MOLECULAR AND CELLULAR BIOLOGY, Mar. 1982, p. 221-232
0270-7306/82/030221-12$02.00/0

Vol. 2, No. 3

Construction, Replication, and Chromatin Structure of TRP1 RI Circle, a Multiple-Copy Synthetic Plasmid Derived from *Saccharomyces cerevisiae* Chromosomal DNA

VIRGINIA A. ZAKIAN[1]* AND JOHN F. SCOTT[2]†

Hutchinson Cancer Research Center, Seattle, Washington 98104[1]; and Molecular Biology Institute, University of California, Los Angeles, California 90024[2]

Received 4 August 1981/Accepted 1 October 1981

Transformation studies with *Saccharomyces cerevisiae* (bakers' yeast) have identified DNA sequences which permit extrachromosomal maintenance of recombinant DNA plasmids in transformed cells. It has been hypothesized that such sequences (called ARS for autonomously replicating sequence) serve as initiation sites for DNA replication in recombinant DNA plasmids and that they represent the normal sites for initiation of replication in yeast chromosomal DNA. We have constructed a novel plasmid called TRP1 RI Circle which consists solely of 1,453 base pairs of yeast chromosomal DNA. TRP1 RI Circle contains both the *TRP1* gene and a sequence called *ARS1*. This plasmid is found in 100 to 200 copies per cell and is relatively stable during both mitotic and meiotic cell cycles. Replication of TRP1 RI Circle requires the products of the same genes (*CDC28*, *CDC4*, *CDC7*, and *CDC8*) required for replication of chromosomal DNA. Like chromosomal DNA, its replication does not occur in cells arrested in the G1 phase of the cell cycle by incubation with the yeast pheromone α-factor. In addition, TRP1 RI Circle DNA is organized into nucleosomes whose size and spacing are indistinguishable from that of bulk yeast chromatin. These results indicate that TRP1 RI Circle has the replicative and structural properties expected for an origin of replication from yeast chromosomal DNA. Thus, this plasmid is a suitable model for further studies of yeast DNA replication in both cells and cell-free extracts.

Yeast cells can be transformed by recombinant DNA plasmids containing a selectable yeast gene (21). When certain sequences from yeast DNA are cloned into such plasmids, they enable them to transform at high frequency ($\sim 10^3$ to 10^5 transformants per μg; 4, 42) and to be maintained in transformed cells as supercoiled extrachromosomal molecules. It has been hypothesized that sequences which promote high-frequency transformation and extrachromosomal maintenance of recombinant DNA plasmids are serving as initiation sites for DNA replication in plasmid molecules (22, 42). In addition, it has been suggested that these sequences normally serve as initiation sites for DNA replication in the intact chromosome.

Replication of chromosomal DNA in yeast requires the products of a number of cell cycle (*CDC*) genes. The products of *CDC28*, *CDC4*, and *CDC7* act sequentially before the initiation of DNA synthesis, whereas the products of *CDC8* and *CDC21* are required continuously during the S phase (18–20). The product of only one of these genes (*CDC21*), thymidylate synthetase, has been identified (5). Replication of the multiple-copy endogenous plasmid of yeast called 2μ DNA also depends on these gene products (26, 32). In contrast, replication of yeast mitochondrial DNA occurs in the absence of an active *CDC28*, *CDC4*, or *CDC7* gene product (13, 30) and in the presence of the yeast pheromone α-factor (14, 31), which inhibits replication of both chromosomal and 2μ DNAs (8, 26). Moreover, both yeast chromosomal and 2μ DNAs are organized in typical nucleosomal subunits (25, 27, 28), whereas mitochondrial DNA is not believed to be associated with histones (9, 33).

In this paper we describe the construction and stability properties of a multiple-copy extrachromosomal plasmid called TRP1 RI Circle, a plasmid comprised solely of yeast DNA. TRP1 RI Circle was constructed by the self-ligation of a 1.45-kilobase (kb) *Eco*RI fragment from yeast chromosomal DNA which bears both the structural gene for *TRP1* and a sequence called *ARS1* (autonomously replicating sequence) capable of

† Current address: Department of Microbiology, Urbana, IL 61801.

promoting high-frequency transformation and extrachromosomal maintenance of plasmid DNAs (41). We show that the plasmid exists in 100 to 200 copies per cell and is maintained with high stability during both mitotic and meiotic cell cycles. In addition, we report that (i) replication of the TRP1 RI Circle depends on the same gene products required for replication of yeast chromosomal DNA and (ii) TRP1 RI Circle is organized into a typical nucleosomal structure. Because these properties are those expected for a chromosomal origin of replication, our data support the hypothesis that sequences which promote high-frequency transformation and extrachromosomal maintenance of plasmid DNAs function as initiation sites for DNA replication.

MATERIALS AND METHODS

Strains and growth conditions. The strains used or constructed (or both) in this work are listed in Table 1. The techniques and growth media used for genetic constructions and analysis were essentially as described previously (39). The following media were used. YMHU contains (per liter) 0.67 g of yeast nitrogen base without amino acids or ammonium sulfate (Difco Laboratories), 5 g of $(NH_4)_2SO_4$, 20 g of glucose, 40 mg of histidine-hydrochloride, and 20 mg of uracil. YEPD contains (per liter) 20 g of glucose, 20 g of peptone (Difco), and 10 g of yeast extract (Difco). Y complete minus tryptophan contains (per liter) 20 g of glucose; 1.2 of yeast nitrogen base; 2.5 g of $(NH_4)_2SO_4$; 10 g of succinic acid; 6 g of NaOH; 0.1 g each of adenine, lysine, arginine, threonine, and cysteine; 0.05 g each of histidine, tyrosine, leucine, methionine, serine, valine, isoleucine, phenylalanine, and proline; and 3 mg of uracil (pH 5.8). For the α-factor experiment, less NaOH was added to Y complete minus tryptophan to adjust the pH to 3.5.

Purification of TRP1 RI Circle DNA. JSY7/T cells were grown to a density of approximately 2×10^8 cells per ml in YEPD or to a density of 2×10^7 cells per ml in YMHU. The cells were harvested by centrifugation (3,000 rpm, 45 min, 4°C, IEC centrifuge, 6-liter rotor). Most of the medium was removed, and the cells were recentrifuged (10,000 rpm, 10 min, 4°C, Beckman J2-21 centrifuge, JA 14 rotor). Cells were suspended (to a density of approximately 2×10^{10} per ml for cells grown in YEPD or 2×10^9 per ml for cells grown in YMHU) in a solution containing 50 mM potassium phosphate (pH 7.8), 0.9 M sorbitol, and 50 mM EDTA, frozen in liquid nitrogen, and stored at −70°C. The cell suspension was thawed, Zymolyase-60,000 (Kirin Brewery Co., Japan) was added to a concentration of 0.5 mg/ml, and the cells were incubated for 1 h at 0°C

TABLE 1. *S. cerevisiae* strains used[a]

Strain	Genotype	Source
α*trp1*	*matα trp1*	Lynna Hereford
198	*mata ade1,2 ura1 his7 tyr1 lys2 cdc8-1*	Leland Hartwell
314	*mata ade1,2 ura1 his7 tyr1 lys2 cdc4-1*	Leland Hartwell
SR668-2	*matα tyr1 leu2 trp1 ade1 cyh2 cdc28-4*	Steven Reed
NNY1	*matα ura3 trp1 Δhis3-1/cir*^{0}	Stewart Scherer
JSY7/T	*matα ura3 trp1 Δhis3-1*/TRP1 RI Circle *cir*^{0}	This work
JSY9/T	*matα trp1*/TRP1 RI Circle	This work
JSY11/7	*matα trp1*/YRp7	This work
JSY12/T	*matα ura3 Δhis3-1 trp1*/TRP1 RI Circle *cir*^{0}	This work
JSY15/7	*matα ura3 Δhis3-1 trp1*/YRp7 *cir*^{0}	This work
JSY65	*matα ade his trp1 cdc8-1*	This work
JSY202	*mata ura1 tyr1 ade lys2 trp1*	This work
JSY204	*mata ura1 tyr1 ade lys2 trp1 cdc4-1*	This work
RH15e2/T	*mata ura cdc7-4 trp1*/TRP1 RI Circle	Robert Hice
JSY322/T	*mata ura Δhis3-1 trp1*/TRP1 RI Circle	This work
JSY334/T	*mata ura his cdc4-1 trp1*/TRP1 RI Circle	This work
JSY487/T	*mata tyr1 his ura cdc28-4 trp1*/TRP1 RI Circle	This work
JSY488/T	*mata tyr1 ura cdc28-4 trp1*/TRP1 RI Circle	This work
JSY542/T	*mata ura cdc8-1 trp1*/TRP1 RI Circle	This work

