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Genetics

Characterization of 2-μm DNA of *Saccharomyces cerevisiae* by restriction fragment analysis and integration in an *Escherichia coli* plasmid

(cytoplasmic DNA/episomal DNA/reversed duplication/heteroduplex mapping)

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ABSTRACT Electrophoretic analysis of *Eco*RI and *Hin*dIII restriction fragments of 2-μm supercoiled DNA of *Saccharomyces cerevisiae* indicated that this class of DNA is heterogeneous and probably consists of two types of molecules. Integration of the 2-μm yeast DNA in *E. coli* plasmid pCR1 directly showed the existence of two types of molecules, as each of these could be individually inserted into separate bacterial plasmids. The difference between the two types of 2-μm circles is due to an inversion of about 1.6×10^6 daltons. The inversion is flanked by a reversed duplicated sequence of 0.45×10^6 daltons. Possible implications of this structure are discussed.

Saccharomyces cerevisiae contains a class of closed circular duplex DNA molecules having a monomeric circumference of 2 μm and exhibiting the density of nuclear DNA (1, 2). This circular DNA, located outside the mitochondria (2) and nucleus (3), may be associated with a membrane fraction (4), and is here designated as episomal DNA. Analysis of the renaturation kinetics has shown that all 2-μm molecules have essentially the same base sequence without detectable repeated sequences (5).

In this paper we report the integration of yeast episomal DNA in the *Escherichia coli* plasmid pCR1, which carries a gene conferring resistance to kanamycin. This has allowed us to separate and analyze two types of 2-μm DNA molecules that are identical in size and density, but produce different restriction fragments upon digestion with *Eco*RI or *Hin*dIII endonuclease.

MATERIALS AND METHODS

Strains and DNA Preparations. *E. coli* 490 ($recA^-$, r_k^-, m_k^-) was obtained from G. Hobom. The pCR1 kanamycin resistance plasmid was isolated as closed circular DNA (6) from *E. coli* C600 transformed with this plasmid, after exposure of the cells to chloramphenicol (7). Yeast episomal DNA was isolated from *S. cerevisiae* H1 as described (2) by fractionation of protoplast lysates in two successive CsCl–ethidium bromide equilibrium gradients. Ethidium was removed with Dowex 50 WX8 H^+ at pH 7.

Enzyme Reactions. The *Eco*RI restriction endonuclease was purified and assayed as described by Green *et al.* (8); purification was stopped after the DEAE-cellulose step. T4 DNA ligase and *Hin*dIII endonuclease were purchased from Miles Labs Ltd. Samples were incubated with *Hin*dIII endonuclease in 50 mM NaCl, 6 mM Tris·HCl (pH 7.5), 6 mM $MgCl_2$, and 0.01% bovine serum albumin for 1 hr at 37°. *Eco*RI-digested pCR1 (0.25 μg) and 2 μg of a limited *Eco*RI digest of yeast episomal DNA (80% linear DNA of 2-μm length) were ligated in 100 μl (9). The reaction was followed by gel electrophoresis. After completion (24 hr) the ligation mixture was dialyzed extensively (10), and used for transformation of *E. coli* 490.

Transformation and Selection were performed according to Cohen *et al.* (10), except that the cells were grown in enriched P medium (11), supplemented with L-methionine (20 μg/ml), thymine (2 μg/ml), and 0.1 volume of L-broth (12). Kanamycin-resistant colonies were screened for integrated yeast episomal DNA by colony hybridization (13). We simplified this procedure by floating the colony filter (after wetting the edge with liquid paraffin) on the solutions used for lysis and fixation. The colonies were hybridized with [^{3}H]RNA synthesized with *E. coli* RNA polymerase (holoenzyme, kindly provided by H. Blüthmann) and yeast episomal DNA as template under the conditions described (14). The washed and ribonuclease-treated filters (13) were impregnated with a solution of 10% 2,5-diphenyloxazole (PPO) in ether (15) (100 μl/25-mm filter). Flash activated Kodak Royal RPS 54 x-ray film was exposed to the filter for 4 days at −70 °. Positive colonies were grown up for isolation and analysis of plasmid DNA.

