The EMBO Journal vol.6 no.13 pp.4205–4212, 1987

Antagonistic controls regulate copy number of the yeast 2μ plasmid

James A.H.Murray, Maurizio Scarpa[1], Nicoletta Rossi[2] and Gianni Cesareni

EMBL, Postfach 10.2209, 6900 Heidelberg, FRG

[1]On leave of absence from Dip.di Pediatria, Università di Padova, Italia
[2]On leave of absence from Dip.di Biologia, II Università di Roma, Italia

Communicated by G.Cesareni

The endogenous 2μ plasmid of yeast encodes a site-specific recombinase FLP that can cause an amplification of plasmid copy number. Using strains overexpressing 2μ plasmid proteins from chromosomal constructs to disrupt the normal balance of gene products, we show here that copy number is controlled by regulating the transcript level of *FLP*. Expression of *FLP* is negatively regulated over a 100-fold range by the joint action of the plasmid-encoded REP1 and REP2 proteins, which also have a role in plasmid partition. We also show that the product of the fourth plasmid open reading frame *D* increases *FLP* expression by relieving the repression caused by REP1 and REP2. This is the first demonstration of a function for this gene, which we call *RAF*. The transcription of *RAF* is also repressed by REP1 and REP2 acting together, but requires a higher level for complete inhibition than that required to repress *FLP*. Copy number is therefore negatively regulated by REP1–REP2 concentration both by direct repression of *FLP* and indirectly, by control of the positive element, the anti-repressor RAF. We propose that these antagonistic regulatory mechanisms amplify the signal produced by a small fall in copy number.
Key words: Saccharomyces cerevisiae/overexpression/galactose promoter/lethal phenotype

Introduction

The yeast 2μ plasmid is the one of the few eukaryotic replicons understood in any molecular detail (reviewed Murray, 1987). It is found in ~75% of the natural isolates of the budding yeast *Saccharomyces cerevisiae* and confers no overt phenotype apart from a slight reduction in growth rate. It is perhaps best regarded as a 'molecular parasite' (Futcher and Cox, 1983; Mead *et al.*, 1986), which uses its host's normal machinery for DNA replication (Livingston and Kupfer, 1977; Zakian *et al.*, 1979) but, lacking any means of horizontal transfer except mating (Futcher and Cox, 1983), must ensure efficient plasmid maintenance by encoding products necessary for plasmid partition during cell division and for copy number control.

The 2μ plasmid is a circular DNA molecule of 6.3 kb (Hartley and Donelson, 1980) present in multiple copies (30–200/cell Clark-Walker and Miklos, 1974; Futcher and Cox, 1984; Mead *et al.*, 1986). Although it lacks a centromere, this nuclear DNA molecule shares many of the properties of larger chromosomes making it a useful tool for the investigation of DNA replication and the interaction of DNA with other nuclear structures (Livingston and Kupfer, 1977; Zakian *et al.*, 1979; Jazwinski and Edelman, 1979; Jong and Scott, 1985; Taketo *et al.*, 1980; Wu *et al.*, 1987). It is also the basis of many vectors used for recombinant DNA research in yeast (Broach, 1983; Parent *et al.*, 1985), all of which are, however, less stable than the wild-type plasmid (Futcher and Cox, 1984). This has prompted further research into the molecular mechanisms of 2μ maintenance.

The 2μ plasmid (Figure 1a) has two unique regions separated by perfect inverted repeats (IRs) of 0.6 kb that are a substrate for the plasmid-encoded site-specific recombinase FLP. FLP-mediated recombination between these inverted repeats changes the relative orientation of the unique regions. If this recombination event occurs during DNA replication between a repeat that has already replicated because of its proximity to the unique origin of replication (*ori*), and the as-yet-unreplicated repeat on the other side of the plasmid, the previously converging replication forks now chase each other around the molecule spooling out multimeric plasmid until further FLP crossovers terminate and resolve the process (Futcher, 1986; Volkert and Broach, 1986). This unique mechanism of increasing copy number does not breach the presumed rule of eukaryotic DNA replication that only one initiation event is allowed for each origin in one cell cycle and contrasts sharply with the mechanism known for all other plasmids, which are prokaryotic and regulate copy number by controlling the frequency of DNA replication initiation (reviewed Scott, 1984).

In addition to FLP, 2μ plasmid has the open reading frames (ORF) *REP1*, *REP2* and *D*. The REP1 and REP2 gene products act together on the *cis*-locus *STB* to ensure equi-partitioning of plasmids during cell division (Kikuchi, 1983; Veit and Fangman, 1985; Cashmore *et al.*, 1986; Murray and Cesareni, 1986; also called *REP3*, Jayaram *et al.*, 1983; 1985). In the absence of this system, plasmids fail to enter the daughter bud from the mother cell and are therefore rapidly lost from the population (Murray and Szostak, 1983). The REP1 protein co-purifies with a yeast nuclear matrix fraction (Wu *et al.*, 1987) and may therefore act by providing dispersed binding sites for plasmid molecules in a nuclear structure that is equally shared at mitosis. The function of the *D* gene product was as yet unknown, although it is transcribed into a polyadenylated RNA that encompasses the ORF and whose 5′ end lies near its start. It therefore seemed likely that the *D* gene is translated into a protein (Sutton and Broach, 1985).

We have analysed gene regulation and copy number control of the 2μ plasmid by constructing strains that inducibly overexpress plasmid gene products from a chromosomal locus and using these either to disrupt the normal balance of plasmid proteins, or to complement mutant plasmids. This reveals that *FLP* expression regulates plasmid copy number and that its control involves all three of the other plasmid products REP1, REP2 and D. Based on the function that we demonstrate for the product of the *D* ORF, we propose the name RAF for this gene, an acronym of REP Antagonizing Factor or Recombinase Activating Factor.