[a] Strains α*trp1*, 198, 314, SR668-2, NNY1, and RH15e2/T were generously provided by the persons indicated. Strain NNY1 was the product of the transplacement of *Δhis3-1* into the yeast chromosome which was described, but not named, by Scherer and Davis (36). The remaining strains were constructed in this work. Symbols used in the genotype after "/" refer to extrachromosomal plasmids. In the strain names " /T" or " /7" indicates that the strain contains TRP1 RI Circle or YRp7, respectively. JSY7/T was constructed by transformation of NNY1 with the ligation mixture in which TRP1 RI Circle was first formed. The TRP1 RI Circle DNA purified from JSY7/T (as described in the text) was used to transform NNY1 to yield JSY12/T and to transform α*trp1* to yield JSY9/T. JSY11/7 and JSY15/7 were YRp7 transformants of α*trp1* and NNY1, respectively. JSY65 was a haploid progeny from a diploid formed between α*trp1* and 198. JSY202 and JSY204 were progeny of a cross between α*trp1* and 314. JSY12/T was then mated with JSY202 and JSY204 to yield haploid progeny containing TRP1 RI Circle, JSY322/T, and JSY334/T, respectively. JSY322/T was then mated with SR668-2 and sporulated to yield haploid strains containing TRP1 RI Circle (JSY487/T and JSY488/T). JSY322/T was also crossed with JSY65 to yield JSY542/T.

until spheroplasts were formed. Sodium dodecyl sulfate was added to a concentration of 1%, the lysate was mixed well by inversion, and potassium acetate was added to 1 M. The mixture was chilled on ice overnight (approximately 16 h), and cell debris, large DNA, and sodium dodecyl sulfate were pelleted by centrifugation (JA 20 rotor, 15,000 rpm, 4°C, 30 min). The supernatant was extracted twice with chloroform-phenol (1:1, wt/wt) and twice with diethyl ether. Phases were separated by centrifugation (JA 20 rotor, 23°C, 10,000 rpm, 10 min) for both the chloroform-phenol extractions and for the first diethyl ether extraction. After the first ether extraction, which produced a gelatinous material at the phase interface, both liquid phases were decanted into a separatory funnel (leaving the gel interface behind). Phases were separated in the funnel, and the aqueous phase was reextracted with ether. Two volumes of 100% ethanol were added to the aqueous phase, and the mixture was stored at −20°C for 1 h. The precipitate was collected by centrifugation (JA 14 rotor, 15,000 rpm, 4°C, 30 min), drained, dried at room temperature, and redissolved in 4.0 ml of 10 mM Tris-hydrochloride (pH 7.5)–1 mM EDTA containing 50 μg of RNase A (Worthington Diagnostics) per ml. The mixture was digested at 37°C for 15 min and then adjusted to 150 mM NaCl. Debris was removed by centrifugation (Eppendorf microfuge, 5 min, 23°C), and the supernatant removed. The pellet was successively resuspended four times in 10 mM Tris-hydrochloride (pH 7.5)–1 mM EDTA–150 mM NaCl followed by centrifugation each time to remove debris. Supernatants containing plasmid were pooled, adjusted to a density of 1.54 g/ml using cesium chloride and 1 mg of ethidium bromide per ml, and centrifuged to equilibrium (42,000 rpm, 48 h, 20°C, Ti 60 rotor, Beckman Instruments, Inc.). The gradient was collected into 25 fractions, and samples of each fraction were extracted four times with equal volumes of 2-propanol saturated with a 1.54-g/ml density solution of CsCl in 10 mM Tris-hydrochloride (pH 7.5)–1 mM EDTA. The samples were diluted fourfold with 10 mM Tris-hydrochloride (pH 7.5)–1 mM EDTA, precipitated by the addition of 0.1 volume of 3 M sodium acetate (pH 5.5) and 2 volumes of 2-propanol, chilled in a dry ice-ethanol bath for 10 min, collected by centrifugation in an Eppendorf microfuge (10 min, 4°C), and examined by agarose gel electrophoresis. Fractions containing TRP1 RI Circle DNA were pooled, extracted with 2-propanol, and precipitated as described above. This DNA was used for restriction enzyme analysis (see Fig. 2). TRP1 RI Circle DNA for use in yeast transformations was further purified by recentrifugation in CsCl-ethidium bromide gradients as above.

Recombinant DNA manipulations and yeast transformation. YRp7 plasmid DNA was prepared from *Escherichia coli* cells incubated for 14 to 16 h with 160 μg of chloramphenicol per ml. Lysis of cells was done essentially as described previously (23). Cleared lysates were centrifuged to equilibrium in two successive ethidium bromide-CsCl gradients as described by Bazarel and Helinski (2). The regions of the gradients containing plasmid DNA were visualized by illumination with UV light and removed by side puncture. The DNA was recovered as described for pooled TRP1 RI Circle fractions (see above).

Yeast transformations were done essentially as described previously (21), except that the spheroplast-DNA mixture containing 40% polyethylene glycol was plated directly in regeneration agar. All recombinant DNA manipulations were done in compliance with the National Institutes of Health guidelines for recombinant DNA research. After purification and retransformation into *Saccharomyces cerevisiae* strains the TRP1 RI Circle is exempt from the recombinant DNA guidelines since it contains only *S. cerevisiae* DNA. Restriction enzymes were obtained from Boehringer-Mannheim Corp., Bethesda Research Laboratories, and New England Biolabs and used as directed. T4 DNA ligase was from P-L Biochemicals.

Radiolabeling of cells and extraction procedures for copy number determinations and replication experiments. Cultures were grown at 23 to 25°C for about six generations in medium containing 1 μCi of [2-^{14}C]uracil per ml to a density of about 6×10^6 cells per ml. In most experiments, cells were removed by filtration from the medium containing [^{14}C]uracil and then suspended in an equal volume of fresh medium containing 10 μCi of [6-^{3}H]uracil. In the experiment with strain JSY334/T (*cdc4*), [^{3}H]uracil was added directly to the medium containing [^{14}C]uracil. The cultures were returned to 25°C for 10 min and then divided into two portions. One portion was maintained at 25°C, and the other was either transferred to 37°C (for strains JSY322/T, RH15e2/T, JSY334/T, and JSY542/T) or to 38°C (for strains JSY487/T and JSY488/T) or maintained at 25°C in the presence of sufficient α-factor (partially purified by the procedure described in reference 16) to arrest cells for two generations (strain RH15e2/T, α-factor experiment). At appropriate intervals, samples (30 to 85 ml), were removed, pelleted by centrifugation, and washed two times in cold distilled water. The samples were processed by the procedure of Davis et al. (15), except that spheroplasts were formed by using 450 μg of Zymolyase 60,000 per ml in SCE (1 M sorbitol, 0.1 M sodium citrate, 0.06 M EDTA, pH 7.0) containing 13 mM β-mercaptoethanol. After lysis and addition of sodium acetate, preparations were maintained on ice for 30 min. Samples were incubated with 200 μg of DNase-free pancreatic RNase for 1 h at 37°C and then run on 0.7% agarose gels as described previously (46). DNA in the individual lanes was treated with 0.25 N HCl (44) to facilitate transfer of large and supercoiled DNAs, denatured in 0.5 M NaOH 1.5 M NaCl (two washes, 40 min each), neutralized, and then transferred to nitrocellulose (Schleicher & Schuell Co., 0.45 μm) by the procedure of Southern (40). The nitrocellulose strips were rinsed in 70% ethanol, dried, cut into segments 0.5 cm in length, and monitored for ^{3}H and ^{14}C counts per minute (toluene-Omnifluor; New England Nuclear Corp.).

Chromatin analysis. All manipulations for chromatin analysis were done essentially as described for the strain lacking 2μ DNA (28), except that SHC was used instead of SPC (SHC contains 1 M sorbitol, 20 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid [pH 7.5], 0.1 mM $CaCl_2$), and the spheroplasts were frozen as pellets in liquid nitrogen and stored at −70°C for several weeks before lysis. Spheroplasts were thawed and lysed by resuspension in a hypotonic buffer containing 5 mM potassium phosphate (pH 7.2), 0.1 mM $CaCl_2$, and 1 mM phenylmethyl sulfonyl fluoride. After removal of a sample from each lysate

for the 0 time point, micrococcal nuclease (Worthington; 150 U/ml) was added. Additional samples were removed after 3, 6, 12, 24, and 60 min at 37°C. Reactions were stopped, and DNA was extracted and subjected to electrophoresis in agarose gels as described by Nelson and Fangman (28) and in the legend to Fig. 5. The gel was acid treated, denatured, and neutralized, and the DNA was transferred to diazotized paper as described previously (44), except that transfer was carried out overnight with a small-pore plastic sponge serving as a wick. Diazotized paper was prepared as described previously (1). YRp7 DNA was nick translated with ^{32}P (34), and 10^6 cpm was used for hybridizations, which were carried out as described previously (44). Autoradiography was for 12 h with Kodak XRP-1 film and a Du Pont Quanta III b intensifying screen at −70°C.

