European Science Foundation

Standing Committee for Life and Environmental Sciences (LESC)

ESF/LESC EXPLORATORY WORKSHOP ON:

Understanding chromosome behaviour: prospects for constructing chromosome-based vectors for gene therapy

MEETING REPORT:

The meeting was held September 27-30th, 2001, at Schloss Elmau, near Klais, an idyllic setting in the Bavarian Alps.

The meeting was opened by Annamaria Rosina, on behalf of the ESF. Annamaria explained the structure of the organisation and the ethos behind ESF funding to enhance collaboration and communication between scientist is specific disciplines across Europe. Different ESF initiatives were described including Workshop programmes, Networks and Eurocore consortia.

Dr Jackson, the convenor, introduced the scientific objective of the meeting. A global overview of nuclear architecture was presented and specific points of focus for furtuer developments identified. A summary of the scope and objectives of the meeting is given below:

Initiatives to sequence the human genome have already provided a wealth of detail about our genetic makeup. However, the process of gene expression is so complex that there is little prospect of using sequence information alone to predict levels and patterns of gene expression in different cell types. Though the basic steps needed to activate gene expression are now well defined, it is also clear that the efficiency and precision of this process can be influenced by higher order nuclear organization. This is evident from the dramatic differences in patterns and levels of gene expression that are often seen when transgenes are transcribed from unnatural chromosomal sites. This and other observations imply that different chromosomal sites - and perhaps different nuclear positions - have quite different functional capabilities. Clearly, a much more detailed analysis of chromatin function is needed if we are to understand how to reliably manipulate expression from ectopic genes in human cells.

With this in mind, the objective of this workshop is to assemble a group of predominantly European researchers in order to perform a detailed appraisal of our present knowledge of chromosome structure and nuclear architecture. We will assess critical features that link nuclear structure and function and discuss the most productive experimental systems to model natural chromosome behaviour. Finally, we will focus on the possibility of developing gene expression vehicles for use in human gene therapy and discuss the prospects of mimicking natural patterns of gene expression from extra-chromosomal vectors.

A further 20 research presentations were arranged into 6 thematic sessions - see programme attached.

In line with the desire to stimulate wide discussion and an open exchange of views, sessions where structure with talks of approximately 20 minutes followed by 10 minutes for discussion. Consecutive sessions were punctuated by coffee breaks to encourage further discourse. This strategy was clearly effective, with many detailed and informative discussions developing as a result.

The scope of the science covered by the workshop is indicated in the attached abstract for each presentation. Each of the talks presented was of a high calibre, in line with the international standing of those present. An additional description of the science presented at the meeting was prepared by Wendy Bickmore for publication in Genome Biology. A copy of this report is also attached.

The final session of the meeting was arranged to discuss the possibility of submitting a proposal to the ESF to establish an ESF network in chromosome biology. There was unanimous enthusiasm for this idea. It was agreed that Dr Jackson would lead this initiative. A proposal was subsequently compiled and submitted to the ESF on November 30th. A copy of the network proposal is also attached.

From an organisational point of view we believe that the meeting was also and outstanding success. The meeting was held at Schloss Elmau in the Bavarian Alps. Situated about 100km south of Munich this venue provided an ideal setting with a truly idyllic backdrop. The hotel and conference facilities were first rate, providing a high standard of accommodation and cuisine. This environment undoubtedly contributed to an extremely warm and friendly ambience that encouraged and stimulated both scientific and personal interactions amongst those present. This success of this aspect of the meeting has been commented on by many of those who attended. Below is an example of a letter of thanks from a postdoctoral attendee:

X-Sieve: cmu-sieve 2.0
From: <hulsmann@embl-heidelberg.de>
To: Dean.Jackson@umist.ac.uk
Subject: Elmau-meeting programme
Date: Tue, 02 Oct 2001 15:44:13 +0200 (CEST)

Dear Dean,
I would like to use this opportunity to also thank you for letting me participate in the seminars. Compared to the meetings I went before (especially the txn meeting at EMBL one year ago) this one was exceptional in many respects. I really liked the informal atmosphere created by the people and it was nice that there was so much time to discuss (not only) scientific issues. Actually it was a pity that it was only for two days since I would have liked to talk to more people. So I hope Hans and you will organise many more meetings in the future! And maybe I will be able to present something related to chromatin organisation at some point although I still haven t decided what I am interested in.
All the best Bastian.

