Functional analysis of the yeast plasmid partition locus *STB*

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Derivatives of the yeast 2μ plasmid with the *cis*-acting locus *STB* (also called *REP3*) are stably maintained if two plasmid-encoded proteins are present in *trans*. There are conflicting reports of both the extent of *STB* and its possible involvement in plasmid partition or copy number control. We have resolved the controversy by constructing 2μ derivatives with a conditional *STB* function, and showing that when *STB* is inactivated plasmids become concentrated in a small fraction of the population although the total number of plasmids remains unaltered. Moreover we show that *STB* consists of two functionally distinct domains which we call *STB*-proximal and *STB*-distal relative to the origin of replication. Although *STB*-proximal is sufficient for proper partitioning, this function is severely disrupted by active transcription from neighbouring sequences. *STB*-distal is important to protect *STB*-proximal and *ORI* from such transcription, and can be efficiently replaced by a 94-bp terminator fragment in an orientation-dependent manner. We find that *STB*-distal contains an additional element which depresses transcription from upstream promoters. We also describe the phenomenon of replication inhibition which we believe can explain the anomalous instability of some yeast plasmids.

Key words: 2μ plasmid/partition/replication inhibition/*Saccharomyces cerevisiae*/conditional mutants

Introduction

The 2μ is a cryptic nuclear plasmid that confers no phenotype beyond the ability to maintain itself at ~30–100 copies per cell. Its maintenance system is efficient, since 2μ is widespread in wild and laboratory strains of *Saccharomyces cerevisiae* and the appearance of plasmid-less [*cir*⁰] cells in a plasmid-containing [*cir*⁺] population is rare (Futcher and Cox, 1983).

Although 2μ is an autonomous circle of DNA, its replication is dependent on normal cell functions in so far that it is limited to a single initiation during S-phase (Zakian *et al.*, 1979) and requires certain *CDC* functions (Petes and Williamson, 1975; Livingston and Kupfer, 1977).

The sequence of 2μ (Hartley and Donelson, 1980) reveals that its 6318 bp contain four main open reading frames (ORF) and two perfect inverted repeats of 599 bp that divide the plasmid into two unique regions (Figure 1, upper). Functional analysis showed that FLP, the product of the largest ORF (A), catalyses site-specific recombination between inverted repeats either on different molecules, resulting in multimers, or on the same molecule giving rise to two isomeric forms A and B which differ in the relative orientation of the unique regions. Futcher (1986) proposed a model of amplification involving this system, a suggestion supported by the recent results of Volkert and Broach (1986).

The products of the B and C ORFs act in *trans* on the partition locus *STB* (Kikuchi, 1983; also called *REP3*; Jayaram *et al.*, 1983). In the absence of B or C proteins, or when *STB* is disrupted, 2μ derivatives are rapidly lost due to the failure to efficiently segregate plasmids into the bud at mitosis, a phenotype normal for plasmids based on a chromosomal autonomously replicating sequence (*ARS*; Murray and Szostak, 1983). Indeed such plasmids can be stabilized in a [*cir*⁺] strain by the inclusion of *STB* sequences, indicating that the origin of replication of 2μ does not have important plasmid specific features (Kikuchi, 1983).

We have studied the organisation of *STB*, which for convenience we divide into two domains lying either side of the *Hpa*I site. *STB*-proximal lies proximal to *ORI* between the *Hpa*I and *Ava*I sites, and *STB*-distal is distal to *ORI* between the *Pst*I and *Hpa*I sites (Figure 1, lower). *STB*-proximal is characterized by five tandem direct repeats of 62 bp with 82–97% homology and little chromatin structure. *STB*-distal includes the 3' end of the D ORF and a transcription terminator (Sutton and Broach, 1985).

There have been conflicting reports of the extent and function of the *STB* region. Jayaram *et al.* (1983) reported that the whole of *STB* (*REP3*) from the *Pst*I to the *Ava*I sites was required as

Fig. 1. 2μ plasmid B form and enlargement of the *STB*–*ORI* region. The ORFs A–D are indicated by arrows; the A, B and C ORFs are also called FLP, REP1 and REP2 respectively. The inverted repeats are shown as solid bars, and the functions of the *cis*-acting loci *ORI* (cross-hatch) and *STB* are described in the text. Note that numbering here is for the B form; elsewhere in the text and Figure 3 it refers to the A form to facilitate comparison with other authors.