RESULTS

Construction and structure of TRP1 RI Circle. A 1.45-kb *Eco*RI restriction fragment from yeast chromosomal DNA containing the *TRP1* gene and a sequence called *ARS1* was excised from the yeast vector YRp7 (42) and ligated at low DNA concentration (2 μg/ml) to favor intramolecular circularization and the elimination of pBR322 DNA (Fig. 1). The ligation mixture was used to transform a *trp1* strain of yeast (NNY1, Table 1), and transformants were selected by their ability to grow in the absence of tryptophan. Transformants were obtained at a frequency of approximately 1,000/μg of *TRP1-ARS1* *Eco*RI fragment present in the ligation mixture. DNA was isolated from the transformed cells (JSY7/T) and analyzed by agarose gel electrophoresis (Fig. 2). Transformed cells were found to contain multiple copies of a 1.45-kb supercoiled plasmid. TRP1 RI Circle was present in comparable amounts in strains containing (*cir*$^+$) or lacking (*cir*^{0}) 2μ DNA, the endogenous yeast plasmid (Fig. 2, lanes C and E). The TRP1 RI Circle plasmid was purified from JSY7/T cells, and its structure was verified by cleavage with restriction endonucleases. Digestion with *Eco*RI alone or with a combination of *Eco*RI and *Hin*dIII yielded the fragments expected for a plasmid consisting solely of the 1.45-kb yeast *Eco*RI fragment present in YRp7 (Fig. 2, lanes G through J). Purified TRP1 RI Circle DNA transformed *trp1* strains (NNY1 and α*trp1*) at a frequency of 7,000 to 8,000 transformants per μg of DNA.

Copy number of TRP1 RI Circle. The number of copies of 2μ DNA and TRP1 RI Circle per cell at 25°C during the log phase of growth (Table 2) was determined in six strains from the percentage of [^{14}C]uracil counts per minute detected in purified plasmid DNAs after their separation on agarose gels and transfer to nitrocellulose (Fig. 3B). (A nonspecific DNA precursor was used for isotopic labeling because yeast cells can not utilize thymidine.) For all except the *cdc28* strains (JSY487/T and JSY488/T), the copy number of 2μ DNA averaged 40 molecules per cell (range of 27 to 64), and the copy number for TRP1 RI Circle averaged 146 molecules per cell (range of 97 to 203). (For strains JSY322/T and

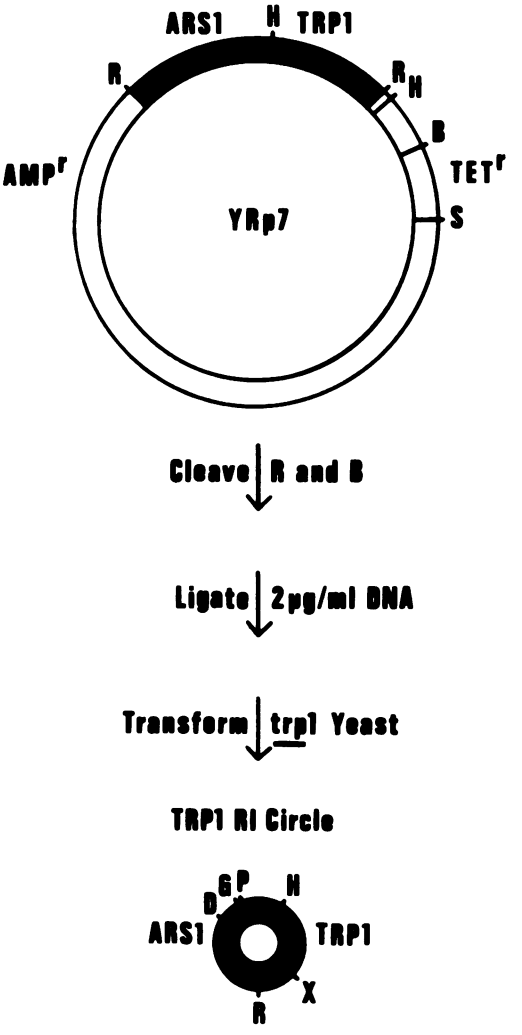

FIG. 1. Construction of TRP1 RI Circle. Four micrograms of YRp7 DNA (42) purified from *E. coli* as described in the text was digested to completion with a combination of *Eco*RI and *Bam*HI restriction endonucleases. The digestion mixture was heated at 60°C for 10 min and then diluted to a concentration of 2 μg/ml in ligation buffer containing 50 mM Tris-hydrochloride (pH 7.8), 10 mM $MgCl_2$, 1 mM ATP, 20 mM dithiothreitol, and 0.01 Weiss unit of T4 DNA ligase. The ligation mixture was incubated at 4°C for 2 days and used to transform a *trp1* strain of yeast (NNY1). Transformants were selected by their ability to grow in the absence of tryptophan. Symbols for restriction enzyme cleavage sites are as follows: R, *Eco*RI; H, *Hin*dIII; B, *Bam*HI; P, *Pst*I; X, *Xba*I; D, *Hin*dII; and G, *Bgl*II.

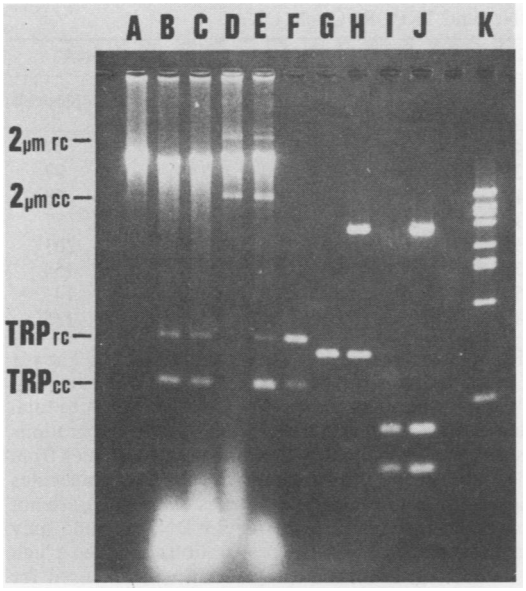

FIG. 2. Structure of TRP1 RI Circle. DNA samples were separated on a 1% agarose gel in TBE buffer containing (per liter) 10.8 g of Trizma base (Sigma), 5.5 g of boric acid, and 0.93 g of disodium EDTA. Electrophoresis was for 4 h at 2.5 V/cm. The gel was stained with 0.5 μg of ethidium bromide per ml in the same buffer and photographed by using transillumination with UV light. Lanes A through E contain total DNA from the following strains: A, NNY1; B, JSY7/T; C, JSY12/T; D, *αtrp1*; E, JSY9/T. These DNAs were from cells grown to midlog phase (approximately 10^8 cells per ml) prepared essentially as described in reference 39, except NNY1 DNA, which was purified as described in the legend to Fig. 5. Lanes F through J contain purified plasmid DNAs as follows: F, TRP1 RI Circle; G, *Eco*RI-digested TRP1 RI Circle; H, *Eco*RI-digested YRp7; I, *Eco*RI- and *Hin*dIII-digested TRP1 RI Circle; J, *Eco*RI- and *Hin*dIII-digested YRp7; and K, *Hin*dIII-digested adenovirus 5 DNA. The YRp7 and TRP1 RI Circle DNAs were purified as described in the text. The positions of covalently closed supercoiled 2μ plasmid DNA (2μm cc), relaxed circle 2μ DNA (2μm rc), covalently closed supercoiled TRP1 RI Circle DNA (TRP cc), and relaxed TRP1 RI Circle DNA (TRP rc) are indicated.

RH15e2/T, in which copy numbers were determined in two independent experiments, the average of the values from the two experiments was used as the strain value.) If the values from the *cdc28* strains are included, the average number of molecules per cell averaged 30 for 2μ DNA and 120 for TRP1 RI Circle DNA. Our copy number values for 2μ DNA are similar to reported values: one study reported about 60 copies per haploid cell (12), and another reported a range of 20 to 53 copies per haploid cell (38). Because our values for 2μ DNA agree with reported values and because they are reproducible within a strain (JSY332/T and RH15e2/T, Table 2), we assume that our extraction procedure is reasonably quantitative for all DNA species in the cell.