Electron Microscopy. DNA was recovered from gel bands with the "freeze and squeeze" method (16). DNA samples were spread from 50% formamide (17) onto distilled water, using a modified microdrop spreading procedure (18). Heteroduplexes were prepared and spread as described (17). Molecules were measured with a Hewlett-Packard digitizer connected to a Hewlett-Packard model 9830 A computer.

RESULTS

Gel analysis of intact yeast episomal DNA

Closed circular episomal DNA, purified via two sequential CsCl-ethidium bromide gradients, was analyzed on agarose gels (19). All preparations contained the main bands, 3 and 5, visible in Fig. 1b, that represent, respectively, the supercoiled and relaxed form of the 2-μm DNA circle. The size of the open circular molecules, measured with phage PM2 relaxed circles (3.1 μm, ref. 20) as standard, was 2.0 μm, in agreement with reported values of 1.88–2.2 μm (1–3, 21, 22). A thin band migrated just ahead of the open circular band in most gels of episomal DNA (Fig. 1b, band 4). In electron micrographs this band was shown to consist of supercoiled DNA of about 4 μm in length. Most preparations contained the minor bands 1 and 2 (Fig. 1b), whose positions suggest that they are the relaxed form of the 4-μm molecule and the supercoiled form of the 6-μm circular DNA class, respectively. Both 4-μm and 6-μm circles have been previously observed in episomal DNA (4, 21, 22).

The migration rate of closed circular DNAs in 0.5% agarose plus ethidium bromide (100 μg/ml) has been shown to be inversely proportional to the logarithm of the molecular weight (19). We have used this gel system to measure the molecular weight of supercoiled 2-μm DNA with pCR1 DNA, phage PM2 DNA, and ColE1 DNA as markers (Fig. 1 h and i). The intact 2-μm DNA migrated slightly faster than the supercoiled ColE1 DNA, corresponding to a molecular weight of 4.1×10^6. This

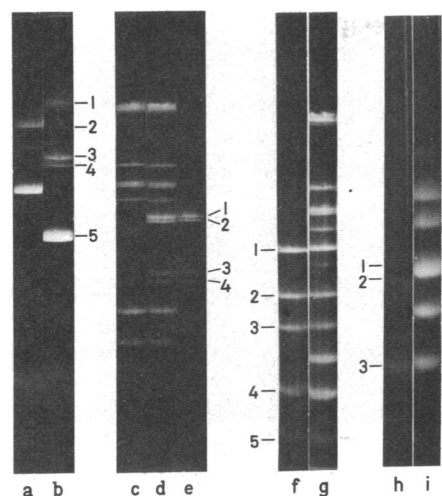

FIG. 1. Agarose gel analysis of yeast episomal DNA and marker DNAs. Electrophoresis was carried out in agarose tube gels (5 × 90 mm) at constant voltage of 3 V/cm at 18° and continued until the bromophenol blue marker left the gel. Gels a–g contained 0.7% agarose, 0.5 μg of ethidium bromide per ml, and buffer (19). Gels h and i contained 0.5% agarose and 100 μg of ethidium bromide (19). Gels were photographed on Polaroid type 55 P/N film, with 366 nm light and a yellow and red filter. Bands on different gels are numbered arbitrarily. (a) Phage PM2 DNA; (b) yeast episomal DNA; (c) *Eco*RI digest of λDNA and *Bsu* digest of λdvl DNA; (d) as in gel e plus markers as in gel c; (e) *Eco*RI digest of yeast episomal DNA; (f) *Hin*dIII digest of yeast episomal DNA; (g) as in gel f plus markers as in gel c; (h) yeast episomal DNA showing supercoiled 2-μm DNA (band 3), open circular 2-μm DNA (band 1), and supercoiled 4-μm DNA (band 2); (i) yeast episomal DNA plus markers ColE1 DNA (4.2 × 10^6, ref. 23), PM2 DNA (6 × 10^6), and pCR1 DNA (8.5 × 10^6).

value is in agreement with the 2.0-μm length measured in electron micrographs. The presumptive closed circular dimer of 4-μm length ran at the leading edge of the pCR1 supercoil band, which indicates a molecular weight of 8.2 × 10^6.

