Circular DNA of a yeast episome with two inverted repeats: Structural analysis by a restriction enzyme and electron microscopy*

(II episode/DNA dimers/rearrangements of sequence)

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ABSTRACT Small circular DNA molecules from genetically characterized clones of Saccharomyces cerevisiae have been studied by restriction endonuclease analysis and electron microscopy. The circular monomers (6000 bases) are shown to contain two inverted repeats of the same sequence (600 bases) situated opposite each other along the perimeter. Four endonuclease EcoRI fragments are obtained in 1:1:1:1 stoichiometry, and their sum gives a length of about 12,000 bases. The two large fragments and the two small ones differ from each other by 200 bases. We propose a model for the structure of the monomer molecule. Two classes of monomers can be generated by intramolecular recombinations within inverted repeats; they differ by the relative orientation of nonrepetitive segments. The structure of dimers as predicted by the model is verified by self-renaturation of single-stranded circles. Inverted repeats in circular molecules may be related to the insertion release faculty of II episode in the chromosomes.

We have recently demonstrated a novel class of hereditary factors responsible for drug resistance in yeast Saccharomyces cerevisiae. By some criteria (2.2 meiotic segregation, centromere linkage) they behave like nuclear genes, while by other criteria (loss during vegetative multiplication, apparent migration from one chromosome to another) they are reminiscent of bacterial plasmids and episomes. The presence of one of these factors, II, which confers resistance to oligomycin, shows a direct correlation with the presence of 2.2 μm covalently closed circular DNA molecules. We hypothesized that this circular DNA may play an informational role in the biogenesis and/or structure of membranes (1). Following our previous results and for the sake of simplicity we shall call this 2.2 μm circular DNA "II DNA."

Experiments reported here were designed to analyze the structure of the II circular molecules isolated from genetically characterized clones. We show by restriction enzyme analysis and by electron microscopy that all molecules have two inverted repeats situated opposite each other on the perimeter of the circle. This arrangement may be related to the insertion-release faculty of factor II in the chromosomes.

MATERIALS AND METHODS

Yeast Strains. Circular DNA was purified from the strain DR19 clone T3 which carries the genetic determinants II and T" and is resistant to oligomycin, venturicidin, chloramphenicol, cycloheximide, and triethylth (1).

Purification of II Circular DNA. II DNA extracted as in ref. 1 was further purified by band velocity sedimentation. The DNA sample (1 ml) was dialyzed in standard saline citrate (SSC; 0.15 M NaCl, 0.015 M sodium citrate, pH 7.0). Sodium sarconate (Sarkosyl) was added to a final concentration of 0.5% and 20 μl of Pronase (Calbiochem) at 2 mg/ml were added (the Pronase solution was preincubated 30 min at 37°). The mixture was incubated 30 min at 37° and then layered onto 2.2 ml of CsCl solution at a density ρ = 1.410 g/ml, deposed on a bottom layer of CsCl (ρ = 1.700 g/ml) in a SW50 cellulose nitrate tube. The CsCl was dissolved in 5 mM sodium citrate containing 40 μg/ml of ethidium bromide. Centrifugation in a Beckman instrument with an SW50 rotor was at 38,000 rpm for 7 hours at 15°. The fluorescent band at the interphase was collected.

The purity of the DNA is shown by two criteria: (i) a homogeneous buoyant density in CsCl gradient (ρ = 1.698 ± 0.002 g/ml); (ii) electron microscopy, which shows the presence of circular molecules only (see below).

Purification of Restriction Endonuclease EcoRI. The enzyme was purified according to Thomas and Davis (2) from the bacterial strain RY 13, Yoshimori (3) (provided by the Recherche Concertée sur Programme no. 385).

EcoRI Reactions. The purified DNA was dialyzed in 0.1 M Tris-HCl at pH 7.5, 1 mM Na2EDTA, and 10 mM MgSO4, DNA was digested with EcoRI endonuclease for 30-60 min at 37°.

Agarose Gel Electrophoresis. Solutions of 1% agarose were prepared and electrophoresis was carried out essentially as described by Helling et al. (4). The gels were illuminated at 365 nm and photographed through a Kodak Wratten filter no. 23. Negatives were scanned with a Joyce Loebel densitometer.

