Isolation and partial characterization of homologous sequences of ribosomal genes (rDNA) in *Blastocladiella emersonii*

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ABSTRACT. The definition and the characterization of replication origins regions in higher eukaryotes are still controversial. The initiation of the replication is site-specific in some systems but seems to occur in large regions in others. Because of its *in tandem* organization, reducing the area to the restricted space that codifies an unit of transcription, rDNA regions are attractive models to study replication origins. In this work we isolated and started to characterize a clone that contains a ribosomal sequence from the aquatic fungus *B. emersonii*, Be97M20. Southern blots showed several sites for the restrition enzymes Eco RI, HindIII and SalI. A northern blot of total RNA, hybridized against a probe made from Be97M20, confirmed its homology with the ribosomal 18S gene. The detailed characterization, including complete restriction map, subcloning, sequence and analysis on bidimensional gels will provide important information about the structure and dynamics of this region.

Key words: *Blastocladiella emersonii*, rDNA, ribosomal genes.

RESUMO. Isolamento e caracterização parcial de sequências homólogas a genes ribossomais (rDNA) em *Blastocladiella emersonii*. A definição e a caracterização de regiões de origens de replicação nos eucariotos superiores são ainda controversas. A iniciação da replicação é sítio-específica em alguns sistemas e, em outros, parece estar contida em regiões extensas. Regiões rDNA são modelos atrativos para o estudo de origens de replicação pela sua organização *in tandem*, reduzindo a área de estudo para o espaço restrito que codifica uma unidade de transcrição. Neste trabalho nós isolamos e caracterizamos parcialmente um clone que contém uma sequência ribossomal do fungo aquático *Blastocladiella emersonii*, Be97M20. Southern blots mostraram diversos sítios para enzimas de restrição Eco RI, HindIII e SalI. Northern blot de RNA total hibridado contra uma sonda feita com Be97M20 confirmou a sua homologia com o gene ribossomal 18S. A caracterização detalhada, incluindo o mapeamento de restrição completo, subclonagem, sequenciamento e análise em géis bidimensionais proverão informações adicionais importantes sobre a estrutura e dinâmica desta região.

Palavras-chave: *Blastocladiella emersonii*, rDNA, genes ribossomais.

Introduction

The characterization of replication origins regions in higher eukaryotes is a important unanswered question (Biamonti *et al.*, 2003; Gerbi *et al.*, 2003). Ribosomal genes have been studied in a wide range of organisms and they have been useful for evolutionary, phylogenetic, developmental, ecological, phytopathological, transcriptional and replicational studies (Muller *et al.*, 2000; Ghosh *et al.*, 2003; Maric *et al.*, 2003; Pantou *et al.*, 2003). High conservation between rDNA regions has been observed in relation to sequence, structure and copy numbers. Ribosomal genes regions are attractive models to study the origin of DNA replication, because they are organized *in tandem*, reducing the area to the restricted space that encodes one transcription unit. The 45S rDNA genes are moderately repeated in the genome, with the number of copies between 100 and 300 per haploid genome. The sequences encode 5.8S, 18S, 28S and spacer rRNAs, which are organized into groups and transcribed as a single unit (Garber *et al.*, 1988;

Tsuge *et al.*, 1989; Pasero and Marilley, 1993). During transcription, some of these regions give rise to structures called nucleolar organizing regions, NORs, found in one or more *loci* in the chromosomes (Futuyma, 1992). Electron microscopy studies suggest that the initiation of rDNA replication is restricted to the intergenic spaces in protozoa and metazoans (references in Bérnard *et al.*, 1995). Bidimensional gels analyses have confirmed these observations and revealed that the initiation is site-specific in *Saccharomyces cerevisiae* (Brewer and Frangman, 1998; Linkens and Huberman, 1990, Ivessa and Zakian, 2002, Pasero *et al.*, 2002) and *Physarum polycephalum* (Bérnard *et al.*, 1995). By contrast, at initial embryogenesis of *Xenopus* sp eggs, rRNA genes are not transcribed and replication initiation occurs at intervals of 9-12 Kb, independently of specific sequences. However, the replication initiation sites became restricted to the intergenic spaces when the transcription of rRNA genes is activated at the end of the blastula and the beginning of the gastrula (Hyrien *et al.*, 1995). In human cells, multiple initiation sites have been found in extensive zones of 31 Kb, into not transcribed spacer regions (Bérnard *et al.*, 1995).