Restriction map of episomal DNA

Yeast episomal DNA was incubated with *Eco*RI endonuclease and the products were examined by agarose gel electrophoresis. Fig. 1d and e show that the episomal DNA digest contained four DNA fragments that were resistant to further digestion by *Eco*RI. The molecular weights of the fragments were: 2.45 × 10^6, 2.35 × 10^6, 1.55 × 10^6, and 1.45 × 10^6, as estimated from their positions relative to internal markers (Fig. 2). The total molecular weight of about 8 × 10^6 suggested the existence of two types of 2-μm DNA molecules, referred to as type 14 and type 23, each giving rise to two *Eco*RI fragments (fragments 1 + 4 and 2 + 3, respectively).

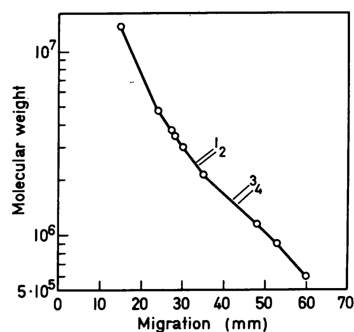

FIG. 2. Electrophoretic mobility of the *Eco*RI fragments of yeast episomal DNA electrophoresed in Fig. 1d. Molecular weights of *Eco*RI fragments of λDNA: 13.7, 4.74, 3.73, 3.48, 3.02, and 2.13 × 10^6 (24) and *Bsu* fragments of λdvl: 1.15, 0.9, and 0.6 × 10^6 (25) present as internal markers.

Gel electrophoresis of *Eco*RI digests of preparations containing 10% 4-μm supercoiled DNA and the other minor bands shown in Fig. 1b did not reveal any extra DNA fragment bands, which suggests that the class of closed circular molecules of 4-μm length are multimers of the 2-μm molecules.

The *Hin*dIII endonuclease digest of yeast episomal DNA contained five fragments (Fig. 1f and g) of the following molecular weights: 2.48, 1.77, 1.44, 0.9, and 0.6 × 10^6. The total molecular weight was approximately 7.2 × 10^6, which is 0.8 × 10^6 less than the 8 × 10^6 measured for the *Eco*RI fragments. This difference can be explained by the occurrence of the 0.9 × 10^6 fragment in both types of 2-μm circles, which is indicated by the relatively higher quantity of DNA in the 0.9 × 10^6 fragment band.

The locations of the restriction sites indicated in Fig. 3 have been derived from digestion of isolated *Eco*RI fragments by *Hin*dIII and from double digestion of 2-μm DNA by both *Hin*dIII and *Eco*RI (Table 1). Comparison of *Hin*dIII fragments with those of the double digest (Table 1) shows that the *Eco*RI sites are located in close proximity to *Hin*dIII sites. Definite orientation, however, cannot be derived from these data, but can be concluded from restriction fragments of recombinant DNA molecules analyzed below.

The different sizes of restriction fragments from two circles of presumably identical base sequence as concluded from the kinetic complexity (5), can be accounted for by postulating an inversion of at least 1.8 × 10^6 that contains two *Hin*dIII sites (H1 and H2) and one *Eco*RI site (RIA), as visualized in Fig. 3.

Table 1. Molecular weights × 10^{-6} of restriction fragments of 2-μm DNA

Type of 2-μm DNA	*Eco*RI digest: Fragment no.*	*Eco*RI digest: M_r	*Eco*RI digest: M_r after *Hin*dIII cleavage	*Hin*dIII digest: M_r	*Hin*dIII digest: Map position*	*Eco*RI + *Hin*dIII digest (M_r)
14	1	2.45 →	1.70 + 0.90	1.77	H2-H3	1.70
	4	1.45 →	1.40	1.44	H1-H3	1.40
				0.90	H1-H2	0.90
23	2	2.35 →	2.35	2.48	H1-H3	2.35
	3	1.55 →	0.90 + 0.60	0.90	H1-H2	0.90
				0.60	H2-H3	0.60

M_r = molecular weight.
* See Fig. 4.

