MOLECULAR AND CELLULAR BIOLOGY, Sept. 1985, p. 2466–2475
0270-7306/85/092466-10$02.00/0

Vol. 5, No. 9

Properties of *REP3*: a *cis*-Acting Locus Required for Stable Propagation of the *Saccharomyces cerevisiae* Plasmid 2μm Circle

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Received 31 December 1984/Accepted 15 May 1985

Stable propagation of the yeast plasmid 2μm requires an origin of replication, a *cis*-active locus designated *REP3*, and two plasmid-encoded proteins which are the products of the *REP1* and *REP2* genes. The three *REP* loci appear to constitute a partitioning system, ensuring equal distribution of plasmid molecules to mother and daughter cells after mitosis. We have localized the *REP3* site completely within a segment of five-and-one-half direct tandem repeats of a 62-base-pair unit, bordered by *Hpa*I and *Ava*I restriction sites within the large unique region of the 2μm genome. In addition, we find that the repeated elements are functionally distinct. Only a subset of the repeats is necessary to promote full partitioning activity. The other repeats appear to promote plasmid transcription. These results are discussed in the context of a model of plasmid copy control involving titration of a plasmid-specific protein by the repeated elements within *REP3*.

As an autonomously propagating genetic element, the multicopy yeast plasmid 2μm circle can be viewed as a chromosomal replicon on which are superimposed plasmid-encoded stability functions. Under steady-state growth conditions, the plasmid behaves essentially as a chromosomal replicon: each plasmid replicates once and only once during each cell cycle, using the same enzymatic machinery used to replicate chromosomal DNA (27, 32, 37). Despite stringent control of its replication, the 2μm circle is stable during mitotic growth and is transmitted efficiently to all haploid progeny after meiosis (5, 16). Many hybrid plasmids constructed from 2μm circle sequences also manifest high mitotic and meiotic stability (9, 17, 23, 24, 29). The high stability of hybrid 2μm plasmids stands in marked contrast to hybrid plasmids constructed from *ARS* fragments, sequences that promote autonomous replication in yeast cells and that are likely candidates as chromosomal replicons. Such plasmids are frequently not transmitted to daughter cells after mitosis and thus exhibit substantial mitotic instability. In addition, most *ARS* plasmids are not transmitted to spores after meiosis. Current evidence suggests that the relatively high stability of the 2μm circle and of hybrid plasmids derived from it can be attributed to a plasmid-encoded partitioning system and to the ability of the plasmid to increase copy number when its copy number is low (23, 24).

In recent studies, the 2μm circle-encoded components that are necessary for stable plasmid propagation have been defined (23, 24). This stability system consists of two *trans*-acting functions and two *cis*-acting functions (Fig. 1). The *trans*-acting functions correspond to two proteins encoded by plasmid genes designated *REP1* and *REP2* (corresponding to reading frames B and C, respectively, of Hartley and Donelson [21]). One of the *cis*-acting functions is the replication origin of the plasmid, which has been localized to a region of less than 85 base pairs (bp) (10, 23). The second *cis*-acting site is located several hundred bp away from the origin and is, perhaps, the site at which the *REP* proteins act. This site has been called the *REP3* or *STB* locus.

The *REP3* locus has several unusual structural and functional features. It encompasses a region of the plasmid consisting of a 62-bp element arranged in an array of five-and-one-half direct tandem repeats. These repeated elements are not absolutely identical but, rather, exhibit cross-homology of between 75 and 90%. *REP3* also resides in a region of the genome corresponding to the 3′ end of several plasmid-derived RNAs as well as a promoter sequence for a transcript of unknown function (A. Sutton and J. R. Broach, Mol. Cell. Biol., in press). In addition, the segment of DNA spanning *REP3* is bereft of nucleosomes in vivo (26). Finally, Kikuchi has determined that the stabilizing activity of *REP3* is independent of its distance from and orientation to the origin of replication and, in fact, is active with origin sequences other than that from 2μm circle (24).

Our earlier analysis left some ambiguity as to the functional limits of the DNA segment that constitutes the *REP3* locus (23). Thus, we were not previously able to define the precise location of the *REP3* locus or to ascertain which if any of the above structural features plays an integral role in *REP3* function. In this paper, we provide a more refined analysis of this locus and demonstrate that the segment of DNA encompassing the repeated 62-bp element is sufficient to elicit *REP3* function. In addition, we find that the repeated elements are functionally distinct. Only a subset of the repeats is necessary to promote full partitioning activity. The other repeats appear to promote plasmid transcription. These results are discussed in the context of a model of plasmid copy control involving titration of a plasmid-specific protein by the repeated elements within *REP3*.

MATERIALS AND METHODS

Strains. *Escherichia coli* C600 (*thr-1 thi-1 leuB6 supE44 lacY1 tonA21 hsdR hsdM*$^+$) or HB101 (*recA13 ara-14 proA2 lacY1 galK2 supE44 xyl-5 mtl-1 r*B$^-$*m*B$^-$) was used for construction, propagation, and amplification of hybrid plasmids.

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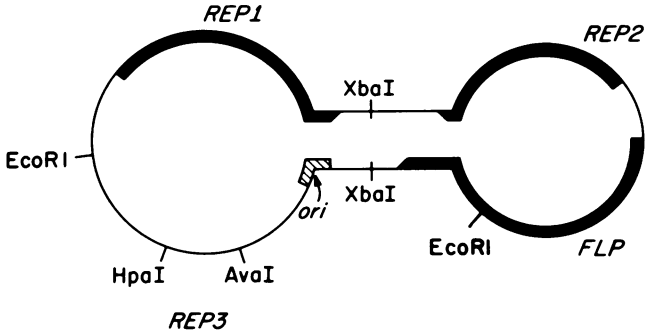

FIG. 1. Genetic components of the 2μm circle stability system. Genetic elements comprising the stability system of the 2μm circle are indicated on a schematic diagram of the B form of the plasmid genome. These elements include two *trans*-active loci, *REP1* and *REP2*, the *cis*-active locus, *REP3*, and the origin of replication (hatched box). The location of a third plasmid gene, *FLP*, is also shown (tapered arrows correspond to the 3′ end of the coding regions).

The following yeast strains were used in this study: DC04 [cir$^+$] and [cir^0] (*MAT***a** *ade1 leu2-04*), S150-2B [cir$^+$] and [cir^0] (*MAT***a** *leu2-3 leu2-112 his3 ura3-52 trp1*), and K207 [cir$^+$] and [cir^0] (*HML***a** *MAT***a** *HMR***a** *mar1 leu2 his2 his3 trp1*). The symbols [cir$^+$] and [cir^0] refer to strains that contain or lack the endogenous 2μm circle, respectively. We have determined that the stabilities of plasmids under nonselective growth are independent of the host strains employed to measure them; that is, a plasmid carrying the *LEU2* marker showed the same stability irrespective of whether it was resident in strain DC04, SI50-2B, or K207. Stabilities of the *HIS3*- or *TRP1*-containing plasmids were determined in S150-2B or in K207, respectively. Rich (YEPD) and synthetic media have been previously described (12).

