MOLECULAR AND CELLULAR BIOLOGY, July 1989, p. 2914–2921
0270-7306/89/072914-08$02.00/0

Vol. 9, No. 7

The OBF1 Protein and Its DNA-Binding Site Are Important for the Function of an Autonomously Replicating Sequence in *Saccharomyces cerevisiae*†

SCOTT S. WALKER,[1] STEPHEN C. FRANCESCONI,[1] BIK-KWOON TYE,[2] AND SHLOMO EISENBERG[1]*

Department of Microbiology, School of Medicine, The University of Connecticut Health Center, Farmington, Connecticut 06032,[1] and Section of Biochemistry, Molecular and Cell Biology, Cornell University, Ithaca, New York 14853[2]

Received 9 January 1989/Accepted 4 April 1989

The autonomously replicating sequence *ARS121* was cloned as a 480-base-pair (bp) long DNA fragment that confers on plasmids autonomous replication in *Saccharomyces cerevisiae*. This fragment contains two OBF1-binding sites (sites I and II) of different affinities, as identified by a gel mobility shift assay and footprint analysis. Nucleotide substitutions (16 to 18 bp) within either of the two sites obliterated detectable in vitro OBF1 binding to the mutagenized site. Linker substitution (6 bp) mutations within the high-affinity site I showed effects similar to those of the complete substitution, whereas DNA mutagenized outside the binding site bound OBF1 normally. We also tested the mitotic stability of centromeric plasmids bearing wild-type and mutagenized copies of *ARS121*. Both deletion of the sites and the extensive base alterations within either of the two OBF1-binding sites reduced the percentage of plasmid-containing cells in the population from about 88% to 50 to 63% under selective growth and from about 46% to 15 to 20% after 10 to 12 generations of nonselective growth. Furthermore, linker (6 bp) substitutions within site I, the high-affinity binding site, showed similar deficiencies in plasmid stability. In contrast, plasmids containing linker substitutions in sequences contiguous to site I displayed wild-type stability. In addition, plasmid copy number analysis indicated that the instability probably resulted not from nondisjunction during mitosis but rather from inefficient plasmid replication. The results strongly support the notion that the OBF1-binding sites and the OBF1 protein are important for normal *ARS* function as an origin of replication.

The replication of eucaryotic chromosomal DNA proceeds by numerous initiations along a chromosome, generating multiple replicons. It is believed that in *Saccharomyces cerevisiae* the initiation sites at replicons are specific and correspond to the autonomously replicating sequences (*ARS*s). *ARS*s were isolated as DNA fragments that confer on plasmids bearing them the ability to replicate autonomously in yeast cells (34). Both in vitro (5, 17, 22) and in vivo (1, 3, 6, 19, 27, 30, 36) experiments, in particular the recent studies carried out by Brewer and Fangman (2) and Huberman et al. (14), indicate that *ARS*s incorporated into plasmids function as origins of replication. Recently, Huberman et al. (15) have located, in a 22.5-kilobase-pair (kbp) stretch of chromosome III, a functional DNA replication origin within several hundred base pairs of an *ARS* element, strongly suggesting that *ARS*s are also the origins of chromosomal replicons. Attempts were made to identify and isolate proteins that interact with DNA elements at the *ARS*, assuming that such proteins may have a role in the function of the *ARS* as an origin of replication. For example, Shore et al. (33) and Buchman et al. (4) reported the identification of a DNA-binding activity in partially purified extracts, SBF-B and ABF-I, respectively, which interacted specifically with a DNA sequence located in domain B of *ARS1* and the functionally unrelated domain B of *HMRE*. By DNA competition analysis, Buchman et al. (4) have also shown that ABF-I recognizes sequences in *ARS*s that are found in the *HMRI* and *HMLI* loci. Furthermore, a protein with similar DNA-binding properties has now been purified as a 134- to 135-kilodalton polypeptide (9, 35). So far, however, a clear link between the binding of purified ABF-I to its recognition sites and *ARS* activity has not been demonstrated. We have also isolated an *ARS* DNA-binding protein, OBF1, which appears to bind to a broad spectrum of *ARS*s (10, 12). The purification to near homogeneity and characterization of this protein are reported in the accompanying paper (12). In this report, we describe the binding properties of OBF1 to *ARS121*; by using in vitro site-directed mutagenesis, we demonstrate a high, direct correlation between the ability of OBF1 to bind to the ARS and the ability of the *ARS* to function efficiently in vivo.

MATERIALS AND METHODS

Strains and growth conditions. *Escherichia coli* HB101 was used for transformation and plasmid propagation (25). *S. cerevisiae* 8534-8C (*MATα leu2-3 -112 his4Δ34 ura3-52*) was used for all plasmid transformation and maintenance studies (24). Bacteria were grown in LB broth or on LB plates at 37°C. Medium was supplemented with ampicillin (100 μg/ml) when appropriate. Yeast cells were grown at 30°C in YPD (rich) and SD (minimal) media, prepared as described elsewhere (32). SD was always supplemented with histidine (20 μg/ml) and leucine (30 μg/ml). Uracil was added at 50 μg/ml when needed.

Plasmid constructions and DNA manipulations. Plasmid YRp121, which contains 6.6 kbp of yeast DNA (Fig. 1A), including *ARS121* and the OBF1-binding sites, has been described previously (7). The shuttle vector YCp5-3 was constructed by inserting the 2.0-kbp fragment containing *CEN3* from pYe(*CEN3*)41 (8) into *Bam*HI- and *Hind*III-cleaved YIp5. This plasmid contains the *S. cerevisiae* selectable marker *URA3*. The *ARS121*- and *ARS1*-containing

* Corresponding author.

† We dedicate this paper to Arthur Kornberg in honor of his 70th birthday.