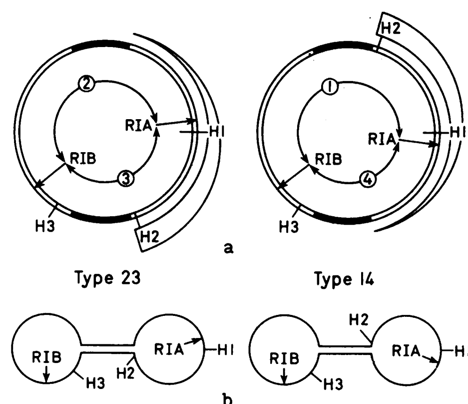

FIG. 3. (a) Schematic outline of double-stranded yeast 2-μm DNA types 14 and 23. The inversion that differentiates between types 14 and 23 is indicated with the half crescent. The duplication sites are black. Numbers 1–4 refer to the *Eco*RI fragments; RIA and RIB refer to *Eco*RI sites; H1, H2, and H3 refer to *Hin*dIII sites. (b) Schemes of reannealed single-stranded yeast 2-μm DNA.

A similar model for yeast 2-μm DNA was presented by M. Guerineau at the 10th FEBS Meeting, Paris, 1975.

Electron microscopy of reannealed 2-μm DNA and its *Eco*RI fragments

To visualize the postulated inversion depicted in Fig. 3, we denatured and renatured a 2-μm DNA preparation. Most molecules that contained single- and double-stranded regions, however, were dumb-bell structures with two single-stranded loops of approximately 0.80 μm and a double-stranded stem of 0.23 μm (Fig. 4a, schematically drawn in Fig. 3). Apparently the single-stranded molecules contain a reversed noncontiguous duplication which renatures fast. The single-stranded loops then behave like closed circles and thus cannot renature with complementary loops of other molecules. This made heteroduplex formation between types 14 and 23 rare. The dumb-bells with long stem (1.4 μm, Fig. 4b) must arise from 4-μm molecules, since these structures occur frequently in reannealed 4-μm DNA, a preparation that, in addition, contained structures expected for homo- and heterodimers of 2-μm molecules in tandem or head-to-head arrangement, most probably generated by recombination (H. D. Royer, unpublished observations).

None of the isolated *Eco*RI fragments, when renatured, gave rise to loop formation, which indicated that the duplicated sequences are in separate *Eco*RI fragments and the RI sites are located in the loops. To determine the size of the inversion we, therefore, made heteroduplexes between *Eco*RI fragments 1 and 2 (Fig. 4c). These heteroduplexes are double-stranded at one end (RIB site) and have two single strands at the other end terminated at the RIA site. The lengths of the single strands were approximately 0.45 and 0.40 μm, and that of the double strand was 0.84 μm (means from 10 heteroduplexes). We can, therefore, conclude that the inversion encompasses one total loop and that the RIA site is located close to the middle of this loop as drawn in Fig. 3.

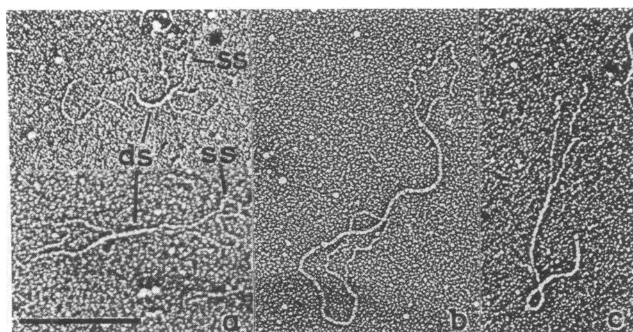

FIG. 4. (a) Self-annealed 2-μm molecule. Two single-stranded (ss) loops are connected with a double-stranded (ds) stem. (b) Self-annealed 4-μm dimer. (c) Heteroduplex of *Eco*RI fragments 1 and 2. Bar is 0.5 μm.

