Understanding nuclear organization: when information becomes knowledge

Workshop on Nuclear Organization

Dean Jackson

Manchester University, Manchester, UK

The Third Elmau Conference on Nuclear Organization: From Basic Science to Application took place at Schloss Elmau, Germany, between 7 and 10 October 2004. The workshop was organized by H. Lipps, S. Gasser and W. Deppert and was sponsored by Applied Biosystems, Bitplane, Boehringer-Ingelheim, Carl-Zeiss AG, EMBO, GATC Biotech AG, KFI Foundation, Leica Microsystems, Roche and Schering Foundation.

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The EMBO workshop on Nuclear Organization provided an excellent forum for exploring the links between information, controversy and knowledge. This meeting was the third in a series instigated by Hans Lipps, to explore how our understanding of nuclear organization might contribute to the design of nonchromosomal vector systems for gene therapy. With this emphasis in mind, the meeting was structured to explore the present state-of-the-art research on chromosome architecture and nuclear organization, with added emphasis on behaviour in living cells, vector design and therapeutic applications.

Chromosome structure

Chromosomes have key structural components that determine crucial aspects of their biological behaviour. It is logical to assume that the functions provided by these elements—centromeres, telomeres and origins of DNA replication (ori)—will be required by ectopic gene delivery systems that could be used for biomedical applications. A detailed understanding of the natural structures is thus required to design counterparts that might be used to mimic their natural behaviour.

Centromeres dictate the equal distribution of sister chromatids to daughter cells. W. Earnshaw (Edinburgh, UK) emphasized the precision with which mitosis is choreographed through the behaviour of kinetochores, microtubules, the mitotic spindle and the mitotic checkpoints (Vagnarelli & Earnshaw, 2004). Using a proteomic analysis as a starting point, he presented a vision of the behaviour of a novel protein, Borealin, that associates in a holo-complex with inner centromere protein (INCENP), aurora B and survivin. Using RNA interference (RNAi), Borealin was shown to be involved in correct metaphase alignment, and, together with survivin, provided spindle checkpoint function. Elegant live-cell imaging was used to show how defects in the kinetochore and general defects in chromosome structure due to loss of condensins or cohesins can disrupt the efficacy of mitosis.

The ends of linear chromosomes are protected by telomeres, which have a unique DNA architecture that serves to 'seal' the ends of the chromosomes (Vega *et al.*, 2003). A special telomere-dependent replication complex called telomerase is needed to copy telomeres during chromosome duplication and to prevent chromosome shortening. As cells undergo differentiation, telomerase

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Introduction

En route to Elmau, a thought captured my attention. In science, the goal must be knowledge, or understanding. However, inevitably, we generate information. In many fields of biological research, it seems that precious few experiments support any immediate or profound insight. At times it might even appear that the accumulation of information actually drives controversy and hampers understanding. Can this really be so? If so, can controversy have a part in driving understanding?

The Faculty of Life Science, Manchester University, Jackson's Mill, Sackville Street, Manchester M60 1QD, UK.

Tel: +44 (0)161 200 4255; Fax: +44 (0)161 236 4255;
E-mail: dean.jackson@man.ac.uk

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activity declines and chromosome shortening provides a sensor of cell ageing and contributes to the regulation of cell senescence.

V. Zakian (Princeton, NJ, USA) presented an elegant account of how yeast genetics can be used to interrogate telomerase structure–function relationships. She described the cell-cycle regulation of telomerase function and explained how the telomerase components Est1 and Est2, together with the telomerase RNA-based interaction between the DNA-binding protein Ku and the helicase Pif1, interact to regulate telomere length. E. Gilson (Lyon, France) extended this story by focusing on the roles of the subtelomeric chromatin in the *cis*-regulation of telomerase activity. Using a yeast model system, the subtelomeric chromatin was modified to manipulate the number of binding sites for two subtelomeric proteins, Tbf1 and Reb1. Binding of these proteins was shown to regulate telomere length in cells that carry a deletion of *Tel1*, which encodes a phosphatidylinositol-3-OH kinase (PI(3)K) known to be involved in telomere stability.

