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AUTHOR(S):

UEDA, Mitsuyoshi; HIKIDA, Masaki; ATOMI, Haruyuki; TANAKA, Atsuo

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Isolation of the Gene Encoding Yeast Peroxisomal Isocitrate Lyase by a Combination of the Plaque Hybridization with Non-Radioactive Probes and the Amplification of Phages in a Small Scale

By

Mitsuyoshi UEDA*, Masaki HIKIDA*, Haruyuki ATOMI*
and Atsuo TANAKA*

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Abstract

A genomic DNA encoding isocitrate lyase, one of peroxisomal enzymes, was successfully isolated from an *n*-alkane-utilizable yeast genomic library prepared in a λEMBL phage by a combination of the plaque hybridization with a non-radioactive, biotin labeled, cDNA and the amplification of the phages in a small scale. Three clones, partially overlapping, with sizes of about 11, 13 and 16 kbp respectively were finally obtained. The genomic Southern blot analysis using the biotin-labeled probe suggested the presence of one isocitrate lyase gene in the genomic DNA.

1. INTRODUCTION

Radioactive labeling of nucleic acids has been an indispensable technique in the development of genetic engineering, in spite of its inconvenience inherent to the handling and stability of radioisotopes. Therefore, much effort has been made to exploit available non-radioactive probes. In a series of experiments of

* Department of Industrial Chemistry

Abbreviations: 20 ×SSC, NaCl 175.3 g, sodium citrate 88.2 g/liter (pH 7.0), (5 ×SSC, 2 ×SSC, 1 ×SSC and 0.1 ×SSC were prepared from the 20 ×SSC solution with 4, 10, 20 and 200 times dilution.) : 2 ×SSCP, NaCl 14 g, sodium citrate 8.8 g, KH_2PO_4 3.54 g, sodium EDTA 0.74 g/liter (pH 7.4) : 50 ×Denhardt's solution, Ficoll 400 10 g, polyvinylpyrrolidone 10 g, bovine serum albumin 10 g/liter: LB medium, Bacto-tryptone 10 g, Bacto-yeast extract 5 g, NaCl 5 g, glucose 1 g/liter (pH 7.5) : NZY medium, NaCl 5 g, $MgSO_4 \cdot 7H_2O$ 2 g, yeast extract 5 g, NZ amine 10 g/liter (pH 7.5) : SM solution, NaCl 5.8 g, $MgSO_4 \cdot 7H_2O$ 2 g, 1 M Tris-HCl (pH 7.5) 50 ml, gelatin 2 g/liter: TE, 10 mM Tris-HCl (pH 8.0), 1 mM sodium EDTA.

gene cloning, if a process of screening could be easily carried out with safety, studies using DNA manipulation may be more popular and familiar.

As for methods using non-radioactive probes, antibodies conjugated with peroxidase, alkaline phosphatase or β-galactosidase have been used to screen clones from cDNA libraries prepared in phages,[1] and also avidin conjugated with peroxidase or alkaline phosphatase to screen clones from colonies of *E. coli*.[2] In comparison with these examples, screening from genomic libraries prepared in phages with non-radioactive labeling probes is difficult because of a lower content of phage DNA in one plaque than that of *E. coli* plasmid DNA in one colony, and of the inconvenience to isolate and identify the inserted DNA in phage DNAs.

This paper describes a convenient isolation method of the gene encoding one of peroxisomal enzymes from the yeast genomic library by a combination of a plaque hybridization method with non-radioactive probes and a convenient amplification of phages in a small scale.

2. MATERIALS AND METHODS

Preparation of the yeast genomic DNA library in λEMBL phage.

