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Application of In Situ PCR to Yeast Cells for Screening YAC Libraries

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ABSTRACT

We have used *in situ* PCR technology in yeast cells with the ultimate goal of cloning and screening genomic yeast artificial chromosome (YAC) libraries. The target sequences in YAC clones were amplified "in situ" in yeast cells by the same set of microsatellite primers used in solution-based PCR screening. The method is fast and sensitive and obviates the steps required for individual isolation of DNAs from hundreds to thousands of YAC clones and thus has an advantage over conventional solution-based PCR screening. This approach can conceivably be applied to the products of automated robotic workstations.

INTRODUCTION

In situ polymerase chain reaction (PCR) is an extremely sensitive morphological technique used to detect minute quantities of DNA or RNA in tissue sections or intact cells of eukary-

otic and prokaryotic systems. This technology is derived from the combination of conventional PCR widely used by molecular biologists and *in situ* hybridization commonly used by morphologists and geneticists (12). Here, we demonstrate *in situ* PCR technology, to our knowledge, for the first time in yeast cells with the ultimate goal of cloning and screening genomic yeast artificial chromosome (YAC) libraries. The advent of cloning in yeast vectors was a boon to research groups interested in genome mapping of many organisms, including, for instance, human, mouse, *Drosophila* and *Caenorhabditis elegans* (2,3,6,8,10), but screening YAC libraries has proven to be much more tedious and time-consuming than screening recombinant libraries constructed in the more conventional plasmid, lambda and cosmid vectors in bacteria. The current strategy to screen YAC libraries is based on solution PCR and requires the physical isolation of DNA and culturing of tens of thousands of individual clones. A typical YAC library of 30 000 clones can be screened by a "super" and "sub-pool" scheme, which requires approximately 100 PCRs to reach the coordinates of a single YAC clone of interest (10). The technical approach described here obviates the individual isolation of DNAs from thousands of clones and can easily be applied to the products of automated robotic workstations (13). DNA micro-array technology developed in many genomic systems, including human, mouse and yeast, requires individual isolation of DNAs from hundreds to thousands of clones before their transfer and further analysis on glass slides (1,4,7). *In situ* amplification of target sequences directly in yeast cells bypasses this time-consuming step and provides a simple and powerful approach to analyze micro-arrayed libraries by detecting fluorescently labeled *in situ* PCR products within cells using fluorescent microscopy. With this technology, it is conceivable to transfer the entire YAC library on a single slide and screen by a single PCR. Since instrumentation for automated detection of fluorescence signals from rapidly developing DNA micro-array systems is now commercially available, the yeast *in situ* PCR technology has the

potential to be useful in high-throughput approaches to genome analysis.

MATERIALS AND METHODS

Growth and Attachment of Cells on Glass Slides

The AB1380 strain of yeast cells was grown in selective medium [0.17% yeast nitrogen base (wt/vol), 0.5% (NH₄)₂SO₄ (wt/vol), 1% casein (wt/vol) and 0.01% adenine hemi-sulfate (wt/vol)] (Becton Dickinson Microbiology Systems, Sparks, MD, USA) and, since yeast cells do not otherwise adhere to glass, attached on silane-coated glass slides (gamma methacryloxypropyl-trimethoxysilane; ONCOR, Gaithersburg, MD, USA) or Concanavalin A (Con A) (20 µg/mL, type IV; Sigma, St. Louis, MO, USA). Although silane was used initially for attachment, glycolipids and glycans of the yeast cell wall appeared to have a stronger binding interaction with Con A-treated slides, which were soaked in 20 µg/mL Con A for 2–18 h. Except for initial experiments described in Figure 1, attachment and growth of cells were performed only on Con A-treated slides. Cells attached to Con A-treated slides were washed with distilled water and fixed with fresh methanol acetic acid mixture (3:1 vol/vol) for 30 min to 1 h. This procedure, similar to that used in standard cytogenetic procedures, does not lyse the yeast cell wall, but appears to extract both cellular and nuclear membranes so that yeast cells become permeable to reagents used in *in situ* PCR. The fixative was replaced once with a fresh aliquot after 15 min, and the slides were air-dried. In initial experiments, 0.5–1 U of lyticase (Sigma) in 0.01 M sodium phosphate containing 50% glycerol was also used along with TSB buffer (0.1 M Tris, pH 7.4, 1 M Sorbitol and 14 mM βmetOH) (Sigma) to digest the cell wall and permeabilize attached yeast cells. The treatment time ranged between 30 min and 2 h. The cells were rinsed with phosphate-buffered saline (PBS) after treatment. Even with the low concentrations of lyticase, significant number of cells were detached from the glass slides during subsequent treatments.