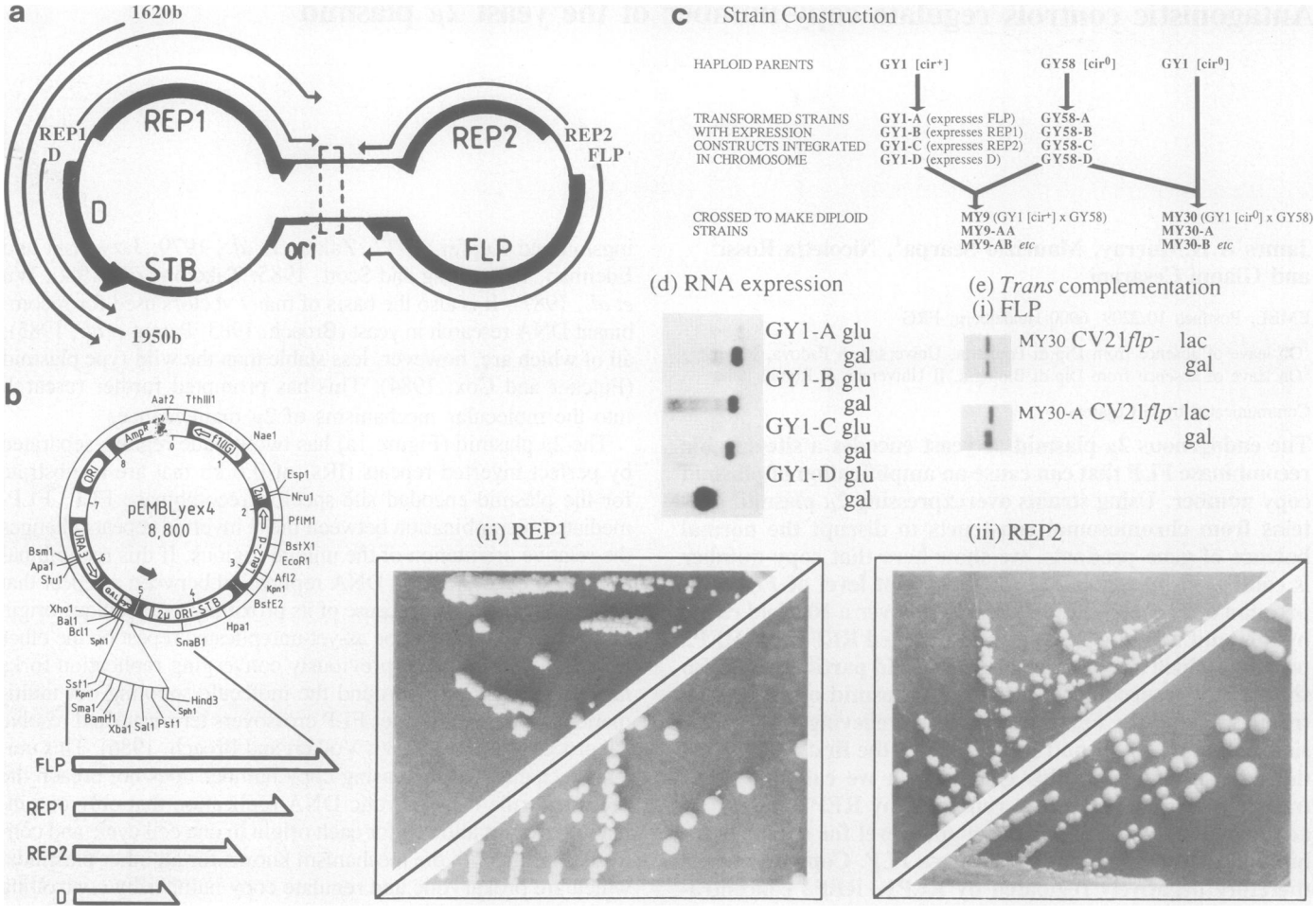

Fig. 1. (**a**) Genetic and transcription map of the B form of the 2μ plasmid drawn with the inverted repeats aligned. In the A form the relative orientations of the two halves are inverted relative to each other by FLP recombination within the boxed region. The 1620-base and 1950-base transcripts are not thought to have a protein coding function. (**b**) The galactose inducible yeast expression plasmid pEMBLyex4 (Cesareni and Murray, 1987) was used to construct vectors expressing 2μ genes. pEMBLyex4 contains a hybrid *GAL-CYC1* promoter (Guarente *et al.*, 1982; large arrow), a polylinker and beyond this a transcription terminator in 2μ sequences (double line). This, and 2μ sequences allowing autonomous replication in yeast, were deleted during construction to give integrating vectors. Linearization with *Apa*I, *Bsm*I or *Stu*I was used to direct integration into the *URA3* locus. The defective *leu2-d* allele is ineffective as an integrated copy allowing such strains to be transformed again using selection for *LEU2*. The f1 origin allows the preparation of single strand DNA from f1 superinfected *E.coli* (Dente *et al.*, 1983). (**c**) Construction and relationships of strains is described in more detail in Materials and methods. Suffixes indicate expression construct in each *URA3* locus A = pyexFLPΔ, B = pyexREP1Δ, C = pyexREP2Δ, D = pyexDΔ. (**d**) Northern analysis showing inducible expression of RNA. RNA was prepared at OD_{590} = 0.5 after overnight growth in glucose or galactose media, hybridized to the oligo-labelled *Xho*I−*Bam*HI *CYC1* fragment of pEMBLyex4. (**e**) *Trans*-complementation (i) FLP. Southern blot of *Eco*RV digested DNA extracted from MY30-A transformed with CV21 (see text) or CV21*flp*$^-$ (constructed by removal of an *Sph*I site) grown in selective lactate or galactose media. The 2μ origin probe hybridizes to two bands only if FLP inversion has occurred to give both isomers: for CV21*flp*$^-$ only seen when galactose induces FLP expression from the chromosomal construct. (ii) Plasmids with an insertion in a REP gene are poorly partitioned and give sectored colonies on selective plates. The defect of the plasmid Tn*5*-1′ (*rep1*$^-$*REP2*$^+$; see text and Materials and methods) is complemented in the strain GY58-B on galactose (lower) but not glucose (upper) selective plates and (iii) similarly galactose dependent REP2 complementation occurs in GY58-C transformed with Tn*5*-55′ (*REP1*$^+$ *rep2*$^-$).

Results

Trans-complementation

Since the 2μ plasmid confers no selectable phenotype, it is usually studied using recombinant shuttle derivatives that carry a yeast selectable marker and sequences allowing propagation in *Escherichia coli*. However, all such plasmids are lost at least two orders of magnitude faster than the wild-type (Futcher and Cox, 1984), suggesting either that there are no truly non-essential regions into which foreign DNA can be inserted, or that extra DNA is itself deleterious. To analyse copy number control, we therefore adopted the strategy of disrupting the regulation of the wild-type 2μ plasmid by overexpressing, from inducible chromosomal constructs, either one or two of the 2μ gene products. This approach deliberately creates reversible non-physiological conditions to observe the response of 2μ control mechanisms and is therefore analogous to the use of temperature sensitive mutants in genetic analysis.

The constructs were obtained by inserting fragments of the 2μ plasmid chromosome corresponding to the four ORFs (A, B, C and D corresponding to the genes *FLP, REP1, REP2* and *RAF* respectively) into the expression vector pEMBLyex4 (described in Figure 1b and in Materials and methods) that uses the efficient galactose inducible promoter and by integrating the resulting recombinant plasmids into the *URA3* locus on chromosome V of haploid strains of a and α mating type. These were crossed to make an isogenic series of [cir$^+$] and [cir^0] strains that differ only in the expression constructs they contain (Figure 1c; note that strains containing 2μ plasmid are designated [cir$^+$] and those without [cir^0]). The diploid [cir$^+$] wild-type is MY9 and the derivatives are designated, for example, MY9-AC (in this