The *Candida tropicalis* nuclear DNA was prepared according to the methods of Cryer *et al.*[3] and Kamiryo *et al.*[4] The total yeast genomic DNA was partially digested with Sau 3 AI. The digestion was carried out until the size of the fragments became to be 10 kbp – 20 kbp. In the meantime, λEMBL 3 was excised at the Bam HI site and used as a vector for the preparation of the yeast genomic DNA library. DNA fragments excised from the genomic DNA with Sau 3 AI were dephosphorylated with bacterial alkaline phosphatase and inserted into the λEMBL 3 vector DNA. The constructed DNAs were packed in phage coat proteins using a packaging kit (STRATAGENE, La Jolla, CA, USA) and transfected to *Escherichia coli*. We were successful in constructing the yeast library with 7×10^5 plaques/μg DNA.

Preparation of biotin-labeled DNAs.

cDNA encoding isocitrate lyase isolated from the λgt 11 cDNA library with anti-isocitrate lyase antiserum[5] was labeled with biotin 11-dUTP using the nick translation system (BRL Inc., Gaithersburg, MD, USA) and used as the probes at 150 – 200 ng/ml.

Screening with the biotin-labeled probe DNA.

The screening of clones was carried out according to the method of Benton and Davis[6] with the following modifications. Cells (*E. coli* P 2392) were infected

with λEMBL 3 phages, plated in a top agar (NZY medium containing 0.7% agar), and then spread on 90 mm diameter agar-plates prepared with NZY medium. After the cells were grown for at least 12 h at 37℃ to form large plaques, the plates were placed at 4℃ for 2 h, and then phages were transferred to a nitrocellulose filter by placing the dry filter (Millipore, 0.45 μm) on the lawn of cells for 15 min at 25℃. During this adsorption, the filters and plates were marked for orientation. The filters, carefully lifted up, were dried for 30 min at 25℃. Phages were denatured and fixed *in situ* by dipping the filters in a solution containing 0.5 M NaOH and 1.5 M NaCl for 10 min. The filters were then neutralized by dipping twice in a 0.5 M Tris-HCl buffer (pH 7.5) containing 1.5 M NaCl for 10 min. Thereafter, the filters were soaked in 1 ml/filter of 1 ×SSC containing 1 mg proteinase K (Merck, Darmstadt, FRG) at 37℃ for 1 h to remove the proteins of the phages. The filters were washed twice every 5 min in a 0.2 M Tris-HCl buffer (pH 7.4) containing 2 ×SSCP, blotted, dried for 60 min at 25℃, and baked at 80℃ for 3 h. After baking, the filters were soaked in a 5 ×SSC solution followed by pre-hybridization. The solution (per 10 filters) for pre-hybridization was composed of 5 ml of 100% formamide (deionized form), 2.5 ml of 20 ×SSC, 1.0 ml of 500 mM sodium phosphate buffer (pH 6.5), 0.25 ml of 10 mg /ml salmon sperm DNA (Sigma, St. Louis, MO, USA), 0.1 ml of 10% SDS, 1.0 ml of 50 ×Denhardt's solution, 0.5 g of Dextran sulfate (Pharmacia, Uppsala, Sweden) and 0.15 ml of sterilized H_2O. This solution was first boiled for 10 min, immediately chilled on ice for 10 min and then used. The solution for pre-hybridization and the filters were packed in a plastic-sealed bag and incubated for 3 h at 42℃. The filters were transferred from the solution for pre-hybridization to the solution for hybridization in which sterilized water in the solution for pre-hybridization was substituted by the biotin-labeled cDNA solution (150 − 200 ng /ml) for isocitrate lyase. Then the bag was sealed and incubated for 21 h at 42℃. After hybridization, the filters were taken out and washed in a large volume (10 to 15 ml per filter) of 2 ×SSC containing 0.1% SDS at 25℃ for 20 min, and then for 30 min in 0.1 ×SSC containing 0.1% SDS at 50℃. Next, the filters were soaked in buffer 1 (100 mM Tris-HCl (pH 7.5), 1.0 M NaCl, 2 mM $MgCl_2$, 0.05% Triton X-100 and 3 % bovine serum albumin (Fraction V, Wako, Osaka, Japan)) for 20 min at 42℃ to decrease the background for colorimetry. The filters were then soaked in buffer 1 containing 30 μl of 0.5 μg/μl streptavidin-alkaline phosphatase (ZYMED Lab. Inc. San Francisco, CA, USA) for 10 min at 25℃ and washed with buffer 1 for 1 h at 25℃ followed by washing with buffer 2 (100 mM Tris-HCl (pH 9.5), 1.0 M NaCl, 5 mM $MgCl_2$) for 20 min at 25℃. Finally, the filters were soaked in 10 ml of buffer 3 (100 mM Tris-HCl (pH 9.5), 100 mM NaCl,

