Rapid method for construction of yeast artificial chromosome human DNA libraries involving the trapping of cells in agarose films

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A simple method for the molecular cloning of fragments of more than one hundred kilobase pairs of exogenous DNA, by the encapsulation of cells in agarose beads, was reported previously for the construction of a human genomic DNA library in a yeast artificial chromosome (YAC) vector (in situ YAC construction) [1]. The efficiency of this procedure is impaired by the step in which agarose beads that contain human DNA fragments are melted before transformation. The incomplete solubility of the ligated human DNA fragment-YAC vector often results in lower than desirable frequencies of transformation. To overcome this problem we have developed a new improved method that involves use of an agarose film. The technical manipulations involved in the construction of clones of very large segments of human DNA are discussed.

YAC construction, in situ; Agarose film; Human chromosome 21 DNA library

1. INTRODUCTION

A system for the cloning of large fragments of exogenous DNA as artificial chromosomes in yeast has recently been described [1,2]. Attempts are being made to apply this technology to the construction of libraries of human chromosomes or of the total human genome [1–3]. For the construction of yeast artificial chromosome (YAC) libraries with large DNA inserts, the first critical step is the extraction of intact genomic DNA from the source material. The intact genomic DNA is extremely sensitive to physical forces during the manipulations required for cloning. Furthermore, technical skill is essential for extraction of DNA and partial digestion with restriction enzymes [2,3]. The initial successful attempts at cloning large segments of human DNA in YACs involved the use of agarose beads, such that cells were entrapped in beads of low-melting agarose to obtain the intact DNA molecules. The preparation of high molecular weight DNA from cells imbedding in agarose beads was based on a modification of the original work by Jackson et al. [4]. It reproducibly allows the preparation of very large fragments of DNA without shearing during isolation [1]. Extensive efforts have been made to construct a human chromosome 21 YAC library using the agarose bead method from a hybrid cell line, 153E9a3, a hybrid between Chinese hamster ovary (CHO) cells and human lymphocyte chromosome 21 [5]. However, we have noticed several problems during the construction of the YAC library, as follows. First, the extensive washing and repeated pipetting to break up the agarose beads often caused a 50% loss of recovery of final bead DNA products. Second, agarose beads of less than 10 μm in diameter were hard to melt before transformation. These problems resulted in incomplete digestion of the agarose matrix by agarase, which in turn reduced the amount of free DNA fragment transformed into host yeast spheroplasts. Third, entrapped cells were frequently aggregated in the agarose beads which also reduced the efficiency of DNA extraction.

To overcome these problems, we have developed a new method of DNA extraction that we call the agarose film method. We have used this improved cloning technique to prepare very large fragments of DNA as YAC recombinants and compared each step of this cloning procedure with the analogous step in the agarose bead procedure.

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2. MATERIALS AND METHODS

2.1. *Yeast strains, plasmids and chemicals*

Saccharomyces cerevisiae strain AB1380 (Mat-a, ade2-1, can 1-100, lys2-1, trp1, ura3, his5[psi+]), pYAC55, and the HY-1 YAC clone (125-kb DNA fragment from EB virus transformed human peripheral lymphocytes (CGM1)) were kindly provided by M. Olson [2]. Restriction enzymes, bacterial alkaline phosphatase (BAP) and DNA ligase were purchased from Toyobo, Tokyo, Japan. All other chemicals were from Sigma Chemical Co., St. Louis, MO, USA.

2.2. *Cells and tissue culture*

The Chinese hamster ovary cell-human chromosome 21 hybrid cell line, 153E9a3 [5], was given to us by D. Patterson and cultured in a Ham's F12 medium (Nissui, Tokyo) supplemented with 7% dialyzed fetal bovine serum (FBS; Hyclone, Logan, UT, USA). 1×10^7 153E9a3 cells were radiolabeled with 1 mCi of [^{3}H]thymidine (20 Ci/mmol; Amersham, Buckinghamshire, UK). The radioactivity of

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DNA entrapped in agarose beads (or films) and the concentration of DNA were measured for calculation of the specific activity of DNA in encapsulated beads (or films) as described elsewhere [1]. We estimated that there were about 50 μg of genomic DNA per ml of agarose beads or films that contained approximately 8×10^6 cells.

