Instituto Juan March
de Estudios e Investigaciones

58 CENTRO DE REUNIONES
INTERNACIONALES SOBRE BIOLOGÍA

Workshop on

Chromosome Behaviour: The Structure and Function of Telomeres and Centromeres

Organized by

B. J. Trask, C. Tyler-Smith, F. Azorín and A. Villasante

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The lectures summarized in this publication were presented by their authors at a workshop held on the 23rd through the 25th of September 1996, at the Instituto Juan March.

Depósito legal: M. 40.175/1996
Impresión: Ediciones Peninsular. Tomelloso, 27. 28026 Madrid.

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INTRODUCTION

The functional and structural analysis of the chromosomal elements responsible for the stability of the eukaryotic chromosome is essential for the understanding of the mechanisms involved in normal chromosome inheritance. During the last few years, significant progress has been made in this field. The purpose of this workshop was to bring together recent results on the structure and function of two essential chromosomal structures: telomeres and centromeres, compiling data from a large variety of organisms, ranging from lower eukaryotes to mammals. Telomeres are the specialised protein-DNA complexes present at the ends of eukaryotic chromosomes. In most organisms, telomeric DNA consists of short sequence motifs that typically contain tracts of three or four guanines. The G-rich strand of telomeric DNA has the ability to fold into four-stranded DNA structures. In this workshop, the atomic structure of tetra-stranded DNA was presented, as obtained by NMR and X-ray crystallography. The effects of adenine substitution on the folding of the G-rich strand into tetra-stranded DNA was discussed. Finally, the ability of the complementary C-rich strand to form an intercalated tetra-stranded complex was also reported and details of this structure as well as of the factors influencing its stability were presented.

It is well known that proper telomere function requires the recruitment of sequence-specific DNA-binding proteins. An invaluable tool to investigate the interactions of telomeric sequences and proteins is the yeast *Saccharomyces cerevisiae*. At the meeting the atomic structure of the complex formed by telomeric DNA with the DNA binding domain of RAP1, the major telomere binding protein of the yeast *S. cerevisiae*, was presented. These studies provide important insights into how proteins recognize telomeric DNA sequences. The telomeres of *S. cerevisiae* are the best characterized in terms of both structure and function. Much less is known about the structural proteins that interact with telomeric DNA in *Schizosaccharomyces pombe*. However, its chromosome structure and dynamics is more closely related to higher eukaryotes. Work on *S. pombe* telomeres, mainly describing the interactions of specific proteins with telomeric DNA, was presented. Some of the features of telomeric binding proteins (myb domain) appear to be highly conserved from yeast to humans.

Telomeres have a special chromatin structure which results in gene silencing. It was shown that, in the yeast *S. cerevisiae*, the proteins associated with telomeric heterochromatin (RAP1, SIR3, SIR4) show an heterogeneous nuclear distribution.

The question of how telomeres are replicated was also addressed at the meeting. Data presented indicated that, in addition to elongation of the G-strand by telomerase, the telomere replication also involves a specific degradation of the C-strand. Recently, the relationship between telomerase activity and age has been the subject of active research. The results presented using a *S. cerevisiae* model system, suggest that telomeres may influence the length of the yeast life span but may not be involved in the aging process *per se*.

As previously mentioned, results presented focused on several organisms including ciliated protozoa and *Drosophila*. Most eukaryotic telomeres are made by G-rich repeats which are generated by telomerase. A contrast is represented by *Drosophila*, where telomeres do not have those simple repeats; instead telomere-specific transposable elements are found. Data on telomerase-independent mechanisms for telomere maintenance in *Drosophila* were presented.

Interest was shown not only on the very end of chromosomes, but also on several structural and functional aspects of the subtelomeric regions. Results presented at the meeting indicate that DNA near end of human chromosomes has large blocks of duplicated material and is highly polymorphic. Furthermore, the large-scale polymorphic duplications can contain genes, such as members of the olfactory receptor gene family.

Unlike telomeres, where functional sequences are well conserved, the repeated sequences found at many centromeres vary widely between species. The identity of the functional sequence has been well characterized only in yeast.

There has been debate about whether the small centromere of *S. cerevisiae* provides a model for the larger centromeres of most other organisms: are *cerevisiae*-like "magic sequences" embedded in the centromeric repeats of other organisms?. In his summary of the meeting, John Carbon identified the demise of the "magic sequence" hypothesis as one of its major conclusions. New evidence presented at the workshop suggested that repeated sequences alone are sufficient to form the centromeres of multicellular eukaryotes.

Schizosaccharomyces pombe can be seen as providing a model for the centromeric DNA of higher eukaryotes, and the identification of the *S. pombe* centromere binding protein Abp1 with extensive homology to the mammalian centromere protein CENP-B allows the model to be extended to include proteins as well. The centromeric DNA of a *Drosophila* minichromosome has now been characterised: only satellite DNAs and transposons were found. None of these sequences were detected at all other *Drosophila* centromeres and each sequence had non-centromeric locations. These findings, together with the observation that deleted minichromosomes lacking the centromeric DNA can show surprising stability, led to the thought-provoking suggestion that there may be nothing at all special about the centromeric DNA: perhaps, after suitable epigenetic activation, any sequence whatsoever can show centromeric activity.

The relevance of some structural features of *Drosophila* centromeric satellites and the functions of specific proteins that interact with mammalian centromeres were also discussed. Workers on mammalian chromosomes have been trying for years to emulate the success of those working with yeast, and create mammalian artificial chromosomes starting from DNA elements. The first reports of success from this approach provided some of the highlights of the meeting. Evidence is mounting that alphoid satellite DNA introduced into a cell may be sufficient to form a human centromere. Although more work is still required, the techniques are now at hand to create functioning human minichromosomes. Chromosome based vectors will allow the cis-acting DNA requirements for mammalian chromosome function to be rigorously defined. Furthermore, synthetic chromosomes could be extremely useful tools in the field of gene therapy.

In conclusion, findings reported at the workshop provided new insights into this dynamic field, giving a stimulating overview on the structure and function of two essential chromosomal structures. Undoubtedly, detailed function and structure may vary considerably from system to system; however, the workshop provided a wonderful opportunity to learn and to reflect about the possible links of chromosome behaviour among species, from yeast to human.

Session I: Telomere organization and function: lower eukaryotes
Chairperson: Daniela Rhodes

TELOMERE MAINTENANCE IN SACCHAROMYCES CEREVISIAE

(V.A. Zakian, Department of Molecular Biology, Princeton University, Princeton NJ 08544)

Saccharomyces chromosomes end in ~300 bps of $C_{1\text{-}3}A/TG_{1\text{-}3}$ DNA. After telomeres are replicated by conventional semi-conservative replication, which occurs at the end of S phase, terminal restriction fragments from yeast chromosomes or linear plasmids acquire single-strand $TG_{1\text{-}3}$ tails, estimated to be ~50-100 bases in length. These $TG_{1\text{-}3}$-tails are generated on ends replicated by both leading and lagging strand synthesis by a telomerase-independent mechanism. The $TG_{1\text{-}3}$-tails are probably generated by degradation of the $C_{1\text{-}3}A$ strand of yeast telomeric DNA (Wellinger et al., 1996 Cell 85:423). Cells carrying mutations in the essential gene *CDC13* arrest in G2 phase when grown at restrictive temperatures. In *cdc13-1* arrested cells, the strand running 5' to 3' from the telomere towards the centromere is degraded (Garvik et al., 1995 Mol. Cell. Biol. 15:6128). These data suggest that Cdc13p limits the cell cycle regulated $C_{1\text{-}3}A$-strand degradation that occurs at the end of S phase. To determine if Cdc13p prevents degradation by binding to telomeric DNA, Cdc13p was produced in *E. coli* or over-produced in yeast and tested for its ability to bind to telomeric DNA, using a gel shift mobility assay. Both *E. coli* and yeast generated Cdc13p bind specifically to single strand $TG_{1\text{-}3}$ DNA. This binding is not competed by duplex telomeric DNA, single strand $C_{1\text{-}3}A$ DNA, or RNA. Over-expression of Cdc13p causes telomere shortening. Genes near yeast telomeres are transcriptionally repressed, a phenomenon called telomere position effect, TPE. Cells expressing the temperature sensitive version of Cdc13p encoded by *cdc13-1* cells have reduced TPE when grown at semi-permissive temperatures. Together with previous data, the results suggest that Cdc13p binds to telomeric DNA *in vivo* where it regulates the accessibility of telomeric DNA to replication, degradation, and transcription.

Workshop on chromosome behavior, Madrid, 1996

The sequestering of Sir3 and Sir4 at telomere clusters and the role of concentration gradients in regulating SIR-mediated gene repression in yeast

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A novel technique for combined immunofluorescence / *in situ* hybridization on fixed budding yeast cells is shown to maintain the three dimensional structure of the nucleus as monitored by focal sections of cells labeled with fluorescent probes and by staining with a nuclear pore antibody. Within the resolution of these immunodetection techniques, we show that proteins encoded by the *SIR3*, *SIR4* and *RAP1* genes colocalize in a statistically significant manner with Y' telomere associated DNA sequences, in fewer than ten foci per diploid nucleus. This suggests that telomeres are clustered in vegetatively growing cells, and that proteins essential for telomeric silencing are concentrated at their sites of action, i.e. at telomeres and subtelomeric regions.

Transcriptional repression at the silent mating type loci in yeast requires the targeting of Sir proteins through specific interactions formed at *cis*-acting silencer elements. We show here that a reporter gene flanked by two functional silencers is not repressed when integrated at more than 200kb from a telomere. Repression is restored by creation of a new telomere 13kb from the integrated reporter, or by elevated expression of *SIR1*, *SIR3* and/or *SIR4*. Coupled expression represses in an additive manner. When overexpressed, Sir3 and Sir4 are dispersed throughout the nucleoplasm, in contrast to wild type cells where they are clustered in a limited number of foci. Efficient silencer function at internal loci thus seems to require either proximity to a pool of concentrated Sir proteins, i.e. proximity to telomeres, or delocalization of the silencing factors.

The localization of Sir proteins to telomeres appears to require interactions among Sir3, Sir4 and the C-terminal domain of Rap1. In the absence of Sir3, Sir4 is found dispersed throughout the nucleus, while in the absence of Sir4, Sir3 is localized mainly to the nucleolus. Evidence that the pool of Sir4 at telomeres is in equilibrium with a nontelomeric pool is suggested from the characterization of proteins that interact with the N-terminal domain of Sir4. Two factors, Sif2 and Sif3, bind tightly to the Sir4 N-terminal domain as monitored by two-hybrid analyses. When overexpressed, Sif2 weakens telomeric silencing, while its disruption improves repression at both telomeric and silent mating type loci. Sif2 is not localized to telomeres and genetic analyses suggest that it keeps the non-telomeric concentration of Sir4 in check.

THE ORGANIZATION OF INTERNAL TELOMERIC REPEATS IN THE DEVELOPING MACRONUCLEUS OF HYPOTRICHOUS CILIATES

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The macronucleus of hypotrichous ciliates contains millions of individual short DNA-molecules each carrying an expression cassette and sequences required for the replication as a linear DNA-molecule. These molecules are created after sexual reproduction in a series of morphologically well defined events. After fusion of two haploid micronuclei the diploid zygote nucleus divides mitotically and one nucleus becomes a micronucleus, the other differentiates into the macronucleus. Macronuclear development includes the formation of polytene chromosomes, degradation of these chromosomes, elimination of DNA and fragmentation into short gene-sized DNA-molecules. In the micronuclear genome macronuclear specific sequences occur in clusters, very often their coding region is interrupted by short non-coding sequences, (IES, internal eliminated sequences) or transposon-like elements which are excised during polytene chromosome formation and macronuclear precursor sequences are never associated with sequences homologous to the telomeric repeat. Thus, in order to create a functional macronuclear DNA-molecule at least the following processes have to occur: Excision of IES and transposon-like elements, specific fragmentation of the genome and de novo addition of telomeric repeats (for review: Lipps and Eder, 1996).