Plasmids. Plasmids used to identify the boundaries of *REP3* all carry a defined portion of the 2μm circle genome cloned into a vector consisting of pBR322 plus either *LEU2*, *HIS3*, or *TRP1* as a yeast-selectable marker (see Fig. 3 and 4). The specific constructions of several of these plasmids have been described previously; references are given in the appropriate figure legends. The series of plasmids with designations p18 or 17.1 was constructed specifically for this study. These plasmids contain specific 2μm circle segments cloned into either plasmid pSZ62 (31) or plasmid YRp17.1 (constructed by D. T. Stinchcomb, C. Mann, and R. W. Davis, Stanford University, Stanford, Calif.) (see Fig. 2 and 3, respectively). Plasmid pSZ62 consists of pBR322 carrying a *Bam*HI fragment spanning the yeast *HIS3* gene. Plasmid YRp17.1 is derived from plasmid YRp17 (34) and consists of pBR322 plus an *Eco*RI fragment spanning *ARS1* and *TRP1* and a *Hin*dIII fragment spanning *URA3*. Salient to our purposes, plasmid YRp17.1 has unique *Eco*RI, *Sal*I, *Ava*I, and *Pvu*II sites. For each construction, the 2μm circle fragment of interest was purified by electrophoresis on low-gelling agarose (25) and ligated to the appropriate vector plasmid digested with the desired restriction enzyme(s). The specific constructions used in this study are diagrammed in Fig. 2.

Plasmid pMJ20 consists of the entire 2μm circle genome with pBR322 inserted into the *Eco*RI site of the small unique region. Plasmids XHO3, XHO4, XHO5, and XHO15 are previously described deletion derivatives of plasmid pMJ20, each carrying a small defined deletion around the *Hpa*I site in the large unique region of the 2μm circle (23). An *Xho*I linker was inserted at the site of the deletion in each plasmid. The *Sal*I-*Xho*I fragment spanning *LEU2* was then inserted into the *Sal*I site in the pBR322 moiety of each plasmid.

Plasmids XHO5-PAB120 and XHO5-PAB120′ contain the yeast *CYC1* transcription terminator introduced in opposite orientations into the *Xho*I site of plasmid XHO5. We excised the terminator as a 120-bp *Bam*HI-*Hin*dIII fragment from plasmid pAB120 (provided by K. Zaret and F. Sherman, University of Rochester, Rochester, N.Y.). We rendered the ends of the purified fragment flush by incubation with the Klenow fragment of *E. coli* DNA polymerase I plus all four deoxynucleoside triphosphates and then ligated this fragment to *Xho*I-digested plasmid XHO5 DNA, similarly treated with Klenow enzyme.

Plasmid XHO5-GAL10 contains the bidirectional yeast transcriptional promoter from the *GAL1* and *GAL10* genes, cloned into the *Xho*I site of plasmid XHO5. We obtained the promoter as a 1,400-bp *Sal*I-to-*Xho*I fragment as follows. Plasmid pNN78 (35) carries a 2-kilobase-pair yeast genomic fragment spanning the divergent *GAL1* and *GAL10* promoter. In derivative 4 of this plasmid we have inserted a *Sal*I site between the transcriptional and translational initiation sites 5′ to the *GAL10* coding region (11). An *Hpa*I site lies in the *GAL1* coding region, 1,400 bp upstream from the *GAL10* transcriptional initiation site. We ligated *Xho*I octanucleotide synthetic linkers to *Hpa*I-digested plasmid pNN78#4DNA. After ligation, we digested the DNA with *Xho*I plus *Sal*I and then ligated the recovered 1,400-bp fragment to *Xho*I-digested plasmid XHO5 DNA.

We obtained plasmid XHO5-GAL10-pAB120 by ligating the 120-bp terminator fragment from plasmid pAB120 to *Xho*I-digested XHO5-GAL10 DNA, after rendering the ends of the plasmid and the purified fragment flush as above.

General procedures. Transformation of *E. coli* was performed by the method of Mandel and Higa (28). Yeast transformations were performed as described by Beggs (3). Restriction enzymes, BAL 31, and T4 DNA ligase were purchased from New England Biolabs or from Bethesda Research Laboratories, and enzyme reactions were carried out as recommended by the suppliers. Polyadenylated RNA was purified from yeast cells, fractionated, immobilized, and probed as described previously (7; Sutton and Broach, in press).

Stability assay. A yeast transformant containing a test plasmid was grown overnight in 5 ml of selective medium (synthetic medium lacking that nutritional requirement specified by the yeast gene carried on the test plasmid) to a density of approximately 5×10^7 cells per ml. From this preculture, 5 μl was inoculated into 5 ml of YEPD medium, and the strain was grown for approximately 10 generations (to approximately 10^8 cells per ml) at 30°C. Appropriate dilutions of this culture were plated on YEPD plates to yield roughly 250 to 500 colonies per plate. After 48 h of incubation at 30°C, the plates were replicated onto selective plates. The stability of the plasmid is expressed as the percentage of cells harboring the plasmid-borne marker. In the case of some plasmids containing the *HIS3* marker (see Results), stability measurements were carried out in a *his3*$^-$ *leu2*$^-$ host in which *REP1* and *REP2* functions were provided, not from native 2μm circle but from plasmid pCV20, a *LEU2*-plus-2μm circle hybrid plasmid lacking the 2μm circle recombination system. Nonselective medium, in these cases, contained histidine but not leucine. Plasmid stability was measured as the percentage of Leu$^+$ cells that were also His$^+$. The stability value for each plasmid tested represents