5 mM- $MgCl_2$) containing 40 μl of nitro blue tetrazolium (75 mg/ml ‡70% (v/v) dimethylformamide) (Wako, Osaka, Japan) and 40 μl of 5-bromo-4-chloro-3-indolyl phosphate (50 mg/ml dimethylformamide) (Sigma, St. Louis, MO, USA) in the dark. The signals appeared 5 to 10 min after the reaction started. The reaction was terminated by soaking the filters in buffer 4 (10 mM Tris-HCl (pH 7.5), 1.0 mM EDTA). Positive candidate clones were picked up and rescreenings were carried out with the same method until all the plaques on the plate had positive signals.

Convenient isolation of λ EMBL phage DNA.

SM solution (5 ml) was poured on a plate covered with plaques all having positive signals, and then 160 μl of $CHCl_3$ was added. The plates were incubated for 16 h at 4 ℃. The solution on the plates was transferred to a Corex tube (Corning, NY, USA), and $CHCl_3$ was added to give 5 % (v/v). This tube was centrifuged at 8,000 rpm for 15 min (KUBOTA KR-180 type, RA-3 rotor, Tokyo, Japan) at 0 ℃. By this amplification of the single phage, a titer about 1.6×10^9 pfu was obtained. This amplified phage solution was infected to the cells (*E. coli* P 2392) at moi 0.2 and incubated at 37℃ for 15 min. The cells transfected with the phage (0.1 ml) were inoculated in 4 ml of a medium composed of 10 g NZ-amine, 5 g NaCl, 5 g Bacto-yeast extract and 2 g $MgSO_4 \cdot 7H_2O$/liter (pH 7.5),[7] and cultivated with shaking at 37℃ for 12 h. After cultivation, 0.1 ml of $CHCl_3$ was added and the cell suspension was shaken again at 37℃ for 20 min. This solution was centrifuged at 8,000 rpm for 15 min at 25℃. The supernatant obtained was transferred to a Corex tube, added by 1 μl of 10 mg/ml RNA nuclease (Sigma, St. Louis, MO, USA) and 5 μl of 1 mg/ml DNA nuclease (Sigma, St. Louis, MO, USA), and incubated for 30 min at 37℃. After the reaction, 5 ml of buffer A (SM solution containing 20 % (w/v) polyethylene glycol 6000 and 2 M NaCl) was added. The solution was mixed well and placed in ice water for 1 h. Phage particles were obtained as a pellet by centrifugation at 9,500 rpm (RA-3 rotor) for 20 min at 4 ℃. After removing buffer A thoroughly, 0.5 ml of SM buffer was added and the pellet was solubilized completely. This solution was added in a sample tube, mixed with 5 μl of 10 % SDS and 5 μl of 0.5 M EDTA (pH 8.0) and incubated at 68℃ for 15 min. The DNA of the phages was extracted with 500 μl of water-saturated phenol, followed with 500 μl of $CHCl_3$. The DNA was precipitated by addition of iso-propanol (500 μl) and was kept at − 70℃ for 20 min. After rinsing with 70 % ethanol and lyophilization, the DNA was solubilized with 50 μl of TE solution containing 2 μl of 10 mg/ml RNA nuclease (DNA nuclease-free). This solution (10 μl) was enough for detecting the inserted DNA or sub-cloning. For sub-cloning, digested DNAs were elec-

