# Chromosome associations in budding yeast caused by integrated tandemly repeated transgenes

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## Summary

**The binding of GFP-tagged tetracycline repressor (TetR) molecules to chromosomally integrated tetracycline operator (*tet*O) sequence repeats has been used as a system to study chromosome behaviour microscopically in vivo. We found that these integrated transgenes influence the architecture of yeast interphase nuclei, as chromosomal loci with tandem repeats of exogenous *tet*O sequences are frequently associated. These associations occur only if TetR molecules are present. *tet*O tandem repeats associate regardless of their chromosomal context. When they are present at a proximal and a distal chromosomal position, they perturb the normal polarized Rabl-arrangement of chromosome arms by recruiting chromosome ends to the centromeric pole of the nucleus. Associations are established at $G_1$ and are reduced during S-phase and mitosis. This system may serve as a model for the role of DNA sequence-specific binding proteins in imposing nonrandom distribution of chromosomes within the nucleus.**

Key words: Yeast, Chromosomes, Nucleus, Nuclear architecture, GFP, Pairing, FISH, Mitosis

## Introduction

The distribution of chromosomes within interphase nuclei is not random. First, in most species there exists a polarized arrangement of chromosome arms, the so-called Rabl orientation, where centromeres are assembled near one pole of the nucleus, while telomeres point towards the opposite pole [for examples from various organisms, see the following references (Foe and Alberts, 1985; Fussell, 1987; Dong and Jiang, 1998; Zickler and Kleckner, 1998; Jin et al., 2000; Goto et al., 2001)]. Second, there exist clear cases of somatic homologous pairing, especially in dipteran insects (Metz, 1916). Somatic pairing or transient homologous associations have been claimed to occur also in other organisms including budding yeast (Burgess and Kleckner, 1999; Burgess et al., 1999). Under certain circumstances, non-allelic chromosomal loci tend to co-localize within the nucleus (Nikiforova et al., 2000; Abranches et al., 2000). Moreover, specific chromosome regions often reside in subcompartments of the nucleus. Telomeres tend to be positioned near the nuclear periphery (Gotta et al., 1996). Tandem repeat regions often fuse into clusters described as ectopic heterochromatin pairing. These spatial relationships between specific chromosomal regions may underlie epigenetic phenomenons such as gene silencing or co-ordinated expression of genes or of the alleles of a gene (transvection) (Henikoff, 1997; Marshall et al., 1997a; Lamond and Earnshaw, 1998; Gasser, 2001).

Since in most cell types it is impossible to trace individual chromosomes during interphase, fluorescence in situ hybridization has been used to label chromosomes or parts thereof to study their positions inside nuclei. As a means to investigate chromosome distribution and behaviour in living cells of various organisms, the *lac*O/LacI-GFP system was introduced (Straight et al., 1996; Belmont and Straight, 1998; Belmont, 2001). Also, the similar *tet*O/TetR-GFP system was used to study the segregation behaviour of chromosomes in live yeast cells (Michaelis et al., 1997). Both systems are based on the transgenic expression of a bacterial regulating protein fused to green fluorescent protein (GFP). The fusion protein then binds to a target DNA sequence, of which many copies are tandemly integrated into a specified chromosomal region, at which the GFP tags produce microscopically visible fluorescence.

When we attempted to adapt the *tet*O/TetR-GFP system to study various aspects of chromosomal organization within *S. cerevisiae* interphase nuclei, we observed that integration of *tet*O repeats into chromosomes promotes the association of the target loci. Here, we describe the nature of these associations and their effect on the architecture of interphase nuclei. We also discuss whether mechanisms similar to those by which *tet*O repeats associate, could play a role in various nonrandom chromosomal interactions with putative functions in DNA repair and epigenetic regulation of gene expression.

## Materials and Methods

### Strains with chromosomal GFP markers

Yeast strains have been constructed that show chromosome site-specific labels by green fluorescent protein (GFP). These strains synthesize a bacterial tetracycline repressor (TetR)-GFP fusion protein and have multiple copies of the tetracycline operator (*tet*O) sequence inserted in specific chromosomal loci. TetR molecules with GFP tags bind to the *tet*O tracts and elicit fluorescence at the target site. The original haploid strain, constructed by Rafal Ciosk, is described elsewhere (Michaelis et al., 1997). In short, a tandem repeat of 112 copies of a 50 bp segment containing the 20 bp *tet*O sequence (Gossen and Bujard, 1992) was triple integrated into the *URA3* locus on the left arm of chromosome *V*, 35 kb away from the centromere

