Preparation of centromeric heterochromatin by restriction endonuclease digestion of mouse L929 cells

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Abstract. When L929 cells in metaphase are digested with either Eco RI or Alu I, chromatin containing about 85% of the DNA is released. DNA from the Alu I- and Eco RI-resistant chromatin is enriched 6.8- and 3.7-fold, respectively, in satellite sequences. Analysis by electron microscopy of these digests reveals the existence of structures containing condensed heterochromatin and kinetochores. When these preparations are incubated with anticentromere serum from a human CREST scleroderma patient and then with rhodamine-conjugated antihuman IgG, fluorescence appears in the form of paired dots, the same pattern found in whole metaphase chromosomes. The fluorescent staining pattern, the electron microscopy, and the enrichment of satellite DNA sequences together support the conclusion that the Eco RI- and Alu I-resistant structures contain centromeres. We anticipate that these preparations will be useful in studies of the interactions between centromeric heterochromatin, kinetochores, and microtubules.

Introduction

Metaphase chromosomes of most eucaryotic organisms possess a primary constriction of condensed chromatin defined by classical geneticists as the centromere. Although a functional definition for the centromere has been supplied by the isolation of yeast sequences which confer segregation (Clarke and Carbon 1980), we have adhered to the cytogenetic definition of the term (e.g., Bostock and Sumner 1978). During metaphase, the two chromatids are held together in this region, and one of a pair of trilamellar structures, the kinetochores, is associated with each chromatid. Microtubule bundles attach the chromosome to the mitotic apparatus at the kinetochore. Hence, the centromere is apparently involved in chromatid pairing and chromosome segregation.

In most higher eucaryotes, centromere regions contain highly repeated DNA sequences (Eckhardt 1975), and DNA has been implicated in the ability of kinetochores to nucleate microtubule assembly (Pepper and Brinkley 1980). In addition, direct association of chromatin with microtubules was observed by Ris and Witt (1981). In mice, centromere regions contain a relatively large region of condensed chromatin, and the highly repetitive 1.691 g/cm² satellite DNA hybridizes in situ to the centromere regions of all of the chromosomes except the Y (e.g., Pardue and Gall 1970). These two properties make mouse centromeres attractive structures for molecular investigation because their large size allows analysis by light microscopy, and the satellite DNA provides an easy initial assay during purification. The molecular composition and architecture of the centromere region is poorly understood. Nevertheless, it has been proposed that the association of mitotic and meiotic spindle to chromosomes is mediated by specific DNA sequences which bind to specific microtubule-associated proteins (Lindsay 1975). These sequences might include those which direct chromosome segregation.

Independently segregating DNA sequences have been identified in yeast by Clarke and Carbon (1980). Due to the small size of the yeast genome and the fact that it is well mapped genetically, it was possible to “walk” along the chromosome from known genes to the centromere. In addition, the availability of an extrachromosomal, autonomously replicating vector made it possible to select cloned sequences for their ability to be stably maintained in the absence of integration. This approach is not feasible in mammals because the genome is huge compared to the yeast genome (about 150 times larger), it is not as finely mapped, and a suitable mammalian vector which replicates autonomously without integration into host DNA is not available. Therefore, our approach toward investigating centromeres in mammalian cells is based on the development of a method of purification of mouse centromeric heterochromatin from metaphase chromosomes. This was accomplished by selective nuclease digestion and solubilization of chromosome arms. We chose to use metaphase chromosomes rather than interphase chromatin as starting material for the purification of centromeric heterochromatin because this material can be analyzed both biochemically and microscopically during purification steps. Furthermore, there is no evidence that heterochromatin isolated from interphase nuclei represents all the material in the centromere regions of metaphase chromosomes and only that material. Thus, interphase-derived heterochromatin (Mazrimas et al. 1979; Zhang and Horz 1982) is of limited utility in the dissection of macromolecular interactions between centromeric heterochromatin, kinetochores, and the mitotic spindle.

Previous studies have shown that centromere regions of metaphase chromosomes are relatively resistant to the action of pancreatic DNase I (Luykx 1965; Rattner et al. 1978), and DNase I digestion can be used to prepare centromere regions from mouse metaphase chromosomes (Rattner et al. 1978). In these preparations, however, the
DNA is low molecular weight and highly nicked. Since pericentric mouse satellite DNA is almost totally resistant to cleavage by Eco RI and Alu I (Hörz et al. 1974) and because restriction enzyme digestion yields relatively high-molecular-weight, unnicked DNA fragments, we developed a method of selective digestion of chromosomal arms using these two enzymes. This yields preparations enriched in centromeric heterochromatin containing high-molecular-weight DNA which is amenable to buoyant density and restriction enzyme analysis and which should contain DNA sequences required for chromosome segregation.