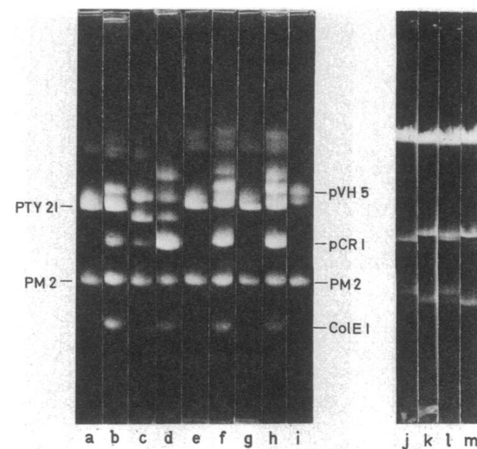

FIG. 5. Agarose gel electrophoresis of recombinant plasmid DNAs containing yeast 2-μm DNA. Experimental conditions were as described for Fig. 1h and i. (a) PTY21 and PM2 DNA. (b) PTY21 plus markers (molecular weights in parentheses): ColE1 (4.2×10^6), PM2 (6×10^6), pCR1 (8.5×10^6), pVH5 DNA (ColE1-*trp*, 14.8×10^6, ref. 26). (c) PTY30 and PM2 DNA. (d) PTY30 plus markers as in gel b. (e) PTY36 and PM2 DNA. (f) PTY36 plus markers as in gel b. (g) PTY39 plus PM2 DNA. (h) PTY39 plus markers as in gel b. (i) PM2 plus pVH5 DNA. (j–m) Agarose gel electrophoresis of *Eco*RI digests of recombinant plasmid DNA. Conditions were the same as in Fig. 1a. (j) PTY21. (k) PTY36. (l) PTY21 plus PTY36. (m) PTY39.

Construction and characterization of recombinant plasmids

Plasmid pCR1, which is a derivative of the ColE1-*kan* plasmid pML2 (26) in which one of the two *Eco*RI sites has been deleted, was joined to yeast 2-μm DNA as described in *Materials and Methods*. *E. coli* 490 was transformed with the dialyzed ligation mixture by the procedure described by Cohen *et al.* (10). The cells were transformed with a frequency of 3×10^{-5}, as calculated from the numbers of kanamycin-resistant cells and total viable cells present after 90 min of incubation in L-broth. About 7×10^4 transformed bacteria were obtained per μg of DNA, a yield comparable to that obtained with closed circular R-factor DNA (10). Colony hybridization (13), with [^{3}H]RNA synthesized on episomal DNA, showed that about 20% of the transformants contained yeast episomal DNA.

Plasmid DNAs from five transformants containing yeast episomal DNA (PTY9, 21, 30, 36, and 39) were isolated as closed circular DNA (6, 7) with yields of 0.5–1 mg of DNA per g of bacteria (wet weight), indicating that the integrated episomal DNA did not interfere severely with plasmid replication. The molecular weights were determined by gel electrophoresis (Fig. 5). Three of the analyzed plasmids, PTY21, PTY36, and PTY39, had a molecular weight of about 12×10^6, which is approximately the molecular weight expected for plasmid pCR1 (8.5×10^6) plus the 2-μm yeast molecule.

On *Eco*RI digestion all plasmids yielded a fragment of 8.5×10^6, the molecular weight of linear pCR1 DNA. In addition plasmid PTY21 yielded two DNA fragments with electrophoretic mobilities corresponding to those of *Eco*RI fragments 2 and 3 of yeast 2-μm DNA, whereas PTY36 and PTY39 both

