ABSENCE OF 2 μm DNA SEQUENCES IN *SACCHAROMYCES CEREVISIAE* Y 379-5D

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1. Introduction

Several strains of *Saccharomyces cerevisiae* are known to contain 50–100 small circular duplex DNA molecules per cell [1,2]. They have a genetic complexity equivalent to 4×10^6 daltons [3], a contour length of 2 μm or multiples of this and a long duplication–inversion comprising 20% of the monomer length [4,5]. Their biological function is unknown, although it has been suggested that they can code for resistance to certain antibiotics [6].

In prokaryotes some DNA sections flanked by a duplication–inversion can be transferred from one genome to another [7]. We were interested to see whether a similar phenomenon occurs in eukaryotes and have studied whether 2 μm DNA is present in nuclear DNA in an integrated form. Experiments recently reported by Cameron et al. [8] showed that in *S. cerevisiae* strains containing free 2 μm DNA circles, no specifically integrated 2 μm DNA sequences could be found. Livingston [9] has reported that from *S. cerevisiae* Y 379-5D no 2 μm DNA circles could be isolated by ethidium–CsCl equilibrium gradient centrifugation. We have studied whether in this *S. cerevisiae* strain 2 μm DNA sequences, integrated in nuclear DNA, do occur. If 2 μm DNA codes for essential proteins and no free 2 μm DNA circles are present, we would expect to find these sequences in the nuclear DNA of this strain in an integrated form.

2. Methods and materials

S. cerevisiae Y 379-5D was obtained from Dr C. P. Hollenberg (Max-Planck Institut für Biologie, Tübingen, FRG). Cells were grown on lactate medium and protoplasts were prepared as described [2]. Protoplasts were resuspended in 50 mM Tris–HCl (pH 8.0), 50 mM EDTA and gently lysed to avoid shear forces by addition of Na-laurylsarcosinate to a final concentration of 1%. Saturated NaI was added to the lysate to a density of 1.53 g/cm^3 and ethidium bromide to a concentration of 5 μg/ml. The DNA was spun to equilibrium in a Spinco 65 angle rotor for 48 h at 20°C and 45 000 rev/min. Since 2 μm DNA has the same density as nuclear DNA [2], the main DNA band was collected from the NaI gradients avoiding the mtDNA band. There is no evidence in yeast for DNA species banding at a position different from the known nuclear and mtDNAs. Ethidium was removed by extraction with amylalcohol (3-methylbutan-1-ol) and the preparation was dialysed against 10 mM Tris–HCl (pH 7.5). RNA was digested with 30 μg pancreatic ribonuclease per ml for 1 h at 37°C. Sodium dodecylsulphate was then added to 0.1% and the preparation incubated with 100 μg pronase per ml for 2.5 h at 37°C. The mixture was extracted successively with phenol/cresol, isobutanol and ether. The solution was dialysed extensively against 10 mM Tris–HCl (pH 7.5). The purified DNA was greater than 30×10^6 daltons as determined by agarose gel electrophoresis and suitable DNA markers.

2 μm DNA was purified from *S. cerevisiae* JS1-3D by two successive ethidium–CsCl gradient centrifugations [2]. Rabbit DNA was a gift from Dr A. J. Jeffreys. Yeast 17 S ^{32}P-labelled ribosomal DNA (rRNA) was a gift from Drs C. Klootwijk and P. de Jonge (Laboratory of Biochemistry, Free University, Amsterdam) and had spec. act. of 200 000 cpm/μg. Endonuclease *Eco*RI was a gift from

Professor C. Weissmann (Institut für Molekularbiologie II, University of Zurich, Switzerland) through Dr R. A. Flavell.

Purified PTY-63 DNA [5] was a gift from Dr C. P. Hollenberg. This is a recombinant plasmid of pCR1 DNA (8.5×10^6 daltons) containing 2 μm DNA. The DNA was labelled in vitro by nick-translation with DNA polymerase I from *Escherichia coli* [10] to spec. act. 14×10^6 cpm/μg. In vitro labelling, restriction endonuclease digestions, gel electrophoresis, transfer of DNA to nitrocellulose filters and filter hybridization (omitting the low salt washes) were carried out as described by Jeffreys and Flavell [11].

3. Results

It has been shown that *S. cerevisiae* Y 379-5D does not contain the normal content of 2 μm DNA circles