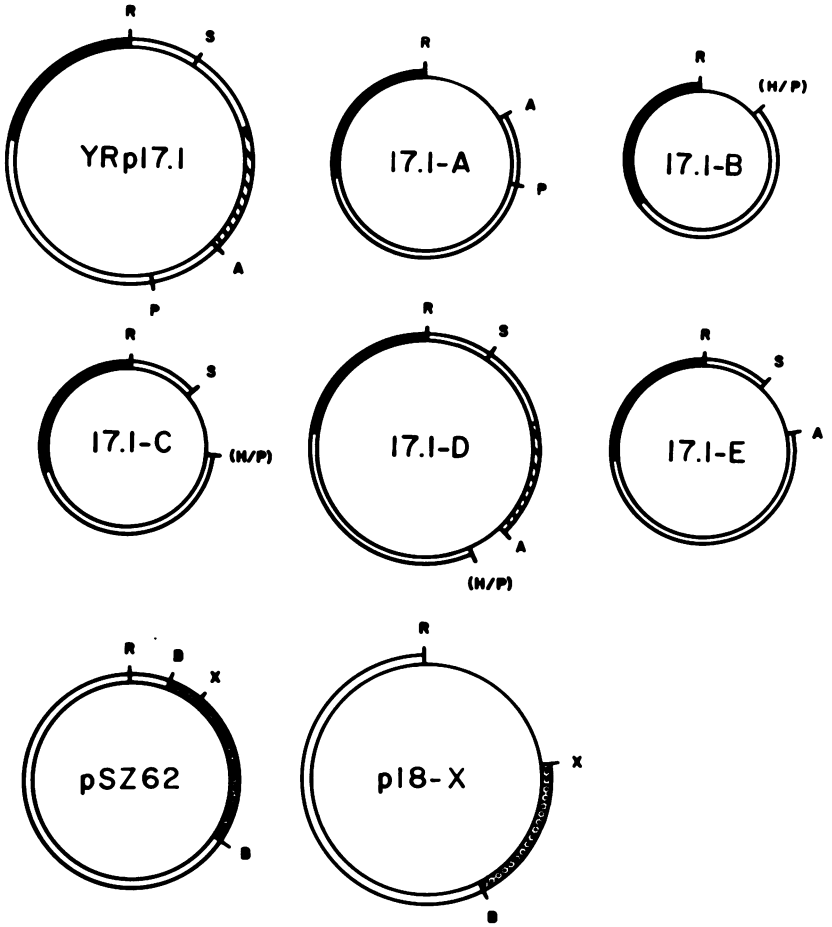

FIG. 2. Plasmids for localizing *REP3* activity. Schematic diagrams of the plasmids used in this study indicate the relative positions of pBR322 sequences (open double lines), *TRP1/ARS1* sequences (filled double lines), *URA3* sequences (hatched lines), *HIS3* sequences (dotted lines), and 2μm circle sequences (single lines). Plasmids 17.1A through 17.1E are derived from plasmid YRp17.1 by insertion at the indicated sites of the 2μm circle fragments diagrammed in Fig. 4. Plasmid p18-X was obtained by insertion of the 2μm circle fragments diagrammed in Fig. 3B into the *Xho*I-*Eco*RI sites of plasmid pSZ62. Restriction enzyme site abbreviations: R, *Eco*RI; S, *Sal*I; A, *Ava*I; H, *Hpa*I; P, *Pvu*II; X, *Xho*I.

results from stability determinations from at least two separate transformants, often conducted at different times. The values reported represent the average of all assays performed for the indicated plasmid. The values from the different determinations for any one plasmid differed by less than 15% from the reported value.

RESULTS

Assay for *REP3* function. We have shown that 2μm circle hybrid plasmids exhibit relatively high stability if they contain both the 2μm origin of replication and the three plasmid loci that constitute the copy control and partitioning system (23). Removal of the origin of replication from such a plasmid renders it incapable of transforming recipient yeast strains. Removal of any one of the other components of the copy control and partitioning system (Fig. 1) significantly reduces the stability of the plasmid without, however, impairing its transforming capability (9, 23, 24).

In experiments described in this paper, we have used stability as an assay for *REP3* activity. In each case, the test plasmid contains a fragment of 2μm circle DNA to be assessed for *REP3* function, as well as a selectable marker and a segment of DNA conferring autonomous replication. After introducing the plasmid into isogenic recipient strains that either lack or harbor endogenous 2μm circle ([cir^o] or [cir$^+$]), we determined the stability of the plasmid in both strains. Our measure of stability is the percentage of plasmid-bearing cells present in the population after 10 generations of growth of a transformant in nonselective conditions (23; see also Materials and Methods). In the [cir^o] strain, we expect all the test plasmids to be highly unstable regardless of the presence or absence of *REP3*, since neither of the *trans*-active *REP* functions is present. On the other hand, in the [cir$^+$] strain, endogenous 2μm circle plasmids provide *REP1* and *REP2* products in *trans*. Thus, a test plasmid that contains an intact *REP3* locus should exhibit a high level of stability, whereas a *rep3*$^-$ plasmid would exhibit the same low stability as it does in the [cir^o] strain.

In some cases, a slightly modified strategy was used for plasmids that contain an inverted repeat of the 2μm circle. Since such plasmids are substrates for the 2μm circle site-specific recombination system mediated by the plasmid-encoded *FLP* gene (8), they can potentially integrate into the endogenous 2μm circle and thereby acquire, at least transiently, a functional *REP3* locus. To circumvent this potentially obfuscating event, we conducted stability measurements of such plasmids in a host strain devoid of the

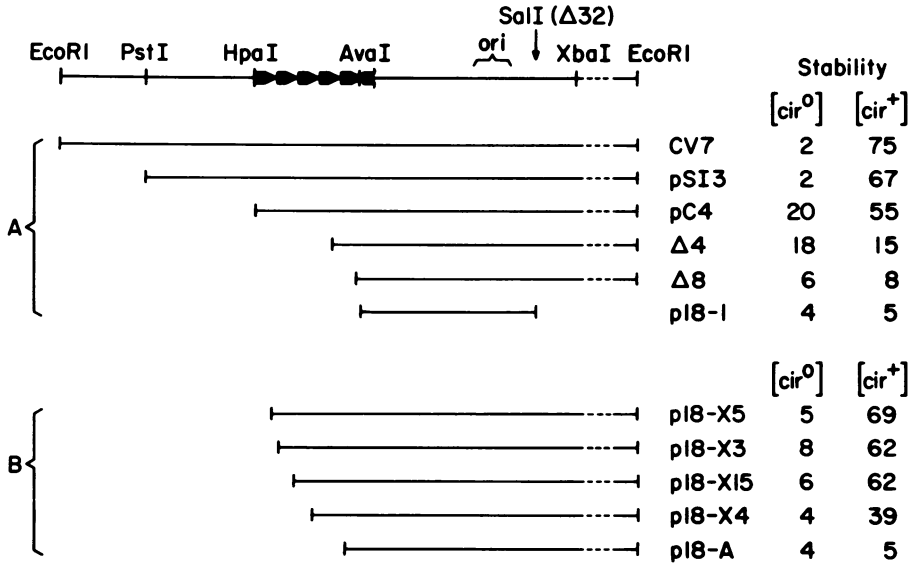

FIG. 3. Identification of the left-hand boundary of the *REP3* locus. The upper line represents the smaller *Eco*RI fragment of the B form of the 2μm circle, which spans the origin of replication (ori) and *REP3*. The positions of the repeated 62-bp element are indicated by the filled arrows. The location of the *Sal*I linker insertion in plasmid pL2-Δ32 (23) is also indicated. The portion of this 2μm circle fragment carried on each of several plasmids is indicated below the diagram, immediately to the left of each plasmid designation. The first five plasmids listed carry the yeast *LEU2* gene as a selectable marker; the last six carry the yeast *HIS3* gene. The detailed structures of plasmids CV7, Δ4, and Δ8 have been previously described (9), as have those of plasmids pSI3 and pC4 (6). Plasmids in group B were constructed by cloning the indicated *Xho*I-to-*Eco*RI or *Ava*I-to-*Eco*RI fragment from plasmids XHO3, XHO4, XHO5, or XHO15 (see Materials and Methods) into pSZ62 DNA digested with *Xho*I plus *Eco*RI. Plasmid p18-1 was constructed by cloning the indicated *Ava*I-to-*Sal*I fragment from plasmid pL2-Δ32 into pSZ62 DNA digested with *Ava*I-plus-*Sal*I. The mitotic stabilities of each of the plasmids in group A were determined in strains S150-2B [cir°] and [cir+] as described in the text and are listed immediately to the right of the plasmid designation. Stabilities of plasmids in group B were determined in strains S150-2B [cir°] and S150-2B [pCV20].

wild-type 2μm circle but harboring plasmid pCV20, a hybrid plasmid carrying the entire 2μm circle genome but in which the *FLP* gene is inactivated. Thus, plasmid pCV20 provides *trans*-active *REP* functions but does not recombine with the test plasmid.