In Situ PCR

We have modified the in situ PCR protocol by Troyer et al. (14). The primer sets D18MIT58 and D18MIT35 were obtained from Research Genetics (Huntsville, AL, USA) and were also used in solution-based PCR to screen the Princeton-Whitehead mouse YAC library to select mouse-chromosome-18-specific clones, designated YAC18-25 and YAC18-49. The sizes of CA repeats amplified with D18MIT58 and D18MIT35 were 186 and 136 bp, respectively, in the C57BL/6J strain of

mice whose genome was used for the YAC library preparation (10). Reaction mixtures (50 μ L) consisted of 200 μ M each of dATP, dCTP and dGTP; 100 μ M dTTP; 100 μ M biotin-dUTP (Sigma) (when biotin-dCTP instead of dUTP is used 100 μ M dCTP was in the reaction mixture), 0.16 μ M of each PCR primer, 2.5 U of AmpliTaq[®] DNA Polymerase (PE Biosystems, Foster City, CA, USA), 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM MgCl₂, 0.001% (wt/vol) gelatin. Gene-Cone[™] chambers (Gene Tec, Durham, NC, USA) were used to eliminate the need

Figure 1. Detection of in situ PCR products on YAC clones YAC18-25 and YAC18-49 using primer pairs D18MIT35 and D18MIT58. YAC clones YAC18-25 and YAC18-49 were isolated from the Princeton-Whitehead mouse YAC library using the dinucleotide marker primer pair designated D18MIT35. Further PCR analysis of these clones with additional dinucleotide repeat markers showed that YAC18-25 can be amplified with both D18MIT35 and D18MIT58, while Y18-49 does not contain the genomic region amplified by D18MIT58. The YAC clones are members of a contig on mouse chromosome 18. Panels A and B show the in situ PCR on YAC18-25, and Panels C and D show reactions on YAC18-49 using primer sets D18MIT35 (A and C) and D18MIT58 (B and D). Cells on silane-coated slides (ONCOR) were treated with 0.1 mg/mL lyticase at 37°C, dehydrated with ethanol series and amplified by in situ PCR. The 50- μ L reaction mixture consisted of 200 μ M of each of dATP, dCTP and dGTP; 100 μ M dTTP; 100 μ M Biotin-dUTP, 0.16 μ M of each primer, 2.5 U of AmpliTaq DNA Polymerase, 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM MgCl₂, 0.001% (wt/vol) gelatin. PCR products were detected with FITC-labeled avidin, biotinylated goat anti-avidin and with Avidin-DCS-FITC. Cells were counter-stained with PI. As expected, YAC18-25 is positive (i.e., green fluorescence present) using both PCR primer pairs (D18MIT35 and 58), while YAC18-49 is positive with D18MIT35 but negative (red PI counterstain only) with D18MIT58.

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for coverslips and inconvenient usage of sealers on slides containing fixed yeast cells. A Gene-Cone chamber encloses a 15-mm diameter specimen area on a microscope slide to control evaporative loss of reagents during thermal cycling. In this system, the PCR reagents are pipetted into the top opening, and the top opening is sealed with an adhesive film that is impermeable to water vapor but accommodates pressure changes in the chamber. The self-adhesive Gene-Cone chamber was placed over dry and fixed yeast cells, and the glass slide was heated to 95°C for 5 min before the reaction mixture was added to the chamber. The 50 µL of reaction mixture were added into the Gene-Cone Chamber, and the slides were incubated for 1 additional minute at 95°C before the top of the chamber was sealed with the self-adhesive top seal to prevent breakage of the seal from heat pressure. Slides were placed on a slide adapter, which includes a thin heating platter. The platter transfers and spreads heat evenly to slide samples. A spring-pressure platter placed on top of slides applies light pressure to ensure intimate contact of the slide, heating platter and the heat block, thus eliminating air pockets that will alter heat transfer. A GeneAmp® PCR System 9600 Thermal Cycler (PE Biosystems) was used with the cycling profile of 94°C for 3 min, 58°C for 1 min and 72°C for 1 min followed by 24 cycles of 92°C for 1 min, 58°C for 1 min and 72°C for 1 min. Following the reaction, slides were removed from the thermal cycler and allowed to cool to room temperature. Gene-Cone chambers were removed by lifting the extended tab and peeling the chamber and adhesive off the slide.