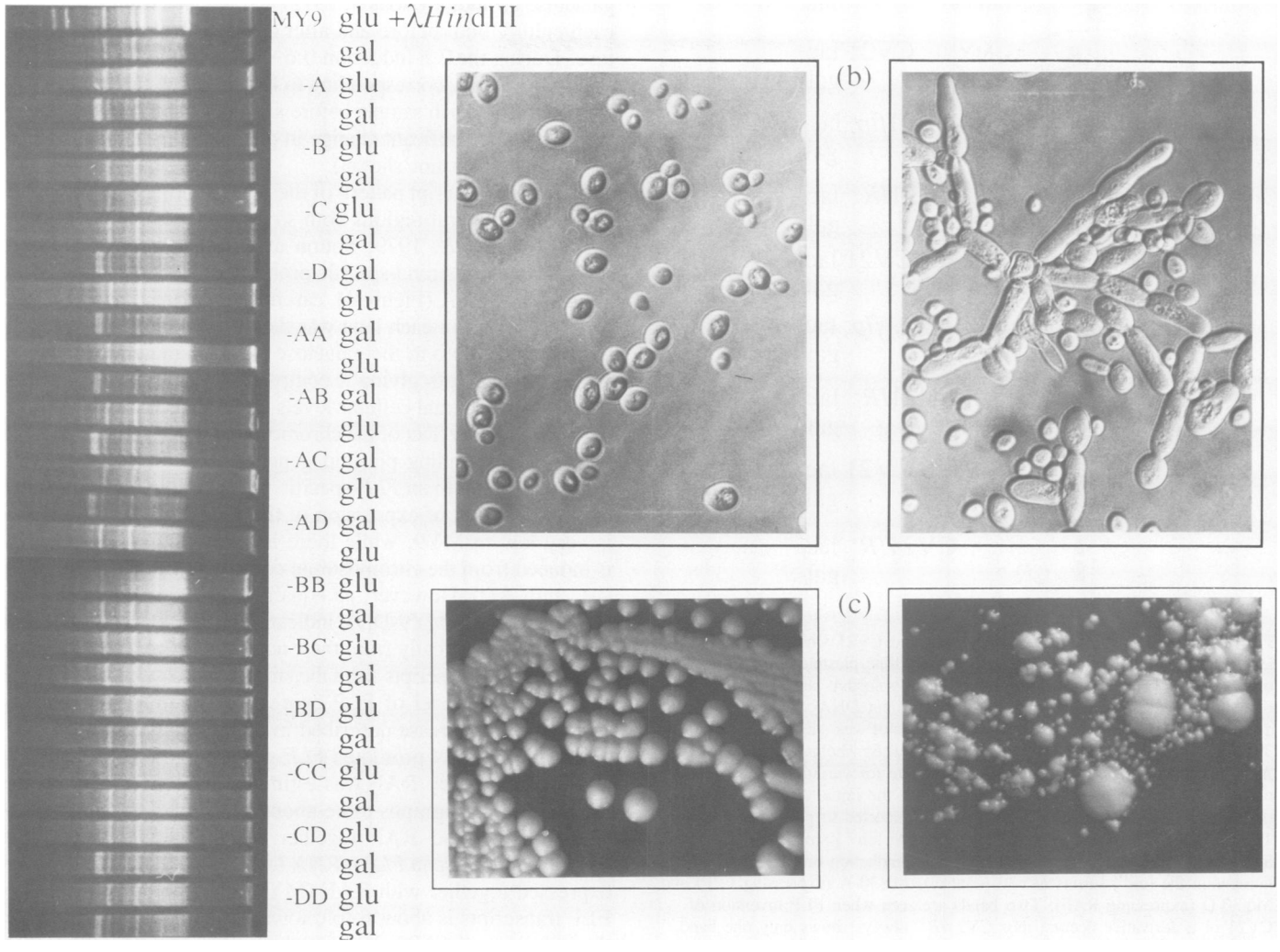

Fig. 2. (a) Ethidium bromide stained total DNA extracted after overnight growth of MY9 strains in glucose or galactose complete media (from equal inocula of lactate grown cells), digested with *Xba*I and run on a 0.8% agarose gel (origin to the right). The prominent new galactose induced bands correspond to 2μ bands of 3076 and 3242 bp; rDNA bands of 3598 and 5536 bp are apparent in every lane. The first lane also contains a λ *Hin*dIII marker.
(b) Photomicrographs using a 64× objective and differential interference contrast of MY9-AA after overnight growth in complete glucose (left) and galactose media (right), showing the dramatically altered cell morphology when 2μ plasmid copy number is increased. (c) Photographs of colonies of MY9-AA pregrown in complete galactose media and then streaked on complete glucose (left) and galactose (right) plates.

case expressing FLP and REP2). MY30 is a [cir^0] equivalent of MY9. The expression of the chromosomal constructs is undetectable on glucose or lactate media but strongly induced by growth on galactose (Figure 1d). We tested the *trans*-activity of the three proteins whose functions were known, by showing galactose dependent inversion of a *flp*$^-$ recombinant plasmid [Figure 1e(i)] and complementing *in trans* the stability defect of a *repl*$^-$ *REP2*$^+$ plasmid in the strain GY58-B (expressing REP1) and a REP1$^+$ *rep2*$^-$ plasmid in GY58-C [expressing REP2; Figure 1e(ii) and (iii)] during growth on galactose.

Copy number

Electrophoresis of DNA extracted from the MY9 strains after overnight growth on glucose (repressing) or galactose (inducing) media and stained with ethidium bromide, shows two prominent new bands in cultures in which *FLP* expression was induced (Figure 2a). These bands are not visible under normal conditions and correspond to a dramatic increase in 2μ plasmid copy number of at least 10-fold. The two other major bands are from the repeated cluster of ribosomal DNA genes. An equal increase in copy number was seen when RAF was overexpressed (MY9-D, -CD, -DD) except for the concomitant induction of REP1 and RAF (MY9-BD). Southern analysis of the same gel (data not shown) confirms that the bands are from 2μ plasmid and that overexpression of other 2μ plasmid genes for about eight generations does not noticeably affect copy number.

On examining the cultures with a very high 2μ plasmid copy number we found that they contain many cells which are abnormally large and elongated, often with multiple buds (Figure 2b). These resemble the terminal phenotypes of some cell cycle mutants at their restrictive temperature (Hartwell *et al.*, 1973). Moreover the colonies formed by clones that overexpress FLP or RAF are very variable in size and often sectored on galactose plates (Figure 2c). Large colonies growing out have lost the 2μ plasmid (data not shown). A nuclear mutation *nib1* (Holm, 1982) that results in an elevated 2μ plasmid copy number also gives rise to enlarged cells and sectored colonies with a 'nibbled' appearance, although in this case the cells do not show the abnormal budding pattern. We have not observed such phenotypes in [cir^0] strains expressing any 2μ plasmid protein. Others have noted that the cell cycle length of enlarged cells was twice as

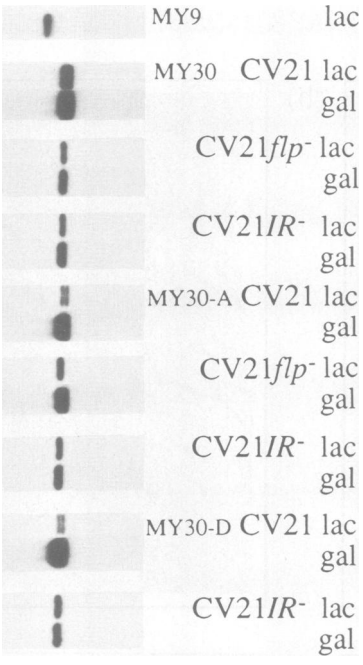

Fig. 3. Increase of copy number of the plasmid CV21 when FLP or RAF levels are elevated is not seen in the non-invertible plasmid CV21*IR*⁻ (see text). Strains were grown to near saturation in selective lactate or galactose (inducing) media and Southern blots of *Eco*RV cut DNA were hybridized to a probe of the 2μ *Pst*I–*Sna*BI fragment. The blot was stripped by boiling 30 min in TE/1% SDS and rehybridized to a yeast ribosomal probe. Appropriate exposures were used to compensate for variation in the amount of DNA loaded. Note that there is variation in the initial (lactate) copy number, but that in each case it responds as expected to galactose induction. The first lane shows the 2μ copy number in the [cir⁺] strain MY9. Subsequent lanes show the effect of galactose induction on plasmid copy number in the [cir⁰] strains MY30 (control), MY30-A (expressing FLP) and MY30-D (expressing RAF). Two bands are seen when FLP inversion of CV21 or a derivative occurs; thus CV21*IR*⁻ always shows only one band, as does CV21*flp*⁻ except when FLP is provided *in trans* (MY30-A gal).

long and that pedigree analysis indicated extensive cell death (A.E.Reynolds, personal communication).

To show that the RAF-induced increase in copy number requires an active FLP inversion system, we constructed a derivative of the plasmid CV21 (Broach *et al.*, 1982; CV21 is *FLP*⁺ *REP1*⁺ *REP2*⁺ *raf*⁻, being 2μ with pBR322 and *LEU2* inserted in the *D* ORF) which cannot invert because *Xba*I sites in the FLP recognition site are removed (Broach *et al.*, 1982). In the diploid [cir⁰] strains MY30-A and MY30-D this plasmid CV21*IR*⁻ was unable to increase its copy number in response to FLP or RAF induction, whereas the copy number of the control plasmid CV21 increased in both cases (Figure 3). Since CV21 is *raf*⁻, this result also shows that amplification does not require RAF. We also constructed CV21*flp*⁻, a derivative of CV21 with a 4-bp deletion in the *FLP* gene, which also increases its copy numbers when FLP is induced.