Fig. 2. Construction and details of *STB*-distal plasmids (a) pY24 is the *Pst*I–*Xba*I 2μ fragment from YEp24 (Botstein *et al.*, 1979) spanning *STB* and *ORI* inserted into the same sites in pUC19 (Yanish-Perron *et al.*, 1985). The 2μ fragment was excised using the flanking polylinker restriction sites *Hind*III and *Bam*HI and inserted into the same sites in pEMBLy21 (Baldari and Cesareni, 1985), a *URA3* integrating vector with no other yeast origin of replication. pY45 is the same as pY26 except the 94-bp *Eco*RII fragment containing the terminator is deleted from *STB*-distal. pY56 was constructed from pY26 by cutting with *Sph*I and *Hpa*I and re-circularising after flush-ending with Klenow polymerase. *GAL* derivatives were constructed by inserting an 890-bp *Hinc*II fragment from pEMBLyex2 (Baldari *et al.*, in preparation) giving galactose-inducible expression in yeast (essentially the *Hinc*II–*Bam*HI fragment of G2; Guarente, 1983) into the filled *Hind*III sites of plasmids pY26, pY45 and pY56. This fragment has 239 bp of the 3' end of *URA3* (cross-hatch), 365 bp of the *GAL*I–*GAL*10 intergenic region (*GAL* UAS, open bar), 243 bp of the *CYC*1 non-translated leader (slant-hatch) and part of the pEMBL18⁺ polylinker (Dente *et al.*, 1985). LAG plasmids (not shown) are equivalent to *GAL*, but the *Hinc*II fragment is oriented to direct transcription away from *STB*. (b) The 2μ *STB*–*ORI* region and, below, details of 2μ sequences (black bar) in pY26 and derivatives. pY120 and pY121 differ only in the orientation of the terminator (as shown by arrows) and were constructed by cloning the 94-bp *Eco*RII *STB*-distal terminator fragment in the pY56-GAL polylinker downstream of the *GAL*–*CYC*1 promoter. pY122 (not shown) was similarly constructed using the 164-bp *Bam*HI–*Cla*I terminator fragment from pΔGA23-284 (Yarger *et al.*, 1986). The orientation tested was such that any residual *URA3* promoter activity of the terminator would read into *STB*. H3 = *Hind*III, S = *Sph*I, P = *Pst*I, R2 = *Eco*RII, H = *Hpa*I, A = *Ava*I, X = *Xba*I.

part of an amplification system involving B and C proteins (called REP1 and REP2). On the other hand Kikuchi (1983) showed that *STB* allowed effective partitioning of *ARS* based plasmids when B and C products were present but did not increase the average plasmid copy number. Both *STB*-proximal and *STB*-distal were absolutely required. In a later analysis Jayaram *et al.* (1985) suggest that only the *Hpa*I–*Ava*I fragment (*STB*-proximal) is required and that it is indeed involved in plasmid partition, a conclusion supported by Cashmore *et al.* (1986).

We have examined the roles played by different elements in

STB on copy number and partition of 2μ derivatives, and we present data which demonstrate that, although *STB*-proximal is sufficient to confer stability on 2μ derivatives, *STB*-distal contains elements which are important for the normal partition function of *STB*-proximal.

Results

Effect of *STB*-distal on the maintenance of 2μ derivatives

Since 2μ itself confers no phenotype on [cir⁺] cells, it is con-

Table I. Stability and copy number of *STB*-distal plasmids

	% Ura ⁺ selective growth (initial %) ^a			% Loss per generation ^b <i>i</i> %		Average copy number per cell ^c		Average copy number per [plasmid] ⁺ (ura ⁺) cell	
	glu	glu	gal	glu	gal	glu	gal	glu	gal
pY26	72	31	43	7.9 (6.2–8.9)	4.8 (3.0–6.2)	7 (5–11)	nd	23 (16–37)	nd
pY26-GAL	78	46	39	4.9 (3.2–8.1)	6.1 (4.8–7.8)	17 (9–25)	18 (9–31)	49 (25–74)	46 (22–77)
pY45	71	34	37	6.8 (5.0–8.4)	5.8 (4.1–7.4)	8 (4–12)	nd	23 (11–35)	nd
pY45-GAL	71	44	18	4.7 (4.2–5.5)	12.6 (10.2–14.8)	18 (7–27)	23 (18–30)	42 (17–61)	109 (83–142)
pY56	63	19	24	11.0 (9.2–13.2)	8.8 (6.8–10.4)	14 (7–21)	nd	64 (34–107)	nd
pY56-GAL	60	37	2	4.4 (2.6–5.7)	31.3 (28–34)	11 (4–15)	6 (3–11)	26 (10–37)	314 (165–550)
pY120	62	36	30	4.9 (3.7–6.0)	6.0 (5.6–6.3)	12 (10–14)	12 (7–17)	31 (26–36)	43 (26–63)
pY121	54	32	6	4.5 (4.1–4.8)	19.0 (18–20)	8 (6–10)	6 (5–8)	31 (24–38)	160 (127–203)
pY122	51	28	8	5.4 (5.3–5.4)	15.0 (14–16)	29 (19–49)	13 (10–15)	96 (65–164)	166 (130–184)

^aSee Materials and methods.^bResults quoted are the average of four independent experiments except for pY120 series (2) with the maximum and minimum values below. *i*% = *i* × 10².^cBetween three and 11 autoradiograph scans are averaged with maximum and minimum values below. nd = not done.

Fig. 3. (a) *STB* showing Δ Xho deletions and their stability as a percentage of plasmid-containing cells after a period of non-selective growth. Δ Xho plasmids are CV20 [entire 2 μ B form, with pBR322 and *LEU2* inserted in the *EcoRI* site of the *A(FLP)* gene] with small deletions in *STB*. All data from Jayaram *et al.*, 1983; numbering refers to 2 μ A form. (b) Sequence of the *EcoRII*–*EcoRII* fragment showing precise endpoints of Δ Xho deletions (Jayaram *et al.*, 1983, 1985) and the two terminators 1 (square boxes) and 2 (pentagrams) and respective polyadenylation sites (arrows; Sutton and Broach, 1985). (c) Sequence of the *EcoRII*–*HpaI* fragment with the primary (A) and secondary (B) blocks of homology to sequences between the *A(FLP)* and *C(REP2)* genes. Although the homology is less for the three lower cases, a consensus TC(T rich)₁₃ATCTTG emerges.