Detection of In Situ PCR Products by Indirect Fluorescence

Slides were immersed in 2× standard saline citrate (SSC) for 2 min, then in PBS for 1 min. Each slide then was treated with 100 µL of 1:400 dilution of Avidin-DCS-FITC (Vector Laboratories, Burlingame, CA, USA) in 1× SSC, 1% bovine serum albumin (BSA) and 0.001% Tween®-20 (antibody buffer; Sigma). Slides were incubated at 37°C for 20–30 min in a moist chamber. Slides were washed with three

changes of 4× SSC, 0.01% Tween-20 (washing buffer) at room temperature for 5 min and blocked with 100 µL of 1× SSC, 3% BSA (blocking solution). One hundred microliters of 1:100 dilution of biotinylated goat anti-avidin (Vector Laboratories) in antibody buffer were applied to the slides, and they were incubated at 37°C for 20–30 min. Slides were washed and blocked as before and incubated with 100 µL of a 1:400 dilution of Avidin-DCS-FITC in antibody buffer at 37°C for 20–30 min. Slides were washed with washing buffer and counter-stained with 25 µL of VECTASHIELD® anti-fade (Vector Laboratories) containing 10 µg/mL propidium iodide (PI) (Sigma). Slides were examined with a Model BH2 Fluorescence Photomicroscope (Olympus, Lake Success, NY, USA) with the epifluorescence attachment and pictures were taken on Kodak 400 film (Eastman Kodak, Rochester, NY, USA).

RESULTS AND DISCUSSION

In Situ PCR in Yeast Cells

We have performed in situ PCR in YAC clones, which were isolated from the Princeton-Whitehead mouse YAC library (10). We chose to use a well-characterized pair of two YAC clones, which map to the D1 region of mouse chromosome 18 (YAC18-25 and YAC18-49). Both YAC clones were isolated by conventional PCR screening using a set of primers specific for a dinucleotide repeat, designated D18MIT35. The clones were shown by fluorescence in situ hybridization (FISH) to be non-chimeric and specific for mouse chromosome 18 (unpublished). Further analysis of these clones with additional PCR primer pairs has shown that Y18-25 is amplifiable with both D18MIT35 and a second primer set designated D18MIT58, while Y18-49 is not amplified with D18MIT58 primer set. These findings suggest the clones form an overlapping contig in the D18MIT35 locus. This selectivity of the primers on this pair of YAC clones allowed us to determine the specificity of the in situ PCRs in YAC-containing yeast cells.

An in situ PCR technique modified from direct in situ single-copy PCR

(DISC-PCR) (14) for mapping microsatellite sequences to porcine chromosomes was used. In this technique, chromosomal regions on porcine chromosomes were amplified on glass slides by using microsatellite sequence primers. Subsequently, amplified regions were detected non-isotopically to assign and orient a linkage group to pig chromosomes. Here, we have attached yeast cells containing YAC18-49 and YAC18-25 clones on glass slides and amplified the specific microsatellite sequences of the mouse genome in situ in the presence of the yeast genomic DNA background. In control solution-based PCR, yeast genomic DNA is not amplified by these mouse chromosome specific primers (data not shown). The key steps in in situ PCR are fixing yeast cells and making the cell wall and membranes permeable to primers, polymerase and free nucleotides, yet being capable of retaining the PCR products within the cell without significant leakage. Buffered formalin and ethanol have been used for fixation of various tissue samples and bacterial cells before subjecting them to in situ PCR (5,9,11). We have found that 3:1 mixture of methanol:acetic acid, as used in standard mammalian cytogenetic techniques, not only provides an excellent fixation procedure but also permeabilizes the membranes for in situ PCR reagents. Methanol:acetic acid fixative can keep the cell wall intact so that amplification products can be confined within the cells without significant leakage. In initial experiments, low concentrations of lyticase, which digests yeast cell wall, were also used. While lyticase effectively made the cells permeable, at the same time, it interfered with the attachment of cells to a glass surface. Since the essential part of the screening technique involves the strong attachment of cells on a glass surface, lyticase treatment of cells was eliminated as an alternative procedure in later stages of experiments.

