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Total Synthesis of a Functional Designer Eukaryotic Chromosome

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Abstract

Rapid advances in DNA synthesis techniques have made it possible to engineer viruses, biochemical pathways and assemble bacterial genomes. Here, we report the synthesis of a functional 272,871 bp designer eukaryotic chromosome, *synIII*, which is based on the 316,617 bp native *Saccharomyces cerevisiae* chromosome *III*. Changes to *synIII* include TAG/TAA stop-codon replacements, deletion of subtelomeric regions, introns, tRNAs, transposons and silent mating loci as well as insertion of loxPsym sites to enable genome scrambling. *SynIII* is functional in *S. cerevisiae*. Scrambling of the chromosome in a heterozygous diploid reveals a large increase in “a mater” derivatives resulting from loss of the *MAT α* allele on *synIII*. The total synthesis of *synIII* represents the first complete design and synthesis of a eukaryotic chromosome, establishing *S. cerevisiae* as the basis for designer eukaryotic genome biology.

Saccharomyces cerevisiae has a genome size of ~12 megabases (MBs) distributed among 16 chromosomes. The entire genome encodes ~6000 genes of which ~5000 are individually nonessential (1). Which of these non-essential genes are simultaneously dispensable? While a number of studies have successfully mapped pairwise “synthetic lethal” interactions between gene knockouts, those methods do not scale well to 3 or more gene combinations because the number of combinations rises exponentially. Our approach to address this question is to produce a synthetic yeast genome with all nonessential genes flanked by loxPsym sites to enable inducible evolution and genome reduction (a process referred to as SCRaMBLEing) *in vivo* (2, 3). The availability of a fully synthetic *S. cerevisiae* genome will allow direct testing of evolutionary questions such as “what is the maximum number of

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We dedicate this publication to the memory of Har Gobind Khorana, who synthesized the first yeast tRNA gene.

Author Contributions

N.A., H.M., J.S.B., J.D.B. and S.C. designed experiments. J.D.B. and S.M.R. designed *synIII*. N.A., H.M., L.A.M., S.R., G.S., S.M.R., J.S.D., Z.K., Y.C., Z.G., V.L., S.M., K.K., N.A., G.F. and S.C. performed experiments. N.A., H.M., G.S., R.K., J.D.B. and S.C. analyzed data. N.A., H.M., J.D.B. and S.C. wrote the manuscript. JHU Build-A-Genome course students (author names denoted with*) synthesized most of the building blocks (BBs) for *synIII*; H.M., G.S., S.M.R., J.S.D., L.Z.S., E.M.C., Y.C., K.Z., J.S.H., M.H., J.T. and J.D.B. taught the Build-A-Genome course. S.C. led the effort on the construction and assembly of *synIII*.

The *synIII* sequences have been deposited at GenBank with accession numbers KJ463385 (the “as designed” reference sequence version 3.3_41) and KC880027 (the actual physical sequence in strain HMSY011, sequence version 3.3_42).

The authors declare no competing financial interests.

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nonessential genes that can be deleted without a catastrophic loss of fitness?” and “what is the catalog of viable 3-gene, 4-gene, ... n-gene deletions that survive under a given growth condition?” that are not otherwise easily approachable in a systematic unbiased fashion. Engineering and synthesis of viral and bacterial genomes have been reported in the literature (4–11). An international group of scientists, has embarked on constructing a designer eukaryotic genome, Sc2.0 (www.syntheticyeast.org), and here we report the total synthesis of the first complete designer yeast chromosome.

