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Physical Monitoring of Mating Type Switching in *Saccharomyces cerevisiae*

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The kinetics of mating type switching in *Saccharomyces cerevisiae* can be followed at the DNA level by using a galactose-inducible *HO* (*GAL-HO*) gene to initiate the event in synchronously growing cells. From the time that *HO* endonuclease cleaves *MAT*a until the detection of *MAT*α DNA took 60 min. When unbudded G1-phase cells were induced, switching obeyed the rules established for normal homothallic cells; both the induced cell and its daughter switched to the opposite mating type in "pairs." In the presence of the DNA synthesis inhibitor hydroxyurea, *HO*-induced cleavage occurred but cells failed to complete switching. In these blocked cells, the *HO*-cut ends of *MAT*a remained stable for at least 3 h. Upon removal of hydroxyurea, the cells completed the switch in approximately 1 h. The same kinetics of *MAT* switching were also seen in asynchronous cultures and when synchronously growing cells were induced at different times of the cell cycle. Thus, the only restriction that confined normal homothallic switching to the G1 phase of the cell cycle was the expression of *HO* endonuclease. Further evidence that galactose-induced cells can switch in the G2 phase of the cell cycle was the observation that these cells did not always switch in pairs. This suggests that two chromatids, both cleaved with *HO* endonuclease, can interact independently with the donors *HML*α and *HMR*a.

Homothallic mating type switching in *Saccharomyces cerevisiae* is an efficient mitotic gene conversion event by which cells interconvert between **a** and α mating types (reviewed in references 10, 20, and 25). The process is initiated by a double-strand break at the *MAT* locus (33) created by the action of the *HO* endonuclease (21, 22). In essence, *MAT* switching can be thought of as a specialized double-strand break repair event (10, 20, 25, 33, 35) in which the donor acts as a template for new DNA synthesis. Repair of the double-strand break at *MAT* results in the substitution of *MAT* Y**a** or Yα sequences with nonhomologous sequences derived from one of two unexpressed mating type loci, *HML* and *HMR*. The three mating type loci have regions of homology, X and Z1, which flank the allele-specific Y region; *MAT* and *HML* have two regions of additional homology, W and Z2 (2, 34) (see Fig. 2A).

A single switching event gives rise to a pair of switched cells, suggesting that this event normally occurs in G1 phase, prior to replication of the chromosomal DNA (12). The observation of Nasmyth (26) that *HO* expression is limited to the G1 phase lends support to this conclusion. It is not clear, however, if limitation of switching to G1 can be explained solely on the basis of periodic *HO* expression or whether accessibility of *MAT* to the *HO* endonuclease is also restricted in a cell cycle-dependent fashion.

The present study was undertaken for two reasons: (i) analysis of mating type switching at the DNA level, from initiation to completion, could be used to determine the kinetics of switching and the time interval during which DNA intermediates should be sought; and (ii) physical monitoring of switching coupled with genetic analysis to delineate the relationship between mating type switching, *HO* endonuclease expression, and the cell cycle. For these purposes, we have used heterothallic (*ho*) cells carrying a plasmid-borne copy of a *GAL-HO* fusion gene (16). In homothallic (*HO*) cells, the *HO* gene is under tight transcriptional control and is transcribed neither in daughter cells (26) nor in cells expressing both **a** and α mating type information (17). Sequences located 5′ to the *HO* mRNA start site have been identified as being responsible for regulation of *HO* transcription (27–30). In the *GAL-HO* fusion gene, these sequences have been replaced by 365 base pairs (bp) derived from the *GAL10* regulatory region to create a galactose-inducible *HO* endonuclease (16). Jensen and Herskowitz (16) have shown that when the *HO* gene is expressed from the *GAL10* promoter, its transcription is released from both mother-daughter and **a**/α control.

We have examined mating type switching, both physically and genetically, in synchronous and asynchronous populations of cells. We show that *HO* endonuclease expression from the *GAL10* promoter is cell cycle independent, as is accessibility of the *MAT* locus to the endonuclease. Furthermore, we demonstrate that initiation of mating type switching out of the G1 phase does not alter the kinetics of repair of the double-strand break. We found mating type switching in these cells to be a surprisingly slow recombinational event and discuss possible rate-limiting steps with respect to the molecular mechanism of the event.

MATERIALS AND METHODS

Strains. *S. cerevisiae* R166 was obtained by transformation of BWG1-7A (*ho HML*α *MAT***a** *HMR***a** *leu2-3,112 ura3-52 his4-519 ade1-100 GAL*) with the *GAL-HO* plasmid. BWG1-7A was a gift from L. Guarente. R126 (*ho HML*α *MAT***a** *hmr-3*Δ *leu2-3,112 ura3-52 trp1 thr4 GAL*) was constructed in this laboratory. The *hmr-3*Δ allele was originally obtained from A. J. S. Klar (18). Yeast cells were transformed with lithium acetate (13).

Plasmids. All strains carried the *GAL-HO* plasmid (*URA3 ARS1 CEN4 GAL-HO*) described by Jensen and Herskowitz (16). Other plasmids used have the following structures. pJH3 has the 3.5-kilobase (kb) *Eco*RI-*Hin*dIII *MAT***a** fragment (2) inserted between the *Eco*RI and *Hin*dIII sites of pBR322. pJH4 contains a 300-bp *Alu*I Yα fragment (2) in the

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*Hpa*I site of pBR322. pCW1-1 contains a 4.1-kb *Hin*dIII *MAT*α fragment inserted into the *Hin*dIII site, a 2.2-kb *Xho*I-*Sal*I *LEU2* fragment (1) at the *Sal*I site, and a 1.45-kb *Eco*RI *TRP1-ARS1* fragment (36) at the *Eco*RI site of pBR322. pCW2-6 has 1.6 kb of *MAT*-specific sequences derived from the *Hin*dIII *MAT* fragment by deleting the W, X, Y, and Z sequences; the resulting 1.6-kb *Hin*dIII fragment is inserted into the *Hin*dIII site of pGEM3 (Promega Biotec). pCW5-6 contains a 246-bp *Nde*I-*Eco*RV Yα fragment cloned between the *Hin*cII and *Sma*I sites in the multiple cloning site of pGEM3.

