Enhancement of Telomere-Plasmid Segregation by the X-Telomere Associated Sequence in *Saccharomyces cerevisiae* Involves *SIR2*, *SIR3*, *SIR4* and *ABF1*

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ABSTRACT

We have previously shown that circular replicating plasmids that carry yeast telomere repeat sequence ($TG_{1\text{-}3}$) tracts segregate efficiently relative to analogous plasmids lacking the $TG_{1\text{-}3}$ tract and this efficient segregation is dependent upon *RAP1*. While a long $TG_{1\text{-}3}$ tract is sufficient to improve plasmid segregation, the segregation efficiency of telomere plasmids (TEL-plasmids) is enhanced when the X-Telomere Associated Sequence (X-TAS) is also included on the plasmids. We now demonstrate that the enhancement of TEL-plasmid segregation by the X-TAS depends on *SIR2*, *SIR3*, *SIR4* and *ABF1* in *trans* and requires the Abf1p-binding site within the X-TAS. Mutation of the Abf1p-binding site within the X-TAS results in TEL-plasmids that are no longer affected by mutations in *SIR2*, *SIR3* or *SIR4*, despite the fact that other Abf1p-binding sites are present on the plasmid. Mutation of the ARS consensus sequence within the X-TAS converts the X-TAS from an enhancer element to a negative element that interferes with TEL-plasmid segregation in a *SIR*-dependent manner. Thus, telomere associated sequences interact with $TG_{1\text{-}3}$ tracts on the plasmid, suggesting that the TASs have an active role in modulating telomere function.

LINEAR chromosomes terminate in telomeres, specialized structures composed of repetitive DNA and associated proteins (reviewed in BLACKBURN 1991; ZAKIAN 1989). In *Saccharomyces cerevisiae*, terminal telomere repeat sequences are composed of double stranded $(dTG_{1\text{-}3}/dC_{1\text{-}3}A)_n$ termed $TG_{1\text{-}3}$ tracts. There are two classes of *T*elomere-*A*ssociated *S*equences (TASs) located just internal to the terminal $TG_{1\text{-}3}$ tracts: Y'-Telomere Associated Sequences (Y'-TASs) and X-Telomere Associated Sequences (X-TASs) (CHAN and TYE 1983b; LOUIS and HABER 1992).

Although TASs are present in the chromosomes of most eukaryotes (reviewed in ZAKIAN 1989), the function of TASs is not evident. In *S. cerevisiae*, both X and Y' TASs include sequences that can function as Autonomously Replicating Sequences (ARSs) on plasmids (CHAN and TYE 1983b). However, there is no requirement for ARS function near the telomere on artificial chromosomes (WELLINGER and ZAKIAN 1989), and removal of TASs from native chromosome derivatives does not have major effects on chromosome stability (MURRAY and SZOSTAK 1986). In *Drosophila*, TASs may assume the function of telomeres since chromosomes lacking telomere repeat sequence tracts are stable (BIESSMANN *et al.* 1992; LEVIS 1989).

In *S. cerevisiae*, telomeres and the silent mating type loci (*HML* and *HMR*) share some common features. Genes inserted adjacent to a telomere become transcriptionally repressed, a property termed telomere position effect (GOTTSCHLING *et al.* 1990). Genes located within the *HM* loci are also transcriptionally repressed (reviewed in ALBERTS and STERNGLANZ 1990; LAURENSON and RINE 1992; PILLUS 1991). Transcriptional repression at both telomeres and the *HM* loci requires the products of *SIR2*, *SIR3* and *SIR4*, (APARICIO *et al.* 1991; IVY *et al.* 1986; KLAR *et al.* 1979; MARSHALL *et al.* 1987; RINE and HERSKOWITZ 1987). Silencing at the HM loci and at yeast telomeres is thought to involve the establishment and maintenance of an inaccessible or closed chromatin structure (reviewed in LAURENSON and RINE 1992; SANDELL and ZAKIAN 1992). There is no evidence that Sir2p, Sir3p or Sir4p bind specific DNA sequences (BUCHMAN *et al.* 1988a; SHORE *et al.* 1987). Histones in strains overexpressing Sir2p are hypoacetylated, suggesting that Sir2p may be a protein deacetylase (BRAUNSTEIN *et al.* 1993). Sir3p dosage influences the degree of silencing at telomeres (RENAULD *et al.* 1993). Sir4p is a very large, hydrophilic protein (180 kDa) that shares some homology with the coiled-coil domain of the human nuclear lamins (DIFFLEY and STILLMAN 1989; MARSHALL *et al.* 1987). Sir3p and Sir4p appear to interact since overexpression of Sir3p can suppress a *sir4* mutation (IVY *et al.* 1986).

At the *HM* loci there are different combinations of three binding sites (Abf1p-binding site, Rap1p-binding site, and ARS consensus sequence (ARSC)) that are important for transcriptional silencing (BRAND *et al.* 1987; MAHONEY *et al.* 1991). These sequences are also found at telomeres: ARSs within TASs generally include an ARSC (CHAN and TYE 1983a, b) and many

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also contain an Abf1p-binding site (BISWAS and BISWAS 1990; EISENBERG *et al.* 1988).

Abf1p and Rap1p are essential, multifunctional DNA-binding proteins involved in the transcriptional activation of many genes (BRINDLE *et al.* 1990; BUCHMAN *et al.* 1988a, b; CHAMBERS *et al.* 1990; DELLASETA *et al.* 1990a, b; HALFTER *et al.* 1989a, b; HUET *et al.* 1985; RHODE *et al.* 1990; SHORE and NASMYTH 1987; SHORE *et al.* 1987; VIGNAIS *et al.* 1987). Abf1p-binding sites are located within most ARSs and some Abf1p-binding sites enhance replication at these ARSs (BUCHMAN *et al.* 1988a; CAMPBELL and NEWLON 1991; DIFFLEY and STILLMAN 1988; WALKER *et al.* 1990). Rap1p-binding sites are found upstream of many genes and are present within the $TG_{1\text{-}3}$ repeats at telomeres (BERMAN *et al.* 1986; BUCHMAN *et al.* 1988a; LONGTINE *et al.* 1989; SHORE and NASMYTH 1987). *rap1* mutants and cells overexpressing *RAP1* have altered telomere tract length, suggesting that Rap1p is involved in $TG_{1\text{-}3}$ tract length regulation (CONRAD *et al.* 1990; KYRION *et al.* 1992; LUSTIG *et al.* 1990).

The ARSC is found within most functional ARSs and is necessary, but not sufficient, for replication initiation (reviewed in CAMPBELL and NEWLON 1991). A number of factors that interact specifically with the ARS consensus have been identified (BELL and STILLMAN 1992; DIFFLEY and COCKER 1992; HOFMANN and GASSER 1991; SCHMIDT *et al.* 1991). Two of these origin recognition complex proteins (ORCs) have been isolated in a screen for genes important for silencing at HMR (LAURENSON and RINE 1992).

