New insights into the chromosome cycle

Conference on the Replication & Segregation of Chromosomes

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duplication, as the epigenetic patterns of histone modifications throughout the genome must also be copied faithfully. Moreover, chromosome replication is coupled to other processes such as the establishment of cohesion between the resultant sister chromatids, which facilitates mitosis and promotes the repair of DNA breaks by homologous recombination (Fig 1). Many of these processes have not yet been reconstituted using purified proteins, and much remains to be discovered. The EMBO meeting on the Replication & Segregation of Chromosomes in Geilo, Norway, brought together people working on these issues in diverse eukaryotic and prokaryotic species. Here, we provide an overview of some meeting highlights, but space constraints prevent us from covering all of the talks. We therefore apologize to those whose work we have not been able to discuss.

Origins and the initiation of DNA replication

Chromosome replication begins at sites called origins that are bound by initiator proteins, which allow recruitment of the replicative DNA helicase and that contribute to the initial unwinding of the origin. In *Escherichia coli*, the initiator protein DnaA forms multimeric complexes that bind to the origin and cause local unwinding within AT-rich sequences. T. Katayama (Fukuoka, Japan) described how the tetrameric DiaA protein binds to many DnaA molecules and stimulates their cooperative assembly onto origin DNA (Keyamura *et al*, 2007). A model for initiation was described in which multimeric DnaA forms a spiral with a central pore that accommodates origin DNA, and promotes DNA unwinding in the presence of ATP through the interaction of residues on the surface of the pore with single-stranded origin DNA within the AT-rich region (Ozaki *et al*, 2008).

Many bacteria have just one chromosome, but others such as *Vibrio cholerae* have two chromosomes. Previous studies have shown that DnaA regulates the origin on chromosome I; however, the situation for chromosome II is more complicated as it also requires another factor known as RctB. Y. Yamaichi (Boston, USA) showed that RctB binds to the origin of chromosome II and promotes local unwinding. Although it lacks conventional ATP-binding motifs, RctB is able to bind and hydrolyse ATP *in vitro*. Surprisingly, however, ATP was found to inhibit the binding of RctB to origin DNA, which is in contrast to the situation for DnaA (Duigou *et al*, 2008).

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Introduction

The mechanisms by which cells replicate and segregate their chromosomes have been the subject of intense study for several decades, yet many fundamental questions still remain unanswered. This is partly due to the complexity of these processes, which are regulated so that they are tightly coupled to cell growth and cell division, and have an extremely low error rate, thus allowing the stable inheritance of the genome. The elaborate nature of chromatin in eukaryotic cells further complicates the process of chromosome

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Fig 1 | The chromosome cycle in bacteria and eukaryotes. Replication of the circular chromosome of *Escherichia coli* starts at a single origin and the segregation of sister chromosomes takes place as replication proceeds. In eukaryotes, replication starts at many origins per linear chromosome and cohesin (orange rings) maintains sister chromatid attachment during the process. Chromosome segregation only occurs after cohesin is cleaved in anaphase.

In both prokaryotes and eukaryotes, the initiation of DNA replication must occur only once per cell cycle. The underlying mechanisms that have evolved to prevent re-initiation have been studied extensively in *E. coli* and its close relatives, but are less well known in other families of bacteria. P. Graumann (Freiburg, Germany) discussed the regulation of chromosome replication in *Bacillus subtilis*. Cytological studies have shown that the replication machinery seems to be localized to the centre of the cell during much of the cell cycle (Lemon & Grossman, 1998) and the same is true for DnaA, which is tethered to the machinery by the YabA protein. By contrast, origin DNA only associates with the replication machinery for a brief period, as chromosome replication is initiated, before moving away to other parts of the cell. It thus seems that the spatial sequestration of DnaA might limit initiation to one round per cell cycle in *B. subtilis*.

In eukaryotes, origins are bound by the origin recognition complex (ORC), which is required for recruitment of the MCM (mini-chromosome maintenance) helicase that subsequently unwinds the parental DNA duplex. Replication proteins in Archaea are similar to eukaryotic proteins, but are usually simpler and thus easier to study. D. Wigley (London, UK) described how the Orc1 protein of *Aeropyrum pernix* interacts with origin DNA. A 'winged-helix' domain (Fig 2; blue) at the carboxyl terminus of Orc1 is mainly responsible for DNA binding, and inserts into both major and minor grooves of the origin DNA, widening them and thus distorting the DNA and contributing to its unwinding. Interestingly, however, the amino-terminal 'AAA+' domain (Fig 2; purple) also inserts into the minor groove at a characteristic G-rich sequence within

the origin recognition box elements, bending the DNA and contributing to its melting. The interaction of the AAA+ domain with the G-rich sequence determines the directionality of binding, and thus ensures that Orc1 binds in the appropriate orientation to be able to promote the loading of the MCM helicase onto the 'DNA unwinding element' within the origin (Gaudier *et al*, 2007; Fig 2).