Controlling DNA replication

Although the basic principles of centromere and telomere structure and function are accepted, much less is known about the general properties of replication origins in higher eukaryotes (Gilbert, 2004). These structures are of fundamental importance, as DNA must be duplicated precisely during each division cycle in proliferating cells. Potential origins must be spaced to ensure that the mechanism of activation allows replication to complete during S phase (Hyrien *et al*, 2003). O. Hyrien (Paris, France) emphasized this point using nuclear assembly in a versatile system involving *Xenopus* egg extracts, in which the spacing problem is particularly relevant as S phase has a duration of approximately 20 min. Hyrien explained how the distribution of active origins could be modulated during nuclear assembly by altering the concentration of DNA and extract. By analysing replication forks on DNA fibres, replication is seen to initiate throughout S phase with potential origins broadly distributed. However, S phase completion is ensured through an ataxia-telangiectasia-related (ATR)-dependent checkpoint that introduces local origin interference and adjusts the frequency of initiation to the supply of replication factors.

G. Biamonti (Pavia, Italy) reviewed a well-characterized human origin, located at the lamin B1 gene (Paixao *et al*, 2004). He showed that transplanting this region to ectopic chromosomal loci stimulated replication at the new locus in about 90% of cell clones. This region is known to bind to the origin recognition complex (ORC) and a more detailed molecular deconstruction of the origin defined two short (about 20 bp) AT-rich elements with binding sites for the homeobox protein Hox3. Given the efficiency of origin function at ectopic chromosomal sites, it was surprising that the lamin B1 sequence failed to direct initiation of DNA synthesis from plasmids. A possible explanation for this was presented by R. Knippers (Konstanz, Germany), who showed that a prototype episomal gene therapy vector efficiently performed cell-cycle-regulated DNA synthesis even though no precise origin could be defined. In this case, ORC/mini-chromosome maintenance (MCM) proteins that were responsible for initiation were able to associate with the episome using an efficient DNA sequence-independent mechanism (Schaarschmidt *et al*, 2004). Molecular models for mammalian origins are hard to find, in part because the fidelity of chromosomal origins is usually lost when they are transferred to episomes. Bucking this trend, A. Schepers (Munich, Germany) detailed a high-

Fig 1 | The replication programme. During S phase in mammalian cells, specific regions of the genome are replicated according to a defined temporal and spatial programme. Characteristic patterns of nascent DNA synthesis define early (top), middle (centre) and late (bottom) synthesis. (A–I) Nascent DNA in replication factories labelled in permeabilized HeLa cells by indirect immunolabelling of biotin-dUTP incorporated *in vitro*. The same patterns are seen when human fibroblast (J–L) are labelled with Cy3-dUTP *in vivo* and visualized in living cells. Scale bar, 5 μ m.

throughput approach that has allowed his team to describe six mammalian origins that direct ORC binding and sequence-specific DNA initiation from episomes that incorporate an efficient Epstein–Barr nuclear antigen-mediated mitotic segregation mechanism. These sequences supported initiation both from episomes and the natural chromosomal locus.

DNA synthesis is organized so that specific regions of the genome are replicated at precise times (Fig 1). A key aspect of this process is how replication origins that are close to actively transcribed genes operate at the onset of S phase (Gilbert, 2004). D. Gilbert (Syracuse, NY, USA) described a system to explore links between gene expression and DNA replication using embryonic stem cells that can be induced to differentiate in culture. As cells commit to differentiation they undergo programmed changes in gene expression, which allowed Gilbert and his group to perform a detailed analysis of the crosstalk between genome architecture, gene expression and DNA replication. Gilbert analysed changes in gene expression in about 50 tissue-specific genes during differentiation along the neural lineage. Several genes were identified that switch replication timing from early to late or late to early, depending on transcriptional status. However, within this sample, switches in timing were restricted to genes within AT-rich isochores, which suggests that chromosome architecture imposes constraints on the mechanisms of replication timing.

However the timing of DNA replication is determined, it is generally accepted that chromosome structure and nuclear organization must be crucial for understanding how specific regions of the genome are replicated at predictable and reproducible times, and nuclear positions, during S phase. This type of structure–function relationship leads us to ask: How important is the interphase configuration of chromosomes for chromatin function?