Fig. 4. Northern blot analysis of RNA from CHL2881-1D (*cir*⁺ no plasmid), transformed with the plasmids indicated and grown in selective non-inducing (SDglu) or inducing (SDgal) conditions for the *GAL*–*CYC1* promoter. A single-stranded Sp6 transcript from the 2μ *Pst*I–*Xba*I fragment cloned in pSp6-5 was used to make a probe which specifically detected plasmid transcripts in the direction *Pst*I–*Xba*I (Melton *et al.*, 1984). *B* gene transcripts are also detected on long exposures because of homology to the inverted repeat.

venient to study the behaviour of recombinant plasmids with an insertion of a yeast selectable marker either into the whole 2μ, or into minimal replicon derivatives containing only essential *cis*-acting sequences. We chose the latter approach to eliminate the possible confusion that could arise if deletions near *STB* had an effect on the expression of other genes on the same plasmid. *Trans* factors are therefore supplied by propagation in a [*cir*⁺] host. Plasmid pY26 consists of the *Pst*I–*Xba*I fragment spanning *ORI* and *STB* (Figure 2) inserted in the polylinker of the *URA3* integrating plasmid pEMBLy21 (Baldari and Cesareni, 1985). Since this plasmid does not contain a complete inverted repeat, it is not a substrate for the FLP-site-specific recombination system and does not integrate into the endogenous 2μ population. Thus in a [*cir*⁺] strain pY26 is maintained as an intact, autonomously replicating episome with a copy number of ~30 per plasmid-containing cell and a loss rate in non-selective conditions of ~7% per generation (Table I, measured as described in Materials and methods). This is in broad agreement with published results for similar plasmids (Futcher and Cox, 1984).

We also constructed a deleted derivative of pY26 lacking *STB*-distal (pY56, Figure 2). This plasmid was lost at a somewhat faster rate of ~10% per generation, and was present at ~75 copies per plasmid-containing cell although the average copy number over all cells in the population was much the same as for pY26. This indicated that the function of *STB*-proximal was somewhat less effective in pY56 than in pY26, and moreover since we had affected the distribution but not the total number of plasmids that *STB*-proximal is involved in plasmid segregation.

However the difference we observed between the stabilities of pY26 and pY56 is far less dramatic than was found by Kikuchi (1983) for plasmids that contained equivalent portions of 2μ. In that report, a plasmid carrying all of *STB* (pYK2031) was present in 78% of cells after a period of non-selective growth, whereas a plasmid with only *STB*-proximal (pYK2049) was present in only 7% of cells. Similar examples can be found amongst the results reported by Jayaram *et al.* (1983) for plasmids with deletions of the *Hpa*I–*Pst*I fragment of *STB*-distal.

It therefore seemed likely that *STB*-proximal is influenced by its neighbouring sequences, and that although *STB*-distal is not absolutely required, its function is essential if the 2μ origin fragment is inserted into specific plasmid contexts.

One functional element of STB-distal is a transcription terminator

We used the data of Jayaram *et al.* (1983) to compare the published stability of a series of plasmids with deletions straddling the *Hpa*I site with their reported deletion endpoints in *STB*-distal and *STB*-proximal. We noticed that the instability of these plasmids correlated not with the size of deletion of *STB*-proximal but rather the extent to which *STB*-distal was removed (Figure 3a). Further results of Jayaram *et al.* (1983) for deletions entering *STB*-distal from the *Pst*I site suggested that a substantial drop in stability occurred when a small region around position 2800 was removed. Examination of the DNA sequence in this region (Figure 3b) showed two blocks of good homology to a consensus yeast transcription terminator (Zaret and Sherman, 1982). While this work was in progress Sutton and Broach (1985) have confirmed that transcripts entering *STB*-distal are indeed terminated at this point.

We therefore constructed a further derivative of pY26, in which *STB*-distal remained intact except for a deletion of a 94-bp *Eco*RII fragment from position 2770 to 2864 that completely removes both blocks of terminator homology. Contrary to our expectations, this plasmid (pY45; Figure 2) proved to have an identical loss rate and copy number to pY26 (Table I).

However since we knew that pY56 was not as unstable as the equivalent plasmid of Kikuchi (1983) we reasoned that perhaps there was rather little yeast transcription originating in the bacterial sequences of our plasmids. We therefore inserted a 1-kb fragment containing the *GAL*–*CYC1* hybrid promoter originally derived from plasmid G2 (Guarente, 1983) so that galactose-inducible transcription was directed into *STB* from the distal side. We then compared the loss rates and copy numbers of the resulting plasmids (pY26-GAL, pY45-GAL, pY56-GAL; Figure 2), growing transformed clones in non-selective media containing either glucose or galactose as described in Materials and methods and Table I. We found that while pY26-GAL had the same behaviour with glucose or galactose as carbon source, both pY45-GAL and pY56-GAL were lost at a faster rate in galactose. The effect was more marked for pY56-GAL and was similar to that reported for plasmids that lack *STB* entirely (Futcher and Cox, 1984).

