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Isolation and partial characterization of 2-μm yeast plasmid as a transcriptionally active minichromosome

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Yeast cell extracts from 2-μm-containing strains (cir$^+$) showed higher transcriptional activity than their corresponding isogenic sets (cir$^\circ$). These extracts were used to purify transcriptionally active 2-μm minichromosomes in a sucrose gradient. Minichromosomes were transcribed in vitro and, employing hybridization techniques, the RNA synthesized was shown to present 2-μm-specific sequences. This model system should permit the direct study of transcriptionally active eucaryotic chromatin.

Minichromosome; Transcriptional complex; RNA synthesis; Sarkosyl; (Yeast plasmid)

1. INTRODUCTION

In multicellular organisms only a small fraction of the DNA is transcribed in each differentiated cell (reviews [1,2]). The study of most cellular DNA molecules has been hampered by their large size. Alternative model systems are provided by small, circular DNA molecules, such as simian virus 40 DNA (SV40), isolated and characterized as transcriptional complexes [3,4]; however, only about 1% of these complexes are transcriptionally active (5000 copies per infected cell) [5], and a relatively high proportion of inactive SV40 chromatin (99%) prevents the study of the transcriptionally active SV40 minichromosomes.

The 2-μm yeast plasmid is a double-stranded, circular DNA molecule (6.3 kbp), packaged into nucleosomes by a normal complement of histones [6] and exists in approx. 60 copies per haploid cell [7]. This plasmid interconverts between two distinct structures (forms A and B) that differ in the orientation of one unique region with respect to each other [8]. Since both forms (A and B) of the plasmid are equimolar and actively transcribed [9], at least one out of 30 molecules (more than 3%) should be transcriptionally active; thus, a greater proportion of active chromatin should exist for 2-μm circles as compared to SV40 minichromosomes [4,5]. As a first step in directly defining the components involved in yeast RNA synthesis, we have isolated and partially characterized 2-μm transcriptionally active complexes from yeast.

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2. MATERIALS AND METHODS

Strains of *Saccharomyces carlbergensis* (CDO4) cir$^+$ (containing 2-μm plasmid) and cir (kindly provided by Dr J. Campbell, California Institute of Technology) were grown at 30°C to exponential phase in 500 ml of rich medium YM-1. Spheroplasts (1.5×10^{10}) were obtained [10] and lysed in 5 ml buffer A: 25 mM Tris-HCl (pH 7.9), 1.0 mM EDTA, 0.1 M NaCl, 0.5 mM DTT and 0.25% sarkosyl [3]. The lysate was centrifuged for 30 min at 190000 × g (4°C) and the supernatant separated and assayed (90 μl) for RNA synthesis in vitro. The standard reaction mixture for RNA syn-

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thesis (125 μl final volume), using 5 μCi [^{3}H]UTP (10.9 Ci/mmol) per assay, has been described [4]. The purification of 2-μm minichromosomes was carried out from the *cdc*28 mutant of *S. cerevisiae* (strain 621-4, obtained from L.H. Hartwell's laboratory, Department of Genetics, University of Washington), grown at exponential phase (30°C) in YM-1; to express the mutation, the temperature was raised to 37°C for 90 min; extraction and purification of 2-μm minichromosomes were performed basically according to Livingston and Hahne [6]. The minichromosomes were sedimented in a 10–40% sucrose gradient for 160 min at 164000 × *g* (4°C); the gradient was collected and alternate fractions assayed for RNA synthesis [4]. Identification of 2-μm minichromosomes was achieved using different fractions from the sucrose gradient which were subjected to agarose gel electrophoresis, then transferred to nitrocellulose [11] and hybridized with a radioactive 2-μm plasmid DNA probe purified from CsCl gradients. DNA purification [12], cRNA synthesis [3], RNA purification [13] and DNA-RNA hybridization [14] were carried out as described. Radioactivity was determined in toluene-based scintillation liquid.