Figure 1 shows fluorescent signals of the in situ PCR products on YAC clones YAC18-25 and YAC18-49 using primer sets of D18MIT35 and D18MIT58. Panels A and B show the results of in situ PCR performed on YAC18-25, and Panels C and D show reactions in YAC18-49 using primers sets for

D18MIT35 (A and C) and D18MIT58 (B and D). As expected, YAC18-25 is positive (i.e., green FITC fluorescence signal in cytoplasm) using both PCR primer pairs, while YAC18-49 is positive with D18MIT35 but negative with D18MIT58 (red PI counterstain only). Note that there is a stronger signal with D18MIT35 than with D18MIT58. The D18MIT35 primer set is also more robust in more traditional solution-based PCR. With this experiment, the selectivity of the primer pairs for each clone as observed in solution-based PCR was also successfully shown by in situ PCR

procedure, indicating that the detection technique is specific enough to isolate single clones during YAC library screening. The results shown in Figure 1 were obtained using a lyticase permeabilization treatment. It appears that in situ PCR products often leak out of the nuclear region of yeast cells, while the nuclear membrane stays intact surrounding the nuclear DNA showing bright orange signal (propidium iodide). This has been the typical feature of yeast cells treated with lyticase. In all subsequent experiments in yeast, a simple in situ fixation procedure was used,

Figure 2. Simultaneous in situ PCR of YAC clones YAC18-25 and YAC18-49 positioned in a defined array. YAC18-25 and YAC18-49 were grown separately under selective conditions, washed with PBS once and suspended in PBS. About 1 μ L of each cell suspension was spotted on a Con A-treated slide, in an array of three spots on the first and third rows of YAC18-49 and three spots in the center row of YAC18-25. Cells were fixed with 3:1 mixture of methanol:acetic acid (vol/vol), and in situ PCR was performed with D18MIT58 (positive for YAC18-25; negative for YAC18-49) as described in Materials and Methods. As expected, Y18-25 is positive (bright greenish yellow) for D18MIT58 (middle row), and Y18-49 (top and bottom rows) is negative for the same primers, showing the specificity of the in situ PCR conditions. Individual array members were photographed separately (rather than the entire array in a single field of view) because at the low magnification (10 $\times$) required to include the entire array simultaneously, sufficient fluorescent sensitivity to detect positive signals is not allowed.

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consisting of dipping the slides in a jar of freshly mixed 3:1 methanol:acetic acid fixative followed by air drying (see Materials and Methods). This fixation procedure most probably extracts both nuclear and cellular membranes while preserving the cell wall intact, since the positive fluorescent signal (greenish yellow) is observed throughout nuclear and cytoplasmic regions of yeast cells with less intense staining of nuclear DNA with PI (see below).

In Situ PCR in a Defined Array of Yeast Cells

In the experiment described above, each in situ PCR was performed as a separate reaction in a separate chamber. To demonstrate that in situ PCR is capable of detecting a positive clone from among a background of negative clones, a defined array of YAC18-25 and YAC18-49 clones in predetermined

locations was constructed on a single glass slide (Figure 2). YAC18-25 and YAC18-49 were grown separately in liquid media under selective conditions and spotted on Con A-treated slides with a defined 3×3 array. Three spots in the first and third rows were YAC18-49 cells, and the three spots in the center row were YAC18-25 cells. An in situ PCR was performed on this array in a common chamber containing primer pair D18MIT58 (positive for YAC18-25 but negative for YAC18-49). Figure 2 shows the results. As expected, YAC18-25 is positive (bright greenish yellow) for D18MIT58 (middle row), and YAC18-49 is negative (top and bottom rows) for the same primer pair, again showing the specificity of the in situ PCR performed in a common reaction chamber. The signal can be seen in the cytoplasmic regions of the fixed yeast cells and in the nuclei, presumably due to the extraction of nu-

clear envelope. However, the exterior cell wall/the residual cell membrane appears to retain sufficient PCR product to provide observed fluorescent signal. In situ PCR amplification of YACs bearing mouse genomic regions is not limited to microsatellite sequences, since any PCR primer pair known to be in the genomic region of interest can be used for this analysis. Since the GeneCone chambers enclose an area of 15 mm in diameter, they would not be suitable for screening the entire YAC library spotted on a glass slide. However, relatively bigger in situ PCR chambers enclosing larger areas are now available from various manufacturers. Conceivably, the target sequences in a YAC library can be amplified and detected in as few as 1–2 days after the initial cell attachment. The application of the in situ PCR technique to YAC library screening with the combination of micro-array technology has the potential

to greatly speed YAC library screening.