We examined RNA from yeast carrying these plasmids (Figure 4) and found that all three plasmids produce an abundant galactose-induced RNA. In pY26-GAL this is a short transcript of ~350 bases (measured on 6% acrylamide gel; data not shown) consistent with termination within *STB*-distal, whereas the induced transcripts in pY45-GAL and pY56-GAL are longer showing that transcription proceeds through *STB*-distal and terminates, probably before reaching *ORI*, at a single site around position 3570. To prove that the transcription terminator contained within the *Eco*RII fragment of *STB*-distal was sufficient to protect *STB*-proximal we inserted this fragment between the *GAL* promoter and *STB*-proximal in pY56-GAL in both orientations (Figure 2b). When this fragment was present in the same orientation as it normally is in *STB*-distal (pY120) it effectively protected *STB*-proximal during galactose growth. In the other orientation (pY121) the protection effect was much less marked and galactose induction destabilized the plasmid. This indicates that in the correct orientation this transcription terminator is sufficient to protect *STB*-distal. However, the bidirectional terminator of Yarger *et al.* (1986) derived from a region upstream of the *URA3* gene was less effective than the 2μ *Eco*RII fragment when similarly inserted to create plasmid pY122. We do not know whether this difference reflects a difference in the termination mechanism of these sequences or residual promoter activity within the terminator fragment.

Fig. 5. Northern blot analysis for non-selective cultures of CHL2881-1D grown in non-inducing (YPglu) or inducing (YPgal) media. The double-stranded oligo-labelled probe was the *Xho*I–*Sna*BI fragment of pY120 (Figure 2b; the *Xho*I site at the *GAL*–*CYC1* sequence boundary).

STB-proximal is a partition locus

We also examined the copy numbers of the *GAL* promoter plasmids after non-selective growth in glucose or galactose (Table I and Figure 7). We found that total number of plasmids in the yeast population (measured as the average copy number per cell) did not change significantly between glucose and galactose, and that the plasmid copy number per cell capable of forming *Ura*⁺ colonies was therefore much higher when transcription destabilized *STB*. This effect is dependent on *2μ*-encoded factors since in a [*cir*⁰] strain the stability of pY45-*GAL* is the same in glucose and galactose (data not shown). Transcription does not affect replication in these plasmids, and such conditionally stable plasmids are therefore strong evidence that *STB* is a partition locus involved in plasmid segregation during cell division.

STB-distal also contains a silencer-like element

We have shown that although pY45 has the *Eco*RII terminator fragment deleted from *STB*-distal its loss rate is the same as pY26, whereas pY56, which lacks all of *STB*-distal, is less stable. We also observed that induction of the *GAL* promoter is more destabilizing for pY56-*GAL* than for pY45-*GAL*. This suggested the presence of a further stabilizing element in *STB*-distal in addition to the *Eco*RII terminator. We did not believe this to be further termination activity since even on a long exposure of Figure 4 (data not shown), no short transcripts are observable in pY45-*GAL* grown in galactose. This suggested the presence of an element that exerts a negative influence on upstream promoters.

We examined RNA prepared from non-selective cultures transformed with the various plasmids. In this way we can make direct comparisons between plasmids, since the accumulation of transcripts is the result of transcription from the same number of plasmid molecules in each lane (although their distribution between cells is different). In selective cultures cells losing the plasmid stop growing, so the more unstable the plasmid, the higher is the average copy number.

We found that pY56 shows substantially more transcription of the *STB*-proximal region from the *Hpa*I to the *Sna*BI site than pY26 or pY45, and that the induced *GAL* promoter in pY45-*GAL* is considerably less active than in pY56-*GAL* (Figure 5). This is not simply due to the very close proximity of the *GAL* promoter to *STB*-proximal since pY121 containing the ineffective inverted *Eco*RII terminator between *GAL* and *STB*-proximal also shows a high level of transcription, but suggests the presence of an additional *cis* element in *STB*-distal which reduces transcription from upstream promoters. pY26-*GAL* does not clearly

Fig. 6. Northern blot analysis as Figure 4 but probed with nick-translated pY24 (Figure 2a).

show this effect, but exact comparison is difficult since as a stable plasmid its population distribution is different.

Thus in each case there is a good correlation between the amount of transcription in *STB*-proximal and the rate of plasmid loss, with transcription and instability increasing in the order pY26 = pY45 = pY120 induced < pY56 < pY45-*GAL* induced < pY121 induced < pY56-*GAL* induced. In the case of pY120 we were not able to identify any galactose-induced transcript by Northern analysis. We assume that this transcript is unstable, probably because its predicted size is only 150 bases plus poly(A) tail.

GAL sequences can silence all plasmid transcription

We noticed that plasmids containing the *GAL* promoter seemed consistently more stable under repressing conditions (glucose) than the equivalent non-*GAL* plasmids. In particular pY56-*GAL* behaved identically to pY26-*GAL*, in contrast to their parental plasmids pY26 and pY56. It is also apparent on Figure 4 that the *GAL* plasmids have a lower level of transcription entering *STB*. To test whether this was due to a blocking effect of *GAL* sequences on transcripts originating in upstream bacterial sequences, we used a probe which hybridises to most plasmid transcripts. Figure 6 shows that this is not the case, and repressed *GAL* sequences have a general silencing effect on all plasmid transcription. The arrows indicate the specific *GAL*-induced transcripts picked up by the probe in Figures 4 and 5, illustrating that these abundant transcripts are only a small proportion of the total plasmid transcription. Heavy plasmid transcription is consistent with the results of Marczynski and Jaehning (1985).