Analysis from different systems reveals important clues about the structure and dynamics of replication origin regions. In this context, the aquatic fungus *Blastocladiella emersonii*, an eukaryotic microbial system with highly synchronic growth and cellular differentiation phases, has biochemical ways with reasonable homology with higher eukaryotic cells (Gomes *et al.*, 1979) and it can be a suitable system to study replication origin regions and replicons. In this work, we present the results of the initial characterization of the rDNA region from this microorganism.

Material and methods

Organisms:

Blastocladiella emersonii.

The *Blastocladiella emersonii* used was an American strain kindly provided by Dr. Wilson Roberto Navega Lodi, from the Biochemistry Department, Faculdade de Medicina, *Universidade de São Paulo*, *Ribeirão Preto,* state of *São Paulo*, Brazil, and routinely kept in our laboratory. The maintenance and manipulation conditions of the organism were similar to those described in Soll *et al.* (1969). A detailed description of the evolutionary cycle of *B. emersonii* can be found in Lovett (1975). Briefly, germination, which occurs in 40min at 27ºC, is marked by flagellum retraction, formation of vesicles that migrate to the cellular surface,

mitochondria fragmentation, disintegration of the nuclear cap containing all the ribosomes, formation of the endoplasmic reticulum and the germination tube. In the growth there is an increase in cellular mass, with successive nuclear divisions without cellular division, in which the cell grows in a way similar to a coenocyte. At any growth time, sporulation can be induced by deprivation of amino acids (Corrêa and Lodi, 1986). In this process, an increase is observed in macromolecule degradation activities and a decrease or interruption in biosynthetic pathways, as stable RNA (Corrêa, 1990). Under optical microscopy, five phenotypes are observed: a) the vegetative cell at the moment of sporulation induction, b) the septate zoosporangia, which is characterized by the appearance of a basal septum in the region between the cellular body and the rhizoid, (Corrêa and Lodi, 1986; Peralta and Lodi, 1988), c) the papillate zoosporangia, which presents a cellular appendix, named papillae, from which the future zoospores will leave the mother cell, d) the cleaved zoosporangia, at the end of the process, with rupture of the papillae, e) the empty zoosporangia, represented by the cellular structures, cell wall and rhizoid, after liberation of the zoospores.

Sporulation, especially after the formation of the papillae, is marked by intense modification of the cellular components: formation of the flagellum, fusion of mitochondria, aggregation of the ribosomes, which are involved by a membranous structure around the nucleus called nuclear cap and a complex process of membrane biosynthesis, culminating in the cleavage of the cytoplasm and the individualization of the zoospores inside the mother cell (Lessie and Lovett, 1968; Barstow and Lovett, 1978).

Escherichia coli strains:

DH5α: *supE44 ΔlacU169 (ϕ80lacZΔM15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1* (Studier and Moffatt, 1986).

ED8767: *supE44 supF58 hsdS3 recA56 galK2 galT22 metB1* (Murray *et al*, 1977).

DNA and RNA preparations:

DNA from pure zoospores suspensions was obtained by washing daily culture in solid Petri dishes with SS (0.1M CaCl$_2$, 0.05M Tris maleate pH 6.8), filtrated by commercial filter paper. The suspension of zoospores was centrifuged for 5min at 4,000 rpm, 4ºC. The supernatant was discarded and the zoospores were suspended in 0.02M Tris-HCl, 0.2M NaCl and 0.005M EDTA (ethylenediaminetetraacetic acid) at pH 8.0. After,

the same volume of 0.4M EDTA, pH 8.0, 1% N-lauryl sarcosyl and 10µg/mL RNAse were added. After 20min at room temperature, proteinase K at final concentration of 500µg/mL was added. The material was then incubated for 4 hours at 37°C. Extractions by phenol, phenol/chloroform, chloroform and precipitation using 0.2M NaCl and ethanol were then carried out (Sambrook *et al.*, 1989).