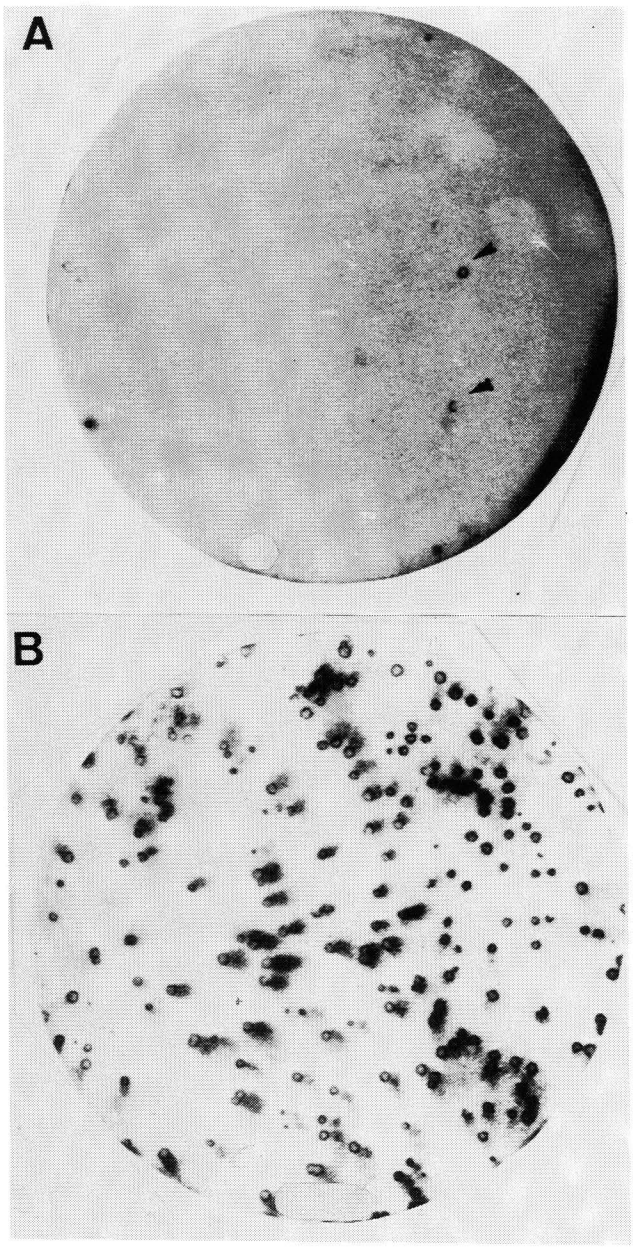

Fig. 1. Screening of a λ EMBL 3 *Candida tropicalis* genomic DNA library for isocitrate lyase clones. (A) A representative nitrocellulose filter of a first screening using the biotin-labeled cDNA for isocitrate lyase. Arrow heads indicate positive signals. (B) A representative filter after plating and screening repeatedly untill all plaques on the plate produced positive signals.

trophoresed on a low-temperature melting agarose gel. Then, the target DNA was excised out, thawed at 65℃ and extracted with water-saturated phenol.

Genomic DNA hybridization analysis.

Aliquots (each 20 μg) of total genomic DNA of *C. tropicalis* were digested with several endonucleases (TOYOBO, Osaka, Japan) and separated by electrophoresis on a 0.8% agarose gel. The DNA fragments in the gel were transferred onto a nitrocellulose filter according to the method of Maniatis *et al.*.[8] Prehybridization (42℃, 3 h) and hybridization (42℃, 18 h) using biotin-labeled probes were carried out by the same method as described above. The washing of filters after hybridization was performed with 2 ×SSC containing 0.1% SDS for 20 min at 25℃ and 0.1 ×SSC containing 0.1% SDS for 30 min at 50℃.