Yeast chromosome *III*, the third smallest in *S. cerevisiae* (316,617 bp), containing the *MAT* locus determining mating type, was the first chromosome sequenced (12). We designed *synIII* according to fitness, genome stability and genetic flexibility principles developed for the Sc2.0 genome (2). The native sequence was edited *in silico* using a series of deletion, insertion, and base substitution changes to produce the desired “designer” sequence (Figures 1, S1, S2 and Supplementary Text). The hierarchical wet-lab workflow used to construct *synIII* (Fig. 2) consisted of three major steps: 1) The 750 bp “building blocks” (BBs) were produced starting from overlapping 60- to 79-mer oligonucleotides and assembled using standard PCR methods (13, 14) by undergraduate students in the Build-A-Genome class at Johns Hopkins University (Fig. 2A) (15). The arbitrary naming scheme for the different sized DNA molecules used in the Sc2.0 project is explained in Fig. S3. 2) The 133 *synIIIL* (+ centromere) BBs and 234 *synIIIR* BBs were assembled into 44 and 83 overlapping DNA “minichunks” of ~2–4 kb respectively (Table S1, Figures 2B and S4) (16, 17). 3) All adjacent minichunks for *synIII* were designed to overlap one another by one BB to facilitate further assembly *in vivo* by homologous recombination in yeast (18, 19). Using an average of 12 minichunks and alternating selectable markers in each experiment, the native sequence of *S. cerevisiae III* was systematically replaced by its *synIII* counterpart in eleven successive rounds of transformation (Fig. 2C; Table S2) (20, 21).

PCRTag analysis (2) revealed the presence of *synIII* synthetic PCRTags and absence of native PCRTags (Fig. 3A; see Supplementary Text, Figures S5, S6 & S7 for complete set of PCRTag analyses). The smaller size of *synIII* and intermediates in its full synthesis as compared to the native yeast chromosome was demonstrated by pulsed-field gel electrophoresis (Figures 3B and S8) (22). Analysis of the intermediate strains revealed that the starting strain had some unexpected rearrangements in at least two chromosomes and that an additional rearrangement occurred during the assembly process; these did not affect *synIII* (Fig. S8). These abnormalities were eliminated through back-crossing the *synIIIL* intermediate strain to strain BY4742 (Table S3), yielding a *MAT α* strain with an electrophoretic karyotype perfectly matching BY4742 but for the expected altered length *III* (compare lane 97 to 97* in Fig. S8). Southern blot analyses using arm-specific radiolabeled probes further verified and validated the structure of the left and right arm telomere ends of *synIII*, which had been specified by the UTC sequence (Fig. S9). Restriction fragment sizes on Southern blots are compatible with the deletion of *HML*, *HMR*, and much of each subtelomere (Fig. S9). This was further confirmed by complete genome sequencing of the *synIII* strain.

DNA sequencing of the *synIII* strain genome revealed sequence differences at ten sites in *synIII* compared to our designed sequence (Table S4). Nine of the changes are base

substitutions or one bp indels. Three of the nine mutations correspond to pre-existing but apparently innocuous mutations in the minichunks and BBs. Of the remainder, two correspond to the wild-type base at this position, and thus may simply reflect inheritance of wild-type sequence. Since PCRTag analysis (Table S5) was the method used to validate transformants during the 11 intermediate construction steps, the recombination events involved are “patchy” transformants with tiny patches of native DNA instead of synthetic sequence that would have been missed during the PCRTag analysis. The remaining four mutations, which must have originated during the integration process, all occur in regions of overlap in the *synIII* minichunks, suggesting that the homologous recombination process may be somewhat error prone relative to baseline error rates (23). The tenth change is the absence of an expected loxPsym site.