We have analyzed internal sequences homologous to the macronuclear telomeric repeats in the micronucleus and the developing macronucleus of *Stylonychia*. There exist about 1500 internal telomeric repeats which are all contained in a very conserved element. This element is bordered by two 2 kb direct repeats. Immediately adjacent to one of these repeats an 18mer $C_4A_4C_4A_4C_2$ telomeric sequence is localized. Sequences homologous to a macronuclear DNA-molecule follow 180 bp downstream of the telomeric bloc. The macronuclear homologous sequence is flanked by the second direct repeat (Stoll et al., 1993). Unlike the TBE (telomere bearing element) described in *Oxytricha fallax* the element found in *Stylonychia* is not excised early during polytene chromosome formation and no ORF showing homology to transposases can be detected (Doak et al., 1993). The high sequence conservation of the *Stylonychia* element suggests that it has some important function for DNA processing during macronuclear development. In fact, part of this element is transcribed during the polytene chromosome stage of macronuclear development yielding an RNA which contains the 18mer telomeric

$(C_4A_4)_2C_2$ sequence and a sequence homologous to a conserved sequence found in the subtelomeric region of all macronuclear DNA-molecules (Maercker and Lipps, 1993). This led us to speculate that this transcript could be involved in the de novo addition of telomeric sequences by acting as a template for the synthesis of G_4T_4-repeats to the 3'-end.

In order to experimentally test this hypothesis we established a transfection system by microinjection for *Stylonychia*. A vector was constructed which contains the micronuclear version of two macronuclear DNA-molecules, one of which was modified by the insertion of a polylinker sequence. When this vector was injected into the developing macronucleus it was correctly fragmented and telomeres were added de novo (Wen et al., 1995). Since this system allows the analysis of DNA-processing within single cells it may prove usefull for the identification of sequences required for correct de novo addition of telomeres.

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Telomere Length is Inversely Correlated to Lifespan in *Saccharomyces cerevisiae*

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The mortality kinetics of a population of yeast cells can be described by the Gompertz equation, the classical definition for aging populations. In this report we demonstrate an inverse correlation between telomere length and lifespan in *Saccharomyces cerevisiae*. Mutations which lengthen telomeres including *rap1s* and *Δrif1* lead to a shortening of lifespan. Furthermore, the significant shortening of lifespan in the *Δrif1* strain was partially suppressed by alleles of *SIR4* which are thought to enhance silencing at AGE, a hypothetical aging gene in yeast. In contrast, overexpression of truncated *TLC1*, the gene encoding the yeast telomerase RNA, leads to a shortening of telomeres and a delay in senescence. Strikingly, this extension in lifespan requires *SIR3* which encodes an essential component of the silencing machinery. These findings suggest that telomeres may set the reproductive capacity of yeast by regulating the levels of genomic silencing within the cell. In contrast to the prevailing telomere hypothesis which proposes that short telomeres lead to cellular senescence, we speculate that the telomere shortening in mammalian cells may not cause senescence but may actually be a cellular response to an age-dependent loss of genomic silencing: telomeres shorten so that cells can continue proliferating.

Telomeric length, chromatin structure and silencing are modulated by SIR3 dosage and the amino terminus of histone H4 in *Sacharomyces cerevisiae*.

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Telomeres have a special DNA and chromatin structure and play an essential role in cell biology stabilizing chromosomes and facilitating complete replication of chromosomal termini. Telomeric DNA usually contains tandem repetitions of a short motif flanked by subtelomeric, middle repetitive sequences. In yeast, telomeres are composed of about 350 bp containing the C1-3A repeat and are usually flanked by two subtelomeric elements: the X element, present in most yeast telomeres, and the Y' element, present only in a subset. A significant number of reports have described that transcriptional activity is negatively affected by the proximity to yeast telomeres. However, to our knowledge, a biological role for this telomeric silencing remains to be established.

We have focused our efforts on the study of yeast telomeric and subtelomeric chromatin structure and its influence on telomeres length and transcriptional silencing. The average chromatin structure of X elements was analyzed in wild type and mutant strains, some of which were known to be affected in telomeric silencing. These mutants lacked SIR3 protein or overexpressed it, bore a deletion of 28 aa in the amino terminus of histone H4 or had substitutions of the 4 acetylatable residues in the amino tail of histone H4. Standard nucleosome arrangements were not detected in the X elements of wild type strains. Instead, regions lacking detectable nucleosomes were found. In addition, some modifications to the wild-type structure were identified in silencing mutants. These mutants also exhibited altered length of the left telomere of chromosome III.

To study transcriptional silencing in a natural context, the levels of expression of the yeast retrotransposon Ty5-1 were analyzed in all the strains mentioned above. These strains contain only one copy of Ty5 near to the left telomere of chromosome III. Little expression was found in wild type strains, in the *sir3* overexpresing mutant and in the mutant affected in histone H4 acetylation. By contrast, clear derepression was observed in the *sir3* deleted mutant and in the mutant lacking the amino tail of histone H4. Therefore, the expression of Ty5-1 was silenced under natural conditions and was affected by previously described modifiers of telomeric silencing. Preliminary experiments have shown expression of Ty5-1 along the cell-cycle in a wild type strain, between G1 and S phase. Since Ty5 elements have been found preferentially in the vicinity of silenced areas like telomeres or *HM* loci, our results suggest a possible biological role for transcriptional telomeric silencing: Ty5 elements could survive in silenced areas keeping its transposition at low levels, thus avoiding undesirable mutagenic stress of the host cells. We are currently testing this hypothesis.

The chromatin structure of the putative Ty5-1 promoter region was analyzed in a wild type strain and in the *sir3* deleted mutant. Specific changes of chromatin structure were detected.

We would like to thank Michael Grunstein, Carol Newlon and Bik Tye for their generosity in supplying us with many strains and plasmids used in this study.

Session II: Telomeric DNA: Structure and protein recognition
Chairperson: Virginia A. Zakian

FOUR-STRANDED HELICES FORMED BY OLIGOGUANINE SEQUENCES

AIH Murchie, F Aboul-ela, G Laughlan§, B Luisi§ and DMJ Lilley

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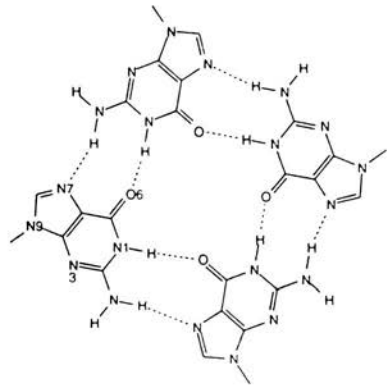
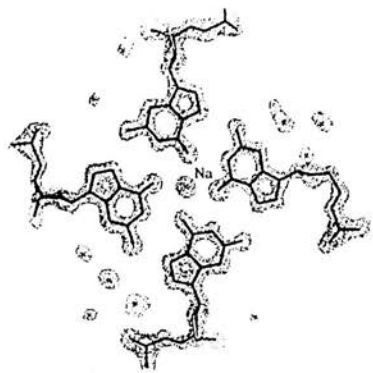
Oligoguanine sequences exhibit a propensity to adopt four-stranded helical structures in the presence of sodium or potassium ions. These are based upon the formation of hydrogen-bonded guanine tetrads (below). When tetraplexes are assembled from four separate strands, they have a strong tendency to do so in a parallel manner, but tetraplexes formed from two or more guanine blocks in the same continuous strand do so in an antiparallel manner.



We have studied the formation of parallel-stranded tetraplexes by short oligoguanine-containing oligonucleotides in the presence of sodium ions by biochemical and biophysical methods. In particular, we have studied the structure formed by the sequence dTG_4T by high resolution NMR in solution, and by crystallography.



NMR studies showed that it adopts a parallel-stranded tetraplex in which the glycosyl bonds are all in the *anti* conformation, and the deoxyribose rings are close to *C2'-endo*. We obtained crystals of dTG_4T that diffract to extremely high resolution. The structure has been solved to 1.2Å (see detail of tetrad, above), and more recently extended to 0.95 Å resolution. The molecular geometry is the expected tetraplex based on guanine tetrads. Seven coordinated sodium atoms were seen on the axis (note electron density at centre of tetrad, above), coordinated by guanine O6 atoms, and the four equivalent grooves are highly hydrated.

Using functional group substitution, we have studied the folding of intramolecular tetraplexes formed by sequences of the kind $d(T_{2\text{ or }4}XXXX)_4$, where X represents guanine, adenine or a base variant. Using a gel electrophoretic assay of folding, we find that only sequences based on G_4 or AG_3 are able to undergo tetraplex folding in the presence of monovalent ions. However, limited substitution of bases by ^{7C}G or ^{7C}A suggests that the latter folds by the formation of three all-guanine tetrads, with the adenines formally forming part of the loops where they would be expected to interact with thymine bases. No evidence for alternative tetrads based on the participation of adenine was found.

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Solution structure of a parallel-stranded tetraplex formed by $d(TG_4T)$ in the presence of sodium ions by nuclear magnetic resonance spectroscopy. F Aboul-ela, A I H Murchie, D G Norman & D M J Lilley *J. Molec. Biol.* 243, 458-471 (1994).

Tetraplex folding of telomere sequences, and the inclusion of adenine bases. A I H Murchie & D M J Lilley *EMBO J.* 13, 993-1001 (1994).

STRUCTURE OF THE YEAST TELOMERIC PROTEIN RAP1 IN COMPLEX WITH DNA: HOW TELOMERIC DNA SEQUENCES ARE RECOGNISED.

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Telomeric DNA typically consists of short, tandemly arranged sequence repeats. In the budding yeast *Saccharomyces Cerevisiae* the telomeric DNA is packaged by the non-histone protein RAP1[1]. The binding of RAP1 to telomeric DNA is involved in both telomere length regulation and transcriptional silencing[2]. The recently determined crystal structure of the RAP1 DNA-binding domain (RAP1DBD) with an 18bp telomeric DNA fragment provides the first insight into the recognition of the telomeric DNA sequences by a protein[3]. RAP1DBD contains two similar domains that bind DNA in a tandem orientation, recognizing two TGTGG tandem repeats in the RAP1 binding site. The two domains are structurally related to the homeodomains and Myb motifs. The cloning of the human telomere repeat binding factor TRF has revealed that this protein also contains a Myb motif[4], suggesting that the recognition of the conserved telomeric sequence repeats may take place via a conserved DNA binding motif.

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Folding of Cytosine-Rich Nucleic Acids

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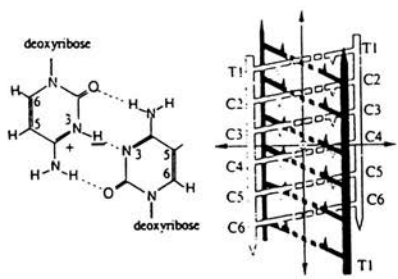
The human genome contains many repeated sequences. Some of the repeated motifs located at centromeric and telomeric sites are cytosine-rich, and may adopt an unusual DNA conformation, called the "i-motif". The stability of this structure relies on the formation of hemiprotonated C.C+ base pairs[1].



A DNA single-stranded short oligonucleotide, which sequence mimicks the cytosine-rich strand of human telomeres $(CCCTAA)_n$ may adopt the i-motif conformation *in vitro* [2,3], whereas a RNA molecule of corresponding sequence does not[4]. Repeated centromeric motifs, such as $(CCTAA)_n$ may also form the i-motif *in vitro*, although this structure is unstable.

Antisense and "anti-gene" strategies are based upon the specific recognition of cellular nucleic acids components (RNA or ds DNA) by single-stranded oligonucleotides. What happens if the antisense agent adopt a folded conformation such as the i-motif ? In a large set of experimental conditions, such a cytosine-rich oligonucleotide is blocked in a inactive conformation[4], and thus unable to associate with its target. Other routinely used nucleic acid modifications (phosphorothioates, 2'Ome) are now investigated.

Optimal i-motif stability occurs at acidic pH, but the formation of this structure is still possible in physiological conditions. This prompted us to search for for nuclear proteins that may recognize this motif. Human cells indeed contain several nuclear proteins that bind specifically to cytosine-rich nucleic acids. These factors are currently under characterization.

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Identification of a telomere binding protein in *S.pombe* and its function in controlling telomere length.

