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Beyond the bounds of evolution: Synthetic chromosomes... How and what for?

Au-delà des limites de l'évolution : les chromosomes synthétiques... Comment et pourquoi faire ?

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ABSTRACT

Chromosome synthesis is still at its early stage. The budding yeast *Saccharomyces cerevisiae* is an organism of choice with respect to this field, thanks to its efficient homologous recombination pathway. By iteratively concatenating short DNA molecules to ultimately generate large sequences of megabase size, these approaches allow piecing together multiple genes and genetic elements in a way designed by an individual prior to their assembly. They therefore hold important promises as a tool to design complex genetic systems or assemble new genetic pathways that allow addressing fundamental and applied questions. The constant drop in DNA synthesis costs, fed by the development of new technologies, opens new perspectives with respect to the conceptual way these questions can be addressed. Thanks to its properties, *S. cerevisiae* may provide solutions for chromosome synthesis in other organisms, in combination with genome editing techniques.

Mots clés :
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RÉSUMÉ

Les chromosomes synthétiques sont une technique récente. La levure de boulangerie *Saccharomyces cerevisiae* est un organisme de choix dans ce domaine, notamment grâce à une voie de recombinaison homologue très efficace. Par concaténation itérative de molécules d'ADN courte, ces approches permettent de générer finalement de grandes séquences de plus d'un million de bases. Les gènes et éléments génétiques présents sur ces molécules sont définis par un individu préalablement à leur assemblage. Elles représentent donc d'importantes promesses comme outils pour concevoir des systèmes génétiques complexes ou assembler de nouvelles voies génétiques qui permettent de répondre à des questions fondamentales et appliquées. La baisse constante des coûts de synthèse d'ADN, alimentée par le développement de nouvelles technologies, ouvre de nouvelles perspectives par rapport à la manière conceptuelle dont ces questions peuvent être abordées. Grace à ses propriétés, *S. cerevisiae* pourrait vraisemblablement représenter une solution à la synthèse de chromosomes dans d'autres d'espèces, notamment en combinaison avec des techniques d'édition de génome.

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1. Introduction

In 1865, Mendel was enunciating the famous laws that rooted the chromosomal heredity theory 50 years or so later. Since then, DNA studies have remained more or less constrained, qualitatively, to the genomes found in the species analyzed. One hundred and fifty years later, in March 2014, new organizations across the world reported in their scientific section the complete synthesis of the first eukaryotic chromosome: baker yeast *Saccharomyces cerevisiae* chromosome 3 [1]. This symbolic and visible step occurred in the continuity of other pioneering works leading to the synthetic reproduction of viral and bacterial genomes, including the 7.5 kb poliovirus [2] and the 1.1 Mb *Mycoplasma genitalium* genome [3,4], paving the way to a new era of chromosomal research. This chromosome represented approximately 2.2% of the total 13.4 million base pairs of DNA encompassed within the 16 chromosomes of this species, but this achievement nevertheless opened important technical and scientific perspectives. Notably, it demonstrated the feasibility and accessibility of these approaches, highlighting their potential and possibly inciting more scientists to exploit them, while they may have appeared utopic to researchers, if not to the public, only a few years ago.

Chromosome synthesis can be included into the larger genome engineering denomination, along with genome editing techniques such as CRISPR/cas9 [5,6] or oligo-mediated approaches [7–9]. These technology-based projects sometimes raise considerable and understandable concerns, all the more reinforced by their occasional association with eugenic threats or to the word "creation", whose religious connotations easily entertain fears and worries. So far, these techniques have either facilitated the ability to introduce discrete modifications in DNA molecules not easily accessible before (such as the human genome), or allowed one to scale up the amount of changes introduced at once in a genome with respect to classical molecular biology techniques used for decades. But these achievements have called for reinforced collaborations between humanities and experimental sciences, so that philosophers, sociologists and jurists work together with biologists to identify and research the impact of future application of these techniques. A pioneering example of such collaborations also comes from the Sc2.0 project, which aims at reassembling the entire yeast genome through the efforts of an international consortium of scientists that have all agreed to a Statement of Ethics and Governance [10]. These social investigations are encouraged and funded by research agencies and institutions around the world, providing expertise and ethical recommendations to policy makers and regulatory bodies [11].

