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Meeting report

From linear genome sequence to three-dimensional organization of the cell nucleus

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A report on the Jackson Laboratory 'Advances in nanostructural genomics II' meeting, Bar Harbor, USA, 3-6 October 2002.

In the modern era of biochemical science, the isolation, identification and characterization of biologically relevant molecules have become routine. The challenge remains, however, to discover how these molecules are assembled within cells to form the dynamic network of molecular machines that supports living organisms. Because molecular machines are only several tens of nanometers in size (for example, a ribosome is about 30 nm wide), new optical methods with both increased sensitivity and higher resolution are required to define the spatial relationships and dynamic interplay between interacting biomolecules. Furthermore, techniques that provide dynamic, three-dimensional images of individual molecules *in vivo* will be necessary if we are to study the detailed organization of 'nanomachines' within the cell. The 'Advances in nanostructural genomics II' meeting at The Jackson Laboratory (Bar Harbor, ME, USA) brought together workers from the diverse areas of research that address these challenges. The topics covered ranged from genome sequence analysis and gene expression and organization of the cell nucleus to high-resolution imaging methods, nanolithography and mathematical modeling.

One of us (R.v.D.) opened the meeting by summarizing the current state of research on gene expression, chromatin structure and nuclear organization, and highlighting the areas that would benefit from a multidisciplinary approach. These include studies of chromatin structure *in vivo*, the function of subnuclear structures, the relationship between

gene clustering and functional nuclear domains, and how the mobility of nuclear components relates to their activities. A newly assembled magneto-microscope was also described; it was developed by a consortium of groups (called 'Cellular Submarine') who came together after the 'Genomics meets nanoscience' meeting last year (Politz and Pombo, *Genome Biol* 2002, **3**:reports4007.1-4007.3). It can guide micro-magnetic beads that have factors or enzymes bound through the nucleus, like an 'intracellular submarine', to measure and/or interfere with nuclear function at the nanoscale level.

Higher-order chromatin organization

The availability of the nearly complete human and mouse genome sequences has increased the need for robust tools for sequence annotation. Carol Bult (The Jackson Laboratory, Bar Harbor, USA) and Ross Hardison (Pennsylvania State University, University Park, USA) described the development of annotation methods, such as those available at the websites of the Penn State Bioinformatics Group [<http://bio.cse.psu.edu/>] and of the UCSC Genome Bioinformatics Group [<http://www.genome.ucsc.edu>]; they pointed out that currently no single search program can consistently identify all coding sequences within a region, and that the detection of functional non-coding regions is even more difficult. As Tim O'Brien (also at The Jackson Laboratory) explained, more detailed information can currently be obtained from specific genomic regions when computational and genetic methods are combined. As a model for functional genomic analyses he used the complex, 40 Mb large piebald region encoding coat color on mouse chromosome 14. Five coding genes per megabase were found in this region, together with a large number of non-coding

sequences, some of which are essential. Although further studies are needed to determine which of these are associated with biological function, initial studies have shown that experimentally defined auto-regulatory elements and putative transcription-factor-binding sites are contained within these conserved blocks of sequence. Giacomo Cavalli (National Centre for Scientific Research (CNRS), Institute of Human Genetics, Montpellier, France) and Doug Engel (University of Michigan, Ann Arbor, USA) showed that long-range interactions *in trans* between different sequences, such as locus control regions and Polycomb repressor elements, affected gene activity in *Drosophila* and mouse. Cavalli demonstrated that these sequences physically interact within the three-dimensional space of the *Drosophila* nucleus, and Engel echoed the idea that our definition of a gene must change, if we wish it to include all sequences that control a particular gene's transcription.