Denaturation and Self-Renaturation of II DNA. The II covalently closed circles (form I = FII) were converted into open circles with a single-strand break (form II = FII) by x-irradiation. The proportion of molecules converted from FII to FII was about 60% at a dose of 3000 rads (30 kJ/kg). The II DNA was then dialyzed against 20 mM NaCl, 5 mM Na2EDTA, pH 7.4 at 4° for 36 hr. Denaturation and self-renaturation were achieved by two techniques. No significant differences between them were observed. In the first procedure, the II DNA was denatured with alkali as described by Schnös and Inman (5). The high pH buffer was adjusted to pH 11.4 with NaOH; 7 μl of the DNA solution (5 μg/ml) were mixed with 3 μl of high pH buffer, allowed to stand 1 hr at room temperature, and then cooled in an ice bath. The cold solution was diluted with 10 μl of formamide and 2 μl of 0.1% cytochrome c (type III) and al-
and the arguments in favor of this appellation are given in the next section. Molecules 8 μm long were extremely rare.

After alkali denaturation two new types of molecules appeared: circular single-stranded molecules and linear ones. Single-stranded circles can be distinguished from double-stranded ones by a clear difference in contrast and by a more sinuous form (Fig. 1A and B). Furthermore, single-stranded circles appeared slightly longer (2.4 μm) than the double-stranded ones measured on the same grids, in agreement with Inman and Schnös (9).

Self-renaturation of previously denatured circles produces interesting results. When low concentrations of Π DNA were denatured and then allowed to renature to favor intrastrand rather than interstrand duplex formation, characteristic dumbbell figures were observed (Fig. 1). All have a central double-stranded region and two large apical single-stranded loops. Thirty-six such dumbbell molecules were measured. The average contour length was found to be 2.4 μm (SEM = 0.07 μm). In each of these molecules, the two single-stranded loops are significantly different in contour length: the smaller loop measures 0.92 μm (SEM = 0.02 μm) and the larger measures 1.10 μm (SEM = 0.02 μm). The double-stranded region between them is 20 μm long, equivalent to a single-stranded length of 20 × 0.20 = 4.00 μm (SEM = 0.03 μm). It can, therefore, be deduced that the double-stranded monomer contains two inverted repeats of the same sequence. In the denatured molecules, these two repeats anneal perfectly to give the 0.2-μm-long double-stranded region. Each sequence is about 600 base pairs long [assuming 1 μm = 3000 bases (3 kb)] and the two sequences are separated by DNA which has no apparent self-complementarity. Sixteen self-renatured single-stranded dimers were found whose contour length was 4.4 μm. These also displayed a dumbbell shape (Fig. 1F) with two apical single-stranded loops similar in length to the loops observed in self-renatured monomers. In between these terminal loops either a continuous stretch of duplex DNA was observed (Fig. 1F and schematic Fig. 2) or two duplex regions each 0.22 μm long separated in the middle by two single-stranded segments 0.80 μm long each (Fig. 1E). Dimers of the first type were more frequently observed than those of the second type. Therefore dimer molecules have the same basic structure as monomers, i.e., two inverted repeats of the same sequence in the native double-stranded molecule. However, an important difference exists between the lengths of the sequences in these two forms. In monomers the repeats are 0.6 kb long, while in the most frequently observed dimers the repeats are 3.9 kb long (Fig. 2).

Analysis of EcoRI endonuclease fragments of Π circular DNA

When native purified Π circles are analyzed by agarose gel electrophoresis, five bands are observed (Fig. 3A). Electron
microscopy shows that the major band (aI) corresponds to the closed circular monomers (FI) and that the band aII contains open circles (FII). The band b, which is the weakest, consists of 3 μm circles and the bands cl and cII correspond respectively to closed and open 4.4 μm circular dimers.

After digestion by EcoRI, four fragments are observed (Fig. 3B) and all the bands corresponding to the native circles have disappeared. The new bands consist only of linear molecules as verified by electron microscopy. Densitometer tracings of a typical slab are presented in Fig. 4A. No changes in the distribution patterns occur with prolonged digestion (1–3 hr) or with higher concentrations (5X) of EcoRI enzyme.