To check for negative effects of modifications on fitness of *synIII*-containing strains from the wild type (BY4742), we examined colony size, growth curves, and morphology under various conditions. A growth curve analysis established that *synIII* and the isogenic native strain had no detectable fitness difference (Fig. S10). The strains were also indistinguishable from each other on colony size tests (Fig. 3C), indicating that defects in fitness attributable to the *synIII* intermediate or *synIII* are very modest, with only one condition out of 21 (high sorbitol) showing a subtle fitness defect for *synIII* (Fig. S11). Cell morphology of all intermediate strains was similar to that of wild type (Fig. S12) except that during replacement round R3 (giving rise to strain 219 kb-*synIII*), a very low frequency (~1% of cells) of morphologically abnormal buds were observed (Fig. S12). We performed transcript profiling to identify possible changes in gene expression across *synIII* or genome-wide due to synonymous substitutions, introduction of loxPsym sites, and other changes. While ten loci are differentially expressed at genome-wide significance ($p < 7.4 \times 10^{-6}$ for 5% family-wise error rate based on 6756 loci with at least one mapped read, and also corresponding to 1% false-discovery rate), eight of these correspond to loci intentionally deleted from *synIII*. The remaining two loci are *HSP30* on *synIII*, ~16-fold down, and *PCL1* on native chromosome XIV, ~16-fold up (Fig. S13).

The inclusion of hundreds of designed changes in the synthetic chromosome, including the removal of 11 tRNA genes, said to be important sites of cohesin loading, might result in subtle or overt destabilizing effects on the synthetic chromosome; alternatively, removal of repetitive DNA sequences might increase stability by reducing the likelihood of “ectopic” recombination events involving two different repeat copies. Because of the 98 loxPsym sites added to *synIII* (and all the other changes), it was important to evaluate the genome integrity and the loss rate of the chromosome in the absence of Cre expression. PCRTag analysis revealed that *synIII* is stable over 125 mitotic generations in 30 independent lineages (Fig. 4A). To evaluate the loss rate of *synIII*, we used the “a-like faker” assay in which *MATa* cells carrying *synIII* were monitored for acquiring the ability to mate as *MATa* cells, a consequence of losing chromosome III (24). Despite the extensive chromosome engineering, the frequency of *MATa/synIII* loss was not significantly different from that of the wild-type control (Fig. 4B).

It is not known whether cohesin accumulation at a tRNA gene region directly depends on the presence of the tRNA gene, nor its effect on chromosome stability is clear. We compared

the map of cohesin binding sites on native chromosome and *synIII* using ChIPseq analysis (Fig. S14). The overall cohesin binding pattern is quite similar between the two chromosomes. However, at three tRNA genes that show a prominent peak in the native chromosome, that peak is reduced or in one case, the glutamine tRNA gene tQ(UUG)C, completely absent from *synIII* (Fig. S14). Thus, we conclude that tRNA genes and their documented interactions with both cohesin and condensin (25, 26) are dispensable for high levels of chromosome stability. We also compared the replication dynamics of *synIII* and native *III* (Supplementary Text, Table S9 and Fig. S15) and saw few dramatic changes in dynamics in spite of several ARS sequences having been deleted.

SCRaMbLEing in haploid strains containing chromosome *synIII*, leads to lethality via essential gene loss (Fig. S16). We looked for more subtle effects of SCRaMbLE in a heterozygous *MATa/a* (mating incompetent) diploid strain with a synthetic *MATa* chromosome and a native *MATa* chromosome (Fig S17). We introduced the Cre-EBD plasmid into such strains as well as to WT *MATa/a* diploids, and very briefly induced with estradiol. In spite of the minimal level of SCRaMbLEing induced, we observed a massive increase in the frequency of “a mater” derivatives in the native*III*/*synIII* heterozygous strains (Figures 4C & S18). Such a-mater derivatives can arise from the loss of the *MATa* locus, because such “MAT-less” strains express a-specific genes. PCRTAg mapping of several such derivatives showed that these variants had indeed lost different sections of *synIII*, all of which included the *MAT* locus (Fig. S18).