3. RESULTS AND DISCUSSION

Screening of clones harbouring isocitrate lyase DNA

By the first screening using a biotin-labeled cDNA probe (*ca.* 1000 bp) reported in the previous paper,[5] 15 positive plaques were detected from about 90,000 plaques. In Fig. 1-A, one representative filter with positive signals is shown. Positive plaques were seen as navy-blue and doughnut-shaped rings. One of the plaques with a positive signal was picked up and rescreened several times until all the plaques became positive (Fig. 1-B). With this method, clones harbouring other peroxisomal enzyme DNAs were also isolated using the respective biotin-labeled cDNAs (data not shown). Generally, the amount of DNA in one plaque is suggested to be one-order lower than that in one colony transformed with foreign DNAs. However, our method proved to be valid for the plaque hybridization to screen the target gene from the yeast genomic library with cDNA probes. In this case, the navy-blue positive plaques made it easy to recognize the positive clones, because the background can be made clear by shortening the color-developing reaction period. When a biotin-labeled probe is used to screen candidate clones by the plaque hybridization method, it should be noticed that the following procedures are essential: (1) deproteination with proteinase K to prevent a false signal appearance by phage proteins, (2) prehybridization for 3 h at 42℃ with the solution containing dextran sulfate, and (3) blocking of a non-specific binding site by biotin or avidin using 3% bovine serum albumin solution.

Isolation and evaluation of inserted DNAs from phages

Purified phages from a single plaque were infected to *E. coli* cells and amplified by the convenient method described in 'MATERIALS AND METHODS'.

