Authors

Jack A. Vincent, Toshio Tsukiyama

Fred Hutchinson Cancer Research Center, 1100 Fairview Ave N, Seattle, 98109. Tel 206 667 4996

1. Experiment Design

1a) Goal

Determine the kinetics of DNA replication in the presence of Methyl methanesulfonate (MMS) in a chromatin-remodeling mutant (*isw2 nhp10*)

1b) Description

The signal form newly replicated DNA was compared to the signal from unreplicated DNA at time-points throughout a single S phase to determine percent replication for each locus on the microarray

1c) Keywords

Chromatin remodeling, replication, MMS, *isw2 nhp10*, density transfer

1d) Experimental Factors

Newly replicated DNA vs unreplicated DNA collected from the same S phase sample

1e) Experiment design

Genomic DNA isolated from samples taken at 30, 45, 60, 90, and 120 minutes (wild type) or 45, 60, 90, 120, and 150 minutes (*isw2 nhp10*) after release from G1 arrest (in media containing 0.015% MMS) was digested by restriction enzyme and fractionated into 2 pools: DNA that was replicated in the latest S-phase, and DNA that had not yet been replicated. DNA from each pool was oppositely labeled and co-hybridized to a microarray.

1f) Quality Control Steps

Dye swaps were performed for each sample

2. Samples, extract preparation, and labeling

2a) Organism

S.cerevisiae; W1588-4c background

2b) Genotypes

WT
YTT1831 (*MAT***a** *ade2-1*::pRS402 *can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 RAD5+*)

isw2 nhp10 double mutant
YTT3306 (*MAT***a** *ade2-1*::pRS402 *can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 RAD5+ isw2*::NatMX *nhp10*::HphMX)

2c) Hybridizations

Hybridization ID	Time point (min)	genotype	Replicated DNA label	Unreplicated DNA label
wt_30min_a	30	WT	Cy5	Cy3
wt_30min_b	30	WT	Cy3	Cy5
wt_45min_a	45	WT	Cy5	Cy3
wt_45min_b	45	WT	Cy3	Cy5
wt_60min_a	60	WT	Cy5	Cy3
wt_60min_b	60	WT	Cy3	Cy5
wt_90min_a	90	WT	Cy5	Cy3
wt_90min_b	90	WT	Cy3	Cy5
wt_120min_a	120	WT	Cy5	Cy3
wt_120min_b	120	WT	Cy3	Cy5
in_45min_a	45	*isw2 nhp10*	Cy5	Cy3
in_60min_a	60	*isw2 nhp10*	Cy5	Cy3
in_60min_b	60	*isw2 nhp10*	Cy3	Cy5
in_90min_a	90	*isw2 nhp10*	Cy5	Cy3
in_90min_b	90	*isw2 nhp10*	Cy3	Cy5
in_120min_a	120	*isw2 nhp10*	Cy5	Cy3
in_120min_b	120	*isw2 nhp10*	Cy3	Cy5
in_150min_a	150	*isw2 nhp10*	Cy5	Cy3
in_150min_b	150	*isw2 nhp10*	Cy3	Cy5

2d) Sample treatment
All samples were subject to treatment with 0.015% MMS.

2e) Preparation of Hybridization extract

Genomic DNA isolation: Cells were grown at 30°C for a minimum of 7 generations in minimal medium containing ^{13}C and ^{15}N as the sole carbon and nitrogen sources (dense media) until the optical density reached 0.25 - 0.30 at 600 nm. Cells were then synchronized with α-factor for 105 minutes, filtered, transferred to complete (YC) media containing ^{12}C and ^{14}N (light media), then

incubated in the continued presence of α-factor for 75 minutes prior to release (conditioning phase). MMS was added to 0.015% 15 minutes into the conditioning phase. After the conditioning phase, cells were filtered, washed with warm light media, and released in light media in the presence of 0.015% MMS. Samples were collected at the indicated times after release and genomic DNA was isolated via bead beating and phenol extraction. Genomic DNA was then digested to completion with EcoRI.

Separation of newly replicated DNA from unreplicated DNA: Light isotope (replicated) DNA was separated from heavy isotope (unreplicated DNA) by CsCl gradient centrifugation as described at http://fangman-brewer.genetics.washington.edu/density_transfer.html. After drip fractionation, fractions containing replicated and unreplicated DNA were determined by slot blot followed by hybridization with radiolabeled (and fragmented) genomic DNA. Replicated and unreplicated DNA fractions were each pooled separately. Three fractions from the middle of the gradient, containing both replicated and unreplicated DNA, were excluded from the pooled samples. DNA was retrieved by ethanol precipitation.