RNA fractions were obtained from suspensions of zoospores centrifuged for 5min at 4,000 rpm, 4°C. The same volume of iced lise buffer (0.01M tris-HCl pH 9.0, 2% SDS, 0.05M EDTA, 5% ethanol) was added and the material was homogenized. Ten volumes of reagent Trizol® (Gibco-BRL) were added to the homogenized material, mixed quickly in a vortex and centrifuged for 10min at 14,000 rpm, 4°C, to remove debris. The supernatant was collected, transferred to a clean tube and centrifuged for 5min, 14,000 rpm at room temperature. Chloroform, corresponding to 20% of the volume used by the reagent Trizol®, was added, and the tube was agitated in the vortex until an emulsion was obtained. After incubation for 2min at room temperature, the emulsion was centrifuged for 15min at 14,000 rpm, 4°C and the aqueous phase containing the RNA was collected. The RNA was then precipitated by isopropanol in the ratio of half the volume of Trizol® and incubated for 10min at room temperature. The precipitated RNA was collected after centrifugation for 10min at 14,000 rpm and 4°C. The precipitate was air dried and suspended in 50µl of autoclaved water treated with 0.1% DEPC (diethyl pyrocarbonate).

Extraction of cosmidial DNA

Identified recombinant clones in pTCF cosmid were obtained from genomic library in *E. coli* ED8767 transformed strain (kindly made in the Debatisse-Butin laboratory at Pasteur Institute, Paris, France). Cosmid DNA preparation, unlike the plasmid DNA, cannot be extracted by the CTAB method because it does not inactivate endogenous DNAses, which co-precipitate with the cosmids. The alkaline method was then used, with some adaptations in relation to Sambrook *et al.* (1989). In short, 100mL of transformed bacteria culture was incubated in LB/ampiciline 50µg/mL medium for 16 hours at 37°C under vigorous agitation. The culture was centrifuged at 4°C for 5min, 4,000 rpm. The supernatant was discarded and the medium excess removed by drying the inverted tube on a filter paper. The sediment was suspended in 5mL of iced solution I (0.05M glucose, 0.025M Tris-HCl pH

8.9, 0.01M EDTA). Some lysozyme crystals were added and the solution was incubated for 10min at room temperature. After the addition of 15mL of solution II [0.2N NaOH, 1% SDS (sodium dodecylsulphate)], under delicate agitation, incubation proceeded in the ice for 10min. Then, 11 mL of iced solution III (60mL of 5M potassium acetate, 11.5mL glacial acetic acid, 28.5mL deionized water) was added, maintaining delicate agitation. After a new incubation in ice for 30min, the material was centrifuged at 4°C for 15min, 4,500 rpm. The aqueous phase was separated by filtration and the extraction carried out using phenol, phenol/chloroform and chloroform. The aqueous phase was collected and precipitated using 0.6 volumes of isopropanol and incubated in ice for 30min. The DNA was recovered by centrifugation at 4°C for 30min, 4,500 rpm. The DNA was then washed using iced 70% ethanol, suspended in 300µl of TE pH 8.0 containing 10µg/mL RNAse and incubated for 30min at room temperature.

Quantification of the nucleic acids was carried out by spectrophotometric readings of the samples at 260 and 280nm (Sambrook *et al.*, 1989). Total DNA and the recombinant DNA clones were digested by restriction enzymes from Pharmacia, Gibco-BRL and Biolabs. The digestions were carried out using the manufacturing recommendations at 37°C for 2 hours.