2. Yeast as experimental model

It is not surprising that the first entirely synthetic eukaryotic chromosome belonged to the yeast *S. cerevisiae*. This single cell organism, which has accompanied men for thousands of years thanks to its usage in bread making and fermentation processes, proved well suited to the molecular and formal genetics studies during the 20th century. In addition, the important gene density of its genome put it at the forefront of the genomics field early on. In 1989, a European consortium initiated the sequencing of chromosome III, completed in 1992 [12]. The entire genome was released in 1996 [13], making yeast the first sequenced eukaryotic organism and paving the way to genomic studies [14]. Twenty years later, taking advantage of yeast as a model organism with respect to eukaryotic chromosome synthesis appeared again particularly appealing: indeed, besides their density in genes, yeast chromosomes are also particularly prone to undergo homologous recombination. Thanks to the development of adequate techniques [15], this property allows researchers to replace the native DNA molecule with DNA molecules synthetized *in vitro* over dozens of kb in a single step (Fig. 1). The native chromosome sequence can then be iteratively replaced by a "synthetic" counterpart. For instance, the Sc2 project tackled first the right arm of chromosome 9 [16], then the entire chromosome 3 [1]. While it took approximately six years for the first full chromosome to be synthetized, other chromosomes were expected to be completed at a faster pace, reflecting the experience obtained with the chromosome 3 assembly project and also resulting from alternative, faster strategies. Then, they will be combined together into the same yeast strain, with the ultimate objective to reconstitute a fully synthetic eukaryotic genome. Besides replacing the native chromosome, assembly in yeast also allows the generation of new chromosomes carrying DNA sequences from other species, reminiscent of yeast artificial chromosomes (YAC [17]) that proved very convenient to generate genetic maps of the human genome more than two decades ago [18,19]. The publication of the synthetic chromosome 3 of *S. cerevisiae* by the Sc2.0 consortium represented a landmark towards the full replacement of the yeast genome by a synthetic counterpart. This international consortium, coordinated by Jef Boeke (New York University), is somehow organized similarly to the original sequencing project, with chromosomes projects being distributed to institutions over the world. This organization allows exploring different strategies as well as promoting the approach over the world, with for instance some partners developing teaching classes around the project and involving students on the synthesis/assembly aspects, while others purchasing directly DNA from companies. Besides NYU and Johns Hopkins University in the USA, research institutes in China (Beijing Genomic Institute, Tianjin University) or in Europe (University of Edinburgh, Imperial College London) all contribute to this effort (full list on http://syntheticyeast.org/collaborators/).

3. Examples of applications

The ability to assemble synthetic chromosomes in yeast considerably enriches the already vast catalog of experimental techniques and applications available in this species. Indeed, the variety of "designs", *i.e.* the genetic composition of the DNA molecules introduced into the genome, appears only limited by one's imagination and questions. Modifications can be introduced in the genome,

