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Molecular Characterization of Flow-Sorted Mammalian Centromeres

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Abstract

This is the final report of a three-year, Laboratory Directed Research and Development (LDRD) project at the Los Alamos National Laboratory (LANL). The project involved experiments directed towards developing a molecular characterization of the centromere region of mammalian chromosomes. Attempts to purify this essential chromosomal locus by conventional methods have thus far been unsuccessful. However, preliminary data obtained in collaboration with the National Flow Cytometry Resource (NFCR) showed that it is possible to purify a chromosome fragment that is present in certain cultured mouse cell lines and has all the properties expected of an intact centromere region. To begin sorting this minichromosome for the identification of proteins preferentially associated with centromere regions, standard buffers utilized in chromosome sorting were evaluated for potential effects on maintenance of chromosomal proteins during sorting. The data indicate that the presence of several buffer constituents results in the extraction of all but a few chromosomal proteins. The subsequent use of a magnesium sulfate buffer resulted in the sorting of mouse chromosomes that do not suffer a significant loss of proteins. Several DNA stains were also evaluated for causing protein dissociation, but no significant losses were observed. Although flow-sorted chromosomes have been used extensively for DNA analysis and cloning, this is a pioneering effort by the NFCR, and its collaborators, to exploit chromosome sorting capabilities for the analysis of chromosomal proteins.

Background and Research Objectives

The centromere regions of the chromosomes of higher organisms represent less than 20% of the entire chromosome, on average, and are distinguishable as a highly condensed structure composed of DNA and a collection of proteins that are, as yet, relatively poorly characterized. One *bona fide* heterochromatin-enriched protein (HP1) identified in *Drosophila* shares homology with a protein involved in gene repression

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(polycomb) in the same system, suggesting that some centromere-associated proteins serve more than one function: differential condensation and regulation of gene expression. By analogy, similar multifunctional proteins are likely to be associated with mammalian centromeres. Interestingly, one conserved nonhistone chromosomal protein (HMG-I/Y) that is concentrated in centromeric heterochromatin is required for positive regulation of several diverse genes. Although a few centromere-restricted proteins (identified by their presence in autoimmune patients' sera) have been characterized in considerable molecular detail, their precise functions remain to be elucidated. In addition to heterochromatin-associated proteins that may play a role in differential condensation, centromeres are composed of DNA sequences and other proteins responsible for the biological function of the centromere -- chromosome segregation during mitosis and meiosis. This function has only been dissected at the molecular level in the yeasts because of small genome sizes and relative simplicity of centromeres and, more recently, in *Drosophila*. At the present time, we do not know how much of the information available for the yeast systems will serve as a model for how a vertebrate centromere is constructed and functions.

A thorough molecular description of the components of centromere regions requires purification of these chromosome segments in quantities sufficient for biochemical analyses. Mammalian centromeres have never been purified. Several years ago, as a peripheral observation to an ongoing project, one of us reported the existence of a chromosome fragment in cultured mouse NIH 3T3 cells that appears to be an intact centromere region [1]. It is the same size as a typical centromere and it can segregate normally. Since it is a fraction of the size of mouse chromosomes, it was suspected that it might be separable and, therefore, purified based on DNA content via flow cytometry. We succeeded in isolating this "minichromosome" from a cultured mouse cell line by fluorescence-activated chromosome sorting.

We next directed our investigations toward a molecular dissection of novel centromere-enriched proteins associated with this minichromosome. To achieve this goal, we first had to evaluate whether or not the standard protocols for chromosome sorting would dissociate chromosomal proteins. Detailed analysis of various buffers showed that this was the case. Different DNA binding fluorochromes used in sorting were tested for dissociability with no obvious disruptions observed. Individual components were evaluated for their ability to dissociate proteins.

Importance to LANL's Science and Technology Base and National R&D Needs

The results of this work are of importance to our fundamental understanding of fundamental genome organization and the structure of chromosomes, and to applications-driven projects such as bioagent detection. Molecular dissection and characterization of mammalian centromere regions will provide the basis for understanding fundamental mechanisms underlying chromosome dynamics such as differential condensation, regulation of gene expression and chromosome segregation. This is the first time the National Flow Cytometry Resource (NFCR) has directed its chromosome sorting capabilities toward protein, rather than DNA sequence, analysis. These studies provide baseline data that will serve researchers who can exploit the facilities for protein analysis.

In the context of the Human Genome Project, the information obtained in these studies will be useful in defining the molecular basis of hereditary defects that are characterized by aberrant centromere structure and/or function. Finally, the identification of functional mammalian centromere components represents an essential step in the construction of an artificial mammalian chromosome that could be used in basic research and as a vector for gene therapy.

The ability to detect different classes of proteins in combination with DNA probes is a critical technology for the detection and identification of biological agents. These assays will rely on ultrasensitive flow cytometric techniques and will require all classes of proteins to be retained for reliable identification of microbes and other biologicals. These studies provide us with critical information about protocols that will be used for the preparation of samples in which proteins must be stabilized and fluorescently tagged for accurate characterization of biological particles.

Scientific Approach and Accomplishments

The major goal of this project was to employ flow cytometry to isolate large quantities of both intact mouse chromosomes and a chromosome fragment derived from centromere regions in order to biochemically compare the protein composition of the two fractions, as a novel approach to identify proteins preferentially associated with centromeres that could play a role in centromere higher order structure and/or function.