In all these assays, the presence of a functional *REP3* locus on a plasmid was heralded solely by an increase in the stability of the plasmid in response to the presence of *REP1* and *REP2* functions. By using this criterion, any spurious effect on the basal level of stability resulting from specific constructions or from the presence of other sequences can be distinguished from events specifically affecting *REP3* integrity and function. It should be appreciated that this assay is not intended to be quantitative. The fact that we obtained consistent results in duplicate experiments conducted at different times and the fact that we assessed merely the difference in the behavior of the plasmid in isogenic [cir°] and [cir+] strains vouchsafe the validity of the assay for providing a simple indication of the presence or absence of a functional *REP3* locus.

Determination of the left-hand boundary of the *REP3* locus. By using a series of plasmids, each containing a different segment of 2μm circle DNA from that region of the genome to which the *REP3* locus had been approximately localized, we were able to obtain an initial indication of the functional limits of the locus. The portion of the 2μm circle genome carried on each plasmid is diagrammed in Fig. 3. In addition, each of the plasmids examined contains the 2μm origin of replication and either *LEU2* or *HIS3* as a selectable marker. The mitotic stabilities of the plasmids in group A were determined in both [cir°] and [cir+] strains, while those in group B were determined in a [cir°] strain and in one lacking 2μm but harboring plasmid pCV20. As mentioned previously, plasmid pCV20 provides *trans*-active *REP* functions but will not recombine with the test plasmid.

Results of stability studies on the plasmids in group A indicate that sequences to the left of the *Pst*I site are not essential for *REP3* function but sequences between *Hpa*I and *Ava*I sites are necessary. For reasons that we do not fully understand, plasmids pC4 and Δ4 exhibited a moderately high stability even in the absence of *trans*-acting *REP* functions. However, the stability of pC4 was increased almost to that of plasmids containing an intact *REP3* locus in the presence of the *REP* functions while that of plasmid Δ4 was not stimulated. Thus, we can tentatively conclude that the *REP3* locus resides entirely to the right of the *Hpa*I site.

To confirm these results and to refine the position of the left-hand boundary, we generated a series of plasmids containing small, nested deletions around the *Hpa*I site. We inserted *Xho*I octanucleotide linkers at the site of the deletion and then cloned onto the *HIS3* plasmid pSZ62 a fragment from each deletion plasmid that extends from the deletion endpoint to an *Eco*RI site located distal to the origin of replication. The portion of the 2μm circle carried on each of the resultant plasmids is indicated in Fig. 3B. As a consequence of the construction protocol, vector sequences abutting the *REP3* region are the same in each plasmid. In addition, we tested the plasmids for stability in a strain in which *trans*-active *REP* functions were provided by a plasmid, pCV20, that could not recombine with the test plasmids. Thus, the possibility of artifacts is minimized.

The results of the stability tests (Fig. 3B, right) confirm that sequences to the left of the *Hpa*I site are not required for *REP3* function, whereas removal of all 2μm sequences to the left of the *Ava*I site abolishes the ability of the plasmid to increase stability in response to *REP* proteins. Thus, at least

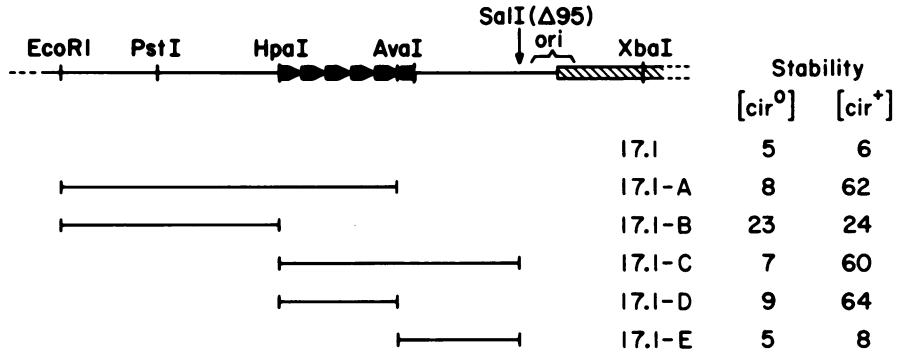

FIG. 4. Functional limits of the *REP3* locus. The upper line shows a portion of the 2μm circle genome spanning the *REP3* locus and the origin of replication. Filled arrows indicate 62-bp repeat units, and the hatched line indicates the inverted repeat region. The location of the origin of replication (ori) is shown, as is the position of the *Sal*I linker insertion in plasmid pL2-Δ95 (23). Those restriction fragments indicated below the genomic diagram were cloned separately onto plasmid YRp17.1 as described in Materials and Methods to yield plasmids 17.1-A through 17.1-E. The mitotic stabilities of these derived plasmids as well as that of the parent YRp17.1 plasmid were determined in strains K207 [cir°] and [cir+] and are given to the right of the plasmid designations.

some of the five-and-one-half repeats located between the *Hpa*I and *Ava*I sites are necessary for *REP3* activity. Results obtained with plasmids p18-X15 and p18-X4 demonstrate that removal of up to two repeat units does not significantly reduce the integrity of the locus. A marked reduction in stimulation of stability is observed only with plasmids from which three repeat units or more have been removed.

Determination of the right-hand boundary of the *REP3* locus. Kikuchi demonstrated that the 2μm stability system can act to stabilize plasmids constructed from chromosomal autonomously replicating sequences (*ARS* plasmids) independent of the presence of the 2μm circle origin of replication (24). We have taken advantage of this fact to define the right-hand boundary of the *REP3* locus.

Plasmid YRp17.1 carries the autonomously replicating sequence *ARS1* as well as the yeast genes *TRP1* and *URA3* and portions of the bacterial plasmid pBR322. Consistent with numerous previous observations, we found that plasmid YRp17.1 is mitotically unstable in [cir+] as well as [cir°] strains (Fig. 4). We inserted into plasmid YRp17.1 various 2μm circle DNA fragments from the vicinity of the *REP3* locus but lacking the origin of replication. These constructions were facilitated by the availability of plasmid pL2-Δ95, in which we deleted the 2μm origin and replaced it with a *Sal*I synthetic linker (23). We assessed the stabilities of the resulting YRp17.1 derivatives in isogenic [cir°] and [cir+] strains. In Fig. 4, we have indicated the portion of the 2μm circle present on each plasmid and the stability values obtained with that plasmid in each of the two strains. In the [cir°] strain, most of the plasmids exhibit essentially the same low stability as seen with the parent plasmid YRp17.1. In the [cir+] strain, however, those plasmids that contain a fragment of the 2μm circle that includes the *Hpa*I-to-*Ava*I segment show high stability while those that lack this sequence display low stability. Therefore, we can conclude that the *REP3* locus is contained completely within this 300-bp region. For unknown reasons, plasmid 17.1-B exhibits its relatively high stability in the [cir°] strain, but since the stability is not increased in the [cir+] strain, this property of the plasmid is apparently not a function of the 2μm circle *REP* system.