2.3. *Contour clamped homogenous electric field (CHEF) gel electrophoresis*

CHEF gel electrophoresis was performed as described elsewhere [6,7], using a Pulsaphor electrophoresis unit (Pharmacia LKB Biotechnology, Uppsala, Sweden). 1% agarose gels in 0.5 × TBE (45 mM Tris-borate, 45 mM boric acid, 1 mM EDTA (pH 8.0)) were prepared by pouring 130 ml of agarose into a 15 × 15 cm^2 frame. Electrophoresis was carried out in 0.5 × TBE as running buffer at a constant temperature of 12°C, maintained by recirculation of the buffer through a heat exchanger, with a constant voltage of 170 V for 20 h. The stepped switching interval was 60 s for the initial 12 h and 90 s for the following 8 h. Under these conditions DNA molecules from approximately 20 to 1500 kb can easily be separated. Yeast cells were embedded and lysed in agarose (agarose plugs) by the protocol of Carle and Olson [8].

2.4. *Preparation of agarose beads and films that contain human DNA*

Preparation of agarose beads from the cultured cells followed the method described previously [1] except that 1×10^8 1531E9a3 cells were used. For the preparation of agarose film, cells (1×10^8 cells) were scraped from flasks and washed in 5 ml PBS(−) (3 mM KCl, 1.5 mM KH_2PO_4, 0.14 M NaCl, 8 mM Na_2HPO_4, pH 6.8). The suspension of cells (5 ml in PBS(−)) was warmed to 50°C in a water bath and then mixed with 5 ml of 1.0% low melting point agarose (BRL, Gaithersburg, MD, USA) prewarmed to 50°C. Each 500 μl of cell-agarose suspension was poured into a siliconized test tube (inner diameter, 16 mm; length, 170 mm) and allowed to form an even coat all over the inner surface of the test tube, for the preparation of a uniform cell-agarose membrane (film), by rotating the inclined test tube and immediately cooling it on ice so that the agarose film solidified. Films were collected by scraping from the insides of the tubes, washed in cold PBS(−) by pipetting to break them up into smaller pieces and pelleted by centrifugation at 400 × *g* for 10 min. Films were resuspended in 20 ml of 1% (w/v) SDS, 25 mM EDTA (pH 8.0), 50 μg/ml proteinase K (Merk, Darmstadt, FRG) and incubated overnight at 45°C. After digestion with proteinase K, the films were resuspended in 20 ml TE (10 mM Tris-HCl (pH 8.0), 1 mM EDTA (pH 8.0)) that contained 0.1 mM phenylmethylsulfonylfluoride (PMSF) and dispersed well. They were pelleted by centrifugation and then washed several times in TE before storage at 4°C.

2.5. *Digestion with restriction enzyme*

In most cases 100 μl of beads or film in suspension (5 μg DNA) were washed twice in 1 ml of 1 × Eag I buffer (150 mM NaCl, 10 mM Tris-HCl (pH 8.0), 10 mM $MgCl_2$, 1 mM DTT (Toyobo Co., Tokyo), 100 μg/ml BSA (Boehringer-Mannheim, Mannheim, FRG)) and an appropriate concentration of the enzyme (New England Biolabs, Beverly, MA, USA) was added to 200 μl of the total digestion mixture. The reaction was incubated at 37°C for 2 h and stopped by addition of 1 ml of TE.