Extraction of plasmid DNA and obtention of pPHR102 insert:

The extraction of the plasmid DNA was carried out to produce the probe pPHR102. Culture at 3mLof *E. coli* DH5α transformed by plasmid pPHR102 was incubated overnight in LB medium (Sambrook *et al*, 1989) at 37°C and centrifuged for 5min at 10,000 rpm at room temperature. The sediment was suspended in STET (8% saccharose, 0.05M EDTA, 0.5% Triton X-100, 0.05M trishydroximethyl aminometane, pH 8.0) and lysozyme at 50µg/mL and incubated for 5min at room temperature. After this period, the preparation was boiled for 1min and centrifuged for 10min at 14,000 rpm at room temperature. The chromosomic DNA and the proteins that form the gelatinous sediment were removed using a sterilized toothpick and 12µl of 5% CTAB (cethyl trimethyl ammonium bromide) was added. After centrifugation at 14,000 rpm for 5min at room temperature, the sediment was suspended in 300µl of 1.2M NaCl, 700µl of absolute ethanol and centrifuged for 15 s at 14,000 rpm, 4°C. The precipitated plasmid DNA was suspended in 50µl TE (0.01M tris-HCl, 0.001M

EDTA, pH 8.0) plus 10μg/mL RNAse and incubated for 30min at room temperature. After quantification, an aliquot of the plasmid was digested by Hind III to obtain the insert corresponding to the largest part of the primary transcript of the ribosomal gene. After electrophoresis in 0.7% agarose gel, the band corresponding to the insert HGRF55 was electroelucted and extracted by phenol, phenol/chloroform and chloroform. After digestion, the pPHR102 insert was used to prepare the probe.

Southern and northern blots:

DNA samples were applied in 0.7% agarose TBE gel. After electrophoresis, the gels were photographed and the migration distances of the standard lambda DNA Hind III were measured and noted. The gels were incubated in 0.24N HCl for 20min under agitation, washed with water and incubated in 0.5N NaOH and 1.5M NaCl for 40min. The capillary system described by Sambrook *et al.* (1989) to transfer DNA to N+ nylon membrane (Amersham, UK) was used. Electrophoresis of the RNA samples was carried out in denaturing 1% agarose with 1x MOPS buffer: 0,02M MOPS [3-(N-morpholino) propanesulfonic acid]; 0,005M sodium acetate; 0,0001M EDTA; pH 7,0, 6% formaldehyde, 60V. The transfer was processed by capillarity (Sagerström and Sive , 1996).

Hybridization:

Ribosomal probe preparation used on Southern blot essays was carried out using the Hind III/Hind III 5-Kb insert of the plasmid pPHR102, kindly provided by Richard Braun, Bern, Switzerland, containing the largest part of the primary transcript of the rRNA gene of *Physarum policephalum*. *Blastocladiella emersonii* Be97M20 clone probed by pPHR102 was obtained from a pTCF cosmide genome library and it was used as probe in northern blot analysis. Labeled probes were carried out using the Random Primer Kit (Pharmacia) and dCTP [32]P (Amersham). Labeled DNA was separated from free deoxynucleotides in a Sephadex G50 column (Pharmacia) packed in a 1-mL syringe with TE. Probe aliquots were submitted to a beta liquid scintilator for specific radioactivity quantification. *In situ* hybridization was probed by Sal I Be97M20 digest, labeled by Random Primer labeling kit and biotin 11-dUTP (Boehringer Mannheim).

Nylon membranes pre-hybridized for approximately 2 hours at 65°C in hybridization buffer (0.5M sodium phosphate pH 7.2, 7% SDS, 0.001M EDTA, 1% bovine serum albumin; Church and Gilbert, 1984) were incubated for about 16-18 hours in a hybridization oven at 65°C with agitation. After hybridization, the membranes were washed in 2X SSC (0.3M NaCl, 0.03M trisodium citrate pH 7.0) plus 0.5% SDS for 5min at room temperature and in 1X SSC plus 0.5% SDS for 30min at 50°C. Hybridized washed membranes were covered by plastic film and placed in a hypercassette with Hyperfilm autoradiographic film (Amersham), where they were kept at -70°C. Exposure time varied according to the specific radioactivity of the probe and the quantity of material in the membranes. After exposure, the films were developed using a Dektol developer and a fixing bath, both Kodak.