Insertions near the origin can effect replication

We were also interested in investigating the effect of inducing transcription through *ORI*, so we inserted the same *GAL* promoter fragment used to construct the other *GAL* plasmids into the unique *Sna*BI site of pY26-*GAL* that separates *STB* from *ORI*. In one orientation (plasmid *ORI*-*GAL*) transcription was directed towards *ORI*, in the other (*STB*-*GAL*) towards the proximal side of *STB*.

Table II. Stability and copy number of replication inhibition plasmids

	% Ura ⁺ selective growth (initial %) ^a		% Ura ⁺ after ~10 generations non-selective growth (final %)		% Loss per generation <i>i</i> %		Average copy number per cell		Average copy number per [plasmid] ⁺ (ura ⁺) cell	
	glu		glu	gal	glu	gal	glu	gal	glu	gal
ORI-GAL	73		54	6	3.8 (3.6–4.0)	24.0 (23–25)	18 (14–21)	7 (7–8)	36 (29–43)	123 (116–130)
ORI-TER	73		53	3	3.7 (3.0–4.3)	27.4 (27–28)	9 (7–10)	1.1 (0.7–1.4)	16 (13–19)	34 (23–46)
STB-GAL	46		16	2	10.8 (9.9–11.7)	32.2 (28–34)	2.4 (1.0–3.4)	0.2 (0.1–0.3)	17 (8–25)	10 (5–15)
STB-TER	50		18	0.4	8.2 (5.8–10.5)	41.5 (35–48)	2.1 (1.1–3.0)	<0.1 ^b	11 (5–16)	^b
pY26-LAG	68		51	61	2.3 (1.5–3.1)	0.6 (0.5–0.6)	6 (5–7)	5 (4–6)	12 (9–14)	9 (7–11)
pY45-LAG	72		46	48	4.6 (3.3–5.8)	4.3 (3.7–4.8)	6 (5–7)	6 (4–7)	11 (9–14)	11 (8–13)

^aSee notes to Table I.^bPlasmid band not measurable above background.

We found that ORI-GAL behaved like pY26-GAL on glucose, indicating that *STB* can be separated from *ORI* by a further 900 bases without adverse effect (Table II). Induction of transcription through *ORI* (shown by the appearance of a stable transcript, Figure 4) caused a substantial increase in loss rate. STB-GAL however had a lower stability on glucose as a carbon source, which was further exacerbated by growth on galactose. No stable transcript could be detected on galactose (data not shown).

STB-GAL, and to some extent ORI-GAL, exhibit a phenomenon we call replication inhibition characterized by the population of plasmids increasing more slowly than the population of cells due to the failure to replicate each plasmid molecule every cell cycle. It is detected because an unstable plasmid does not show an elevated copy number per plasmid-containing cell, or by a low average copy number per cell. We will discuss this further in the next section.

We constructed four further plasmids to investigate replication inhibition. ORI-TER and STB-TER have the same *Eco*RII terminator fragment from *STB*-distal that stabilised pY120 inserted downstream of the *GAL* promoter in ORI-GAL and STB-GAL, respectively. This failed to stabilise either plasmid, and in fact increased the inhibition effect. We also demonstrated that the proximity of *GAL* sequences to *STB* is not in itself destabilizing by constructing pY26-LAG and pY45-LAG which are identical to pY26-GAL and pY45-GAL but with the *GAL* promoter in the opposite orientations, so that *GAL* upstream activation sequences lie close to *STB*. As shown in Table II these plasmids are very stable.

We therefore conclude that the primary cause of replication inhibition is interference by particular sequences although the effect can be exacerbated by transcription.

Discussion

Stability and copy number

The maintenance characteristics of any plasmid are determined by the interaction of its replication and partition systems. Replication produces new plasmid copies, which are partitioned between cells during division. If each plasmid molecule replicates on average more than once per cell cycle amplification occurs, and the plasmid:cell ratio increases. We have also described the phenomenon of replication inhibition in which this ratio decreases. All three mechanisms can contribute to the stability of the plasmid, a phenotypic characteristic which refers to the rate at which the plasmid is lost, and to the observed copy number. The

Fig. 7. Autoradiographs were scanned for copy number measurements, choosing for each lane appropriate exposures within the film's linear response. Total yeast DNA extracted from the non-selective stability measurement cultures (Materials and methods) was digested with *Bam*HI and *Hind*III (except ORI-GAL and ORI-TER also with *Xho*I), blotted and probed with oligo-labelled *Hind*III–*Sst*I fragment from the 5' end of *URA3* which is present at one copy on each plasmid. CHL2881-1D (cir⁺ no plasmid) contains two *URA3* hybridising bands; where one co-migrated with the plasmid band it was subtracted before calculating the copy number.

molecular mechanism and significance of partition and amplification will differ for multicopy and single copy plasmids and on whether replication is tied to the cell cycle.