Media. Cells were grown in YEP (1% [wt/vol] yeast extract, 2% [wt/vol] Bacto-peptone [Difco]) supplemented with dextrose (2% [wt/vol]; YEPD) or lactic acid (3.15% [wt/vol], pH 5.5; YEPL). To induce the *GAL-HO* gene, galactose (2%, wt/vol) was added to YEPL medium. Synthetic complete medium lacking uracil (SC-ura) was made by the method of Sherman et al. (31). Bacto-agar (Difco) was added at 2% (wt/vol) for solid medium.

Cell cycle arrest. (i) α-Factor (Sigma Chemical Co.; 1 mg/ml in ethanol solution) was added to exponentially growing YEPL cultures (2×10^6 to 4×10^6 cells per ml) to give a final concentration of 2.5 μg/ml. Incubation was continued until >90% of cells were unbudded. Cells were recovered on a sterile nitrocellulose disk (0.45 μm pore size; Millipore Corp.) by vacuum filtration. The disk was washed with fresh YEPL (warmed to 30°C) and suspended in an equal volume of warmed YEPL. (ii) Hydroxyurea (HU; Sigma Chemical Co., 1.5 M solution in water) was added to logarithmic YEPL cultures to give a final concentration of 150 mM. The cultures were incubated for 4 h at 30°C, and the cells were recovered as described above.

DAPI staining. Diamidinophenyl indole (DAPI) was used to fix and stain cells as described by Williamson and Fennel (38).

***HO* induction.** A 1- to 2-day-old SC-ura culture was used to inoculate YEPL to a cell density of 1×10^5 to 4×10^5 cells per ml. The cultures were incubated overnight at 30°C with vigorous aeration to a final cell density of 2×10^6 to 4×10^6 cells per ml. Galactose was added as a 20% (wt/vol) solution, and incubation was continued.

***HO* endonuclease assay.** Culture (2 ml, 4×10^6 to 8×10^6 cells) was added to 1 ml of sterile glycerol on ice, frozen in dry ice, and stored at −80°C. The cells were thawed on ice, crude extracts were prepared, and assays were carried out as described by Nasmyth (26), except that electrophoresis was done in agarose gels (0.8% agarose, 40 mM Tris-acetate, 2 mM EDTA, pH 8.0) rather than acrylamide. The substrate used for the assays, pCW1-1, was linearized with *Nco*I, and the 3′ ends were ^{32}P-labeled with T4 DNA polymerase (New England Biolabs) as described by Maniatis et al. (24).

Modified pedigree analysis. An asynchronous culture was induced for *HO* endonuclease expression for 1 h, after which time glucose was added. The cells were immediately recovered, washed, and resuspended in YEPD. A streak of cells was laid down on a YEPD plate, and either single unbudded cells (G1) or cells with small buds (non-G1) were micromanipulated away from the streak. After incubation at 30°C, the mother and daughter cells were separated and allowed to grow into colonies. These colonies were then tested for mating type.

DNA extraction. From 15 to 20 ml of culture (5×10^7 to 10×10^7 cells) was collected onto an 0.45-μm-pore filter, washed with 20 ml of (TE 10 mM Tris hydrochloride, 1 mM EDTA, pH 7.4), and resuspended in 0.5 ml of 100 mM Tris hydrochloride (pH 8.0)–50 mM EDTA–2% sodium dodecyl sulfate in a 1.5-ml microcentrifuge tube. Glass beads (0.45 to 0.5 mm diameter, acid washed and baked) were added to the level of the meniscus, 0.5 ml of TE-saturated phenol was added, and the tube was mixed in a Vortex mixer twice for 20 s each and cooled on ice. The aqueous phase was subsequently extracted with equal volumes of TE-saturated phenol-chloroform-isoamyl alcohol (25:24:1) and chloroform-isoamyl alcohol (24:1), made 2 M in ammonium acetate, and the DNA was precipitated with 2 volumes of isopropanol. The precipitate was pelleted, dissolved in TE, treated with pancreatic RNase (20 μg/ml, 30 min, 37°C) and proteinase K (50 μg/ml, 60°C, 60 min), phenol-chloroform extracted, and ethanol precipitated. The purified DNA was dissolved in TE and stored at −20°C.

Southern analysis. Restriction endonuclease-digested DNA was electrophoresed, transferred to nitrocellulose filters (Schleicher and Schuell), hybridized to radioactively labeled DNA probe, washed, and autoradiographed by standard procedures (24). Nick-translated DNA probes were prepared by standard procedures (24). Some DNA was transferred to the nylon membrane Zeta Probe (Bio-Rad) as specified by the manufacturer and hybridized to radioactively labeled RNA probe by the procedure of Church and Gilbert (7). RNA probes were prepared as specified by the manufacturer (Promega Biotec).

RESULTS

Galactose-induced mating type switching is a slow event. The initiation and completion of *MAT* switching are marked by the cleavage of *MAT* by *HO* endonuclease and the subsequent replacement of mating type-specific sequences with those of the opposite mating type. These events can readily be observed by Southern blot analysis of DNA taken from synchronized cultures induced for *HO* expression.