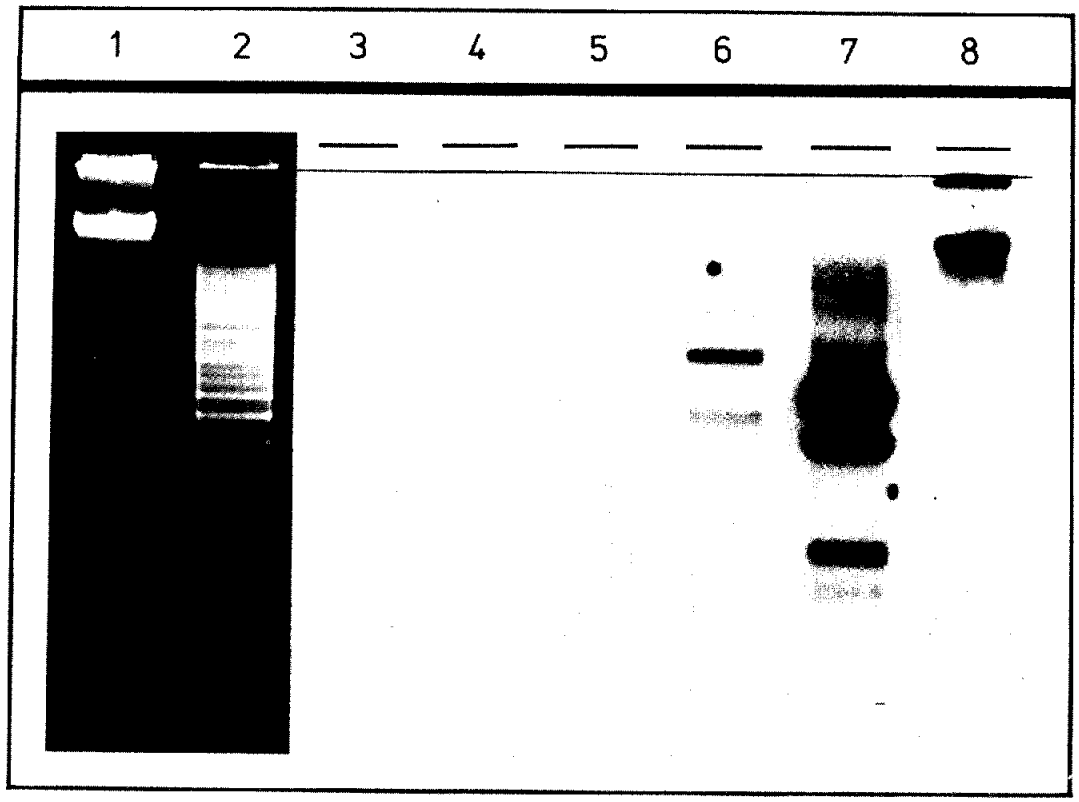

Fig.1. Hybridization of ^{32}P-labelled PTY-63 DNA to yeast nuclear DNA fragmented with *Eco*RI. 2 μg nuclear DNA was fragmented with *Eco*RI and the DNA electrophoresed on 0.7% agarose gels. Slot 1 shows the fluorescence of unfragmented DNA, slot 2 the fluorescence of *Eco*RI fragmented DNA. The DNA from agarose strips like that shown in slot 2 was transferred to nitrocellulose and hybridized with 12×10^6 cpm of ^{32}P-labelled PTY-63 DNA. Slots 3–7 show autoradiograms of the nitrocellulose strips. Slot 3 contained unfragmented DNA, slot 4 *Eco*RI (4 μl) fragmented DNA, slot 5 *Eco*RI (8 μl) fragmented DNA and slot 6 *Eco*RI (4 μl) fragmented DNA to which 2 μm DNA was added before digestion in an amount of 0.2 copy per haploid nuclear DNA (0.2 ng 2 μm DNA). Only two of the four *Eco*RI 2 μm DNA fragments can be seen after autoradiography, since the bands of 2.45 and 2.35×10^6 daltons and the bands of 1.55 and 1.45×10^6 daltons merged due to deliberate overexposure to visualize possible bands in the other slots. The faint band with the highest molecular weight in slot 6 is a 2 μm DNA partial fragment. The DNA on strips 7 and 8 were hybridized with 6×10^6 cpm of 17 S ^{32}P-labelled rRNA. Slot 7 contained *Eco*RI (8 μl) fragmented DNA and slot 8 unfragmented RNA. The minor bands in slot 7 are probably due to hybridization of 17 S rRNA to DNA containing sequences for 26 S rRNA [14] or to 26 S RNA contamination of the 17 S rRNA preparation.

as compared with other yeast strains [9]. We have investigated whether 2 μm DNA sequences are present in this strain in a reduced number of copies, either as free DNA circles or in an integrated state in the nuclear DNA.