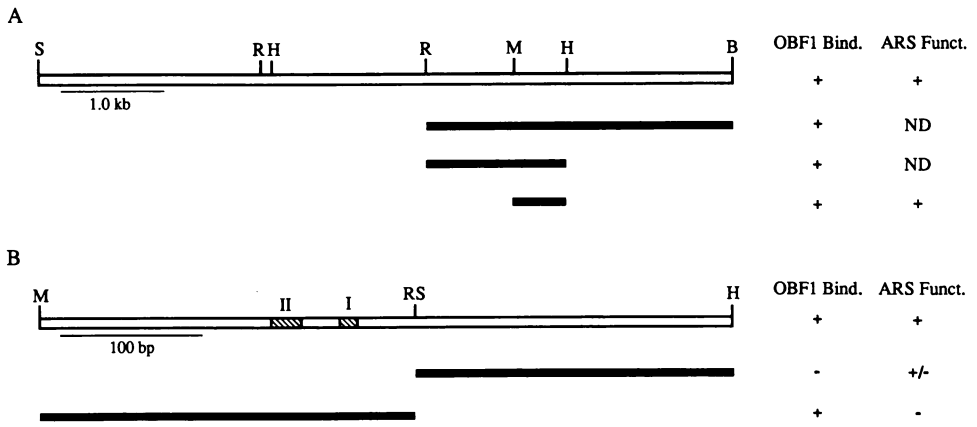

FIG. 1. Cloning of the ARS- and OBF1-binding activities in YRp121. YRp121 was isolated as described previously (7). (A) Restriction endonuclease cleavage map of the yeast DNA contained in YRp121. This 6.6-kbp insert was dissected solely on the basis of the ability of a DNA fragment to bind OBF1 in an agarose gel mobility retardation assay (see Materials and Methods). ■, Cloned DNA fragments. The 480-bp *Msp*I-*Hin*dIII fragment was analyzed for *ARS* function by cloning it into an integrative yeast plasmid and testing for high-frequency transformation. (B) Cloning of the 480-bp *Hin*dIII-*Msp*I fragment shown in panel A. Fragments were cloned as described in Materials and Methods and tested for *ARS* function as described above for the 480-bp *Msp*I-*Hin*dIII fragment. Symbol and abbreviations: ▨, locations of the two OBF1-binding sites in *ARS121*; ND, not determined; B, *Bam*HI; H, *Hin*dIII; M, *Msp*I; R, *Eco*RI; RS, *Rsa*I; S, *Sal*I.

derivatives of YCp5-3 were created by ligating DNA fragments into the *Eco*RI and *Hin*dIII sites. YCp5AB121 was constructed by first inserting the 480-bp *Msp*I-*Hin*dIII fragment from YRp121 (Fig. 1) into pUC19 at the *Acc*I and *Hin*dIII sites and then removing the *Eco*RI-*Hin*dIII fragment and inserting it into YCp5-3. YCp5A121 was constructed by ligating the 210-bp *Hin*dIII-*Rsa*I fragment (Fig. 1B) into the mp19 polylinker of pUC19 at the *Hin*dIII and *Sma*I sites, followed by insertion into YCp5-3. YCp5B121 was constructed similarly by cloning the 270-bp *Rsa*I-*Msp*I fragment (Fig. 1B) first into pUC19 and then into YCp5-3. YCp5A1 contains the 380-bp *Hin*dIII-*Hin*cII *ARS1* fragment. Plasmid DNA was prepared from *E. coli* cultures by the standard alkaline lysis procedure (25). Plasmids were used to transform yeast cells to uracil prototrophy by the lithium acetate procedure (16).

DNA footprinting. DNA sequences protected from digestion with DNase I by the binding of OBF1 were identified as described previously (18). The nucleotide sequence within the protected regions was determined by chemical cleavage sequencing (26).

OBF1 purification and binding assay. The DNA-binding protein OBF1 was purified to homogeneity as described elsewhere (12). The agarose gel mobility retardation assay for protein-DNA binding used in this study was essentially as described for the purification of OBF1. OBF1 binding substrates were radioactively labeled by using reverse transcriptase and [α-^{32}P]dATP. For each reaction, 4.5 fmol of the labeled binding substrate was used along with the indicated amount of purified OBF1 in a 20-μl total reaction volume. Free and bound DNA fragments were separated on a 1.4% agarose gel at 95 mA. Gels were then dried and subjected to autoradiography as described elsewhere (10). To determine the percentage of DNA shifted, autoradiographic images were quantitated on a model 620 video densitometer with associated computer software (Bio-Rad Laboratories).

Oligonucleotide-directed mutagenesis. Specific nucleotides in *ARS121* were altered by the method of Kunkel (23), using the Bio-Rad Muta-Gene in vitro mutagenesis kit to generate recombinant M13mp18. In addition to the mutagenic oligonucleotide, the M13 universal sequencing primer (17-mer) was included in each reaction. This modification increased the frequency of the desired mutation. Oligonucleotides were synthesized in a Cyclone DNA synthesizer (Biosearch, Inc.). Typically, oligonucleotides were constructed such that the region to be modified was flanked on each side by 12 homologous nucleotides. Bacteriophage were initially screened for the mutagenized sequence by plaque hybridization on Colony/Plaque Screen (Dupont, NEN Research Products) membranes probed with the mutagenic oligonucleotide. Oligonucleotides were radioactively labeled by using T4 polynucleotide kinase and [γ-^{32}P]ATP. Filters were handled according to the instructions of the manufacturer. Chain termination sequencing (31) confirmed the presence of each sequence modification. Mutations were then cloned into the *Eco*RI and *Hin*dIII sites of YCp5-3.

Analysis of plasmid mitotic stability. Plasmid mitotic stability analyses were performed essentially as previously described (21). Single colonies were inoculated in SD medium lacking uracil (selective). Stationary-phase cultures were then used to inoculate separate tubes of SD with and without uracil at 10^4 cells per ml. Cultures in nonselective medium were grown for 10 to 12 generations at 30°C, diluted in sterile water, and plated in triplicate on SD plates lacking uracil and on YPD (rich) plates. Plates were incubated at 30°C until colonies arose. Mitotic stability was calculated by dividing the average number of Ura$^+$ colonies at a given dilution by the average number of colonies on the YPD plates at the same dilution. Stabilities are expressed as percentage of total population bearing the plasmid. Mitotic stability determinations were done on at least six independent transformants for each plasmid construct. Selective cultures were grown to approximately 10^7 cells per ml and then diluted and plated as described above. Mitotic stability was calculated as described for transformants grown nonselectively.

Determination of plasmid copy number. A single colony was inoculated, and the culture was grown to saturation in SD medium without uracil. These cells were then subcultured at 10^4/ml in 25 ml of SD containing uracil and grown nonselectively for 10 to 12 generations. A sample was diluted and plated for determination of percent Ura$^+$ cells as described above. Total DNA was then isolated by the guani-