Recently, a cell suspension version of in situ PCR has been applied to bacteria to identify and characterize genetically single bacterial cells in natural systems (9). Lysozyme and proteinase K were used to permeabilize the bacterial cells, and in situ PCR was performed on bacterial cells while they were in suspension. Such techniques have limitations in that enzymatic treatment of the cell wall with lysozyme or lyticase (at least in case of yeast cells) also interferes with the attachment of cells to glass slides. A micro-scaled version of YAC libraries as described in this report utilizes glass slides as a support system, and the tight attachment of yeast cells to these slides is essential for the maintenance of defined locations required for micro-array technology. The fixation and permeabilization technique described here therefore has an advantage for analyzing genomic or expression libraries in yeast or bacterial hosts that are attached to glass in a micro-array format.

We have also developed FISH in yeast cells as an alternative way of screening replica plates generated by high-density replication systems (unpublished). Although the speed and the efficiency of both screening strategies are similar, FISH of yeast cells requires rather large DNA probes for the region of interest as opposed to pairs of primers required for screening with in situ PCR. Nevertheless, both strategies when combined with micro-array technology promise advancements in maintenance and screening of YAC libraries, which are a major resource for the mapping of mouse and human genomes and are essential tools for the human genome project.

REFERENCES

1. **Brown, P.O. and L. Hartwell.** 1998. Genomics and human disease - variations on variation. *Nature Genet.* 18:91-93.
2. **Burke, D.T., G.F. Carle and M.V. Olsen.** 1987. Cloning of large segments of exogenous DNA into yeast by means of artificial chromosome vectors. *Science* 236:806-812.
3. **Burke, D.T. and M.V. Olson.** 1991. Preparation of clone libraries in yeast artificial chromosome vectors. *Methods Enzymol.* 194:251-270.
4. **Cheung, V.G., J.P. Gregg, K.J. Gogolin-Ewens, J. Bandong, C.A. Stanley, L. Baker, M.J. Higgings, N.J. Nowak et al.** 1998. Linkage-disequilibrium mapping without genotyping. *Nature Genet.* 18:225-230.
5. **Chieu, K.P., S.H. Cohen, D.W. Morris and G.W. Jordan.** 1992. Intracellular amplification of proviral DNA in tissue sections using polymerase chain reaction. *J. Histochem. Cytochem.* 40:333-341.
6. **Coulson, A., R. Waterston, J. Kiff, J. Sulston and Y. Kohara.** 1988. Genome linking with yeast artificial chromosomes. *Nature* 335:184-186.
7. **De Risi, J.L., V.R. Iyer and P.O. Brown.** 1997. Exploring the metabolic and genetic control of gene expression on a genomic scale. *Science* 278:680-686.
8. **Garza, D., J.W. Ajioka, D.T. Burke and D.L. Hartl.** 1989. Mapping the Drosophila genome with yeast artificial chromosomes. *Science* 246:641-646.
9. **Hodson, R.E., W.A. Dustman, R.A. Garg and M.A. Moran.** 1995. In situ PCR for visualization of microscale distribution of specific genes and gene products in prokaryotic communities. *Appl. Environ. Microbiol.* 61:4074-4082.
10. **Kusumi, K., J.S. Smith, J.A. Segre, D.S. Koos and E.S. Lander.** 1993. Construction of a large insert yeast artificial chromosome library of the mouse genome. *Mamm. Genome* 4:391-392.
11. **Nuovo, G.J. and S.J. Silverstein.** 1988. Comparison of formalin, buffered formalin and Bovin's fixation on the detection of human papilloma virus DNA from genital lesions. *Lab. Invest.* 59:720-724.
12. **Nuovo, G.J.** 1995. In situ PCR. *Protocols and methods. PCR Methods Appl.* 4:151-167.
13. **Olsen, A.S., J. Combs, E. Garcia, J. Elliott, C. Amemiya, P. de Jong and G. Threadgill.** 1993. Automated production of high density cosmid and YAC colony filters using a robotic workstation. *BioTechniques* 14:116-123.
14. **Troyer, D.L., D.W. Goad, H. Xie, G.A. Rohrer, L.J. Alexander and C.W. Beattie.** 1994. Use of direct in situ single copy (DISC) PCR to physically map five porcine microsatellites. *Cytogenet. Cell Genet.* 67:199-204.

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