**Table 1. Yeast strains used in this study**

| Strain name | Relevant genotype | Features* |
|---|---|---|
| SK1 (FKY1) | *MAT***a**/*MAT*α, HO/HO | Wildtype |
| NKY857 | *MAT***a**, ho::*LYS2, lys2, leu2, his4, ura3* | Haploid SK1 |
| SLY911 | *MAT*α, ho::*LYS2, lys2, leu2, ura3, leu2*::prom$_{URA3}$::*tetR*::*GFP*::*LEU2, ura3*::*tet*O-*URA3, BMH1*::*tet*O::*URA3*; derived from FKY806 | *tet*O repeats trans-CEN/TEL |
| FKY1012 | *MAT***a**/*MAT*α, HO/HO, *leu2*::prom$_{URA3}$::*tetR*::*GFP*::*LEU2*/*LEU2, ura3*::*tet*O-*URA3*/*ura3*::*tet*O-*URA3* | *tet*O repeats CEN/CEN |
| FKY1024 | *MAT***a**/*MAT*α, HO/HO, *leu2*/*leu2, ura3*/*ura3, leu2*::prom$_{URA3}$::*tetR*::*GFP*::*LEU2*/*LEU2, BMH1*::*tet*O::*URA3*/*BMH1*::*tet*O::*URA3* | *tet*O repeats TEL/TEL |
| FKY806 | *MAT***a**/*MAT*α, ho::*LYS2*/ho::*LYS2, lys2*/*lys2, leu2*::prom$_{URA3}$::*tetR*::*GFP*::*LEU2*/*LEU2, ura3*::*tet*O-*URA3*/*URA3, BMH1*/*BMH1*::*tet*O::*URA3* | *tet*O repeats trans-CEN/TEL |
| SLY1662 | *MAT***a**/*MAT*α, ho/ho, *ura3*::*tet*O::*URA3*/*URA3, BMH1*/*BMH1*::*tet*O::*URA3* | *tet*O repeats trans-CEN/TEL, no *tetR* present |
| SLY1663 | *MAT***a**/*MAT*α, ho/ho, *ura3*/*ura3, BMH1*::*tet*O::*URA3*/*BMH1*::*tet*O::*URA3* | *tet*O repeats TEL/TEL, no *tetR* present |
| SLY1664 | MAT**a**, ho, leu2, ura3::tetO::URA3, BMH1::tetO::URA3 | tetO repeats CEN/TEL, no tetR present |

*For the chromosomal localization of tetO repeats see also Fig. 1B.

(Fig. 1A). In a second strain, approximately 500 copies of *tet*O were integrated into the *BMH1* locus, 30 kb from the right end of chromosome *V* (Ciosk et al., 1998) (R. Ciosk, personal communication). A construct with *tet*R expressed under the *URA3* promoter (prom$_{URA3}$) was integrated into the *LEU2* locus on chromosome *III* (Fig. 1A).

These strains were backcrossed at least five times to strain SK1 (Kane and Roth, 1974) by using a haploid derivative of SK1 (NKY857, kindly provided by Nancy Kleckner, Harvard University, Cambridge, MA); diploid strains FKY806, FKY1012 and FKY1024 with homozygous and heterozygous centromere-near and telomere-near *tet*O integrations (Table 1; Fig. 1B) were constructed by crossing. The strains were microscopically checked for the presence of chromosomal GFP dots. Strains SLY1662 and SLY1663 carry heterozygous CEN and TEL *tet*O inserts and homozygous TEL *tet*O inserts, respectively, but they are missing the *tet*R construct. Therefore they do not show chromosomal GFP dots and *tet*O loci have to be detected by FISH (see below). Strains SLY1662 and SLY1663 were derived from *leu2* spores of strains FKY1012 and FKY1024 that were crossed to wild-type haploids. *leu2 tet*O segregants were selected and the presence of *tet*O sequences at the desired locations was tested by PCR.

### Culture conditions and cytological preparation

Yeast cells were grown in liquid YPD medium. For some experiments, cells were arrested in S-phase by treatment with hydroxyurea (10 mg/ml) for 3 hours. Arrest was confirmed by microscopic examination of bud formation and by immunolabelling of the spindle (see below).

Living cells were collected by centrifugation and resuspended in YPD:glycerol 3:1 on a slide for microscopy. For the semi-spreading procedure, which is described in detail elsewhere (Jin et al., 2000), samples of 3-5 ml were taken and formaldehyde was added directly to the culture medium at a final concentration of 4%. Fixation was performed at room temperature for 30-60 min. After rinsing twice in 2% KAc, cell pellets were resuspended in 500 μl 2% KAc, and 10 μl 0.5 M dithiotreitol and 14 μl of a Zymolyase 100T (Seikagaku Co., Tokyo) stock solution (10 mg/ml) were added. After the digestion of the cell walls for 20 minutes at 37°C, the cells were washed in 2% KAc and resuspended in suitable volumes of 2% KAc. 20 μl of this cell suspension were put on a slide and mixed with 80 μl detergent (1% aqueous solution of Lipsol; LIP Ltd, Shipley, UK) and 120 μl fixative (4% paraformaldehyde and 3.4% sucrose in distilled water). The mixture was then spread out with a glass rod and left to solidify in a chemical hood. For microscopy, the slides were washed in water (5 minutes), air dried and nuclei were stained with DAPI (1 μg/ml).

For testing whether the *tet*O sequence repeat number is sufficiently stable during mitotic growth, a single colony of strain FKY806 was transferred from a plate into 1 litre of liquid medium and grown to stationary phase. The number of cells in the culture was found to be $1.5\times10^{11}$ cells, which means that cells have on average gone through 37 mitotic cycles. 10 μl of the suspension ($1.5\times10^6$ cells) were used to inoculate 10 ml of presporulation medium, grown for another seven generations (to a density of ~$2\times10^7$ cells/ml) and then transferred to sporulation medium. Cells were fixed as described (Williamson et al., 1983) and the number of GFP signals per pachytene nucleus was determined.