Long-range interactions were further discussed by Job Dekker (Harvard University, Cambridge, USA) who presented a novel high-throughput method, chromosome conformation capture or '3Cs', used to study the conformations of chromosomal sequences *in vivo*. In brief, sequences that interact *in vivo* are fixed within whole nuclei, chromatin is digested, and crosslinked fragments are ligated at low concentration to promote intra-molecular ligation. Ligated DNA fragments were quantified by PCR using oligonucleotides spanning a genomic area of interest. Dekker used this method to determine the frequencies of physical interactions between pairs of genomic loci on chromosome III of *Saccharomyces cerevisiae* and found the chromosome to be quite flexible with a circular configuration of only 300 nm in diameter; the most active domains occurred in more extended conformations. Siegfried Janz (National Cancer Institute, National Institutes of Health (NIH), Bethesda, USA) and Michael Difilippantonio (Center for Cancer Research, NIH, Bethesda, USA) discussed interchromosomal interactions that result in oncogenic chromosome translocations and gene amplifications. They used the murine t(12;15) translocation as a model for human Burkitt's lymphoma, in which interchromosomal DNA rearrangements lead to aberrant expression of the oncogene *c-myc* and to increased propensity for chromosome segregation errors and tumorigenesis. Difilippantonio reported that the joining of non-homologous broken chromosome ends may trigger the aberrant DNA rearrangement, as non-homologous end joining is involved in repairing programmed double-strand breaks in DNA that occur naturally during lymphocyte development, for example, when V, D and J gene segments in the immunoglobulin loci are rearranged to create the variable antigen receptor region (VDJ recombination), or when the gene segments that define different antibody classes are recombined (isotype switching). Janz also studied the fine structure of the t(12;15) translocation but used mice that have intact non-homologous end-joining pathways by using *c-myc* knock-ins, which he argued can

mimic translocations between the *c-myc* and the immunoglobulin H (IgH) gene more accurately than previous mouse models. He found that DNA rearrangements were most frequently caused by aberrant isotype switching, and rarely by VDJ recombination.

Understanding the complexities of chromatin and its interactions with chromatin-modifying factors were highlighted in a thought-provoking talk by Chris Woodcock (University of Massachusetts, Amherst, USA). If we are to fully understand the nucleus we will need to describe its biochemical and biophysical parameters. We have begun to understand the substrates (chromatin being perhaps the main one), the catalysts (enzymes that act on the substrates), and the kinetics of interaction, but it remains a daunting challenge to understand the complexity of the kinetic parameters of a long polymer with local modifications that is acted upon only locally by specific catalysts. Woodcock highlighted that a wide range of techniques will be necessary (with examples from his own work on the yeast SWI/SNF nucleosome remodeling complex) if we are to define chromatin structure at four levels - at the primary, nucleosome level; the secondary level of the 30 nm DNA fibre; the tertiary folding beyond the 30 nm fibre; and the quaternary structure of the whole chromosome within the nucleus. Astrid Visser (Scripps Research Institute, La Jolla, USA) discussed the need for combined efforts to sequence the histone modification 'code', which posits that different combinations of histone modifications signal different chromatin states, to give insights into higher-order chromatin structure. This could include capture of specific chromatin conformations, followed by linearization of the chromatin and reading of the histone code. Chromatin configurations could also be captured either after DNA stretching and visualization by light or electron microscopy, or possibly with technology that might allow sequencing the histone code in the future.

Improvements in nanotechnology

Although research on nanostructures is one of the most rapidly growing fields in modern science, the lack of higher-resolution substrate patterning methods has prevented direct examination of these structures at the level of resolution needed to study macromolecular activity within the cell. Joachim Spatz (University of Heidelberg, Germany) demonstrated that non-conventional methods of patterning surfaces onto glass coverslips allow the preparation of 5 nm gold dots that are separated by distances of 30-100 nm - a scale at which molecular interactions within and between cells can be monitored.