Cells of strain R166 (*ho HML*α *MAT***a** *HMR***a**), which carries the *GAL-HO* plasmid, were synchronized in G1 phase with α-factor as described in Materials and Methods. The arrested cells were harvested by filtration and released from the G1 block into YEP-lactate medium plus galactose to induce *HO* expression. After 30 min, the cells were washed and resuspended in YEPD. Samples were removed from the culture at various times before and after galactose induction. *HO* endonuclease activity was assayed in an in vitro assay (26; Materials and Methods). *HO* endonuclease activity was detected in the cell extracts after the 30-min galactose induction period. At this time greater than 80% of the cells were unbudded and presumably in G1. Consistent with the observation of Nasmyth that *HO* is rapidly turned over (26), we found that *HO* endonuclease was no longer detectable 30 min after transferring cells from galactose (YEPGal) to glucose (YEPD) medium (Fig. 1). In a parallel experiment, DNA was extracted from synchronized, induced cells to examine the sequence of events in homothallic mating type switching. Cells were induced for 45 min and then resuspended in glucose-containing medium (YEPD). The DNA was digested with *Hin*dIII and examined by Southern hybridization to ^{32}P-labeled RNA probes specific for *MAT* (pCW2-6, Fig. 2B) and Yα (pCW5-6, Fig. 2C). A 4.0-kb *MAT***a** restriction fragment was initially detected (Fig. 2B). Coincident with the appearance of *HO* endonuclease activity in the cell extracts, cutting of the 4.0-kb *MAT***a** fragment to generate two subfragments of 2.8 and 1.2 kb was observed. The product of a switch, a 4.1-kb *MAT*α fragment, was first detectable 60 min after removal of galactose (Fig. 2C). Thus, from *HO* cutting of *MAT***a** to the appearance of

the *MAT*α allele, approximately 1 h was required to complete a *MAT* conversion event initiated in the G1 phase of the cell cycle.

It was possible that the use of α-factor as a means of synchronizing the cells perturbed the system so as to alter the kinetics of switching. We therefore repeated this experiment without α-factor synchronization. The results were the same: the 4.1-kb *MAT*α *Hin*dIII fragment appeared approximately 60 min after *HO* cleavage of *MAT***a** in these asynchronous cultures (data not shown).

The galactose-induced *MAT* switching that we monitored at the DNA level appeared to be normal by all genetic criteria. As previously shown by Jensen and Herskowitz (16), G1-phase cells induced briefly in galactose switch in "pairs"; that is, the induced cell gives rise to two cells with the same new mating type. This was also true with our strains. G1 (unbudded) cells of strain R166 were induced for 60 min, washed with glucose (YEPD) medium, and streaked on a YEPD plate. Individual cells were observed microscopically to divide once into two progeny cells, which were separated by micromanipulation and allowed to grow into colonies. Among 82 such pairs, 59 gave rise to two *MAT*α colonies, while a single pair contained one **a** and one α mating colony. The remaining 22 pairs (27%) either did not switch at all or else replaced *MAT***a** with Y**a** sequences from *HMR***a**. Thus, the pair rule established for normal homothallic cells (12) was obeyed in 59 of 60 cases, even though the time required for switching appeared to be sufficient for the cells to pass through S into G2 phase. This point will be discussed further in the Discussion.

Mating type switching in the presence of continuous *HO* expression. The fact that synchronous and asynchronous cultures switched *MAT* with the same kinetics suggested that *MAT* could be switched in non-G1 phases of the cell cycle. The *GAL-HO* fusion gene lacks the upstream sequences necessary for cell cycle regulation of *HO* transcription (27–29), and expression of the *HO* endonuclease in cells carrying the *GAL-HO* plasmid should be independent of the cell cycle. We carried out several experiments to examine the cell cycle dependence of *MAT* conversion.

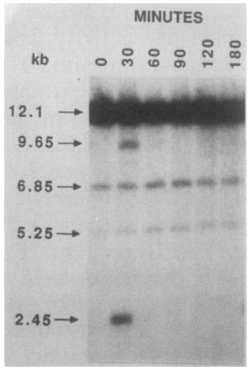

FIG. 1. Detection of *HO* endonuclease activity in crude cell extracts. α-Factor-synchronized cells were induced for *HO* endonuclease expression for 30 min and then washed into YEPD. Samples were removed at various times, and crude cell extracts were assayed for *HO* activity as described in Materials and Methods. The plasmid substrate pCW1-1 was linearized and ^{32}P end labeled. Cleavage by the *HO* endonuclease yielded two subfragments of 9.65 and 2.45 kb. The two minor subfragments of 6.85 and 5.25 kb were generated by cleavage of the plasmid by a second, constitutively expressed endonuclease activity (most likely *Sce*II [20]).