### Fluorescence in situ hybridization (FISH) and immunostaining

To obtain FISH probes for the *tet*O integration regions, either immediately adjacent chromosomal sequences or the *tet*O sequence itself were PCR-amplified. FISH probes on chromosome *V* were produced by PCR using the Expand Long Template PCR System (Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturer's instructions. Appropriate primers were designed based on the published yeast genomic sequence (*Saccharomyces* Genome Database http://genome-www.stanford.edu/Saccharomyces). For the amplification of products of around 10 kb the following conditions were applied: 2 minutes at 94°C; 10 cycles with 10 seconds at 94°C, 30 seconds at 58°C, 8 minutes at 68°C; 20 cycles with 10 seconds at 94°C, 30 seconds at 58°C, 8 minutes at 68°C with an increment of 20 seconds per cycle and a final extension step of 10 minutes. The amplified PCR products were purified using the Gel Extraction Kit QIAEX II (Qiagen, Valencia, CA) and subsequently labelled by nick translation either with Cy3-dUTP (red; Amersham, Little Chalfont, UK) or Fluorescein-12-dUTP (green; Roche Diagnostics GmbH, Mannheim, Germany) as described previously (Loidl et al., 1998). The chromosomal localizations of FISH probes are shown in Fig. 1C. For generating FISH probes for the *tet*O repeats themselves, the sequence was PCR-amplified from the plasmid pCM189 containing *tet*O palindromic units (Gari et al., 1997) and simultaneously labelled with Cy3-dUTP.

Labelled probes were dissolved in hybridization solution (50% formamide, 10% dextran sulfate, 2× SSC) to a final concentration of approximately 30 ng/μl. After 5 minutes of denaturation at 95°C, the probes were dropped onto slides, denatured for 10 minutes at 80°C and hybridized for 48 hours at 37°C. Post-hybridization washes were performed in 50% formamide/2× SSC (37°C), 2× SSC (37°C) and 1× SSC (room temperature) for 5 minutes each. Finally, slides were mounted in Vectashield anti-fading medium (Vector Laboratories, Burlingame, CA) supplemented with 0.5 μg/ml DAPI (4′6-diamidino-2-phenylindole) as DNA-specific counterstain.

Microtubules and the spindle pole body (SPB) were immunolabelled according to a standard protocol (Pringle et al., 1991) with the monoclonal rat anti-yeast tubulin antibody YOL1/34 (Kilmartin et al., 1982) purchased from Serotec, Kidlington, UK). Slides were washed twice for 3 minutes in 1× PBS (130 mM NaCl,

7 mM $Na_2HPO_4$, 3 mM $NaH_2PO_4$, pH 7.5), excessive liquid was drained and the slides were incubated with a drop of primary antibody (diluted 1:200 in 1× PBS) under a coverslip at 4°C overnight. After three 3 minute washes in 1× PBS, slides were incubated with FITC- or TRITC-conjugated secondary antibody for 120 minutes at room temperature. The slides were then washed three times for 3 minutes in 1× PBS. Cells were postfixed for 10 minutes in paraformaldehyde fixative (see above), washed for 3 minutes in distilled water and either mounted under a coverslip in Vectashield supplemented with DAPI or airdried and subjected to the standard FISH procedure (see above).

Slides were evaluated with a ZEISS Axioplan II epifluorescence microscope equipped with appropriate filter combinations for FITC, Cy3 and DAPI. Images were captured separately for the different fluorochromes using a computer-controlled cooled CCD camera (Photometrics, Tucson AZ), and pseudocolored and merged with the help of the IPLab image analysis software (Scanalytics, Fairfax, VA). GFP or FISH signals were classified as associated if they were lying side-by-side and touching, or if they were merged to form a single spot.

## Results

### Exogenous *tet*O tracts at allelic and ectopic positions are frequently associated in yeast nuclei

Strains with two tracts of exogenous repeated *tet*O sequences inserted at certain chromosomal positions (Fig. 1B) were used to study the relative positions of these loci within interphase nuclei. The *tet*O repeats were visualized by binding of ectopically expressed bacterial TetR protein with GFP fused to it. In two diploid strains, two *tet*O tracts were inserted at allelic locations either close to the centromere on the left arm (CEN/CEN) or close to the telomere of the right arm (TEL/TEL) of the two chromosomes *V*. In another diploid strain, *tet*O was inserted near the centromere of one and near the telomere of the other chromosome *V* (trans-CEN/TEL). In a haploid strain *tet*O arrays were integrated at both the centromeric and the telomeric locus on chromosome *V* (cis-CEN/TEL).

Strains with *tet*O repeats at two allelic positions showed a single GFP signal in 67.2±6.1% (CEN/CEN) and 74.2±4.6% (TEL/TEL) ($n$=600 each) of living cells. Comparable high levels of GFP signal associations were observed in cells treated according to the semi-spreading procedure (61.8±7.1% for CEN/CEN and 71.1±6.8% for TEL/TEL, $n$=1400 each; Fig. 2A; Fig. 3). To test whether the association of signals reflects the normal relative positions of these chromosomal loci or if they are promoted by *tet*O inserts, the corresponding chromosomal regions were labelled by FISH (probes 1 and 2, Fig. 1C) in a yeast strain without inserted *tet*O repeats and the frequencies of signal associations were counted. Centromeric regions were associated in 33.7±4.3% and telomeric regions in 26.3±3.1% of the 250 nuclei scored. The association of homologous centromeres is elevated compared with other regions because of the general clustering of centromeres (Jin et al., 1998) but still less frequent than in the presence of *tet*O repeats. The average association frequency for other homologous chromosome regions (13 sites on 8 different chromosomes tested) was found to be about 18% (Q. Jin, J.F. and J.L., unpublished).