The rate of loss of 2μ derivatives is measured by counting the ratio of colony formers on selective and non-selective plates after a number of generations growing in non-selective liquid media. This has normally been reported as a percentage (Jayaram *et al.*, 1983; Kikuchi, 1983; Cashmore *et al.*, 1986), but this makes comparisons between authors difficult. Furthermore we have observed considerable differences for various plasmids in the proportion of plasmid-containing cells under selective conditions (Tables I and II) and hence in the original inoculum, and therefore favour a method (based on Futcher and Cox, 1983, 1984) in which the instability *i*, the rate of production of [plasmid]⁰ cell per [plasmid]⁺ cell per generation is calculated as described in Materials and methods. $i\% = i \times 10^2$ is therefore the percentage loss per generation.

The copy number of 2μ plasmids is measured by extracting DNA and comparing plasmid bands with a chromosomal standard either by hybridization (Jayaram *et al.*, 1983; Kikuchi, 1983; Veit and Fangman, 1985; Cashmore *et al.*, 1986) or by direct scanning of ethidium bromide-stained gels (Broach, 1983; Futcher and Cox, 1984). We have used the hybridization technique of Cashmore *et al.* (1986) but have differed from all previous authors in measuring copy number under non-selective condi-

tions, allowing us to measure copy number and rate of loss for the same population (Figure 7 and Materials and methods). This approach means we can clearly distinguish between changes in copy number caused by amplification or inhibition and those caused by non-disjunction since, by calculating copy number both as a population average and an average per plasmid-containing cell, we can observe the efficiency of replication.

The plasmids STB-GAL and pY56-GAL illustrate the importance of this integrated approach. 50% of cells in STB-GAL transformed colonies contained the plasmid, which dropped to 2% after 11 generations in complete galactose media. Similarly the proportion of cells carrying pY56-GAL fell from 60% to 2% during the same experiment. We could clearly show that different mechanisms were responsible for this instability only because we could directly compare the total plasmid pool with total cell numbers and show that in the case of STB-GAL the replication origin was less efficiently utilized.

Transcription effects

We have shown that transcription can reduce the partition efficiency of 2μ derivatives. This could be due either to direct disruption caused by the passage of the RNA polymerase II complex on the formation of other protein–DNA complexes, or to the confinement of plasmids to dominant sites that favour transcription over other processes. Circumstantial evidence in this direction might be drawn from the ability of Sp6 polymerase to transcribe through the *Xenopus* 5S RNA gene complex without disrupting it (Wolffe *et al.*, 1986). Transcription also disrupts the centromere function of CEN-ARS plasmids resulting in an increased copy number from one to 10–100 copies and a decreased stability (Chlebowicz-Śledziowska and Śledziwski, 1985).

Replication inhibition

We have also shown that replication can be indirectly affected by transcription, since the close proximity of promoter sequences, particularly when activated, causes plasmid instability due to a reduction in the rate of successful replication initiation or completion. We call this phenomenon replication inhibition and have shown that it explains the anomalous instability of certain 2μ -derived plasmids. This specific inhibition of replication in the *cis*-configuration has been previously described in bacteria but, in contrast to our results, the effect could be rescued by blocking transcription. This suggests that in bacteria transcription itself is interfering, whereas in 2μ DNA structure may be important. Thus the copy number of ColE1 vectors containing a strong promoter is decreased (Stueber and Bujard, 1982; Adams and Hatfield, 1984; Lambert and Reznikoff, 1985) unless a good transcription terminator is inserted downstream (Gentz *et al.*, 1981). Replication of plasmids from the chromosomal origin of *Bacillus subtilis* is inhibited by a promoter-containing sequence *sar* unless the –35 region is deleted and transcription reduced (Seiki *et al.*, 1982), and replication of *Escherichia coli* *oriC* plasmids is adversely affected by *lac* promoter transcription unless the *trp* attenuator is also present (Tanaka and Hiraga, 1985).

STB deletion plasmids

Using 2μ derivatives with conditional STB function we have shown that in such minimal replicon plasmids STB is involved only in partition and does not have a role in amplification. This agrees with Cashmore *et al.* (1986) and with later conclusions of Jayaram *et al.* (1985). Differences in plasmid copy number are due to ineffective segregation caused by transcription. It is interesting that the elevated copy number caused by selection on

a less stable plasmid is persistent, since pY56 still has a slightly higher average than pY26 even after 10 generations of non-selective growth (Table I).