A plot of the molecular weights of λ fragments versus electrophoretic mobility is given in Fig. 4B. Helling et al. (4) have shown that under these conditions the logarithm of molecular weight is proportional to the mobility. The molecular weights for yeast circular DNA fragments calculated from the plot are: 2.65, 2.50, 1.35, and 1.20 × 10⁶.

As shown by Fig. 4, the four EcoRI fragments of II circles are in the same molar proportions. Therefore, the expected molecular weight of the original II circular molecules would be 7.7 × 10⁶ when calculated by the addition of all four fragments. This value, however, is about twice that calculated from electron microscopic measurements of monomeric circles (1, 8, 10-14). Furthermore, the two large fragments and the two small ones differ from each other by the same molecular weight of 150,000.

**DISCUSSION**

Main findings of the present work are: (a) Monomeric circles (6 kb) have two inverted repeats of the same sequence (about 0.6 kb each), which are approximately facing each other on the perimeter of the circle. (b) Dimeric circles also have inverted repeats but these can be much longer (about 4 kb each) than the length expected on the basis of addition. (c) Four EcoRI fragments are obtained in 1:1 stoichiometry by digesting a population of monomers and dimers. The sum of the four fragments gives a length of about 12 kb, or twice the length of a monomeric circle. The two large fragments and the two small ones differ from each other by the same length of 0.2 kb.

We would like to propose a model for the structure of II DNA circles (referred to as monomers and dimers), since they represent most of the population. The 3 μm circles will be considered separately. The model is based on intra- and intermolecular recombinations which can occur within inverted repeats.
and on the rearrangements of sequence to which they can lead.

**Rearrangements of Sequence Through Intramolecular Recombination Within Monomeric Circles.** In Fig. 5, the type A molecule is a double-stranded monomeric circle with two inverted repeats of the same sequence (designated ABC) situated opposite each other. The ABC sequence of one strand can be paired with the a'b'c' sequence of the other strand. If, within this paired region, a reciprocal exchange of strands occurs, a novel molecule of type B will emerge. The two types of molecules differ only with respect to the relative orientation of the two DNA segments located between the inverted repeat sequences.

**Rearrangements of Sequence Through Intermolecular Recombination Between Monomers.** Dimer molecules can be formed by recombination between monomer types A × A, B × B, and A × B. Two ways of pairing and recombination can occur for each of these. This is depicted in Fig. 6 where monomers of the type A are considered and recombination occurs within repetitive segments ABC. It is apparent that the arrangement of noncomplementary sequences in the upper dimer DEF GHI DEF GHI is different from that in the lower dimer (DEF i'h'g' f'e'd' GHI) no matter which strand is considered. This is due to the fact that the respective arrangement of parental monomers is different in the upper pairing from that in the lower one. In a similar manner, pairing and recombination between monomers of type B will lead to two types of dimers, one of which will be identical in sequence to a dimer derived from A × A pairings. Altogether five types of dimers can be formed, and no other types of dimers will be produced if recombination occurs within segments DEF or GHI instead of taking place within repetitive segments ABC.

It follows that different lengths of self-complementary sequences are, therefore, created within single strands of a dimer. It is clear that the structure of the DX dimer in Fig. 7 is such that the self-complementary regions are only those that preexisted in the parental monomers, i.e., the two ABC, c'b'a' segments. However, the DY dimer has a much longer and continuous self-complementary sequence on each single strand, since the nonself-complementary sequences of the monomer (i.e., DEF and f'e'd' or GHI and i'h'g') are now inserted as inverted repeats between the preexisting inverted repeats ABC and c'b'a' (Fig. 7).

**The Model Compared with the Experimental Results.** The model explains most of the present findings and makes a certain number of predictions that can be tested. Although based essentially on the study of self-annealed single-stranded monomers, it explains in a satisfactory manner: (a) existence of two types of dimers. In the first type (Dimer DX in Figs. 6 and 7 to be compared to the microphotograph Fig. 1E) the self-complementary regions are only the preexisting repeats from the

![Fig. 5](image1.png)

**Fig. 5.** Model of intramolecular recombination within monomeric circles. The letters ABC and a'b'c' represent the inverted repeat sequence. The letters DEF and GHI represent the two DNA segments located between the inverted repeat sequences. The arrows represent the EcoRI sites.