In total, 35 scientist attended the meeting. Besides those invited speakers supported by the ESF the meeting was attended by a number of postgraduate and postdoctoral scientists working on different aspects of chromosome biology and gene therapy. Six scientists working for companies with related interests also attended.

Perhaps, the only negative aspect of the meeting followed the unfortunate events of September 11th in New York. Principally as a result of the prospect that international air travel might be severely curtailed in the wake of these events, 2 US speakers and 3 participants from Australia felt that to travel to Europe at that time would be ill-advised. Though this was disappointing, we do not believe their absence had a significant bearing on the successful outcome of the Workshop.

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Understanding chromosome behaviour:

prospects for constructing chromosome-based vectors for gene therapy

PROGRAMME

Schloss Elmau, Germany

27-30 September 2001

Convenors: D. Jackson# and H. Lipps*

 # University of Manchester, Institute of Science and Technology (UMIST), United Kingdom

** Institute for Cell Biology, University of Witten/Herdecke, Germany*

Additional support was kindly provided by *"Applied Biosystems"*, *"Boehringer Ingelheim"*, *"Miltenyi Biotec"* and *"Roche"*

Thursday 27 September

Afternoon Arrival of participants

Evening Welcome drinks and buffet

Introduction **ESF/LESC Presentation**
Annamaria Rosina, ESF Standing committee for Life And Environmental Sciences

Aims and scope of the meeting
Dean Jackson, Conveynor

Friday 28 September

Session 1 **Gene and Chromatin structure**
Chair - Juergen Bode

Speakers: Dirk Schübeler - Gene activation and repression in red cells - endogenous loci, ectopic sites and genomic approaches (45 min Plenary lecture sponsored by Applied Biosystems).

Genevieve Almouzni - Chromatin assembly from nucleosome formation to defining heterochromatin compartment.

Francisco Antequera - Organization of mammalian CpG island promoters.

Session 2 **Chromatin domains and the nuclear matrix**
Chair - Wendy Bickmore

Speakers: Niall Dillon - Characterisation of a mammalian functional gene expression domain.

Juergen Bode - Definition of autonomously regulated chromatin domains and utilization of their active elements.

Frank Fakelmayer - How SAR-binding proteins recognize their target.

Uli Laemmli - Boundary elements in chromatin.

Session 3 **Chromosomal elements involved in replication and Maintenance**
Chair - Hans Lipps

Speakers: William Brown - Assaying centromeric DNA using novel site specific integrases and engineering mini-chromosomes.

Fernando Azorin - The contribution of satellite DNAs to the structure and function of centric heterochromatin.

Beppe Biamonti - Phosphorylation and subnuclear localisation of human DNA ligase I: a model mechanism to control the activity of enzymes involved in DNA metabolism.

Saturday 29 September

Session 4 **Chromosome structure and artificial chromosomes**
Chair - George Dickson

Speakers: Dani Zink - The dynamic interplay between DNA and the replication machinery in living cells.

Christine Farr - Generation of a series of mini-chromosomes in DT40: dissection of the human X centromere.

Peter Marynen - Mitotic and meiotic stability in mice of a chromosomal vector derived from a human small accessory chromosome.

Pierluigi Donini - A MAC for the pig.

Session 5 **Nuclear architecture**
Chair - Dean Jackson

Speakers Wendy Bickmore - The dynamic organisation of genes and chromosomes in the nuclei of human cells.

Matthias Merkenschlager - Nuclear organisation and gene silencing in lymphocytes.

Chris Cremer - New approaches to the quantitative light optical analysis and biocomputing of human nuclear genome structure.