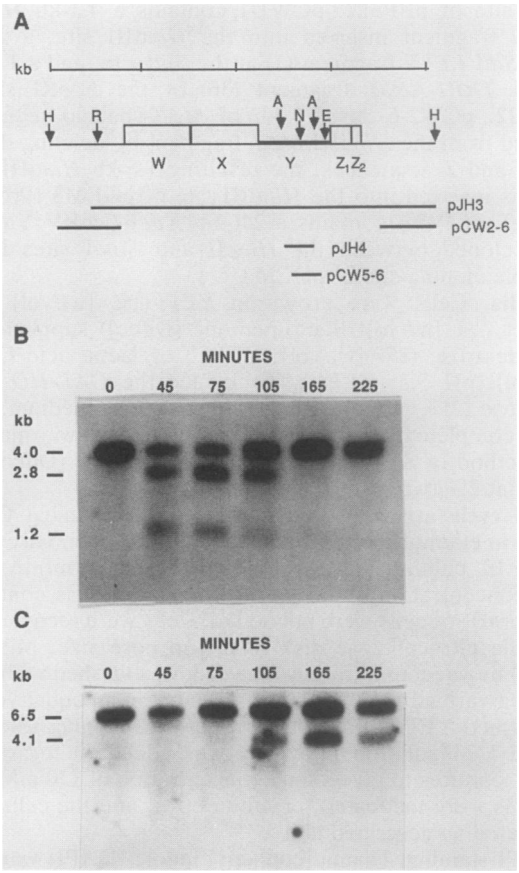

FIG. 2. (A) Partial restriction map of the 4.1-kb *MAT*α locus. *Hin*dIII (H), *Eco*RI (R), *Alu*I (A), *Nde*I (N), and *Eco*RV (E) sites are shown. The *HO* endonuclease cleaves the *MAT* locus at the border between Y and Z. The bars represent regions used as probes in Southern analysis (see Materials and Methods). (B) Southern analysis of R166 DNA. Cells were induced for *HO* endonuclease for 45 min, and samples were taken at 30, 60, 120, and 180 min after the cells were washed into YEPD. DNA was digested with *Hin*dIII, electrophoretically separated on a 0.5% agarose gel, and blotted to a nylon membrane (Zeta Probe). The blot was probed with ^{32}P-labeled *MAT*-specific RNA (pCW2-6). The 4.0-kb band corresponds to the *MAT***a** locus, and the 2.8- and 1.2-kb bands correspond to the *HO* endonuclease cleavage products of *MAT***a**. (C) The Southern blot in panel B was washed free of the *MAT*-specific probe and hybridized with ^{32}P-labeled Yα-specific RNA. The 4.1-kb band corresponds to the *MAT*α locus, and the 6.5-kb band is the *HML*α fragment, which also hybridized to the Yα probe.

An α-factor-arrested culture of R166 was released from the cell cycle block and incubated in the presence of galactose for more than 3 h, during which time the cells completed more than one cell cycle. Cell cycle progress was followed by monitoring nuclear morphology by DAPI staining (Table 1). *HO* endonuclease was first detected in extracts from cells taken 30 to 45 min after addition of galactose (Fig. 3A). The level of activity seen reached a plateau after 60 to 90 min. Thus, *HO* endonuclease appears to be expressed continuously throughout the cell cycle.

The proportion of cells committed to switching was followed genetically as well as physically. At each time point, cells were plated onto YEPD to turn off further transcription of *HO*. Single colonies from the YEPD plates were tested for mating type (Table 2). A 15-min incubation in the presence of

TABLE 1. Percentage of cells in various stages of the cell cycle upon release from α-factor[a]

Class	% of cells in class at time (min) postrelease							
	0	30	60	90	120	150	180	240
I	88	80	83	42	26	19	17	21
II	3	9	9	51	53	41	25	47
III	1	4	2	3	17	29	40	15
IV	8	7	6	4	4	11	18	17

[a] YEPL-grown R166 cells were arrested at the G1 stage of the cell cycle with α-factor. After these cells were washed free of α-factor, galactose was added to 2% and samples were taken to monitor cell cycle progress. Cells were stained with DAPI (39) and assigned to a class after microscopic examination. Classes: I, unbudded mononucleate; II, budded with a single nucleus in the mother cell; III, budded with the nucleus in the isthmus; IV, budded binucleate.

TABLE 2. Mating phenotypes of colonies derived from cells grown in galactose[a]

Time in galactose (min)	Mating type (% of total cells)		
	a-Maters	α-Maters	Nonmaters[b]
0	98.1	0	1.9
15	79.7	20.3	0
30	46.4	52.2	1.4
45	37.9	62.1	0
60	31.9	68.1	0
90	36.6	62	1.4
120	43.1	53.8	3.1

[a] Galactose was added to an α-factor-synchronized culture of R166. Cells were removed at various times and plated on YEPD. Colonies were tested for both mating type and Ura phenotype. The numbers given are expressed as a percentage of Ura$^+$ (p*GAL-HO*-containing) colonies. The values given for cells not treated with galactose (0 min in galactose) are derived from several independent experiments.

[b] Nonmaters presumably arose from matings between **a** and α cells subsequent to plating.

galactose was sufficient to induce switching of 20% of the cells. By 60 min, 68% of the cells had been committed to switch, presumably by *HO* endonuclease cleavage at *MAT*. After longer periods of *HO* induction, the percentage of α mating colonies decreased; by 240 min only 20% were α mating. Further evidence presented below suggests that cells which had already switched from *MAT***a** to *MAT*α were induced to switch back to *MAT***a**. However, the high level of *HO* endonuclease expression after long periods of induction made it difficult to assess this observation (see below).