Two different strains bearing the same allele of *cdc28* (*cdc28-4*, JSY487/T, and JSY488/T) have reduced amounts of 2μ DNA (11 copies per cell) and probably lower amounts of TRP1 RI Circle DNA (an average of 67 copies per cell) at permissive temperatures. Copy numbers for both plasmids appear to be high in the *cdc4* strain (JSY334/T; 64 copies of 2μ DNA, 203 copies of TRP1 RI Circle DNA). These data suggest that the copy number of extrachromosomal plasmids can be influenced by the genetic background of the strain containing them.

Growth rate of cells containing TRP1 RI Circle. NNY1 cells grown in YMHU containing 20 μg of tryptophan per ml at 30°C were found to have a doubling time of 126 min. JSY7/T cells (containing TRP1 RI Circle) grown in YMHU (no tryptophan) under the same conditions had a doubling time of 120 min. These data indicate that the presence of more than 100 copies of a chromosomal structural gene (*TRP1*) and a putative chromosomal origin (*ARS1*) on extrachromosomal plasmids does not adversely affect the growth rate of cells. For comparison, JSY15/7 cells (containing YRp7) exhibited a population doubling time of approximately 390 min under the same conditions.

Mitotic stability of TRP1 RI Circle. The mitotic stability of TRP1 RI Circle in transformed cells was determined in two strains, one which contained 2μ DNA (JSY9/T, cir^+) and one which did not (JSY12/T, cir^0). The stability of the Trp^+ phenotype in these strains was compared with that of the same strains transformed by YRp7 (JSY11/7, cir^+; and JSY15/7, cir^0). The four strains were grown at 23°C for approximately 15 generations to stationary phase in YMHU. Samples of each culture were diluted and plated on media containing tryptophan. After colonies had formed, they were replica plated to media lacking tryptophan. Fewer than 5% of the JSY11/7 cells and 12% of the JSY15/7 cells were Trp^+ (retained YRp7), whereas 86% of the JSY9/T cells and 80% of the JSY12/T cells were Trp^+ (retained TRP1 RI Circle). The strains containing TRP1 RI Circle were also diluted into YEPD (a rich medium containing tryptophan) and grown at 23°C to stationary phase (approximately 15 generations), and the percentage of Trp^+ cells was determined. Again, a high percentage of cells retained the TRP1 RI Circle (80% in JSY9/T and 73% in JSY12/T). Thus, in comparison with YRp7 the TRP1 RI Circle is stable during mitotic growth. Indeed, its stability is comparable to that for plasmids containing a yeast centromere (11).

TABLE 2. Copy number of 2μ DNA and TRP1 RI Circle DNA

Strain[a]	2μ DNA		TRP1 RI Circle DNA	
	% of total DNA	Copies/cell	% of total DNA	Copies/cell
JSY322/T (temperature resistant, 25°C)	1.4 ± 0.7 (4)	31	1.0 ± 0.7 (4)	97
JSY322/T (temperature resistant, 37°C)	1.3 ± 0.1 (2)	29	1.0 ± 0.7 (4)	97
JSY487/T (*cdc28*)	0.5 ± 0.1 (4)	11	0.6 ± 0.2 (4)	58
JSY488/T (*cdc28*)	0.5 ± 0.1 (3)	11	0.8 ± 0.1 (3)	77
JSY334/T (*cdc4*)	2.9 ± 1.8 (3)	64	2.1 ± 1.0 (4)	203
RH15e2/T (*cdc7*) I	1.2 ± 0.5 (5)	27	1.6 ± 1.2 (5)	154
RH15e2/T (*cdc7*) II	1.3 ± 0.8 (5)	29	1.5 ± 0.9 (5)	145
JSY542/T (*cdc8*)	1.7 ± 0.5 (4)	38	1.4 ± 0.3 (3)	135

[a] Cells were grown in Y complete minus tryptophan which contains 3 μg of uracil per ml at 23 to 25°C. The pH of the medium was 5.8 for all cultures except RH15e2/T (*cdc7*) II, where the pH was 3.5. The percentage of plasmid DNA per cell was determined from the ratio of ^{14}C counts per minute in purified plasmid DNA to total ^{14}C counts per minute in DNA after transfer to nitrocellulose. The number of independent DNA preparations used to compute the average value is given in parentheses after the percentage value. The range of values from different preparations is indicated. For TRP1 RI Circle copy number determinations included molecules migrating at both the nicked and supercoiled positions (Fig. 3A). Because nicked molecules of 2μ DNA are not resolved from bulk DNA under our gel conditions, copy number determinations for 2μ DNA include only supercoiled molecules and are therefore sometimes underestimated. If a given DNA preparation contained a high proportion of TRP1 RI Circle DNA in the nicked form (≥ 35% of total), that preparation was not used for determining copy number of 2μ DNA. Plasmid copy number for RH15e2/T was determined during both the *cdc7* experiment (I) and the α-factor experiment (II). Values used for sizes of the yeast haploid genome, 2μ DNA, and TRP1 RI Circle were, respectively, 14,000 (Fangman and Zakian, in press), 6.32 (17), and 1.45 (43) kb.

Meiotic stability and segregation of TRP1 RI Circle. Yeast strains containing TRP1 RI Circle were mated with other *trp1* strains to form diploids. Diploids containing TRP1 RI Circle were selected on the basis of complementation of unlinked markers and by their ability to grow in the absence of tryptophan. The diploids were sporulated at 23°C; haploid spores were separated by sonication to obtain random spores or by tetrad dissection and were germinated by standard techniques (39). Sporulation and germination were carried out in the presence of tryptophan (i.e., without selection for TRP1 RI Circle). The genotypes of the haploid clones were determined by standard genetic tests, and the presence of TRP1 RI Circle was inferred by a Trp+ phenotype. Very few of the asci obtained under these conditions contained four spores. The spores in five of five complete four-spore tetrads derived from such four-spore asci displayed a segregation pattern of 4 Trp^+:0 Trp^-. Of 84 dissected spores, 76% were Trp^+. In addition, from a total of 300 haploid clones generated as random spores from six crosses, 78% were Trp^+. In contrast, YRp7 is not transmitted to spores when meiosis takes place in media containing tryptophan and is transmitted to only ~25% of spores when meiosis takes place in media lacking tryptophan (24). Thus, in comparison with YRp7, TRP1 RI Circle is relatively stable during meiosis. We have exploited the ease with which TRP1 RI Circle can be introduced into new *trp1* strains by genetic crosses to construct a set of *cdc*/TRP1 RI Circle strains which were used for the replication analyses (Table 1 and above).

Replication of TRP1 RI Circle. Cultures were prelabeled with [^{14}C]uracil for about six generations to uniformly label DNA. Cells were removed from the medium containing [^{14}C]uracil and incubated in fresh medium containing [^{3}H]uracil under both permissive and restrictive conditions for the equivalent of about two generations for an unarrested population. Replication of both 2μ DNA and TRP1 RI Circle DNA was monitored by determining the $^{3}H/^{14}C$ ratio of DNA molecules purified by agarose gel electrophoresis (Fig. 3A). An increase in $^{3}H/^{14}C$ ratio for TRP1 RI Circle and for 2μ DNAs was used as an index of replication (Fig. 4). Transfer of DNA to nitrocellulose before determination of [^{3}H]- and [^{14}C]uracil counts was necessary to eliminate the uracil counts in contaminating RNA. The [^{14}C]uracil profile after such a transfer (Fig. 3B) demonstrates that RNA is not retained by the procedures used.

Yeast cells are arrested in the G1 phase by both mating pheromones and by defects in several cell cycle genes. The products of cell cycle genes *CDC28*, *CDC4*, and *CDC7* act late in the G1 phase (35) in a sequential and dependent pathway (19, 20) that leads to entry of cells into the S phase. α-Factor arrests cells of a mating type at the same point in the cell cycle as a defect in the *CDC28* gene. The product of the *CDC8* gene is required throughout the S phase.