In addition to ORC, loading of the MCM helicase at origins of DNA replication in eukaryotic cells requires two other factors, CDC6 (cell division cycle 6) and CDT1 (chromatin licensing and DNA replication factor 1). In vertebrates, the activity of CDT1 is inhibited for much of the cell cycle through its binding to another factor, geminin, thereby ensuring that loading of the MCM helicase at origins occurs just once per cell cycle. Interestingly, geminin also has a separate function during development, probably acting as a regulator of transcription. As described by C. Gutierrez (Madrid, Spain), another regulator of CDT1 in the plant *Arabidopsis thaliana* might also have such a dual role: the homeobox transcription factor GL2 controls the determination of cell fate in the root epidermis. Surprisingly, overexpression of CDT1 was found to cause an increase in the level of GL2. To investigate how this might work, a two-hybrid screen was designed to search for partners of CDT1, which identified the protein GEM (GL2 expression modulator). GEM binds to a component of the GL2 transcriptional regulatory complex and CDT1 competes for this interaction (Caro *et al*, 2007). Therefore, it seems that GEM could be analogous to the vertebrate geminin protein, although the two proteins are not related in their amino-acid sequences; however, whether GEM actually regulates CDT1 during DNA replication remains to be shown (Caro & Gutierrez, 2007).

Once the MCM helicase has been loaded at the origins of DNA replication, initiation is triggered by the consecutive action of two kinases: cyclin-dependent kinase and CDC7 kinase. Previous work has indicated that MCM is an important target of CDC7 and, although the consequences of MCM phosphorylation remain to be determined, might include structural changes within the helicase complex or promotion of the recruitment of other factors. H. Masai (Tokyo, Japan) described a surprising observation about Hsk1, the orthologue of CDC7 in the fission yeast *Schizosaccharomyces pombe*. The *hsk1* gene is essential in fission yeast, as it is required to help activate the MCM helicase during the initiation of chromosome replication. A screen for mutations that suppressed the lethal effects of *hsk1Δ* identified mutations in another gene, *mrc1*, which encodes a factor recruited to the MCM helicase at DNA replication forks. Mrc1 is required for the activation of a 'checkpoint' response when problems occur during chromosome replication, and it has also been shown to be important for establishing the normal rate of progression of DNA replication forks in budding yeast. So far, it is unclear why the absence of Mrc1 might suppress the effects of Hsk1; however, one possibility suggested by Masai is that Mrc1 somehow inhibits initiation and Hsk1 antagonizes this effect. It will be interesting to see whether other kinases can fulfil the function of Hsk1 in the absence of Mrc1, or whether initiation under those conditions is truly independent of the phosphorylations that are normally catalysed by Hsk1.

Each eukaryotic chromosome is replicated from many different origins that are activated at various times during S phase of the cell cycle. This provides an insurance mechanism that helps to ensure that replication of the genome can still be completed even if there are problems at DNA replication forks from early origins. In higher eukaryotes, replication might actually occur at many sites within an

'initiation zone', rather than at one discrete site within a particular origin. When problems arise during replication—for example, in response to DNA damage—defects in DNA synthesis at the earliest origins are detected and lead to activation of checkpoint kinases that stabilize the stalled forks, delay mitosis and also block the firing of new origins. J. Blow (Dundee, UK) discussed the fact that many copies of the MCM helicase are normally loaded around origins, and these seem to provide another aspect of the failsafe mechanism when things go wrong at DNA replication forks. Normally, the first replication forks to be established within the initiation zone displace the excess MCM complexes, which represent 'dormant' sites where initiation could have occurred but usually does not. However, if damage causes the early forks to stall, then the dormant MCM is able to support the establishment of new forks, thus increasing the chances that replication will be completed once the damage has been dealt with. Curiously, it seems that dormant MCM within a particular initiation zone might not be subject to inhibition by the S-phase checkpoint, as the frequency of initiation events within an initiation zone actually increases when forks stall. By contrast, the checkpoint might inhibit the activation of new origin clusters in other initiation zones (Ge *et al*, 2007). It will be interesting to discover how this discrimination is achieved, and whether it involves changes in epigenetic modifications around active or inactive initiation zones. It will also be interesting to see whether the failsafe mechanism provided by the dormant MCM complexes around an initiation zone is defective in particular cancer cells, in which MCM loading might be less efficient than in normal cells.