The *REP3* locus exhibits promoter activity. From our previous Northern analysis of 2μm circle transcription and from our data obtained by S1 analysis, we have found that a number of transcripts either end or begin in the vicinity of the *Hpa*I site in the large unique region of the 2μm circle plasmid (7; Sutton and Broach, in press). These include the 1,950-base transcript, whose 5′ end should lie near the *Hpa*I site, and the 1,620-base and D transcripts, which have 3′ ends between the *Pst*I and *Hpa*I sites (Fig. 5). As mentioned in the previous section, we have constructed plasmids containing small deletions centered around this *Hpa*I site (23). Subclones from these plasmids were used to generate the p18 series of plasmids that, as described in the previous section, provide a definition of the left-hand limit of the *REP3* locus. We have examined the transcription pattern of plasmids containing these deletions as a means of evaluating the extent to which *REP3* influences expression of transcripts in its vicinity.

We isolated polyadenylated RNA from a [cir°] strain either harboring plasmid CV20, which consists of the entire 2μm circle genome with pBR322 plus *LEU2* cloned into the small unique region, or harboring individual members of the XHO series of plasmids. These plasmids are identical to plasmid CV20 except for deletions around the *Hpa*I site, whose endpoints are indicated in Fig. 5. We fractionated samples of RNA from these strains on denaturing agarose gels and transferred the fractionated RNA to diazobenzyloxymethyl-paper. We then hybridized the immobilized RNA with one strand of a DNA fragment derived from the *REP1* coding region, the location of which is shown in Fig. 5. The strand hybridized to transcripts that extend leftward into the *REP1* gene, that is, to the 1,950- and 1,325-base transcripts.

The results of the transcription analysis are presented in Fig. 5. We found that plasmid CV20 gave rise to the 1,950- and 1,325-base transcripts at relative levels essentially identical to those seen in our previous analysis of transcripts from the endogenous 2μm circle (7). In strains harboring plasmid XHO4, no 1,950-base transcript is present, even though a normal level of the 1,325-base transcript was observed. Similarly, the 1,950-base transcript is absent in yeast cells containing plasmid XHO5, although the 1,325-base transcript, as well as a transcript somewhat smaller than 1,950 bases, is present. Finally, in strains harboring plasmid XHO15, the 1,325-base transcript and a transcript intermediate in size between the 1,950-base RNA and that seen in yeast cells containing plasmid XHO5 are present, while the 1,950-base transcript is absent.

In addition to variations in transcript sizes generated by the different plasmids, there are also notable differences in the relative transcript levels. In strains harboring either plasmid XHO5 or XHO15, the level of the larger transcript

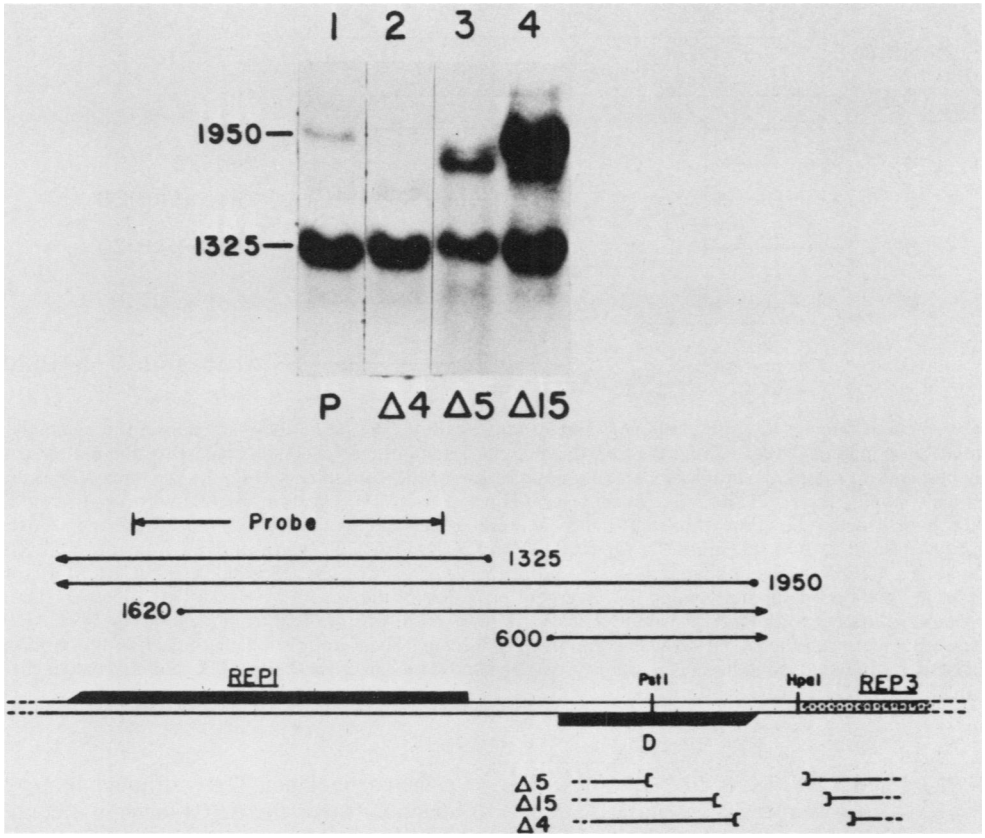

FIG. 5. Promoter activity of the *REP3* locus. (Top) Total polyadenylated RNA was isolated from strain DC04 [cir°] harboring either plasmid pCV20 (P, lane 1), plasmid XHO4 (Δ4, lane 2), plasmid XHO5 (Δ5, lane 3), or plasmid XHO15 (Δ15, lane 4). The XHO plasmids are identical to plasmid pCV20 save for small deletions spanning the *Hpa*I site, the exact endpoints of which are shown in the diagram in the bottom portion of the figure. RNA samples (0.01 mg) were fractionated on a 2.0% methyl mercury–agarose gel, transferred to diazobenzyloxymethyl-paper, and probed with a 1,314-bp *Hin*dIII fragment from the 2μm circle genome, as noted in the diagram in the bottom portion of the figure. The DNA probe was labeled on only one strand by appropriate treatment with exonuclease III, Klenow polymerase, and ^{32}P-labeled deoxynucleoside triphosphates (Sutton and Broach, in press). Only the 1,950- and 1,325-base transcripts hybridized to this single-strand probe. (Bottom) Schematic representation of the large unique region of the 2μm circle (open double line), on which the locations of open reading frames (filled lines, tapered at the 3′ end) and the location of the *REP3* region (mottled line) are indicated. The map positions of transcripts (designated by transcript size in bp) and the source of the probe used in the filter hybridization presented in the upper portion of the figure are shown above this diagram. The precise positions of the 5′ and 3′ ends of all the transcripts have been determined (Sutton and Broach, in press) except for the 5′ ends of the 1,620- and 1,950-base transcripts. The sequences deleted in the three deletion plasmid derivatives of plasmid pCV20 are indicated below the diagram.

was approximately equal to that of the 1,325-base transcript. This is in contrast to the situation in strains harboring plasmid CV20 or the authentic 2μm circle, in which the steady-state level of the 1,950-base transcript was approximately 20 times lower than that of the 1,325-base transcript. The reduced level of the 1,325-base transcript in strains harboring plasmid XHO5 correlates with the reduced level of plasmid in such strains compared to that in strains containing plasmid CV20 or XHO15 (23). The 1,325-base transcript level, on a per genome basis, was approximately equivalent for plasmids XHO5 and XHO15. We conclude that deletions removing sequences around the *Hpa*I site have profound effects on the sizes and amounts of the RNA species generated from the 1,950-base transcription unit. The implications of these results are elaborated upon in the Discussion section.