Initial chromosome sorts, utilizing buffers developed by Los Alamos researchers for chromosome isolations used for genomic cloning, resulted in samples devoid of detectable amounts of protein [2]. We investigated the possibility that the combinations of

DNA binding fluorochromes used in chromosome sorting might cause protein dissociation since it has been shown that one dye, Hoechst 33258, can compete for binding of at least one chromosomal protein [3]. A series of sorted and unsorted samples were run on SDS-PAGE to test for the presence of proteins when the chromosomes were exposed to several combinations of the most commonly used stains -- chromomycin A3 (CA3), Hoechst 33258 (HO) and propidium iodide (PI). Samples were normalized by loading protein associated with equal amounts of DNA from sorted and unsorted samples in each lane. Upon silver staining of the polyacrylamide gels, detectable quantities of protein were observed only with the unsorted chromosomes (Figure 1). With the exception of some high-molecular weight scaffold proteins, the sorted chromosome samples were consistently devoid of protein bands -- regardless of the combination of DNA stains used. We determined that chromosomal proteins were being displaced from the DNA into the supernatant prior to sorting. However, this was not due to competition for binding between the DNA sorting fluorochromes and proteins. As a result, the buffers used were focused on.

The chromosome isolation and sorting buffers had previously been used extensively in the harvesting of chromosomal DNA for subsequent use in developing libraries and genomic sequencing. These studies represent the first attempt to utilize the NFCR facilities for work oriented toward protein analysis. An extensive analysis of each component in the sorting buffer showed that the presence of EDTA, EGTA and 2-mercaptoethanol resulted in extraction of all but a few chromosomal scaffold proteins. To effectively demonstrate this point, chromosomes isolated from freshly lysed cells were pelleted in a modified buffer lacking the disruptive components (Figure 2). The chromosome pellets not only retained protein bands but also displayed an enrichment of bands from 30-50 kDa not seen among the cytoplasmic proteins of the supernatants.

Since chromosomal proteins are extracted during isolation in the standard buffer, why do chromosomes retain their structure when viewed by fluorescence microscopy after sorting? We hypothesize that spermine and spermidine, present in the buffers, exchange for proteins resulting in protein-depleted, highly compacted chromosome-like structures.

Chromosome sorting for protein analysis is a novel approach, as is evidenced by the publication of only two papers using the technique in the last twelve years [4,5]. A buffer containing 10 mM MgSO₄, 10 mM KCl, and 5 mM HEPES (pH 8.0) proved to solve the problem since chromosomes were sorted without any apparent loss of proteins. An SDS-PAGE analysis of these chromosomes stained with DNA binding fluorochromes (CA3, HO and PI) did not reveal, prior to sorting, any apparent differences in protein loss. Nor was any apparent difference seen when we employed a novel sorting strategy by

"staining" the chromosomes with a fluorescein-labeled antibody against histone H1 (Figure 3). We can reproducibly observe a large number of polypeptides, in addition to the histones, by SDS-PAGE and silver-staining of gels containing proteins released from approximately 200,000 chromosomes per lane.

Finally, the addition of dithiothreitol (DTT) to the sorting buffer to improve the resolution of the chromosomes during sorting was not accompanied by protein loss. With these complications taken care of, it should not be as difficult to carry out protein analysis as long as a supply of approximately 500,000 microchromosomes can be obtained per experiment. We are now in a position to attack the major goal of this project, and we plan to submit a grant proposal for continued support of this work.

References

- [1] Hamkalo, B.A., Farnham, P.J., Johnston, R., and Schimke, R.T. "Ultrastructural features of minute chromosomes in a methotrexate-resistant mouse 3T3 cell line." **Proc. Natl. Acad. Sci. USA**, 82:1126 (1985).
- [2] Cram, L.S., Campbell, M., Fawcett, J.J., and Deaven, L.L. "Polyamine buffer for bivariate human flow cytogenetic analysis and sorting." **Methods in Cell Biology**, 33:377 (1990).
- [3] Radic, M.Z., Lundgren, K., and Hamkalo, B.A. "Curvature of mouse satellite DNA and condensation of heterochromatin." **Cell**, 50:1101 (1987).
- [4] Trask, B., van den Engh, G., Gray, J., Vanderlaan, M., and Turner, B. "Immunofluorescent detection of histone 2B on metaphase chromosomes using flow cytometry." **Chromosoma**, 90:295 (1984).
- [5] Levy, H.P., Schultz, R.A., Ordonez, J.V. and Cohen, M.M. "Anti-kinetochore staining for single laser, bivariate flow sorting of Indian Muntjac chromosomes." **Cytometry**, 12:695 (1991).

Figure 1. Silver stained SDS-PAGEs of A) unsorted and B) sorted chromosomes;. Fifteen micrograms of chromosomes loaded in each lane.

Lane 1--chromosomes stained with CA3 only

Lane 2--chromosomes stained with CA3 and HO

Lane 3--chromosomes stained with CA3 and PI

Lane 4--chromosomes stained with CA3, HO and PI

Figure 2. A mouse chromosome prep isolated from freshly lysed cells was pelleted in chromosome isolation buffer lacking EDTA and 2-mercaptoethanol. Increasing volumes of resuspended pellet (P) and supernatant (S) were run on the SDS-PAGE and silver stained.

MW--molecular weight markers

C--a control of 100,000 unsorted mouse chromosomes

Figure 3. SDS-PAGE analysis of chromosomes sorted with the magnesium sulfate buffer, in the presence of several DNA binding fluorochromes. Two hundred thousand chromosomes were loaded in each lane.

MW--molecular weight markers

T--total human H1 (used as an MW marker)

Lane 1--sorted with CA3 and HO

Lane 2--sorted with CA3

Lane 3--sorted with HO

Lane 4--sorted with PI

Lane 5--sorted with a histone H1 antibody and PI