Rapid advances in microfluidics and microinstrumentation are also taking place. Gabriel Lopez (University of New Mexico, Albuquerque, USA) described the development of high-throughput microfluidic devices that allow cells that are cultured in 96-well plates to be washed completely between

medium changes, by using flow, rather than diffusion, as the mixing impetus. Scott Collins (University of Maine, Orono, USA) discussed refined instrumentation for single-cell experiments, such as microperfusion chambers that can be used to mimic arteries in arteriosclerosis studies and optical traps that can be used to manipulate individual cells. He also discussed a nano-sequencing technique in which DNA is electrophoresed through a nanopore and the sequence determined by measuring the current modulation caused by different bases as they move through the pore. Currently, however, only purines and pyrimidines can be differentiated. Watt Webb (Cornell University, Ithaca, USA) demonstrated that the activity of single polymerase molecules that are immobilized inside a zero-mode waveguide (subwavelength holes in a metal film) can be followed by timing the fluorescence bursts that are emitted from labeled nucleotide analogs that enter the active site of the enzyme and are incorporated into the growing DNA strand. This technique is also being developed into a new DNA sequencing method.

Magnetic tweezers are a promising tool in cell biology; they allow objects inside cells to be moved while the forces exerted by biological systems in the pico- to nano-Newton range are measured. In contrast to optical tweezers, they do not use laser light that is potentially harmful, particularly when objects are below the micrometer size range. Gunther Reiss (University of Bielefeld, Germany) described a magnetic trap with which the two-dimensional and three-dimensional position of magnetic nanoparticles can be controlled with high sensitivity and precision.

Horst Weller (University of Hamburg, Germany) and Avi Ulmann (Brooklyn Polytechnic Institute, New York, USA) discussed how nanoparticles can be used for biolabeling and new surface chemistry techniques, for example, biotin-conjugated particles can be used to visualize streptavidin that is used in immunolabeling procedures or that is attached to a glass coverslip. Advances in the chemical synthesis of highly luminescent semiconductor nanocrystals, such as gold nanocrystals coated with silica, often referred to as quantum dots, allow the development of alternative fluorescent or luminescent labels that can be used instead of standard organic dyes, such as fluorescein isothiocyanate (FITC).

Improvements in microscopy

Conventional fluorescence microscopy can be used to determine the positions of objects only when they are separated by distances greater than a few hundred nanometers - a restriction imposed by the diffraction limit of light. In contrast, fluorescence resonance energy transfer (FRET) techniques can be used to measure distances within one to ten nanometers. To visualize objects whose distance lies between these limits, alternative techniques are being developed. One of us (M.S.) discussed the use of dyes, such as Cy5 and JF9, that have different fluorescence life times

and of time-resolved fluorescence spectroscopy to distinguish and measure distances between two dye molecules that are separated by less than 30 nm. With fluorescence correlation spectroscopy (FCS) several properties of fluorescent labels can be measured and the mobility of single labeled molecules in live cells can be monitored. Samuel T. Hess (National Institute of Child Health and Human Development, NIH, Bethesda, USA) and Thomas Weidemann (German Cancer Research Center, Heidelberg, Germany) used this technique to study the properties of proteins tagged with green fluorescence protein (GFP) and their intracellular mobility in living cells. Hess discussed different properties of fluorescent proteins and their application as biological markers. For example, enhanced GFP (eGFP) flickers between dark and bright states at low pH, such that it may be used as a pH sensor. Weidemann used FCS to determine the concentration of nucleosomes labeled with H2B-eYFP in the HeLa cell nucleus and in mitotic chromosomes.

Other new microscopy techniques, such as laser holography and X-ray microscopy, are being used to study the three-dimensional organization of the nucleus. Jürgen Kreuzer (Dalhousie University, Halifax, Canada) showed that laser holography allows the acquisition of detailed images of living organisms without prior labeling. The image not only looks 'real' but appears to float in space and to move when the viewer moves, as with a real object. So far, Kreuzer has imaged heart cells and diatoms, and has tracked swimming algae using this technique. Günther Schmahl (University of Göttingen, Germany) and Gerd Schneider (Lawrence Berkeley National Laboratory, Berkeley, USA) discussed the application of X-ray microscopy to studying the nuclear distribution of the *Drosophila* dosage compensation protein MSL-1, which elevates the transcriptional activity of the X chromosome in males. Structural preservation is excellent under cryogenic conditions and the natural contrast between macromolecules and water in the cell allows the visualization of fine structural details. The distribution of immunogold stained MSL-1 protein was resolved to a level of about 60 nm; for example, the protein was observed to localize close to, but not within, the nucleolus. Michael Grunze (University of Heidelberg, Germany) discussed developments in the use of newly available synchrotron radiation sources of high brilliance to yield X rays and other types of radiation for electron- and soft X-ray-based microscopy of biological systems. With such a source of radiation, separate imaging of different chemical elements may become possible and we may see the development of novel types of microscopic imaging with spatial resolution at the atomic level.