Session 6 **Towards the clinic**
Chair - Pirka Donini

Speakers: Hans Lipps - Non-viral episomal vectors for mammalain cells: exploiting chromosomal strategies.

George Dickson - Gene therapy for neuromuscular and cardiovascular disease (45 min).

Walter Zimmerli - Fundamental research and application: The genetic fix. Epistemological and Ethical Aspects (45 min).

Sunday 30 September

Session 7 **Round-table discussion**

The organisers - Concluding remarks. Prospects for developing closer European collaboration and networks.

Departure

ABSTRACTS

Gene activation and repression in red cells - endogenous loci, ectopic sites and genomic approaches

Dirk Schübeler.

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Our research focuses on how transcription is regulated on the level of transcriptional activators, chromatin structure, LCR/enhancer function and nuclear organization.

1) Analysis of the native human and mouse β globin locus control region. Using homologous and site-specific recombination we have recently deleted the endogenous human and mouse β globin LCR and shown that the LCR, while required for transcriptional activation of the genes, is not required for an open chromatin structure of the locus. We will present chromatin-immunoprecipitation (ChIP) analyses of wildtype and LCR deletion mutants and discuss the influence of the LCR on the degree of histone acetylation in the mouse and human loci.

2) To understand the mechanism by which DNA methylation represses transcription in ectopic genomic sites we have adapted a recombinase based targeting system to introduce in vitro methylated DNA into the genome. Using this system we have analyzed the influence of methylation on chromatin structure and protein binding in various constructs, as well as the influence of enhancers on maintenance of the methylated state.

3) Recent advances in DNA-microarray technology allow the analysis of the expression of thousands of genes simultaneously. We will discuss strategies and present preliminary results regarding how this technology can be used to monitor on a genomic scale changes in chromatin structure, DNA-methylation and timing of DNA replication.

Chromatin assembly from nucleosome formation to defining heterochromatin compartment

Christèle Maison*, Delphine Bailly*, Antoine H.F.M. Peters**, Jean-Pierre Quivy*, Angela Taddei*, Monika Lachner**, Thomas Jenuwein** and Geneviève Almouzni*.

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Higher-order chromatin structure has been linked to the post-translational modification of histone tails, such as acetylation, phosphorylation and methylation. Combinations of these modifications have been proposed to provide a mark recognised by specific proteins. This is exemplified, in both mammalian cells and fission yeast, by the hypoacetylated constitutive pericentric heterochromatin, which is transcriptionally inert and contributes to centromere organization. Methylation of lys 9 of histone H3 also appears essential for the functional organization of these chromosomal domains. This modification is specifically recognised by HP1 proteins (Heterochromatin Protein 1), components of pericentric heterochromatin and implicated in gene silencing and centromere function. A general principle has begun to emerge in which H3-K9 methylation could act as a marking system to establish and maintain stably repressed regions and heterochromatin subdomains.

We will present our data concerning the quality and stability of pericentric heterochromatin organization in interphase nuclei of mouse cells, a model in which a specific higher order structure dependent on both modification of histone tails and a novel RNA component will be discussed.

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Organization of mammalian CpG island promoters

Francisco Antequera.

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Mammalian promoters belong to two different categories in terms of base composition and DNA methylation. In human and mouse, approximately half of them have a G+C content and a methylation pattern that are undistinguishable form bulk DNA and are invariably associated with tissue-specific genes. The other half is associated with CpG islands, which are regions devoid of methylation and have a G+C content higher than the genome average. All housekeeping genes and many tissue-specific genes fall within this group. We have studied the relationship between promoters and CpG islands using in vivo footprinting and expression analysis. Our results show that mouse and human CpG island promoters show a disparate pattern of protein-DNA interactions at regulatory regions of genes whose genomic organization, sequence and expression pattern are highly conserved between both organisms. Despite these species-specific differences, a unifying picture emerges from the precise confinement of protein-DNA interactions between the 5' boundary of the CpG islands and the transcription initiation site. This finding allows direct localization of promoters on genomic sequences and is relevant given the availability of the human genome sequence and the difficulty to identify regulatory regions on the basis of sequence homology with other organisms.