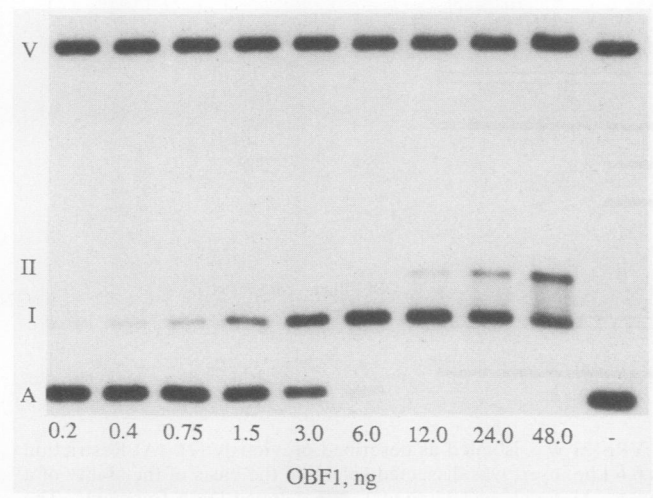

FIG. 2. Direct binding of OBF1 to *ARS121*, suggesting the presence of two protein-binding sites. The binding substrate, YCp5AB121, was cleaved with *Eco*RI and *Hin*dIII, radioactively labeled, and incubated with or without OBF1 as described in Materials and Methods. Purified OBF1 was added to the reactions in the amounts indicated for each lane. DNA in each reaction was electrophoretically resolved and identified by autoradiography as described in Materials and Methods. Positions of free (A) and protein-bound (I and II) *ARS121* fragments and of the vector (V), YCp5-3, are shown on the left.

dine hydrochloride lysis method as described previously (13). DNA (2 to 4 μg) from cells bearing a particular plasmid was digested to completion with *Eco*RI, subjected to electrophoresis on a 0.75% agarose gel, and transferred to a GeneScreen Plus filter (Dupont, NEN) as described by the manufacturer. The filter was probed with a 505-bp *Acc*I fragment of the yeast *URA3* gene radiolabeled by the random hexanucleotide primer technique described previously (11). The filter was then subjected to autoradiography. To estimate plasmid copy number, the autoradiogram was analyzed by densitometry to determine the ratio of probe hybridization to the plasmid *URA3* gene and the chromosomal *ura3-52* allele. Densitometric scans were conducted as described for the OBF1-binding assay (above).

RESULTS

Isolation of *ARS121* DNA. *ARS121* DNA was first isolated as a 6.6-kbp yeast DNA insert (Fig. 1A) able to confer on plasmids the ability to replicate autonomously in vivo. We previously observed that this DNA competed effectively for OBF1 with the telomeric *ARS120* (10, 12). To delimit further the region responsible for OBF1 binding, the 6.6-kbp insert was digested with restriction endonucleases to produce DNA fragments, which were tested for the presence of OBF1-binding sites by gel retardation experiments. Using this approach, we were able to localize the binding site for OBF1 to a 480-bp region situated on the 6.6-kbp insert between the *Hin*dIII and *Msp*I restriction sites (Fig. 1A). When subcloned into a plasmid containing a yeast selectable marker, this DNA fragment (Fig. 1B), to which we refer as *ARS121*, displayed *ARS* activity.

Two OBF1-binding sites in *ARS121*. In a direct DNA-binding assay with purified OBF1, *ARS121* yielded two retarded DNA bands upon electrophoresis in agarose gels (Fig. 2). This result suggested the presence of two OBF1-binding sites in the DNA, in contrast to the interaction of OBF1 with *ARS120*, for which only one OBF1-binding site was identified (10). To locate precisely the position of these binding sites, we performed footprinting analysis by limited digestion with DNase I. Indeed, two regions in the DNA, separated by 30 nucleotides, were protected from DNase I digestion (Fig. 3). Although the DNA sequences of these sites revealed similar nucleotide sequences, the DNA band shift assay in agarose gels suggested that the two sites differed in affinity for OBF1. Upon titration with OBF1, the second shift of the *ARS* was observed only after a major portion of the DNA had shifted to the first position (Fig. 2). In addition, smearing of radioactively labeled material in the area between the two retarded bands was observed, implying that the second complex is unstable and dissociates during electrophoresis. These results also suggest that the sites probably interact with OBF1 in a noncooperative fashion. To examine the binding of OBF1 to individual sites, we constructed substitution mutations whereby DNA sequences in sites I and II were replaced by another 18- and 16-bp sequence, respectively. These substitutions, obtained by in vitro site-directed mutagenesis, are depicted in Fig. 3. Titration of OBF1 in binding reactions containing mutagenized DNAs *b1*, *b2*, and *b12* (Fig. 4A and B) clearly demonstrated that (i) DNA substitutions within both sites, *b12*, obliterated detectable binding of OBF1 (Fig. 4B) and (ii) the affinity of binding was much higher to site I than to site II, since 25- to 50-fold-higher levels of OBF1 were needed to shift the same amount of *b1* (site I mutant) as *b2* (site II mutant) DNA. Surprisingly, DNA sequence alterations in site II (*b2*) appeared to enhance the affinity of OBF1 to site I. This probably occurred because of an increased ratio of OBF1 to the amount of binding-site DNA in the reaction, resulting from the inactivation of site II for binding.

To delimit further the size of site I necessary for binding, we constructed linker (6 bp) substitution mutations across the site I region (Fig. 5). Three such mutations, *ls1*, *ls2*, and *ls3*, affected drastically the binding of OBF1 (Fig. 6B). About 10 to 12 times more OBF1 was needed to retard the mobility of mutagenized than of wild-type DNA in agarose gels. The remaining binding observed was a result of OBF1 binding to site II and perhaps some residual binding to the mutated site I, since overall binding to *ls1*, *ls2*, and *ls3* DNAs (Fig. 6B) was somewhat more efficient than to *b1* DNA (Fig. 4B). In contrast, sequence alterations outside the footprinted region (*ls0*, *ls4*, and *ls5*), on the 5′ and 3′ ends, did not reduce OBF1 binding to site I (Fig. 6A). Interestingly, in the *ls4* mutant, four nucleotides within the 3′ end of the footprinted region were also substituted. OBF1, however, bound the *ls4* DNA with at least the same efficiency as wild-type DNA. These results delimited the size of site I to no more than 18 nucleotides. Furthermore, OBF1 bound with greater affinity to *ls0*, *ls4*, and *ls5* than to wild-type DNA (Fig. 6A), suggesting that in addition to the primary sequence of the recognition site, a higher-order DNA structure may play a role in OBF1 binding.