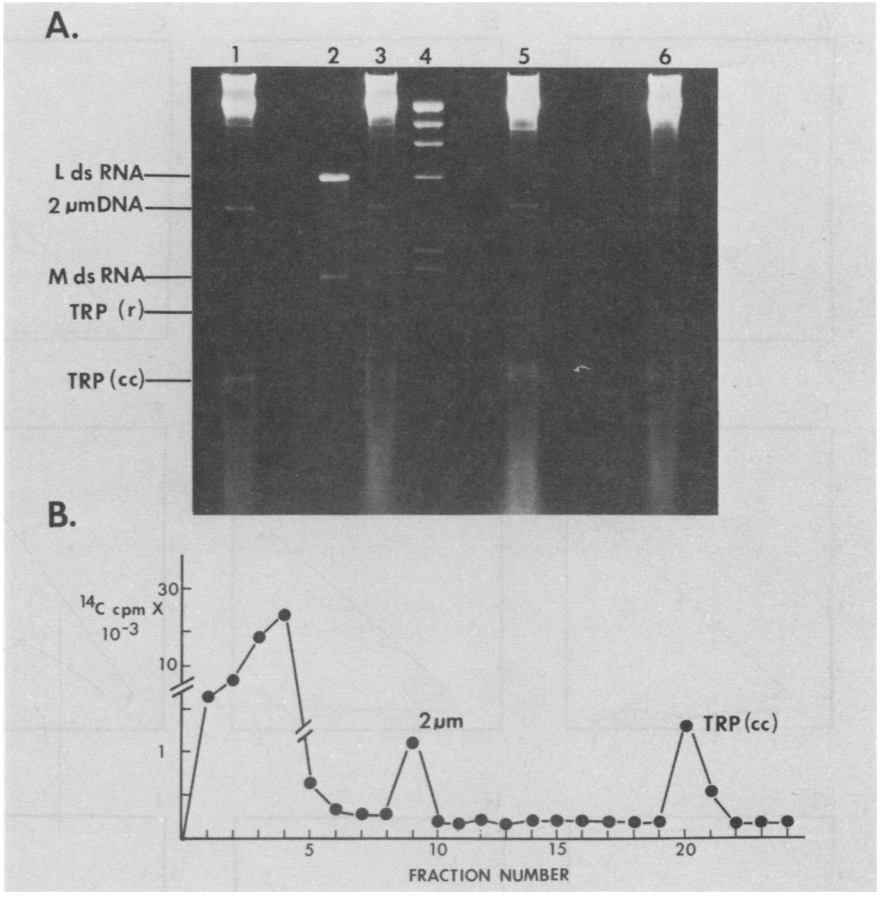

FIG. 3. Purification of plasmid DNAs by agarose gel electrophoresis and transfer to nitrocellulose. A, Ethidium bromide-stained 0.7% agarose gel of nucleic acids extracted from strain JSY334/T (*cdc4*) grown at 25°C (lanes 1 and 5) and 37°C (lanes 3 and 6). Samples were removed at 0.8 generations (lanes 1 and 3) and 1.2 generations (lanes 5 and 6) and extracted as described in the text. Lane 2 contains purified L and M double-stranded RNAs (a gift from R. Wickner), which are multicopy genetic elements found in the cytoplasms of most strains of *S. cerevisiae* (reviewed in reference 7). Both species of double-stranded RNAs are eliminated by RNase treatment before electrophoresis. Lane 4 contains *Hin*dIII-cleaved λ DNA. The positions of covalently closed 2μ plasmid DNA (2μm), relaxed TRP1 RI Circle DNA [Trp(r)], and covalently closed TRP1 RI Circle DNA [TRP(cc)] are indicated. B, Profile of [^{14}C]uracil counts per minute (cpm) after transfer to nitrocellulose. The DNAs in individual wells from agarose gels were transferred to nitrocellulose (40). After transfer of DNA, each strip of nitrocellulose was cut into segments 0.5 cm in length, and the ^{14}C and ^{3}H counts per minute in each segment were determined.

The data in Fig. 4 clearly show that replication of TRP1 RI Circle, like that of chromosomal DNA, is dependent on the products of the *CDC28*, *CDC4*, *CDC7*, and *CDC8* genes: TRP1 RI Circle is replicated at permissive temperatures in all of these *cdc* strains, whereas little or no synthesis of TRP1 RI Circle was detected at restrictive temperatures (Fig. 4B, C, F, G, and H). Synthesis of TRP1 RI Circle was only inhibited at 37°C in *cdc* strains: in the temperature-resistant strain JSY322/T, replication of TRP1 RI Circle occurred equally well at 25 and 37°C (Fig. 4A). In addition, synthesis of TRP1 RI Circle did not occur in cells incubated with α-factor (Fig. 4D). In all experiments, the pattern of replication for 2μ DNA was also monitored and found to be similar to that of TRP1 RI Circle (Fig. 4E and I).

In both *cdc28* strains (JSY487/T and JSY488/T) at 25°C, the copy number of 2μ and TRP1 RI Circle DNAs was lower than in the other *cdc* strains (Table 2). Moreover, during incubation at 38°C (restrictive temperature for *cdc28*), the amount of 2μ and TRP1 RI Circle DNAs recovered per cell decreased. After the equivalent of two generations at 38°C in strain JSY487/T, no