2.6. *Ligation of DNA in agarose beads or film and the pYAC vector*

The pYAC vector was prepared by digestion with *Bam*HI and *Not*I and dephosphorylated with BAP phosphatase as described elsewhere [1,2]. After 100 μl of suspension of beads or film has been washed once with H_2O, 10 μl (10 μg) of pYAC vector, 20 μl of 10 × ligation buffer (500 mM Tris-HCl (pH 8.0), 70 mM $MgCl_2$, 10 mM DTT), 2 μl of 100 mM ATP, 10 μl of T4 ligase (5 U/μl; Toyobo, Tokyo) and 58 μl of H_2O were added and then the mixture was incubated at 15°C for 5 h. The beads or film were washed twice with TE by centrifugation and melted at the indicated temperature for 15 min after addition of 40 μl of 0.5 M EDTA (pH 8.0), 8 μl of 5 M NaCl and 252 μl of H_2O. The melted beads or films were incubated at 37°C overnight after addition of various amounts of agarase (5 U/μl in 50% glycerol; Calbiochem Corp., San Diego, CA, USA) and immediately used for transformation.

2.7. *Transformation of yeast spheroplasts*

The yeast cells (ABL380) were grown overnight with moderate aeration in 50 ml of YPD medium (1% yeast extract, 2% peptone, 2% glucose, pH 5.8) to 1.5×10^7 cells per ml. Spheroplast preparation from this range of cell concentrations did not have a significant change of the transformation frequency. The cells were washed successively with 50 ml of H_2O and 50 ml of 1 M sorbitol (Fisher Scientific, Fair Lawn, NJ, USA) and resuspended in 15 ml SCED (1 M sorbitol, 0.1 M sodium citrate (pH 5.8), 10 mM EDTA, 10 mM DTT). 100 μl of Zymolyase (Kirin, Tokyo; 2 mg/ml) were added and the mixture was incubated at 30°C with occasional shaking. The preparation of spheroplasts was followed by measuring the decrease in turbidity at 660 nm of a 10-fold dilution of cells in H_2O. When spheroplasting had proceeded to 60–70% of completion, cells were harvested by centrifugation for 4 min. The following steps were and should be carried out gently and with care. The spheroplasts were washed twice with 20 ml of STC (1 M sorbitol, 10 mM Tris-HCl (pH 7.5), 10 mM $CaCl_2$) by resuspension and centrifugation for 4 min. The cell pellets were resuspended in 2 ml of STC. One ml of the suspension of spheroplast cells was added to the ligated DNA-pYAC55 vector-agarose beads or film. Sonicated salmon sperm DNA was added as carrier to give a total concentration of DNA of 100 μg. After standing at room temperature for 10 min, the mixture was supplemented with 10 ml of PEG (10 mM Tris-HCl (pH 7.5), 10 mM $CaCl_2$, 20% polyethyleneglycol 8000 (Fisher Scientific, Fair Lawn, NJ, USA)). The spheroplasts were harvested by centrifugation for 4 min after standing for another 10 min. The pellets were resuspended in 2 ml of SOS (1 M sorbitol, 7 mM $CaCl_2$, 0.27% (w/v) uracil, 0.25% yeast extract, 0.5% bactopeptone, 0.5% glucose), and incubated for 30 min at 30°C. Individual aliquots (200 μl) of the suspension of spheroplasts in SOS were added to 8 ml of TOP agar (1 M sorbitol, 2.5% agarose in SD medium [9]) kept at 50°C. After gentle mixing of the spheroplasts and TOP agar the mixture was immediately poured onto SORB plates (SD plates that contained 1 M sorbitol and 2% glucose) and incubated at 30°C. Colonies were first plated on single drop-out regeneration plates (−Ura), and then picked up on double-selection (−Ura-Trp) plates. Transformation procedures were followed as described [10] with some modifications.