Characterisation of a mammalian functional gene expression domain

Niall Dillon.

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The region that contains a eukaryotic gene or genes and associated cis-regulatory sequences is often described as a functional domain. In order to understand the relationship between the structure and function of domains it is important characterise their functional components and to relate the properties of these elements to overall spatial organisation. We are using the mouse 5-VpreB1 locus as a model system to study the organisation of a mammalian gene expression domain. A total of 12 DNase I hypersensitive sites (HS) have been mapped within the 19 kb region that is sufficient to give full position insensitive expression of the genes. Analysis of transgenes that have integrated into pericentromeric heterochromatin has demonstrated that there are at least two activities that can initiate the chromatin reorganisation that precedes gene activation. Following this initial chromatin disruption event, a second separate step is required for transcriptional activation. By examining the behaviour of the heterochromatic transgene under conditions of reduced factor dosage, we have obtained evidence that binding of transcription factors to specific sequences is directly involved in the earliest stages of higher order chromatin reorganisation prior to gene activation.

Definition of autonomously regulated chromatin domains and utilization of their active elements

J. Bode [1)], A. Baer [1)], E. Ernst [1)], S. Goetze [1)], A. Knopp [1)], K. Nehlsen [1)], C. Benham[2)]

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S/MARs have been discovered more than a decade ago and have been defined as DNA-elements staying attached to or associating with the nuclear skeleton after the extraction of the histones and soluble factors from eukaryotic nuclei (1). While S/MARs do not conform to any obvious sequence consensus, their recognition is governed by structural features, most significantly a propensity to expose single strands under negative superhelical tension. This property has been used to localize S/MARs in SIDD (stress-induced duplex destabilization) profiles, to explain their illegitimate recombination potential (4), to predict their participation in transcription and replication processes and to guide their design for various biological and biotechnological applications.

The first widely accepted activity of S/MARs was the augmentation of transcription initiation rates which is distinct from enhancement (1). Since then a variety of additional functions have been delineated acting on transcriptional competence, and providing enhancer or origin of replication support. This overlap of functions has made difficult the unambiguous demonstration of any of these components. It was originally for this reason that we have refined techniques based on site-specific recombination systems like Flp/FRT (3,7). With these techniques, complete

chromatin domains cannot only be decomposed but also be elaborated at a predefined chromosomal locus. Our most advanced system, the recombinase-mediated cassette exchange (Flp-RMCE) permits the mutual exchange of cassettes which are flanked by an FRT-site and an FRT-mutant, resp. (3,7).
So far the stable modification of target cells is mostly achieved by integrating vectors(2). For gene therapy purposes, derivatives of retroviral vectors have found extensive use although their expression may be rapidly silenced. While methylation-dependent silencing can be controlled by the presence of S/MARs(1), major recent efforts have been devoted to the use of S/MAR-ori sequences from the human genome to obtain vectors which replicate autonomously providing a stable and high-level expression. We refer to the contribution by H.J. Lipps who describes present efforts to develop a prototype episomal vector the properties of which depend on a series of intricate ori-S/MAR interactions (5,6).

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2 - A. Baer, D. Schübeler and J. Bode (2000). Transcriptional Properties of genomic transgene integration sites marked by electroporation or retroviral infection. Biochemistry 39, 7041-7049.
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5 - J. Bode, C. P. Fetzer, K. Nehlsen, M. Scinteie, Bok-Hee Hinrich, A.Baiker, C. Piechazcek, C. Benham and H. J. Lipps (2001). The Hitchhiking Principle: Optimizing episomal vectors for the use in gene therapy and biotechnology. Int. J. Gene Ther. Mol. Biol 6, 33-46. www.gtmb.org
6 - H. J. Lipps and J. Bode (2001). Exploiting chromosomal and viral strategies: The design of safe and efficient non-viral gene transfer systems. Curr. Opin. Mol. Therapeut. 3, 133-141.
7 - A. Baer and J. Bode. Coping with kinetic and thermodynamic barriers: RMCE, an efficient strategy for the targeted integration of transgenes. Curr. Opin. Biotech. (2001 in press).