Importance of the OBF1-binding sites for *ARS* function. *ARS* activity is identified by the ability of a DNA sequence to confer upon a plasmid autonomous replication in vivo. Moreover, differences in *ARS* activity can be measured by determining the frequency of yeast transformation as well as assessing the mitotic stability of a plasmid in the cell. To evaluate the contribution of the OBF1-binding site to *ARS* function, the *ARS121* DNA fragment (Fig. 1B) was cut with *Rsa*I endonuclease, and the resulting *Msp*I-*Rsa*I and *Hin*dIII-*Rsa*I fragments were each subcloned into YCp5-3 (a

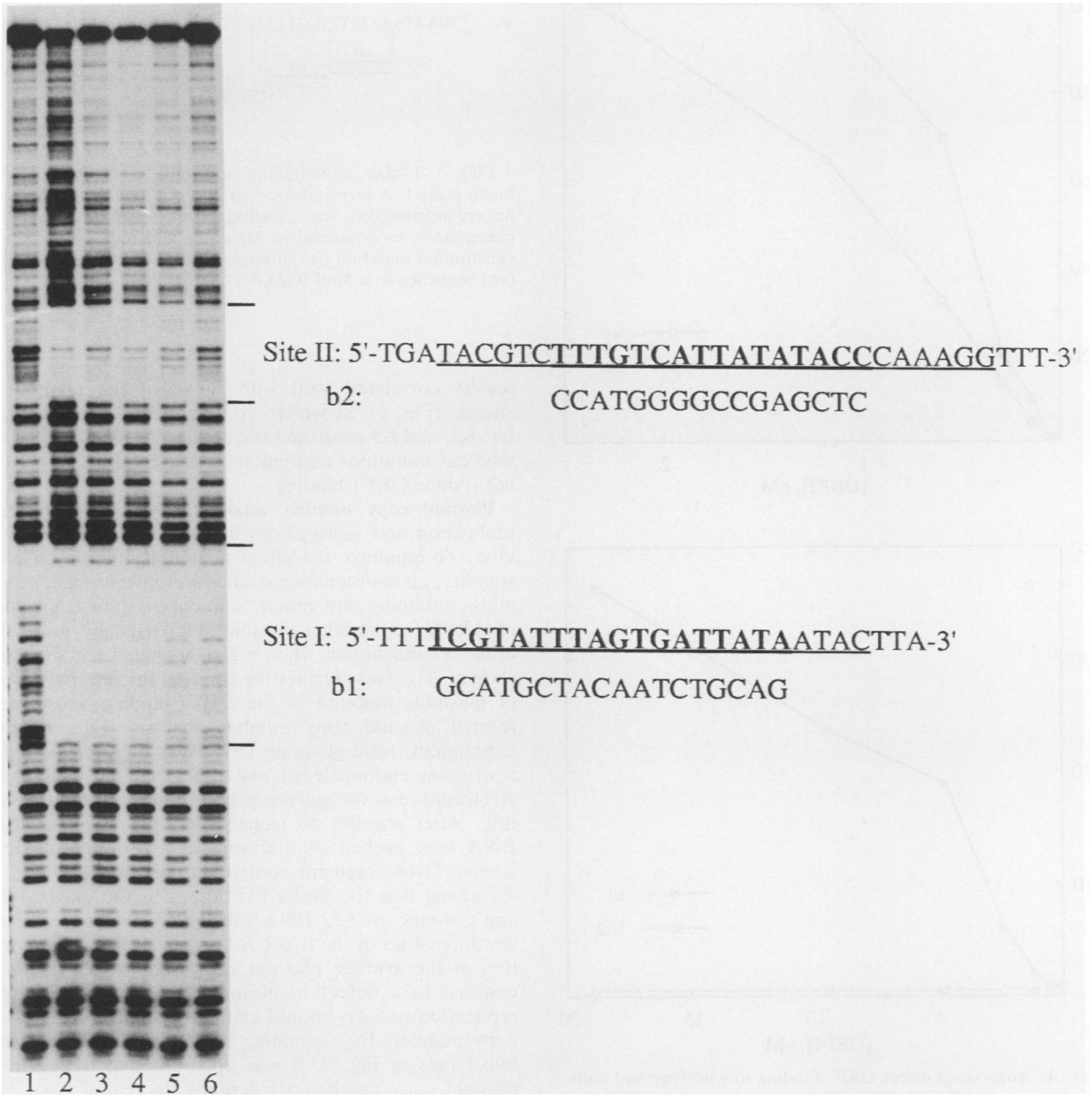

FIG. 3. Identification of the two OBF1-binding sites in *ARS121*. Footprinting analysis of the regions of *ARS121* protected from DNase I digestion by bound OBF1 was done as described in Materials and Methods. Lanes: 1, reaction without OBF1 protein; 2 through 6, reactions with 132, 66, 14, 2.8, and 0.7 ng, respectively, of OBF1. The nucleotide sequences of sites I and II are shown on the right. The regions protected from DNase I digestion are underlined. Sequences are shown in the same 5'-to-3' orientation as in Fig. 1B. Below each of the two binding-site sequences are the oligonucleotide-directed sequence alterations. Mutation *b1* is a substitution of 18 bp of site I (bold type); mutation *b2* is a substitution of 16 bp of site II (bold type); Mutation *b12* is *ARS121* containing both *b1* and *b2* sequence alterations.

shuttle vector containing *CEN3*), yielding plasmids YCp5B121 and YCp5A121, respectively. In a transformation test, only plasmid YCp5A121 was able to transform yeast cells at a high frequency. YCp5B121 did not yield transformants. However, plasmid maintenance studies indicated that plasmid YCp5A121, which does not contain sites I and II, was considerably less stable than YCp5AB121 (Fig. 7), which includes the entire *Msp*I-*Hin*dIII fragment described in Fig. 1B. Therefore, important information that affects *ARS121* activity is encoded in the *Msp*I-*Rsa*I region. The OBF1-binding sites are situated within this region and therefore may be important for plasmid stability. To test this possibility, we analyzed the mitotic stability of the mutagenized derivatives of YCp5AB121 described above. Indeed, plasmid maintenance analysis (Fig. 7) performed with the *b1* mutant showed a reduced level of stability in comparison with the wild-type *ARS*. A reduced level of stability, although slightly higher than that of *b1* in nonselective medium, was also seen with the *b2* mutant. The double mutant *b12* showed stability lower than those of *b1* and *b2* and comparable to that of YCp5A121, a plasmid with the binding sites deleted (Fig. 7). These results imply that the DNA sequence information important for *ARS* function includes sites I and II. To assess the relationship between the ability