***REP3* activity is affected by adjacent sequences.** Results presented in previous sections demonstrate that the *REP3* locus resides entirely between the *Ava*I and *Hpa*I site on the 2μm circle genome. That is, these sequences are sufficient to promote full stabilization of a plasmid in response to the 2μm circle-encoded *REP* functions. These results stand in apparent contrast to our previous report, in which we examined the stability of the XHO plasmids described in the previous section (23). The XHO plasmids carry the entire 2μm genome with both *REP* genes intact. Thus, for alterations around the *REP3* locus, the stability of any of the plasmids in a [cir°] strain should reflect the level of *REP3* function in that plasmid. The regions deleted from two of these plasmids and the stabilities of the resultant plasmids in yeast cells are shown in Fig. 6. As we have previously reported, plasmid XHO3 exhibits a stability equivalent to that of the parental wild-type plasmid, whereas the stability of plasmid XHO5 is substantially reduced (23). The reduced stability of plasmid XHO5 is apparently due to a *cis* defect, since propagation of the plasmid in a [cir$^+$] strain does not significantly enhance its stability (M. Jayaram and J. R. Broach, unpublished observations). In addition, the absence of *REP3* activity resulting from the XHO5 deletion is not the consequence of the specific junction created at the site of the deletion, since a plasmid with a deletion extending from the left-hand boundary of XHO5 to the right-hand boundary of XHO3 also

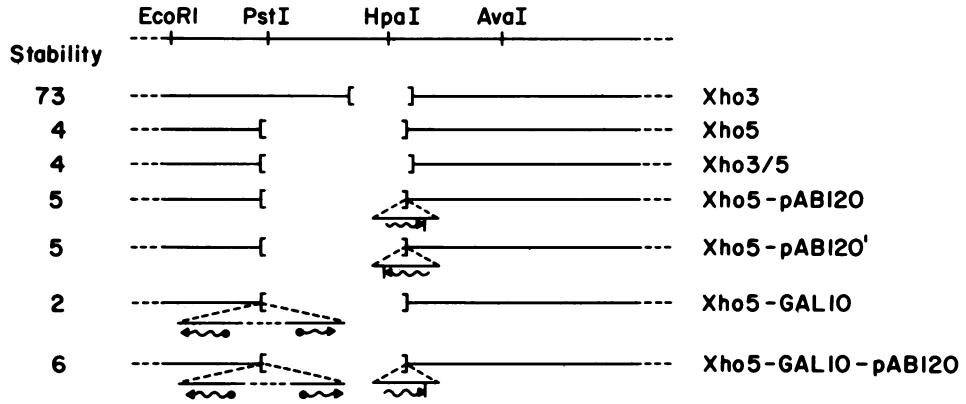

FIG. 6. The *REP3* locus is subject to context effects. The structure around the *REP3* locus in each of the plasmids listed on the right is shown beneath a restriction map of the *REP3* region of the 2μm circle. Each of these plasmids consists of the entire 2μm circle genome with *LEU2* plus pBR322 cloned into the *Eco*RI site in the small unique region of the 2μm moiety (Fig. 1). Plasmids XHO3 and XHO5, containing the indicated deletions spanning the *Hpa*I site, have been described previously (23). Plasmid XHO3/5 was constructed from plasmids XHO3 and XHO5 to yield a hybrid with a deletion whose left-hand endpoint is the same as that of plasmid XHO5 and whose right-hand endpoint is the same as that of plasmid XHO3. Plasmids XHO5-pAB120 and XHO5-pAB120′ were derived from plasmid XHO5 by insertion, in opposite orientations, of a 120-bp fragment spanning the 3′ end of the yeast *CYC1* gene. Plasmid XHO5-GAL10 was derived from plasmid XHO5 by insertion of a 1,400-bp fragment spanning the divergent promoter of the yeast *GAL1* and *GAL10* genes. The fragment is oriented with the *GAL10* promoter directed to the left. Plasmid XHO5-GAL10-pAB120 was derived from plasmid XHO5-GAL10 by insertion of the *CYC1* terminator sequence between the *GAL1* sequence and the *REP3* locus. The mitotic stability of each of these plasmids was determined in strain DC04 [cir°] grown on glucose media or, for the last two plasmids, on galactose media. The stability values are shown to the left of the plasmid diagrams.

exhibits loss of *REP3* activity (Fig. 6, line 3). Thus, this analysis would suggest that sequences essential to *REP3* activity lie between the *Hpa*I and *Pst*I sites. Paradoxically, though, as we have shown in Fig. 3, sequences extending rightward from the deletion endpoint of XHO5 exhibited full *REP3*$^+$ activity when cloned onto plasmid pSZ62. That is, the same sequences that exhibited *REP3*$^+$ activity in one plasmid were not functional when present in a second plasmid. Thus, we tentatively conclude that *REP3* activity is sensitive to the nucleotide context in which the locus resides.

Those sequences to the left of the *Hpa*I site in the 2μm circle that are deleted in plasmid XHO5 include signals for transcriptional initiation and transcriptional termination (7; Sutton and Broach, in press). To assess whether loss of either or both of these elements is responsible for the *rep3*$^-$ phenotype of plasmid XHO5, we constructed and tested the plasmids shown in Fig. 6. Plasmids XHO5-pAB120 and XHO5-pAB120′ contain a 120-bp sequence encompassing the region corresponding to the 3′ end of the yeast *CYC1* transcript inserted in opposite orientations at the site of the deletion in plasmid XHO5. This insertion yields truncation and polyadenylation of transcripts entering the region from the left (as the sequence is drawn in Fig. 6; data not shown). Thus, the sequence from *CYC1* appears to replace functionally the polyadenylation sequences removed by the deletion (Sutton and Broach, in press). Nonetheless, neither of these hybrid plasmids exhibits an increase in stability over that of plasmid XHO5. Plasmid XHO5-GAL10 carries at the site of the insertion the bidirectional promoter for the *GAL1* and *GAL10* genes. However, even on galactose medium, when this promoter is transcriptionally active (23), the plasmid shows no increase in stability over that of plasmid XHO5. Finally, we constructed plasmid XHO5-GAL10-pAB120, which contains both the bidirectional promoter and the *CYC1* terminator at the deletion site. As indicated, this plasmid also failed to display increased stability, even on galactose medium. Thus, although it appears that the XHO5 deletion places the *REP3* locus in a context that inhibits its activity, we have not been able by reconstruction experiments to identify the nature of the sequences in this region that normally insulate the locus from this inhibitory environment.