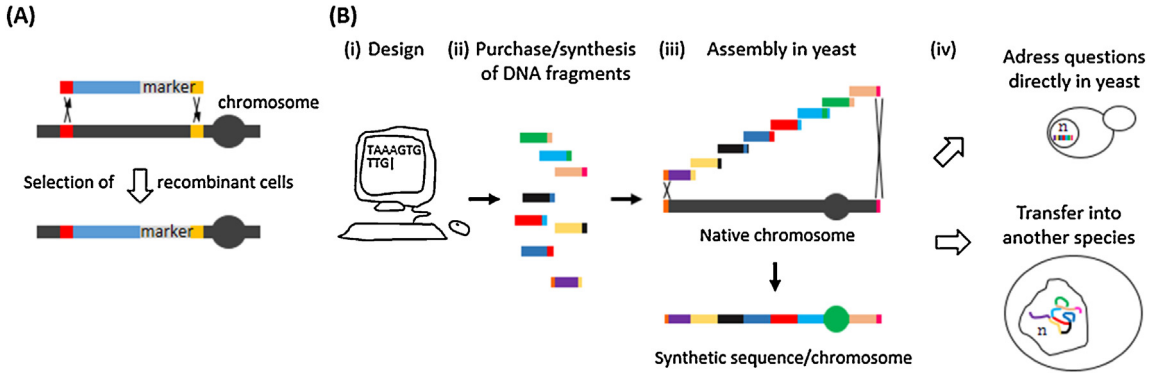

Fig. 1. Principles of chromosome assembly in budding yeast. (**A**) Schematic representation of homologous recombination event between two pairs of identical sequences (red and green squares) present on an extrachromosomal molecule, introduced into the nucleus through transformation, and on the chromosome. The DNA material (in blue) flanked by these sequences on the extrachromosomal molecule will be integrated within the chromosome, replacing the DNA molecule flanked by the similar identical regions. Selection of the event is insured thanks to the marker gene (in grey) transferred into the chromosome during the recombination event as well. (**B**) Illustration of large DNA synthesis through homologous recombination (in yeast). (i) The sequence is designed on a computer, and (ii) fragments of DNA corresponding to this sequence are purchased or synthetized *de novo* using oligonucleotides. The sizes of these fragments can vary, but range within a few kb long. Fragments carry overlapping identical extremities that allow homologous recombination to concatenate them once transformed (iii) in the yeast nucleus. The sequence introduced between the two flanking extremities (in pink, which have counterparts along the yeast chromosome) can correspond to yeast DNA, but also to other species. Several fragments are introduced at once (for instance, ten). Markers appropriately positioned allows selecting for the recombinant cells, and iterative transformation can lead to full replacement or assembly of Mb size regions. To verify that the native sequence is replaced by the synthetic blocks, a PCR-Tags strategy can be used similar to the one used in the Sc2.0 design [1,16]. These short sequences are specific to either the native or synthetic sequence: performing PCR using the corresponding primers allows testing for the presence and absence of the synthetic and natural sequence, respectively. Depending on the number and sizes of blocks transformed into the yeast, the efficiency of the transformation will vary, but typically screening of a few dozen clones leads to a handful of good candidates having integrated all the blocks. The synthetic region can therefore be gradually assembled, eventually replacing the native chromosome. (iv) Upon completion, the synthetic chromosome can either be exploited in yeast, to address specific questions of interest, or the synthetic region can also be isolated and transferred into the genome of another organism (n: nucleus).

so that the slightly (or more heavily) modified natural sequence can for instance be exploited to investigate basic biological processes. With the constant drop in DNA synthesis costs (announcements of future objectives down to $ 0.03/pb were recently made [20], though current prices start at ~$0.15 for kb-size molecules, but can vary strongly with the length and complexity of the sequence), the development and democratization of synthetic chromosomes assembly paves the way to the generation of complex experimental genetic systems larger than the one allowed by current techniques. Besides the cost, the time necessary to transfer DNA molecules into a yeast genome remains reasonable, with 30 kb or more being routinely transferred at each transformation step. The major time constraint remains the validation of the transfer, and most importantly the debugging step, *i.e.* identifying and solving unexpected problems resulting from the DNA sequence inserted into the genome.

Nevertheless, the approach is likely to allow testing new hypothesis at unprecedented scales, resolution, or complexity, leading to fundamental discoveries. For instance, the Sc2.0 chromosome design leaves the protein-coding sequences unaffected, resulting in a genome sequence differing essentially from the native one for its lack of repeated elements [1,16]. These repeats, including tRNA (that will be ultimately grouped on a supernumerary chromosome in the final strain), transposable elements, and rDNA cluster, have been shown to influence chromosome stability as well as driving, to some extent, genome spatial organization. The consequences of their absence can for instance be studied from these perspectives. The work on the synthetic chromosome III showed that no major differences could be detected regarding replication timing, *i.e.* that despite three replication origins were removed, the chromosome duplicate in a timely manner, similar to the wild-type case. Also, tRNA genes correlate with cohesin enrichment sites [21], and therefore their absence could potentially have a deleterious effect on chromosome stability. However, patterns of cohesin enrichment were not significantly altered in the synthetic chromosome 3. Overall, this first synthetic chromosome, which presents a conservative design, behaved essentially as a normal, wild-type chromosome [1]. The analysis of the 3D organization of these chromosomes is ongoing (RK, unpublished), which will put a specific focus on the importance of the missing elements on these characteristics. In addition to these modifications, an inducible site-specific recombination system was introduced in the synthetic region. This system (called SCRaMbLE for synthetic chromosome rearrangement and modification by lox-P mediated evolution), consists in adding short (~35 bp) symmetrical loxP sequences sites at multiple intergenic positions [16]. In the presence of the CreI recombinase protein, two loxP sites colliding with each other will recombine in either orientation. If these sites are not in allelic positions, this will result in the generation of a broad variety of chromosomal rearrangements including duplications, deletions, and translocation, leading to the spontaneous generation of a broad range of genomic structures. This inducible assay aims at increasing genome plasticity to perform a deeper exploration of the space of the genome structures, consequently increasing the phenotypic diversity. An in-depth analysis of 64 strains recovered from the SCRaMbLE induction of 43 loxP sites