The kinetics of switching when *HO* was continuously induced (Fig. 3B) can be compared with the events presented in Fig. 2, where *HO* was induced for only 30 min. As before, 45 min after addition of galactose and coincident with the appearance of *HO* activity in cell-free extracts, cutting of the 4.0-kb *Hin*dIII *MAT***a** fragment to generate two subfragments of 2.8 and 1.2 kb (Fig. 3B) was observed. By 90 min more than 50% of the *MAT***a** DNA had been cleaved. At 120 min, when switching to *MAT*α was detected, another band of 2.9 kb appeared. This fragment was the expected size for *HO* cleavage of *MAT*α. The appearance of this fragment indicates that when *HO* endonuclease was continuously expressed, the product of switching, *MAT*α, could itself be

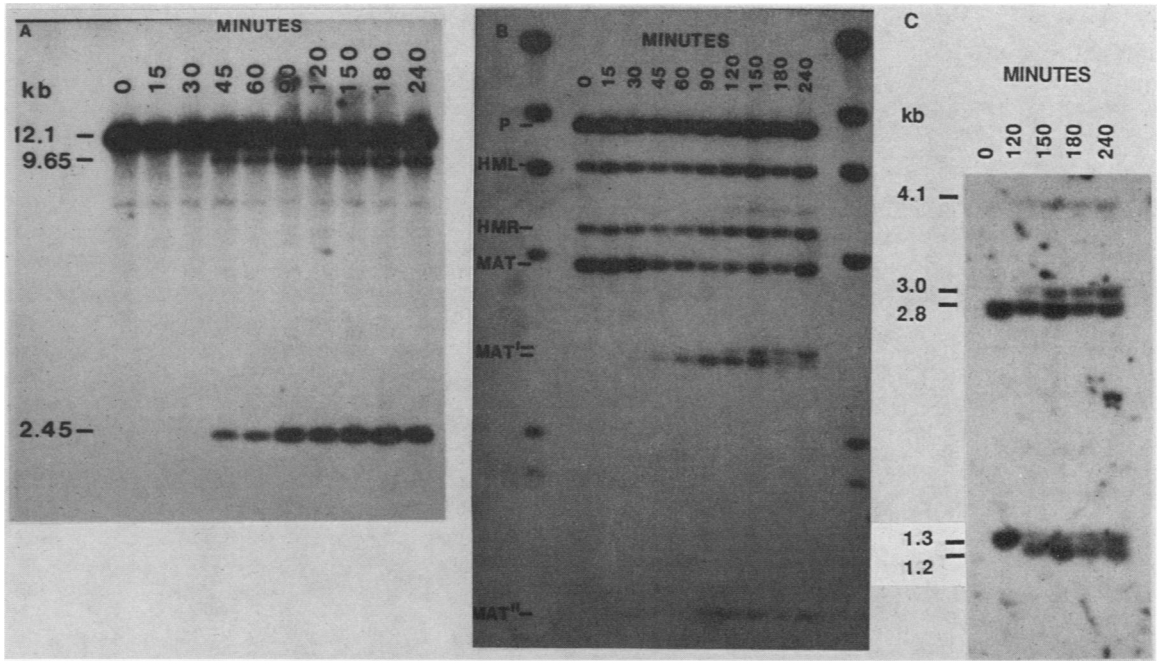

FIG. 3. An α-Factor-synchronized culture of R166 was induced for *HO* endonuclease, and samples were taken at intervals after the addition of galactose. (A) Detection of *HO* endonuclease activity in crude cell extracts. See the legend to Fig. 1 for details. (B) Southern analysis of *Hin*dIII-restricted DNA. DNA was electrophoretically separated on a 0.5% agarose gel, Southern blotted to a nitrocellulose membrane, and hybridized with ^{32}P-labeled pJH3. Restriction fragments corresponding to the p*GAL-HO* plasmid (8.3 kb) and *HML*α (6.5 kb), *HMR***a** (4.8 kb), and *MAT***a** (4.0 kb) are indicated. *HO* cleavage of *MAT***a**, seen at 45 min, generated two subfragments of 2.8 kb (*MAT'*) and 1.2 kb (*MAT''*). *HO* cleavage of the switched *MAT*α allele, seen at 120 min, generated subfragments of 2.9 kb (*MAT'*) and 1.2 kb (*MAT''*). (C) DNA from min 0, 120, 150, 180, and 240 time points was digested with both *Hin*dIII and *Bgl*II, electrophoresed on a 0.5% agarose gel, Southern blotted to nitrocellulose, and hybridized with ^{32}P-labeled pCW2-6. Bands corresponding to *MAT*α (4.1 kb), *HO*-cleaved *MAT*α (2.9 and 1.2 kb), and *Hin*dIII-*Bgl*II-digested *MAT***a** (2.7 and 1.3 kb) can be seen. The 1.2-kb *HO*-cleaved *MAT***a** fragment comigrated with that from *MAT*α.

cleaved before completion of the cell cycle. At 120 min, more than 96% of the cells had not completed mitosis and nearly 80% of them had not yet initiated mitosis (Table 1).

We confirmed the identity of the 2.9-kb fragment as being derived from *HO* cleavage of *MAT*α in two ways. First, this band hybridized to a Yα-specific probe (data not shown). Second, as the *MAT***a** allele contained a unique *Bgl*II site that was absent in the *MAT*α allele, it was also possible to distinguish *MAT*α from *MAT***a** in DNA digested with both *Hin*dIII and *Bgl*II (Fig. 3C). When such a digest was probed with ^{32}P-labeled *MAT*-specific plasmid DNA, the 120-min sample showed restriction fragments of both 4.1 and 2.9 kb, corresponding to intact and *HO*-cleaved *MAT*α, respectively. The distal *HO* endonuclease cleavage products of *MAT***a** and *MAT*α were indistinguishable. Thus, when *HO* endonuclease was present at the G2 phase of the cell cycle, there was no apparent delay between the appearance of the switched *MAT* allele and its *HO* cleavage product. This does not necessarily mean that the *MAT* locus is equally susceptible to *HO* cleavage in post-G1-phase cells, as under these conditions (but not when *HO* was induced for shorter times), we also observed some cleavage of the *HML*α locus (see below).