Nuclear biology

Kevin Sullivan (Scripps Research Institute) summarized the current understanding of the DNA sequence and protein content of kinetochores, which form at the centromeres of mitotic chromosomes. Although centromeres are rich in satellite DNA, the sequence itself does not specify the function of

centromeres, which is instead inherited epigenetically. CENP-A, a histone-like protein that binds alphoid satellite DNA, is made during G2 phase of the cell cycle and incorporated into the inner kinetochore plate until mitosis. Using stretched DNA fibers, it was found that centromeres contain a mixture of CENP-A and histone 4. The Aurora B kinase, which phosphorylates CENP-A and H3, assembles at pericentric heterochromatin in a CENP-A-dependent process; Aurora moves to other chromosomal regions only after phosphorylation is complete at this site. This mechanism ensures an epigenetic mark of centromeres. Jim McNally (National Cancer Institute) addressed the relationship between transcriptional activation and chromatin decondensation. Several genetically engineered systems that consist of clusters of tandemly repeated genes display such relationship, but this behavior could be specific to artificially amplified gene clusters. McNally therefore looked for naturally occurring clusters of genes that are transcriptionally coactivated, for example, clusters that are controlled by extracellular signals. Such a cluster was found on mouse chromosome 22 in a microarray gene-expression analysis, and McNally showed that this naturally occurring cluster shows a decondensation after transcriptional activation that is very similar to that of the artificial gene clusters.

Active RNA polymerase I transcription sites are spatially organized into superstructures within the nucleolus. Making use of quantitative high-resolution light and electron microscopy, another of us (A.P.) reported that polymerases II and III also seem to form clusters of approximately eight enzymes in the nucleoplasm; such 'factories' are about 50 nm in diameter and each is surrounded by a cloud of nascent RNA. Antibodies against phosphorylated (active) polymerase II provided evidence that the factories contain different forms of polymerase.

The main cytological features of a eukaryotic nucleus are the nucleolus, chromosome territories, and the ill-defined interchromatin space. Thoru Pederson (University of Massachusetts Medical School, Worcester, USA) reviewed our current understanding of the interchromatin space, focusing mainly on the diffusion properties of RNA and other particles. He argued that interchromatin domains are active regions that are defined by and simultaneously interact with genes from different chromosomes. One of us (J.P.) showed that both the RNA and proteins that make up the signal recognition particle (SRP), which is required for targeting membrane and secretory proteins to membranes, are present within nucleoli, suggesting that the assembly of SRP might occur in this compartment. SRP RNA was found concentrated in subnucleolar domains that do not overlap with sites of ribosome assembly, suggesting that the nucleolus contains previously unrecognized domains devoted to novel functions.

Christoph Cremer (University of Heidelberg) first described that small, gene-dense chromosomal territories are located

near the center of the nucleus, whereas small, gene-poor chromosomal territories are located near the periphery. He also found that larger chromosomes tend to be located closer to the periphery of the nucleus. He explained that current modeling techniques cannot account for all of these observations. In an overview of new and forthcoming approaches in the nuclear structure field, he then discussed new microscopic technologies that are being developed in his group that will allow structural analysis to a precision level of 10 nm. Cremer stressed that beyond the development of new microscopy methods, quantitative modeling of biological structures and processes must be refined and developed, and better methods for labeling biomolecules must be discovered if we are to visualize specific proteins and RNA and DNA sequences *in vivo* with minimal disturbance of biological structures. So far, we have only had a glimpse at cellular structures. But with the rapid development of methods that allow us to study nanostructures, we may soon get a clearer picture.