Circular yeast plasmids that contain only autonomously replicating sequences (ARS-plasmids) are not segregated efficiently and are only present in a small percentage of cells, even under selection for the plasmid. Under nonselective conditions ARS-plasmids are rapidly lost (>20% per division, loss rate >0.20). Yeast plasmids that segregate more efficiently than ARS-plasmids require specific DNA sequences, *in cis*, and specific gene products, *in trans*. The 2-μm plasmids include the *STB/REP3* site which is recognized by the Rep1p and Rep2p gene products encoded by the endogenous 2-μm plasmid (BROACH and VOLKERT 1991). CEN-plasmids include a functional centromere sequence and are bound by factors also important for chromosomal centromere function (reviewed in CARBON and CLARKE 1990).

Plasmids carrying telomeric DNA (including a TAS) and plasmids carrying the *HMR E* region are stabilized due to improved plasmid segregation (KIMMERLY and RINE 1987; LONGTINE *et al.* 1992). Efficient segregation of either of these plasmids involves Sir2p, Sir3p and Sir4p (KIMMERLY and RINE 1987; LONGTINE *et al.* 1993). While plasmids carrying telomeric DNA retain some segregation function in *sir2*, *sir3* or *sir4* mutant strains (LONGTINE *et al.* 1993), *HMR E*-plasmid segregation is completely dependent upon *SIR2*, *SIR3* and *SIR4* (KIMMERLY and RINE 1987; LONGTINE *et al.* 1993). Conversely, these plasmids are affected differently in some *rap1* mutant strains: the segregation of plasmids carrying telomeric DNA is dramatically reduced while the segregation of *HMR E*-plasmids is not reduced in these strains (LONGTINE *et al.* 1992).

In our previous studies, we used the term "TRS-plasmid" to refer to plasmids carrying *t*elomere *r*epeat *s*equences ($TG_{1\text{-}3}$ tracts) (LONGTINE *et al.* 1992, 1993). In this study we discovered that these plasmids have two components which interact differently with different gene products. Accordingly, we now use the term "TEL-plasmid" (instead of "TRS-plasmid") to refer to any plasmid carrying telomere repeats whether or not they include telomere associated sequences. There are two types of TEL-plasmids: T+X plasmids contain $TG_{1\text{-}3}$ tracts and an X-TAS, including an ARS within the X-TAS (X-ARS), and T-plasmids include $TG_{1\text{-}3}$ tracts as the only telomeric sequence on the plasmid and are replicated by a nontelomere-derived ARS. T-plasmids segregate more efficiently than ARS plasmids and T+X-plasmids segregate more efficiently than T-plasmids (LONGTINE *et al.* 1992), suggesting that the X-TAS enhances the segregation of T+X-plasmids. In this paper, we demonstrate that T+X-plasmid segregation requires the products of *SIR2*, *SIR3*, *SIR4* and *ABF1*, while T-plasmid segregation does not require these gene products. The Abf1p-binding site within the X-TAS is required for the enhancement of TEL-plasmid segregation by *SIR2*, *SIR3* and *SIR4*. Mutation of the ARS consensus within the X-TAS disrupts TEL-plasmid segregation in a *SIR*-dependent manner. In contrast, *RAP1* is required for the segregation of all T+X-plasmids and T-plasmids.

MATERIALS AND METHODS

***Escherichia coli* strains and DNA manipulations:** *E. coli* strains used were MC1061 {*ΔaraD139*, *Δ(ara-leu)7697*, *Δ(lac)X74*, *galU*$^-$, *galK*$^-$, *hsr*$^-$, *hsm*$^+$, *strA*} (CASADABAN and COHEN 1980), XL1-blue {*recA1*, *endA1*, *gyrA96*, *thi*, *hsdR17*, *supE44*, *relA1*, *lac* [F′, *proAB*, *lacIq*, *ZΔM15*, Tn*10*(tetR)]} (Stratagene), CJ236 {*dut-1*, *ung-1*, *relA1* [pCJ105(Cmr)]} (KUNKEL *et al.* 1987) and MC1066F′ {*hsd(r*$^-$, *m*$^-$), *trpC9830*, *leuB600*, *pyrF*::Tn*5*(Km^R), *lacΔx74*, *strA galU*, *galK*, [F′*proAB*, *lacI*^{q}, *lacZΔM15* Tn*10*]}. MC1066F′ was constructed by mobilization of the F′ factor from XL1-blue into MC1066 (CASADABAN *et al.* 1983). Plasmid DNA was isolated using standard protocols (AUSUBEL *et al.* 1989) and *E. coli* cells were transformed by electroporation (DOWER *et al.* 1988).

Restriction enzymes (Promega and New England Biolabs), T4 DNA polymerase and T4 DNA ligase (New England Biolabs) were used according to the manufacturer's instructions. The DNA sequence was determined using Sequenase 2.0 (United States Biochemical) according to manufacturer's instructions.

Yeast strains, media and plasmids: The genotype and source of yeast strains used in this work are listed in Table 1. Media for *S. cerevisiae* were prepared according to standard methods (ROSE *et al.* 1990).

Plasmids used in this study are illustrated in Figure 1. The

TABLE 1

***S. cerevisiae* strains**

Strains	Alias	Genotype	Source
YJB42	S150-2B [cir^0]	*MAT***a** *ura3-52 leu2-3,112 his3Δ [trp1-289]^1* [cir^0]	J. BROACH
YJB43	S150-2B [cir$^+$]	*MAT***a** *ura3-52 leu2-3,112 his3Δ trp1-289*	J. BROACH
YJB46		YJB42 *sir3Δ 1::LEU2*	J. BROACH
YJB203		YJB42 *ade2Δ 1*	LONGTINE *et al.* (1993)
YJB306		YJB203 *sir2Δ 2::HIS3*	LONGTINE *et al* (1993)
YJB202		YJB203 *sir3Δ 1::LEU2*	This work
YJB307		YJB203 *sir4Δ 2::HIS3*	LONGTINE *et al.* (1993)
YJB234	JCA30	*MAT* **a** *trp1Δ his3Δ 200 ura3-52 lys2-801, ade2-1, gal$^-$, ABF1-HIS3* [cir+]	RHODE *et al.* (1992)
YJB236	JCA35	YJB234 *abf1-5*	RHODE *et al* (1992)
YJB209	YDS2	*MATα ade2-1 can1-100 his3-11,-15 leu2-3,-112 trp1-1 ura3-1* [cir$^+$]	KURTZ and SHORE (1991)
YJB208	YDS410	YDS2 *rap1-5*	KURTZ and SHORE (1991)

construction of pYET1, pETACC, pEXACC, pEXACC-Δ2 μm, pYET-0, pRL51 and pRLTet have been described previously (LONGTINE *et al.* 1992). pRL51-ARS1 and pRLTet-ARS1 were derived from pRL51 and pRLTet, respectively, by replacement of the entire X-TAS fragment with an *ARS1-TRP1* fragment from YRp17, using *in vivo* recombination (MA *et al.* 1987).