DISCUSSION

Plasmid functions promoting stable propagation. Results from several recent studies suggest that the 2μm circle propagates stably because plasmid molecules partition reasonably equally between mother and daughter cells after mitosis, because the 2μm circle has the potential to amplify and because cellular copy levels of the plasmid are actively maintained. As discussed below, it appears that these three processes, partitioning, amplification, and copy control, are catalyzed by plasmid-encoded products.

Murray and Szostak have demonstrated that hybrid 2μm circle plasmids lacking a complete *REP* system exhibit, in a manner similar to that observed with hybrid plasmids constructed from chromosomal *ARS* elements, a high level of mitotic nondisjunction (29). Specifically, plasmid-bearing cells, even those that apparently contain a large number of plasmids, frequently fail to transmit plasmid molecules to their daughter progeny after mitosis. In other words, such hybrid 2μm circle plasmids not only fail to partition evenly at mitosis but in fact fail to do so in a highly nonrandom fashion. In contrast, plasmids in which the 2μm circle *REP* system is intact do not exhibit this highly asymmetric inheritance pattern but distribute themselves equitably between mother and daughter cells after mitosis. In addition, Kikuchi observed that the 2μm circle *REP* system could enhance the stability of a plasmid without necessarily increasing its total copy level within the population (24). These results, taken together, suggest that the *REP* system actively

ensures equal distribution of plasmid molecules to mother and daughter cells after mitosis.

The 2μm circle plasmid also possesses an ability to amplify copy number. Sigurdson et al. have demonstrated that when a cell formerly lacking the 2μm circle acquires the plasmid by cytoduction, it most often receives only one copy of the plasmid (33). Nonetheless, by the time such a cytoductant has grown into a colony, each cell in the colony contains the normal 50 to 100 copies of the plasmid. Thus, even though the replication of the plasmid is normally under stringent cell cycle control, under certain circumstances the plasmid population can increase faster than that of the host. Recent evidence suggests that this amplification potential requires at least the 2μm circle-encoded site specific recombination system (A. W. Murray and J. W. Szostak, personal communication). The roles of other plasmid components in this process are unknown. The manner by which this process is regulated is also unknown.

Finally, the 2μm circle plasmid appears to manifest copy control. That is, the specific number of plasmid molecules per cell is apparently actively set, presumably by the plasmid itself. This is most clearly demonstrated by the fact that the presence of a hybrid 2μm circle plasmid reduces the number of copies of endogenous 2μm circle in a cell (18). Similarly, the copy level of hybrid 2μm circle plasmids with an intact *REP* system is lower in [cir$^+$] than [cir^o] strains (23). This aspect of incompatibility, that the sum of the copy numbers of two related plasmids in a cell in which both are present is approximately equal to the copy number of either of the plasmids when that plasmid is present alone, is a property of bacterial plasmids that share copy control systems. However, it should be noted that although the 2μm circle plasmid apparently possesses a copy control mechanism, maintenance of 2μm circle copy levels is not as rigid as that of bacterial plasmids. Substantial variation in the plasmid copy number of a plasmid-bearing strain can be observed at the colony level, as judged by in situ β-galactosidase assays of strains carrying a *LacZ* fusion gene on a 2μm circle vector (M. Casadaban, personal communication; J. Broach, unpublished data) or by direct copy level measurements in clonal cultures (17). This indicates that the actual plasmid level in individual cells varies over a wide range around a mean and that drift in copy level toward the mean occurs relatively slowly.

Partitioning activity of *REP3*. The properties of the *REP3* locus as defined by studies described in this and previous (23, 24) reports provide some insights into the role it plays in the above processes. First, *REP3* is active only in *cis*. That is, it stabilizes only those plasmids in which it is resident. Second, its activity is independent of the particular *ARS* element responsible for replication of the plasmid. It is active in conjunction not only with *ARS1* and the 2μm circle *ARS*, as demonstrated by Kikuchi (24) and in this study, but also with the E and I site *ARS*s from *HML* (data not shown). Third, *REP3* is independent of its orientation with respect to and its distance from the plasmid *ARS* element. This has been demonstrated by Kikuchi (24) and confirmed in this study in that the location and orientation of the *REP3* sequences differed in the various plasmid constructs in which *REP3* was present. Finally, *REP3* possesses no *ARS* activity itself. In all these respects, *REP3* would appear to act independently of the replication system of the plasmid; rather, its properties are consistent with it being a passive site for attachment to or interaction with a partitioning system. This assumption is reinforced by the similarity of the above-mentioned properties of *REP3* and those of cloned centromere sequences isolated from yeast cells (4, 15) or those of the *sopC* locus of the partitioning system of F plasmids of *E. coli* (30). However, eucaryotic enhancer elements also exhibit orientation and distance independence and are active with heterologous as well as homologous transcription initiation sites (1, 2, 20, 22). Therefore, by analogy, it is still possible, although not necessarily likely, that *REP3* exerts its influence through some form of transcriptional activation of the origin of replication in the plasmid in which it is resident.

Assuming that *REP3* serves as the site on the 2μm circle plasmid through which the *REP* proteins act to ensure equipartitioning of the plasmid at meiosis and mitosis, one can envision at least two general models by which such a partitioning system could work. First, the *REP* proteins could function as a bivalent connector, promoting attachment of the plasmid, via the *REP3* locus, to some component, such as the spindle apparatus, that actively segregates to the two cells at mitosis. On the other hand, the highly nonrandom segregation of *rep*$^-$ plasmids suggests that such plasmids are not free to diffuse throughout the nucleus after S phase but, rather, are stuck or sequestered at some site that remains predominantly with the mother cell at mitosis. Thus, the *REP* proteins may act merely to pry plasmid molecules loose from this site of sequestration so that they might be free to distribute themselves randomly throughout the nucleus. Given the relatively high copy number of the 2μm circle in the cell, random distribution of plasmids in the nucleoplasm would almost invariably ensure that both mother and daughter cells would acquire plasmids after mitosis. In this model, *REP3* would presumably act as the handle on the plasmid that is used by the *REP* proteins to effect this desequestration.