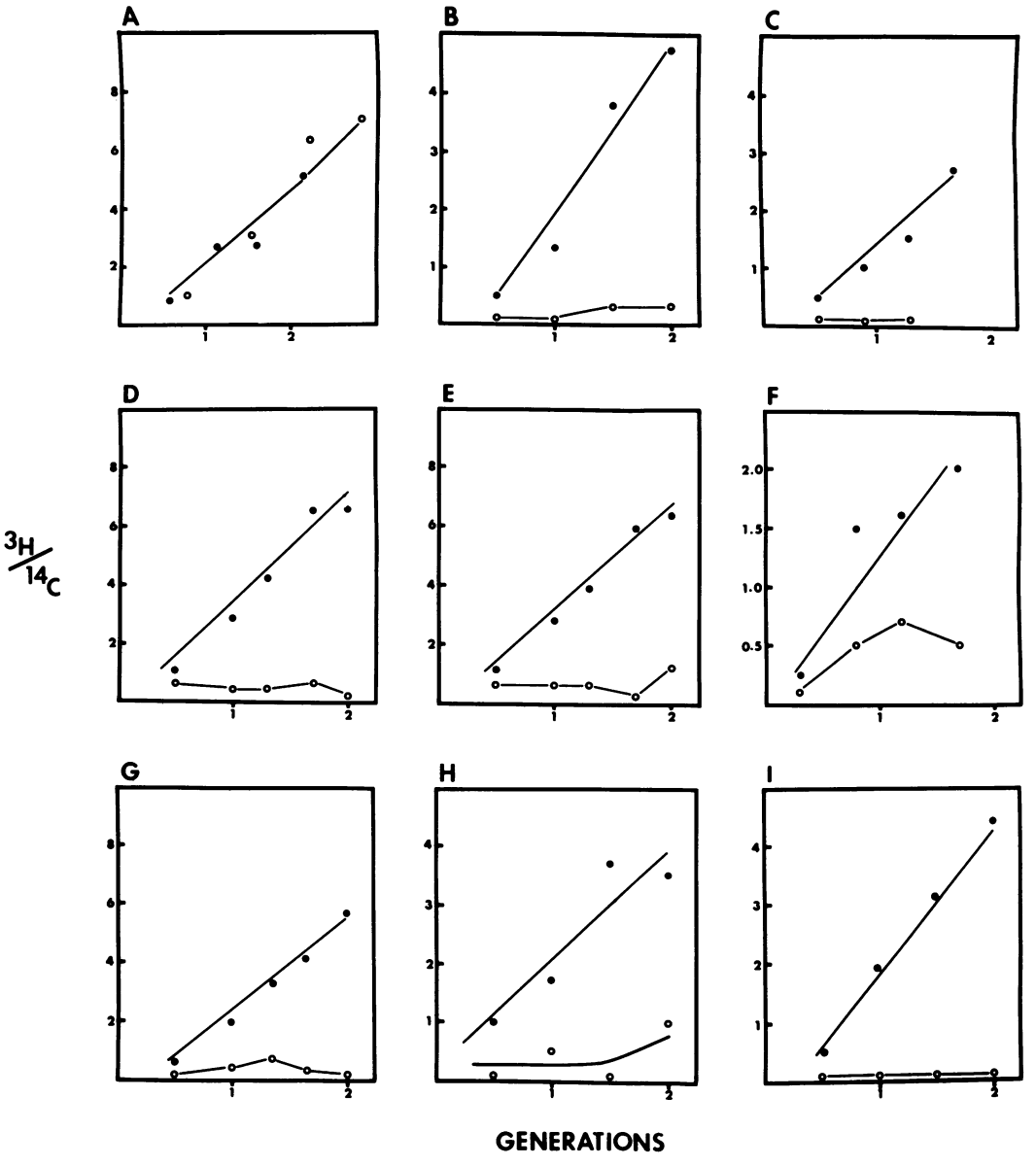

FIG. 4. Replication of TRP1 RI Circle and 2μ DNAs. Cultures were labeled with [^{14}C]uracil for about six generations at 23 to 25°C. For strain JSY334/T (F), [^{3}H]uracil was added directly to the ^{14}C-labeled cells. In all other experiments, cells were transferred to fresh medium containing [^{3}H]uracil. Cultures were divided into two portions; one portion was maintained at 23 to 25°C (permissive temperature), and the other portion was transferred to restrictive conditions (37 or 38°C [A, B, C, F, G, H, and I] or incubation with α-factor [D and E]). The number of cells per milliliter was monitored in the control populations at 25°C (and in strain JSY322/T at 37°C) to determine generation time in an unarrested population. Samples were removed and processed throughout a period corresponding to approximately two generations in the unarrested culture. The plasmid species were purified by agarose gel electrophoresis and transferred to nitrocellulose, and their $^3H/^{14}C$ ratios were determined. The number of [^{14}C]uracil counts per minute ranged from 110 to 7,100 in a purifed nucleic acid species. The panels represent replication of (A) TRP1 RI Circle DNA in JSY322/T (temperature resistant); (B) TRP1 RI Circle DNA in JSY488/T (*cdc28*); (C) TRP1 RI Circle DNA in JSY487/T (*cdc28*); (D) TRP1 RI Circle DNA in RH15e2/T (α-factor); (E) 2μ DNA in RH15e2/T (α-factor); (F) TRP1 RI Circle DNA in JSY334/T (*cdc4*); (G) TRP1 RI Circle DNA in RH15e2/T (*cdc7*); (H) TRP1 RI Circle DNA in JSY542/T (*cdc8*); (I) 2μ DNA in JSY542/T (*cdc8*). Symbols: ●, $^3H/^{14}C$ ratios at 23 to 25°C; ○, $^3H/^{14}C$ ratios under restrictive conditions (37°C for panels A, F, G, H, and I; 38°C for panels B and C; α-factor for panels D and E).

^{14}C counts per minute were detected in the position of TRP1 RI Circle DNA. No detectable peak of ^{14}C counts per minute was seen in the position of 2μ DNA after 2 generations at 38°C for strain JSY488/T and after 1.5 generations for strain JSY487/T. Thus, 2μ and TRP1 RI Circle DNAs not only require the *CDC28* product for replication, but they also appear to be selectively degraded in *cdc28* strains maintained at restrictive temperatures. It is possible that *cdc28-4* strains have low copy numbers of extrachromosomal plasmids because these plasmids are degraded in cells arrested in the G1 phase of the cell cycle by either temperature (as shown here) or by maintenance in stationary-phase cultures.

Chromatin structure. Strains NNY1 and JSY12/T were grown to midlog phase in YMHU (supplemented with 40 μg of tryptophan per ml for NNY1). Spheroplasts were formed, frozen in liquid nitrogen, and stored at −70°C. Spheroplasts were thawed, lysed by suspension in hypotonic buffer, and digested with micrococcal nuclease, and the DNA samples were purified. DNA fragments were separated on agarose gels, transferred to diazotized paper, hybridized with ^{32}P-labeled YRp7 DNA, and autoradiographed (Fig. 5). The nucleosomal DNA fragments derived from the single chromosomal copy of *TRP1-ARS1* DNA are not detected by the exposures used (NNY1; Fig. 5, lanes 1 through 6). Therefore, the DNA fragments generated by micrococcal nuclease digestion and detected by autoradiography after hybridization with YRp7 represent the fragments derived from TRP1 RI Circle. This pattern of fragments was indistinguishable from that detected by ethidium bromide staining of the gel (representing the bulk of yeast chromatin). The average size of the DNA fragments protected after 12 min of digestion with nuclease was 156 base pairs per nucleosome (data not shown), a size compatible with published values (~160 base pairs; 16a). On the longer gel (Fig. 5C and D), the ladder of fragments detected by autoradiography continues to a fragment of the size that would have contained eight nucleosomes, with the full-length linear plasmid band being found in the appropriate position for a fragment which would have contained nine nucleosomes. Therefore, the TRP1 RI Circle is organized into nucleosomes comparable to the bulk of yeast chromatin and contains at least eight and probably nine nucleosomes per mini-chromatin circle.

DISCUSSION

Yeast transformation studies with recombinant DNA plasmids have identified a class of DNA sequences (*ARS*s) which promote high-frequency transformation and extrachromosomal maintenance of plasmid DNAs. From analogy with procaryotic systems, these properties are those expected for sequences which serve as initiation sites for DNA replication. The evidence supporting the hypothesis that *ARS*s function as initiation sites for DNA replication in both recombinant DNA plasmids and in intact DNA molecules includes the following. (i) A specific region from the endogenous yeast plasmid 2μ DNA functions as an *ARS* in transformation studies (6) and also appears to contain the site used most frequently for initiation of DNA replication in intact 2μ DNA (28a). (ii) Sequences capable of high-frequency transformation occur about once in 30 to 40 kb of yeast chromosomal DNA (3, 10), a spacing similar to the spacing of initiation sites detected in small molecules of chromosomal DNA by electron microscopy (29). (iii) A subfragment containing *ARS1* from the 1.45-kb *Eco*RI *TRP1* fragment is used preferentially as a template for DNA synthesis in vitro in comparison with the adjacent subfragment (37). (iv) The region in 2μ DNA which functions as an *ARS* in transformation studies may also be used as an initiation site for DNA replication in vitro (42a).

In this paper we describe the construction of a novel yeast plasmid TRP1 RI Circle (Fig. 1), consisting solely of a 1.45-kb segment derived from yeast chromosomal DNA. This plasmid contains both the *TRP1* gene and a sequence called *ARS1* (41) and is present in 100 to 200 copies per cell. In contrast to YRp7 (a plasmid containing the 1.45-kb *Eco*RI fragment in pBR322), TRP1 RI Circle is stably maintained in the absence of selective pressure during both mitotic and meiotic cell cycles. Indeed, its stability is similar to that of plasmids which contain yeast centromere DNA. However, during meiosis the TRP1 RI Circle usually segregates 4 Trp^+:0 Trp^- to the haploid progeny, whereas plasmids containing centromere DNA segregate predominately 2 Trp^+:2 Trp^- (11). Moreover, a fragment about 10 kb from the TRP1 gene on chromosome 4 which exhibits centromere activity has been identified (Stinchcomb, Mann, and Davis, manuscript in preparation). Thus, the stability of TRP1 RI Circle is not due to its containing a centromere. It is also unlikely that the stability of TRP1 RI Circle can be attributed solely to its high copy number. Other synthetic plasmids exist in comparable numbers per cell but are highly unstable; for example, pXEY26, a synthetic plasmid with a stability similar to that of YRp7 (45), is found in 200 copies per cell in some strains (Zakian and Kupfer, manuscript in preparation). Likewise, the removal of pBR322 is not sufficient to explain the properties of TRP1 RI Circle: when a 10.1-kb fragment containing the *HIS3* gene of yeast is inserted into