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In order to study telomere function in the eukaryotic model organism, *Schizosaccharomyces pombe*, we have performed a one-hybrid screen for *S.pombe* proteins that bind specifically to the telomere sequence. This screen identified a single protein, FTP. FTP is a novel protein containing at its C-terminus a region of homology with the Myb DNA binding domain, a domain also found in the human telomere repeat factor (Chong *et al.*, 1995 *Science* **270**). Disruption of *FTP* leads to a dramatic increase in telomere length, from a terminal restriction fragment size of ~300bp to nearly 5000 bp. This telomere length increase is accompanied by derepression of transcription of genes located adjacent to *S.pombe* telomeres (reduced position effect; Nimmo *et al.*, 1994 *EMBO J.* **13**). Finally, our chromatin mapping experiments indicate an unusual structure at the telomeres involving stably bound nonhistone proteins; this chromatin structure is altered in the *FTP*-disrupted strain. Thus, FTP may control telomerase activity either by interacting with telomerase subunits or, perhaps more likely, by controlling the accessibility of telomeric DNA.

THE TELOBOX, A MYB-RELATED TELOMERIC DNA BINDING MOTIF FOUND IN PROTEINS FROM YEAST, PLANTS AND HUMAN.

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The *Saccharomyces cerevisiae* TTAGGG-Binding Factor 1 (Tbf1) was identified and cloned through its ability to interact with vertebrate telomeric repeats *in vitro*. We showed that a sequence of 60 amino acids located in its C-terminus is critical for DNA binding. This sequence exhibits homologies with Myb repeats and is particularly well conserved among the following proteins.

- Five proteins from plants, two of which are known to bind telomeric-related sequences within promoter regions : the maize *Shrunken* initiator -binding protein IBP1 and the parsley BoxP-binding factor BPF1.

- Two proteins from human, which correspond to the previously described human TTAGGG binding factor (TRF) and a yet unknown protein called TB. The cDNA encoding TB was isolated and sequenced. Truncated forms of TRF and TB, mainly constituted by the Myb-related sequence, are sufficient to bind the human telomeric repeats specifically. We propose to call the particular Myb-related motif found in these proteins the « telobox ».

In summary, this work reveals the existence of two human telomere-associated proteins, TRF and TB, sharing a TTAGGG binding domain (telobox) at their C-terminus. Interestingly, the sequences of TRF and TB diverge outside the telobox, suggesting that these proteins perform separate functions at telomeres.

Session III: Telomere organization and function: higher eukaryotes
Chairperson: Huntington F. Willard

LARGE-SCALE POLYMORPHIC DUPLICATIONS OF SUBTELOMERIC DNA

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The sequence at the ends of human chromosomes does not shift abruptly from the $(T_2AG_3)_n$ repeat shared by all chromosomes to unique, chromosome-specific DNA. Instead, the subtelomeric regions of many chromosomes contain sequences that are duplicated on other chromosomes (see references). We have used FISH (fluorescence in situ hybridization), PCR, Southern blotting, and DNA sequencing to analyze the subtelomeric DNA of several chromosomes.

Our results reveal several surprising features of the subtelomeric regions: (a) the blocks of DNA sequence shared by different chromosomes can be quite large (>50 kbp) and highly similar, (b) these large-scale duplications are highly polymorphic, i.e., they may be present or absent on a given chromosome in normal individuals, (c) different blocks are organized in a complex patchwork that may differ among chromosomes, and (d) the large-scale polymorphic duplications can contain genes, such as members of the olfactory receptor gene family.

The 40-kbp segment cloned in cosmid 7501 from human chromosome 19 illustrates several of these properties. This sequence maps by FISH near the ends of 3q, 15q, and 19p in each of 44 individuals sampled from 5 diverse human populations. All but 4 individuals carry 7501-sequence on both homologs of these chromosomes (7501-sequence was present on only one homolog of 15 in 3 individuals and of 19 in one individual). These results suggest that the events leading to the duplication onto these 3 chromosomes preceded the spread of modern man.

In addition to these 3 common sites, the 7501-block is also present on other chromosomes in some individuals. The number of copies varies among normal individuals from 7 to 11 per diploid cell. A total of 14 subtelomeric sites (2q, 3q, 5q, 6p, 6q, 7p, 8p, 9q, 11p, 15q, 16p, 16q, 19p, 19q) were found to contain the cosmid's sequence in at least one of the 44 individuals. Small portions of the 7501 sequence were also observed on 4 additional chromosomes in some individuals. 7501 positive alleles were rare on some chromosomes and were detected only in a single individual, but were moderately frequent on other chromosomes (e.g., 64% of 88 chromosomes 11). The frequency of 7501-positive alleles of some chromosomes (e.g., 7p) varied among human populations. The bulk of the 7501-block is present on only one chromosome in chimpanzee and gorilla. This chromosome is the ortholog of human chromosome 4, which does not carry the sequence in any of the humans analyzed. These results suggest that the 7501-block has undergone very recent duplications onto and/or deletions from some chromosome ends.

These different chromosomes share at least 40-kbp of sequence. Chromosomes 3, 15, and 19 isolated in somatic cell hybrids are positive, with a few minor exceptions, for PCR assays identifying 17 locations across 7501. The similarity among the paralogs is indicated by the fact that probes from several portions of 7501 each identify a single band on Southern blots of EcoRI-

digested human DNA, despite their presence on 3 or more different chromosomes in each sample. Remarkably, we have found so far no differences in 2.3 kbp of sequenced DNA or the restriction map encompassing ~40 kbp from one allele each of chromosomes 3 and 19. This similarity suggests that the duplications leading to some copies occurred relatively recently (<1 Mya).

This duplicated segment contains three regions with homology to members of the olfactory receptor gene family. It is not yet known if the OLFR-sequences are transcribed into functioning proteins or if variations in the location or copy number of these sequences have a phenotypic consequence.

It is clear that the patchwork of duplicated blocks will frustrate attempts to map the subtelomeric regions using techniques that were developed for and honed on single-copy regions of the genome. Our preliminary efforts to walk from 7501 have linked the maps of 19 (41%) of the 46 chromosome ends and an interstitial site at 2q13-14. The 7501-block is flanked by sequences that are duplicated on even larger, overlapping subsets of chromosomes (a different set on either side). Polymorphism is also observed in the chromosomal distribution of these flanking sequences.

Our observations indicate that subtelomeric regions owe their complex organization to a series of events that have duplicated, transferred, deleted, and rearranged large blocks of DNA. We hypothesize that large blocks of telomeric sequence translocated periodically from one chromosome to another. The number of paralogous copies could have increased if block-containing alleles of both donor and recipient chromosomes were passed on to subsequent generations. Deletions, single-base mutations, and additional inter-chromosomal duplications could have resulted in the current jumble of blocks shared among different subsets of chromosomes. Our data suggest that at least some of these events took place recently, i.e., since the split of human from other primates and during the evolution of modern man.

It is perhaps not a coincidence that members of the OLFR family are found in such unstable regions of the genome. The ability to duplicate and diversify the repertoire of OLFR genes relatively rapidly may be under positive selection. By duplicating and rearranging the most distal ends of chromosomes, new OLFR genes could be generated without losing the original gene or negatively affecting dosage-sensitive genes located more internally. Indeed, FISH mapping of other OLFR-containing clones reveals a strong bias for subtelomeric locations.

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TELOMERES AND A POSSIBLE ORIGIN FOR TRANSPOSABLE ELEMENTS

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Telomeres (chromosome ends) have important roles in chromosome replication, in nuclear division, and probably in cell-type-specific organization in interphase nuclei. In most eukaryotes, telomeres are generated by an enzyme, telomerase, that reverse transcribes a small segment of RNA repeatedly to yield a chain of short DNA repeats on chromosome ends. These very simple repeats are not found in Drosophila. Instead two telomere-specific transposable elements, *HeT-A* and *TART,* are present in multiple copies on normal chromosomes and also capable of "healing" broken chromosome ends. *HeT-A* and *TART* form chains of repeats on the ends of Drosophila chromosomes but these repeats are much longer and more complex than those generated by telomerase. *HeT-A* and *TART* transpose only to chromosome ends. They appear to be retrotransposons transposing by means of a polyadenlyated RNA (non-LTR retrotransposons); however both *HeT-A* and *TART* have novel structural features which may be related to their telomere functions.

Transposable elements are abundant in the genomes of higher organisms but are usually thought to affect cells only incidentally, by transposing in or near a gene and affecting its expression. *HeT-A* and *TART* are the first transposable elements with obvious roles in chromosome structure. We hypothesize that these elements are evolutionarily related to telomerase; in both cases an enzyme extends the end of a chromosome by adding DNA copied from an RNA template. If this hypothesis is correct, studies of the Drosophila telomere transposons offer important comparisons to help understand telomere structure and function. A second implication of this hypothesis is that other retrotransposable elements and retroviruses may also have evolved from telomerase. This evolution need not have occurred only in Drosophila, although in other organisms the new products have not replaced the endogenous telomerase. (Oncogenes have arisen from cellular genes. Perhaps viruses that carry oncogenes have also arisen from cellular genetic mechanisms--telomerase and other cellular mechanisms yet to be discovered.)

Elements that transpose by means of a poly(A)+ RNA intermediate cannot take an external promoter to a new site. Some non-LTR retroposons, e.g. *Jockey* and LINE-1, solve this problem by an internal promoter. *HeT-A* has 0.8 kb of 5' non-coding sequences that might contain an internal promoter similar to the other promoters. We have tested the ability of the *HeT-A* 5' sequences to promote transcription of a β-galactosidase reporter gene in transiently transfected Drosophila cells. The *HeT-A* 5' sequences did not show any promoter activity in this assay. Surprisingly, we have found promoter activity in the 3' end of *HeT-A*. Since *HeT-A* elements are often found in head-to-tail tandem arrays, adjacent elements could collaborate in transcription. Our results suggest that the upstream element directs transcription of its 3' neighbor. The 3' end of the upstream element is at least partially analogous, both in structure and in function, to the long terminal repeats (LTRs) that serve as promoters for other classes of retroelements. The oligo(A) forming the proximal junction between each *HeT-A* element and the chromosome is typical of non-LTR retrotransposons yet the promoter activity of the upstream repeat may be a primative LTR, giving a glimpse of how LTRs evolved. Since *HeT-A*

elements are found only in heterochromatin, the *HeT-A* promoter must be active in heterochromatin. This is the first heterochromatic promoter to be molecularly characterized.

The *HeT-A* retrotransposon is distinguished by its exclusively telomeric location, and by the fact that, unlike other retrotransposons, it does not encode its own reverse transcriptase. *HeT-A* coding sequences diverge significantly, even between elements within the same genome. Such rapid divergence has been noted previously in studies of *gag* genes from other retroelements. Sequence comparisons indicate that the entire *HeT-A* coding region codes for *gag* protein, with regions of similarity to other insect retrotransposon *gag* proteins found throughout the open reading frame. Similarity is most striking in the zinc knuckle region, a region characteristic of *gag* genes of most replication-competent retroelements. We identify a subgroup of insect non-LTR retrotransposons with three zinc knuckles of the form: (1) $CX_2CX_4HX_4C$, (2) $CX_2CX_3HX_4C$, (3) $CX_2CX_3HX_6C$. The first and third knuckles are invariant; but the second shows some differences between members of this subgroup. This subgroup includes *HeT-A* and a second Drosophila telomeric retrotransposon, TART. Unlike other *gag* genes, *HeT-A* requires a -1 frameshift for complete translation. Such frameshifts are common between the *gag* and *pol* sequences of retroviruses but have not before been seen within a *gag* sequence. The frameshift allows *HeT-A* to encode two polypeptides; this mechanism may substitute for the post-translational cleavage that creates multiple *gag* polypeptides in retroviruses. *D. melanogaster HeT-A* coding sequences have a polymorphic region with insertions/deletions of 1-31 codons and many nucleotide changes. None of these changes interrupt the open reading frame, arguing that only elements with translatable ORFs can be incorporated into the chromosomes. Perhaps *HeT-A* translation products act in cis to target the RNA to chromosome ends.

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Session IV: Centromere organization and function (I)
Chairperson: Huntington F. Willard

Centromere structure and function: fungal model systems. John Carbon, Department of Molecular, Cellular & Developmental Biology, University of California, Santa Barbara, CA, 93106, USA.