Also in a strain with the *tet*O arrays at non-allelic positions on the two chromosomes *V* (trans-CEN/TEL), and in a haploid strain containing both *tet*O tracts on the same chromosome (cis-CEN/TEL), associations of *tet*O sites were frequent (51.4±2.7% and 55.9±7.2%; $n$=1400 each) (Fig. 3). This is

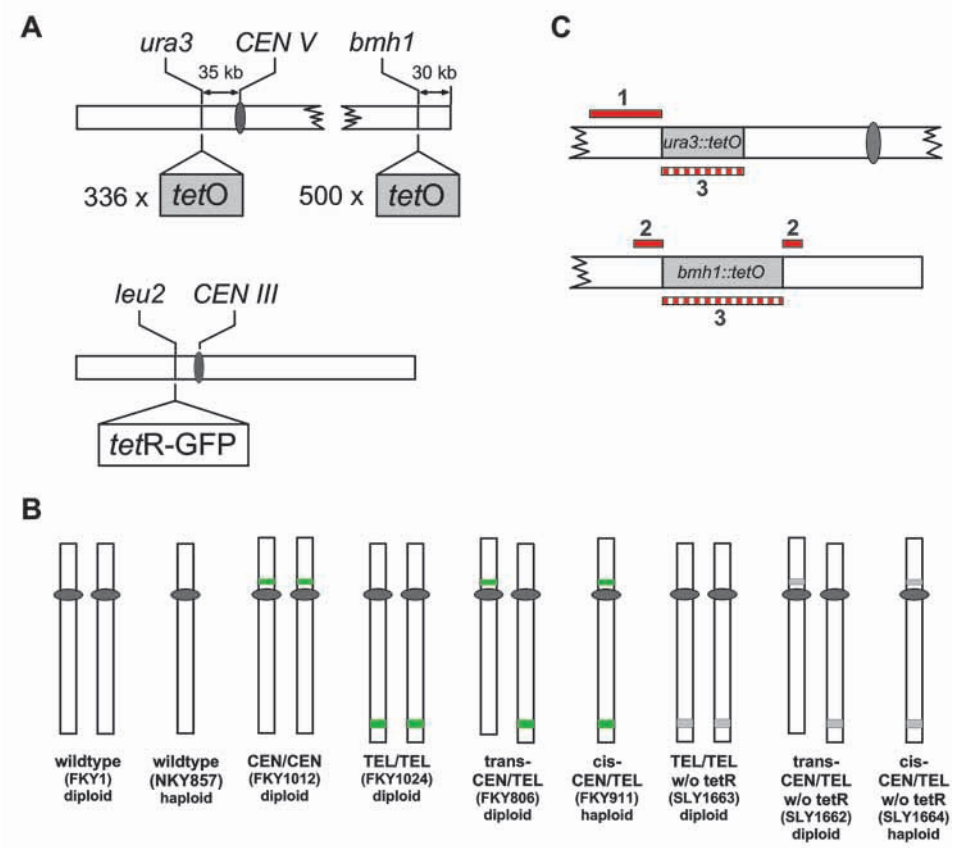


**Fig. 1.** Maps of *tet*O integration sites and FISH probes on chromosome *V*. (A) One *tet*O tract is integrated 35 kb away from the centromere on the short arm at the *URA3* locus. The other integration site is 30 kb away from the right telomere at the *BMH1* locus. (B) Yeast strains with different *tet*O inserts used in the experiments. Diploid strains are homozygous for CEN *tet*O or TEL *tet*O or heterozygous for the two inserts on chromosomes *V*. A haploid strain has both inserts in the same chromosome (cis). These strain constructs exist both as versions expressing and not expressing TetR-GFP (grey ovals, centromeres; green rectangles, integrated *tet*O sites in the presence of TetR; grey rectangles, integrated *tet*O sites in the absence of TetR). (C) Map of FISH probes for regions adjacent to centromeric (1), telomeric (2) *tet*O insertion sites, and for the *tet*O sequence (3).