We were able to provide additional support for the presence of centromeres in these preparations by taking advantage of the recent demonstration that certain autoimmune-disease patients with the CREST scleroderma syndrome produce antibodies to centromeres (Moroi et al. 1980). Thus, antisera from these people can be used to stain centromeres by immunofluorescence. We have adopted this procedure as an assay for mouse centromeres in our purification procedure. Our results show that antisera from a human CREST patient binds specifically to heterochromatin.

Materials and methods

Preparation of radioactively labeled metaphase cells

Monolayers of L929 cells were grown to semiconfluency in Joklik's modified minimal essential medium (Gibco) supplemented with 10% fetal calf serum (KC Biologicals) in Corning T150 flasks. They were then labeled with 2.5 μCi/ml 3H-thymidine (NEN) for 7 h followed by 6 h of recovery in unlabeled medium and 11 h of incubation with colcemid (Gibco) at a concentration of 100 ng/ml. Metaphase cells were harvested by selective detachment, pelleted at 2,500 g for 5 min, washed in Joklik's minimal medium, repelleted, and resuspended in medium. This yields populations of cells with more than 99% metaphases.

Restriction endonuclease digestion of metaphase cells

Washed metaphase cells were resuspended in medium at a concentration of approximately 10^6 cells/ml and mixed with 3.5 x 10^{-5} units/cell of either Eco RI (BRL) or Alu I (New England Biolabs). Then 2 ml of this mixture was placed in a 5-cm-diameter plastic petri dish and Triton X-100 (Sigma) was immediately added dropwise to a final concentration of 0.02%. Solubilized chromatin was separated from resistant chromatin by pelleting at 4,000 g for 10 min. Radioactivity in pellets and supernatants was measured by liquid scintillation counting in Aquasol (NEN).

DNA purification

DNA was isolated from mouse L929 cells according to the method of Manuelidis (1977). Total DNA was purified through two successive CsCl-Hoechst 33258 equilibrium bandings, and main band DNA was purified by three successive gradients. Hoechst 33258 was from Hoechst America and CsCl was from EM.

Analytical equilibrium sedimentation

Purified DNA was banded in an An-G rotor in a Beckman Model E analytical ultracentrifuge at 44,000 rpm for 24 h when absorbance was scanned at 263 nm.

Restriction enzyme digestion

DNA samples were digested with restriction enzymes from New England Biolabs or BRL according to the suppliers' directions. Digested samples were mixed with an equal volume of 80% glycerol, 0.1 M EDTA and subjected to electrophoresis through 1.4% or 0.5% agarose in 0.04 M Tris-HCl, 5 mM sodium acetate, 1 mM EDTA (pH 7.6) on a 20 x 20 x 1 cm horizontal submarine gel at about 28 V for 15 h. Gels were stained in 2 μg/ml ethidium bromide and photographed with Polaroid type 55 film. DNA fragment size estimations were made by comparing migration distances with those of restricted λX174 replicative form DNA.

Electron microscopy

Metaphase chromosomes and chromosome fragments were deposited on carbon-coated copper electron microscope grids by sedimenting through 0.5 M sucrose according to the method of Rattner et al. (1975).

Immunofluorescence

Chromosomes for immunofluorescent staining were obtained by placing 10 μl metaphase cells at 10^6 cells/ml and 10 μl of 1% NP40, 10 mM Tris (pH 8.3) on a clean, siliconized coverslip and touching a clean slide to the coverslip to pick it up. The slide and coverslip were inverted, and lysis proceeded at room temperature for 6 min. Eco RI-resistant chromatin was obtained by digesting metaphase cells on clean coverslips in Petri dishes as described above. After 15 min fixation with 3.7% formaldehyde in PBS (8.0 g NaCl, 2.16 g Na2HPO4, 0.2 g KH2PO4, and 0.2 g KCl/liter, pH 7.2), slides and coverslips were washed thoroughly in PBS and stained sequentially with a 1:40 dilution of sleroderma serum, which has been characterized by Cox et al. (1980), for 30 min at 37°C and then a 1:25 dilution of rhodamine-conjugated goat antihuman IgG (Cappel) for 20 min at 37°C. Some slides and coverslips were stained in an aqueous solution of 0.5 μg Hoechst 33258/ml for 10 min and then rinsed thoroughly in PBS and water. Coverslips were mounted with Gelvatol (Rodriguez and Deinhardt 1960). Slides were viewed with a Zeiss Photomicroscope III microscope equipped with fluorescence illumination with a 100X phase objective. Photographs were made by exposing Kodak Tri-X pan (ASA 400) and developing with Diafine (Acufine, Inc).

Results

A fraction of metaphase chromatin resistant to digestion by Eco RI and Alu I

We attempted to release chromosomal arms from centromere regions by restriction endonuclease digestion of intact labeled metaphase cells, taking advantage of the inability...