RNA analysis

We investigated the transcriptional regulation of 2μ plasmid genes by comparing the transcript pattern of the endogenous plasmid pool in the [cir⁺] MY9 strains during growth on lactate and galactose. Since prolonged induction of FLP or RAF causes an increase in copy number, which would complicate the interpretation of results, we used a shorter pulse of galactose induction. Cells were grown in lactate-containing media to relieve glucose catabolite repression and allowed rapid and full induction of the galactose promoter (Adams, 1972) and RNA was prepared from each culture both before and after a two hour pulse with galactose. During the 2 h induction 0.6–0.8 cell doublings occurred (data not shown), corresponding to less than one cell cycle. DNA extracted from each sample before and after the induction showed there was no significant change in plasmid copy number during this period (data not shown).

Since the transcript pattern of the 2μ plasmid is relatively complex and in several regions both strands are transcribed (Figure 1a; Broach *et al.*, 1979; Sutton and Broach, 1985), we used several different strand-specific probes on five duplicate northern blots of total RNA (Figure 4), carefully checking that the amount of RNA loaded in each lane was the same. Note that these probes hybridize both to the galactose-induced and endogenous 2μ plasmid transcripts giving a comparison of the level of induction with the normal cellular levels but at the same time obscuring any possible effect of the chromosomal gene on the expression of the corresponding plasmid gene.

Hybridization to an *FLP*-specific probe (Figure 4a) shows that the normal level of expression of the 1425-base *FLP* transcript is very low in MY9, while there is a strong signal when FLP is induced from the chromosomal construction (MY9-AA, -AB, -AC and -AD). However, an equally strong signal is seen when RAF is induced (MY9-DD), indicating that *FLP* transcripts have increased dramatically from their normal level to about the same abundance as transcripts from the strong galactose promoter. This shows that the level of FLP expression required to obtain the copy number increase described in the previous section can occur from normally promoted *FLP* genes. The total induction of *FLP* expression by RAF was estimated by comparison of preflashed autoradiographs to be about 170-fold. The induction of excess REP2 and RAF together in MY9-CD results in an equivalent increase in *FLP* mRNA levels, but when REP1 is overexpressed together with RAF (MY9-BD), the accumulation of FLP transcripts is about 6-fold lower. Thus REP1 anatagonizes the induction of *FLP* by RAF, which would explain the lack of significant copy number increase observed after overnight growth of this strain.

Hybridization to the *RAF* probe (Figure 4d) shows a substantial reduction in *RAF* mRNA when REP1 and REP2 are overexpressed (MY9-BC). This is in contrast to the result obtained with the *FLP* probe, which, on a long exposure (data not shown), shows there is no reduction in steady state *FLP* transcript levels in response to elevated REP1–REP2 levels. No effect on *RAF* mRNA levels is seen in MY9-BB or MY9-CC indicating that REP1 and REP2 act interdependently to produce this effect. We therefore conclude that *RAF* is negatively regulated by the simultaneous presence of excess REP1 and REP2.

Hybridization to the *REP1* probe (Figure 4b) shows that *REP1* transcripts are relatively abundant in [cir⁺] cells and that the expression of additional REP1 from the chromosomal construct increases the total amount of *REP1* mRNA about 3- to 5-fold above the normal level. There is a consistent increase in *REP1* transcript level when RAF is induced (see MY9-AD, -CD and -DD lanes); this is a direct effect of RAF and not a consequence of a subsequent FLP increase, since the level is normal in MY9-AA. RAF induction also causes a small but reproducible increase in 1950-base transcript levels (Figure 4e and longer exposures of Figure 4b; not shown). We also noted that the 1950-base transcript, that presumably has no protein coding function (Figure 1a), is reduced on induction of REP1 and REP2 together. A short, previously unreported transcript of ~600 bases that lies within the *RAF* coding region is also detected by the 1950-base transcript

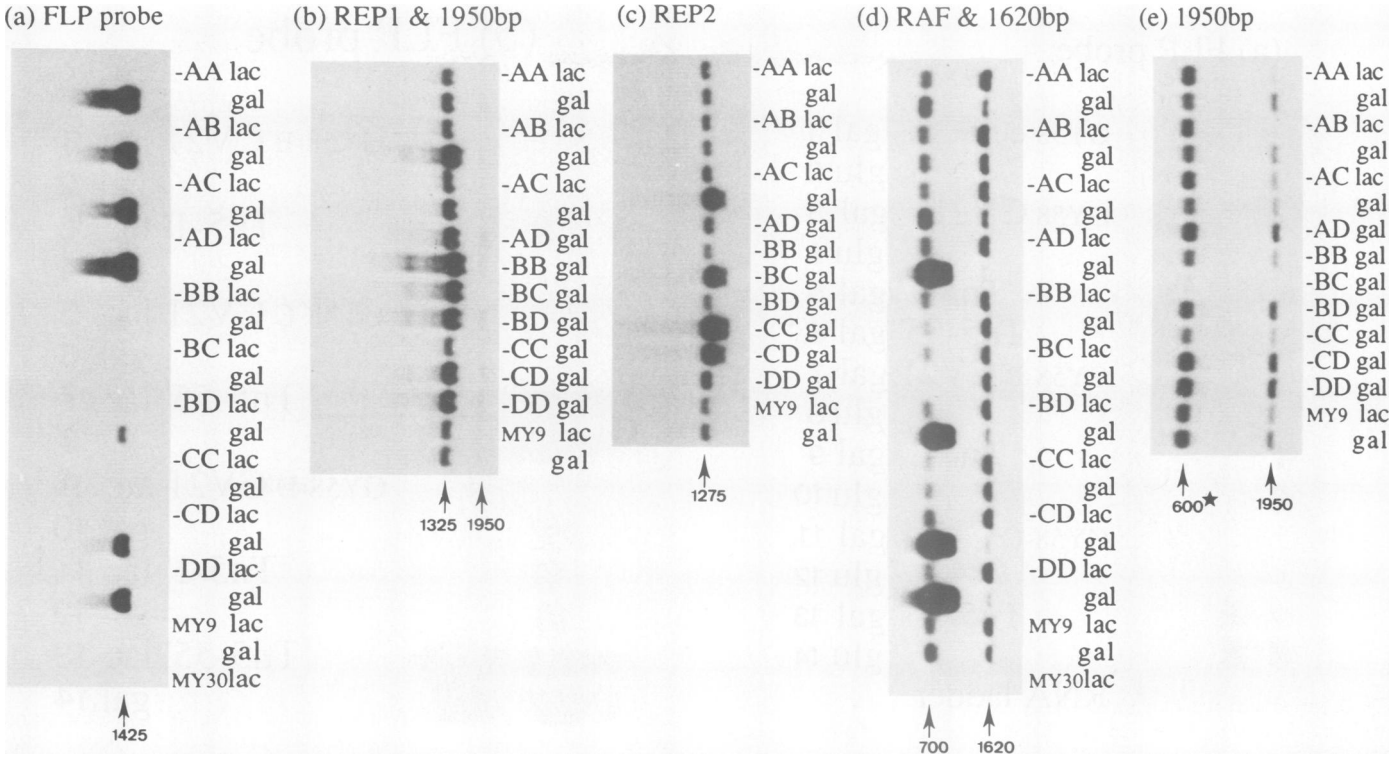

Fig. 4. Northern analysis of duplicate filters of RNA prepared from the diploid MY9[cir$^+$] strains before (lac) and after (gal) a 2-h pulse of galactose induction. MY30 is an isogenic [cir^0] control. The probe is indicated above each blot (see also Materials and methods) and hybridizes to the endogenous 2μ transcripts and to the appropriate galactose induced transcript. The sizes of transcripts are marked. The star indicates a previously unreported 2μ transcript (see text).

probe, but not by the REP1 probe. This suggests that the 600-base transcript may have a 5′ end co-terminal with the 1950-base transcript and a 3′ end before the REP1 gene.