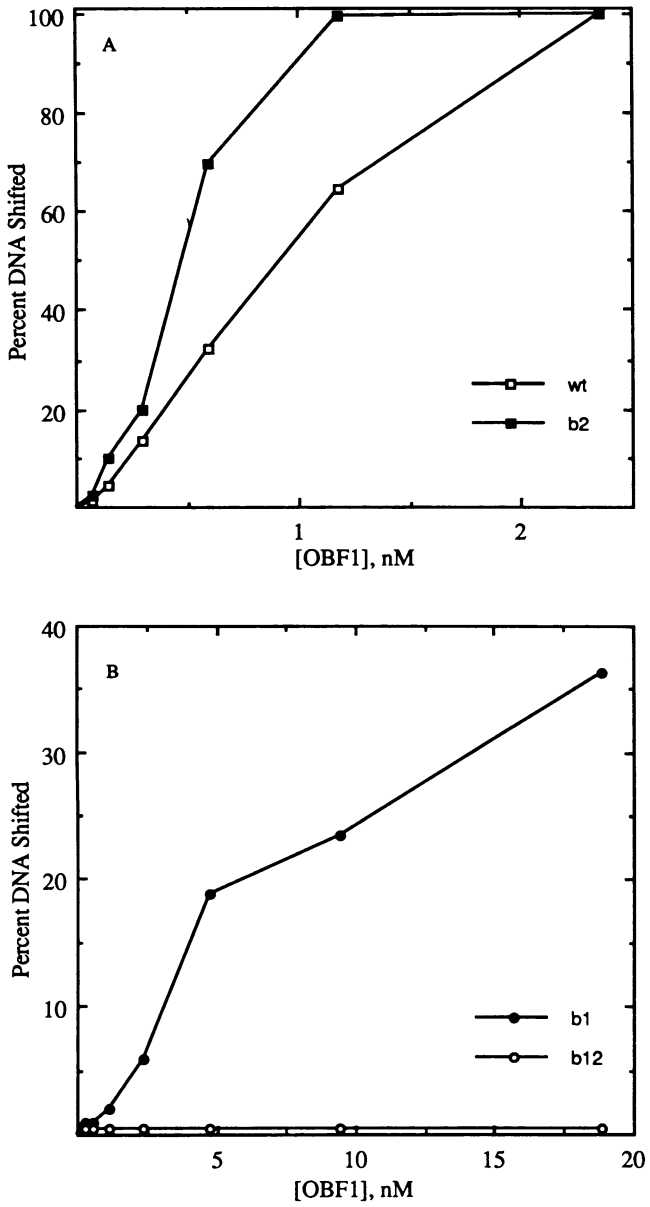

FIG. 4. Analysis of direct OBF1 binding to wild-type and mutagenized copies of *ARS121*. Plasmids bearing each binding substrate were cleaved, radiolabeled, incubated with OBF1, and electrophoretically resolved as described for Fig. 2. To determine the percentage of protein-bound DNA, the autoradiographic images, similar to those depicted in Fig. 2, were quantitated by densitometry. OBF1-bound *ARS121* DNA is expressed as percentage of total *ARS121* DNA (shifted, position I and, where appropriate, position II; unshifted, position A; Fig. 2). (A) OBF1-binding reactions containing wild-type (wt) or *b2* (site II mutation) *ARS121* DNA. At higher OBF1 concentrations, all DNA remained in the shifted position(s). (B) OBF1-binding reactions containing the site I mutation (*b1*) and the double mutation (*b12*).

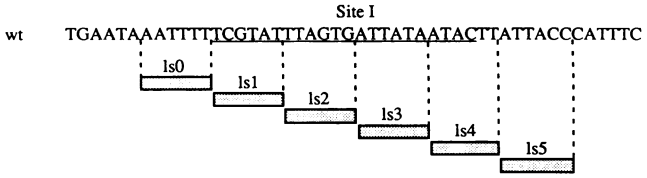

FIG. 5. Linker substitution mutagenesis of *ARS121* OBF1-binding site I. A series of six restriction endonuclease cleavage site linker substitutions was constructed by oligonucleotide-directed mutagenesis as described in Materials and Methods. Each linker substitution mutation (*ls0* through *ls5*) is a change of the wild-type (wt) sequence to a *Sac*I (GAGCTC) restriction site (boxes).

of OBF1 to bind to *ARS121* and *ARS* activity, we performed plasmid stability studies with mutations generated by linker substitution across the footprinted region of site I. Mutants *ls1*, *ls2*, and *ls3* had, like *b1*, lower stability when grown in either selective or nonselective medium (Fig. 8). In contrast, mutants *ls0*, *ls4*, and *ls5*, which are outside the footprinted region, appeared to have wild-type stability levels. These results correlated well with those of the OBF1-binding studies (Fig. 6), in which we demonstrated that mutations *ls1*, *ls2*, and *ls3* abolished the ability of OBF1 to bind site I, whereas mutations contiguous to the footprinted region did not reduce OBF1 binding.

Plasmid copy number analysis. Two major processes, replication and segregation, can affect plasmid stability in vivo. To minimize the effect of nondisjunction on plasmid stability, all maintenance studies were performed with plasmids containing centromeric sequences. In fact, the stability of *ARS121* containing plasmids increased by more than 2 orders of magnitude when it also included *CEN3* (data not shown). To assess further the cause of the relative instability of plasmids mutated in the OBF1-binding sites, we performed plasmid copy number analysis (Fig. 9). In this experiment, total genomic DNA was digested with *Eco*RI restriction endonuclease and the DNA was subjected to electrophoresis on agarose gels, followed by Southern blotting. After transfer to GeneScreen Plus membranes, the DNA was probed by hybridization with a radioactively labeled DNA fragment containing part of the *URA3* gene. Assuming that the probe hybridizes to the plasmid *URA3* and genomic *ura3-52* DNA with equal efficiencies, the relative intensities of the two *URA*-containing bands are indicators of the average plasmid copy number per genome. In contrast to a defect in plasmid segregation, an impaired replication process should cause a decrease in the average copy number. By examining the relative intensities of the two bands in Fig. 9, it was apparent that (i) the average plasmid copy number per genome decreased from 3.3 for plasmid YCp5AB121 to 1.6 for *b2*, 0.9 for *b1*, 0.6 for the *b12* double mutant, and 0.3 for YCp5A121 (OBF1-binding sites deleted), and (ii) surprisingly, in the cells that had retained the Ura^+ phenotype, we calculated that plasmid YCp5AB121 was maintained at about 8 copies per cell despite the presence of *CEN3*, which previously had been shown to regulate, by an unknown mechanism, a low plasmid copy number (37). The average copy number per Ura^+ cell also decreased in cells containing the mutated *ARS121* to 5.5, 4.9, and 2.7 for *b1*, *b12*, and YCp5A121, respectively. Incidentally, plasmid YCp5A1 (*ARS1*) included as a reference in these experiments, was maintained at a copy number of 1 to 2 per Ura^+ cell. We do not understand why plasmids bearing *ARS121* were maintained at a multicopy level. However, since the average copy number per genome and per Ura^+ cell decreased when *ARS121* was mutagenized, we interpret these results as meaning that plasmid instability is primarily the result of reduced efficiency of plasmid replication.