Chromosome territories

About 100 years ago, Boveri first explored the concept of interphase chromosome territories (reviewed by Cremer & Cremer, 2001). He believed that the chromosomes that were visible during mitosis occupied a restricted nuclear area during interphase, and the chromatin from different chromosomes did not mix. This was confirmed when multicolour fluorescence *in situ* hybridization (M-FISH) first allowed the individual territories to be visualized in fixed cells. M-FISH is now a routine experimental tool and, in principle, the concept of territories remains intact. However, as with many biological principles, the devil is in the detail (Fig 2). During the past five years, a small number of studies have shown that defined chromosomal domains within highly active, gene-rich, regions might be found to lie outside the apparent boundaries of their chromosome territory. A. Pombo (London, UK) described how high-resolution, high-sensitivity FISH on ultra-thin cryosections can be used to determine if this is a general or special case. This technically challenging approach provides an opportunity to look in detail at DNA mixing across the boundaries of adjacent chromosomes. The advantage of this technology is that the increased sensitivity allows the use of commercially available chromosomal probes, or micro-dissected sub-chromosomal probes labelled with biotin or digoxigenin and indirectly labelled with cyan dyes, whereas previous studies had used specific probes (for example, cosmid) to label defined chromosomal loci. When chromosomal probes were co-hybridized to pairs of chromosomes in activated human lymphocytes, the mixing across the boundaries of adjacent chromosomes was in the range 0.2–6%. In these cells, it is estimated that about 15% of the nuclear volume contains mixed territories. The functional significance of this remains to be explored, although it is notable that the degree of intermingling for each pair of chromosomes correlates with the frequency of ionizing radiation-induced translocations in particular cells.

As chromatin from different chromosomes is not intermingled during mitosis, mixing across the borders of chromosome territories must occur in proliferating cells as nuclei re-form after mitosis. A crucial question is whether chromatin assumes a stable intermingled state, or whether certain parts of the genome have properties that allow local diffusion of the chromatin fibre. Although this is probably critical to function, it is notable that our understanding of chromatin dynamics in higher eukaryotes is entirely rudimentary. This is true despite the fact that many studies confirm the link between gene activity and nuclear location. One example was presented by D. Zink (Munich, Germany), who described the organization of three adjacent gene loci on human chromosome 7 that are localized independently of each other in functionally distinct nuclear regions (Zink *et al*, 2004). The distribution of these loci is dictated by their individual patterns of gene expression such that transcriptional activity dictates nuclear position and not vice versa. It appears that individual genes are not arranged in a specific way that dictates function but rather that genes can be targeted to specific nuclear sites by a transcription-dependent mechanism.

Chromatin dynamics

In higher eukaryotes, rapid chromatin movements seem to be constrained by the organization of chromosome territories and are restricted to zones of about 1 μm (Chubb *et al*, 2002; Fig 3). S. Gasser (Basel, Switzerland) explained how chromosomal loci of the simple eukaryote *Saccharomyces cerevisiae* could be tagged using green fluorescent protein (GFP)–Lac repressor fusion protein bound to *lac* operator sequences to evaluate chromatin movement (Gasser,

Fig 2 | Chromosome territories. In mammalian cells, individual chromosomes appear to occupy a restricted nuclear volume—a chromosome territory. (A) An example in which a probe to human chromosome 11 (short arm, 11p) has been hybridized to target sequences in a human lymphocyte. Two 11p territories (green) lie against the nuclear periphery. However, when a shorter telomeric probe is used—the subtelomeric region on 11p contains a highly active gene cluster—this region (red foci) is seen to be located outside the territory as defined by the general 11p probe. Although distinct territories can be defined, DNA from the corresponding chromosomal region is able to escape from the territory under some circumstances. (B) The typical arrangement of the same regions as in (A) in human fibroblasts. For details, see Mahy *et al*, 2002, reproduced by copyright permission of The Rockefeller University Press. (C) Low- and high-resolution models for the architecture of chromosome territories. The low-resolution model depicts how the chromosome axis is displaced in space. The high-resolution model emphasizes how DNA foci of approximately 0.5 Mbp DNA contribute to the structure. Different colours represent individual territories. For details, see Cremer & Cremer, 2001, reproduced by copyright permission from Macmillan.