![Fig. 6](image2.png)

**Fig. 6.** Model of intermolecular recombination between two monomers. The two pathways of pairing and recombination between the inverted repeat sequences of two type A monomers are depicted. The same letters are used as in Fig. 5.

![Fig. 7](image3.png)

**Fig. 7.** Expected self-annealing patterns of the single-stranded monomers and dimers. The corresponding double-stranded monomers and dimers are depicted in Figs. 5 and 6. The two possibilities for self-annealing of the dimer DY are given.
parental monomers. In the second type (Dimer DY in Figs. 6 and 7, to be compared to the microphotograph in Fig. 1F), the self-complementary regions are much longer and continuous. (b) The fact that apical single-stranded loops observed after self-renaturation of single-stranded circles have similar lengths in monomers and dimers (Fig. 2). Furthermore, the model reconciles restriction enzyme analysis and electron microscopy data, which otherwise appear contradictory. Taken at their face value, the results of endonuclease digestion indicate that the molecules are heterogeneous in sequence. The presence of all four fragments in 1:1 stoichiometry rules out the hypothesis that this heterogeneity is due to monomers being different from dimers, for the latter represent only a small fraction of the total DNA mass. The main prediction of the model is the existence of two molecular types, A and B, whose sequences are rearranged. If these are present in 1:1 stoichiometry, all of the restriction enzyme data can be explained. It suffices to postulate that there are two EcoRI sites per monomer, and that each site is located in the DNA segment situated between the inverted repeat sequences (Fig. 5). Cleavage at the two sites (1/1' and F/f') of the molecule B should give two fragments: the longest and the shortest one. Cleavage at the same sites of the molecule A should give two fragments intermediate but unequal in length. The sum total of each pair of fragments should be the same and should correspond to the length of the monomer. This is precisely what is found (see Fig. 4): 2.65 + 1.20 = 2.50 + 1.35 = 3.85 × 10^6 daltons. This sum is compatible with the monomer length expected from electron microscopy measurements. It should be stressed that according to our model the digestion of dimers should lead to the same fragments as those obtained from monomers. Furthermore, there is a symmetry axis between the two inverted repeats which permits the localization of the two EcoRI cleavage sites on the monomeric circle. These two sites cannot be located within the inverted repeats (ABC) or within one and the same inter-repeat segment (either DEF or GHI). In both cases the molecules A and B would generate fragments of the same length. The two sites have to be located one within the DEF segment and the other within the GHI one. The lengths of the fragments observed (Fig. 4) indicate that one of the sites is distant by some 125 base pairs from the symmetry axis between the two inverted repeats ||(2.65 − 2.50) × 10^6 daltons)/600 daltons per base pair]/2 = 125.

It is our feeling that the model explains all the presently known facts with one minor exception. Very few circles of intermediate length between monomers and dimers were observed (Fig 3, band b). After digestion this band disappears, but the sensitivity is low. From electron microscopy we know that these cm circles also have a central self-complementary segment (not shown). This suggest that the three cm circles may arise by rearrangement of the same sequences as those present in monomers and dimers. The rearrangement mechanism is, however, less obvious.

Most of our work has been done on circles purified from a mutant clone that displays a variety of drug resistances (1). We have also studied circles isolated from a wild-type strain sensitive to all the drugs. Very similar results, in terms of electron microscopy and EcoRI fragments, were found. The acquisition of drug resistances, therefore, is not due to rearrangements or insertions of large fragments into the II circles.

Inverted repeat sequences present in bacterial plasmids have been shown to promote the insertion and/or the translocation of defined genetic units at several specific sites either on the plasmid or on the bacterial chromosome (15–18). For these reasons, they are called insertion sequences. Inverted repeat sequences have also been described in bacterial (19) and animal virus (20). The existence of such inverted repeats in II molecules may be responsible for their unusual genetic properties (1). It will be interesting to see if the II DNA may constitute a useful vector, and yeast a suitable host, for genetic engineering of eukaryotes.

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