To generate pYET1-μabf1, an Abf1p-binding site in the X-TAS was mutated by oligonucleotide directed mutagenesis (KUNKEL *et al.* 1987) of pRAXACC to yield pRAXACC-μabf1. (pRAXACC was constructed by insertion of the *Bam*HI-*Eco*RI X-TAS fragment from pEXACC (LONGTINE *et al.* 1992) into the *Bam*HI and *Eco*RI sites within the polylinker of pASZ10 (STOTZ and LINDER 1990)). pYET1-μabf1 was made by transfer of the μabf1-*Xho*I region of pRAXACC-μabf1 to pYET1 by *in vivo* recombination (MA *et al.* 1987). Plasmid DNA from transformants was screened to identify the appropriate recombinants.

The wild-type X-TAS, 5′-TCGC**ATCATT**ATGC**ACG**GC ACTTGCCTCAGC-3′, includes an Abf1p consensus sequence (bold) (BUCHMAN *et al.* 1988a). In addition there are two nucleotides (double underlined) that are not in the published sequence (BUTTON and ASTELL 1986). We used oligonucleotide μABFXho, 5′-TCGC**ATCAT**TATCTcgaGCTTGCCTCAGC-3′, to replace the region of the Abf1p site that is most important for binding Abf1p (bold ACG in wild-type above (DELLASETA *et al.* 1990b)) with a *Xho*I restriction site (single underline). Changed nucleotides are indicated as lower-case letters. DNA sequence analysis confirmed that the μabf1 site is 5′-TCGCATCATTATCTCGAGCTTGCCTCAGC-3′. When this same combination of nucleotide changes is present in the Abf1p-binding site at *YP2-TUB*, the altered site (mir1) does not bind Abf1p (HALFTER *et al.* 1989b). In addition, gel retardation assays with a labeled pRAXACC X-TAS fragment confirmed that the pRAXACC-μabf1 site does not compete for binding to the Abf1p-binding site in the X-TAS of pRAXACC.

pYET1-μarsc was generated by two step polymerase chain reaction (PCR) (KAMMANN *et al.* 1989). In the first step, the μXARS-*Sma*I primer (5′ TAAAATCACCTAcccgggAAAATATTCTAC-3′, where the cccggg is replacing the wild-type ARS consensus) and the M13 universal sequencing primer (USB, Cleveland) were used with pRAXACC as the template in PCR to produce a fragment of the X-TAS carrying the mutated ARS consensus (μarsc). In the second step, *Eco*RI-digested-pRAXACC and the PCR product from the first step were gel purified and used as templates along with the M13 universal primer and the M13 reverse sequencing primer (USB, Cleveland). The product of the second PCR was cotransformed into yeast along with *Eco*RI-digested YEPlac195 (GIETZ and SUGINO 1988) to obtain pEXACC-μarsc by *in vivo* recombination (MA *et al.* 1987). The DNA sequence of the resulting plasmid included two additional nucleotide changes within the region covered by XARS-*Sma*I. Thus, in pEXACC-μarsc, the wild-type sequence 5′TAAAATCACCTAAACATAAAAATATTCTAC-3′ (ARS consensus is underlined) was changed to 5′ TAAAAT-CAaaTAcccgggAAAATATTCTAC-3′ (mutated bases are in lower case). Despite extensive screening, we did not recover clones carrying XARS-*Sma*I that did not also suffer deletions and/or rearrangements elsewhere in the plasmid. pYET1-μarsc was derived from pEXACC-μarsc and pYET1 by *in vivo* recombination (MA *et al.* 1987).

Plasmid stability measurements: Plasmid loss rate (*lr*) is defined: $lr = 2[1 - exp\{(1/n)\,ln(F_1/F_0)\}]$ where F_0 and F_1, respectively, correspond to the fraction of plasmid bearing cells before and after nonselective growth and where n is the number of generations between measurements F_0 and F_1. Loss rates were determined as previously described (LONGTINE *et al.* 1992). A minimum of four independent loss rate measurements were performed and the rank sum test (SNEDECOR and COCHRAN 1980) was used to evaluate statistical significance. Loss rate measurements for *abf1-5* and *rap1-5* mutants were performed on cells grown on plates to minimize cell clumping (LONGTINE *et al.* 1992).

RESULTS

The X-TAS enhances the segregation of TEL-plasmids: In previous studies, we observed that a TEL-plasmid that included a long $TG_{1\text{-}3}$ tract and 1.1 kb of X-TAS segregated efficiently (pYET1, loss rate 0.03) while a derivative of the plasmid that lacked most of the X-TAS was lost at a higher rate (pETACC, loss rate 0.10) (LONGTINE *et al.* 1992). The loss rate of pETACC was intermediate between the loss rate of pYET1 and the loss rate of a derivative ARS plasmid that lacked the $TG_{1\text{-}3}$ tract and retained the X-TAS, including the X-ARS (pYET-0, loss rate 0.20) (LONGTINE *et al.* 1992). These results suggested that a long $TG_{1\text{-}3}$ tract is sufficient to improve plasmid segregation and that the presence of the adjacent X-TAS enhances the segregation ability of telomere repeat sequences.

To test whether the X-TAS enhancement of TEL-plasmid segregation was dependent upon the relative positions of the X-TAS and the $TG_{1\text{-}3}$ tract on the plasmids, we compared the loss rates of T– and T+X-plasmids in which the X-TAS is not adjacent to the $TG_{1\text{-}3}$