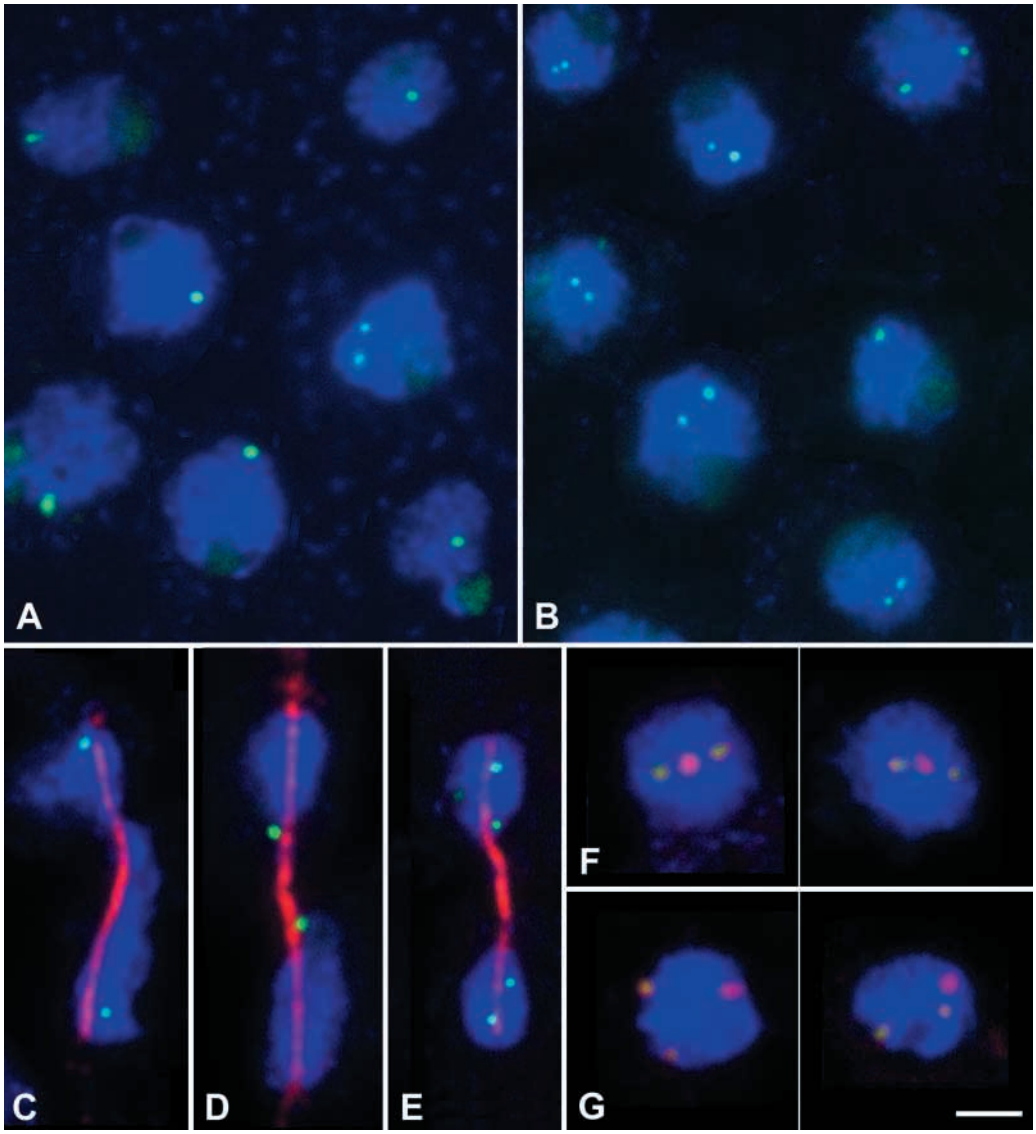


**Fig. 2.** Association and intranuclear organization of chromosomal *tet*O inserts in yeast nuclei. (A) In a diploid strain, two *tet*O repeats associate in the presence of TetR molecules and form a single signal in most cells, irrespective of their chromosomal positions. (B) In hydroxyurea-arrested nuclei, *tet*O associations are reduced and two separate GFP signals are seen in most nuclei. (C-E) Association behaviour of tetO repeats during anaphase of diploid cells. (C) Centromere-near *tet*O repeats (CEN/CEN) remain mostly associated and form a single GFP spot near the two spindle poles. (D) Telomeric regions (TEL/TEL) also remain mostly associated and appear as a single signal in the two daughter nuclei. (E) Ectopic *tet*O inserts (one near the centromere, the other near the telomere: trans-CEN/TEL), which are frequently associated in interphase, are mostly separate in anaphase cells and appear as two GFP signals in both daughter nuclei. (F,G) Recruitment of the telomere-near *tet*O repeat to the centromeric pole in trans-CEN/TEL nuclei. FISH with probes for the centromere-near (F, probe 1) and telomere-near (G, probe 2) *tet*O insertion site in wildtype (left) and the trans-CEN/TEL strain (right). Centromeres are always close to the SPB. In the wildtype, both telomeres are far from the SPB, whereas in the trans-CEN/TEL strain, one telomere is usually close to the SPB (see also Fig. 5). green, GFP; red, immunostained spindle and SPBs; orange, FISH signals. Bar, 2 μm.

remarkable because in yeast interphase nuclei there exists a roughly parallel orientation of chromosome arms with all centromeres clustered at one pole and the chromosome ends assembling near the opposite pole (Jin et al., 1998; Jin et al., 2000).

Since the false impression of association or fusion of GFP signals could be generated by signal loss caused by mitotic intrachromosomal recombination, we checked the stability of inserted *tet*O repeats. Cultures of the trans-CEN/TEL strain (FKY806), which had been grown for an average of at least 40 mitotic generations, were sporulated and the number of signals was determined in pachytene nuclei. Homologous synapsis at pachytene relocates the non-allelic regions with inserted *tet*O repeats to different regions on the bivalent and thus suppresses their fusion (J.L., unpublished). Of 100 arbitrarily selected pachytene nuclei, 91% showed two separate CEN-GFP/TEL-GFP signals and 9% showed a single signal. There was no nucleus without any signal, and in nuclei with one signal this

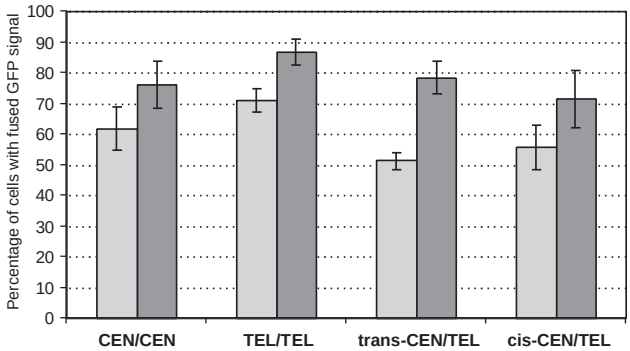


**Fig. 3.** Frequencies of *tet*O association in different GFP-TetR-expressing yeast strains (see Fig. 1B) in exponential (light grey bars) and stationary (dark grey bars) cultures. For each strain the experiment was repeated 14 times with 100 nuclei evaluted in each.

was strong, suggesting that it was produced by two fused *tet*O repeats (presumably due to the looping back of the bivalent on itself). Thus there was no notable loss of signals during extended vegetative growth and we can safely assume that the presence of a single signal per nucleus in the experiments, which were all done on fresh cultures, is not normally due to the loss of *tet* operator sequences and reduced GFP signal intensity.