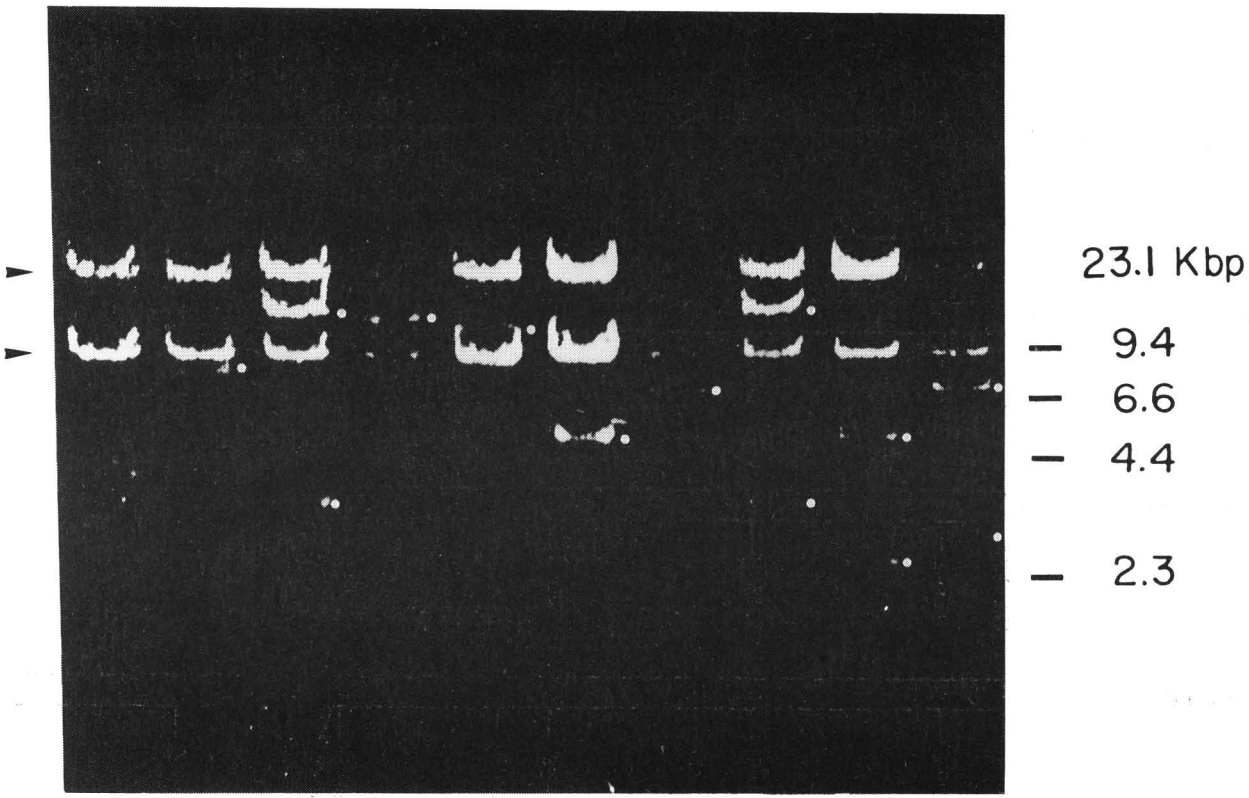

Fig. 2. A representative electrophoresis of inserted foreign DNAs in arm DNAs of λ EMBL phages isolated by the convenient amplification method. Arrow heads indicate the bands of 20 kbp and 9 kbp derived from λ EMBL 3 arms after digestion with Sal I. Other bands (white dots) are inserted DNAs in each clone.

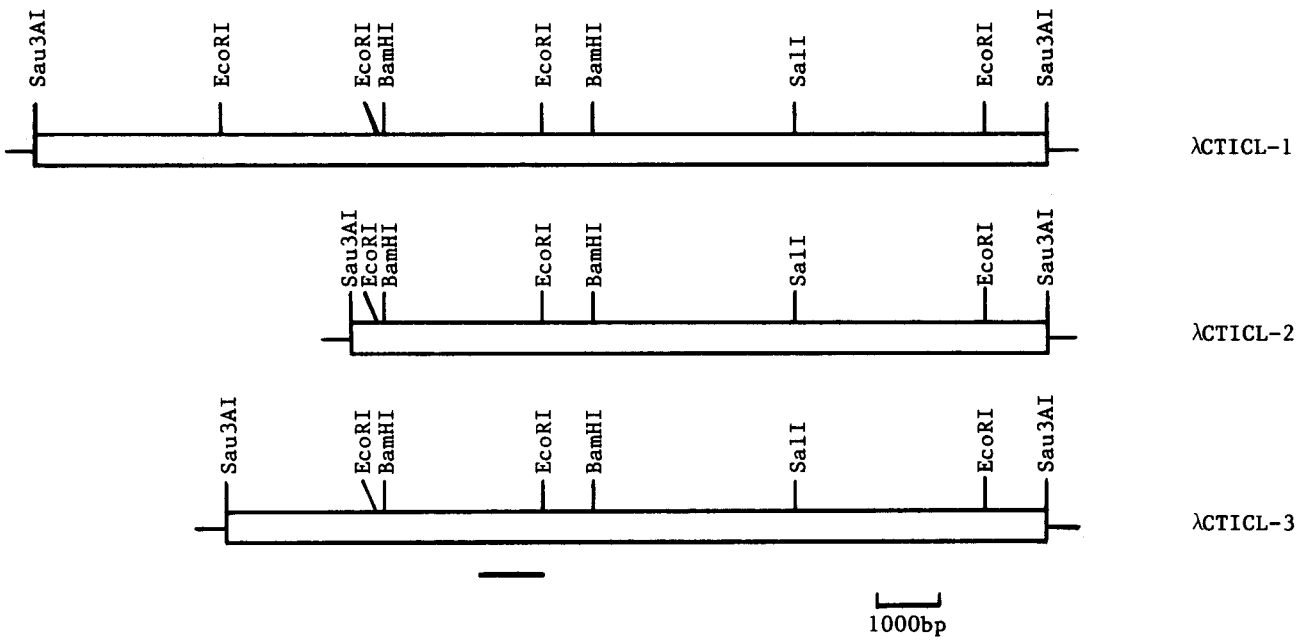

Fig. 3. Restriction maps of the representative DNAs for isocitrate lyase isolated. A bar expressed the location of cDNA for isocitrate lyase used as the probe. The size of λCTICL-1 is about 16 kbp and included all other DNAs described in the text.