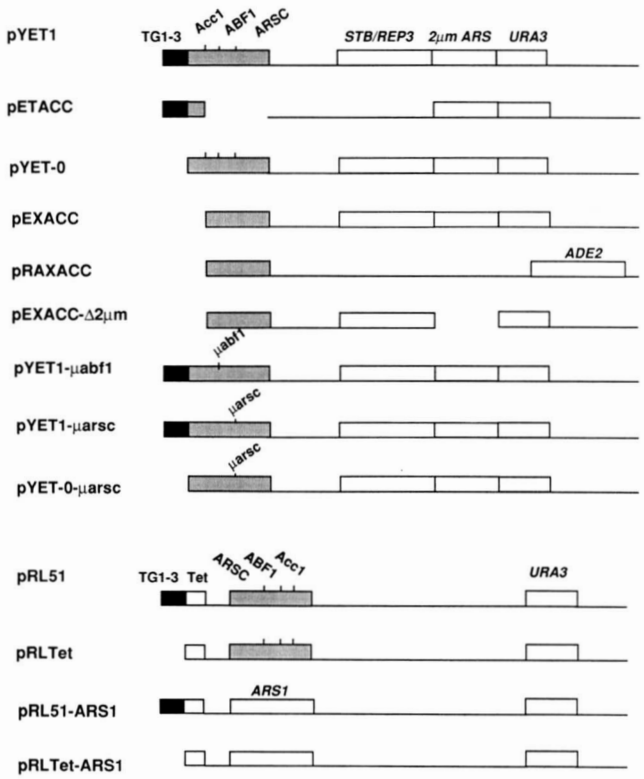

FIGURE 1.—Plasmids used in the study. All the plasmids are depicted as being linearized at the $TG_{1\text{-}3}$/bacterial plasmid vector junction. The telomere repeat sequences are oriented such that the chromosomal terminus would be at the left end of the fragments. The thin lines indicate bacterial vector sequences. The shaded box indicates X-TAS. $TG_{1\text{-}3}$ tracts are depicted as dark boxes. The *Acc*1 restriction site used for some of the constructions is illustrated. Abf1, the Abf1p-binding site within the X-TAS; ARSC, the ARS core consensus sequence within the X-TAS. Boxes are yeast genes *URA3* and *ADE2*, the *ARS1* sequence, and the *STB/REP3* stabilization site and 2-μm ARS sequences, both derived from 2-μm plasmids. pYET1 includes the 1.2-kb telomere proximal portion of the X-TAS and the adjacent 365 bp of $TG_{1\text{-}3}$ from the left arm of chromosome *III* (BUTTON and ASTELL 1986) inserted in YEplac195, a 2 μm-based vector (GIETZ and SUGINO 1988). pETACC, pYET-0, pEXACC and pEXACC-Δ2 μm are deletion derivatives of pYET1 (LONGTINE *et al.* 1992). pRAXACC was derived from pEXACC. The gaps in the figure indicate regions deleted from the plasmids. pYET1-μabf1, pYET1-μarsc and pYET-0-μarsc carry the mutated Abf1p-binding site (μabf1) or the mutated ARS consensus sequence (μarsc). pRL51 includes 400 bp of $TG_{1\text{-}3}$ and 1.2 kb of X-TAS. The X-TAS is located 1.9 kb from the $TG_{1\text{-}3}$ on pRL51. pRLTet is identical to pRL51 except that it lacks yeast $TG_{1\text{-}3}$ (LONGTINE *et al.* 1992). Both pRL51 and pRLTet include a short tract of *Tetrahymena* telomere repeat sequence (Tet). pRL51-ARS1 and pRLTet-ARS1 were derived from pRL51 and pRLTet, respectively, by replacement of the X-TAS with an *ARS1-TRP1* fragment.

tract (Figure 2). In pRL51, the X-TAS is located 1.9 kb from the $TG_{1\text{-}3}$ and is inverted relative to its orientation in the chromosome and in pYET1 (Figure 1) and the X-ARS is the only ARS on the plasmid. pRL51-ARS1 is a T-plasmid derived from pRL51; the X-TAS sequence has been replaced with *ARS1* (Figure 1). The loss rate of the

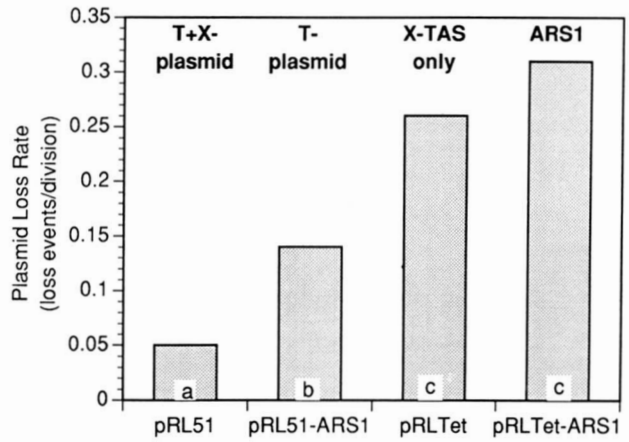

FIGURE 2.—Enhancement of TEL-plasmid segregation by the X-TAS. Median loss rate values of TEL-plasmids and their controls in strain YJB42. a, b and c indicate distinct classes of loss rates that are significantly different from each other (5% level).

T-plasmid (pRL51-ARS1) was significantly higher than the loss rate of the T+X-plasmid (pRL51), and was significantly lower than the loss rates of analogous ARS plasmids that carry either *ARS1* (pRLTet-ARS1) or the X-ARS alone (pRLTet) (Figure 2). Thus, the X-TAS enhances the segregation of TEL-plasmids even when the X-TAS is not adjacent to the $TG_{1\text{-}3}$ tract. We previously demonstrated that a series of related plasmids that have the identical $TG_{1\text{-}3}$ tract inserted at different distances and orientations from the X-TAS (pRL53, pRLTet-53a and pRLTet-53b) all have similar loss rates (LONGTINE *et al.* 1992). All of these T+X-plasmids were more stable (loss rates ≤ 0.08) than the analogous T-plasmid measured in this study (pRL51-ARS1, loss rate 0.14, Figure 2), demonstrating that the X-TAS enhancement of TEL-plasmid segregation is independent of the distance between, and relative orientation of, the X-TAS and $TG_{1\text{-}3}$ tract. In addition, the ability of the X-TAS to enhance TEL-plasmid segregation occurs when the X-ARS is the only ARS on the plasmid (pRL51, Table 1) as well as when there is an additional ARS sequence on the plasmid (pYET1, (LONGTINE *et al.* 1992)).

X-enhancement of TEL-plasmid segregation requires *SIR2*, *SIR3* and *SIR4*: Previous work from this lab showed that mutations in *SIR2*, *SIR3* or *SIR4* cause a T+X-plasmid to become less stable. The loss rate of T+X-plasmid pRL51 ($TG_{1\text{-}3}$ and TAS sequences not adjacent) in *sir* mutant strains was intermediate between the loss rate of this T+X-plasmid in wild-type cells and the loss rate the analogous ARS plasmid in wild-type cells (LONGTINE *et al.* 1993). Similarly, the loss rate of T+X-plasmid pYET1 ($TG_{1\text{-}3}$ and TAS sequences adjacent) in *sir* mutant strains is intermediate between the loss rate of pYET1 in wild-type cells and the loss rate of pYET-0, the analogous ARS plasmid, in wild-type cells (Figure 3). Thus, *sir* mutations influence different T+X-plasmids in the same way, irrespective of the relative orientation and distance of the $TG_{1\text{-}3}$ tracts and X-TAS