2002). By monitoring absolute movement in real time using sampling times as short as 1.5 s, it is evident that gene domains are highly mobile and frequently show movements of >500 nm over a 10-s time-frame (Fig 3). Telomeres and centromeres, by contrast, are relatively immobile. Single telomeres can be tagged in living cells and have been shown to be in contact with the nuclear membrane: even though movements can be monitored, these occur in close contact with the nuclear periphery. Telomeres are bound at the membrane through a complex that incorporates silent information regulators (Sir) and Ku. In yeast strains deleted for *Sir4* and *Ku70*, the peripheral telomere tethering is lost and telomeres become dynamic and randomly disordered (Taddei *et al*, 2004). Extending this study, Gasser explained that telomeres are very variable in both structure and behaviour, such that changes in the structure of subtelomeric chromatin influence the extent and dynamics of association at the nuclear periphery. Intriguingly, by staining both telomeres of single chromosomes it is possible to monitor the movement of linked telomeres, which appear to move together in the same nuclear space. The striking mobility of transcribed genes is ATP-dependent and increases by recruitment of the strong activator virus protein 16 from herpes simplex virus (VP16) through ATP-dependent chromatin remodelling. This implies that chromatin fluidity is linked to transcriptional status.

Fig 3 | Visualizing chromatin dynamics. Chromatin dynamics can be monitored in living cells through the stable association of Lac repressor–green fluorescent protein (GFP) with clusters of target sites. Tagged loci in mammalian cells (arrowheads, top panel) show constrained movement, with specific loci appearing to vibrate in zones of less than 1 µm over many minutes. The extent of movement correlates with gene activity (graph). For details, see Chubb *et al*, 2002, reproduced by permission from Elsevier. In yeast, similar absolute movements are seen when the position of a specific locus is recorded at 1.5-s intervals (bottom right; red lines show displacement during each recording interval; the white ring marks the nuclear periphery). Yeast chromatin is highly dynamic, with frequent bursts of movement at about 0.5 µm/10 s or faster. Absolute movements are similar in the two cases; however, although a yeast locus in open chromatin might sample all of the accessible nuclear space over a period of minutes, a mammalian locus in equivalent chromatin is exposed to <1% of the nuclear volume. For details, see Gasser, 2002, reproduced with permission © (2002) AAAS.

The nuclear matrix and lamina

The influence of chromatin dynamics on gene function raises a perennial anxiety in nuclear structure circles—the nuclear matrix question (reviewed by Jackson, 2003). The rapid chromatin movements seen in yeast are clearly against the concept that chromatin in eukaryotic cells will be stably associated with a fixed nuclear structure. However, differences between yeast and higher eukaryotes are notable: yeast do not have nuclear filament proteins such as the nuclear lamins and regulate genome architecture in other ways. Even so, the simple mechanisms used by yeast have an impact on crucial aspects of chromatin function; replication timing is a good example, with chromosomal loci at the nuclear periphery in G1 phase of the cell cycle set up as late-firing origins. It is possible that related, but more complex, regulatory mechanisms have evolved in multicellular eukaryotes. One way of approaching this question was described by F. Fackelmayer (Hamburg, Germany), who showed how an important nuclear matrix protein, nuclear scaffold associated factor A (SAF-A), is implicated in nuclear structure and function. First, using pseudonuclei assembled in *Xenopus* egg extracts, Fackelmayer showed that SAF-A provides an architectural framework on which active replication factories are assembled. The SAF-A filaments form a nuclear reticulum, even

when DNA is removed. If SAF-A assembly is disrupted using antibodies, DNA synthesis is inhibited. A dominant-negative approach can be used to show that the same is true in cultured cells. Expressing the C280 SAF-A deletion mutant prevents endogenous SAF-A from assembling into networks and inhibits DNA synthesis. Consistent with a proposed role as a structural component, SAF-A linked to GFP, and standard recovery from photo-bleaching techniques showed the protein to be immobile over periods of ~1 h.

The lamin proteins provide the only known protein-based filament network in mammalian nuclei (Gruenbaum *et al*, 2003). The lamin proteins are type V intermediate filament proteins, which, unlike other family members, assemble branched filaments. These filaments provide a cage-like framework that is fixed to the inner nuclear membrane; lamin proteins are also found throughout the nucleus. The nuclear lamina and lamin-associated proteins have essential roles in chromatin organization and cell division. R. Foisner (Vienna, Austria) described in detail the roles of lamin-associated proteins and analysed their behaviour during nuclear reorganizations that were associated with mitosis. Y. Gruenbaum (Jerusalem, Israel) demonstrated how the simple multicellular model organism *Caenorhabditis elegans*—having a single lamin gene, well-characterized associated proteins and an intensively studied developmental programme—provides a wonderful system to explore the functions of the nuclear lamina.