Centromeres in budding yeasts (*S. cerevisiae* and *K. lactis*) are relatively simple structures, containing about 125 bp of DNA (the *CEN* locus) with specific binding sites for five known proteins (1). The *CEN* DNA plus associated proteins binds to a single microtubule to bring about chromatid separation during mitosis and meiosis. In contrast, centromeres in fission yeast (*S. pombe*) are more complex, containing several kilobases of DNA, and bind to three microtubules. Centromeres in the filamentous fungus, *Neurospora crassa*, are still more complex, containing 400-500 kb of DNA (2). Although the small budding yeast centromere contains no repetitive DNA sequences, the larger centromeres in fission yeast, filamentous fungi and animal cells contain many repetitive DNA sequences, some of which are essential for proper centromere function. The repetitive DNA sequences within the centromere DNA of *N. crassa* consist, in part, of many copies of several different classes of retroposons. The functional role of these retroposon DNAs in centromere function is still unclear.

Centromere activity in budding yeast is mediated by a multisubunit protein complex (CBF3), which binds to the essential CDEIII region of the *CEN* DNA locus (3). This complex contains four components (Cbf2p/Ndc10p, 110 kD; Cbf3Bp, 64 kD; Ctf13p, 58 kD; and Skp1p, 23 kD). The genes specifying these four proteins are all essential in yeast (1). The CBF3 complex can be reconstituted in functional form from the four subunits prepared by over-expression in various systems (*E. coli*, *Pichia pastoris*, insect cells) (4). Binding to *CEN* DNA requires the complete complex, and at least one of the subunits (110 kD) must be phosphorylated for binding to occur. The role of each of the subunits in centromere function is still unclear, although the 64 kD subunit contains a Zn-finger motif typical of DNA binding proteins (5), and the 110 kD subunit contains sequence motifs typical of protein kinases. The 110 kD subunit, prepared in pure form by over-expression in *P. pastoris*, contains an in vitro autophosphorylation activity and will catalyze the phosphorylation of BSA (collaborative experiments with Claire Fouquet, UCSB, & Johannes Lechner, Univ. of Regensberg). In the presence of the yeast kinesin Kar3p, CBF3 will mediate the attachment of *CEN* DNA to microtubules in vitro, and, in the presence of ATP, will move the DNA in a plus-to-minus direction on the microtubules, the same direction that chromatids move during anaphase separation (6,7). Evidence indicates that one important role of the centromere protein complex CBF3 is to regulate attachment and movement of the *CEN* DNA on microtubules.

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THE FISSION YEAST MODEL FOR THE HIGHER EUKARYOTIC CENTROMERE.

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Centromeric DNA of the fission yeast *Schizosaccharomyces pombe* is considerably more complex than that of the budding yeasts and shares some properties with higher eukaryotic centromeres. The three centromeres in *S. pombe* consist of 40 to 100 kilobases pairs (kb) of DNA organized, differently on each of the three chromosomes, into distinct classes of centromere-specific repeats, which are further arranged into a large inverted repeat (1, 2). The center of the centromeric inverted repeat, or central core, contains 4-7 kb of non-homologous sequences, which, when present in *S. pombe* on a minichromosome carrying K-type centromeric repeat sequences, impart considerable mitotic and meiotic centromere function to the minichromosome (3, 4). Thus, the two DNA elements, the K-type repeat and the central core, are critical for centromere function. Minichromosomes carrying centromeric DNA with certain deletions within either of these critical elements are the targets of a novel epigenetic mechanism that affects centromere function in vivo (5, V. Ngan & L. Clarke, unpublished). This epigenetic system results in the conversion of the centromere on the minichromosome from an inactive to an active state and probably involves specific folding of centromere components into a higher order chromatin structure (5, 6).

Using both biochemical and genetic strategies, we have begun to identify and examine *S. pombe* proteins that interact specifically with centromeric DNA. DNA affinity chromatography with a small fragment from the *cen2* centromeric central core has yielded a nearly pure 60 kDa protein (M. Baum & L. Clarke, unpublished). The p60 fraction binds to at least three non-identical fragments within *cen2* central core DNA and two other regions within the K-type repeat, indicating multiple binding sites for this protein in *S. pombe* centromeric DNA. In an alternate approach to identify centromere proteins, we have screened an *S. pombe* genomic library in a multiple copy vector for genes that in high dosage increase the loss rate of a normally stable *cen1* linear minichromosome (D. Halverson, J. Carbon & L. Clarke, unpublished). One gene identified in this assay specifies a protein with considerable homology to the human centromere protein CENP-B (7) and is identical to the fission yeast gene *abp1* (8). Affinity purified p60 appears to be identical to Abp1p, because specific DNA binding activity is enriched in overexpressing strains and antibody to Abp1p (kindly provided by J.-K. Lee & J. Hurwitz) causes a supershift of the centromeric DNA fragments bound by p60. Interestingly, Abp1p and CENP-B contain many regions with significant homology to transposase-like proteins and in both *Drosophila* (9) and *Neurospora* (E. Cambareri & J. Carbon, unpublished) there are transposable elements in the pericentric and/or centromeric DNA. The identification of a CENP-B-related *S. pombe* protein that binds specifically in vitro to centromeric DNA and affects chromosome segregation in vivo further confirms the validity of the *S. pombe* model for the higher eukaryotic centromere.

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Molecular-genetics of chromosome inheritance in *Drosophila*

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Chromosome replication and transmission are essential for the inheritance of genetic traits, but the mechanisms responsible for these processes remain poorly understood in higher organisms. Despite recent advances in this important field, major gaps exist in our understanding of both the *cis* and *trans*-acting components of inheritance. We have developed *Dp1187* as a model system for studying chromosome structure and function in Drosophila. In the last two years, we have completed molecular-genetic studies that localized the centromeric DNA and meiotic homologue disjunction elements within *Dp1187*, investigated the fine structure and sequence composition of the centromere, and pursued two new directions: analyses of genes and proteins required in *trans* for normal inheritance, and investigations of the importance of chromosomal and nuclear organization to gene expression.

Centromere localization and heterochromatin structure

The centromere, which appears as a constriction in metaphase chromosomes, is associated with the kinetochore and serves as the key attachment site to the spindle during mitosis and meiosis. Higher eukaryotic centromeres are embedded in large blocks of heterochromatin. Repeated DNA has made molecular-genetic analyses of heterochromatin and centromeres in higher eukaryotes extremely difficult. We have used the molecular accessibility of *Dp1187* minichromosomes to demonstrate that Drosophila contains a surprising amount of substructure in the centric heterochromatin. Pulsed-field restriction mapping revealed that *Dp1187* heterochromatin contains 50-200 kb regions that contain single copy and/or middle-repetitive DNA (the "complex islands" *Tahiti, Moorea, Bora Bora* and *Maupiti*) separated by 100-300 kb blocks of highly-repeated simple satellite sequences (Le, Duricka and Karpen, 1995).

Analyses of the transmission behavior of *Dp1187* deletion derivatives localized sequences necessary for chromosome inheritance within the centric heterochromatin. The fully-functional centromere is contained within a 420 kb region, which includes significant amounts of complex DNA, specifically the islands *BoraBora* and *Maupiti*. Surprisingly, the amount of centromeric DNA required for normal transmission differs among division types and between the sexes (Murphy and Karpen, 1995b). In the past year we have made significant progress in our understanding of the fine structure and sequence composition of the centromere. The only centric heterochromatin in the 620 kb derivative *γ1230* corresponds to the 420 kb fully-functional centromere. Cloning and mapping analyses of pulsed field gel-purified *γ1230* DNA have revealed the presence of two simple repetitive sequences that compose most of the functional centromere (AAGAG and AATAT),10 transposable elements, and a novel AT-rich sequence, in an unusual organization. The satellite DNA and the transposable elements are found at many chromosomal sites that do not form centromeres, and sensitive FISH analyses demonstrate that the major satellite components are not present at all Drosophila centromeres. Thus, centromere formation and function may not depend solely on primary DNA sequence, and instead may be regulated by secondary structure and epigenetic mechanisms (Sun, Wahlstrom and Karpen, in preparation).

Studies performed in collaboration with Byron Williams and Mike Goldberg (Cornell) support the idea that centromere formation is regulated in Drosophila. We examined the effects of deleting portions of the centromeric DNA on the localization of the centromere-associated protein ZW10, using indirect immunofluorescence of spermatocytes. Two regions of *Dp1187* are capable of localizing the ZW10 protein: the centric heterochromatin and acentric fragments composed of subtelomeric heterochromatin and euchromatin (originally derived from the tip of the X). The binding of the centromere marker protein ZW10 to acentric *Dp1187* derivatives strongly suggests that these fragments are weakly transmitted (Murphy and Karpen, 1995b) by a microtubule-based mechanism, and have the ability to serve as sites for "neocentromere" formation. Neocentromere activity is propagated through multiple cell and fly generations, even in the absence of "normal" centromeric DNA. Interestingly, the same region present in a normal X, and newly-created acentric fragments from the X tip, do not display neocentromeric activity or localize ZW10. We propose that a regulatory mechanism normally restricts centromere activity to a single site on the chromosome (lateral inhibition), or that centromere function spreads into adjacent regions (see PEV studies, below) and is then propagated by an epigenetic mechanism (Williams, Murphy, Goldberg and Karpen, submitted).

Trans-acting genes and proteins involved in inheritance

Elucidating centromere function requires identification of the gene products that promote chromosome inheritance in *trans*, and ultimately determining how they interact with the centromeric DNA to perform their essential functions. We have initiated genetic, molecular, and cell biological experiments designed to identify genes and gene products that promote normal inheritance by interacting with centromeric DNA, and to determine the proteins' properties, functions, and regulation in the cell cycle.

Interactions between a motor protein and extracentromeric DNA are required for transmission

We have developed a new genetic method for identifying and studying genes involved in chromosome inheritance. Normally, deleting extra-centromeric regions or reducing the dosage of inheritance genes (as in a *mutation*/+ heterozygote) does not produce defective inheritance phenotypes. However, genetic analyses indicate that transmission of *Dp1187* is sensitive to the dosage of *nod*, a kinesin-like gene required for the meiotic transmission of achiasmate chromosomes. Minichromosome deletions displayed increased loss rates in females heterozygous for loss-of-function alleles of *nod* (*nod*/+). The structures of *nod*-sensitive deletions indicate that multiple regions of *Dp1187* interact genetically with *nod* to promote normal chromosome transmission. Interestingly, most *nod* interactions are observed with regions that are not essential for centromere function. Independent biochemical and cytological studies (Afshar et al., Cell 81, 129(1995)) suggest that the genetic/functional *nod*+ interactions mapped in our study involve direct binding of DNA sequences by NOD protein. We propose that normal chromosome transmission requires anti-poleward forces generated outside the kinetochore, perhaps to maintain tension on kinetochore microtubules and stabilize the attachment of achiasmate chromosomes to the metaphase spindle (Murphy and Karpen, 1995a).

Sensitized minichromosome assays identify genes that interact with the functional centromere

The general utility of the sensitive minichromosome approach to the identification and analysis of Drosophila genes involved in centromere function was demonstrated with a series of known meiotic and mitotic mutations. Three heterozygous genotypes (in addition to *nod* /+, see above) had strong effects on minichromosome transmission. Interestingly, 2 of these loci encode kinesin-like proteins (*ncd* , *klp3A*), and one (*mei-S332*) is known to affect sister chromatid cohesion Four other loci had moderate effects, and only 3 loci had weak or no dominant effects on minichromosome transmission (Cook, Murphy, Nguyen and Karpen, submitted).

The conserved heterochromatic location of centromeres suggests that intrinsic properties of heterochromatin may play a role in centromere function. We used the "sensitized" minichromosome assay to determine if known modifiers of heterochromatin-induced position-effect variegation (PEV, see below) include genes that function to promote inheritance. Mutations (kindly provided by Gunter Reuter) in three *Su(var)* loci (of 6 tested) had moderate to strong effects on the transmission of a sensitized *Dp1187* derivative, including two independent mutant alleles of *Su(var) 2-10*. There are over 100 loci known to modify PEV, which may provide an unexpected, rich source of inheritance genes.

Genetic interaction studies were performed with a series of *Dp1187* derivatives in *ncd*, *klp3A*, *mei-S332*, and *Su(var) 2-10* heterozygous females. NOD was shown to promote transmission through interactions with most of the chromosome (see above). In contrast, *Su(var) 2-10*, *mei-S332*, *klp3A* and *ncd* were found to promote *Dp1187* transmission in females by interacting with the 420 kb minimal functional centromere. Our results demonstrate that transmission tests with the *Dp1187* deletion series is an efficient and effective method for identifying genes that interact genetically with the functional Drosophila centromere, and are using this method in de novo genetic screens for novel inheritance genes.

