient for three digestion points.

Purification of DNA

After lysing the nuclei with EDTA and SDS as described (13), yeast tRNA was added to a final concentration of 5 $\mu\text{g}/\text{ml}$ and samples were treated overnight with proteinase K (100 $\mu\text{g}/\text{ml}$) at 37°C. The samples were extracted twice with chloroform: isoamyl alcohol (24:1 vol/vol). To the final aqueous phase was added 1 vol of 5 M ammonium acetate followed by 3 vol of ethanol. The DNA was

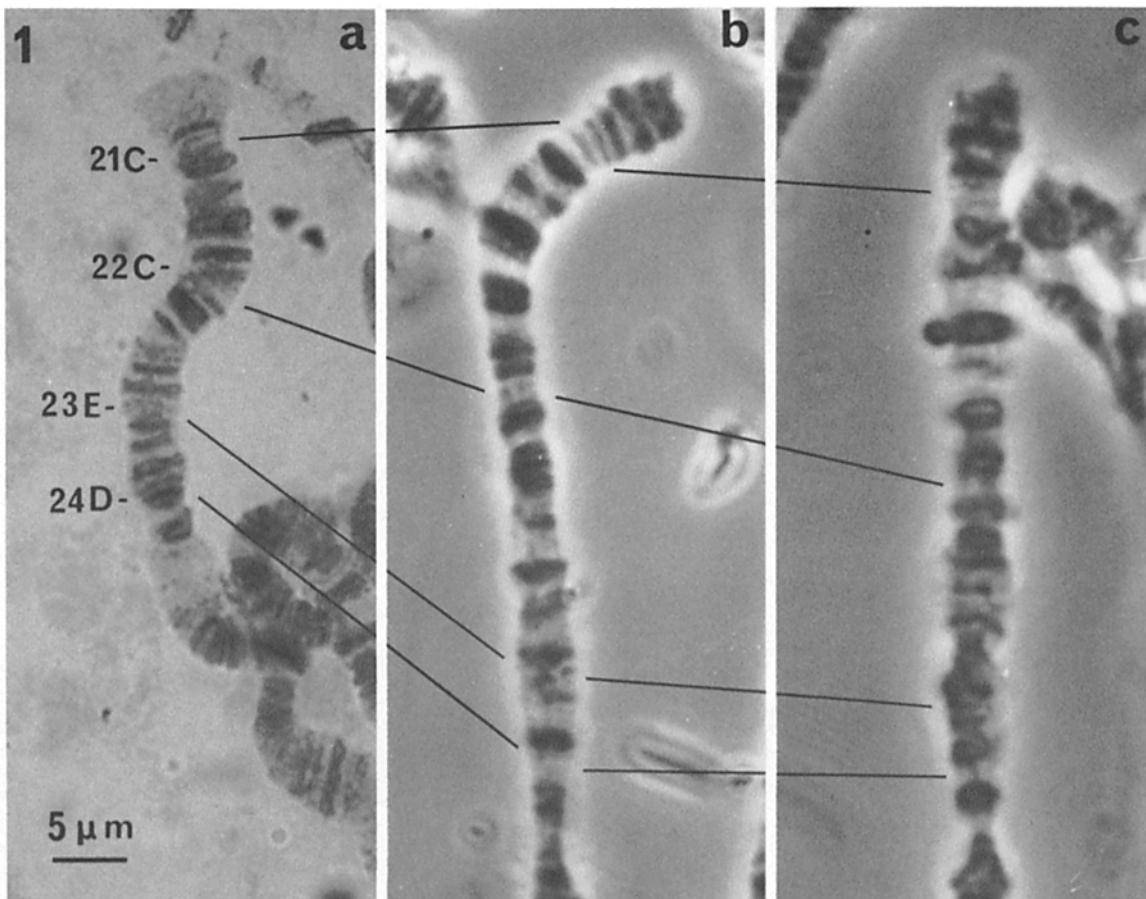


FIGURE 1 A light micrograph comparison of the distal region of chromosome arm 2L isolated by (a) the classical 45% acetic acid squashing procedure, stained with aceto orcein and photographed using bright-field illumination and a green filter, showing some swelling of the chromosome such as the puffed region near the chromosome terminus; (b) the micromanipulative procedure of Hill and Watt (8) using the saline of D'Angelo (9) containing 0.05% formaldehyde, and (c) the same micromanipulative procedure using buffer A of Hewish and Burgoyne (10). The chromosomes in *b* and *c* were unstained and photographed using phase-contrast optics, and appear to have structure likely to be in the native state. $\times 2,000$.

precipitated overnight at -20°C , then pelleted by centrifugation and resuspended in TNE (10 mM Tris-HCl, pH 7.4, 5 mM NaCl, 1 mM EDTA).

Gel Electrophoresis and Southern Transfer

The procedures followed were again those of Wu et al. (13). The recombinant probe for 5S RNA gene was 12D1 (Artavanis-Tsakonas et al. [14]); that for the $\rho = 1.688 \text{ g/cm}^3$ satellite was mDm107, a pBR322 recombinant plasmid with several copies of the 359 bp simple sequence repeat (Hsieh and Brutlag [15]).

RESULTS AND DISCUSSION

A comparison was made of the distal region of chromosome arm 2L isolated by (a) the classical acid squashing procedure, (b) micromanipulation in D'Angelo physiological saline containing 0.05% formaldehyde, and (c) micromanipulation in buffer A (Fig. 1). Corresponding structures in the light micro-