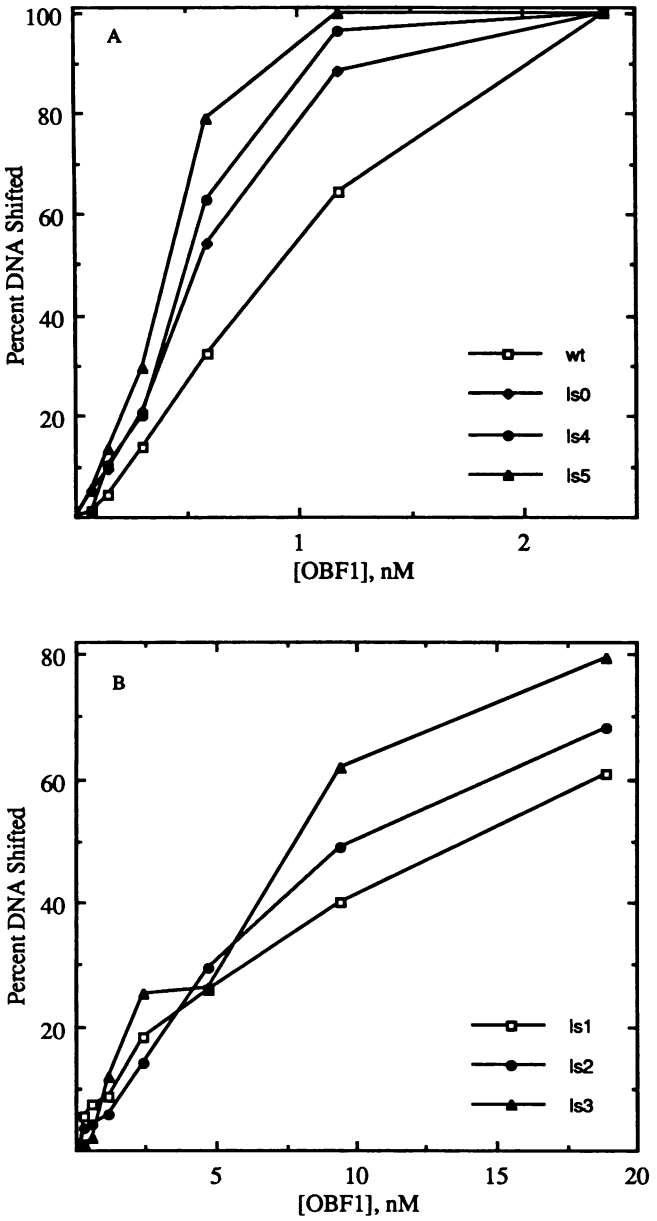

FIG. 6. OBF1 binding to mutants generated by linker substitution in site I. The DNA substrates were prepared and the binding reactions were performed as described for Fig. 4. (A) OBF1 binding to wild-type (wt) *ARS121* and mutant DNAs *ls0*, *ls4*, and *ls5*; (B) OBF1 binding to mutant DNAs *ls1*, *ls2*, and *ls3*. Mutations *ls0* through *ls5* were generated as described for Fig. 5.

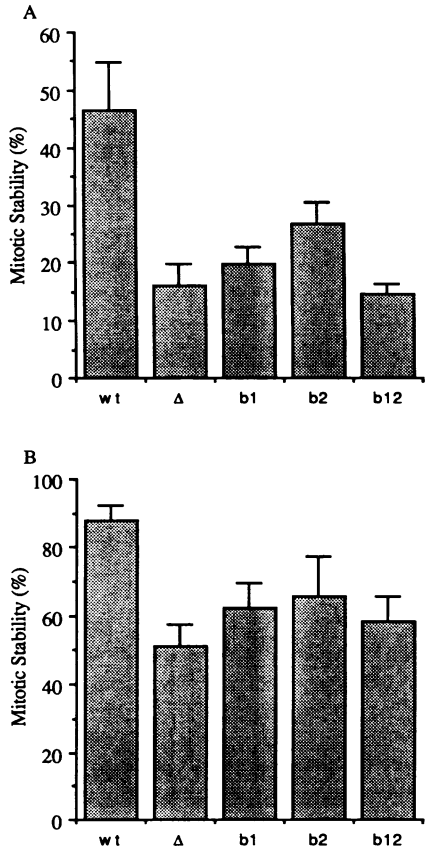

FIG. 7. Effects of mutagenesis of sites I and II on plasmid stability. Deletion of sites I and II (Δ) and the *b1*, *b2*, and *b12* mutations in *ARS121* were obtained as described in Materials and Methods and the legends to Fig. 1 and 3. Mitotic stabilities for these plasmids were determined as described in Materials and Methods. (A) Mitotic stability in nonselective medium; (B) mitotic stability in selective medium. The mitotic stability of plasmid YCp5A1 (*ARS1*) in an identical experiment was about 66.5% in selective and 20% in nonselective medium.

DISCUSSION

We have described a new *ARS*, *ARS121*, in which we have defined, by in vitro site-directed mutagenesis, a DNA element that is important for normal *ARS* function. Two elements, a low- and a high-affinity OBF1-binding site, were identified, one of which (site I) is no longer than 18 nucleotides. Mutagenizing these sites did not abolish but rather reduced the efficiency of *ARS121* activity. Therefore, the OBF1-binding sites are not absolutely required but instead exert a stimulatory effect on *ARS121* function. Interestingly, mutagenesis of either one of the binding sites impeded *ARS121* activity, implying that both are important. These results also suggest that essential elements for *ARS* function, still to be defined, are situated within the *Rsa*I-*Hin*dIII region of *ARS121*. Since all of our plasmid maintenance studies were performed with plasmids bearing centromeric sequences, and since the relative instability of mutated plasmids gives rise to a net plasmid loss in cell culture, we believe that the OBF1-binding sites affect *ARS* function through replication rather than segregation. Furthermore, considering the multicopy nature of *ARS121*-bearing plasmids, this effect is more substantial than is reflected by the stability analysis. Most likely, these sites enhance the efficiency of initiation of replication at the *ARS121* origin. We also found a remarkably strong correlation between the ability of the purified OBF1 to bind to the *ARS* and the in vivo activity of the *ARS*. In other words, only mutant *ARS* DNA that obliterated OBF1 binding also reduced the efficiency of the *ARS*. These results strongly support the notion that the OBF1 protein fulfills a function in vivo in stimulating the initiation of replication at the *ARS121* origin, perhaps analogous to the function of the mammalian nuclear factors NF-I and NF-III in the replication of adenovirus DNA (28, 29). Such a protein may affect the initiation indirectly by physically inhibiting nucleosomal assembly at the *ARS*, keeping the origin exposed for interactions with other initi-