Drosophila homologues of yeast and mammalian centromere proteins

Common attributes of eukaryotic centromeres suggest that protein components may be more highly conserved than the underlying DNA sequences. We are complementing the genetic analyses described above by using degenerate PCR to clone Drosophila homologues of several kinetochore components identified initially in yeasts or mammals. We have obtained clones of Drosophila homologues of several genes, including Skp1, a component of the *S. cerevisiae* kinetochore. Molecular-genetic analyses will be performed in the next year to determine if DmSkp1 and other homologues act as *bona fide* centromere proteins in Drosophila.

Centric Heterochromatin and the Efficiency of Meiotic Disjunction

The pairing and segregation of homologous chromosomes during meiosis I is essential to ensure that gametes and progeny contain the proper number and type of chromosomes. We have determined the chromosomal requirements for achiasmate (nonexchange) homologue disjunction in Drosophila female meiosis I, using a series of molecularly-defined minichromosome deletion derivatives. Efficient disjunction requires 1000 kb of overlap in the centric heterochromatin, and is not affected by homologous euchromatin or overall size differences. Disjunction efficiency decreases linearly as heterochromatic homology is reduced from 1000 to 430 kb of overlap. Additional observations demonstrate that heterochromatin is not acting solely to promote chromosome movement or spindle attachment. Most regions required for disjunction lie outside the fully functional centromere, increased dosage of *nod+* rescues abnormal minichromosome transmission but not disjunction defects, and minichromosome loss does not accompany aberrant disjunction. We conclude that centric heterochromatin contains multiple pairing elements that act additively to initiate and/or maintain the proper alignment of achiasmate chromosomes in meiosis I. We speculate that meiotic pairing is mediated by intrinsic heterochromatic features/components, rather than proteins solely dedicated to this process (Karpen, Le and Le, 1996).

The role of nuclear organization and chromosome structure in gene expression

Studies of position effect variegation (PEV) demonstrate the importance of long-range influences on gene expression in higher eukaryotes. Terminal deletions of *Dp1187* dramatically increase PEV of *yellow* (Tower et al., Genetics 133, 347-359 (1993)) and we have discovered that this PEV can be suppressed by a second minichromosome in *trans*. Experiments monitoring the *trans* -suppressive abilities of minichromosomes that contain molecularly characterized deletions demonstrate that *trans*-suppression does not require the presence of heterochromatin in the second minichromosome, and is not occurring via a form of "transvection known to act at the *yellow* locus. *Trans*-suppression does require the presence of the *yellow* gene region in the suppressing minichromosome, and overall chromosome homology and orientation of the two minichromosomes. We propose two mechanisms for *trans*-suppression, which are not mutually exclusive: chromosome position within the nucleus may affect gene expression, and gene expression may be altered by proximity to a telomere and telomeric proteins (Donaldson and Karpen, submitted).

We have also used *Dp1187* derivatives to study the *cis* effects of specific regions of heterochromatin on gene expression. Experiments with deletions and rearrangements indicate that proximity to a functional centromere decreases expression more than proximity to other regions of heterochromatin. This data suggests that proteins exist that form specific heterochromatic structures within the centromere, and that this heterochromatic structure can affect the expression of a nearby gene. There are significant implications of these results to our understanding of centromere assembly and function (see above). Models for both centromere-induced and *trans*-suppressed variegation are currently being tested with cytological localization and molecular-genetic methodologies.

Recent Publications

Le, M.-H., Duricka, D. and Karpen, G.H. (1995). Islands of complex DNA are widespread in *Drosophila melanogaster* centric heterochromatin. Genetics *141*, 283-303.

Murphy, T. and Karpen, G.H. (1995a). Interactions between the *nod+* kinesin-like gene and extra-centromeric sequences are required for transmission of a Drosophila minichromosome. Cell *81*, 139-148.

Murphy, T. and Karpen, G.H. (1995b). Identification of the DNA required for centromere function in a Drosophila minichromosome. Cell *82*, 599-609.

Karpen, G.H., Le, M-H. and Le, H. (1996). Centric heterochromatin and the efficiency of achiasmate disjunction in Drosophila female meiosis. Science *273*, 118-122.

Donaldson, K.M. and Karpen, G.H. *Trans*-suppression of terminal deficiency-associated position effect variegation in a Drosophila minichromosome (submitted).

Cook, K., Murphy, T.D., Nguyen, T. and Karpen, G.H. Identification of *trans*-acting genes that interact with the *Drosophila melanogaster* centromere using centromere-defective minichromosomes (submitted).

Session V: Centromere organization and function (II)
Chairperson: Gary Karpen

STRUCTURAL STUDIES OF CENTROMERIC HETEROCHROMATIN IN DROSOPHILA

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CENTRO DE BIOLOGIA MOLECULAR "SEVERO OCHOA" (CSIC-UAM)

The chromosomes of *Drosophila melanogaster* offer a good model system for the analysis of the centromeric heterochromatin of higher eukaryotes. Drosophila chromosomes have a hemispherical kinetochore with bundles microtubules attached as in mammals, and, more important, the existing collection of mitotic mutants in *Drosophila melanogaster* opens the possibility of studying the effects of functions in *trans* upon minichromosomes reintroduced into flies.

Unlike telomeric sequences, centromeric sequences evolve rapidly. It is not generally expected that different species will share similar satellite DNA sequences. However, it would be expected that features of centromere structure that are important for function may be conserved through evolution to some extent. In *D. melanogaster*, the centromeric dodecasatellite ((GTACGGGACCGA) repeats) has been found by *in situ* hybridization only on the third chromosome centromeric region, although homologous sequences have been detected on both autosomes in *D. simulans* and on the second, third and X chromosomes in *D. mauritiana* (1, 2). It has also been shown that the dodecasatellite crosshybridizes to human sequences that are likely to correspond to the centromeric 5 bp satellite 3 family (1). Dodecasatellite repeats have G/C strand asymmetry and contain homopurine tracts. A similar distribution is shown by the human 5bp satellite 3, some centromeric satellites from ruminants, birds and plants, and it is a characteristic of telomeric repeats. Interestingly, telomeric-like sequences are present in centromeric heterochromatin in many vertebrate species, in *Arabidopsis* and in maize. The apparent conservation of the G/C strand asymmetry of these repeated sequences rises the possibility that some centromeric functions could be determined by specific structural characteristics of centromeric satellites. The structural characteristics of dodecasatellite DNA were studied in collaboration with Dr. Azorín's Lab (3).

On the other hand, the structural analysis of a yeast artificial chromosome (YAC) clone enriched in dodecasatellite sequences has led us to find a novel retrotransposon, *Circe*, associated with this satellite in the centromeric region of the third chromosome. Moreover, *Circe* has allowed the isolation of a contig encompassing ~200 kb from the centromeric region of the Y chromosome, providing an entry point into a region from which very little sequence information had been obtained to date. The molecular characterization of the contig has shown the presence of *HeT-A* telomeric retrotransposons close to the centromere of the Y chromosome. The finding of HeT-A sequences at a nontelomeric position was unexpected. However, the telomeric-like arrangement of these elements suggests that the centromeric region of the Y chromosome has been originated from a telomeric region. Whether these sequences have any functional significance or are just useless remnants of chromosome rearrangements which occurred during genome evolution is not known (4).

1 J.P. Abad, M. Carmena, S. Baars, R. Saunders, D. Glover, P. Ludeña, C. Sentis, C. Tyler-Smith and A. Villasante. (1992). "Dodeca-satellite: a conserved GC-rich satellite from the centromeric heterochromatin of *Drosophila melanogaster*". Proc. Natl. Acad. Sci. USA. 89, 4663-4667.

2 M. Carmena, J.P. Abad, A. Villasante and C. González (1993). "The *Drosophila melanogaster dodecasatellite* sequence is closely linked to the centromere and can form connections between sister chromatids during mitosis". J. Cell Sci. 105, 41-50.

3 N. Ferrer, F. Azorín, A. Villasante, C. Gutierrez and J.P. Abad (1995). "Centromeric dodeca-satellite DNA sequences form fold-back structures". J. Mol. Biol. 245, 8-21.

4 A. Losada, J.P. Abad and A. Villasante "Organization of DNA sequences near the centromere of the *Drosophila melanogaster* Y chromosome". Submitted.

THE FORMATION OF FOLD BACK STRUCTURES AT CENTROMERIC SATELLITES

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The structural properties of the *Drosophila* dodeca-satellite have been analyzed. This repeated DNA sequence, which is found at the centromeric region of chromosome 3 in *D.melanogaster*, shows an asymmetric distribution of guanine and cytosine residues resulting in one strand being relatively G-rich. Each dodeca-satellite G-strand repeat contains a GGGA-tract. The dodeca-satellite G-strand is capable of forming intramolecular hairpin structures which show a high melting temperature. These fold-back structures are stabilized by the formation of Watson-Crick C·G pairs as well as non-Watson-Crick G·A pairs, occurring at the level of the GGGA-tracts. The complementary dodeca-satellite C-strand also forms intramolecular hairpins which, in this case, are exclusively stabilized by regular Watson-Crick C·G pairs. However, the C-strand hairpins are significantly less stable, melting at a much lower temperature, than the corresponding G-strand hairpins. These results reveal the important contribution of the non-Watson-Crick G·A interactions to the stabilization of the G-strand hairpins. The formation of fold-back structures has also been detected at other centromeric satellites such as the human 5 bp satellite 3 and the *Drosophila* AAGAG satellite. In these cases, the hairpin structures are mainly stabilized through non-Watson-Crick interactions, principally G·A pairs. The structural behavior of other centromeric satellites will also be discussed.

A strong binding for the dodeca-satellite C-strand was detected on crude nuclear extracts obtained from Schneider SL2 cells. This binding activity was found to correspond to a high molecular weight protein (M= 160 Kd) which has been purified to near homogeneity. A partial sequence corresponding to an internal peptide obtained by digestion with V-8 protease has been obtained and exhibits no significant identity with any other known protein. This protein activity does not show significant affinity for the double-stranded dodeca-satellite DNA or its G-strand. The relative affinity of this protein for different DNA substrates has been determined. The dodeca-satellite C-strand itself competes the binding around 50-fold better than any unspecific ssDNA tested (oligod$(CT)_{20}$, M13 or *E.coli*) and more than 1000-fold better than dsDNA (*E.coli* or polidI-dC). These results suggest a certain degree of specificity in its interaction with the dodeca-satellite C-strand. The possible biological relevance of these results will be discussed.

CENP-E is a minus-end kinetochore motor that is essential for chromosome alignment

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CENP-E is a 312 kD kinetochore associated kinesin-like protein that was identified by a monoclonal antibody that was generated against fractionated Hela chromosome scaffold proteins. Unlike the CENP-A, B and C proteins that are associated with centromeric heterochromatin throughout the cell cycle, CENP-E is only transiently associated with kinetochores. CENP-E accumulates in the cytoplasm of interphase cells and assembles onto kinetochores when cells enter mitosis and remains kinetochore-bound until it is degraded at the end of mitosis. The presence of CENP-E at kinetochores suggests that it contributes to kinetochore force generation and is important for mediating chromosome motility.

We have taken two complementary approaches to analyze the *in vivo* function of CENP-E in tissue culture cells. In one approach, we eliminated CENP-E from kinetochores to a level below current detection limits by microinjecting antibodies that inhibited its assembly onto kinetochores. In a second approach, overexpression of a truncated fragment of CENP-E that contains the domain that specifies kinetochore binding effectively outcompeted the endogenous CENP-E from limiting binding sites within the kinetochore. For both strategies, cells that lacked CENP-E at kinetochores were found to be blocked for extended periods of time in mitosis with unaligned chromosomes. Since the mitotically arrested cells contain a bipolar spindle, failure to align chromosomes must result from defective kinetochore function.