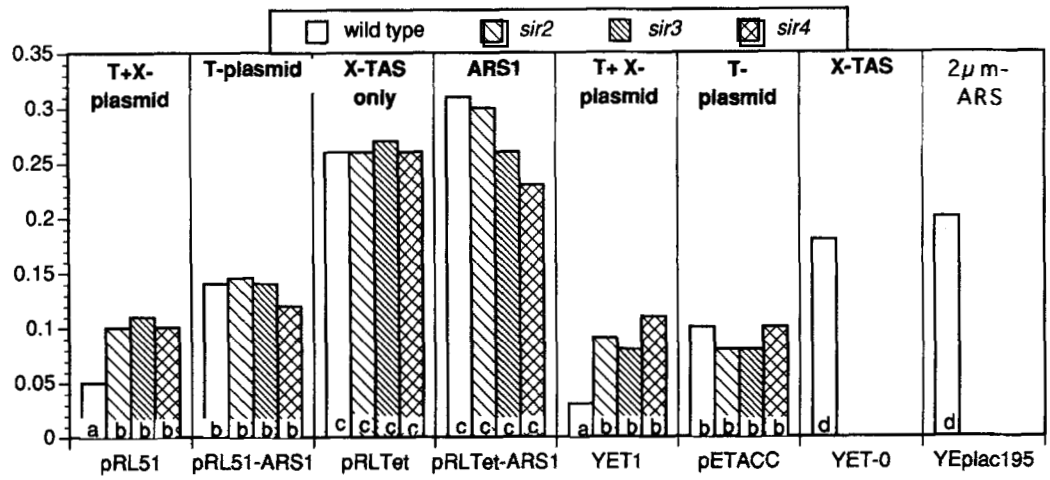

FIGURE 3.—Effects of *sir* mutations on plasmid loss rates. Median loss rate values of TEL-plasmids in isogenic wild type and sir mutant strains. a, b, c and d indicate distinct classes of loss rates that are significantly different from each other (5% level).

on the plasmids.

The intermediate loss rate values of T+X-plasmids in *sir* mutant strains is similar to the loss rate values seen for the analogous T-plasmids in wild-type strains (Figure 2). We analyzed the effects of *sir* mutations on the loss rates of T-plasmids to determine whether the Sir proteins act through the $TG_{1\text{-}3}$. T-plasmids pRL51-ARS1 and pETACC had similar loss rates in wild-type and in isogenic *sir* mutant strains (Figure 3). Furthermore, the T-plasmid loss rates were not significantly changed in isogenic mutant strains carrying two *sir* mutations (*sir2, sir3* or *sir3, sir4)* or in strains carrying complete deletion alleles of either *sir3* or *sir4* (data not shown). Thus, T-plasmid segregation is not dependent upon the presence of one or two *SIR* gene products and the intermediate level of T-plasmid segregation is not due to partial function of some *sir* mutant alleles. These results suggest that the enhancement of T+X-plasmid segregation by the Sir proteins requires the presence of the X-telomere associated sequence.

Role of *ABF1* in TEL-plasmid segregation: There is no evidence that Sir proteins bind DNA directly (BUCHMAN *et al.* 1988a; SHORE *et al.* 1987); therefore, their role in TEL-plasmid segregation may involve other proteins known to bind DNA such as Abf1p and Rap1p. T+X-plasmids include an Abf1p-binding site within the X-ARS element. *SIR*-dependent repression at *HMR E* involves the Abf1p-binding site within the E region (KIMMERLY *et al.* 1988; MCNALLY and RINE 1991). We used a mutant allele of *ABF1* to ask whether Abf1p is involved in TEL-plasmid segregation. *abf1-5* is a temperature sensitive mutant allele that partially impairs the replication function of some ARSs, including ARS1, at 30° (CAMPBELL and NEWLON 1991; RHODE *et al.* 1992). The binding affinity of the *abf1-5* gene product for the Abf1p-binding site is reduced at nonpermissive temperature (RHODE *et al.* 1992).

In the *abf1-5* mutant strain, T+X-plasmid pRL51 was lost 3.3 times more frequently than in the isogenic wild-type strain (Figure 4). The increased loss rate of pRL51 in the *abf1-5* mutant strain could be due to compromised plasmid replication or to decreased TEL-plasmid segregation efficiency. To test the ability of the ARS within the X-TAS (X-ARS) to replicate in *abf1-5* mutant cells, we used two plasmids that require X-ARS replication function for plasmid maintenance. pRL-Tet is an ARS plasmid analogous to pRL51; the X-ARS is the only ARS on the plasmid. It had a high loss rate in wild-type cells and a slightly higher loss rate (1.2 fold) in mutant cells (Figure 4). This result suggests that X-ARS replication may be slightly affected by the *abf1-5* mutation, although the effect was not statistically significant. pEXACC-Δ2 μm includes the 2-μm *STB/REP3* segregation sequence and the X-ARS as the only ARS on the plasmid (Figure 1). In cells carrying the endogenous 2-μm plasmid (cir^+ cells), the 2-μm *REP1* and *REP2* gene products act in *trans* to segregate plasmids that

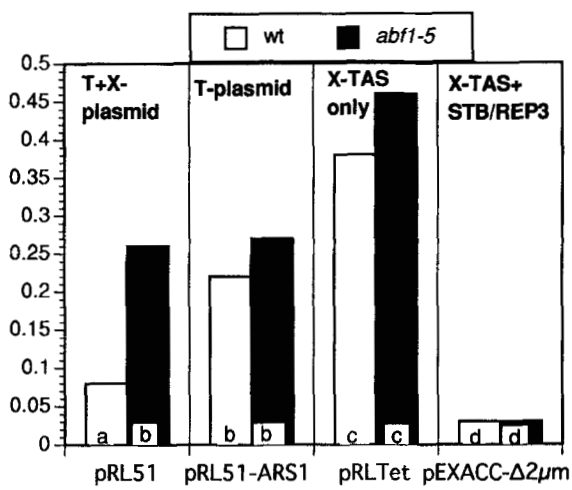

FIGURE 4.—Effects of an *abf1* mutation on plasmid loss rates. Median loss rate values of TEL-plasmids in isogenic wild type and *abf1-5* mutant strains. a, b, c and d indicate distinct classes of loss rates that are significantly different from each other (5% level).

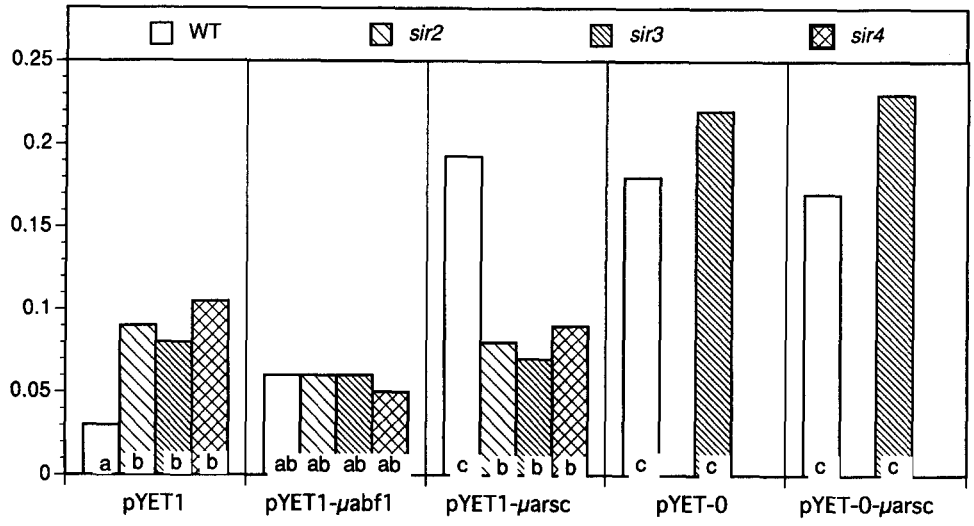

FIGURE 5.—Effects of *cis* mutations μabf1 and μarsc on plasmid loss rates. Median loss rate values of TEL-plasmids in isogenic *sir* mutant strain and wild type. a, b, and c indicate distinct classes of loss rates that are significantly different from each other (5% level). ab indicates that the loss rates are not significantly different from loss rates in either the a or b classes.

carry *STB/REP3 in cis* (reviewed in BROACH and VOLKERT 1991; FUTCHER 1988). pEXACC-Δ2μm has a low loss rate in wild-type *cir*$^+$ cells and in the *abf1-5 cir*$^+$ mutant strain. While the *STB* site may compensate for a slight decrease in replication efficiency, it is clear that the X-ARS can replicate in *abf1-5* mutant cells. Since the *abf1-5* allele causes a significant increase in the loss rate of T+X-plasmid pRL51, the results imply that plasmid segregation function is compromised in *abf1-5* mutant cells.