2.8. *Southern analysis*

CHEF gels were treated with 0.25 N HCl for 15 min, then with a solution of 0.4 N NaOH and 0.6 M NaCl for 15 min, and finally with a solution of 1.5 M NaCl and 0.5 M Tris-HCl (pH 7.5), before transfer to nylon membranes (Pall, Glen Cove, NY, USA) as described elsewhere [11]. Hybridization was carried out in a solution of 1 M NaCl, 10% dextran sulfate and 1.0% sodium dodecyl sulfate with 100 μg/ml of yeast tRNA as carrier and approximately 10^6 cpm/ml of ^{32}P-labeled probe. The probes used were a 280-bp *Bam*HI fragment of BLUR-8 (Alu probe) [12] and *Pvu*II/*Bam*HI digests of pBR 322, radiolabeled with [^{32}P]dCTP with an oligopriming labeling kit (Pharmacia-LKB) [13]. Membranes were washed twice with 2 × SSC at room temperature for 10 min, then once with 2 × SSC, 1.0% SDS at 65°C once for 30 min, and finally once with 0.1 × SSC at room temperature once for 30 min. Autoradiography was performed at −80°C for a day.

3. RESULTS AND DISCUSSION

We recently developed a method using agarose beads for construction of a human genome DNA-YAC library [1]. However, we have sometimes found a lower than anticipated efficiency of transformation. Although we do not know the exact reasons for this lower efficiency,

problems associated with the method are as follows. The preparation of agarose beads is time-consuming; melting of the bead-ligation mixture before transformation is difficult, and loss of some of the final ligated beads occurs during washing. To overcome these problems, we have developed a new rapid method using agarose films. The major advantages of this method are as follows. The time required for preparation of agarose films is approximately half of that required for preparation of agarose beads. Recovery of cells entrapped in agarose film is much higher (90–95% of the total number of cells) than that in the agarose bead method (50–60%). Entrapped DNA in such agarose films shows no apparent degradation of DNA as judged by CHEF even after being stored at 4°C for several months. Fig. 1 shows 153E9a3 cells entrapped in agarose microbeads (A) and in agarose microfilms (B). The cells inside the agarose beads form aggregates, as shown in Fig. 1. The cell aggregates in agarose beads sometimes caused the loss of recovery of DNA after enzyme digestion. By contrast, cells entrapped by agarose film are dispersed independently and distributed equally throughout the film. The agarose membrane (film) formed inside the tube is thin enough (approximately 30 μm thick) to distribute cells evenly in a single layer.

Both partial and complete digestion by Eag I of bead- and film-entrapped 153E9a3 genomic DNA was carried out to compare the size distribution between each type of digested DNA. As shown in Fig. 2, the patterns are almost identical to DNA digested with Eag I. However the hybridization intensity using the *Alu*-probe on digests prepared using agarose film was much stronger in the 150- to 300-kb range than that prepared using agarose beads (Fig. 2B). At the same concentration of enzyme, 150- to 300-kb fragments of DNA were more