How SAR-binding proteins recognize their target

Frank Fackelmayer

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I will talk about our work on the SAF-Box, a conserved protein domain found in many proteins, how it recognizes its target sequences, and what functional consequences this may have.

Boundary elements in chromatin

Ulrich Laemmli.

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Assaying centromeric DNA using novel site specific integrases and engineered mini-chromosomes

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We have used telomere directed chromosome breakage to engineer a range of mini-chromosomes that range is size from 1.6Mb upwards. We have observed that mini-chromosomes with small (~100kb) arrays of alphoid DNA form centromeres and segregate accurately in human and chicken cells but fail to do so in mouse cells. These mini-chromosomes thus provide reagents to assay both the DNA sequences and trans-acting factors necessary for accurate chromosome segregation in mouse cells. We are developing the use of a novel unidirectional site specific integrase from the *Streptomyces* phage C31 to promote integration of cloned DNA into a test mini-chromosome.

The contribution of satellite DNAs to the structure and function of centric heterochromatin

Fernando Azorin.

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The relaive contributions of DNA sequence and chromatin structure to centromere function will be discussed.

Phosphorylation and subnuclear localisation of human DNA ligase I: a model mechanism to control the activity of enzymes involved in DNA metabolism.

Alessandra Montecucco, Rossella Rossi, Giovanni Ferrari, Maria Rosa Lidonnici, Chiara Ferrandi and Giuseppe Biamonti

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DNA ligase I is a key enzyme involved in DNA replication and in DNA repair pathways. Its ability to participate to different aspects of DNA metabolism relies on protein motifs located in the N-terminal regulatory domain of the protein. Indeed, the recruitment of human DNA ligase I (hLigI) to the replication factories is mediated by a short replication factory targeting sequence (RFTS) that corresponds to an evolutionary conserved PCNA binding site. The molecular mechanisms underlying the dynamics of replication factories are still poorly understood. However, the dispersal of hLigI and PCNA from replication factories in response to chemically-induced double-strand breaks suggests the involvement of checkpoint pathways. Moreover, the redistribution of hLigI, PCNA and RPA in response to etoposide treatments suggests the existence of control mechanisms that link DSBs repair and DNA replication pathways by affecting the subnuclear compartmentalisation of the proteins involved. In accord with the notion that phosphorylation/dephosphorylation cycles can modulate the molecular interactions occurring within multi-protein complexes, we have shown that the phosphorylation status of hLigI is regulated during the cell cycle and in response to etoposide-treatment. We have identified two CDK sites located in the N-terminal domain of hLigI that are sequentially phosphorylated during cell cycle. Phosphorylation of these sites hampers the recruitment of hLigI to replication factories, suggesting that a major role of CDK-mediated phosphorylation of replicative enzyme is to control their dynamic association to replication complexes during S-phase.

The dynamic interplay between DNA and the replication machinery in living cells

Nicolas Sadoni[1], M. Cristina Cardoso[2], Andi Barnea[1], Ernst H.K. Stelzer[3], Heinrich Leonhardt[2], and Daniele Zink[1]

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We investigated the dynamics of DNA replication in living cells. Functional complexes of replication proteins were labeled with GFP-fusion proteins, while DNA was labeled with fluorescent nucleotides. We could show before that large-scale movements are not involved in DNA replication [1,2]. Therefore, we concentrated on the analysis of local replication dynamics. The results showed that, in contrast to favored models [3-6], DNA as well as functional complexes of replication proteins displayed local dynamics. The protein complexes displayed local re-localizations, which were not due to movements of whole complexes, but were due to local processes of assembly and disassembly of replication proteins. While DNA poised to replicate can be translocated into these complexes, nascent DNA was extruded in an aggregated form into adjacent regions.
Furthermore, the results showed that 93% of DNA aggregates, which replicated at a defined time point during S-phase, stably maintained their replication timing. These results strongly support the hypothesis, that chromosomes are organized into stable DNA aggregates (subchromosomal foci), which display a defined replication timing during S-phase [7-13].