We have also shown that STB-proximal alone is sufficient for the partition function, a conclusion which was also reached by Jayaram *et al.* (1985), although these authors could not fully explain the instability of some constructs with deletions of STB-distal. Our studies show there is a close correlation between the amount of transcription of STB-proximal and the rate of loss. The stability of Δ Xho deletions of Jayaram *et al.* (1983) shown in Figure 3a can also be explained by the extent to which the terminator function has been removed. However a further deletion Δ Xho5 (op. cit.), which extends from position 2632 to 3021 (approximately the *Pst*I site to 55 bp beyond the *Hpa*I site) was shown to have a stability of only 4%, much lower than Δ Xho15 (32%) which also removes the whole of the terminator. This deletion was studied in detail in a later paper (Jayaram *et al.*, 1985) where it was shown that it is a *cis* effect due to the distal end-point of the deletion. It could be stabilized neither by a promoter nor a terminator. Although only percentage stabilities are reported for all these plasmid in Jayaram *et al.* (1985), copy number in selective conditions are given for the Δ Xho plasmids in Jayaram *et al.* (1983). Δ Xho5 is unique among these deletions in combining low stability with a much reduced average copy number over the population. This is the phenotype of replication inhibition. We therefore propose that the instability of Δ Xho5 is due, not as proposed by these authors, to an effect of sequence context on STB-proximal (*REP3*), but to replication inhibition caused by the close proximity of *ORI* to inhibitory sequences. The inability to stabilise Δ Xho5 derivatives with a terminator (Jayaram *et al.*, 1985) is also consistent with this explanation, since we also found no advantageous effect of a terminator on plasmids suffering from replication inhibition.

Kikuchi (1983) also found that STB-proximal was not sufficient to partition the plasmids pYK2049 or pYK2066. These plasmids are derived from YRp16 and the construction is such that the distal side of STB is separated from the *Eco*RI site of pBR322 sequences only by ~670 bases of the 5' end of the *LEU2* gene. Marczyński and Jaehning (1985) have shown that on the closely related plasmid YCp19 there are strong bacterial associated transcripts (BAT) that arise close to the origin of pBR322 and run through the *Amp*^R marker 3' to 5', and terminate in yeast sequences beyond. In Kikuchi's plasmids we predict that BAT transcripts would encounter STB just as GAL transcripts do in our plasmid pY56-GAL. This accounts for the instability of pYK2049 and pYK2066 being as extreme as pY56-GAL under induction.

STB-distal contains a sequence with 'silencer' properties

We have also found evidence for an additional stabilizing activity within STB-distal that acts by reducing transcription from upstream promoters. From our constructions we cannot distinguish whether this element is in the *Pst*I–*Eco*RII or the *Eco*RII–*Hpa*I fragment. However from circumstantial evidence we believe it to lie in the latter. A 2μ transcript of low abundance, ~1950 bp long, begins in or near STB-proximal and runs 3' to 5' through the *D* ORF to terminate at the 3' end of *B* (Sutton and Broach, 1985). Jayaram *et al.* (1985) have shown that the 5' end lies to the right of Δ Xho15 at least 112 bp within STB-proximal, but it is abolished in Δ Xho4 where the deletion extends 144 bp into STB-proximal. They also found elevated levels of this transcript in plasmids Δ Xho5 and Δ Xho15, which suggests that a negative

element lies within the region defined by the deleted sequences. If this element is the same as that we have found, it can act on upstream promoters lying on either side, suggesting it may fulfil at least some of the criteria for a true silencer element (Brand *et al.*, 1985).

Veit and Fangman (1985) have examined the sensitivity of 2μ chromatin to micrococcal nuclease cleavage when isolated from yeast carrying plasmids disrupted in different 2μ genes. The cleavage pattern within *STB*-distal of such plasmids was compared with 2μ , and showed that in the absence of *B* (but not *A*, *C* or *D*) there was a reduced cleavage at the 3' end of the *D* ORF and increased sensitivity at two sites straddling position 2900. This is further suggestive evidence that the silencer lay in the *EcoRII*–*HpaI* fragment.

When we examined the DNA sequence of this region (Figure 3c) we found a sequence of 26 bp between 2918 and 2943 that is 77% homologous to a similar element of 24 bp between the divergently transcribed *FLP(A)* and *C* genes between 5321 and 5298. Moreover there is a second repeat of this element with lower homology starting at 2880 and three further copies upstream of *FLP*. This correlates with a further observation of Veit and Fangman (1985) that the absence of *B* (or *C*) changes the chromatin structure of the 5' region of *FLP*.

We are therefore testing the hypothesis that the silencing element of *STB*-distal is located in these 24-bp repeats, that *B* protein in *trans* is responsible for the silencing effect and that this element also has an influence on transcription of *FLP*. Since we have shown the silencer acts on upstream promoters distal to *STB* it is also possible that it influences the expression of the *D* ORF, and that the element located near *FLP* could also influence the divergently transcribed gene *C*. Jayaram *et al.* (1985) have shown that the level of *B* transcription is not affected in the Δ Xho plasmids which delete the silencer element.

The regulated expression of *FLP* by *B* protein that it is suggested by this model would be interesting since Volkert and Broach (1986) have provided experimental evidence for the idea of Futcher (1986), that *FLP* and the two inverted repeats are involved in a system of 2μ amplification. Recombination between the repeat which replicates early because of its proximity to *ORI* and the other unreplicated repeat gives rise to a replicating molecule in which the replication forks proceed in the same direction producing plasmid multimers which are resolved by subsequent *FLP* recombinations.

We would summarise our understanding of *STB* as follows. Blocks of repeated elements within *STB*-proximal are required as the site of action of *B* and *C* proteins leading to effective plasmid partition. This function is disrupted by transcription from distal sequences, which is prevented by an efficient transcription terminator within *STB*-distal. There is an additional element within *STB*-distal which represses transcription from distal (upstream) promoters. We believe it to be the same element responsible for repressing the 1950-bp transcript that starts within *STB*-proximal in [*circ*⁺] cells which suggests it is silencer-like in being active in either orientation and on diverse promoters. We propose this silencer is a 24 bp sequence which responds to *B* protein, and that a similar element upstream of *FLP* is also involved in transcription repression. This suggests an indirect role for *B* in copy number control since *FLP* is involved in plasmid amplification (Volkert and Broach, 1986). We do not yet understand the details of this mechanism or the possible role of the 1950-bp transcript.