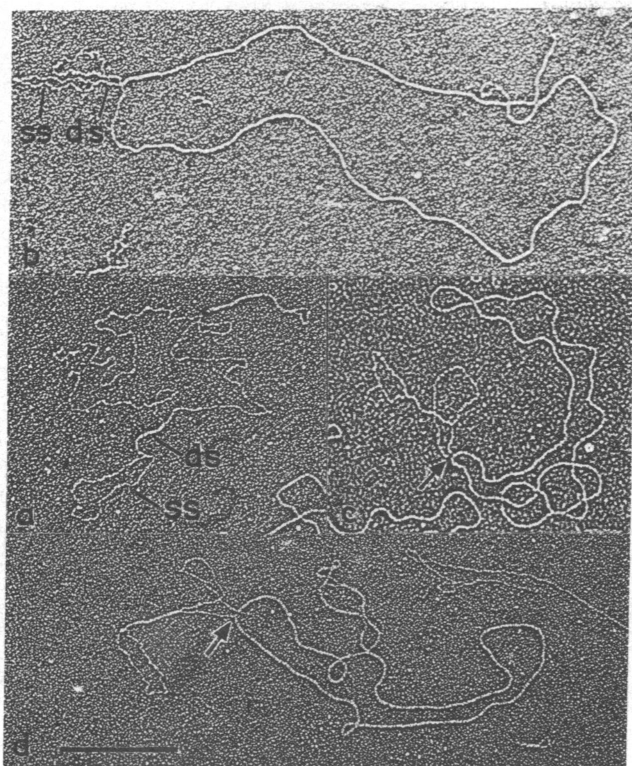

FIG. 6. Homo- and heteroduplexes of PTY plasmids. (a) Single-stranded PTY21 with double-stranded (ds) yeast stem and single-stranded (ss) loop. The palindrome with small loop present in pCR1 is also visible. (b) Homoduplex of PTY21 with ss loops and ds stems from both strands hanging out (see *text* for explanation). (c) Heteroduplex pCR1/PTY21, complete yeast 2-μm strand hangs out in typical dumb-bell structure. Arrow shows connection point. (d) Heteroduplex pCR1/PTY39 as in panel c. Bar is 0.5 μm.

produced two DNA fragments corresponding to *Eco*RI fragments 1 and 4 (Fig. 5j–m). These data provide definite evidence that fragments 2 and 3 and fragments 1 and 4 are part of two different molecules of 2-μm yeast DNA which can be separately integrated into plasmid DNA. PTY9 and PTY30 both contained *Eco*RI fragments 2, 3, and 4. PTY30 DNA consisted of molecules of molecular weight 13.3×10^6, 10.8×10^6, and 8.5×10^6. Upon recloning of PTY9 and PTY30, a homogeneous plasmid DNA population was isolated with a molecular weight of 13.5 $\times 10^6$ and 10×10^6, respectively. PTY9 and PTY30 most likely represented unstable transformants, although improper cloning cannot be excluded.

Because of the peculiar structure of the yeast episomal DNA, its presence in recombinant plasmids could be demonstrated directly in electron micrographs after the plasmid DNA was denatured and reannealed. In Fig. 6 a single-stranded PTY21 with the double-stranded stem and single-stranded loop of yeast 2-μm DNA can be seen. If two complementary strands have renatured (Fig. 6b), the double-stranded stems in the single strands prevent the renaturation of the complementary loops. All stems had about the same length, indicating that branch migration did not occur under these conditions. Without branch migration the loops cannot renature because of the closed circular character they have acquired.

Analysis of heteroduplexes formed between the PTY plasmids and pCR1 revealed the *Eco*RI site on the 2-μm circle used for integration. The three PTY plasmids analyzed, 21, 36, and 39, were all joined at the RIB site, located close (0.12 μm) to the stem (Fig. 6c and d).

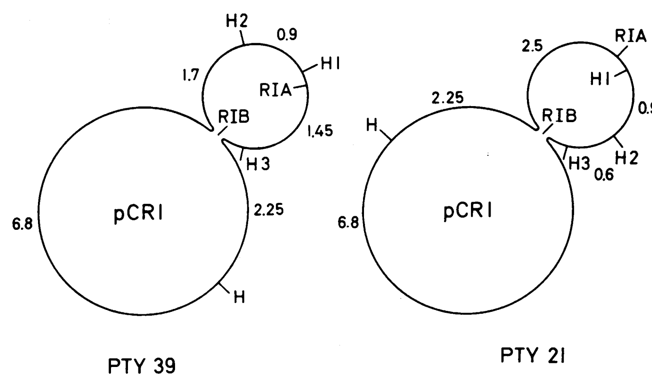

FIG. 7. Locations of restriction sites on PTY21 and PTY39. The *Hin*dIII fragment sizes are indicated in daltons $\times 10^{-6}$.

The orientation of the integrated molecules could be derived from the *Hin*dIII fragments. The single *Hin*dIII and *Eco*RI site on pCR1 are about 2.25×10^6 daltons apart. Both PTY36 and 39, which have integrated type 14, yielded, respectively, fragments of 6.8×10^6 and 8.5×10^6 as largest *Hin*dIII fragments. The 6.8×10^6 fragment had no *Eco*RI site, whereas the 8.5×10^6 fragment was cut by *Eco*RI into two molecules of 6.8 $\times 10^6$ and 1.70×10^6 (see Table 2 for fragment molecular weights of PTY21 and 39). These data are in agreement with the scheme for PTY39 in Fig. 7, and show that H3 has to be located at the RIA side of RIB. The type 14 elements in PTY36 and 39 have opposite orientations. PTY21 yielded *Hin*dIII fragments of 6.8×10^6 (no *Eco*RI site) and 4.75×10^6, cut by *Eco*RI into 2.25×10^6 and 2.50×10^6 fragments. The orientation, therefore, must be as depicted in Fig. 7. The distances H1–RIA and H3–RIB are approximately equal and appear to be about 0.07×10^6 (Table 2).