We have shown that the *REP3* locus consists entirely of tandem direct repeats of a 62-bp element and that functional integrity is not affected by removal of up to two of these units. This contrasts with results from our earlier study and with a report by Kikuchi, in both of which the *REP3* locus was determined to encompass a larger region. However, the discrepancy between this and our previous observation can be explained by the fact that although the *REP3* locus is intact in plasmid XHO5, its activity is masked in that plasmid. It is not clear whether this masking is due to a direct negative influence of contiguous sequences on *REP3* activity, or whether the particular upstream junction of the deletion in plasmid XHO5 generates a *trans*-dominant destabilization effect, through production of an inhibitory subdomain of the D protein, for example. With regard to the observation by Kikuchi, the assignment of *REP3* to a larger domain rests on one construction. As with any element that is defined by being placed in a new context, either by deletion of flanking sequences or by insertion into a new location, the contribution of the new flanking sequences to the activity of the locus can cause some ambiguity in assignment of boundaries. It is possible that the new flanking sequence contributes an essential component to the locus being tested so that the locus appears functional even though all of the sequences that constitute the locus in its normal habitat are not represented in the artificial construct. Alternatively, novel flanking sequences could have an inhibitory activity that would preclude normal function of an otherwise intact locus. Since it seems unlikely that a specific physiological response, namely, the enhancement of plasmid stability in response to *REP* protein, could be adventitiously duplicated in a random juxtaposition of sequence, we feel confident that our current assignment of *REP3* solely to the

repeated units is correct. Our confidence in our current assignment is also enhanced by the fact that we reach the same conclusion with at least five different plasmid constructions.

Promoter activity of *REP3*. We find that promoter activity, in addition to partitioning activity, resides in the region between the *Hpa*I and *Ava*I restriction sites in the large unique region of the 2μm circle plasmid. We have measured the steady-state level of the 1,950-base transcript in cells containing derivatives of the 2μm circle with specific small deletions around the *Hpa*I site. In strains containing any of the three deletion derivatives examined, the 1,950-base transcript is missing. In strains carrying plasmid XHO5 or XHO15 the 1,950-base transcript is replaced by a single additional transcript that is smaller than the 1,950-base transcript in proportion to the amount of DNA removed by the particular deletion. This observation is readily accommodated by assuming that sequences that position the 5′ end of the 1,950-base transcript lie to the right of the right-hand endpoint of the deletion in these plasmids. Given the low steady-state levels of the 1,950-base transcript and the presence of repeated sequences at or near its 5′ end, we were unsuccessful in determining the position of its 5′ end in our previous analysis of the 2μm circle transcript cap sites (Sutton and Broach, in press). However, assuming that the 1,950-base transcript is coterminal with *REP1* mRNA, then our assignment of the 1,950-base transcript cap site near the *Hpa*I site would predict a transcript size of approximately 2,000 bp, a length that is within the error limits of our original transcript size determinations.

If the preceding interpretation of the pattern of production of the 1,950-base transcript is correct, then two additional conclusions can be drawn from our results. First, since no transcripts derived from the 1,950-base transcription unit are present in strains harboring plasmid XHO4, then sequences necessary for production of stable 1,950-base RNA would be expected to lie between the right-hand endpoints of deletions XHO15 and XHO4. These sequences could be required for efficient transcriptional initiation or, less likely, for stable maintenance of the transcript once produced. Second, we can also conclude that sequences required for suppressing the steady-state levels of the 1,950-base transcript lie within sequences deleted in plasmids XHO5 and XHO15. The absence of this sequence in these plasmids would account for the enhanced levels of the transcript seen in strains harboring them. Such a sequence could affect the transcription initiation rate, as a negative enhancer element or as a transcription repressor-binding site. On the other hand, if the sequence lies within the transcript unit, it could reduce the stability or rate of maturation of the 1,950-base transcript.

The presence of a site regulating synthesis of the 1,950-base transcript is consistent with our studies on expression of the *leu2-d* allele from plasmid pJDB219. In this plasmid the promoterless *leu2* gene is fused to 2μm circle sequences so that transcription of the *leu2-d* gene is driven by the 1,950-base promoter (T. Som, A. Sutton, and J. R. Broach, unpublished data). We find that expression of the *leu2-d* gene is 10 to 20 times lower when propagated in a [cir$^+$] strain than when propagated in [cir^o] strains (Som et al., unpublished data). Thus, it appears that synthesis from the promoter of the 1,950-base transcript is repressed in *trans* by 2μm circle-encoded products. The region deleted in plasmids XHO5 and XHO15, whose absence yields high-level synthesis of the 1,950-base transcript, may well be the site at which the 2μm circle proteins interact to repress synthesis of the transcript. It is not clear what function is served by the 1,950-base transcript and, accordingly, the reason for modulation of its expression is even more obscure. Nonetheless, as suggested below, such a regulatory circuitry may be intimately, if currently obscurely, involved in regulating plasmid copy number.

A possible role of *REP3* in plasmid copy control. The region between the *Hpa*I and *Ava*I sites consists of a series of direct tandem repeats of a consensus 62-bp element. The elements are not precisely identical, but, rather, they exhibit homology to each other at a level ranging from 75 to 95%. Our results indicate that these repeat units exhibit some degree of functional variation. That is, deletion of one unit, in plasmid XHO4, yields loss of promoter function even though several other homologous elements are still present in the plasmid. This loss of promoter activity occurs, though, with no apparent reduction in partitioning efficiency.

The organization of the *REP3* locus suggests a model by which copy level regulation may be effected. Previous genetic evidence, as well as results presented in this study, indicates that equipartitioning of 2μm circle plasmids at mitosis requires binding of *REP1* or *REP2* protein to the *REP3* locus (for simplicity, we will assume that the *REP1* protein is the active agent in this process). Our analysis suggests that functional activity with regard to partitioning can be fully manifested by the right-most three repeat elements alone. Our analysis also suggests that the left-most repeat units, although structurally quite similar to the other repeat units, are in fact functionally distinct. That is, the left-most units constitute elements for synthesis and regulation of expression of the 1,950-base transcript while the other units do not. If we postulate a hierarchy of affinity of the *REP1* protein for the different repeat units, with the right-most units exhibiting a higher affinity than the left-most units, then the following scenario is reasonable. At low plasmid copy levels, with a consequent low steady-state level of *REP1* protein, binding to the right-most units occurs with little contemporaneous binding to the left-most units. Thus, equipartitioning is accomplished with little repression and, thus, high-level synthesis of the 1,950-base transcript. On the other hand, at high plasmid levels with attendant high levels of *REP1* synthesis, both sets of sites are filled. Accordingly, equipartitioning is promoted, and repression of synthesis of the 1,950-base transcript occurs. If expression of the 1,950-base transcript were coupled, even in some indirect fashion, with amplification of plasmid copy number, then this system would tend to maintain the plasmid copy number at a fixed level.

The presence of repeated binding domains is a recurrent feature of a number of plasmids and viruses, including plasmid R6K, phage P1, and *E. coli* F factors (13, 19, 36). In the case of *E. coli* F factors, the repeated sequences have been implicated in plasmid incompatibility and, thus, by extension, in plasmid copy control. Thus, it is tempting to speculate that the repeated elements at *REP3* function to titrate protein components as a means by which the plasmid ascertains and responds to cellular copy levels. In no other system, though, has a clear functional distinction between the various repeat units been demonstrated. It will be of obvious interest to assess what, if any, connection exists between the 1,950-base transcript and the 2μm circle amplification system.

ACKNOWLEDGMENTS

We thank Sajida Ismail and Debbie Rodhouse for excellent technical assistance.

This work was supported by grants from the National Institutes of

Health and the National Science Foundation. J.R.B. is an Established Investigator of the American Heart Association, and A.S. is a National Institutes of Health Postdoctoral Fellow.

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