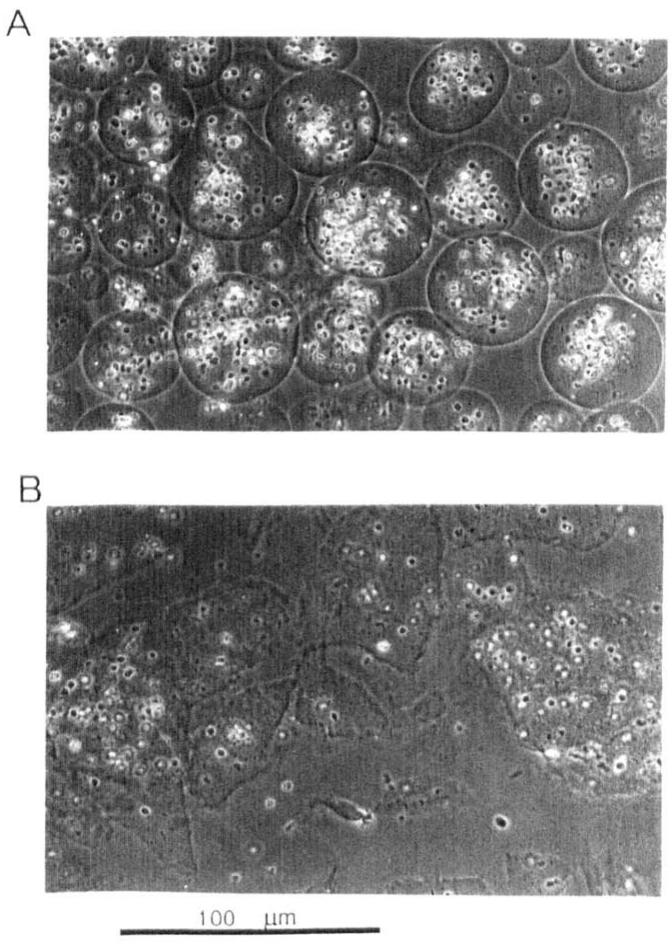

Fig. 1. Phase-contrast microscopy of cells entrapped in agarose beads (A) (see [1]) and in agarose films (B). Bars = 100 μm. The average diameter of agarose beads is between 25 and 75 μm.

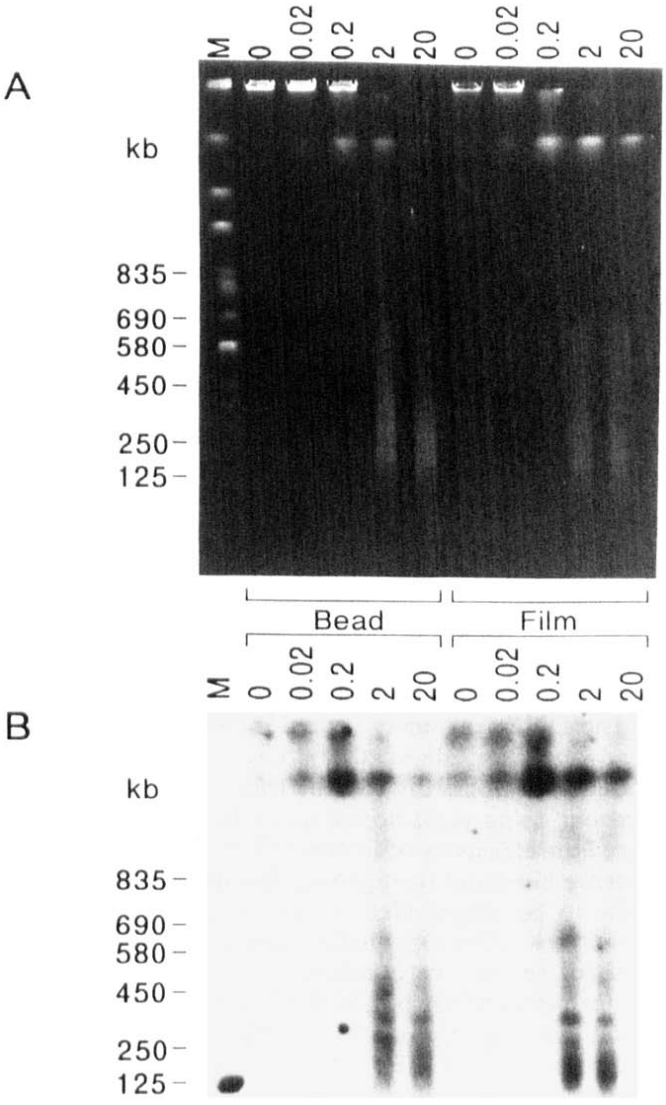

Fig. 2. CHEF gel analysis of hybrid cell (153E9a3) genomic DNA completely and partially digested with Eag I. The genomic DNA (5 μg) was digested with various concentrations of Eag I (0–20 U/μg). (A) Gel stained with ethidium bromide. (B) Southern hybridization with BLUR-8 probe. Lane M (marker) is HY-1.