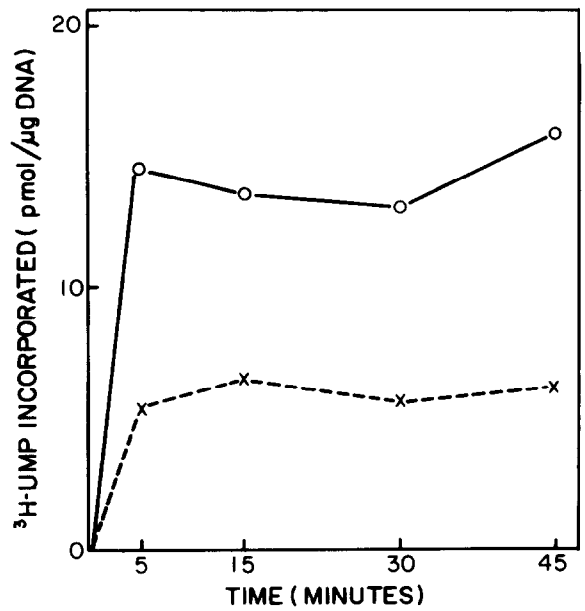

Fig.1. Sarkosyl supernatant transcription from the yeast isogenic set. Spheroplasts of both strains (cir° and cir$^+$) were lysed in buffer A containing 0.25% sarkosyl (see section 2) and aliquots (90 μl) of the clarified supernatants were assayed (30 min at 32°C) for RNA synthesis (see text). Each time point is the average of two determinations. (○—○) cir$^+$ and (×---×) cir°.

3. RESULTS AND DISCUSSION

As a first approach to detect the 2-μm transcriptional complex, we determined the synthesis of RNA in sarkosyl [3,4] containing extracts from a cir$^+$, cir° yeast isogenic set. Fig.1 shows that both strains displayed active endogenous RNA polymerase activity as determined by [^{3}H]UMP incorporation into RNA; transcription was 100% higher in the cir$^+$ strain, suggesting that the presence of 2-μm plasmid was responsible for the increased level of activity in the plasmid-containing strain. Moreover, α-amanitin, a specific inhibitor of yeast nucleoplasmic RNA polymerase B or II [15], reduced the level of transcription approx. 60% in both cases, indicating that most of the product was mRNA (not shown).

The 2-μm plasmid DNA is largely removed from the chromatin when logarithmically growing cells are withdrawn from the cell cycle or when the *cdc*28 gene product is inactivated at the non-permissive temperature [16]. We extracted the 2-μm minichromosomes from a *cdc*28 strain as described by Livingston and Hahne [6] and sedimented them by sucrose gradient centrifugation (see section 2). Elongation of RNA molecules throughout the gradient fractions was measured by in vitro [^{3}H]UMP incorporation and taken as a test for RNA polymerase molecules that have already initiated transcription in vivo. As shown in fig.2A, fractions 8–17 contained most of the endogenous transcriptional activity, cosedimenting (at about 75 S) with the bulk of the yeast 2-μm minichromosomes [6]. In addition, Southern blot analysis of the DNA present in alternate fractions of the gradient was performed using purified yeast ^{32}P-labeled 2-μm plasmid as a probe. Radioactivity was detected (mainly in form II DNA molecules) in samples from fractions 8–17 (fig.2B) where the main transcriptional activity had been found.

The best proof that the transcriptionally active complex is 2-μm-specific was obtained through molecular hybridization experiments. We hybridized in vitro synthesized RNA, derived from