DISCUSSION

The efficient integration of 2-μm yeast DNA in plasmid pCR1 and the normal yields of PTY plasmid DNA after relaxed replication show that yeast episomal DNA can be replicated in *E. coli* at normal rates, if inserted into an *E. coli* plasmid. This replication appears to be faithful, since no difference could be observed between *Eco*RI and *Hin*dIII restriction fragments of 2-μm DNA obtained from relaxed replicated PTY plasmids and those fragments of 2-μm DNA that had been isolated directly from the yeast cell.

Most PTY plasmids yielded only two of the four *Eco*RI fragments present in the digest of a preparation of yeast epi-

Table 2. Molecular weights $\times 10^{-6}$ of restriction fragments of PTY21 and PTY39

Plasmid	*Hin*dIII	*Eco*RI cleavage of single *Hin*dIII fragment
PTY21	6.8	→ 6.8
	4.75	→ 2.50 + 2.25
	0.9	→ 0.9
	0.6	→ 0.6
PTY39	8.5	→ 6.8 + 1.70
	2.35	→ 2.30
	1.45	→ 1.37
	0.9	→ 0.9
pCR1	8.5	→ 6.8 + 2.25

somal DNA. This shows clearly that two forms, types 14 and 23, occur *in vivo*. The size of the inversion that differentiates between types 14 and 23 could be deduced from the *Hin*dIII fragments and visualized in heteroduplexes between *Eco*RI fragments 1 and 2. In these types of heteroduplexes, the inverted region was about 0.8 μm long. This length is about equal to the length of one single-stranded loop in the dumb-bell structure formed in a reannealed single-stranded 2-μm molecule. Most likely, therefore, the inversion encompasses the whole loop and could be generated by a recombination event between the sequences of the reversed duplication in one molecule. A minimum inversion size of 1.8×10^6 (0.9 μm), however, was deduced from the *Hin*dIII restriction map (Fig. 3), assuming that the 0.9×10^6 fragment was included completely in one half of the inversion. This discrepancy can be attributed to (*i*) an underestimation of single-stranded DNA length in electron micrographs or (*ii*) an overestimation of the 0.9×10^6 fragment size. This size is derived from a comigrating λ dvl *bsu* fragment and could be incorrect due to the high adenylate and thymidylate content of the yeast fragment, which can decrease the electrophoretic mobility in agarose gels (24). Yeast 2-μm DNA has the same density as nuclear DNA and probably also its high adenylate and thymidylate content of 60.3% (27). A size of 0.8×10^6 instead of 0.9×10^6 is also in better agreement with the *Hin*dIII products of *Eco*RI fragment 1 (Table 1).

The reversed noncontiguous duplication, as it occurs in the yeast 2-μm molecule, resembles the structure of a tetracycline resistance factor (*tet*^{R}) inserted in phage P22 (28), which is able to integrate at random sites of a genome. If indeed this type of duplication enables random insertion in the case of the *tet*^{R} element, we might assume that the same is true for the 2-μm DNA.

Although no experimental proof exists for this ability to integrate randomly, we realize that bacteria carrying this eukaryotic sequence should be handled with greatest care to prevent escape.

We thank Dr. H. W. Boyer for providing strain *E. coli* RY13, Dr. P. Philippsen for advice regarding the purification of *Eco*RI, Dr. R. E. Streeck for a gift of λ dvl *Bsu* fragments, Dr. D. Y. Thomas for ColE1 DNA and advice during the initial integration experiments, Dr. J. Davies for providing plasmid pCR1, Dr. P. Borst for phage PM2 DNA, Dr. G. F. Meyer for providing electron microscope facilities, Dr. H. Bujard for providing length measurement facilities, Mrs. L. Sanader for skilful technical assistance, and Dr. W. Beermann for his interest and support.

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