The 1620-base transcript (Figure 1a) is also unlikely to have a protein-coding function. RAF induction causes the disappearance of this transcript and the appearance of an abundant novel shorter transcript that hybridizes to the same probe (Figure 4d). We can conclude that this transcript is of 2μ origin and not from the galactose-promoted construct since it is not detected by the *CYC1* probe (Figure 1d), but its function, if any, is unknown.

No regulation of *REP2* transcription by other proteins can be seen in this experiment (Figure 4c), although, as in the case of RAF, REP2 induction causes the appearance of a considerably longer transcript of unknown function that hybridizes to the *REP2* probe.

Direct control of FLP expression

These experiments would have failed to detect any direct negative regulation of *FLP* by plasmid encoded genes if repression were already maximal at normal copy number. We therefore compared the level of *FLP* transcripts in the plasmid CV21 (*FLP*$^+$ *REP1*$^+$ *REP2*$^+$ *raf*$^-$) and two derivatives Tn*5*-1′ and Tn*5*-55′ that have transposon insertions disrupting the *REP*1 and *REP*2 ORFs respectively (Broach *et al.*, 1982; Jayaram *et al.*, 1983). Some caution has to be used in the interpretation of these experiments involving comparison of wild-type and mutant plasmids, since REP1 and REP2 are also involved in plasmid stability. In Figure 5 we compare the wild-type situation, in which most of the cells contain a reasonably uniform plasmid copy number, with mutant cultures where the plasmid is present in a lower proportion of cells at more extreme copy number. However, when we made appropriate correction for the proportion of cells containing the plasmids, both Tn*5*-1′ and Tn*5*-55′ have a higher level of *FLP* expression than CV21 (Figure 5a), suggesting that both REP1 and REP2 are required for normal *FLP* regulation. We used the inducible strains to show that the elevated level of *FLP* expression in *rep* defective strains can be complemented *in trans*. When the strain GY58-B (expressing REP1) was transformed with Tn*5*-1′ (*rep1*$^-$*REP2*$^+$), we observed a high level of FLP expression on lactate, but after three hours of REP1 induction, FLP transcripts fell to a normal level (Figure 5b). A lesser effect was obtained with the strain GY58-C (expressing REP2) and the plasmid Tn*5*-55′ (*REP1*$^+$*rep2*$^-$). Moreover, when GY58-B transformed with Tn*5*-1′, or GY58-C with Tn*5*-55′, was grown in galactose and then shifted to glucose for 6 h, we observed the expected increase in FLP expression as the REP protein level fell (Figure 5a). We therefore conclude that *FLP* is also negatively regulated directly by REP1 and REP2 acting together.

We also noted in this experiment that accumulation of REP1 by prolonged induction causes a minor increase in FLP transcript levels (Figure 5a, lanes 7 and 8), although a shorter induction does not (Figure 5b, lanes 1 and 2).

In a further experiment we asked whether the induction of *RAF* would have an additional positive effect on *FLP* expression in the situation where REP1−REP2 expression was already relieved. We found that, whereas a pulse of *RAF* induction caused a large increase in *FLP* transcripts in GY58-D transformed with the control plasmid CV21 (Figure 5b, lanes 9 and 10), RAF induction had no additional effect on the level of FLP transcripts in GY58-D transformed with Tn*5*-1′ (*REP1*$^+$*rep2*$^-$; lanes 11 and 12) or Tn*5*-55′ (*rep1*$^-$*REP2*$^+$; lanes 13 and 14). RAF therefore acts not as a classical activator of *FLP* expression, but as an anti-repressor of REP1−REP2 repression of *FLP*. We can-

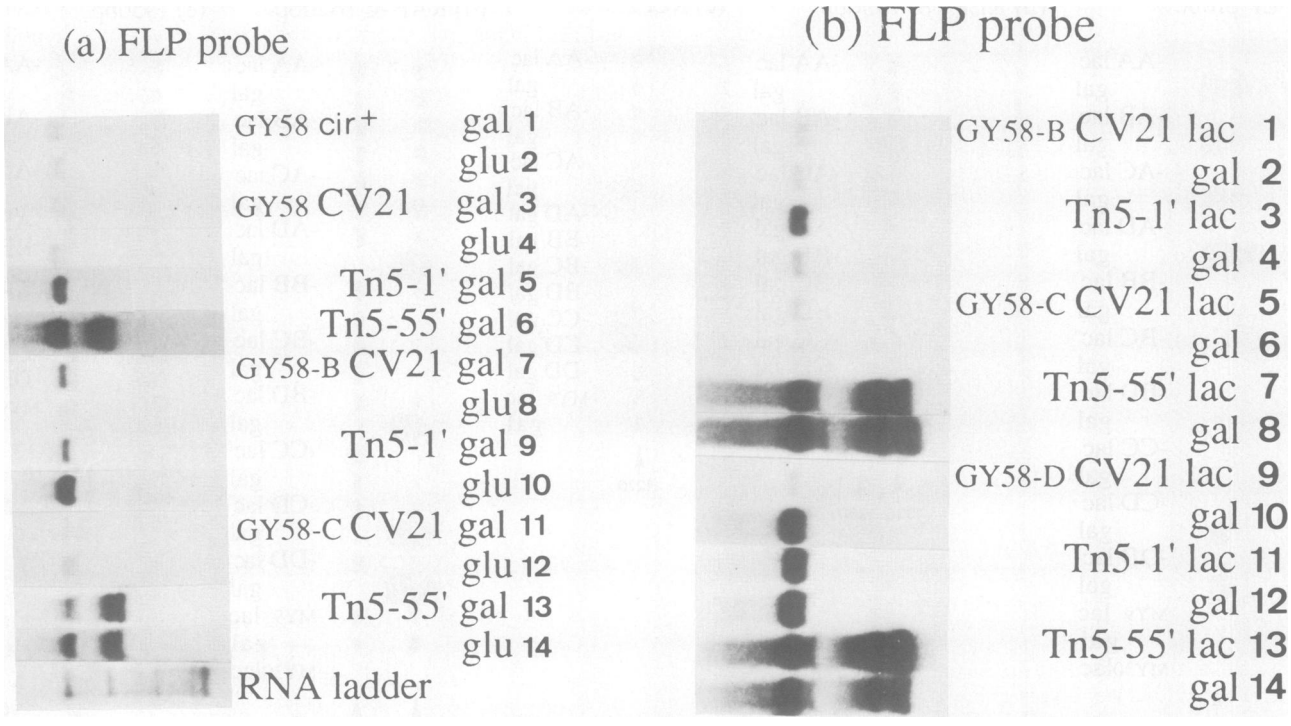

Fig. 5. Repression of *FLP* requires both REP1 and REP2, and RAF is an anti-repressor of the REP1−REP2 repression of *FLP*. Northern analyses, hybridized to *FLP* specific probe, of strains transformed with the plasmids indicated (see text). (**a**) Lanes 1 and 2 show the level of FLP expression in GY58 [cir$^+$] and subsequent lanes the level in GY58 [cir^0] transformed with the plasmids indicated. FLP expression is elevated in cells carrying plasmids with an insertion in REP1 (Tn*5*-1′; lane 5) or REP2 (Tn*5*-55′; lane 6). Expression of the complementing REP protein in GY58-B (lane 9) or GY58-C (lane 13) restores lower *FLP* transcript levels. FLP increases when REP1 or REP2 is withdrawn by a shift to glucose (lanes 10 and 14). Note prolonged REP1 expression causes slight FLP increase (compare lanes 7 and 8). Strains were grown in selective galactose media, shifted to complete galactose media for 2 h, RNA was made from half the sample, and the remainder washed and transferred to complete glucose media for 6 h before RNA preparation. The exposures of each lane are corrected for the number of plasmids present as described in Materials and methods. Tn*5*-55′ shows two extra slower mobility bands from promoters within Tn*5*. The RNA ladder (BRL) shows bands of 9.49, 7.46, 4.40, 2.37 and 1.30 kb. (**b**) The strains were grown in repressing (lactate) selective (-leu) media and RNA was prepared before (lac on figure) and after (gal) a 3 h pulse with 2% galactose. Lanes 9−14 show that induction of *RAF* in GY58-D causes no further increase in *FLP* transcription when either REP1 (Tn*5*-1′) or REP2 (Tn*5*-55′) is absent.

not conclude whether RAF causes its effect on *FLP* expression by reducing the translation of REP1 or REP2, by protein−protein interaction or by competition for DNA binding sites.