Materials and methods

Strains and transformation

The *E. coli* K12 strains 71-18 $\Delta(lac-proAB)$, *thi*, *supE*, [*F'*, *proAB*, *lacI* Δ M15] (Messing *et al.*, 1977) and GC280 (*dam*[−] *dcm*[−]) and the yeast strain CHL2881-1D α *ade2 leu2 ura3 trp1 lys2* [*circ*⁺] (from P. Philippsen) were used. Yeast transformation was according to Altherr *et al.* (1983), except that cells were harvested at OD₅₉₀ 0.3–1.0, and were centrifuged and resuspended in 100 μ l of water before spreading on selective plates.

YPglu and YPgal are 1% Bacto-yeast extract, 2% Bacto-peptone and 2% glucose or 2% galactose respectively. SDglu, SDgal and SDlac are complete synthetic media without uracil (Sherman *et al.*, 1983) with 2% glucose, 2% galactose or 2% lactate and 3% glycerol as carbon source.

Molecular biology

Techniques were according to Maniatis *et al.* (1982) unless otherwise specified. 'Oligo-labelled' probes were according to Feinberg and Vogelstein (1983) using Pharmacia hexamers. Total RNA was extracted (Rubin, 1975) and 7–12 μ g of RNA electrophoresed on 1–1.2% agarose–formaldehyde gels in 1 $\times$ MOPS, 2.2 M formaldehyde buffer, blotted to GeneScreen Plus and hybridised in buffer based on 50% formamide, 10% dextran sulphate (essentially according to manufacturer's instructions). Hybridisation was at 42°C (DNA probes) or 60°C (RNA probes; in this case 100 μ g/ml [*circ*⁰] yeast RNA carrier was also used).

Total yeast DNA was prepared according to Cashmore *et al.* (1986) except that we used 400 mM LiCl instead of 100 mM NaCl in the lysis buffer to reduce SDS precipitation, and the final precipitation was with isopropanol from 2 M ammonium acetate.

Alkaline Southern blots on Zeta-Probe (Reed and Mann, 1985) were pre-hybridised for 5 min in 7% SDS, 500 mM NaPO₄ pH 7.2, 1 mM EDTA at 68°C, hybridised overnight in the same buffer with up to 1.5×10^6 d.p.m./ml of probe, and washed at 65°C in 40 mM NaPO₄, 1% SDS.

Measurement of stability and copy number

We measure plasmid stability and copy number in the same non-selective cultures. Working directly from transformation plates to minimise chromosomal integration, several colonies (to minimise clonal variation, Futcher and Cox, 1984) are resuspended at OD₅₉₀ 0.1–0.3 in SDlac and grown 7 h at 30°C to allow subsequent rapid *GAL* promoter induction (Adams, 1972). Equal amounts of pre-culture are diluted into YPglu and YPgal at $\sim 10^5$ cell/ml and a sample diluted and plated (see below) to determine the initial proportion of plasmid-containing cells (initial %). When these non-selective cultures reach $\sim 10^8$ cells/ml a further dilution and plating is made to determine the proportion of plasmid-containing cells at the end of the period of non-selective growth (final %), and the remainder of the culture frozen at -80°C in 7% dimethyl sulphoxide for further analysis.

Initial and final percentage are determined by serial dilution in phosphate-buffered saline. A mild sonication separates cell clumps which are rare in CHL2881-1D. The final dilution is plated on YPglu and SDglu giving 150–400 colonies per plate. The ratio of colony formers on SDglu/YPglu gives the fraction of cells with plasmid, and the number of generations is calculated from the dilutions. No significant difference was observed when replica plating was used or SDglu + uracil instead of YPglu plates. The instability *i*, the rate at which [plasmid]⁺ cells segregate [plasmid]⁰ cells per generation, was calculated according to the formula

$$i = 1/n \ln [(initial \%)/(final \%)]$$

derived by solving the differential equations $dX/dt = jX$ and $dx/dt = jx - ix$ where x = [plasmid]⁺ cells, X = [plasmid]⁰ cells, j = generation time, n = number of generations in time t , initial % = X/x at start, final % = X/x at end.

This figure is the same as *i* calculated by Futcher and Cox (1983, 1984) using a graphical method, and as the segregation frequency (SF) of Murray and Szostak (1983).

Copy number was measured by preparing DNA from 1 ml of frozen sample (see above), of which one-fifth was used for Southern analysis (Figure 7). Suitable autoradiographs on pre-flashed Kodak X-Omat AR film were scanned with a Helena Quick Scan R&D densitometer and quantified by weighing the peaks of the chromosomal *URA3* and the plasmid *URA3* band to give the copy number per cell. This was corrected using final % to give the copy number per Ura⁺ colony former or [plasmid]⁺ cell. Non-selective RNA preparation (Figure 6) was from further YPglu or YPgal cultures inoculated from the appropriate frozen sample.

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