The *abf1-5* mutation did not cause a significant change in T-plasmid loss rates (pRL51-ARS1, Figure 4). Furthermore, in the *abf1-5* mutant, the T-plasmid and the T+X-plasmid loss rates were not significantly different and were intermediate between the loss rates of the T+X-plasmids in wild-type cells and the loss rates of the analogous ARS plasmid (pRLTet) in both the mutant and wild-type strains. Since the T+X-plasmid segregated like a T-plasmid in the *abf1-5* mutant strain, Abf1p, like Sir2p, Sir3p and Sir4p, is involved in the enhancement of TEL-plasmid segregation by the X-TAS.

The Abf1p-binding site within the X-TAS is involved in the enhancement of TEL-plasmid segregation: Most ARSs include Abf1p-binding sites. T-plasmids include Abf1p-binding sites within the *ARS1* (pRL51-ARS1) or 2-μm ARS (pETACC) present on the plasmids (BUCHMAN *et al.* 1988a). Despite the presence of these Abf1p-binding sites, T-plasmid segregation is not as efficient as T+X-plasmid segregation (Figures 2 and 3). The X-TAS contains a perfect match to the Abf1p-binding site consensus RTCRYNNNNNN*ACG* (where R is purine, Y is pyrimidine and N is any nucleotide) (BUCHMAN *et al.* 1988b; DELLASETA *et al.* 1990a). We mutated the Abf1p-binding site in the X-TAS by replacing the three canonical nucleotides (*ACG*) which are necessary for Abf1p binding to ask whether this site is required for enhanced T+X-plasmid segregation. We constructed pYET1-μabf1, a derivative of pYET1 in which the Abf1p-binding site within the X-ARS no longer contains the nucleotide sequence required for Abf1p binding. This plasmid includes a functional 2-μm ARS in addition to the X-ARS, so that the X-ARS mutation does not abolish plasmid replication. In wild-type cells, the pYET1-μabf1 loss rate was slightly higher than the loss rate of pYET1 (Figure 5). pYET1-μabf1 segregated with similar loss rates in *sir2*, *sir3* and *sir4* mutant strains and in the isogenic wild type strain (Figure 5). Thus, mutation of the X-ARS Abf1p-binding site eliminates the effect of mutations in *SIR2*, *SIR3* or *SIR4* on T+X-plasmid segregation. This result suggests that the Sir proteins interact, directly or indirectly, with the Abf1p-binding site in the X-TAS. Mutations in *sir2*, *sir3*, *sir4* or *abf1* and mutation of the Abf1p-binding site all affect T+X-plasmid segregation in a similar manner; the T+X-plasmid loss rate is increased to an intermediate value that is not significantly different from the loss rate value of T-plasmids.

The role of the ARS consensus sequence within the X-TAS in the enhancement of TEL-plasmid segregation: An ARSC is involved in SIR-dependent repression at the HM loci (BRAND *et al.* 1987; MAHONEY *et al.* 1991; MCNALLY and RINE 1991). We mutated the ARSC within the X-TAS on pYET1 to ask whether this site is required for T+X-plasmid segregation. pYET1-μarsc is derived from pYET1 by replacement of the A/T rich ARSC with a G/C rich sequence (μarsc); this T+X-plasmid includes a functional 2-μm ARS in addition to the mutated X-ARS on the plasmid. In wild-type cells, pYET1-μarsc was lost at a high rate (0.20) indistinguishable from the loss rate of ARS plasmids in this strain (Figure 5). It appears that the 2-μm ARS is fully functional on plasmids carrying the μarsc, since ARS plasmids pYET-0 and pYET-0-μarsc have similar loss rates (Figure 5). This result implies that T+X-plasmid segregation (rather than X-ARS replication) is impaired when the X-ARS is mutated. Interestingly, the *cis* ARSC mutation in pYET1-μarsc affects TEL-plasmid loss rate more dramatically than does deletion of the entire X-TAS, including this ARSC (pETACC, Figure 3). One possible interpretation of this result is that the μarsc mutation abolishes TEL-plasmid segregation by forming

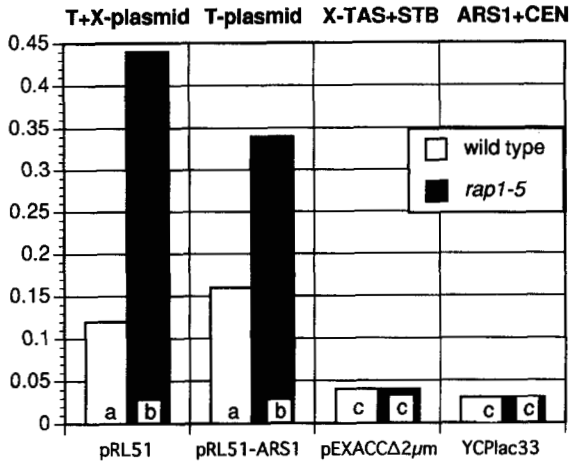

FIGURE 6.—Effect of a *rap1* mutation on TEL-plasmid segregation. Median loss rate values of TEL-plasmids in isogenic wild-type and *rap1-5* mutant strains. a, b and c indicate distinct classes of loss rates that are significantly different from each other (5% level).

a complex in the mutant X-TAS that interferes with (rather than enhances) TEL-plasmid segregation.

In *sir* mutant strains, the pYET1-μarsc loss rate was intermediate (0.07–0.09) between the loss rate of ARS plasmids (≥0.20) and the loss rate of pYET1 in wild-type cells (0.03); it was also similar to the loss rate of pYET1 in these *sir* mutant strains (0.08–0.11). Surprisingly, pYET1-μarsc was more stable in *sir* mutant strains than in the isogenic wild-type strains. It appears that the Sir proteins interact with pYET1-μarsc to mediate the negative effect of X-μarsc on T+X-plasmid segregation. Thus, the Sir proteins influence TEL-plasmid segregation in both negative and positive ways.