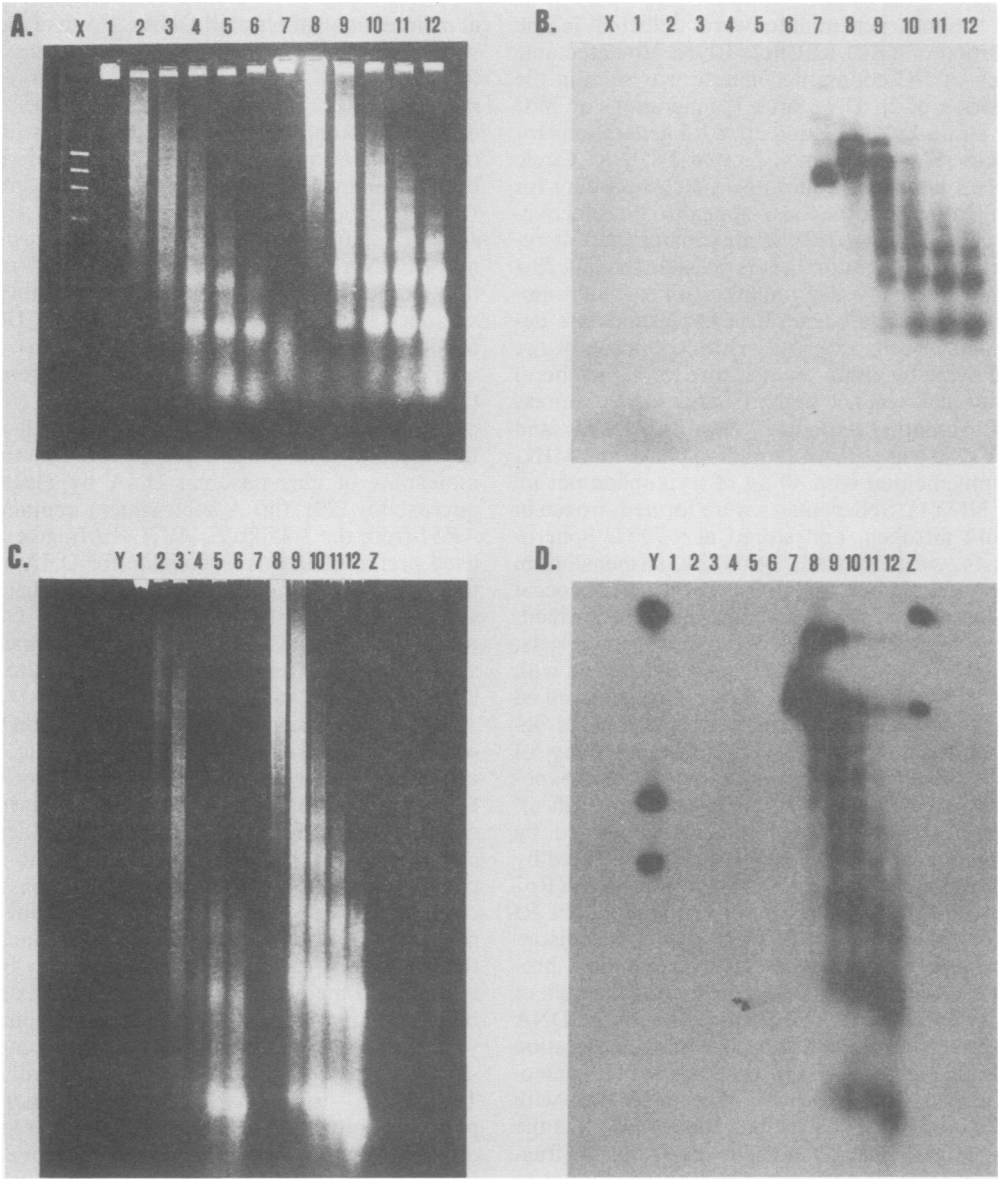

FIG. 5. Chromatin structure of TRP1 RI Circle. A, Ethidium bromide-stained 2% agarose gel run for 5 h at 1.8 V/cm as described in reference 28. Spheroplasts were formed and incubated with micrococcal nuclease as described in the text. Lanes 1 through 6 contain DNA samples from strain NNY1 digested for 0, 3, 6, 12, 24, and 60 min, respectively. Lanes 7 through 12 contain DNA samples from strain JSY12/T digested for 0, 3, 6, 12, 24, and 60 min, respectively. Each lane contains DNA from ~8×10^8 cells. X, *Hae*III-digested ϕ X174; Y, *Eco*RI- and *Hin*dIII-digested YRp7; Z, *Eco*RI-digested YRp7. B, Autoradiograph of the DNA in A after its transfer to diazotized paper and hybridization with ^{32}P-labeled YRp7 DNA. C and D, Same material as in A and B treated in the same manner, except that electrophoresis was for 15 h in TBE buffer (described in the legend to Fig. 2) at 1.25 V/cm.

TRP1 RI Circle, the stability and copy number of the plasmid decrease dramatically (Scott, unpublished results). Therefore, we think it most likely that the stability of TRP1 RI Circle is due to its small size, although the removal of pBR322 and its high copy number may also enhance its stability.

We have also shown that replication of TRP1 RI Circle is under cell cycle control: replication does not occur in cells arrested in the G1 phase

by incubation with α-factor or in the absence of an active *CDC28*, *CDC4*, *CDC7*, or *CDC8* gene product (Fig. 4). These data agree with earlier results which indicated that replication of TRP1 RI Circle DNA requires the *CDC7* gene product and does not occur in the presence of α-factor (Hice and Fangman, unpublished results). The pattern of replication reported here for TRP1 RI Circle, a synthetic plasmid, is identical to that displayed by both yeast chromosomal (19, 20) and 2μ DNAs (26, 32). In contrast, replication of yeast mitochondrial DNA (14, 31) and yeast double-stranded RNAs (47) occurs in cells arrested in the G1 phase of the cell cycle. Moreover, replication of mitochondrial DNA does not require the products of the *CDC28*, *CDC4*, or *CDC7* genes (13, 30).

The dependence of TRP1 RI Circle on the products of *CDC* genes for replication and its failure to replicate in the presence of α-factor can be interpreted to mean that the information required for response to cell cycle control factors is contained within the 1.45-kb *Eco*RI fragment itself. If so, it should be possible to determine the portion of this fragment which responds to these control signals and to isolate mutations in this DNA sequence or secondary mutations in the *cdc* genes (or both) which allow plasmids to replicate under restrictive conditions. Conversely, TRP1 RI Circle may be situated in the nucleus of transformed cells, and this localization may be sufficient to guarantee that the plasmid replicates in a manner identical to that of chromosomal DNA. Thus, the subcellular location of a DNA molecule rather than specific effector sequences on the DNA molecule could determine its replication pattern. In either case, the 1.45-kb TRP1 RI Circle must carry sufficient information either to respond to cell cycle signals or to insure a nuclear location.

In addition we have shown that TRP1 RI Circle is organized into nucleosomes whose size and spacing are indistinguishable from that of nucleosomes in bulk yeast chromatin (Fig. 5). The intracellular form of TRP1 RI Circle probably contains nine nucleosomes, a number sufficient to package fully the DNA molecule. These data suggest either that the transcriptionally active plasmid is covered with nucleosomes or that only a subset of the plasmid molecules are transcriptionally active in each cell.

The replication and structural properties that we have described for TRP1 RI Circle DNA are those expected for an origin of replication derived from yeast chromosomal DNA. These data provide additional circumstantial evidence that sequences capable of promoting high-frequency transformation and extrachromosomal maintenance of recombinant DNA plasmids are acting as initiation sites for DNA replication. These results also indicate that the TRP1 RI Circle, a small, multiple-copy plasmid consisting solely of yeast DNA, organized into nucleosomes, and dependent on the products of *CDC* genes for its replication, will be a tractable template for in vitro studies of chromosomal DNA replication.

ACKNOWLEDGMENTS

We thank R. Hice, S. Scherer, D. Stinchcomb, and K. Struhl for strains and information before publication; M. Lichtenstein for constructing strain JSY65; C. Brajkovich for technical assistance; W. Fangman and H. Weintraub for helpful comments; and H. Devitt for preparing the manuscript.

This work was supported by Public Health Service grants GM27000 (to J.F.S.) and GM26938 (to V.A.Z.) from the National Institutes of Health and by American Cancer Society University of California-Los Angeles grant IN-131 (to J.F.S.).

LITERATURE CITED

1. **Alwine, J. C., D. J. Kemp, B. A. Parker, J. Reiser, J. Renart, G. R. Stark, and G. M. Wahl.** 1979. Detection of specific RNAs or specific fragments of DNA by fractionation in gels and transfer to diazobenzyloxymethyl paper. Methods Enzymol. **68**:220–242.
2. **Bazarel, M., and D. R. Helinski.** 1968. Circular DNA forms of colicinogenic factors E1, E2, and E3 from *Escherichia coli*. J. Mol. Biol. **36**:185–194.
3. **Beach, D., M. Piper, and S. Shall.** 1980. Isolation of chromosomal origins of replication in yeast. Nature (London) **284**:185–187.
4. **Beggs, J. D.** 1978. Transformation of yeast by a replicating hybrid plasmid. Nature (London) **275**:104–109.
5. **Bisson, L., and J. Thorner.** 1977. Thymidine 5'-monophosphate-requiring mutants of *Saccharomyces cerevisiae* are deficient in thymidylate synthetase. J. Bacteriol. **132**:44–50.
6. **Broach, J. R., and J. B. Hicks.** 1980. Replication and recombination functions associated with the yeast plasmid, 2μm circle. Cell **21**:501–508.
7. **Bruenn, J. A.** 1980. Virus-like particles of yeast. Annu. Rev. Microbiol. **34**:49–68.
8. **Bucking-Throm, E., W. Düntze, L. H. Hartwell, and T. R. Manney.** 1973. Reversible arrest of haploid yeast cells at the initiation of DNA synthesis by a diffusible sex factor. Exp. Cell Res. **76**:99–110.
9. **Caron, F., C. Jacq, and J. Rouviere-Yaniv.** 1979. Characterization of a histone-like protein extracted from yeast mitochondria. Proc. Natl. Acad. Sci. U.S.A. **76**:4265–4269.
10. **Chan, C. S. M., and B.-K. Tye.** 1980. The chromosomal replication origins in *Saccharomyces cerevisiae*. Proc. Natl. Acad. Sci. U.S.A. **77**:6329–6333.
11. **Clarke, L., and J. Carbon.** 1980. Isolation of a yeast centromere and construction of functional small circular chromosomes. Nature (London) **287**:504–509.
12. **Clark-Walker, G. D., and G. L. G. Miklos.** 1974. Localization and quantification of circular DNA in yeast. Eur. J. Biochem. **41**:359–365.
13. **Cottrell, S., M. Rabinowitz, and G. S. Getz.** 1973. Mitochondrial deoxyribonucleic acid synthesis in a temperature-sensitive mutant of deoxyribonucleic acid replication of *Saccharomyces cerevisiae*. Biochemistry **12**:4374–4378.
14. **Cryer, D. R., C. D. Goldthwaite, S. Zinker, K. B. Lam, E. Storm, R. Hirschberg, J. Blamire, D. B. Finkelstein, and J. Marmur.** 1974. Studies on nuclear and mitochondrial DNA of *Saccharomyces cerevisiae*. Cold Spring Harbor Symp. Quant. Biol. **38**:17–29.
15. **Davis, R. W., M. Thomas, J. Cameron, T. St. John, S. Scherer, and R. A. Padgett.** 1980. Rapid DNA isolations