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4. Lemon, K. P. & Grossman, A. D. Localization of bacterial DNA polymerase: evidence for a factory model of replication. *Science* **282**, 1516-1519 (1998).
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6. Cook, P. The organization of replication and transcription. *Science* **284**, 1790-1795 (1999).
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Generation of a series of minichromosomes in DT40: dissection of the human X centromere.

Jenny M. Spence, Ricky Critcher, Walter Mills & Christine J. Farr*

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As yet our understanding of mammalian centromeres, telomeres and origins of replication, as well as many other aspects of structure and organisation which may be critical for a fully functional mammalian chromosome, remains poor. As a way of defining these various requirements minichromosome reagents are being developed and analysed. Approaches for minichromosome generation fall into two broad categories: *de novo* assembly from candidate DNA sequences, or the fragmentation of an existing chromosome to reduce it to a minimal size.
Using the latter "top-down" strategy we have systematically generated a series of linear minichromosomes ranging in size from ~2.5 Mb down to 0.5 Mb. These minichromosomes, are based on the human X centromere and have been generated through the targeted introduction of *de novo* telomeres into the alpha and gamma satellite DNAs in the recombination-proficient avian cell line DT40. The size and molecular organisation of these minichromosomes has been determined by PFGE and restriction enzyme mapping. Mitotic stability is being assayed in DT40 and, following transfer, in diploid human fibroblasts ectopically expressing hTERT. The position of centromere proteins and topoisomerase II binding sites on the X alphoid array has been examined.

Mitotic and meiotic stability in mice of a chromosomal vector derived from a human small accessory chromosome

Thierry Voet and Peter Marynen

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A human small accessory chromosome that was mitotically stable in human fibroblasts was developed as a chromosomal vector (HCV) by the introduction of a selectable marker and the 3'- end of an *HPRT* minigene preceded by a *lox*P sequence. The HCV was then transferred to male mouse R1-ES cells and it did segregate properly. Chimeras were generated containing the HCV as an independent chromosome in a proportion of the cells. Part of the male and female offspring of the chimeras did contain the HCV. The HCV was present in >70% of the cells of the F1 HCV+ mice as an independent chromosome with an active centromere and the human *F3* gene was

expressed from the HCV in a human-tissue-specific manner compatible with the hemizygous presence of the transgene. The HCV has been transmitted through the male and female germline for 4 generations and its mitotic and meiotic stability in different mouse strains will be discussed.
Insertion of large genomic DNA fragments (PAC or BAC clones carrying human genes) temporarily destabilises the HCV and results in the amplification of vector sequences and/or amplification of the inserts, in addition to the expected products. Stable clones can be derived with different end products. The possible origin and the potential uses of this phenomenon are being investigated.

A MAC for the pig

Pirka Donini.

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A Mammalian Artificial Chromosome (MAC) was constructed in *Saccharomyces cerevisiae* and transferred to pig cells. The MAC will be used to produce transgenic pigs for possible utilization in xenotransplantation. Starting material for the construction was a YAC with a 300 kb insert containing 260 kb of pig centromeric DNA and 20 kb of pig genomic DNA. One end of the YAC was retrofitted with a fragment containing the Neo gene and 1100 bp of vertebrate telomeric (TTAGGG) DNA.TTAGGG repeats were added to the other end of the YAC by introducing and propagating the YAC in a yeast that had been modified in the RNA subunit of its telomerase in such a way as to cause it to add TTAGGG DNA to the yeast chromosomes.

The yeast strain containing the modified YAC was fused to PK15 pig cells and cells resistant to G418 were selected. A clone, designated d1Neo2, was chosen for study and shown by FISH analysis on metapahase plates to contain a MAC. The MAC was found to be mitotically stable over 34 generations.