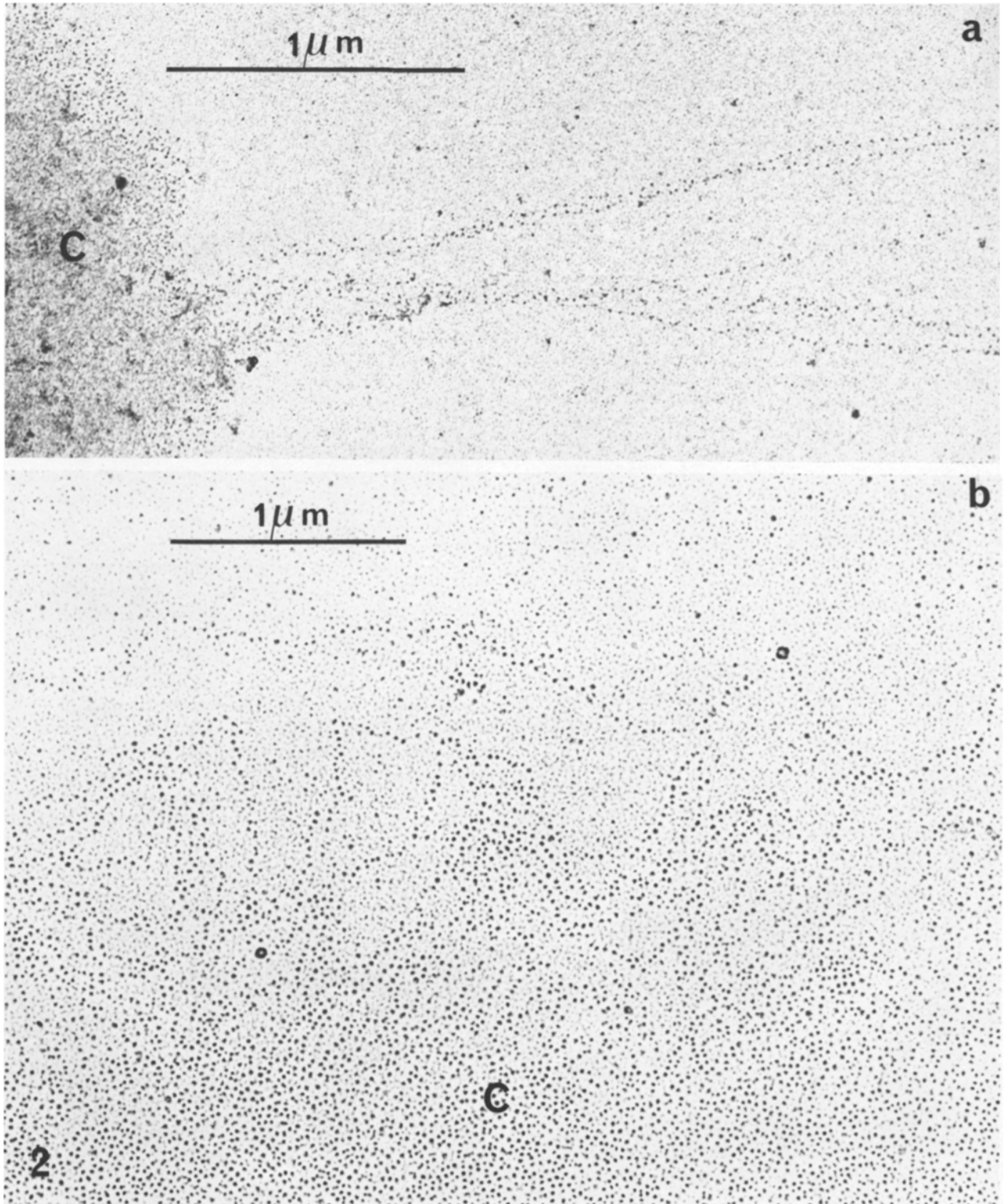


FIGURE 2 Electron micrographs of spread preparations of *Drosophila* salivary chromosomes showing regularly repeating nucleosome organization. The mass of chromosomal material is indicated by C. (a) Stained with ethanolic phosphotungstic acid demonstrating mononucleosomes $\sim 10 \text{ nm}$ diameter. $\times 50,000$. (b) Rotary shadowed with platinum at 7° . $\times 40,000$.

graphs may be correlated on chromosomes that have been isolated in the three different solvents. However, some differences in detail are apparent. For example, the puffed region near the chromosome terminus appears less swollen when the chromosome has not been exposed to 45% acetic acid. In the case of the material in D'Angelo buffer containing 0.05% formaldehyde, it is likely that most macromolecular organization and higher orders of protein structure would be in a native state; low concentrations of formaldehyde of the order of 0.05% have long been known to have minimal, if any, effects on the antigenicity of even quite labile antigens, for example, influenza haemagglutinin (16). In the case of the material isolated simply in buffer A, there seems little doubt that we are observing under the microscope native chromatin structure corresponding to the well-mapped *Drosophila* salivary chromosomes. Furthermore, because of the absence of any acid or covalent-cross-linking fixation, there should be no barrier to swelling these structures by the Miller procedure to resolve nucleoprotein organization at the uninematic level.

An electron microscope examination was made of the material that was micromanipulated in buffer A, as in *c* above, directly onto the surface of a carbon-coated, filmed, glow-discharged electron microscope grid, and then swollen at low ionic strength by the modified Miller procedure as described in Materials and Methods. The result is shown in Fig. 2. There is no doubt that individual nucleoprotein fibers with a characteristic regularly repeating nucleosome organization are being resolved, and that these emanate from regions on the grid where the chromosomes were placed. In Fig. 2*a*, the preparation stained with ethanolic phosphotungstic acid demonstrates nucleosomal particles ~ 10 -nm diameter, 9.7 ± 1.2 nm (SD), separated by variable linker DNA, 26 ± 9 nm (SD). In Fig. 2*b*, the preparation rotary shadowed with platinum at 7° shows greater diameter particles due to deposited platinum. Consistent with the general notions of organization of DNA molecules in these chromosomes, free ends were never observed on the nucleosome bearing fibers.