Analysis of the mitotically blocked cells in real-time by video enhanced DIC microscopy revealed the presence of numerous monopolar chromosomes as well as bipolar chromosomes that failed to maintain a steady position at the spindle equator. Normally, at the onset of mitosis, some chromosomes are positioned between the two spindle poles and they establish rapid bipolar connections and begin to congress towards the equator. However, some chromosomes are positioned much closer to one pole and establish a monopolar connection that is subsequently converted into a bipolar connection when a microtubule emanating from the opposite pole is captured by the unoccupied sister kinetochore. When CENP-E is absent from kinetochores, the monopolar chromosomes are unable to establish a bipolar connection and remain stranded near the pole. Chromosomes that lie between the two poles appear to establish bipolar connections as they oscillate back and forth along the spindle axis. The critical difference is that these bipolar chromosomes that lack CENP-E are unable to position themselves at the spindle equator as they traverse back and forth across the spindle for many hours.

To search for a biochemical explanation for these defective chromosome movements, we characterized the motor properties of CENP-E *in vitro*. Using baculovirus, we expressed the N-terminal kinesin-like motor domain of CENP-E. *In vitro* microtubule gliding assays (in collaboration with Dr. Ed Salmon, University of North Carolina) revealed that the velocity of CENP-E motor was ~2 um/minute at R.T. This rate is ~50-100 fold slower than conventional kinesins that are isolated from squid optic lobe or bovine brain but is consistent with the *in vivo* rates of chromosome movement. We next examined the directionality of the CENP-E motor and found that it moves towards the minus-ends of microtubules, or in a poleward direction within the spindle.

We envision CENP-E to provide two critical roles for chromosome alignment. CENP-E is essential for a monopolar chromosome to establish bipolar connections. We postulate that CENP-E serves to establish stable kinetochore connections with microtubules that traverse across the length of the spindle by using its minus-ended motor activity to exert tension on the microtubule. Once a bipolar connection is established, CENP-E may use its motor activity to pull the chromosome towards the appropriate pole until the chromosome is aligned at the equator. In its absence, bipolar chromosomes exhibit an unrestrained poleward force that prevents chromosome alignment. This suggests that the slow motor activity of CENP-E might be used to restrain other kinetochore motors (i.e. dynein) that may be important for events prior to bipolar attachment.

Heterochromatin and satellite associated proteins binding to the *bw*^{D} insertion of Drosophila

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brown ^{Dominant} (*bw*^{D}) is an insertion of >1 megabase of the repeat AAGAG that interrupts the *Drosophila melanogaster* brown gene coding region and trans-inactivates its homolog. AAGAG repeats are naturally present in the pericentric heterochromatin. *bw*^{D} might sequester the brown locus to a heterochromatic compartment of the nucleus leading to gene inactivation. To investigate the process involved in trans-inactivation of this locus, we looked for proteins that might bind to this region. In wild-type polytene chromosomes, the heterochromatin protein 1 (HP1) is localized primarily to the chromocenter, where the heterochromatin is present. In the *bw*^{D} insertion the 59E1-2 band is split, and an antibody that recognizes HP1 is able to bind to this region, whereas in wild-type chromosomes there is no staining with the antibody in the vicinity of 59E1-2. Mitotic chromosomes from third instar neuroblast were also analyzed for proteins binding to the *bw*^{D} insertion. The brown locus is near the tip of the right arm of chromosome 2. An antibody that specifically binds the GAGA protein decorates this region in mitotic chromosomes, in contrast to wild-type, in which anti-GAGA antibody decorates only the centromeric regions of the chromosomes. Interestingly, mutations in the GAGA protein have no detectable effect in the *bw*^{D} allele.

Chromatin Structure and Expression of Euchromatic Genes in Heterochromatic Domains

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We are investigating the effects of different chromosomal environments on chromatin structure and gene expression in *Drosophila*. We are interested in determining the molecular basis for gene silencing by heterochromatin. When euchromatic genes are brought into juxtaposition with heterochromatin they exhibit gene inactivation in some, but not all, cells. For cell autonomous markers, this gives rise to a variegated pattern of expression known as position effect variegation (PEV). To investigate this phenomenon, a P-element containing two euchromatic genes was mobilized throughout the *Drosophila* genome. The P-element contains the *hsp70-white* gene as a visible marker for gene silencing, and a tagged version of the *hsp26* gene as a well characterized gene suitable for chromatin structure analysis that can be activated in almost all tissues. Following mobilization, a screen for PEV of the *white* gene identified lines with insertions near centromeres, telomeres, and along the small, mostly heterochromatic, fourth chromosome.

A second screen was performed to identify inserts buried "deep" within heterochromatin. After P-element mobilization, the flies were raised at elevated culture temperatures to induce expression of the *hsp70-white* transgene. Two lines were recovered that show some *white* expression when raised at the elevated temperature, but have completely white eyes at normal culture temperatures. These P-elements lie within pericentric heterochromatin. The DNA flanking these insertion sites, as well as other heterochromatic P-element insertion sites, is currently being characterized.

A third mobilization screen has identified P-element inserts along the fourth chromosome that show full expression of the *white* transgene. These results suggest that the fourth chromosome is made up of interspersed heterochromatic and euchromatic domains. This is consistent with the fact that antibodies against heterochromatin protein one (HP1) stain in a banded pattern along the fourth chromosome in polytene cells. Determining the location of all of the fourth chromosome inserts should provide a functional map of these domains and allow a search for boundaries between heterochromatic and euchromatic regions.

Pericentric and fourth chromosome inserts, but not second and third chromosome telomeric inserts, respond to known suppressors of PEV, the *Su(var)s*. These *Su(var)s* include mutations in the gene encoding HP1. Interestingly, certain alleles of *Su(z)2*, thought to be a member of the Polycomb Group, proteins that are involved in down regulation of homeotic loci, suppress second and third chromosome telomeric inserts, but not centromeric and fourth chromosome inserts. This suggests that there are two mutually exclusive sets of packaging proteins, those that are involved in gene silencing at centromeres and the fourth chromosome and those that are involved in gene silencing at the telomeres of the second and third chromosomes. The response to mutations in particular chromosomal proteins may reflect the sequences surrounding the insertion site. Second and third chromosome inserts are located within mini-satellite DNA sequences found at the subtelomeric regions of *Drosophila* chromosomes [collaboration with H. Biessmann (U.C. Irvine), M. Pavlova, B. Levis (Fred Hutchinson Cancer Research Center)].

Three molecular mechanisms have been proposed to explain PEV: (1) DNA elimination, (2) alterations in chromatin packaging, and (3) nuclear compartmentalization. The *hsp26* transgenes in heterochromatin are present in the same copy number as the endogenous *hsp26* gene in diploid tissues, indicating no DNA loss. Restriction enzyme digestion has shown the heterochromatic *hsp26* transgenes are less accessible than euchromatic inserts. Micrococcal nuclease digestion shows the pericentric inserts to be packaged in a more regular nucleosome array then are euchromatic inserts. Translocations of the fourth chromosome telomeric inserts onto the distal regions of the second and third chromosomes result in a weaker PEV phenotype. This suggests that the local concentration of chromosomal proteins near the centromere may have quantitative effects on gene silencing. The transgenes on the translocated fourth chromosomes retain sensitivity to the *Su(var)s*. This suggests that the local chromatin structure, and not position within the nucleus, or proximity to a linked centromere, dictates the response to suppressors. We infer that both local chromatin structure and nuclear position help to determine the degree of silencing observed.

Session VI: Centromere organization and function (III)
Chairperson: Louise Clarke

STRUCTURAL AND FUNCTIONAL STUDIES OF THE HUMAN Y CHROMOSOME CENTROMERE

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We have previously determined the structural organisation of the DNA sequences at the human Y chromosome centromere and localised the region required for mitotic centromere function to an interval of ~500 kb containing the alphoid satellite DNA array and adjacent Yp sequences (1). YAC clones spanning the region have been introduced by spheroplast fusion into a mouse L cell line which supports autonomous replication of the YACs (2). Autonomous circular molecules between ~100 kb and several Mb in size were observed, but all varied in copy number between cells and were lost rapidly in the absence of selection (3), suggesting that an active centromere was not formed. We are now developing an improved assay for chromosome segregation by incorporating the gene for the green fluorescent protein (4) into the structures and observing the fluorescence of living cells in vivo.

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(4) Chalfie, M. et al. (1994) Science 263, 802-805.

Functional aspects of centromeric repeated sequences.

Howard Cooke, Peter Warburton and David Kipling.

In contrast to telomeres where sequence conservation is widespread and obvious the repeated sequences found at many centromeres vary widely in sequence between species and as a result the search for functional mammalian centromeric sequences has not yeilded unequivocal results. A conserved binding site for the centromeric antigen CENP B has been reported previously in human and rodent centromeric satellites and our efforts to determine the extent of the centromeric conservation of this sequence will be reported. Introduction of DNA containing this conserved motif into mammalian cells induces some but not necessarily all aspects of centromeric behaviour suggesting that satellite sequences such as mouse minor satellite and human alphoid DNA may require either further DNA components to form a centromere or epigenetic modification.

Mini-chromosome derivatives of the human Y chromosome.

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We would like to understand the function of human centromeres and to develop chromosome based mammalian vector systems. We have therefore used cloned telomeric DNA to break (1) the human Y chromosome into a series of mini-chromosomes (2, 3). In the first round of breakage (2) we broke the chromosome within the centromeric array of alphoid DNA to generate a pair of acrocentric chromosomes. These segregated accurately at cell division and were retained for many months by cells in culture in the absence of any applied selection. The only major sequence held in common by these chromosomes was the alphoid DNA suggesting but not proving that the alphoid DNA was the major component of the nfunctional centromeric DNA. The long arm acrocentric chromosome occasionally segregated aberrantly at anaphase indicating that other sequences located on the short arm may be required for full centromere function. In second and third round of breakage we have broken the acrocentric chromosomes to produce eight mini-chromosomes which range in size between 2.5Mb and approximately 9Mb (3, 4). Chromosomes as small as 4Mb were stably inherited for 100 population doublings suggesting that the lower limit for mammalian chromosome stability is less than 4Mb.

Targeted chromosome manipulation is inefficient in mammalian cells because of the low frequency of homologous recombination. We have therefore transferred one of our mini-chromosomes, ΔΔhis1 into the chicken bursal line DT40 (5, 6). This led to two lines, one of which contains ΔΔhis1 as linear autonomous molecule. Initial results indicate that ΔΔhis1 is structurally and mitotically stable in DT40 (see poster by Chand et. al.) and is maintained at single copy per cell for 100 population doublings. The stability of human mini-chromosomes in a cell line which mediates a high frequency of homologous recombination suggests that we will be able to further refine our understanding of the sequence requirements for human chromosome function and suggests a route to the development of chromosome based vectors (4).

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Assaying DNA for Replication and Segregation Ability

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In Saccharomyces cerevisiae and Schizosaccharomyces pombe a functional chromosome requires telomeres to cap the ends of the chromosomes, origins of replication called ARSs, and a centromere for segregation. DNA with the necessary sequences can be transfected into these cells and will assemble the correct protein complexes for normal function.

Taking these lower eukaryotes as a model, we are investigating the DNA necessary for mammalian chromosome function by adding DNA elements to yeast artificial chromosome (YAC) clones which are a convenient scaffold on which to build. The DNA is then transferred to mammalian cells to assay for chromosome function. We observe that when YAC DNA is transferred into mouse LA-9 cells by fusion, it often forms extrachromosomal elements in the cells rather than integrating into the mammalian chromosomes. Similarly, when pure yeast DNA was transferred into mouse cells by fusion it also formed extrachromosomal elements, rather than integrating, in some cell lines. These elements are present at high copy number in the cells, and appear as doublets suggesting that they replicate once per S phase. They are however, lost rapidly from the cells if the selection is dropped indicating that they do not segregate. Yeast DNA thus appears to replicate efficiently in mouse cells suggesting that replication will not present a problem when assaying for centromere function.

In one of the cell lines containing yeast DNA, a "de novo" chromosome was seen. This chromosome consists of hundreds of megabases of yeast DNA, has a single centromere, and segregates efficiently. This chromosome was found to have incorporated mouse minor satellite DNA, and a variable amount of mouse major satellite at the functional centromere. Similar "de novo" chromosome formation has been observed by other investigators after introduction of putative centromere DNA and has been interpreted as indicating centromere formation by the input DNA. However, in this case it is clear that the centromere has been acquired from the mouse host cell.