### The association of *tet*O repeats depends on the presence of TetR molecules

To study whether the association of *tet*O sequences reflects an intrinsic property of tandem repeats or if it is caused by the binding of TetR molecules, *tet*O association was evaluated in strains with *tet*O tracts at the centromere- and the telomere-near loci but lacking the transgenic *tet*R construct responsible for TetR-GFP expression. The frequency of associations was tested by FISH with probes specific for regions close to the integration sites of the *tet*O repeats (probes 1 and 2) as well as with a probe specific for the *tet*O sequence (probe 3, see Fig. 1C). The haploid cis-CEN/TEL w/o tetR strain (SLY1664; see Table 1) showed 18.7±6.3% of associated *tet*O regions ($n$=300), which is similar to the frequency of associations of the corresponding chromosome regions in the haploid wild-type strain NKY857 (16.0±4.0%; $n$=300) (Fig. 4). The diploid TEL/TEL w/o tetR strain SLY1663 showed a single FISH signal in 24.8±1.2% of the 200 nuclei inspected, which is comparable with the frequency of the association of the corresponding region in the wild-type strain (SK1) without *tet*O sites (26.3±3.1%; $n$=250). By contrast, when the chromosome regions harbouring the *tet*O repeats in the TetR-GFP-expressing strain FKY 1024 (TEL/TEL) were labelled by FISH, they showed the expected high association of FISH signals (79.4±7.9%; $n$=200).

In another series of experiments, TetR-GFP binding to *tet*O was inhibited by the addition of 12 μg/ml tetracycline to the cell cultures. TetR has a high affinity to tetracycline so that nontoxic amounts can effectively induce inactivation of the repressor (Hillen and Berens, 1994). Similarly, the association frequencies were reduced to the frequency of associations in haploid wild-type cells without the *tet*O insertions (Fig. 4).

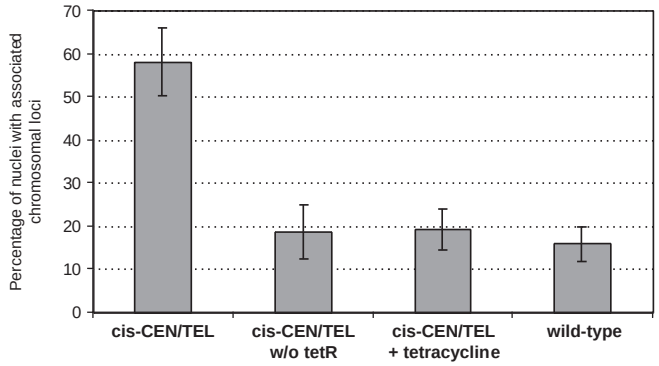


**Fig. 4.** Association frequencies of the centromere-near with the telomere-near region in haploid strains (see Fig. 1B), monitored by adjacent FISH probes (probes 1 and 2, see Fig. 1C). Only in the presence of both the non-allelic *tet*O repeats and the TetR protein is the association high. With *tet*O only, or after inhibition of TetR binding to *tet*O by addition of tetracycline, association is as low as in the wildtype. The experiments were repeated five times and 50 nuclei were evaluated in each.

### The association of tetO repeats is cell cycle-dependent

In stationary cultures (cell density ≥2×10$^8$ cells/ml), associations of *tet*O sites were more frequent than in cycling cultures. In strains with the *tet*O repeats at allelic positions (CEN/CEN and TEL/TEL) the association of the GFP signals reached almost 90% and *tet*O repeats at non-allelic positions (CEN/TEL) were associated in up to nearly 80% of nuclei (Fig. 3). To test whether associations in cycling cultures were cell cycle-dependent, we arrested cells at S-phase by hydroxyurea. These cells showed small buds and short bipolar spindles (Cheeseman et al., 2001). Associations were greatly reduced for all pairs of *tet*O inserts (CEN/CEN, 32.5±2.9%; TEL/TEL, 47.1±7.1%; trans-CEN/TEL, 7.1±1.0%, cis-CEN/TEL, 15.7±1.5%) (Fig. 2B). (We can exclude that the presence of two signals was caused by signal duplication at a single replicated locus, as we never observed three or four signals. Also, we never observed two signals in strains with a single *tet*O repeat.)

The disruption of associations in S-phase was partially restored prior to or during subsequent mitosis. For the identification of anaphase cells, spindles were immunolabelled with an antibody against tubulin. Anaphase cells possessed a long spindle and large buds, and had their elongated nucleus localized half-way through the bud-neck. It was found that CEN/CEN associations were maintained or re-established in both half nuclei in 74% of anaphase cells ($n$=100) (Fig. 2C) and TEL/TEL associations in 64% ($n$=100) (Fig. 2D). By contrast, only 40% ($n$=100) of half nuclei showed CEN/TEL associations (Fig. 2E). It is plausible to assume that the anaphase orientation, with the centromeres migrating with the spindle poles and the telomeres trailing, is detrimental to the re-association of centromere-near and telomere-near *tet*O sites but may promote re-association of allelic *tet*O sites. The highest levels of association of ectopically inserted *tet*O repeats are possibly reached during $G_1$ when the anaphase orientation of chromosome arms is partially relaxed.