Discussion

The 2μ plasmid has evolved two strategies which ensure its long term survival in yeast populations. First it has the REP1−REP2-*STB* partition system to overcome the strong maternal inheritance bias (Murray and Szostak, 1983). Secondly, it has evolved a novel method for recovering from downward fluctuations in copy number that does not depend on altering the rate of initiation of replication, but on reversing the direction of one replication fork, so that the forks chase each other around the circle producing many plasmid copies from a single initiation event. This switch is mediated by the FLP site-specific recombination system. We show that copy number is negatively regulated by controlling the level of *FLP* transcripts, since an increase in the level of FLP results in an increased copy number.

Although overexpression of REP1 and REP2 in the diploid strain MY9-BC does not decrease the level of *FLP* transcripts, other experiments presented here demonstrate that these two products act together to repress *FLP* transcription. This is in keeping with the observation of Veit and Fangman (1985) that the normal chromatin organization of the 5′ end of the *FLP* ORF depends on both REP1 and REP2. In addition, the expression of β-galactosidase from a chromosomally integrated *FLP*-lacZ fusion is negatively regulated by REP1 and REP2 (A.E.Reynolds, A.W.Murray and J.W.Szostak, submitted).

If copy number is controlled by REP1−REP2 repression of *FLP*, a simple prediction is that if either REP1 or REP2 is absent, the repressor should be inactive and the copy number rise. Such experiments are not completely straightforward because REP1 and REP2 are also required for segregation, but when appropriate correction is made the copy number of a *FLP*$^+$*REP1*$^+$*rep2*$^-$ plasmid is six times higher than that of a *FLP*$^+$*REP1*$^+$*REP2*$^+$ plasmid (Veit and Fangman, 1985). RAF, the product of the *D* ORF, has the opposite effect to REP1 and REP2 and increases *FLP* expression and plasmid copy number. Since the overexpression of RAF has no additional effect on *FLP* transcript levels when the repression by REP1−REP2 is already relieved because one of them is absent, it appears that RAF acts by antagonizing the repressing effects of REP1 and REP2. The rapid and large effect of RAF induction on the accumulation of *FLP* transcripts suggests that the regulation is at the level of transcription initiation, although we cannot rule out the possibility of a very efficient messenger stabilization mechanism. RAF is itself regulated by REP1 and REP2 acting together and at normal plasmid copy number a further increase in REP1−REP2 levels reduces *RAF* expression (Figure 4d), although it does not decrease *FLP* expression any more.

Why should copy number be controlled by two antagonistic factors rather than simply by a direct effect of REP1−REP2 concentration alone? It is likely that this strategy gives a greater sen-

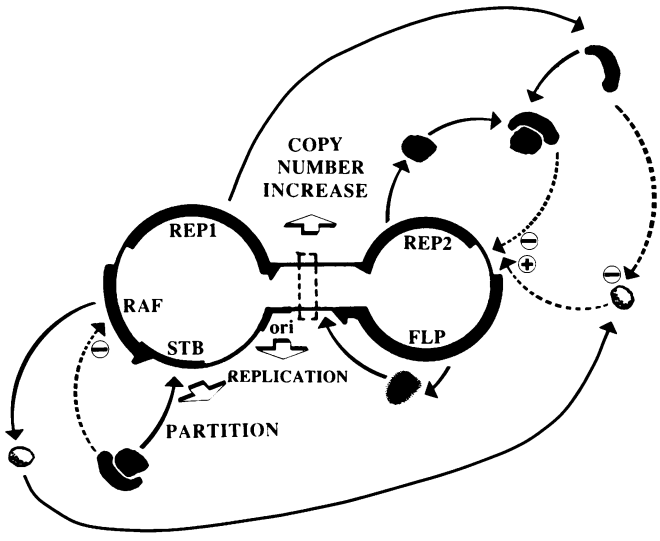

Fig. 6. Model for the roles of FLP, REP1, REP2 and RAF in the independent systems that confer replication, partition and copy number increase (large arrows). Proposed regulatory interactions are shown by dotted lines. REP1 and REP2 are drawn as forming a REP complex, but we have no evidence for this. REP1−REP2 have three roles: they act on STB-*proximal* to mediate partition, and they negatively regulate both *FLP* expression and the expression of the anti-repressor *RAF*. REP1 is indicated directly antagonising RAF induction of *FLP*. There is no pathway by which copy number can be reduced.

sitivity to small fluctuations in copy number, since a fall in copy number and therefore in REP1−REP2 concentration not only directly alleviates the repression of *FLP*, but also allows a higher level of the anti-repressor RAF, thereby amplifying the signal. The different sensitivities of FLP and RAF expression to REP1−REP2 concentration that are suggested by our results may be an integral part of this mechanism.

In a previous analysis (Murray and Cesareni, 1986) we identified two domains in *STB*. *STB*-proximal is the region whose integrity is essential for the partition function of REP1 and REP2 to operate. It contains two tandem direct repeats of 124 bp with 97% homology on which are superimposed five smaller repeats of 62 bp with 78−97% homology. Farther from the origin lies the second domain *STB*-distal that contains not only terminators important for protecting *STB*-proximal, but also a sequence that is capable of repressing transcription from a heterologous promoter upstream. Within this region is a repeated 24-bp motif that is also found upstream of the *FLP* gene. We believe that the REP1−REP2 complex may interact with this sequence to repress transcription, so that the regulation of *RAF* gene expression is mediated from a 'silencer-like' element downstream of the gene (Brand *et al.*, 1985). The interaction at this locus would also conveniently explain the repression by REP1−REP2 of the 1950-base transcript that we observe together with *RAF* repression (Figure 4e). The 1950-base transcript has already been reported to be negatively regulated in response to 2μ plasmid copy number (Jayaram *et al.*, 1985).

In this model REP1−REP2 interact with three distinct plasmid domains with decreasing affinity: first the primary role of REP1−REP2 in partition, interacting with *STB*-proximal, is evident even at low concentration. Secondly, REP1−REP2 interacts with sequences upstream of *FLP* progressively reducing *FLP* expression as copy number increases. Thirdly, with lower affinity REP1−REP2 represses the transcription of *RAF*, perhaps by interacting with sequences in *STB*-distal. These interactions are illustrated in Figure 6. Some of the regulatory mechanisms illustrated in the figure must be considered as working hypotheses and should be confirmed by independent approaches. It is possible that some of the effects that we have identified are only due to non-physiological levels of the relevant gene product after induction in galactose medium. Secondly, our approach would have missed any regulatory circuit that involves the regulation of a gene by its own protein product. Furthermore, in proposing the model of Figure 6, we have not taken into account some observations that we have considered 'minor effects' (negative regulation of the 1620 transcript by *RAF*, elevated levels of REP1 transcripts when RAF is induced) which might turn out to be the features of the regulatory circuit.