***RAP1* interacts with the TRS tract of TEL-plasmids:** Previously, we showed that some temperature sensitive alleles of *RAP1* affect T+X-plasmid loss rates (LONGTINE *et al.* 1992). We measured T-plasmid loss rates in a *rap1-5* mutant strain grown at semipermissive temperature (30°). T-plasmid (pRL51-ARS1) stability was significantly impaired in the *rap1-5* mutant strain (Figure 6). In the *rap1* strain, both T-plasmid and T+X-plasmid segregation were reduced to a similar level (Figure 6).

To control for the possibility that the *rap1-5* mutation affects plasmid replication, we compared the loss rates of an ARS1-CEN plasmid (YCPlac33) in the *rap1-5* mutant and in the isogenic wild-type strain (Figure 6). There was no significant difference in the loss rate of YCPlac33 in the two strains, indicating that the replication of ARS1, like the replication of X-ARS (LONGTINE *et al.* 1992) was not affected in strains carrying the *rap1-5* mutant allele. This result implies that the increased loss rates of both T+X- and T-plasmids in the *rap1-5* mutant strain are due to impaired TEL-plasmid segregation. It should be noted that both these plasmids have high loss rates (comparable to the loss rate of ARS plasmid pRLTet) in the *rap1-5* mutant strain. Thus, unlike mutation in *SIR2*, *SIR3*, *SIR4* and *ABF1*, mutation of *RAP1* completely abolishes the segregation of all T-plasmids as well as T+X-plasmids, suggesting that Rap1p improves TEL-plasmid segregation by interacting with the $TG_{1\text{-}3}$ tracts.

DISCUSSION

We have identified two components that contribute to TEL-plasmid segregation function. One component is the double-stranded $TG_{1\text{-}3}$ tract that requires Rap1p in *trans* for TEL-plasmid segregation. The second component is the X-TAS, including the Abf1p-binding site and the ARSC sequence, that requires *SIR2*, *SIR3*, *SIR4* and *ABF1* in *trans* to enhance the segregation of T+X-plasmids. The X-TAS alone does not function as a segregation sequence, yet when it is present with $TG_{1\text{-}3}$ repeats on the plasmid it enhances plasmid segregation, suggesting that the X-TAS and $TG_{1\text{-}3}$ sequences interact. These results suggest a model (Figure 7) in which Abf1p, bound to the Abf1p-binding site in the X-TAS, mediates the *SIR*-dependent X-TAS enhancement of TEL-plasmid segregation. In addition, there may be interactions between the Sir proteins and proteins bound to other *cis* sites such as the ARSC and the $TG_{1\text{-}3}$ tracts.

Role of gene products required for TEL-plasmid segregation: *RAP1* is required, in *trans*, and multiple Rap1 sites are required, in *cis*, for the segregation both T+X-plasmids and T-plasmids (this work and LONGTINE *et al.* 1992). TEL-plasmid segregation may require Rap1p-Rap1p interactions that are disrupted in the *rap1-5* mutant. We do not know whether Rap1p-Rap1p interactions are limited to DNA sequences in *cis*. These interactions may occur in *trans* between telomeres from different chromosomes to mediate telomere-telomere associations like those observed in cytological studies (reviewed in BLACKBURN and SZOSTAK 1984; GILSON *et al.* 1993; ZAKIAN 1989). It is possible that similar interactions occur between the telomere DNA on TEL-plasmids and at chromosomal telomeres. Such *trans*-interactions between chromosomal telomeres and TEL-plasmids might facilitate the segregation of TEL-plasmids.

Because Rap1p is involved in *SIR*-dependent repression at *HMR* and at telomeres (HARDY *et al.* 1992a; KURTZ and SHORE 1991; KYRION *et al.* 1993; SUSSEL and SHORE 1991), it is possible that, in addition to its role in T-plasmid segregation, Rap1p is also involved in the *SIR*-dependent X-TAS enhancement of T+X-plasmid segregation. While we did not detect any Rap1p/Sirp interactions because the *rap1-5* allele abolishes T-plasmid segregation, we cannot rule out the possibility that Rap1p and Sir proteins interact on T+X-plasmids.

SIR2, *SIR3*, *SIR4* and *ABF1* are required for the X-TAS enhancement of T+X-plasmid segregation. However, these gene products are not required for the segregation of T-plasmids. Thus, mutations in *SIR2*, *SIR3*, *SIR4* and *ABF1* only partially affect T+X-plasmid seg-

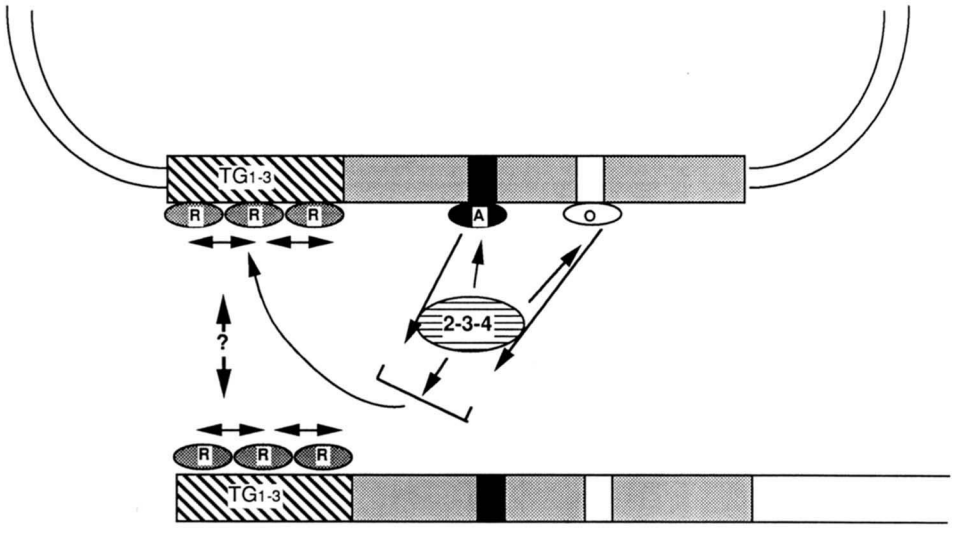

FIGURE 7.—Model for TEL-plasmid segregation. T+X-plasmid (upper portion) showing possible interactions (small arrows) between Abf1p (A) bound at the Abf1p-binding site (black box), origin binding factors including ORC (O) bound at the ARSC (white box) and Sir2p, Sir3p and Sir4p (2–3–4). It is not clear whether these Sir proteins modify Abf1p and/or ORC or whether they interact directly with either of these proteins to enhance plasmid segregation. Small double arrows indicate interactions between Rap1p (R) bound to $TG_{1\text{-}3}$ tracts (diagonal filled boxes). The broad arrow indicates possible associations between chromosomal telomeres and/or between chromosomal telomeres and TEL-plasmids.

regation, because they are required for X-TAS enhancement of TEL-plasmid segregation. T-plasmid segregation does not require these gene products.