The *MAT* locus is susceptible to the *HO* endonuclease outside of the G1 phase. To assess the accessibility of the *MAT* locus at different stages of the cell cycle under transient *HO* induction conditions, another experiment was carried out. An exponential-phase culture of R166 was synchronized with α-factor, and the cells were released from the G1 block into YEPL medium. The culture was then divided into three parts, and incubation was continued. Galactose was immediately added to one culture, which contained 92% unbudded (G1) cells. Galactose was added to the second and third cultures when they reached 46 and 63% budded cells, respectively. Samples were removed from each culture to examine both the induction of *HO* endonuclease activity and the sequence of events at the DNA level. In each of the induced cultures, *HO* activity was first observed in cell extracts 30 min after galactose induction (Fig. 4A, B, and C). Southern analysis of the DNA showed that in each culture *HO* cutting of the *MAT***a** locus was first evident at 30 min. Thus, at a time when *HO* endonuclease expression was still low, the cutting of *MAT* occurred equally well in the G1 and non-G1 cultures. After 90 min of galactose induction, the 2.9-kb band indicative of *HO* cleavage of the newly formed *MAT*α locus was seen in all three cultures (Fig. 4D, E, and F). These data indicate that induction of *HO* expression from the *GAL-HO* gene fusion is cell cycle independent and that the *MAT* locus is accessible

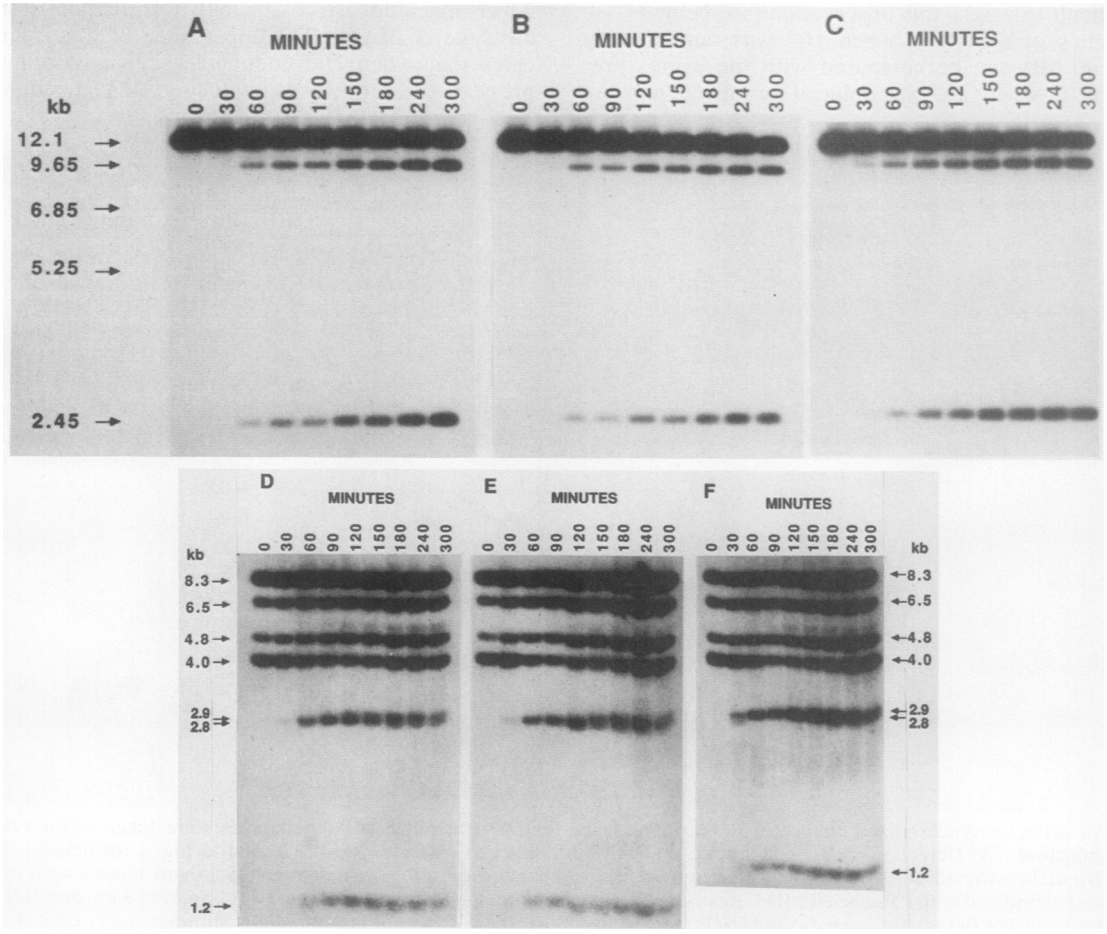

FIG. 4. α-Factor-synchronized cultures were induced for *HO* endonuclease expression at 8% (A,D), 46% (B,E) or 63% (C,F) budded cells. Samples from each of the cultures were removed over the course of 300 min. *HO* endonuclease activity in crude cell extracts was assayed (A,B,C). DNA was restriction digested with *Hin*dIII, electrophoretically separated on a 0.5% agarose gel, Southern blotted to a nitrocellulose membrane, and hybridized with ^{32}P-labeled pJH3 (D,E,F). Arrows: p*GAL-HO* (8.3 kb), *HML*α (6.5 kb), *HMR***a** (4.8 kb), *MAT***a** (4.0 kb), *HO*-cleaved *MAT***a** (2.8 and 1.2 kb), and *HO*-cleaved *MAT*α (2.9 and 1.2 kb).

to cleavage by the *HO* endonuclease in parts of the cell cycle other than G1.