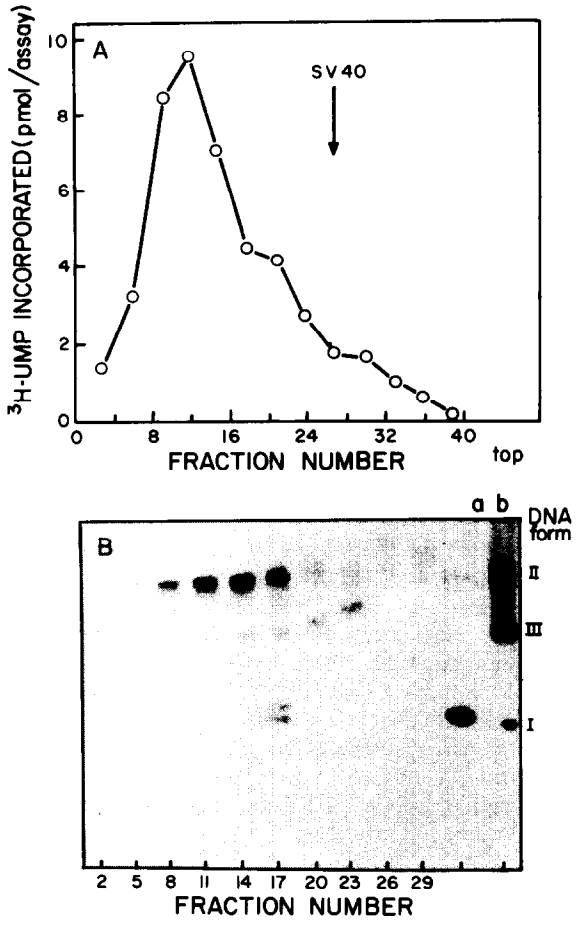

Fig.2. (A) Endogenous transcriptional activity of sucrose gradient fractions (strain 621-4). Minichromosome extraction and sedimentation were performed as described in section 2. Sucrose gradient fractions were assayed for RNA synthesis as indicated in fig.1. The SV40 minichromosome sedimentation coefficient (55 S) was obtained from a parallel centrifugation. (B) Southern blot analysis of 2-μm minichromosomes. Different fractions of the sucrose gradient (20 μl) were heated at 65°C for 5 min with 0.1% SDS (final concentration) and subjected to agarose gel electrophoresis. 2-μm specific DNA sequences were identified in each fraction by Southern hybridization (see text); (a) 2-μm minichromosome input (20 μl cellular extract); (b) CsCl gradient-purified 2-μm DNA containing superhelical (I), relaxed circular (II) and linear (III) DNA forms.

sucrose gradient fractions 8–17 and RNA synthesized from spheroplast crude extracts, to the recombinant plasmid CV19 (a fusion of pBR322, yeast 2-μm and the yeast LEU2 gene) [17]. As a control, 2-μm cRNA was hybridized with CV19, pBR322 and 2-μm yeast DNAs immobilized on nitrocellulose filters (see table 1). It can be seen (table 1) that 2-μm cRNA (positive control), spheroplast RNA, and sucrose gradient-derived RNA hybridized (to CV19 DNA) up to 3.3, 0.03 and 1% of the input, respectively (100, 0.9 and 30.3% of the input, if normalized). Thus, one-third of the sucrose gradient-derived RNA can be taken into account for 2-μm transcriptional com-

Table 1

Hybridization of [^{3}H]RNA synthesized in spheroplast extracts and sucrose gradient fractions

Source	DNA immobilized (75 μg)	[^{3}H]RNA hybridized (cpm)	% input	% corrected
2-μm cRNA (60000 cpm)	CV19	2000	3.3	100[a]
	pBR322	50	0.08	2.4[a]
	2-μm (25 μg)	2720	4.5	100[b]
Spheroplast crude extract (118000 cpm)	CV19	35	0.03	0.9[a]
	pBR322	10	0.01	0.3[a]
Sucrose gradient (63000 cpm)	CV19	640	1.0	30.3[a]
	pBR322	34	0.05	1.5[a]

[a] Corrected factor 30.3 (cRNA from 2-μm hybridized against CV19 DNA)

[b] Corrected factor 22.2 (cRNA from 2-μm hybridized against 2-μm DNA)

Spheroplasts (3×10^{10}) were lysed and centrifuged (see section 2). Half of the supernatant was fractionated by sedimentation as indicated before and the other half kept at 4°C. Both samples (fractions 8–17 of the gradient and whole cells) were employed for in vitro transcription as indicated in the text and the purified [^{3}H]RNA was used for hybridization (in duplicate) as previously described. In this experiment, CV19 DNA was employed as the 2-μm DNA source. Hybridization percentages were calculated from the input and corrected with a factor of 30.3[a] which resulted from the hybridization of 2-μm [^{3}H]cRNA against CV19 DNA (first line) and 22.2[b] when hybridization was against 2-μm DNA (third line)

plexes. It is possible that the low percentage of hybridization obtained could be due to double-stranded RNA formation by full-length 2-μm transcripts synthesized in both orientations from 2-μm DNA forms A and B (see section 1), which prevented hybridization with complementary single-stranded DNA. The above results also indicate that, after sucrose gradient centrifugation, the 2-μm transcriptional complex was purified aprox. 30-fold compared to that present in spheroplasts. From table 1 and fig.2A, we estimated that 2-μm transcription complexes incorporated about 20 pmol UMP/μg DNA. Since the SV40 system (about 1% of the minichromosomes are transcriptionally active) presented a value of about 3 pmol UMP/μg DNA [4,5], it suggests that approx. 6% of the yeast plasmid is in the form of 2-μm transcriptional complexes. This increased proportion of transcriptionally active chromatin (compared to bulk chromatin) should permit one to perform direct comparative experiments between the molecular components of these two populations. We are currently using a chimeric plasmid, pHBS16 [18], which has the strong promoter of alcohol dehydrogenase I, with the hope of increasing even further the percentage of transcriptional complexes among the population of 2-μm minichromosomes.

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