Meeting report

Chromosome replication: from ORC to fork

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A report on the 2001 Eukaryotic DNA Replication meeting, Cold Spring Harbor Laboratory, New York, 5-9 September 2001.

The Cold Spring Harbor Laboratory holds a biennial meeting on eukaryotic DNA replication that provides a broad view of what is happening in this field. The scope of the latest meeting ranged from the mechanistics of replication fork proteins, through tying the cell cycle to replication, to viral replication strategies. Here we pick out some major themes of this exciting meeting, focusing mainly on work that has yet to be published.

Initiation complexes

Previous work has revealed that chromosomal DNA replication is controlled by the sequential assembly of 'pre-replicative complex' (pre-RC) proteins onto specialized DNA sequences, the replication origins, early in the cell cycle. One crucial step in this process is the loading of the minichromosome maintenance (Mcm) proteins Mcm2-Mcm7 onto DNA, which results in the origin becoming 'licensed' for only a single round of DNA replication. Loading of the proteins of the origin recognition complex (ORC) onto replication origins is the earliest known step in pre-RC assembly. Detailed characterization of ORC, which consists of six subunits, Orc1-Orc6, was reported by a number of groups.

Sanjay Vashee (Johns Hopkins University School of Medicine, Baltimore, USA) described the interactions between recombinant human ORC subunits, and proposed that Orc2, Orc3 and Orc4 form a core upon which Orc5 and then Orc1 can be assembled. The reported results suggested that only a minority of Orc6 molecules is associated with this Orc1-Orc5 complex. Consistent with this, Supriya Prasanth (Cold Spring Harbor Laboratory, New York, USA) reported distinct subcellular localization patterns for human Orc6 and Orc2 and Igor Chesnokov (University of California, Berkeley, USA) reported that less than 50% of *Drosophila* Orc6 associates with the other ORC subunits. In both studies, Orc6 was seen to localize to the mid-body, the cytoplasmic bridge between two daughter cells, by telophase. *Drosophila* Orc6 is nevertheless required for ORC to bind specifically to replication origins. Prasanth and Cong-Jun Li (National Institutes of Child Health and Human Development, Bethesda, USA) also showed cell-cycle-dependent disassembly of the ORC complex, suggesting that ORC is not simply a marker for replication origins but is likely to play a complex role in cell-cycle progression.

A number of groups reported the analysis of nucleotide requirements during pre-RC assembly. Several ORC subunits have 'Walker' ATPase motifs, and Chesnokov reported that mutations in the Walker ATPase motif of *Drosophila* Orc1 left the ORC complex unable to bind specifically to origin DNA. In the next step of pre-RC formation, ORC recruits the Cdc6 and Cdt1 proteins, which are themselves required for the assembly of Mcm2-Mcm7 onto DNA. Cdc6 also contains an ATPase motif, and recruitment of yeast Cdc6 is dependent on ATP or non-hydrolysable ATP analogs (Katie Hartland, Imperial Cancer Research Fund, London, UK). Tom Coleman (Fox Chase Cancer Center, Philadelphia, USA) showed that mutations in *Xenopus* Cdc6 that would be expected to block ATP binding also prevented recruitment of Cdc6 to ORC, whereas mutations predicted to allow ATP binding but block ATPase activity prevented loading of Mcm2-Mcm7 but nevertheless allowed recruitment of Cdc6 to the origin. We (J.J.B.) described the reconstitution of loading of Mcm2-Mcm7 using purified proteins (including ORC proteins, Cdt1 and Cdc6), and described nucleotide requirements for this reconstituted assembly reaction that were consistent with the results of Hartland and Coleman. The DNA requirements for Mcm2-Mcm7 loading in *Xenopus* extracts were reported by Johannes Walter (Harvard

Medical School, Boston, USA). He used a system in which DNA is coupled to magnetic beads, and found that ORC, Mcm2-Mcm7 and an additional initiation factor Cdc45 could be loaded onto an 85 bp fragment but not a 71 bp fragment. Increasing the length of the DNA fragments gave no increase in ORC loading, but resulted in increasing amounts of Mcm2-Mcm7.

The Mcm2-Mcm7 proteins are thought to form a DNA helicase that unwinds the DNA ahead of the replication fork. All six Mcm2-Mcm7 proteins have Walker ATPase motifs. Anthony Schwacha (Massachussetts Institute of Technology, Cambridge, USA) described the effects of mutating the Walker A boxes of the budding yeast Mcm2-Mcm7 proteins. Mutation of any one of the six subunits resulted in a dominant-negative phenotype (when overexpressed *in vivo*) and a loss of the complex's ATPase activity (*in vitro*). Analysis of hexamers containing two or more mutant subunits showed that certain combinations restored ATPase activity. Schwacha proposed that Mcm4, Mcm6 and Mcm7 contribute the majority of the ATPase activity, and that the ATPase domains of Mcm2, Mcm3 and Mcm5 fulfil a regulatory function, akin to the ATPase subunits of the F1 proton-pumping ATPase.