A central tenet of this model is that the expression of *REP1* or *REP2* is proportional to copy number and we are currently conducting experiments to test the expression of these genes from their own promoters at different plasmid copy numbers. Cashmore *et al.* (1986) have already shown that one copy of *REP2* at a chromosomal location is sufficient to complement a multicopy *rep2*$^-$ plasmid, whereas one copy of the *REP1* gene does not produce enough product to stabilize many copies of a *rep1*$^-$ plasmid. However, the stability of the *rep1*$^-$ plasmid increases as the number of copies of the integrated *REP1* gene is increased. This suggests that *REP1* is expressed proportionately to 2μ copy number as predicted by the model and that therefore each *REP1* gene copy only produces enough product for the segregation of one, or a small number, of plasmids.

Thus in contrast to copy number control mechanisms evolved by bacterial plasmids, that regulate the number of initiation events, the 2μ plasmid initiates replication every cell cycle in concert with chromosomal DNA and only regulates amplification. It is therefore unable to counter upward fluctuations in copy number, and clones of cells with an elevated 2μ level tend to maintain a high copy number (Holm, 1982; Futcher and Cox, 1984). In fact we might expect the average copy number of the population to drift upwards due to the odd unequal segregation event followed by amplification in the cell inheriting fewer plasmids. This trend is presumably counteracted by the occasional failure of every plasmid origin to initiate replication in every cell cycle, the reduced viability of cells with an elevated copy number and the tendency for partition failures to leave plasmids in the mother cells (Murray and Szostak, 1983) which have a limited potential for further division because of senescence (Mortimer and Johnston, 1959). The very low level of FLP expression in the strain MY9 which is unresponsive to further increase in REP1−REP2 concentration (Figure 3a) may therefore represent the level of site-specific recombination events required to balance these occasional losses or failure to replicate. Further experiments will reveal the molecular basis of the mechanisms that control the 2μ response to changes in plasmid copy number.

Materials and methods

Construction of plasmids

The expression constructs were made using the galactose inducible yeast expression plasmid pEMBLyex4, which was derived from pEMBLyex2 (Baldari *et al.*, 1987) by removal of the *Xba*I site in 2μ sequences (Cesareni and Murray, 1987). Specific constructs were as follows: pyexFLP: *Sph*I−*Xba*I *FLP* fragment from BTYP-2 (Hollenberg, 1978) cloned in the same sites in M13tg131 (Kieny *et al.*, 1983), then into pEMBLyex4 as an *Sst*I−*Pst*I fragment. pyexREP1: *Pvu*II−*Xba*I *REP1* fragment from BTYP-2 in *Eco*RV site of pBGS131$^+$ (Spratt *et al.*, 1986) excised (*Sst*I−*Xba*I) and inserted in pEMBLyex4 in the same sites. *REP2*: *Eco*RV−*Xba*I REP2 fragment from pJBD219 (Beggs, 1978) was cloned in pEMBLex1 (Sollazzo *et al.*, 1985) and oligonucleotide directed mutagenesis used to delete upstream sequences and convert the first four bases of REP2 (ATGG) into an *Nco*I site (CCATGG) giving pex14. This was digested completely with

*Xba*I, partially with *Nco*I, Klenow filled, and the full length REP2 fragment isolated and ligated in LMP agarose (Murray, 1986) to *Pst*I−*Sna*BI cut and Klenow filled pEMBLyex4 giving pyexREP2Δ. pyexD:p82-6B (Hartley and Donelson, 1980) digested with *Bsm*I−*Hpa*I and ligated to *Sst*I−*Sma*I cut pEMBLyex4 in the presence of *Sst*I−*Bsm*I synthetic adaptors (oligos CAATATAAATGCC and CATTTATATTGAGCT). These oligos put the sequence from the equivalent position in *GAL10* just upstream of the ATG and appear to make the transcript more stable than the other constructs (Figure 1d). pyexFLPΔ and pyexREP1Δ are integrating derivatives with 2μ origin deleted by cutting *Pst*I−*Sna*BI, Klenow treatment and recircularization. Similarly pyexDΔ was made by cutting with *Xba*I and *Sna*BI. Only pyexREP1Δ contains any 2μ sequence (23 bp) upstream of predicted ATG translation start (Hartley and Donelson, 1980); all fragments maintain normal polyadenylation and termination sites (Sutton and Broach, 1985).

CV21 consists of the entire 2μ plasmid B form (Figure 1a) with pBR322 and *LEU2* inserted in the *Eco*RI site in the *D* ORF (Broach *et al.*, 1982). Tn*5*-1′ and Tn*5*-55′ are derivatives of CV21 with Tn5 transposon insertions in the *REP1* and *REP2* genes respectively (Jayaram *et al.*, 1983). CV21*flp*$^-$ was constructed by partial digestion of CV21 with *Sph*I (CV21 has two *Sph*I sites, one in pBR322 sequences and one overlapping the translation initiation codon of the *FLP* gene), exonuclease treatment with Klenow polymerase, excision of the linearized plasmid on a low melting point agarose gel, and ligation in the agarose (Murray, 1986). A clone lacking the *Sph*I site in the *FLP* gene was selected. CV21*IR*$^-$ was constructed by removing the *Xba*I sites from the 2μ IRs of CV21, thereby abolishing FLP-mediated recombination (Broach *et al.*, 1982). After complete digestion of CV21 with *Xba*I and blunt ending with Klenow polymerase, a clone lacking *Xba*I sites in which the 2μ fragment was re-inserted in the same orientation as in CV21 was chosen.

Strains

The *E.coli* strains 71/18 (Messing *et al.*, 1977) and RR1ΔM15 (Rüther, 1982) were used in this work. The *S.cerevisiae* strain VB2-20A (α *ade2 leu2 trp1 ura3*), was obtained from P.Philippsen and corresponds to the strain GY1 [cir^0] in our collection. A [cir$^+$] derivative of GY1 [cir^0] was derived by transformation with the plasmid p82-6B+LEU as described by Veit and Fangman (1985). GY58[cir^0] (a *ade1 leu2 trp1 ura3*) was derived from a random spore analysis of GY52, a cross of SC3 (α *gal2 gal10 his3 trp1 ura3* [cir^0] Sigurdson *et al.*, 1981) and DC04a (a *ade1 leu2-04* [cir^0] from Y.G.S.C.Berkeley; Broach and Hicks, 1980). GY58[cir$^+$] was constructed in the same way as GY1[cir$^+$]. MY9 and MY30 are isogenic (*ade1/ADE1 ade2/ADE2 leu2/leu2 trp1/trp1 ura3/ura3*) [cir$^+$] and [cir^0] diploids derived respectively by GY1[cir$^+$] × GY58[cir^0] and GY1[cir0] × GY58[cir^0] crosses. The strains GY58-A to GY58-D were constructed by transformation of GY58[cir^0] with pyexFLPΔ to pyexDΔ. These were crossed to similar transformants of GY1[cir$^+$] to make the MY9 series of strains (see Figure 1c).

Northern analysis

Cultures were grown at 30°C to OD_{590} ~0.5 in complete lactate media; 50 ml culture was harvested for preparation of total RNA (Rubin, 1975) and remainder made 2% in galactose and grown for a further 2 h before RNA preparation. RNA concentration was adjusted to 2 mg/ml, checked, and 12 μg used per lane for northern analysis on GeneScreen *Plus*™ (Murray and Cesareni, 1986). The single strand specific probes were made by *in vitro* T7 or T3 transcription (Morris *et al.*, 1986) in the direction indicated on linearized templates of the following 2μ fragments cloned in Bluescribe (Stratagene). FLP: *Hin*dIII−*Sph*I, REP1 and 1950-base transcript: *Hin*dIII−*Pvu*II, RAF and 1620-base: *Pst*I−*Hin*dIII, 1950-base: as RAF, but in opposite direction. The probe for REP2 was the oligolabelled double strand 163 bp *Nco*I fragment from pex14 (see above; labelled as described by Feinberg and Vogelstein, 1983). Hybridization conditions were as described (Murray and Cesareni, 1986).