The size of the MAC in pig cells, as determined by FISH under confocal microscopy, was found to be approximately 7 Mb, a greater than 20-fold increase over the size of the MAC construct originally present in yeast. MAC d1Neo2 does not enter a FIGE gel programmed for resolving chromosomes in the 3-10 Mb range, indicating that it possesses a chromosomal structure that is not compatible with that of natural linear chromosomes. A preliminary high resolution study of the DNA structure of the MAC carried out by Field Emission in-Lens Scanning Electron Microscopy indicates that the d1Neo2 MAC does not possess the 10 nm fiber chromatin network typical of the DNA of natural chromosomes.

The dynamic organisation of genes and chromosomes in the nuclei of human cells

Wendy A. Bickmore.

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Many levels of chromatin structure are involved in regulating gene expression. Recently, attention has turned to the spatial organisation and functional compartmentalisation of chromosomes, and of the nucleus itself, in the quest to understand how the expression of complex genomes is regulated in health and in disease.
I will show that chromosomes occupy specific positions within the nucleus in both fixed and living cells. Gene-rich human chromosomes general localise towards the interior of the nucleus whereas gene-poor chromosomes are positioned towards the nuclear periphery. This level of organisation is subject to cell cycle regulation. When cells exit the proliferative cycle and enter G0 (quiescence) the positioning of gene-poor chromosomes at the nuclear periphery is rapidly lost, and nuclear order appears to be more random. Signalling pathways that modulate this level of nuclear architecture are being investigated.
In model organisms, and in mammals, it is clear that the nuclear position of some individual genes relates to their gene silencing. However, mammalian genes are generally not organised into operons and so adjacent genes in the genome will often have different patterns of expression. This must place constraints on the possible nuclear position of genes. I will describe the intra-nuclear and intra-chromosomal organisation of contiguous stretches of the human genome and show that, although active and inactive genes can reside side-by side in the nucleus, there is a defined organisation of DNA within interphase chromosome territories. Using lacO arrays integrated into different sites in the human genome I will demonstrate that parts of the genome are constrained to moving within a very small proportion of the nuclear volume.

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Nuclear organisation and gene silencing in lymphocytes

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Our group have a longstanding interest in understanding how patterns of gene expression are established, maintained through cell division, and changed in a progressive and ordered fashion during development. In addition to identifying candidate genes involved in lineage choice, we have performed studies to examine the influence of nuclear location for gene expression. Our experiments combine techniques for fluorescence in situ hybridisation (FISH) that preserve nuclear structure, with high resolution, multi-colour fluorescence microscopy. These studies have shown that many transcriptionally inactive genes are positioned close to constitutive heterochromatin in the nucleus of dividing lymphocytes[1]. This functional 'compartmentalisation' is achieved as cells enter the cell cycle, and appears to be important in maintaining the heritable repression of a subset of genes[2]. Evidence from several studies have suggested that the silencing and recruitment of genes to heterochromatin might be mediated by the DNA binding protein Ikaros. Here we extend these analyses (with 3-dimensional Immuno-DNA and Immuno-RNA FISH techniques) to examine the role of nuclear organisation in specific situations. We examine loci which are monoallelically expressed (such as Ig[4] and imprinted genes), (ii) the behaviour of loci in which expressed and inactive genes which are interspersed (in *cis*)[3], and (iii) the nuclear location of genes which regulate the cell cycle. In addition, the application of a novel PCR-based approach to examine the role of 'replication timing' in gene expression, will be discussed.

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New approaches to the quantitative light optical analysis and biocomputing of human nuclear genome structure

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Far field optical light microscopy with its unique capability for contactless, non-destructive imaging inside thick transparent specimens such as cell nuclei has contributed widely to the present knowledge of the three-dimensional (3D-) architecture of the human genome in the cell nucleus [1]. The results of quantitative light optical analyses, especially confocal data, have been used in computer modelling&simulation approaches of human nuclear genome structure. Computer modelling [2-4] based on experimentally observed features allowed to predict light microscopically observable parameters of chromosome territories, such as telomere-centromere distances, morphological parameters, spatial nuclear chromosome territory distribution and its dynamics, relative translocation rates as a consequence of ionizing radiation induced double strand breaks, etc. In addition, a Brownian dynamics human genome structure computer model allowed to predict the mobility of particles in the nucleus, having e.g. the size of transcription factories, or of mini-chromosomes [5].
A serious problem for the extension of such studies to nuclear genome nanostructure is the limited optical resolution which inhibits rigorous testing of biocomputing models on the ”nanoscale” (e.g. topology of individual small