Changes in the nuclear lamina that arise from genetic defects in lamin genes have been linked to a variety of diseases, called laminopathies (Mounkes & Stewart, 2004). C. Stewart (Frederick, MD, USA) described how transgenic mice could be used to model laminopathies and study the role of lamins in both nuclear architecture and gene expression. A mouse knockout (KO) of the lamin A/C gene (lamin B1 and B2 gene expression provides a residual nuclear lamina) yields mice that develop to term, although death ensues at around 10 weeks due to heart-muscle defects. A precise point mutation, N195K, extends lifespan to approximately 14 weeks, at which point heart conduction defects lead to death from arrhythmic tachycardia. Cell lines from KO mice have a fragile nuclear structure and a weakened cytoskeleton, such that tensile stress induces cell death by apoptosis. Changes in the lamina have a profound impact on chromatin organization at the level of heterochromatin distribution, although a global analysis of gene expression showed that fewer than 20 genes had significant changes in expression levels.

Although the nuclear lamina is known to support essential nuclear functions—a lamin B1 gene KO is embryonic lethal—it is unknown if these are restricted to the nuclear periphery. It is now accepted that lamin proteins are found throughout the nucleus, but how these are structured and whether they provide a filamentous network that could be the basis of a nucleoskeleton is not known. Even so, D. Jackson (Manchester, UK) has described a branched intermediate filament network that ramifies throughout the nucleus of human cells. In Elmau, Jackson described how RNAi techniques had been used to displace each of the nuclear lamin proteins from filaments of the nuclear lamina. During these experiments, changes in the structure of the nuclear lamina were shown to have profound effects on both RNA and DNA synthesis. An almost complete elimination of transcription by RNA polymerase II and an approximately 70% decrease in the number of S-phase cells suggests that a lamin network might contribute to the regulation of chromatin function.

Applying knowledge to vector design

It is likely that rational design of episomal vectors for gene therapy will require a detailed understanding of the spatial and temporal dynamics of chromatin function. Recent studies of the prototype vector pEPI-1 show where progress is being made and where important questions remain. This episomal vector is maintained in proliferating cells in the absence of selection at about five copies per cell. Replication is efficient, although specific origins are not used, and episomes are uniformly segregated to daughter cells; mitotic stability is about 0.99. I. Stehle (Witten, Germany) explained that the key to this behaviour centres on the interplay between gene expression and the function of a matrix attachment region (MAR) in the vector (Jenke *et al*, 2004). Stable gene expression, efficient replication and mitotic segregation are inextricably linked and dependent on MAR function. In spite of this, our present knowledge does not provide a molecular basis to explain the crucial properties of this vector. Perhaps a detailed understanding of MAR function will provide the key.

MARs are known to augment transcription and undergo stress-induced duplex destabilization. This is associated with DNase hypersensitivity and structural changes are thought to contribute to the assembly of protein complexes that regulate gene expression and modulate chromatin fluidity. Historically, MAR function has been used to control long-term, stable expression from genes that are integrated into ectopic chromosomal loci. However, context effects at ectopic loci often complicate the analysis of MAR function. J. Bode (Braunschweig, Germany) explained how this problem can be alleviated using recombination-mediated cassette exchange to construct gene domains at predetermined genomic sites. Using this approach, both natural and synthetic MAR constructs have been used to show the central role of MAR elements in defining the structure and function of gene domains.

Concluding remarks

Elmau 2004 was a thoroughly stimulating conference in a wonderful setting. On reflection, it is necessary to explore how the huge density of information resulting from our endeavours has influenced our basic understanding of how nuclei actually work. The fact that so many fundamental questions remain unresolved is food for thought. How replication origins work, how chromosome structure contributes to defining the spatial and temporal architecture of the replication programme, the impact of different nuclear domains or compartments on chromatin function, and whether function can be regulated by the combined activities of chromosome position, a nuclear sub-structure and chromatin dynamics, have yet to be determined. We have some way to go, although we now have the technologies to confront these problems, reveal the secrets of the nucleus and use this knowledge to design vector systems that will deliver predictable gene expression for biomedical applications.

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