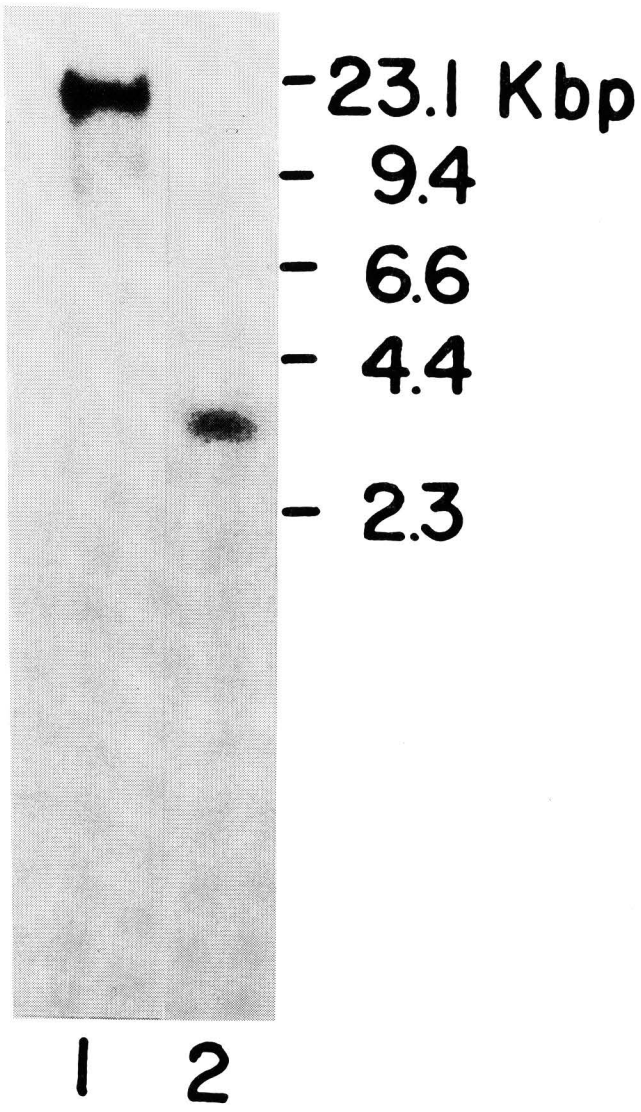

Fig. 4. Genomic Southern blot analysis of *Candida tropicalis* DNA using the biotin-labeled DNA probe for isocitrate lyase. Twenty μg of genomic DNA isolated was digested with Sal I (lane 1) and Bam HI (lane 2), respectively, and electrophoresed.

When the λEMBL phage DNA isolated in a small scale was digested with Sal I, 20 kbp and 9 kbp fragments of λEMBL arm DNA and several DNA fragments were observed on 0.8% agarose gel electrophoresis (Fig. 2). This amplification method to isolate phage DNA was as convenient as the small scale method used

to isolate plasmids from an *E. coli* single colony. Furthermore, the application of low-temperature melting agarose for electrophoresis made it easy to excise only the inserted DNA to carry out sub-cloning of the DNA in, for example, a pUC 19 plasmid. After sub-cloning in the pUC 19 plasmid, restriction mapping of the DNAs obtained was performed. Inserted DNAs obtained were named λCTICL-1, -2 and -3 (Fig. 3). In this case, the longest inserted DNA was about 16 kbp, and other DNA fragments detected were all overlapping to the 16 kbp fragment, judging from the restriction mapping. The combination of screening of the phage library using a biotin-labeled probe and a rapid isolation of DNA would make manipulation of phage DNAs convenient.

Detection of isocitrate lyase gene on the genomic DNA

As the result of a genomic Southern blot analysis with the biotin-labeled DNA after digestion of total yeast DNA with Sal I and Bam HI, whose sites were absent in the probe DNA, one genomic DNA fragment including DNA encoding isocitrate lyase was detected (Fig. 4). In this experiment, the important point was that the addition of dextran sulfate to the solution for pre-hybridization and hybridization made strong signals, when the biotin-labeled probe was used. Even when a probe as small as 140 bp was used, it was possible to detect signals.

Acknowledgements

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