Results and discussion

After hybridization using the *P. polycephalum* rDNA probe pPHR102, 14 clones probably carrying *rDNA* sequences of *B. emersonii* were found. The clones were confirmed by another hybridization procedure and the corresponding cosmide preparations were digested by Sal I. Figure 1 shows the analysis of three of these clones. Be97M20 clone presented five positive bands probed by pPHR102 with estimated molecular weights: 56,700 bp, 18,500 bp, 14,700 bp, 11,800 bp and 7,000 bp. In turn, the clone *S2G11* presented three hybridized bands: 17,100 bp, 9,400 bp and 8,400 bp. The last clone, *S016E5*, presented six bands: 24,900 bp, 11,800 bp, 10,100 bp, 8,700 bp, 5,200 bp and 4,100 bp (Table 1).

Table 1. Estimated weight of bands from ribosomic clones digested by Sal I and probed with pPHR102 in Southern blots.

Clones	Base sizes in pairs					
Be97m20	56700	18500	14700	11700	7000	
S2g11	17100	9400	8400			
S016e5	24900	11800	10100	8700	5200	4100

Southern blots from total DNA of *B. emersonii* digested by Eco RI, Hind III, Sal I, Eco RI/Hind III, Eco RI/Sal I, Hind III/Sal I and Eco RI/Hind III and Sal I, were hybridized with the probe pPHR102 (Figure 2). Total DNA digested by Eco RI resulted in the hybridization of bands with the approximate sizes: 17,100 bp, 12,300 Bp, 10,400 bp and 3,200 bp. After digested by Hind III, the bands observed were: 10,400 bp, 8,500 bp, 6,900 bp, 5,700 bp and 3,900 bp. The digestion by Sal I hybridized with fragments 46,500 bp, 38,000 bp and 14,000 bp. The following bands were obtained through combined digestions by Eco

RI/Hind III: 20,100 bp, 10,800 bp, 8,900 bp, 3,000 bp and 1,700 bp. Eco RI/Sal I rendered bands with 11,600 bp, 8,000 bp, 5,600 bp and 3,000 bp. Conversely, the enzyme combination Hind III/Sal I led to 10,800 bp, 8,500 bp and 6,500 bp bands. Southern blot of total DNA digested by Eco RI/Hind III/Sal I resulted in the hybridization of the bands: 9,400 bp, 7,600 bp, 6,300 bp, 5,400 bp and 3,000 bp (Table 2).
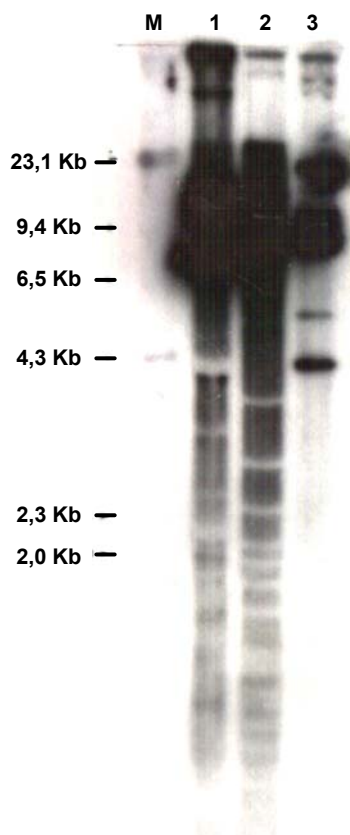

Figure 1. Southern blot of ribosomic clones digested by Sal I. M. molecular weight lambda DNA Hind III; 1: Be97M20; 2: S2G11 and 3: S16E5.

Table 2. Estimated weight of the bands identified by Southern blot of digested genomic DNA from *B. emersonii*. Hybridization has been proceeded against pPHR102 probe.

Enzymes	Base sizes in pairs				
Eco RI	17100	12700	10400	3200	
Hind III	10400	8500	6900	5700	3900
Sal I	46500	38000	14000		
Eco RI / Hind III	20100	10800	8900	3000	1700
Eco RI / Sal I	11600	8000	5600	3000	
Hind III / Sal I	10800	8500	6500		
Eco RI / Hind III / SalI	9400	7600	6300	5400	3000

Northern blot of total RNA from *B. emersonii* probed by Be97M20 DNA indicated that this clone represents only a fraction of the transcript of 18 S rRNA (Figure 3).