When YACs carrying putative centromeric DNA, such as 80 kb of alphoid DNA, are fused into mouse LA-9 cells, they too make extrachromosomal elements with high efficiency. These elements are being analysed for their ability to segregate and to bind a variety of centromeric proteins.

In addition to using the mammalian centromere to achieve stable maintenance of large clones of DNA in mammalian cells, we have introduced the origin of plasmid replication, oriP, from Epstein Barr Virus into circular YACs. A 660 kb circular OriPYAC was transferred into human 293 cells expressing the viral protein EBNA-1 by fusion with yeast spheroplasts. In one of 3 cell lines generated, the 660-kb molecule is maintained as an unrearranged episome. Such OriPYACs are found to be quite stable and are lost at a rate of about 2% per cell division. Our next step is to add mammalian telomere sequences to the ends of linear YACs carrying the oriP element and to determine whether these molecules will form stable linear molecules in human cells expressing EBNA-1.

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Formation of Mitotically Stable Micro-Chromosomes by Transfected Alpha Satellite.

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While there is mounting evidence from a range of studies that alpha satellite DNA is a component of the centromere of normal human chromosomes, much of the data are indirect. Transfected alpha satellite DNA integrated into recipient chromosomes has been shown to display at least some of the properties expected for a centromere; however, to date such DNA has not been shown to lead to formation of stable, autonomously replicating micro-chromosomes consisting of exogenous DNA.

Beginning with a single higher-order repeat of either chromosome 17 or Y chromosome alpha satellite, we have synthesized arrays >150 kb in length consisting solely of tandemly repeated copies of the monomer. The resulting constructs, also containing metabolic markers for selection in either bacteria or mammalian cells, are stable when propagated in E. coli. Alpha satellite DNA from these constructs was ligated in vitro with telomeric DNA and genomic DNA fragments and transfected into human fibrosarcoma HT1080 cells. Neomycin-resistant colonies were selected and analyzed by cytogenetic and molecular assays. While either integration or telomere-truncation events were detected in many colonies by fluorescence in situ hybridization, small micro-chromosomes containing transfected DNA were observed in ~1 in 4 to ~1 in 15 colonies in different experiments. Each micro-chromosome contained alpha satellite DNA from the synthetic arrays in addition to exogenous genomic DNA. Multiple independent micro-chromosomes were evaluated for centromere function. All appeared to demonstrate a functional centromere/kinetochore, as determined by the presence of centromere proteins CENP-C and CENP-E, each of which has previously been associated with active, but not inactive centromeres. Further, each micro-chromosome was completely stable in the absence of selection for periods up to 6 months in culture.

These data support the hypothesis that alpha satellite DNA is an integral component of a functional centromere. Further, the ability to generate large synthetic arrays of alpha satellite from monomers of known sequence should permit further studies to define both the sequence and context requirements for centromere function.

POSTERS

EFFECTS OF 2-AMINOPURINE ON THE ULTRASTRUCTURE OF HeLa CELLS IN MITOSIS

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As was shown by Andreassen and Margolis (1991) cells in mitosis arrested in metaphase by microtubule poisons can overcome this inhibition when they are treated with the protein kinase inhibitor 2-aminopurine (2-AP). We wanted to know what happens when the inhibitor is applied at the transition from interphase to mitosis. The following mitosis was arrested. The spindle was deranged and coul not interact with normally condensed chromosomes which could be found scattered in the cytoplasm frequently surrounded by cisternae of the ER. Normally looking trilaminar kinetochores were only found when they were in contact with microtubules. All kinetochores without microtubules appeared immature. Both types of kinetochores could be seen in the same cell. These results suggest that normal kinetochore development and organization somehow requires the interaction with microtubules. During chromosome condensation obviously definite proteins must be localized to the centromere/kinetochore region to become involved in kinetochore formation.

Reference: Andreassen,P.R. and Margolis,R.L. (1991) J.Cell Sci. 100, 299-310

Terminal long tandem repeats in chromosomes from *Chironomus pallidivittatus*

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In *Chironomus pallidivittatus* the telomeres consist of big blocks (50-200kb) of 340bp tandemly repeated sequences. Such repeats consist of four different well defined subfamilies: M1, D1, D2, D3. M1 is the so- called master unit from which the others have evolved through small mutated regions in each subfamily. Bal 31 digestions and two coloured in situ hybridisations have shown that subfamily D3 is located closest to the chromosome end. Here we provide evidence that these repeats reach the termini of the chromosome. This is demonstrated by PCR amplification of the termini from tails added to DNA extracted from microdissected polytene chomosome ends. We show that DNA is intact within the blocks and that the synthetic tails directly join the 340 bp repeat. Futhermore, the DNA amplified only represents the most distal subfamily allthough all subfamilies are amplified with thw same efficience when digested DNA is used as template. Using plasmid controls we could also show that we did not amplify rare or nonrepresentative DNA termini.
The 340 bp repeats have a G-rich strand, oriented with the 3´-end towards the terminus, like in short telomeric repeats. A termination like the present one has previously been found in ciliate mitochondrial DNA but has so far not been described for nuclear chromosomes.

DT40 - a potential host system for the dissection of human centromeric sequences

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Current knowledge about sequence requirements for complete centromere function in mammals has relied upon studies of naturally occurring chromosomal aberrations, cloned putative centromeric regions in YACs and upon the use of telomere-directed breakage of chromosomes in the alphoid array. We aim to extend the technique of chromosome fragmentation to dissect candidate centromere sequences in a precise and ordered manner. To this aim we have created a series of human Y chromosome derived mini-chromosomes using telomere-directed breakage. Three rounds of fragmentation have generated 8 mini-chromosomes that include deletion derivatives of both the long and short arm and range in size from 8 Mb to 2.5 Mb. We have transferred one of these chromosomes, ΔΔHis1 into DT40, a chicken bursal B cell line that mediates a high rate of homologous recombination. Although the technique of microcell fusion is very inefficient when DT40 is the recipient cell line, we have obtained 2 clones, one of which contains ΔΔHis1 as a linear independant molecule. We are currently assessing the structure and stability of the chromosome in DT40. Initial data suggest that the hybrid has retained the selectable markers and the Y chromosome STSs and that the chromosome is maintained at a single copy per cell. Exploiting the high recombination frequency of DT40, we intend to target candidate centromere sequences by using site-specific recombinases (such as cre-recombinase) to create defined deletions in the minichromosome. In this manner we hope to identify sequences that are necessary, or sufficient, for accurate mitotic segregation of the human Y and possibly other mammalian chromosomes.

Detection, purification and characterization of a 160 Kd protein that preferentially binds the C-strand of the *Drosophila* centromeric dodeca-satellite.

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The *Drosophila* dodeca-satellite, which consists of tandem repeats of an 11-12 bp unit, shows a distinct asymmetric distribution of residues resulting in one of the strands being rich in cytosines (C-strand) while the complementary is enriched in guanines (G-strand). It has been demonstrated by *in situ* hybridization that the dodeca-satellite is present at the centromeric regions of chromosome 3 in *Drosophila melanogaster* and in other chromosomes of other species of *Drosophila* (1,2,3). As a consequence of its peculiar primary sequence, the dodeca-satellite exhibits an interesting structural behaviour. *In vitro*, the dodeca-satellite G-strand forms fold-back structures much more stable than similar structures formed by the C-strand. The higher stability of the fold-backs formed by the G-strand are the consequence of the formation of stable non Watson-Crick base pairs, mainly purine·purine (G·A) pairs, which cannot be formed in the case of the C-strand. The type of molecular interactions determining the formation of these fold-back structures was determined by chemical modification with different reagents (4).

A protein activity has been found which preferentially interacts with the dodeca-satellite C-strand. When crude nuclear extracts, obtained from SL2 cells, were incubated with an 145 bp long double-stranded DNA fragment containing 12 repetitions of the dodeca-satellite or with its individual strands, a strong binding activity was detected for the C-strand but not for the G-strand or the double-stranded fragment. Similar results were obtained when different dodeca-satellite fragments were used. The protein responsible for this binding has been purified to near homogeneity in three chromatographic steps: a pseudo-affinity column (Hi-trap blue Sepharose), an hydrophobic column (phenyl-superose) and an anion-exchange column (Resource-Q). After these steps, positive fractions are highly enriched in a high molecular weight protein (160 Kd) which binds to the dodeca-satellite C-strand. The N-terminal part of the protein is being sequenced. A peptide obtained by digestion with V-8 protease has been sequenced and exhibits no homology with any known protein.

The relative affinity of the protein for different DNA substrates was determined by competition experiments. Unspecific ssDNA (*E.coli* or M13) competes the binding to the C-strand better than unspecific dsDNA (*E.coli*), but much worse than the dodeca-satellite C-strand itself, revealing a certain degree of specificity in its interaction with the C-strand. The protein binds the dodeca-satellite C-strand about 50 fold better than any other ssDNA sequence and more than 1000-fold better than dsDNA.

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Detection of plant telomerase activity

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Telomerase is a ribonucleoprotein enzyme which elongates the G-rich strand of telomeric DNA to compensate for the progressive reduction in its length due to incomplete replication of chromosome ends, which in human somatic cells leads to cell cycle arrest upon shortening of telomeres to a critical length. To examine the possible involvement of telomerase in metabolism of plant genetic material, we used cells of *Nicotiana tabacum* strain TBY-2, a stable long-term culture which has kept a constant pattern of restriction fragments from chromosome termini during its six month period of cultivation in our laboratory. In a direct assay for telomerase, a 5' end-labeled plant telomeric oligonucleotide $^{5'}(TTTAGGG)_6{}^{3'}$ was elongated in a TBY-2 cell extract, showing a pausing pattern which is a characteristic feature of telomerases from other organisms. The elongation was inhibited by RNase A pretreatment of the extract. We conclude that plant cells possess telomerase which is used for maintenance of their telomeres.

Telomeres at 2.25 Å resolution

(How the crystal structure of a RAP1 (DBD) - dsDNA complex was solved)

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Telomeres, the natural ends of the eukaryotic chromosomes, constitute nucleoprotein complexes essential for chromosome structure and function. RAP1 is a multifunctional DNA binding protein from the yeast *Saccharomyces cerevisiae*, where packs the telomeric chromatin. We had biochemically characterized RAP1 and its minimal DNA binding domain (DBD), showing that both distort the telomeric $C_{1\text{-}3}A/TG_{1\text{-}3}$ repeats and also that RAP1 promotes the formation of DNA quadruplexes by the telomeric terminal $TG_{1\text{-}3}$ tails.

We have crystallized a complex between RAP1(DBD) (247 aa residues, 28.8 kDa) and a telomeric dsDNA oligonucleotide (18 bp, C-overhangs) by the hanging-drop vapour difussion method at 20 ºC (20-40% MPD as precipitant and 2mM spermine, 20mM KCl, pH=6.0). The crystals belong to the Trigonal space group $P3_1$ (a=b= 90.61 Å, c= 80.36 Å; $\alpha=\beta=$ 90º, $\gamma=$ 120º) and have two protein-DNA complexes in the asymmetric unit. A native dataset from a crystal diffracting beyond 2.25 Å resolution was collected (CuKα X-ray source, at 95 ºK). MIR-phases were obtained from four derivatives ($HgCl_2$, $K_3UO_2F_5$, Trimethyl-lead acetate and Hg-phosphoro-thioated DNA), with a combined figure of merit of 0.51. Phases were improved by two-fold averaging and solvent flattening (SOLOMON). A model for the telomeric DNA and most of the protein was traced using the molecular graphics program " O". Refinement was performed with X-PLOR, by means of several cycles of positional and restrained B-factor refinement, as well as Molecular Dynamics runs. 103 solvent molecules were identified in Fo-Fc maps and included in the final model. The final R-factor is 21.9% (free R-factor: 29.4%). According to PROCHECK, the protein model shows a rmsd from ideality of 0.009 Å (bond lengths) and 1.363º (bond angles), whereas the values for the DNA component are more relaxed, reflecting the conformational flexibility of DNA. Only residue (Glu416) is just outside of the allowed regions in the Ramachandran Φ / ψ plot.

The three-dimensional structure of the RAP1(DBD)-dsDNA complex and the features of protein-DNA recognition at yeast telomeres will be discussed in this meeting by D. Rhodes in an oral presentation.