Viral model systems have played an important part in the study of eukaryotic DNA replication. In the latent phase, during which it replicates precisely once per cell cycle, Epstein-Barr Virus (EBV) is thought to use the cellular replication machinery. Paolo Norio (Albert Einstein College of Medicine, Bronx, USA) labeled replicating EBV with sequential pulses of different nucleotide analogs and spread the DNA on slides to analyze the resulting replication patterns. This technique identified a large initiation zone with multiple initiation sites, more than one of which may fire on an episome in a single round of replication - an activation pattern reminiscent of initiation zones found at certain chromosomal replication origins. John Yates (Roswell Park Cancer Institute, Buffalo, USA) and Aloys Schepers (Institute of Clinical Molecular Biology and Tumor Genetics, Munich, Germany) presented chromatin immunoprecipitation data showing an association between the viral replication origin (*oriP*) and the cellular ORC and Mcm2-Mcm7 complexes. As on chromosomal DNA, Mcm2 was found to associate with *oriP* during G1 phase and dissociate during S phase of the cell cycle, whereas ORC remained associated throughout the cell cycle; both associations were dependent on the viral Epstein-Barr nuclear antigen (EBNA1) protein. Yates identified an EBNA1 footprint, indicating binding of the protein to DNA at *oriP* (present throughout the cell cycle), but could not detect an ORC footprint. He suggested that ORC may not bind directly to *oriP* DNA, but may bind via EBNA1 or else bind non-uniformly to the origin. Further study of the EBV machinery may well shed light on the way that ORC recognizes chromosomal replication origins.

Silencing and timing

Philippe Pasero (Institut de Génétique Moléculaire, Montpellier, France) described the use of DNA spreading to map replication initiation sites within yeast ribosomal DNA (rDNA). This analysis revealed that in rDNA, active origins tend to be clustered in adjacent repeats, separated by large gaps containing inactive origins. Deletion of the *SIR2* histone deacetylase gene disrupted this clustering of initiation sites. A further link between chromatin structure and replication organization was provided by Liudmilla Rubbi (University of California, Los Angeles, USA), who reported that deletion of the histone deacetylase *RPD3* gene caused late origins located far from the telomeres (internal origins) to fire earlier during S phase, while subtelomeric origins still fired late. We (A.D.D.) presented data showing that deletion of the Ku telomeric chromatin components caused the converse effect, advancing the activation time of telomere-associated origins without affecting internal late origins. The time during S phase at which origins fire was also discussed by Marija Vujcic (Roswell Park Cancer Institute, Buffalo, USA), who tested the effects of chromosome context on origin activation and timing in yeast by transplanting late-firing origins from the *HML* locus (ARS320 and ARS303) to the position of the early-firing origin ARS305. Transplanted ARS320 fired early in S phase, while transplanted ARS303 still fired late. Moving both origins to the location of ARS305 resulted in a late activation time, suggesting that the ARS303 sequence contains a dominant late determinant.

Coordination and regulation

In order to prevent re-replication of DNA in a single cell cycle it is important that loading of Mcm2-Mcm7 (origin licensing) occurs only in late mitosis and G1 phase. These are the cell-cycle stages when cyclin-dependent kinase (CDK) activity is low, and previous work has shown that CDKs inhibit a number of different pre-RC proteins. Ron Laskey (Cambridge University, UK) showed that although the cyclin A can block loading of Mcm2 onto DNA, cyclin E stimulates Mcm2 loading in nuclei of cells exiting from G0 phase. Cyclin A appeared necessary for a subsequent step, possibly the initiation of replication from already licensed origins. As cyclin E plays an essential role in phosphorylation and inactivation of the retinoblastoma (Rb) transcriptional repressor, Rb might mediate the role of cyclin E in promoting origin licensing. Novel roles for Rb during S phase were presented by Dror Avni (Harvard Medical School, Boston, USA), who showed that unlike wild-type cells, $Rb^{-/-}$ cells subjected to γ irradiation underwent re-replication of their DNA. Dephosphorylation of Rb (following S-phase DNA damage) permits its recruitment to origins, where it may play a role in preventing re-replication.

The importance of stalled replication forks in inducing cell-cycle arrest was described by Matthew Michael (Harvard University, Cambridge, USA) in *Xenopus* extracts and by

Maria Marchetti (Roswell Park Cancer Research Institute, Buffalo, USA) in fission yeast. Michael reported that DNA damage induces stalling of replication forks, which in turn delays mitosis by generating a signal that depends on the ATM and Rad3-related (ATR) kinase. Interestingly, both fork stalling and mitotic delay can be relieved by the checkpoint-inhibitor caffeine. Marchetti suggested that the initiation factor Rad4/Cut5 may cooperate with the replication machinery to generate the checkpoint signal when the replication fork encounters DNA damage, and that this is the main mechanism for activating the intra-S-phase checkpoint, rather than by the direct recognition of damaged DNA. A further potential consequence of fork stalling was discussed by Rita Cha (Harvard University, Cambridge, USA), who reported studies on a yeast *mec1* mutant that permanently arrests during S phase with fragmented chromosomes. (Mec1 is a homolog of ATR.) Fragmentation occurred in regions between active replication origins, termed break zones. Moving or deleting origins did not affect the position of break zones, suggesting that they are independently defined genetic elements that may correlate with replication-pause sites.

Although substantial progress was reported at this meeting, several talks emphasized that substantial gaps remain in our understanding of the replication-initiation process. This fact was highlighted by the presentations of Chun Liang (Hong Kong University of Science and Technology, Hong Kong) and Irene Cheng (Cornell University, Ithaca, USA), both of whom reported yeast screens that have implicated a number of new genes in chromosome replication. Finally, Sahba Tabrizifard (University of Medicine and Dentistry of New Jersey, Newark, USA) raised the intriguing possibility of replication mechanisms that may even be independent of origins, with her report of dominant yeast mutants specifically unable to maintain an otherwise stable originless chromosome fragment. Despite the immense progress in the field over the last few years, it appears that there is much still to be done.

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