Non-G1 cells do not always switch in pairs. Expression of *HO* endonuclease during or after chromosomal DNA replication might also be expected to result in a violation of the pair rule (12) of switching. For instance, if the two *MAT*-containing chromatids are cut in G2 phase and interact independently with *HMLα* or *HMR***a**, we would expect to find instances where a mother cell and its daughter had different mating types. We showed earlier (above) that galactose-induced switching of unbudded G1 cells was normal in obeying the pair rule in 59 of 60 cases. When this experiment was carried out with non-G1 cells, nearly 20% of those examined did not switch in pairs. From the same asynchronous culture (60 min of induction) from which the G1 cells were analyzed, 90 non-G1 cells that had a bud that was less than 50% of the area of that of the mother were micromanipulated and allowed to divide. Of the 69 pairs of cells in which at least one of the two progeny switched to *MATα*, 13 (19%) yielded one **a**-mating and one α-mating cell. The occurrence of these nonpaired switches was taken as further evidence that *MAT* switching can be initiated outside of the G1 phase. The occurrence of unpaired switches in non-G1 cells cannot be explained by cleavage of one *MAT* locus and not the other, as shown by the following experiment. Galactose-induced switching was examined in non-G1 cells of genotype *HMLα MAT***a** *hmr3Δ*. In these cells, all double-strand breaks at *MAT* must use *HMLα* as a donor. In this case there were no unpaired switches out of 30 pairs which switched to *MATα*.

Resistance of *HML* and *HMR* to *HO* endonuclease cleavage. Another subfragment of 5.2 kb (R166, Fig. 3 and 4) appeared at low levels at the same time as the cut *MATα* subfragment. This restriction fragment did not show any flanking sequence homology with the *MAT* locus (data not shown), nor was its appearance dependent on cutting of the *MAT* locus, as it appeared with the same kinetics in a strain in which the *MAT* locus had been deleted (data not shown). This restriction band derived from *HO* cleavage of the *HMLα* locus. In R166 this produced two *Hin*dIII fragments of 5.2 and 1.3 kb. In strain R126 (which has a polymorphic *HMLα* *Hin*dIII fragment), the corresponding fragments were 4.7 and 1.3 kb. A 4.7-kb but not a 5.2-kb restriction band was observed in R126 (Fig. 5). The level of *HML* cleavage seen was always low and never approached that seen for either the original or switched *MAT* alleles. Furthermore, this cleavage was only seen after extended periods of *HO* endonuclease induction. It is likely that *HMLα* cleavage is a consequence of overproduction of the *HO* endonuclease. Cleavage of *HMR***a** would produce a fragment of 3.8 kb, which was not detected in any of our experiments. This may reflect differential accessibility of *HMR* and *HML* loci to *HO* endonuclease.

***MAT* switching but not *HO* cleavage is blocked by HU.** Although the switch event took approximately 60 min to be completed, there was no obvious loss of the 2.8-kb Y**a**-containing *MAT* fragment until after the appearance of the switched *MATα* product (Fig. 1B, 2B, and 4D, E, and F). In an attempt to inhibit switching subsequent to cutting but prior to completion of the event, cells were induced in the presence of HU. HU is an inhibitor of DNA synthesis and causes cells to arrest at S phase (32).

A culture of R126 (*ho HMLα MAT***a** *hmr-3Δ*) was blocked at S phase with HU as described in Materials and Methods. The cells were then induced with galactose in the presence of HU, and samples were removed over the course of 240 min. At this time the remaining cells were recovered, washed and resuspended in YEPD, and incubated in the absence of the drug for 70 min. The induction of *HO* endonuclease expression followed the same kinetics as in other experiments and was detectable in cell extracts 30 min after induction (data not shown). Southern analysis of the DNA (Fig. 5) showed that, coincident with the appearance of *HO* activity in cell extracts, the *MAT***a** allele was cleaved. However, generation of a switched *MATα* product was not observed in the presence of the drug. Removal of HU allowed completion of the switch event within 70 min (lane 7), the time previously determined to be required for the conversion of a cleaved to a switched *MAT* locus. Thus, the *HO*-cleaved intermediate appeared to be stable for at least 3 h in HU-blocked cells.

DISCUSSION

The use of a galactose-inducible *HO* gene (16) allowed us to examine the kinetics and cell cycle dependence of yeast mating type switching. When *HO* endonuclease was induced for as little as 30 min, mating type switching was initiated. Galactose-induced switching in G1-phase cells was genetically indistinguishable from normal homothallic switching: cells switched preferentially to the opposite mating type and nearly always switched in pairs. These facts make all the more striking the observation that, when switching was monitored at the DNA level, it took approximately 60 min from the time of *HO* endonuclease cleavage of the *MAT* locus until the appearance of the final switched product.

In fact, the conditions under which we observed *MAT* switching were not fundamentally different from those of normal homothallic conversion. We followed switching in G1 cells after galactose induction for 30 min, followed by transferring the cells to glucose medium to turn off transcription of *HO*. Under these conditions, *HO* endonuclease was no longer detected 30 min after the cells were washed out of the galactose. Nasmyth (26) showed in synchronized populations of homothallic mother cells that *HO* endonuclease activity also persisted for about 30 min in the G1 phase of the