chromatin regions) A recently introduced light microscopic approach, Spectral Precision Distance Microscopy [SPDM; 6 - 9] based on labelling of neighbouring objects with different spectral signatures, spectrally selective registration, high precision position monitoring, and careful calibration of chromatic aberrations, cross talk etc. allows the measurement of positions and mutual distances between the "point-like" fluorescent objects in a range far below the conventional optical resolution criterion ["Abbe-Limit"]. As examples for application of SPDM in nuclear genome structure research, results from an analysis of the BCR-ABL region [10]; of the distribution of genes in the active and inactive X-chromosome territory [11]; and of the Prader-Willi-region [12] are presented. Furthermore, newly developed methods of Spatially Modulated Illumination [SMI] Far field light microscopy allow to measure the diameter of fluorescent targets down to a few tens of nanometers [1 nm = 1 x 10^{-9} m] and to perform distance measurements down to the few nanometer scale, with a precision in the one-nanometer range [13, 14]. This opens an avenue to measure in a quantitative way the condensation and topology of small inactive and active specific chromatin regions down to the few kilobase pair level, and to combine the results obtained with Biocomputing approaches of nuclear genome nanostructure [15]. We expect that such new approaches will contribute to the development of a quantitative, dynamic model of nuclear genome structure from chromosome territories to nucleosomes. Such a model not only would allow to better understand a rapidly growing wealth of quantitative nuclear structure information, but also allow to better estimate the consequences of therapeutical treatments.

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Non-viral episomal vectors for mammalian cells - exploiting chromosomal strategies
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The development of gene transfer systems for therapeutic applications depends to a large part on an understanding of chromosomal elements controlling gene expression, DNA replication and maintenace. The integration of transgenes into chromosomes assures their faithful replication and segregation, but their expression is susceptible to inactivation by the host cell machinery and, in any case, is dependent of the chromosomal context of the integration site. In addition, integrating constructs may cause unwanted and unpredictable mutagenic effects. Episomal constructs should be free from these shortcomings. However, viral-based episomal vectors currently used in mammalian biotechnology require at least one virally encoded protein for replication and maintenance. These virally encoded proteins may cause immunological problems in the host organism and even may lead to transformation of the recipient cells. Therefore the ideal vector for gene therapy should replicate episomally in a variety of mammalian cell types, should not require any virally encoded protein for replication and maintenance and should be stably maintained in the cell in the absence of selection. Until now, progress in this field suffers from an in-depth understanding of these accessory functions, although a number of first generation prototypes have been constructed in the past years which include the construction of mammalian artificial chromosomes using both, the bottom-up as well as the top-down approach. As an immediate solution, small non-viral circular episomal vectors are emerging which not only permit the study of the relevant components in a minimal gene transfer system, but for which a considerable potential for therapeutic applications can be anticipated. The structural and functional requirements for such a small circular non-viral episomal vector will be discussed.

Gene Therapy for Neuromuscular and Cardiovascular Disease

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Skeletal muscle and liver tissues are attractive gene therapy targets in the context of both homologous and heterologous expression of recombinant transgenes. Two areas of application of plasmid and adenovirus vectors will be discussed. Firstly the transfer of dystrophin transgenes into skeletal muscle tissues of the mdx mouse, a preclinical model of gene therapy for the human neuromuscular disease Duchenne muscular dystrophy. Secondly, vector-mediated delivery of transgenes to muscle and liver tissue to provide expression of secreted recombinant plasma lipoproteins, with concomitant inhibition and regression of atherosclerosis in animal models of hyperlipidaemia and cardiovascular disease.

Fundamental research and application: The genetic fix. Epistemological and Ethical Aspects

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