Nuclear DNA was digested with the restriction endonuclease *Eco*RI and the DNA fragments separated by electrophoresis on 0.7% agarose gels. After denaturation the DNA was transferred to nitrocellulose filters and prepared for hybridization. PTY-63 DNA, a recombinant plasmid of pCR1 and a single copy of 2 μm DNA, was in vitro labelled with ^{32}P by nick-translation to spec. act. 14×10^6 cpm/μg and used as a hybridization probe to look for 2 μm DNA sequences in the nuclear DNA digests. Figure 1 summarizes our results. Slots 1 and 2 show the fluorescence of DNA bands before and after complete digestion with restriction endonuclease *Eco*RI. Slots 3–6 show autoradiograms of ^{32}P-labelled PTY-63 DNA hybridized to undigested DNA (slot 3) and to *Eco*RI DNA fragments (slots 4–6). To the sample in slot 6, 2 μm DNA had been added before digestion in an amount equivalent to 0.2 copy of 2 μm DNA per haploid nuclear DNA complement. Hybridization to the specific 2 μm *Eco*RI DNA bands in this slot shows that the hybridization technique is sensitive enough to reveal the presence of small amounts of 2 μm DNA sequences. The results indicate that less than one free 2 μm DNA circle per haploid nuclear DNA complement is present, since no characteristic 2 μm DNA *Eco*RI fragments were found. Our data demonstrate that one 2 μm DNA copy, integrated in a specific site in the nuclear genome, does not occur since no new DNA fusion fragments are present. They also suggest that 2 μm DNA copies, integrated at random in the nuclear DNA, are absent. Since *Eco*RI cuts 2 μm DNA twice, characteristic *Eco*RI 2 μm DNA fragments could arise from randomly integrated 2 μm DNA in submolar yield. These were not found either (similar observations have been made by Kustermann-Kuhn and Hollenberg, personal communication). As a control we hybridized the nitrocellulose filters with yeast 17 S ^{32}P-labelled rRNA. Slot 8 shows weak hybridization with undigested nuclear DNA possibly due to inefficient transfer of high-molecular-weight DNA from gels [12]. Slot 7 shows hybridization with eight DNA fragments derived from the rDNA repeat unit by digestion of the DNA with *Eco*RI [13]. This indicates that the nuclear DNA was fragmented completely and capable of hybridizing.

Table 1
Hybridization of ^{32}P-labelled PTY DNA to yeast nuclear DNA directly immobilized on nitrocellulose filters

DNA on filter	cpm on filter
1. None	632
2. Rabbit	1253
3. *S. cerevisiae*	575
4. *S. cerevisiae* + 0.01 copy of 2 μm DNA	5706
5. + 0.05 copy of 2 μm DNA	29 950
6. + 0.10 copy of 2 μm DNA	47 340

Nitrocellulose Filters were loaded with 50 μg denatured DNA and a number of 2 μm DNA copies per haploid nuclear DNA as indicated in the table assuming a ratio of 1 : 2000 of 2 μm DNA circles to haploid nuclear DNA on a weight basis [16]. Filters were hybridized with 1.4×10^6 cpm of PTY-63 DNA in 2 ml for 65 h at 60°C. Further conditions were as specified for hybridization of Southern strips

We cannot conclude from these experiments that 2 μm DNA sequences are totally absent from this particular *S. cerevisiae* strain. We could possibly have missed a limited number of randomly integrated 2 μm DNA copies in this experimental set-up, for instance when one of the two *Eco*RI sites in 2 μm DNA had been lost. Strains having 2 μm DNA circles with only one *Eco*RI site have been reported [9]. After specific fragmentation with *Eco*RI such 2 μm DNA sequences could be distributed over the whole agarose gel, making the local concentration of 2 μm DNA on the nitrocellulose filters too low to detect by hybridization. In the case of unfragmented DNA the concentration of the 2 μm DNA sequences could be too low to detect on the Southern strips due to inefficiency of transfer. We therefore performed standard DNA–DNA hybridization experiments with nuclear DNA directly immobilized on nitrocellulose filters (table 1). No specific hybridization to *S. cerevisiae* DNA could be detected above the background of filters containing no DNA and filters containing rabbit DNA, while small amounts of added 2 μm DNA are reproducibly detected. In preliminary experiments (De Boer-van den Berg and Tabak, unpublished) we have studied the effect of nuclear DNA on the rate of renaturation of a small amount of in vitro ^{32}P-labelled 2 μm DNA, using S_1 nuclease to discriminate between single-

stranded and double-stranded DNA. We found no acceleration of the rate of renaturation, which again suggests that 2 μm DNA sequences are totally absent in this strain of *S. cerevisiae.*

4. Discussion

We have used two approaches to look for the presence of 2 μm DNA sequences in *S. cerevisiae* strain Y 379-5D. DNA of nuclear density (nuclear DNA and 2 μm DNA possibly present) was purified through NaI gradients. First, the DNA was digested with *Eco*RI and the DNA fragments separated according to size by agarose gel electrophoresis. The fragments were then transferred to nitrocellulose filters and hybridized to in vitro ^{32}P-labelled PTY DNA, a recombinant DNA plasmid containing 2 μm DNA. No hybridization with any DNA fragment was found although reconstruction experiments showed that the presence of 0.1 copy of 2 μm DNA per haploid nuclear DNA complement could be detected by this technique. Secondly, in standard DNA–DNA hybridization experiments with DNA directly immobilized on nitrocellulose filters no specific hybridization was detected either. In these experiments 0.01 copy of 2 μm DNA per cell would have been detected. In preliminary experiments the addition of nuclear DNA to small amounts of ^{32}P-labelled 2 μm DNA also did not accelerate the rate of renaturation of this 2 μm DNA.

We conclude that 2 μm DNA sequences are totally absent in *S. cerevisiae* strain Y 379-5D. If 2 μm DNA carries genetic information, as is suggested by their expression in *E. coli* mini cells [15], this information cannot code for essential functions in the yeast life cycle, since its absence in one yeast strain does not impart any measurable deficiency on the cells.

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