The action and regulation of DNA replication forks

The structure of the *E. coli* replisome has been described, but the more complex eukaryotic replisome is still unknown. Even in bacteria, many questions remain to be answered about the action and regulation of the replisome. A. van Oijen (Boston, MA, USA) described a powerful technique whereby the action of single replication molecules can be visualized. This technique has been applied successfully to the study of T7 viral DNA replication, which only requires four proteins: a DNA helicase, a primase, a DNA polymerase with its processivity factor and a single-strand-binding protein. This work is starting to show how the progression of the fork is coordinated with DNA synthesis. The action of the primase on the lagging strand transiently inhibits the progression of the fork, thus preventing the leading strand from getting ahead (Lee *et al*, 2006). The underlying mechanism remains to be determined, but there are clearly many exciting questions that could be addressed in this way, and it will be interesting to see these studies extended to eukaryotic replisomes using, for example, extracts of *Xenopus* eggs, in which replication forks can be easily assembled *in vitro*.

The three-dimensional organization of the replication machinery throughout the cell or nucleus is poorly understood; however, in eukaryotes it is thought that replication occurs in 'factories' that contain many DNA replication forks. Furthermore, there is evidence to indicate that the action of the two replication forks generated by a single origin is coordinated. D. Sherratt (Oxford, UK), however, argued against the factory model in *E. coli*, in which sister replicons separate from each other after initiation, moving to opposite cell halves while replication proceeds, and returning to mid-cell as replication termination approaches. This suggests that, in *E. coli*, both replication forks are physically separated from each other and follow the path set by the compacted chromosome independently,

Fig 2 | DNA origin recognition and unwinding by an archaeal origin recognition complex. *Aeropyrum pernix* Orc1 proteins bound to the ORB elements would be oriented to place the 'winged helix' domain (blue), which interact with the MCM helicase, facing the duplex unwinding element (DUE) and hence the replication start site. The Orc1–ORB interaction bends DNA and induces its unwinding at the origin. Figure adapted, with permission, from Gaudier M, Schuwirth BS, Westcott SL, Wigley DB (2007) Structural basis of DNA replication origin recognition by an ORC protein. *Science* **317**: 1213–1216. Reprinted with permission from AAAS. ORB, origin recognition box; ORC, origin recognition complex; MCM, minichromosome maintenance.

with no other structure anchoring the replisome to any particular cellular region (Reyes-Lamothe *et al*, 2008).

The mechanisms by which eukaryotic cells preserve DNA replication forks in the presence of DNA damage and maintain the integrity of the genome are still poorly understood. J. Diffley (London, UK) described that, in budding yeast, the Exo1 nuclease is an important target of the S-phase checkpoint in response to DNA damage. In cells lacking the checkpoint, Exo1 contributes to the breakdown of damaged DNA replication forks, therefore leading to a loss of viability. This indicates that the checkpoint normally prevents dangerous factors such as Exo1 from gaining access to DNA replication forks (Segurado & Diffley, 2008).

U. Hubscher (Zurich, Switzerland) discussed how human cells protect DNA replication forks from the harmful effects of the damaged DNA adduct 8-oxo-guanine. The normal replicative DNA polymerases cannot replicate across such damaged DNA; however, cells have repair pathways that could remove the damage instead, as well as various 'bypass' DNA polymerases that are able to replicate across such lesions. Interestingly, the DNA replication factors PCNA (proliferating cell nuclear antigen) and replication protein A—the eukaryotic single-strand-binding protein—stimulate the ability of the bypass DNA polymerase- λ to insert dCTP, instead of dATP, opposite 8-oxo-G, which could potentially lead to a mutation at the damaged site (Maga *et al*, 2007). DNA polymerase- λ is stabilized during cell cycle progression in late S and G2 phase, suggesting that it repairs damaged DNA during and after S phase (Wimmer *et al*, 2008).

Chromosome segregation and cell division

The two sister chromatids generated by chromosome replication are held together until the anaphase stage of mitosis by a process called 'cohesion', which is mediated by a multiprotein complex, cohesin. Previous work has shown that cohesin forms large rings *in vitro*, and it has been suggested that cohesion could result from

individual cohesin rings being present around the two sister chromatids and thus embracing them until anaphase, when the cohesin ring is broken (Fig 1). However, this model has remained controversial, and other possibilities can be envisaged. A.M. Farcas (Oxford, UK) described elegant work performed in K. Nasmyth's laboratory that argues strongly in favour of the entrapment model. Coherent circular minichromosomes were purified from yeast cells expressing modified forms of cohesin that allowed the use of chemical crosslinkers to create covalently closed cohesin rings. By using this approach, dimeric minichromosome-cohesin structures were isolated and found to be resistant to protein denaturation. This indicates that the cohesin rings do indeed concatenate individual minichromosomes (Haering *et al*, 2008).