### The association of *tet*O repeats perturbs normal chromosome arrangement

Although the association of allelic *tet*O repeats caused the alignment of flanking homologous chromosome regions, the global nuclear organization with roughly parallel chromosome arms and clustered centromeres around the SPB (Rabl-like configuration) (Jin et al., 2000), remained unaffected. However, the association of *tet*O arrays at non-allelic positions (trans-CEN/TEL and cis-CEN/TEL) would be expected to lead to the deviation of chromosome *V* from this order. We measured the distances from the SPB of FISH-labelled centromeric (probe 1) and telomeric (probe 2) sites of chromosome *V* in the wild-type and in the trans-CEN/TEL strain. As can be seen from Fig. 5, in the WT centromeric regions were near the SPB and the telomeric regions away from it, as expected. In the trans-CEN/TEL strain, always one of the two telomeric regions was as close to the SPB as the centromere. (By GFP we confirmed that it was the *tet*O-

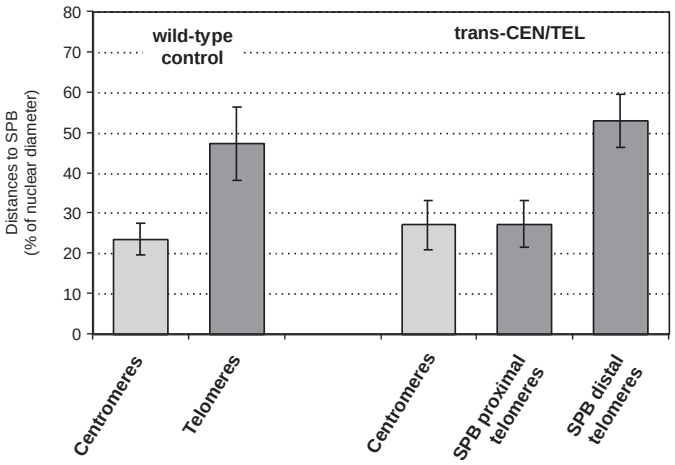


**Fig. 5.** Recruitment of telomeric chromosome regions to the centromeric pole of the nucleus due to *tet*O-mediated centromere-telomere association. The positions of centromeres and telomeres of chromosomes *V* were highlighted by site-specific FISH probes (probes 1 and 2, see Fig. 1C) in different colours, and the SPB (marking the centromeric pole) by simultaneous immunostaining (see Fig. 2F,G). Distances of signals to the SPB were measured and given as a percent of the nuclear diameter.

carrying telomeric region that was near the SPB; data not shown.) Therefore, *tet*O associations cause the right arm of chromosome V to form a loop within the nucleus.

## Discussion

### Possible mechanisms of *tet*O-mediated chromosome associations

We microscopically investigated yeast cells that have exogenous repetitive *tet*O sequences integrated into chromosomes. In these cells, ectopically expressed GFP-TetR fusion protein binds to the *tet*O repeats and highlights them by fluorescence. In strains with *tet*O inserts at two chromosomal loci, we frequently observed the presence of a single GFP signal (Fig. 2A). This could either reflect the close association of the chromosomal regions in which the *tet*O repeats were inserted, or the association of the transgenic sequences themselves.

Somatic pairing as the cause of *tet*O associations would be plausible for *tet*O sequences integrated at allelic chromosome positions and on the condition that an appreciable degree of somatic pairing exists. However, here we showed that associations of chromosome regions with *tet*O repeats are much more frequent than associations in the absence of *tet*O inserts, and that this is true both for allelic and ectopic pairs of *tet*O sites.

Thus, association of chromosome regions is conferred by *tet*O integrations. A previous study found that, in wheat, multiple transgene integration sites were brought together at interphase in spite of their considerable distances on a chromosome; the authors suggested that an ectopic pairing mechanism might act between them (Abranches et al., 2000). In a study similar to ours, Aragon-Alcaide and Strunnikov integrated *tet*O and *lac*O tandem repeats at allelic and ectopic chromosome regions and detected them by the binding of GFP-fused repressor molecules (Aragon-Alcaide and Strunnikov, 2000). They found frequent associations between *tet*O and *lac*O sites, respectively, and reached the conclusion that the repeated arrangement of DNA sequences can promote and stabilize interactions that are based on DNA sequence homology.

Here, we show that the association of *tet*O sequences requires TetR and therefore does not reflect an inherent property of tandem repeats per se. It is likely that TetR-GFP molecules bind to several *tet*O tracts, which physically connects operator sequences and their flanking regions from different chromosomal loci. It had been shown previously in vitro that tetrameric Lac repressor (LacI) can bind two *lac* operators on different DNA molecules simultaneously (Kramer et al., 1987). In addition, it was demonstrated in vivo that a tetramerizing GFP-Lac repressor fusion could hold pairs of sister chromatids together by linking integrated *lac* operator repeats (Straight et al., 1996). Other studies excluded the possibility that associations are generated by the interaction between Lac repressor molecules as they used constructs with the tetramerization domain deleted (Aragon-Alcaide and Strunnikov, 2000; Chen and Matthews, 1992).

TetR binds to *tet*O as a dimer (Hillen and Berens, 1994) but unlike LacI, it does not possess a C-terminal tetramerizing domain, and a TetR dimer can bind only a single *tet*O motif (W. Hillen, personal communication). Therefore it must be assumed that oligomerization, which enables TetR to link separate *tet*O loci, occurs under the special conditions of ectopic expression or expression as a GFP fusion protein. It might be speculated that, also in the case of non-tetramerizing LacI, it is the modification of the protein by the GFP-fusion that causes the association with more than one *lac* operator.