positioned along the right arm of the synthetic chromosome IX showed that, in addition to the relatively simple rearrangements mentioned above, complex structures could be observed [22]. Interestingly, the frequencies of recombination events between distant loxP sites followed the collision frequencies expected from DNA looping theoretical models. The exploration of combinatory chromosome rearrangement events is considerably accelerated and enriched in this system. These pioneering studies, performed with relatively small synthetic regions, show that the SCRaMbLE system performed in strains carrying multiple synthetic chromosomes will undoubtedly broaden the diversity and complexity of the genome structures recovered. Such approaches may be especially valuable regarding targeted evolution experiments aiming at adapting the metabolism to growth conditions of interest. Another interesting perspective is to investigate which genes remain systematically present together in the genome after a SCRaMbLE period: performed over hundreds of clones, one may expect to identify new players in metabolic pathways of interest. In addition, the dynamics of the genome structures under and after selection can be studied, providing for instance insights on the constraints influencing their formation and maintenance over time. Following such strains over time would inform about the adaptation of genome structures to such constraints, providing guidelines for *de novo* design of chromosomes in the future.

Besides such relatively conservative designs resulting in a genetic content marginally modified, large-scale DNA assembly/synthesis is and will increasingly be used to transfer entire metabolic genetic pathways from one or several species into a recipient genome of another species. Such approaches hold promising perspectives, not only with regards to economic interests, since they may be exploited to synthetize chemical compounds at reduced costs, but also because of their innovative potential when it comes to conceive and develop unexpected solutions to environmental or health issues. Recently, *S. cerevisiae* was modified to synthesize opioids, cannabinoids, and other molecules of pharmaceutical interest [23,24]. It must be noted that the genes and metabolic pathways modified and/or transferred so far always correspond to sequences present in other species that are reassembled into yeast. Indeed, although the knowledge about the function and regulation of genes in multiple species is constantly increasing, it remains excessively challenging to design *de novo* a functional gene without copying a sequence that preexists in nature.

4. Future developments

A promising perspective regarding chromosome assembly approaches, which aims at synthetizing large DNA molecules, is to combine them with genome-editing techniques. The combination of both technologies will undeniably provide a considerable boost to chromosomal engineering in a variety of organisms. Budding yeast is likely to provide a convenient solution to such projects. Indeed, it is already possible to assemble efficiently and accurately large pieces of DNA in *S. cerevisiae* and to subsequently isolate and transfer them to other species. Such approaches have successfully been done with bacterial chromosomes, which were transferred from budding yeast into a bacterial host cell where they replaced the original genome [25–27]. In the continuity of such experiments done on unicellular organisms, one can now envision to assemble *de novo* large DNA molecules in yeast, that will be excised and targeted into the genomes of other eukaryotic or prokaryotic species. Obviously, these approaches will not come easily, and will require an important work before being implemented, but they may lead to the generation of *de novo* synthetic chromosomes. However, the potential gain regarding either fundamental discoveries, biosynthesis or biomedical applications suggests they will be investigated in the near future. For instance, *de novo* synthesis of large regions of mammalian genomes could allow developing further human artificial chromosomes (HAC) and new mammalian cell lines, as resources for biomedical research. Strong measures insuring a containment of the synthetic sequences will have to be enforced, as well as regulatory rules and ethic supervision regarding their assembly and usage. The Sc2.0 project, which focuses on the less sensitive yeast genome but which has addressed and has taken into account many of these important issues, will stand as an important landmark in this nascent field.

Disclosure of interest

The author declares that he has no competing interest.

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