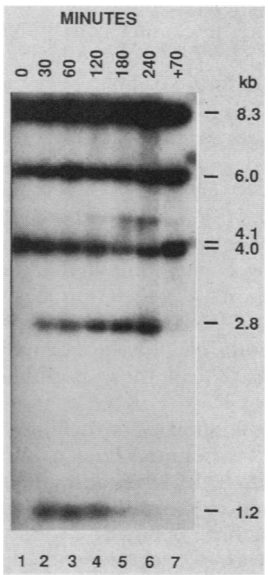

FIG. 5. Galactose was added to HU-blocked cells, and samples of the culture were taken up to 240 min, when the remaining cells were washed into YEPD. The last lane shows DNA extracted from cells after 70 min of growth in YEPD. Bands: p*GAL-HO* (8.3 kb), *HMLα* (6.5 kb), *MAT***a** (4.0 kb), *HO*-cleaved *MAT***a** (2.8 and 1.2 kb).

cell cycle and decreased rapidly when the cells began chromosomal replication. Under the conditions we used, switching took approximately 60 min of a 180-min cell cycle. From cell budding and DAPI staining data (Table 1), it is likely that these cells do not complete *MAT* switching until chromosomal replication is generally completed and the cells are in G2 phase. This is likely to be true also for normal homothallic cells grown in YEPD, as the combined lengths of the S, G2, and M phases remains relatively constant regardless of overall generation time (3, 5, 14, 15). If this is not the case, then the interactions of *MAT* and the donor loci must have been established prior to replication of *MAT*, so that switching still occurred in pairs. It is also possible that replication of *MAT* is delayed until after the completion of switching.

There is, however, a notable difference between our data and those from previous studies of the formation of a double-strand break at *MAT*. Strathern and co-workers (33) first observed *HO* endonuclease cleavage of *MAT* in asynchronous, continuously switching *HO HML***a** *MAT***a** *HMR***a** and *HO HML*α *MAT*α *HMR*α cells. In these cells, they found that less than 2% of the *MAT* DNA was cleaved at any one time. Even accounting for the fact that only mother cells can switch (12) and that only a proportion of these cells are in the late G1 phase, this value does not seem consistent with the persistence of cleaved *MAT* fragments seen in our *GAL-HO* experiments. This raises the possibility that *GAL-HO*-induced switching is slower than in *HO* strains or that switching in continuously switching strains does not accurately mimic the efficiency and/or kinetics of switching in *HO HML*α *MAT***a** *HMR***a** cells. We entertain several possibilities. Possibly, continuous switching accumulates mutations which reduce *MAT* cleavage. Such mutations occur frequently in strains unable to repair double-strand breaks (19, 23, 37) and may also accumulate when cells are compelled to switch every generation. Second, the replacement of *MAT***Ya** with the nonhomologous *MAT*Yα may be an inherently different process than the repair of *MAT***a** to *MAT***a**.

Alternatively, galactose induction of *HO* may occur in such a way that other necessary gene functions are no longer coordinately induced with the endonuclease. Conversely, in continuously switching strains, such activities may be present at higher levels than in cells that have not recently switched. For example, expression of the *RAD54* gene, which is known to be essential for mating type switching (10), has recently been shown to be induced by double-strand DNA breaks (8). Consistent with the idea that other functions need to be induced for the completion of *MAT* switching, we have recently shown that another *HO*-induced event, the formation of a deletion between two flanking regions of non-*MAT* DNA (Rudin and Haber, submitted), is inhibited by the addition of cycloheximide after *HO* endonuclease induction. All of these possibilities are currently being studied.

Cell cycle control of *MAT* switching. In their study of galactose-induced switching, Jensen and Herskowitz (16) reported that *GAL-HO* expression did not cause cells to violate the pair rule in more than 90% of switching events studied. As a possible explanation for this, they suggested that there might be a cell cycle-dependent restriction of *MAT* accessibility to *HO* endonuclease or of some other essential component of the switching process. We have shown that when synchronously growing cells were induced for 30 to 60 min at different stages of the cell cycle, *MAT* cleavage was essentially equivalent whether the cells were in G1 or a later phase in the cell cycle. Thus, we conclude that the restriction of *MAT* switching to the G1 phase in normal homothallic strains is solely attributable to the periodic expression of *HO* endonuclease. In support of this conclusion, we found that a brief induction of *HO* in non-G1 cells yielded two cells of different mating types in 19% of the events studied, in contrast to G1 switching. By using a strain with only one donor locus, we have shown that unpaired switches are not the result of cleavage and switching of only one of the two *MAT* loci in the postreplicative cell. We conclude that both *MAT* loci are cut but sometimes use different donors. In normal homothallic cells, in which switching occurs in G1, *MAT***a** uses *HML*α as a donor 90% of the time and *HMR***a** 10% of the time (18). Thus, the relative proportion of nonpaired events (13 of 69) to paired events (56 of 69) that we saw for non-G1 cells is consistent with independent interaction of the two different *MAT* loci with *HML* and *HMR*.

Fate of Ya DNA replaced by the switch. We note that there is no evidence for degradation of the Ya-containing cut fragment generated by *HO* cleavage of *MAT* in our data. This was most obvious in HU-blocked cells (Fig. 5), where the double-strand break at the *MAT* locus was maintained for more than 3 h before switching was permitted to continue. It is possible that exonucleolytic degradation may proceed rapidly once initiated but that the initiation is a slow step. We infer that removal of *MAT* Y sequences is not the rate-limiting step but must occur rapidly and in conjunction with completion of the switch.

The long interval between the initiation and completion of *MAT* switching in this *GAL-HO* system should make it possible to detect intermediates of recombination. No evidence of intermediate forms subsequent to *HO* cleavage of *MAT* was found. Recently, using strand-specific probes of *MAT* DNA, we identified an apparent strand-specific DNA intermediate in mating type switching which appeared subsequent to *HO* cutting at the *MAT* locus and disappeared upon completion of switching (C. White, B. Connolly, and J. Haber, unpublished observations). Characterization of this intermediate is in progress.

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