The total synthesis of the *synIII* chromosome represents a major step towards the design and complete synthesis of a novel eukaryotic genome structure – using the model *Saccharomyces cerevisiae* as the basis for a synthetic designer genome “Sc2.0”. The many changes made to *synIII*, including intron deletion, tRNA gene removal, and loxPsym sites and PCRTags introduction, do not appear to significantly decrease the fitness or alter the transcriptome or the replication timing of the *synIII* strain, supporting the very pliable nature of the yeast genome and potentially allowing for much more aggressively redesigned future genome versions. Sc2.0 represents just one of myriad possible arbitrary genome designs, and we anticipate that synthetic chromosome design will become a new means of posing specific evolutionary and mechanistic questions about genome structure and function. Rapid advances in synthetic biology coupled with ever decreasing costs of DNA synthesis suggest that it will soon become feasible to engineer new eukaryotic genomes, including plant and animal genomes, with synthetic chromosomes encoding desired functions and phenotypic properties based on specific design principles.

Supplementary Material

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Fig. 1. SynIII design

Representative *synIII* design segments for loxP site insertion (A & B) and stop codon TAG to TAA editing (C) are shown. Green diamonds represent loxP sites embedded in the 3' UTR of non-essential genes and at several other landmarks. Fuchsia circles indicate synthetic stop codons (TAG recoded to TAA). Complete maps of designed *synIII* chromosome with common and systematic ORF names, respectively, are shown in Figures S1 & S2.

Fig. 2. SynIII construction

(A) Building block (BB) synthesis. 750 bp BBs (purple) were synthesized from oligonucleotides at Johns Hopkins University by students in the Build-AGenome course. (B) Assembly of minichunks. 2–4 kb minichunks (yellow) were assembled by homologous recombination in *S. cerevisiae* (Table S1). Adjacent minichunks were designed to encode overlap of one BB to facilitate downstream assembly steps. Minichunks were flanked by a rare cutting restriction enzyme (RE) site, *XmaI* or *NotI*. (C) Direct replacement of native yeast chromosome *III* with pools of synthetic minichunks. Eleven iterative one-step assemblies and replacements of native genomic segments of yeast chromosome *III* were carried out using pools of overlapping synthetic DNA minichunks (Table S2), encoding alternating genetic markers (*LEU2* or *URA3*), which enabled complete replacement of native *III* with *synIII* in yeast.

Fig. 3. Characterization and testing of *synIII* strain

(A) PCRTag analysis (one PCRTags/10 kb) of the left arm of *SynIII* and wild-type yeast (BY4742) DNA is shown. Analysis of the complete set of PCRTags is shown in Figures S4, S5 & S6. (B) Karyotypic analysis of *synIII* and *synIIIL* strains by pulsed-field gel electrophoresis revealed the size reduction of *synIII* and *synIIIL* compared to native *III*. Yeast chromosome numbers are indicated on the right side. *SynIII* (272,871 bp) and native chromosome VI (270,148 bp) co-migrate in the gel. Karyotypic analysis of *synIII* and all intermediate strains is shown in Fig. S8. (C) *SynIII* and *synIIIL* phenotyping on various types of media. Ten-fold serial dilutions of saturated cultures of wild type (BY4742), *synIIIL* and *synIII* strains were plated on the indicated media and temperatures. YPD, yeast extract peptone dextrose; YPGE, yeast extract peptone glycerol ethanol; MMS, methyl methanesulfate. Complete set of *synIII* and *synIIIL* phenotyping under various conditions is shown in Fig. S11.

Fig. 4. Genomic stability of the *synIII* strain
(A) PCRTAg analysis of *synIII* strain after ~125 generations. We assayed for the loss of 58 different segments lacking essential genes in the absence of SCRaMbLEing; no losses were observed after over 200,000 segment-generations analyzed; reported frequency is a maximum estimate of segment loss frequency per generation. (B) Evaluation of the loss rate of *synIII* chromosome using *a*-like faker assay. No significant change in the loss frequency was observed, although the absolute loss rate value is modestly higher in *synIII*. (C) SCRaMbLE leads to a gain of mating type *a* behavior in *synIII* heterozygous diploids. Frequency of *a*-mater and α -mater colonies post-SCRaMbLE (induction with estradiol) in *synIII/III* and *III/III* strains. Complete SCRaMbLE analysis is shown in Fig. S18.