Regions coding rRNAs have been used to study origins of replication sites, allowing structural and functional analysis of the replicons in diverse systems. In the ciliate *Euplotes*, two-dimensional gels have been used to locate replication origins during vegetative growth and in macronuclear development. The results indicated that the telomeres and the promoter region cooperate to recruit replication initiation complexes in the rDNA genes (Tan *et al*, 2003). The origin recognition complex (ORC) is a set of proteins that are highly conserved (Kong *et al*, 2003). Chromatin elements, like nuclear matrix attachment regions (MARs), have a crucial role in DNA replication. MARs identified in nontranscribed spacer (NTS) of silkworm *Attacus ricini* rDNA employ ARS activity in yeast, suggesting that the chromosome structure and the replication origin of rDNA have common features during evolution (Chen *et al*, 2002). The aquatic fungus *B. emersonii* is a organism that presents a special life cycle, genetic and biochemical characteristics similar to higher eukaryotic cells and therefore, it is suitable to be a study model to the origin of replication sites. In this work we have isolated and initiated the characterization of a region that contains rDNA sequences of this microrganism. Hybridization using a *Physarum policephalum* rDNA probe on a genome library of *B. emersonii* resulted in the identification and isolation of clones carrying ribosomal sequences. Southern blot analysis of Be97M20 clone digested by Eco RI, Hind III, Sal I, Eco RI/Hind III, Eco RI/Sal I, Hind III/Sal I and Eco RI/Hind III/Sal I, showed that the region shows various sites of restriction for these enzymes. The complete restriction mapping of this region was not possible because of its complexity. Nevertheless, Southern blots of the clone Be97M20 digested by Sal I and hybridized by pPHR102 probe presented a homologous region to 18S RNA gene (Figure 3). Northern blot has showed that this clone contains a sequence homologue to 18S RNA gene, a unit of transcription not yet described, located upstream to genes 5.8S and 28S (Auwera and Wachte, 1996). A detailed study of this sequence, including subcloning, sequencing, *in situ* hybridization and bidimensional gel analysis are currently in course in our laboratory.
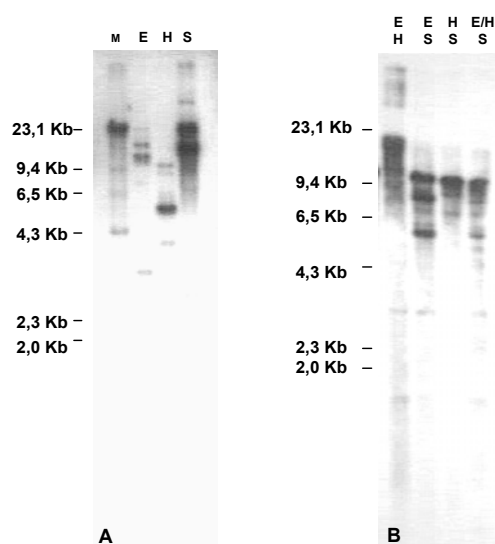
Figure 2. *B. emersonii* genomic DNA digested by restriction enzymes and hybridized against pPHR102 probe. A, line E: DNA digested by Eco RI, line H: Hind III and line S: Sal I. B, line EH: DNA digested by Eco RI and Hind III, line ES: Eco RI and Sal I, line HS: Hind III and Sal I, EHS: Eco RI, Hind III and Sal I.
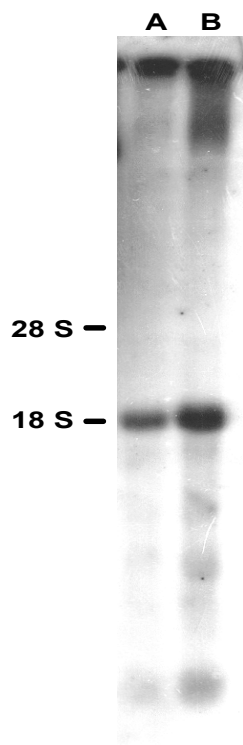
Figure 3. Northern blot of total RNA from *B. emersonii* zoospores hybridized against the Be97M20 probe.

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