F. Uhlmann (London, UK) discussed how cohesion is established during S phase. A simple model is that the cohesin rings are loaded around each chromosome before chromosome replication has occurred, and that the cohesin rings are then somehow preserved around the chromosomes during the process of DNA replication, so that a coherent pair of sister chromatids are produced. It remains to be determined whether the replisome simply passes through cohesin rings when it encounters them, or whether there is transient opening and closing of the cohesin rings. In budding yeast, several factors have been found to be important specifically for the establishment of sister chromatid cohesion during S phase, one of which is an essential acetyl transferase known as Eco1. Uhlmann's group performed a screen for mutations that suppress the lethal effects of inactivating Eco1, which led to the identification of mutations at a specific residue of the cohesin subunit Smc3. This residue was found to be a site of acetylation by Eco1, and the suppressor mutations mimic acetylation. It seems that acetylation of Smc3 by Eco1 stabilizes the cohesin ring, perhaps helping to preserve its integrity around a chromosome when a DNA replication fork replicates that region (Ben-Shahar *et al*, 2008).

As cells enter mitosis, the two sister chromatids become attached at their kinetochores to microtubules from the mitotic spindle, so that they can be subsequently separated during anaphase. It is crucial that the two sister chromatids are separated to opposite poles of the cell, and this is achieved by ensuring that the kinetochores are 'bi-oriented'—that is, each is attached to microtubules from opposite poles of the spindle. The correct bi-orientation of kinetochores generates a tension that is thought to be detected and a necessary pre-condition for the entry into anaphase. T. Tanaka (Dundee, UK) discussed work in budding yeast that showed how cells are able to establish the bi-orientation of sister chromatids on the mitotic spindle. The kinetochores attach first to the side of a microtubule and are then transported towards one pole of the spindle by the kinesin Kar3. The kinetochores are subsequently tethered at the microtubule plus end and pulled poleward by shrinkage of the attached microtubule. It seems that the Dam1 kinetochore protein complex is essential to convert microtubule depolymerization into poleward movement of the kinetochore (Kitamura *et al*, 2007; Tanaka *et al*, 2007). Previous work has shown that the Aurora B protein kinase has an important role in generating the correct bi-orientation of kinetochores on the mitotic spindle. It now seems that the Mps1 kinase is also important in this regard, as it helps to eliminate any erroneous connections between kinetochores and microtubules that do not lead to the generation of tension (Maure *et al*, 2007).

Finally, M. Nollmann (Montpellier, France) discussed a different form of chromosome segregation, which is mediated by bacterial

DNA transporter proteins of the AAA+ family such as FtsK in *E. coli* and SpoIIIE in *B. subtilis*. These factors aid the completion of chromosome segregation during vegetative growth, and are transmembrane proteins that function after cytokinesis to pump double-strand DNA through a central channel. In *B. subtilis*, SpoIIIE is also required during sporulation to pump chromosomal DNA from the mother into the developing spore. Previous studies have shown that FtsK pumps DNA in a single direction by virtue of its interaction with skewed DNA sequences in the bacterial chromosome. By contrast, it was thought that the direction in which SpoIIIE transports DNA was established by the assembly of SpoIIIE on one particular side of the septum, rather than through interaction with specific sequences. However, recent work has shown that SpoIIIE can assemble on either side of the septum, but then a domain at the C terminus interacts with sequences in the chromosomal DNA and causes disassembly of SpoIIIE complexes on the spore side of the septum, so that net transport occurs from the mother into the spore (Ptacin *et al*, 2008).

Conclusions

Despite being two of the oldest topics in molecular biology, the replication and segregation of chromosomes are still not as well understood as other fundamental cellular processes such as transcription or translation. Cells have evolved many complex processes to preserve genome integrity, and DNA replication and segregation must be coordinated with DNA repair, checkpoint pathways, the establishment of cohesion and the maintenance of epigenetics. This EMBO conference on the Replication & Segregation of Chromosomes brought together scientists working on these issues in a range of prokaryotic and eukaryotic organisms. The research presented at the meeting was outstanding and triggered stimulating scientific discussions. As a result, the conference not only delivered many novel concepts and insights to the field, but also opened a large number of new and exciting questions to be challenged in the future.

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