To explore the possibility that the fusion with GFP promotes TetR oligomerization, nuclear *tet*O distribution will be studied in cells that express untagged TetR. Furthermore, *tet*O repeat numbers could be increased to enhance a possible weak tendency of tandemly repeated chromosomal DNA sequences to associate autonomously in the absence of potentially linking regulatory proteins. If transgenes were found to associate, then it would be worth constructing tandem repeat arrays of yeast endogenous sequences to test whether they share this property.

### Ectopic *tet*O associations interfere with the chromosomal order within nuclei

In yeast interphase nuclei, chromosomes are oriented with their centromeres to one pole and the ends towards the opposite pole (Jin et al., 1998). This arrangement is reminiscent of the Rabl-configuration found in various higher eukaryotes. Consequently, loci that are the same distance from the centromere occupy the same latitude of the nucleus with respect to the centromeric pole (Jin et al., 1998). This causes allelic loci to be on average in closer proximity than two randomly selected loci. In fact, FISH labelling of specific chromosomal loci has shown that loci of the same centromeric distance are associated in as many as 24% of nuclei if they are allelic and in slightly less (10%) if they are nonallelic (J.F., Q. Lin, A.L. and J.L., unpublished). Previous studies observed an even higher preference for allelic loci to associate and presented additional evidence for their physical interaction, which were interpreted by a tendency of

homologous chromosomal regions to engage in transient somatic pairing (Burgess et al., 1999; Burgess and Kleckner, 1999). These observations suggest that the nonrandom arrangement of chromosomes within nuclei promotes the association of *tet*O inserts at allelic chromosomal sites.

However, TetR not only links DNA tracts that occupy the same region of the nucleus: if *tet*O repeats are inserted at non-allelic chromosomal loci, TetR-mediated associations are also strong enough to perturb the polarized chromosome arrangement. Whereas normally the roughly parallel orientation of chromosome arms between the centromeric and the telomeric nuclear pole prevails in interphase nuclei, a centromere-near and a telomere-near tandem *tet*O repeat can bring together chromosomal regions from the two opposite nuclear domains. We have shown for chromosome *V* that the chromosome end loops back to the centromeric pole whereas the centromere maintains its position (Fig. 5). This confirms that the centromeres are physically linked to the SPB during interphase, as was proposed previously (Jin et al., 2000), rather than left assembled near the pole as a consequence of the preceding anaphase orientation.

The tethering of centromeric and telomeric chromosome regions via TetR-mediated *tet*O associations implies that these regions must at least transiently contact each other, since a long-range interaction of *tet*O sites is difficult to imagine. This suggests that, in spite of the highly ordered nuclear architecture (Jin et al., 2000), there is sufficient stirring of the nuclear contents. As was shown by Marshall et al., there is a diffusion of chromatin in living cells, which is probably powered by Brownian motion but confined in its extent by microtubules (Marshall et al., 1997b). Furthermore, Heun et al. observed in time-lapse experiments dramatic movements of GFP-tagged chromosomal sites over distances as large as a third of the nuclear diameter, within seconds (Heun et al., 2001). This chromatin motion could have the capacity to lead to the initial contacts of ectopic *tet*O inserts.

### The possible contribution of protein-mediated associations to nuclear architecture

There is an increasing number of cases known where protein-DNA interactions appear to function in the association of specific chromosome regions. The *Drosophila* protein *zeste* can self-associate and can thereby possibly spatially link chromosomal loci to which it binds (Bickel and Pirrotta, 1990). A similar linking effect could be exerted by the sequence-specific DNA-binding members of the mammalian Ikaros family of transcription factors that recognize related DNA sequences and are capable of dimerizing with themselves and other family members (Brown et al., 1997; Cobb et al., 2000; Perdomo et al., 2000). In addition, the ectopic pairing of heterochromatin in *Drosophila* is possibly mediated by the self-association of heterochromatin-binding proteins (Dernburg et al., 1996). The latter authors found that a heterochromatic insertion at the *brown* locus caused its relocalization to the centromeric pole of nuclei due to the physical association of the insert with centromeric heterochromatin.

The question is whether protein-mediated associations of a similar kind play a role in the establishment or maintenance of nonrandom interphase chromosomal arrangement in yeast, and in particular the transient homologous chromosome associations that have been reported to occur in *S. cerevisiae* (Burgess and Kleckner, 1999; Burgess et al., 1999). In this context it may be interesting to note that, like *tet*O associations, these somatic associations are disrupted during S-phase (Burgess et al., 1999). The linkage of *tet*O repeats at ectopic chromosomal sites by TetR is sufficiently strong to perturb the normal polarized orientation of chromosome arms, demonstrating that protein-mediated specific chromosome associations can be quite robust. However, in this case it is likely that the strength of associations results from the tandemly repeated nature of *tet*O inserts providing a large number of binding sites for TetR molecules. Unlike in eukaryotes, tandem repeats are not common in the yeast genome. However, a large number of weak associations along a pair of homologous chromosomes could cause somatic pairing. Cook proposed that promoters, enhancers or other elements of transcription regulation that are part of a DNA polymerizing complex could bind to a homologous DNA molecule in trans (Cook, 1997). Such an event would induce only a weak link between homologous DNAs, but interactions between thousands of such ‘transcription factories’ could act cooperatively to lead to stable somatic pairing.

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