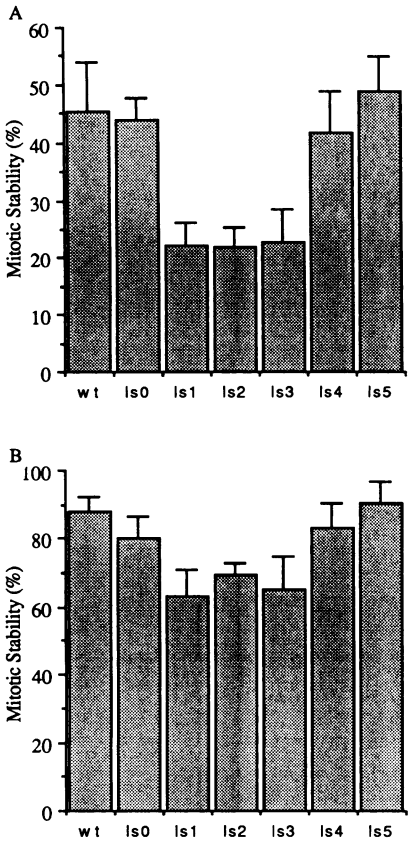

FIG. 8. Mitotic stability of plasmids bearing *ARS121* mutagenized by linker substitution in site I and the adjacent sequences. Mutations *ls0* through *ls5* are shown schematically in Fig. 5. Mitotic stabilities were determined as described in Materials and Methods and the legend to Fig. 8. (A) Mitotic stability in nonselective medium; (B) mitotic stability in selective medium.

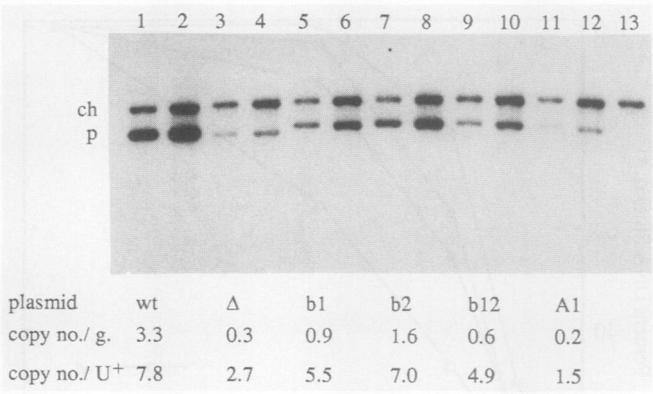

FIG. 9. Plasmid copy determination by genomic Southern blotting. Plasmid copy number was determined as described in Materials and Methods. After digestion of genomic DNA with *Eco*RI and electrophoresis of the DNA on agarose gels, the DNA was transferred to a GeneScreen Plus membrane and probed with a radioactively labeled DNA fragment containing part of the *URA3* gene. To ensure that hybridization of the probe was quantitative, each DNA preparation in the analysis is represented by two samples, one of which contains twice as much total genomic DNA as the other. Lanes: 1 and 2, DNA from YCp5AB121 (wild type)-containing cells; 3 and 4, DNA from YCp5A121 (deletion)-containing cells; 5 and 6, DNA from *b1*-containing cells; 7 and 8, DNA from *b2*-containing cells; 9 and 10, DNA from *b12*-containing cells; 11 and 12, DNA from YCp5A1 (*ARS1*)-containing cells; 13, DNA from 8534-8C cells (no plasmid). p, Position of the plasmid band containing the *URA3*; ch, position of the genomic *ura3-52* allele. The amount of DNA present in the bands was estimated by densitometer scanning, using a Bio-Rad model 620 densitometer. The average copy number per genome (copy no./g.) was calculated from the ratio of plasmid (*URA3*) DNA to chromosomal (*ura3-52*) DNA. To calculate the average copy number per Ura$^+$ cell (copy no./U$^+$), the average copy number per genome was multiplied by a factor corresponding to the reciprocal of the fraction of Ura$^+$ cells in the culture from which the plasmid DNA was isolated.

ation proteins. Alternatively, the involvement of OBF1 may be more direct: (i) while bound to the DNA, OBF1 may serve as an entry site for other proteins through protein-protein interactions; or (ii) bound protein may induce a conformational change in the DNA, facilitating an interaction of the origin with other initiation proteins. It is therefore reasonable to postulate that OBF1 acts at the origin in concert with other proteins. Hence, we believe that elucidation of the function and mechanism of action of OBF1 is contingent on the identification and characterization of other proteins that activate *ARS121* as an origin of replication.

In the accompanying paper (12), we report that OBF1 also binds to *ARS1* and *HMRE*. This binding occurs at sites situated in domain B of *ARS1* and in the functionally unrelated domain B of *HMRE* (unpublished observations), shown by others to bind the ABF-I protein (4, 9, 33, 35) and suggested by others to have a role in the function of these *ARS*s as origins of replication (1, 5, 20, 33). However, a tight link between the ability of purified ABF-I to bind to *ARS1* and *HMRE* and their functions as origins in vivo has not been clearly demonstrated.

In vivo, the OBF1 protein may be involved in stimulating the action of a wide range of *ARS*s; alternatively, its function may be manifested primarily with a unique class of origins, here represented by *ARS121*, in which there is a unique arrangement of a low- and a high-affinity OBF1-binding site. Activation of such a class of origins in the yeast genome might be coordinately regulated during the S phase. Thus, the isolation and characterization of other *ARS*s recognized by OBF1 may prove useful for elucidating the mechanism of action of OBF1 in stimulating the initiation of DNA replication.

ACKNOWLEDGMENTS

We are grateful to Mary J. Osborn for critical review of the manuscript and encouragement throughout the course of this work. We also thank Dan Eisenberg for assistance with the plasmid maintenance studies and Chris Civalier for some of the photography work.

S.C.F. was supported by Public Health Service National Research Service award GM07407 from the National Institutes of Health. This work was supported by American Cancer Society grant MV-368 to S.E.

LITERATURE CITED

1. **Brand, A. H., G. Micklem, and K. Nasmyth.** 1987. A yeast silencer contains sequences that can promote autonomous plasmid replication and transcriptional activation. Cell **51**:709–719.
2. **Brewer, B. J., and W. L. Fangman.** 1987. The localization of replication origins on *ARS* plasmids in *S. cerevisiae*. Cell **51**:463–471.
3. **Broach, J., Y. Y. Li, J. Feldman, M. Jayaram, J. Abraham, K. A. Nasmyth, and J. B. Hicks.** 1983. Localization and sequence analysis of yeast origins of DNA replication. Cold Spring Harbor Symp. Quant. Biol. **47**:1165–1173.
4. **Buchman, A. R., W. J. Kimmerly, J. Rine, and R. Kornberg.**

1988. Two DNA-binding factors recognize specific sequences at silencers, upstream activating sequences, autonomously replicating sequences, and telomeres in *Saccharomyces cerevisiae*. Mol. Cell. Biol. **8**:210–225.