To confirm the presence of nucleosomes in the polytene chromosomes of *Drosophila* salivary glands biochemically we undertook the isolation of sufficient quantities of glands (approximately 350) by hand dissection. The criterion we chose was digestion by micrococcal nuclease. Digestion of native chromatin yields a diagnostic pattern of DNA fragments with sizes in multiples of ~ 200 bp, the result of preferential cleavage in nucleosome linkers. Such a pattern for mono- and oligonucleosomes is clearly shown for total chromatin in Figs. 3*a* and

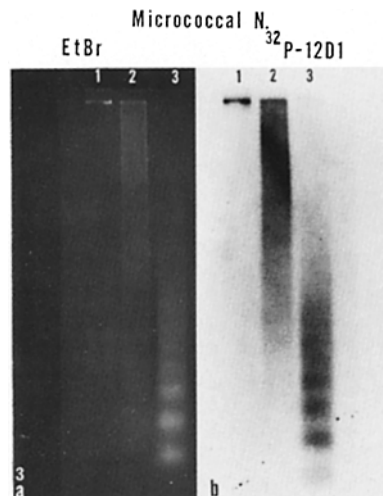


FIGURE 3 Comparison of the general micrococcal nuclease pattern with the pattern from sequences homologous to 5S sequences. (a) 1% agarose gel stained with ethidium bromide. Digestions were carried out for 3 min at 25°C . The concentrations of enzyme for lanes 1, 2, and 3 were 0 U/ml, 59 U/ml and 469 U/ml, respectively. (b) Southern blot of gel a probed with ^{32}P -12D1, a recombinant DNA plasmid containing 5S sequences of *Drosophila*.



FIGURE 4 Comparison of the general micrococcal nuclease digestion pattern with the pattern from sequences homologous to the satellite DNA probe pDm107. (a) 1% agarose gel stained with ethidium bromide as referenced. Digestions were carried out for 3 min at 25°C . The concentrations of enzymes for lanes 1, 2, and 3 were 0 U/ml, 59 U/ml, and 234 U/ml, respectively. Lane 4 is an incomplete HaeIII digest of total genomic DNA. (b) Southern blot of gel a probed with ^{32}P -labeled mDm 107, a recombinant DNA plasmid containing sequences of the 1.688 satellite DNA of *D. melanogaster*.

4*a*. In addition, the specific DNA sequences of 5S genes and a simple satellite ($\rho = 1.688 \text{ g/cm}^3$) have been shown to be liberated in similar families of fragments (Figs. 3*b* and 4*b*). The size of nucleosomes containing satellite sequences is ~ 190 bp with two nucleosomes per satellite sequence repeat, by comparison with an incomplete digestion of genomic DNA with HaeIII which cuts an average of once per sequence repeat (Fig. 4*b*). The results are the same as obtained for the chromatin structure in diploid nuclei from 6–18 h. *Drosophila* embryos (I. L. Cartwright and S. C. R. Elgin, unpublished results).

The above results thus firmly establish, both from electron microscope and biochemical observations, that native *Drosophila* salivary chromosomes exhibit the regularly repeating periodic nucleosomal organization. We are currently attempting to refine our micromanipulative procedures so as to be able to place known chromosomal segments at positions of defined coordinates on the electron microscope grid. With this innovation, and the barrier to Miller dispersal of isolated *Drosophila* salivary chromosomes without the use of dissociating agents bypassed, it should be possible, in the foreseeable future, to examine directly in the electron microscope the nucleoprotein organization and transcription patterns of known mapped loci.

We wish to thank O. L. Miller, Jr., for providing valuable advice. The skilled technical assistance of D. Macpherson and F. Watt is gratefully acknowledged. We thank Iain L. Cartwright for communicating the data on chromatin structure of 5S and satellite regions in diploid nuclei of *D. melanogaster* before publication.

The biochemical work was supported by National Institutes of Health (NIH) grant GM30273 to S. C. R. Elgin. K. Lowenhaupt holds an NIH Postdoctoral Fellowship.

Received for publication 30 July 1981, and in revised form 7 June 1982.

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