Our crystal structure shows, for the first time, the details on how proteins pack and recognize the telomeric DNA repeats at the ends of eukaryotic chromosomes.

A NEW MONOCLONAL ANTIBODY THAT RECOGNIZES CONSERVED SPINDLE COMPONENTS

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Using protein extracts from embryos of *Parascaris* (Nematoda, Ascaridae) as immunogens we have produced a monoclonal antibody (mAb 403) that associates to spindle components. In different *Parascaris* cell types, by indirect immunofluorescence and Western blot analysis, mAb 403 decorates centrosomes, kinetochore-microtubules and centromeric regions and identifies a single protein of 70 kD. mAb 403 recognizes similar antigens in distantly related species as human cells, mouse, *Drosophila melanogaster* and *Caenorhabditis elegans*. A a lambda-ZAP cDNA library of *Parascaris* was constructed and immunoscreened using mAb as a probe. We have isolated a positive clone (403-I) that contains a 2.5 kb insert. We have established that this clone encodes to the authentic 403 antigen by producing policlonal antibodies against the bacterially expressed fusion protein. After sequencing and performing a structural analysis of the predicted protein, our data indicate that the 403 antigen is a coiled-coil myosin-like protein and may play an important role during cell division.

Title: Energetics of primer binding to telomerase from *E. aediculatus*.

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Telomerase is a ribonucleoenzyme which adds the G-rich sequence repeats to the end of linear chromosomes. The RNA moiety serves to align a DNA primer at the active site of the enzyme through base-pairing interactions and provides a template for addition of the repeat sequenc *In vitro*, telomerase functions processively to add multiple repeats to a DNA primer by binding it at second anchor site on the enzyme. We have substituted photo-reactive nucleotide analogs into DNA oligonucleotide primers and, upon irradiation, obtained cross-links to a protein subunit of the ancho site of telomerase from *Euplotes aediculatus*. Photoreactive nucleotides which form the cross-links are 20 and 22 nucleotides upstream of the 3' end of the primer. The 3' end of this primer was shov to be aligned on the RNA template in the active site of the enzyme by the ability of telomerase to incorporate [α-^{32}P]dGTP into the cross-linked primer. These cross-links were used to quantitate tl binding of primer to telomerase, from which we determined the equilibrium dissociation constant. Various oligonucleotide DNA:RNA duplexes were prepared which model the binding of DNA prin to the RNA template. The results of UV melting experiments with these oligonucleotide models w used to measure the contribution of DNA:RNA contacts to primer binding and to estimate, by subtraction, the contribution of protein interactions to the binding of primer to telomerase.

CENTROMERIC SATELLITE DNA IN FISH

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By means of cloning, sequencing, and fluorescence in situ hybridization, we have determined that the EcoRI satellite DNA family is conserved in the 10 sparid species analyzed here. Its conservation, its chromosomal location at the centromere of each chromosome, and its structural features could make this satellite DNA family an important structural and functional element of the centromeres of these species. Monomeric units of this satellite DNA have a consensus length of 187 bp. Its sequence is characterized by a high AT content and the presence of short runs of consecutive AT base pairs.

These monomeric EcoRI repeats also contain three to four copies, depending on the species, of a short sequence reflecting the repetitive duplication and subsequent divergence of an ancestral 9-bp sequence in this family. This sequence motive is conserved in some parts of the monomeric units of the different species studied at the same positions, and, precisely, surrounding the area in which the curvature of the monomeric molecule is greatest. The 9-bp motive is similar to other direct repeat sequences of the centromeric satellite DNAs of other vertebrates, including those of amphibians and mammals.

ANALYSIS OF THE CENTROMERE OF CHROMOSOME TWO OF THE FISSION YEAST: *SCHIZOSACCHAROMYCES POMBE.*

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The centromeres of *S.pombe* contain large regions of repetitive DNA flanking a unique central core (cc) region. The centromeres constitute between 3-15% of each chromosome. *S.pombe* centromeres share some of the features of higher eukaryotic centromeres, however they are both simpler and smaller in size and organization, offering an attractive experimental system.

Position Effect Variegation has been reported at S.pombe centromeres, so we have attempted to characterize the chromatin of the centromere of chromosome two. A method has been developed to map the accessibility of restriction enzyme sites, and thus to assay the 'openness' of the chromatin throughout *CEN2*, this approach has also been used to analyze the effect of three mutations *swi6, clr4, rik1* on chromatin at *CEN2*.

We are also attempting to isolate and characterize the protein components of the *S.pombe* kinetochore. A novel chromatin purification strategy is being developed to accomplish this, and the results of on-going experiments will be discussed.

TRANSCRIPTIONAL ACTIVATION BY Rap1p: GENETIC AND STRUCTURAL STUDIES. Idrissi, F.-Z., Fernández-Larrea, J.B., and Piña, B. Centre d'Investigació i Desenvolupament, CSIC. Jordi Girona 18, 08034 Barcelona, Spain. E-mail: bpcbmc@cid.csic.es.

Rap1p, the yeast telomer binding protein, acts also as activator of many genes encoding ribosomal proteins and glycolytic enzymes, among others. We have investigated the activation potential of Rap1p using artificial enhancer elements based on Rap1p binding sites. UASrpg (5'-ACACCCATACATTT-3') and telomeric (5'-ACACCCACACACCC-3') sequences were cloned in different orientations in front of a minimal *CYC1* promoter. Our results show a clear transcriptional activation dependent on the presence of Rap1p binding sites, showing activation patterns consistent with the know properties of Rap1p-driven natural promoters. In our constructs, UASrpg sequences appear to be more efficient on promoting activation than telomere sequences; in both cases, a strong activation synergism between adjacent sequences is observed.

In vitro assays revealed that neither the observed synergism, nor the lower activation potential of telomere sequences can be explained by the relative affinity of Rap1p for the different constructs. Structural studies of Rap1p/UASrpg and Rap1p/telomeric complexes showed a somewhat different interaction of Rap1p with the two types of binding sites. Our results suggest that, whereas the observed activation synergism between adjacent Rap1p molecules is probably due to cooperative interaction with the target(s) for the transcriptional activation signal, differences in the activation potential among the different types of Rap1p-binding sequences may be related to the intimate structure of the Rap1p-DNA complex.

Two novel alpha satellite DNA blocks near the human chromosome 7 centromere. A. de la Puente1, E. Velasco2, C. Hernández-Chico2, J Cruces1. 1. Dep. de Bioquímica, Facultad de Medicina, Universidad Autónoma, Madrid, Spain. 2. Hospital Ramón y Cajal, Madrid, Spain.

Alpha satellite is the major family of tandemly repeated DNA found at or near the centromeres of primate chromosomes. Alpha satellite is based on a fundamental repeat unit of approximately 171 pb (called monomer), tandemly arranged in a head to tail fashion. These alpha satellite DNA generally display a higher-order organization based on multimers of the 171 pb monomer. Adjacent sequences at the edges of these repeated blocks of the alpha satellite DNA are not fully and their putative contributions to the function of the centromere is still know. We have identified a YAC from the CEPH MkI library which contain two novel blocks the alpha satellite DNA (which we have named Z5 and Z6) separated by 100 kb of non-alphoid sequence. Between the blocks of alphoid sequences there are MER 22 elements, which have been found before only on chromosomes 4 and 19, far from the centromeres, a CpG island, and a (CA)n repeat polymorphism. We have located this region close to the human chromosome 7 centromere by PCR of somatic cell hybrids and linkage analysis.. Z5 contains two inversions interrupting the usual head to tail arrangement of monomers, ending with a tetranuceotide repeat (GAAA)n. The other block, Z6, has an inserted Alu element. Z5 and Z6 do not show the higher-order repeat structure typical of tandemly repeated alpha satellite. We are trying to establish a physical map linking our sequences to the known chromosome 7 centromere repeats, Z1 and Z2. These novel alpha satellite DNA provide new tools to further study the genomic organization of chromosome 7 centromere.

CHROMATID COHESIVENESS AT THE CENTROMERE DURING THE SECOND MEIOTIC DIVISION

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Sister chromatids of mitotic chromosomes remain closely associated along their entire length until their disjunction at the onset of anaphase due to a chromatid cohesiveness acting at the centromere and chromosomes arms. During the first meiotic division the chromatid cohesiveness at chromosome arms is lost but that present at centromeres persists until the chromatid segregation at anaphase-II. In order to investigate this centromere function in detail we have first studied the centromere ultrastructure in serial sections of metaphase-II chromosomes. For this purpose we have chosen grasshopper chromosomes, a model system where we had previously detected by silver staining both sister kinetochores and a connecting strand joining them at the centromere. After conventional procedures for electron microscopy the 'ball and cup' kinetochores are observed as round masses of low density with associated kinetochoric microtubules. Interestingly, some low electron dense fibrillar material extending between both sister kinetochores is observed. This material, the connecting strand, and both kinetochores, are also revealed with different cytochemical procedures for electron microscopy. The EDTA regressive staining method and the Os-PPD technique both preferential for RNPs, as well as the silver staining technique, clearly indicate that there exists some kind of proteic material connecting both sister kinetochores. Additionally, by means of immunofluorescence and using the MPM-2 monoclonal antibody recognizing mitotic phosphoproteins we have detected the presence of a patch of phosphoproteins lying at the centromere inner region. In base to these findings we are proposing a model accounting for the chromatid cohesiveness at the centromere in metaphase-II chromosomes.

Distance-dependence of centromere/kinetochore assembly in dicentric X chromosomes and correlation with malsegregation in anaphase. BA Sullivan and HF Willard. Case Western Reserve University, Cleveland, OH.

Studies in many organisms have indicated that functional dicentrics are typically unstable, being lost due to chromosome bridging/lagging. The stability of dicentric human chromosomes has generally been attributed to inactivation of one of the two centromeres; such chromosomes, therefore, while structurally dicentric, are functionally monocentric. To examine the structure and behavior of active and inactive centromeres, we studied 8 dicentric X isochromosomes (isoX) using indirect immunofluorescence with antibodies against centromere proteins (CENPs) previously implicated in centromere function. In 3 of 7 i(Xq)s and 1 idic(Xp) studied, CENP-C and -E were detected at a single centromere only, consistent with the centromere inactivation hypothesis. In contrast, CENP-C and -E were detected at both centromeres in 4 isoXs, suggesting that such isoXs are functionally dicentric. Notably, the functional status of the two centromeres correlated with the distance between them. Thus, the 4 largest isoXs, with ~16-80 Mb between the centromeres, were functionally monocentric and appeared stable mitotically. However, 4 smaller i(Xq)s, with 8-12 Mb between the centromeres, were functionally dicentric with two CENP-C and -E signals; further, these cases were associated with the greatest degree of mosaicism.

To determine directly if functional dicentrics show an increased frequency of abnormal segregation, we developed an assay to enrich for anaphase/telophase cells after treatment with nocodazole and dihydrocytochalasin B to prevent cytokinesis. Thus, the daughter sets of chromosomes can be visualized directly on the mitotic spindle and segregation of individual chromosomes scored after in situ hybridization. Using this assay, segregation of the normal X, the i(Xq), and a control autosome was scored in >100 anaphase/telophase cells for each cell line. While 3 functionally monocentric i(Xq)s (as well as control chromosomes) segregated normally in >99% of cells, a functionally dicentric i(Xq) showed abnormal segregation (chromosome lag/loss) in ~25% of anaphases scored.

These data (i) demonstrate directly that chromosomes with two functional centromeres encounter segregation difficulties in anaphase, (ii) suggest that mosaicism associated with many functionally monocentric i(Xq)s may reflect loss early in development rather than constitutive mitotic instability, and (iii) provide evidence for a distance-dependence of dicentric chromosome structure and centromere/ kinetochore assembly and behavior.

List of Invited Speakers

Workshop on

CHROMOSOME BEHAVIOUR: THE STRUCTURE AND FUNCTION OF TELOMERES AND CENTROMERES

List of Invited Speakers

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Workshop on

CHROMOSOME BEHAVIOUR: THE STRUCTURE AND FUNCTION OF TELOMERES AND CENTROMERES

List of Participants

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The lectures summarized in this publication were presented by their authors at a workshop held on the 23rd through the 25th of September 1996, at the Instituto Juan March.

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