for enzymatic and hybridization analysis. Methods Enzymol. 65:404–411.
16. **Düntze, W., D. Stotzler, E. Bucking-Throm, and S. Kalbitzer.** 1973. Purification and partial characterization of α-factor, a mating type specific inhibitor of cell reproduction from *Saccharomyces cerevisiae*. Eur. J. Biochem. 35:357–365.
16a. **Fangman, W. L., and V. A. Zakian.** 1982. Genome structure and replication, p. 27–58. *In* J. Broach, E. Jones, and J. Strathern (ed.), The molecular biology of the yeast *Saccharomyces cerevisiae*. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
17. **Hartley, J. L., and J. E. Donelson.** 1980. Nucleotide sequence of the yeast plasmid. Nature (London) 286:860–864.
18. **Hartwell, L. H.** 1973. Three additional genes required for deoxyribonucleic acid synthesis in *Saccharomyces cerevisiae*. J. Bacteriol. 115:966–974.
19. **Hartwell, L. H.** 1976. Sequential function of gene products relative to DNA synthesis in the yeast cell cycle. J. Mol. Biol. 104:803–817.
20. **Hereford, L. M., and L. H. Hartwell.** 1974. Sequential gene function in the initiation of *Saccharomyces cerevisiae* DNA synthesis. J. Mol. Biol. 84:445–461.
21. **Hinnen, A., J. B. Hicks, and G. R. Fink.** 1978. Transformation of yeast. Proc. Natl. Acad. Sci. U.S.A. 75:1929–1933.
22. **Hsiao, C.-L., and J. Carbon.** 1979. High frequency transformation of yeast by plasmids containing the cloned *ARG4* gene. Proc. Natl. Acad. Sci. U.S.A. 76:3829–3833.
23. **Katz, L., D. T. Kingsbury, and D. R. Helinski.** 1973. Stimulation by cyclic adenosine monophosphate of plasmid deoxyribonucleic acid replication and catabolite repression of the plasmid deoxyribonucleic acid-protein relaxation complex. J. Bacteriol. 114:577–591.
24. **Kingsman, A. J., L. Clarke, R. K. Mortimer, and J. Carbon.** 1979. Replication in *Saccharomyces cerevisiae* of plasmid pBR313 carrying DNA from the yeast *trp*-1 region. Gene 7:141–152.
25. **Livingston, D. M., and S. Hahne.** 1979. Isolation of a condensed, intracellular form of the 2μm DNA plasmid of *Saccharomyces cerevisiae*. Proc. Natl. Acad. Sci. U.S.A. 76:3727–3731.
26. **Livingston, D. M., and D. M. Kupfer.** 1977. Control of *Saccharomyces cerevisiae* 2μm DNA replication by cell division cycle genes that control nuclear DNA replication. J. Mol. Biol. 116:249–260.
27. **Lohr, D., and K. E. Van Holde.** 1975. Yeast chromatin subunit structure. Science 188:165–166.
28. **Nelson, R. G., and W. L. Fangman.** 1979. Nucleosome organization of the yeast 2μm DNA plasmid: a eukaryotic minichromosome. Proc. Natl. Acad. Sci. U.S.A. 76:6515–6519.
28a. **Newlon, C. S., R. J. Devenish, P. A. Suci, and C. J. Roffis.** 1981. Replication origins used in vivo in yeast. ICN-UCLA Symp. Mol. Cell. Biol. 21:501–516.
29. **Newlon, C. S., and W. Burke.** 1980. Replication of small chromosomal DNAs in yeast. ICN-UCLA Symp. Mol. Cell. Biol. 19:399–409.
30. **Newlon, C. S., and W. L. Fangman.** 1975. Mitochondrial DNA synthesis in cell cycle mutants of *Saccharomyces cerevisiae*. Cell 5:423–428.
31. **Petes, T. D., and W. L. Fangman.** 1973. Preferential synthesis of yeast mitochondrial DNA in α-factor arrested cells. Biochem. Biophys. Res. Commun. 55:603–609.
32. **Petes, T. D., and D. H. Williamson.** 1975. Replicating circular DNA molecules in yeast. Cell 4:249–253.
33. **Potter, D. A., J. M. Fostel, M. Berninger, M. L. Pardue, and T. R. Cech.** 1980. DNA-protein interactions in the *Drosophila melanogaster* mitochondrial genome as deduced from trimethylpsoralen crosslinking patterns. Proc. Natl. Acad. Sci. U.S.A. 77:4118–4122.
34. **Rigby, P. W. J., M. Dieckmann, C. Rhodes, and P. Berg.** 1977. Labeling deoxyribonucleic acid to high specific activity *in vitro* by nick translation with DNA polymerase I. J. Mol. Biol. 113:737–751.
35. **Rivin, C. J., and W. L. Fangman.** 1980. Cell cycle phase expansion in nitrogen-limited cultures of *Saccharomyces cerevisiae*. J. Cell Biol. 85:96–107.
36. **Scherer, S., and R. W. Davis.** 1979. Replacement of chromosome segments with altered DNA sequences constructed *in vitro*. Proc. Natl. Acad. Sci. U.S.A. 76:4951–4955.
37. **Scott, J. F.** 1980. Preferential utilization of a yeast chromosomal replication origin as template for enzymatic DNA synthesis. ICN-UCLA Symp. Mol. Cell. Biol. 19:379–388.
38. **Seligy, V. L., D. Y. Thomas, and B. L. A. Miki.** 1980. *Saccharomyces cerevisiae* plasmid, Scp or 2μm: intracellular distribution, stability, and nucleosomal-like packaging. Nucleic Acids Res. 8:3371–3391.
39. **Sherman, F., G. R. Fink, and C. W. Lawrence.** 1979. Methods in yeast genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
40. **Southern, E.** 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. 93:503–517.
41. **Stinchcomb, D. T., K. Struhl, and R. W. Davis.** 1979. Isolation and characterization of a yeast chromosomal replicator. Nature (London) 282:39–43.
42. **Struhl, K., D. T. Stinchcomb, S. Scherer, and R. W. Davis.** 1979. High-frequency transformation of yeast: autonomous replication of hybrid DNA molecules. Proc. Natl. Acad. Sci. U.S.A. 76:1035–1039.
42a. **Sugino, A., H. Kojo, B. D. Greenberg, P. O. Brown, and K. C. Kim.** 1981. In vitro replication of yeast 2μm plasmid DNA. ICN-UCLA Symp. Mol. Cell. Biol. 21:529–553.
43. **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.
44. **Wahl, G. M., M. Stern, and G. R. Stark.** 1979. Efficient transfer of large DNA fragments from agarose gels to diazobenzyloxy-methyl-paper and rapid hybridization by using dextran sulfate. Proc. Natl. Acad. Sci. U.S.A. 76:3683–3687.
45. **Zakian, V. A.** 1981. Origin of replication from *Xenopus laevis* mitochondrial DNA promotes high-frequency transformation of yeast. Proc. Natl. Acad. Sci. U.S.A. 78:3128–3132.
46. **Zakian, V. A., B. J. Brewer, and W. L. Fangman.** 1979. Replication of each copy of yeast 2 micron DNA plasmid occurs during the S-phase. Cell 17:923–934.
47. **Zakian, V. A., D. W. Wagner, and W. L. Fangman.** 1981. Yeast L double-stranded RNA is synthesized during the G1 phase but not the S phase of the cell cycle. Mol. Cell. Biol. 1:673–679.