2f) Labeling

0.5ug of each sample was labeled with either Cy3 or Cy5-dUTP (GE Biosciences). 21ul DNA solution was heat denatured at 100°C for 5 min then quick chilled in an ice slurry. The following was added while samples were still on ice: 5ul 10x dNTP Mix (1.2mM each dATP, dCTP, dGTP; 0.6mM dTTP; 10mM Tris 8.0), 3ul Cy-dUTP, 1ul exo- Klenow (50000 U/ml, New England Biolabs). Samples were incubated at 37°C for 1hr. Reaction was stopped by adding 5ul 0.5M EDTA. 60ul of 1mg/ml tRNA was added to each reaction and unincorporated nucleotides were removed using a Biospin 30 column (BioRad). Oppositely labeled samples (i.e. Cy3-replicated and Cy5-unreplicated) from the same timepoint were combined for cohybridization. 20ul polydA::dT (1mg/ml; Sigma) was added to each probe mix, then DNA was precipitated after addition of 1/10 volume 3M NaOAc and 2 volumes 100% ethanol.

3. Array Design

3a) Array name

FHCRC Yeast Amplicon v3.1, GEO Platform GPL1914

3b) array design

- platform type:
  spotted

- array provider:

in-house FHCRC

- surface type:
  glass

- surface type name:
  in-house coated poly-lysine FHCRC slides

- physical dimensions of slides:
  40 x 18 mm

- number of elements on the array:
  6300 (approximately)

- reference system allowing to locate each element:

Elements are spotted by blocks (16 blocks total) of elements. The blocks have referenced coordinates on the array.

- production protocol:

Yeast cDNA microarrays were constructed employing a set of ~6200 orf-specific PCR primer pairs (Research Genetics, Huntsville, AL), which were used to amplify each open reading frame (orf) of the yeast genome. Individual PCR products were verified as unique via gel electrophoresis and purified using ArrayIt™ 96-well PCR purification kits (TeleChem International, Sunnyvale, CA). Purified PCR products were mechanically "spotted" in 3X SSC (450 mM sodium cloride and 45 mM sodium citrate, pH 7.0) onto poly-lysine coated microscope slides using an OmniGrid high-precision robotic gridder (GeneMachines, San Carlo, CA).

<u>3c) Spot information</u>

- simple or composite:
  simple

- element type:
  PCR products

- single or double stranded:
  double

- spot dimension:
  ~130 micrometers

- generation protocol
  PCR using the Research Genetics primer set and genomic DNA

- attachment type
  electrostatic

3d) Specific properties of each spot on the array

- element type:
  PCR products

- PCR primer information
  The PCR primers used for generating the elements are identified by MIPS ORF names.

- approximate length:
  up to 1Kb

4. Hybridization procedures and parameters

- hybridization buffer:
  3X SSC + 10%SDS + 1 mg/mL poly(dA)

- blocking agent:
  no prehybridization

- slide blocking:
  no prehybridization

- probe blocking:
  1mg/mL polydA during hybridization

- wash procedure:
  wash1: 1X SSC, 0.03% SDS
  wash2: 1X SSC dip slides 15 times
  wash3: 0.2X SSC: shake slides 75rpm for 20minutes
  wash4: 0.05X SSC: shake slides 75rpm for 10 minutes
  spin-dry slides in centrifuge 50g for 5 minutes.

- quantity of labelled target used:
  all material generated from 0.5 ug of EcoRI digested genomic DNA

- time, concentration, volume, temperature:
  16h, 26 microliters at 63°C

- Hybridization instrument:
  Manual, TelChem hybridization chambers in waterbath

5. Measurement data and specifications

4a) Scanning hardware
GenePix 4000B scanner (Molecular Devices)

4b) Image analysis
GenePix Pro v6.0 (Molecular Devices)

4b) Normalization (determination of % replication)
File = Percent_replication.txt

Percent replication for each spot was determined as described[1]. The calculation of percent replication at each time point is dependent on two measurements; the percentage of total genomic replication in the population at each time point and the number of cycling cells over the entire time course.

- Total genomic % replication:

The total percent replication for the genome was determined after fractionation of each CsCl gradient. Fractions were blotted to a nylon membrane and then hybridized to a radiolabeled genomic probe (as described above). Percent replication was determined by quantifying the signal from each fraction (by phosphorimager), plotting the value for each fraction, and then comparing the area under the curve corresponding to replicated and unreplicated fractions (see http://fangman-brewer.genetics.washington.edu/density_transfer.html). The total % replication for each sample was as follows:

Time point (min)	% genomic replication WT	% genomic replication *isw2 nhp10*
30	12%	-
45	31%	14%
60	45%	20%
90	66%	27%
120	81%	32%
150	-	35%

- Ratio of cycling cells:

The ratio of cycling cells to non-cycling cells was estimated from the maximum percentage of budded cells obtained during the time course. The maximum percent budded was 94% for WT and 86% for *isw2 nhp10*.

- Smoothing:

Smoothing of normalized data for data figures was done as described[1].

1. Alvino, G. M. et al. Replication in Hydroxyurea: It's a matter of time. *Mol Cell Biol* (2007).