Role of *cis* sites required for X-TAS enhancement of TEL-plasmid segregation: The Abf1p-binding site within the X-ARS is necessary for *SIR*-dependent X-TAS enhancement of TEL-plasmid segregation. Abf1p-binding sites in other contexts on the plasmid are not sufficient for the enhancement of T-plasmid segregation. T-plasmids include *ARS1* or 2-μm ARS for plasmid replication. Although there are Abf1p-binding sites within both *ARS1* and the 2-μm ARS, T-plasmids segregate at the same efficiency in wild type and *sir* mutant strains. The Abf1p-binding site within the X-TAS must be in a specific context that permits Abf1p to bind and mediate the *SIR*-dependent enhancement of TEL-plasmid segregation. On T+X-plasmids, Abf1p presumably binds at the Abf1p-binding site. *sir* mutants and the Abf1p-binding site mutation have similar effects on TEL-plasmids suggesting that the Sir proteins interact with Abf1p bound at the Abf1p-binding site (Figure 7).

The ARSC within the X-ARS is necessary for X-TAS enhancement of TEL-plasmid segregation. ARSC sequences bind a number of factors (BELL and STILLMAN 1992; DIFFLEY and COCKER 1992; HOFMANN and GASSER 1991; SCHMIDT *et al.* 1991). At HMRE, components of the complex that binds ARS regions (ORC) are involved in silencing as well (LAURENSON and RINE 1992). Sir-dependent interactions at the mutant ARSC site (μarsc) actively interfere with T+X-plasmid stability in wild-type cells: plasmids carrying μarsc are more stable in *sir* mutant strains than they are in wild-type strains. While in wild-type strains the μarsc T+X-plasmid has a higher loss rate than T-plasmids, in *sir* mutant strains the μarsc T+X-plasmid is as stable as a T-plasmid. The μarsc does not affect the function of a wild-type ARS on the plasmid: the 2-μm ARS on pYET-0-μarsc is fully functional (Figure 5). The interference in TEL-plasmid segregation observed with pYET1-μarsc is clearly a Sir-dependent interaction, suggesting that the ARSC is also involved in the Sir-dependent enhancement of T+X-plasmid segregation. Perhaps ORC components also participate in the X-TAS enhancement of TEL-plasmid segregation: they might form a complex that can either enhance (or, with μarsc, interfere with) TEL-plasmid segregation.

The Abf1p-binding site mutation and the ARSC mutation have different effects on T+X-plasmid behavior in wild-type strains. The μabf1 *cis* mutation is epistatic to the *sir* mutations while the *sir* mutations are epistatic to the μarsc *cis* mutation. Perhaps, the Abf1p-binding site and the ARSC have different roles in telomere function. At *HMR E*, the Abf1p-binding site and the ARSC site also have different roles in silencing: mutations in *RAP1* or *RIF1* have different effects in *HMR E-ΔARS* strains and *HMR E-ΔABF* strains (HARDY *et al.* 1992a, b; SUSSEL and SHORE 1991). The role of the Sir proteins could be to modify Abf1p and factors that bind at the ARSC. Alternatively, Sir proteins may interact directly by binding the proteins that recognize these sites.

Mechanisms of TEL-plasmid segregation: We previously suggested two possible mechanisms for TEL-plasmid segregation: attachment to a nuclear structure that facilitates plasmid segregation and more compact packaging of plasmid DNA that may facilitate improved plasmid diffusion and/or improved plasmid attachment to a segregating structure (LONGTINE *et al.* 1992, 1993). We now propose that the nuclear attachment mechanism operates on all TEL-plasmids, while a compaction mechanism may operate only on T+X-plasmids. We suggest that attachment of plasmids to a nuclear structure that segregates is directed by the $TG_{1\text{-}3}$ repeats complexed with multiple Rap1 proteins in both T+X-plasmids and T-plasmids. It is tempting to speculate that TEL-plasmids segregate by association of the plasmids with chromosomal telomeres and that these associations are mediated through interactions between $TG_{1\text{-}3}$/

Rap1p complexes on the plasmids and similar complexes on chromosomal telomeres (Figure 7). Rap1p localizes to telomeres, to the nuclear periphery, and to a small number of spots (4–8) per nucleus (KLEIN *et al.* 1992), implying that a number of telomeres are co-localizing or associating at each spot. If TEL-plasmids co-localize with chromosomal telomeres, plasmid-telomere interactions might reflect telomere-telomere associations that occur between chromosomal telomeres.

We propose that on T+X-plasmids Sir-dependent assembly of a tighter chromatin structure causes more compact packaging of the plasmids. Since Sir proteins are involved in the formation of an inaccessible chromatin structure at telomeres and at the HM loci, it is likely that this mechanism operates on T+X-plasmids to mediate X-TAS enhancement of T+X-plasmid segregation. By forming an altered chromatin structure, plasmid diffusion may be more efficient. Alternatively, alterations in the chromatin structure may increase the availability of specific binding sites that are involved in plasmid segregation, thereby enhancing the segregation of the plasmids.

Alterations in replication have been observed to influence plasmid segregation (HOUTTEMAN and ELDER 1993; KIPLING *et al.* 1991). TG_{1-3} tracts on linear CEN-plasmids cause plasmid replication to be significantly delayed in S-phase while TG_{1-3} tracts on circular CEN-plasmids cause plasmid replication to be slightly delayed (FERGUSON *et al.* 1991; FERGUSON and FANGMAN 1992). TG_{1-3} tracts on T-plasmids might alter T-plasmid replication in some way that influences plasmid segregation.

Chromosomal TAS functions: While telomere associated sequences are present at the ends of most eukaryotic chromosomes, their function remains unknown. We have used circular plasmids to demonstrate an interaction between two telomere components, X-TAS and TG_{1-3} tracts, that are located in adjacent positions within the genome. This interaction does not appear to be dependent upon either the relative distance between, or the orientation of, the two sequences, suggesting that X-TAS and TG_{1-3} tracts are able to interact when they are not adjacent in the genome (*e.g.*, when separated by one or more Y'-TASs). Perhaps at chromosomal telomeres the TASs interact in a similar, Sir-dependent manner to enhance the functions of telomere repeats at chromosome ends.

TPE, which is abolished in *sir* mutant strains, does not require the presence of TASs on the particular telomere ends where telomere position effect and chromatin accessibility were measured (GOTTSCHLING 1992; GOTTSCHLING *et al.* 1990; WRIGHT and SHAY 1992). In contrast, T-plasmids (that also have no TAS) are not affected by the same *sir* mutations. If TASs mediate Sir-dependent interactions, then perhaps associations between a number of chromosomal telomeres bring together TASs and TG_{1-3} tracts from different chromosomal telomeres. Under these conditions, TASs and TG_{1-3} tracts might interact in *trans* to establish and/or maintain a closed chromatin structure at telomeres. Consistent with this scenario, mutations in *SIR3* or *SIR4* cause a loss of telomere-telomere associations (GILSON *et al.* 1993; PALLADINO *et al.* 1993).

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