5. **Celniker, S. E., and J. L. Campbell.** 1982. Yeast DNA replication *in vitro*: initiation and elongation events mimic *in vitro* processes. Cell **31**:201–213.
6. **Celniker, S. E., K. Sweder, F. Srienc, J. E. Bailey, and J. L. Campbell.** 1984. Deletion mutations affecting autonomously replicating sequence *ARS1* of *Saccharomyces cerevisiae*. Mol. Cell. Biol. **4**:2455–2466.
7. **Chan, C. S. M., and B. K. Tye.** 1980. Autonomously replicating sequences in *Saccharomyces cerevisiae*. Proc. Natl. Acad. Sci. USA **77**:6329–6333.
8. **Clarke, L., and J. Carbon.** 1980. Isolation of a yeast centromere and construction of functional small circular chromosomes. Nature (London) **287**:504–509.
9. **Diffley, J. F. X., and B. Stillman.** 1988. Purification of a yeast protein that binds to origins of DNA replication and a transcriptional silencer. Proc. Natl. Acad. Sci. USA **85**:2120–2124.
10. **Eisenberg, S., C. Civalier, and B. K. Tye.** 1988. Specific interaction between a *Saccharomyces cerevisiae* protein and a DNA element associated with certain autonomously replicating sequences. Proc. Natl. Acad. Sci. USA **85**:743–746.
11. **Feinberg, A. P., and B. Vogelstein.** 1983. A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. Anal. Biochem. **132**:6–13.
12. **Francesconi, S. C., and S. Eisenberg.** 1989. Purification and characterization of OBF1: a *Saccharomyces cerevisiae* protein that binds to autonomously replicating sequences. Mol. Cell. Biol. **9**:2906–2913.
13. **Holm, C., D. W. Meeks-Wagner, W. L. Fangman, and D. Botstein.** 1986. A rapid, efficient method for isolating DNA from yeast. Gene **42**:169–173.
14. **Huberman, J. L., D. L. Spotila, K. A. Nawotka, S. M. El-Assouli, and R. Davies.** 1987. The in vivo replication origin of the yeast 2 μm plasmid. Cell **51**:473–481.
15. **Huberman, J. L., J. Zhu, R. L. Davies, and C. S. Newlon.** 1988. Close association of a DNA replication origin and an *ARS* element on chromosome III of the yeast, *Saccharomyces cerevisiae*. Nucleic Acids Res. **16**:6373–6383.
16. **Ito, H., Y. Jukuda, K. Murata, and A. Kimura.** 1983. Transformation of intact yeast cells treated with alkali cations. J. Bacteriol. **153**:163–168.
17. **Jazwinski, M. S., A. Niedzwiecka, and G. M. Edelman.** 1983. *In vitro* association of a replication complex with a yeast chromosomal replicator. J. Biol. Chem. **258**:2754–2757.
18. **Johnson, A. D., B. J. Meyer, and M. Ptashne.** 1979. Interactions between DNA-bound repressors govern regulation by the λ phage repressor. Proc. Natl. Acad. Sci. USA **76**:5061–5065.
19. **Kearsy, S.** 1984. Structural requirements for the function of a yeast chromosomal replicator. Cell **37**:299–307.
20. **Kimmerly, W., A. Buchman, R. Kornberg, and J. Rine.** 1988. Roles of two DNA-binding proteins in replication, segregation and transcriptional repression mediated by a yeast silencer. EMBO J. **7**:2241–2253.
21. **Kimmerly, W., and J. Rine.** 1987. Replication and segregation of plasmids containing *cis*-acting regulatory sites of silent mating-type genes in *Saccharomyces cerevisiae* are controlled by the *SIR* genes. Mol. Cell. Biol. **7**:4225–4237.
22. **Kojo, H., B. D. Greenberg, and A. Sugino.** 1981. Yeast 2-μm plasmid DNA replication *in vitro*: origin and direction. Proc. Natl. Acad. Sci. USA **78**:7261–7265.
23. **Kunkel, T. A.** 1985. Rapid and efficient site-specific mutagenesis without phenotypic selection. Proc. Natl. Acad. Sci. USA **82**:488–492.
24. **Maine, G. T., P. Sinha, and B.-K. Tye.** 1984. Mutants of *S. cerevisiae* defective in the maintenance of minichromosomes. Genetics **106**:365–385.
25. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
26. **Maxam, A. M., and W. Gilbert.** 1980. Sequencing end-labeled DNA with base-specific chemical cleavages. Methods Enzymol. **65**:499–560.
27. **Newlon, C. S., R. J. Devenish, P. A. Suci, and C. J. Roffis.** 1981. Replication origins used in yeast. ICN-UCLA Symp. Mol. Cell. Biol. **22**:501–516.
28. **O'Neill, E. A., and T. J. Kelly.** 1988. Purification and characterization of nuclear factor III (origin recognition protein C), a sequence-specific DNA binding protein required for efficient initiation of adenovirus DNA replication. J. Biol. Chem. **263**: 931–937.
29. **Rosenfeld, P. J., and T. J. Kelly.** 1986. Purification of nuclear factor I by DNA recognition site affinity chromatography. J. Biol. Chem. **261**:1398–1408.
30. **Saffer, L. D., and O. L. Miller, Jr.** 1986. Electron microscopic study of *Saccharomyces cerevisiae* DNA chromatin replication. Mol. Cell. Biol. **6**:1148–1157.
31. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-termination inhibitors. Proc. Natl. Acad. Sci. USA **74**:5463–5467.
32. **Sherman, F., and G. R. Fink.** 1986. Methods in yeast genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
33. **Shore, D., D. J. Stillman, A. H. Brand, and K. A. Nasmyth.** 1987. Identification of silencer binding proteins from yeast: possible roles in *SIR* control and DNA replication. EMBO J. **6**:461–467.
34. **Stinchcomb, D. T., K. Struhl, and R. W. Davies.** 1979. Isolation and characterization of a yeast chromosomal replicator. Nature (London) **282**:39–43.
35. **Sweder, K. S., P. R. Rhode, and J. L. Campbell.** 1988. Purification and characterization of proteins that bind to yeast *ARSs*. J. Biol. Chem. **263**:17270–17277.
36. **Tschumper, G., and J. Carbon.** 1980. Sequence of a yeast DNA fragment containing a chromosomal replicator and the *TRP 1* gene. Gene **10**:157–166.
37. **Tschumper, G., and J. Carbon.** 1983. Copy number control by a yeast centromere. Gene **23**:221–232.