Correction of exposures for Figure 5 was done using a dot blot hybridization of samples of the same RNA to a pAT153 (Twigg and Sherratt, 1980) probe that detects abundant transcripts starting within the pBR322 region of CV21 (Marczynski and Jaehning, 1985; Murray and Cesareni, 1986). These transcripts are assumed to be unregulated. This dot blot filter was exposed on the same autoradiograph as the *FLP* hybridized northern transfer filters shown in Figure 5 and an exposure was chosen for each track such that the equivalent control spot gave an optical density reading of 0.5−0.8 on a Melico/Photolog black/white transmission densitometer.

Southern analysis

DNA was prepared as described (Murray and Cesareni, 1986). Agarose gels (0.8%) were run in Tris-acetate buffer and transferred to GeneScreen *Plus*™ membranes and hybridized according to the manufacturer's instructions.

Acknowledgements

We thank B.Sproat and P.Neuner for the oligonucleotides, B.Veit and M.Jayaram for plasmids and V.Allan for helping with the photomicrographs. Some of the work described here was supported by Progetto Finalizzato Ingegneria Genetica e Basi Molecolari delle Malattie Ereditarie of CNR, Rome.

References

Adams,B.G. (1972) *J. Bacteriol.*, **111**, 308−315.
Baldari,C., Murray,J.A.H., Ghiara,P., Cesareni,G. and Galeotti,C.L. (1987) *EMBO J.*, **6**, 229−234.
Beggs,J.D. (1978) *Nature*, **275**, 104−109.
Brand,A.H., Breeden,L., Abraham,J., Sternglanz,R. and Nasmyth,K. (1985) *Cell*, **41**, 41−48.
Broach,J.R. (1983) *Methods Enzymol.*, **101**, 307−325.
Broach,J.R. and Hicks,J.B. (1980) *Cell*, **21**, 501−508.
Broach,J.R., Atkins,J.F., McGill,C. and Chow,L. (1979) *Cell*, **16**, 827−839.
Broach,J.R., Guarascio,V.R. and Jayaram,M. (1982) *Cell*, **29**, 227−234.
Cashmore,A.M., Albury,M.S., Hadfield,C. and Meacock,P.A. (1986) *Mol. Gen. Genet.*, **203**, 154−162.
Cesareni,G. and Murray,J.A.H. (1987) In Setlow,J.K. (ed.), *Genetic Engineering: Principles and Methods Volume 9*. Plenum Press, NY, pp. 135−154.
Clark-Walker,G.D. and Miklos,G.L.G. (1974) *Eur. J. Biochem.*, **41**, 359−365.
Dente,L., Cesareni,G. and Cortese,R. (1983) *Nucleic Acids Res.*, **11**, 1645−1655.
Feinberg,A.P. and Vogelstein,B. (1983) *Anal. Biochem.*, **132**, 6−13.
Futcher,A.B. (1986) *J. Theor. Biol.*, **119**, 197−204.
Futcher,A.B. and Cox,B.S. (1983) *J. Bacteriol.*, **154**, 612−622.
Futcher,A.B. and Cox,B.S. (1984) *J. Bacteriol.*, **157**, 283−290.
Guarente,L., Yocum,R.R. and Gifford,P. (1982) *Proc. Natl. Acad. Sci. USA*, **79**, 7410−7414.
Hartley,J.L. and Donelson,J.E. (1980) *Nature*, **286**, 860−864.
Hartwell,L.H., Mortimer,R.K., Culotti,J. and Culotti,M. (1973) *Genetics*, **74**, 267−286.
Hollenberg,C.P. (1978) *Mol. Gen. Genet.*, **162**, 23−34.
Holm,C. (1982) *Cell*, **29**, 585−594.
Jayaram,M., Li,Y.-Y. and Broach,J.R. (1983) *Cell*, **34**, 95−104.
Jayaram,M., Sutton,A. and Broach,J.R. (1985) *Mol. Cell. Biol.*, **5**, 2466−2475.
Jazwinski,S.M. and Edelman,G.M. (1979) *Proc. Natl. Acad. Sci. USA*, **76**, 1223−1227.
Jong,A.Y.S. and Scott,J.F. (1985) *Nucleic Acids Res.*, **13**, 2943−2958.
Kieny,M.P., Lathe,R. and Lecocq,J.P. (1983) *Gene*, **26**, 91−99.
Kikuchi,Y. (1983) *Cell*, **35**, 487−493.
Livingston,D.M. and Kupfer,D.M. (1977) *J. Mol. Biol.*, **116**, 249−260.
Marczynski,G.T. and Jaehning,J.A. (1985) *Nucleic Acids Res.*, **13**, 8487−8506.
Mead,D.J., Gardner,D.C.J. and Oliver,S.G. (1986) *Mol. Gen. Genet.*, **205**, 417−421.
Messing,J., Gronenborn,B., Müller-Hill,B. and Hofschneider,P.H. (1977) *Proc. Natl. Acad. Sci. USA*, **74**, 3642−3646.
Morris,C.E., Klement,J.F. and McAllister,W.T. (1986) *Gene*, **41**, 193−200.
Mortimer,R.K. and Johnston,J.R. (1959) *Nature*, **183**, 1751−1752.
Murray,A.W. and Szostak,J.W. (1983) *Cell*, **34**, 961−970.
Murray,J.A.H. (1986) *Nucleic Acids Res.*, **14**, 10118.
Murray,J.A.H. (1987) *Mol. Microbiol.*, **1**, 1−4.
Murray,J.A.H. and Cesareni,G. (1986) *EMBO J.*, **5**, 3391−3399.
Parent,S.A., Fenimore,C.M. and Bostian,K.A. (1985) *Yeast*, **1**, 83−138.
Rubin,G.M. (1975) *Methods Cell Biol.*, **XII**, 45−64.
Rüther,U. (1982) *Nucleic Acids Res.*, **10**, 5765−5772.
Scott,J.R. (1984) *Microbiol. Rev.*, **48**, 1−23.
Sigurdson,D.C., Gaarder,M.E. and Livingston,D. (1981) *Mol. Gen. Genet.*, **183**, 59−65.
Sollazzo,M., Frank,R. and Cesareni,G. (1985) *Gene*, **37**, 199−206.
Spratt,B.G., Hedge,P.J., Heesen,S.t., Edelman,A. and Broome-Smith,J.K. (1986) *Gene*, **41**, 337′−342.
Sutton,A. and Broach,J.R. (1985) *Mol. Cell. Biol.*, **5**, 2770−2780.
Taketo,M., Jazwinski,S.M. and Edelman,G.M. (1980) *Proc. Natl. Acad. Sci. USA*, **77**, 3144−3148.
Twigg,A.J. and Sherratt,D. (1980) *Nature*, **283**, 216−218.
Veit,B.E. and Fangman,W.L. (1985) *Mol. Cell. Biol.*, **5**, 2190−2196.
Volkert,F.C. and Broach,J.R. (1986) *Cell*, **46**, 541−550.
Wu,L.-C.C., Fisher,P.A. and Broach,J.R. (1987) *J. Biol. Chem.*, **262**, 883−891.
Zakian,V.A., Brewer,B.J. and Fangman,W.L. (1979) *Cell*, **17**, 923−934.

Received on August 7, 1987; revised on September 24, 1987