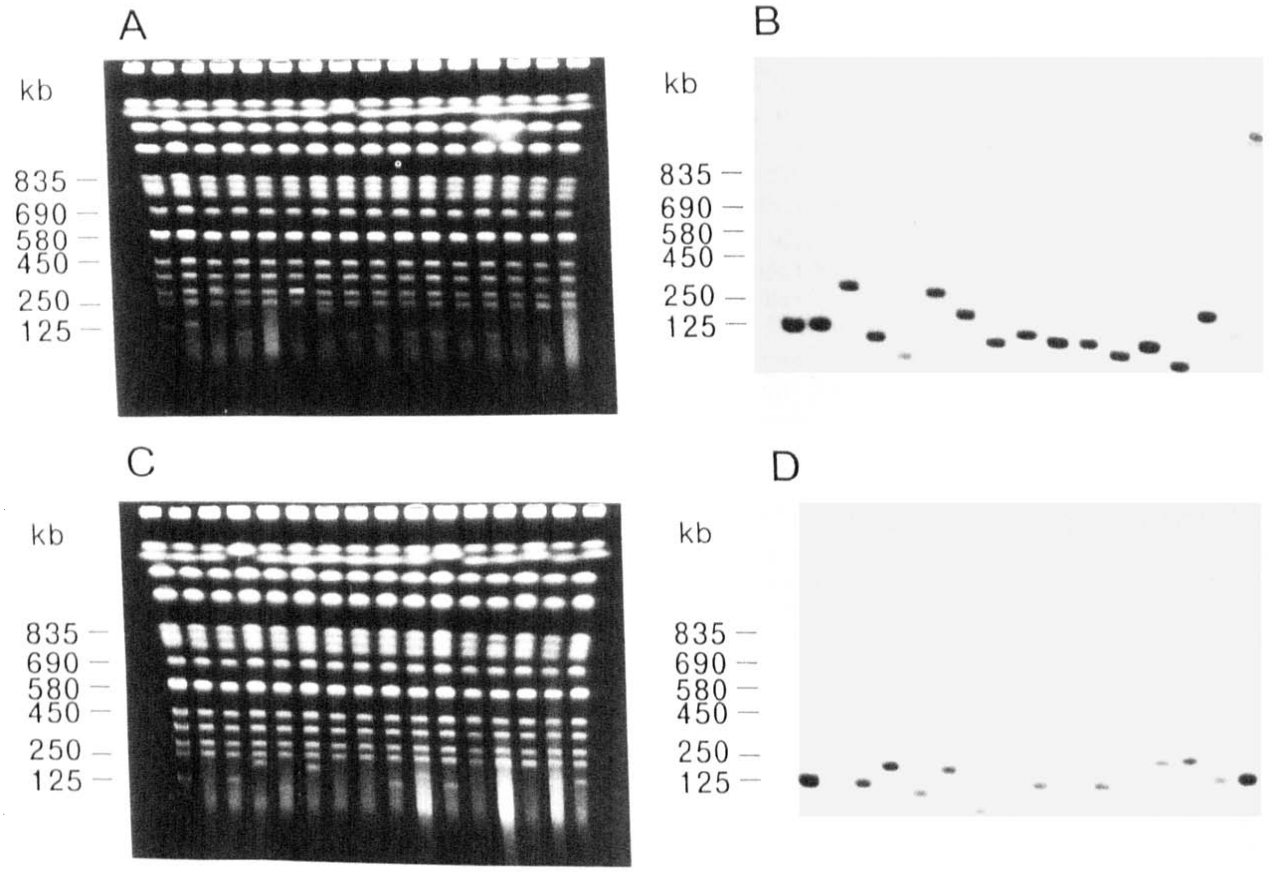

Fig. 3. CHEF gel electrophoresis of YAC clones. Chromosomal DNAs from randomly picked colonies derived by the agarose bead method (A) and the film method (C) were stained with ethidium bromide and the corresponding filters after transfer were probed with pBR322 (B,D). The lane on the left side of each gel contained HY-1.

easily prepared by the agarose film method than by the agarose bead method. The liberation by agarase of DNAs entrapped in agarose film is very simple and reproducibly generates DNA fragments of suitable sizes for molecular cloning with the pYAC vector.

The DNA fragments from 153E9a3 cells in agarose beads or film, digested by Eag I, were ligated to pYAC55 vector arms and the transformation was carried out using yeast host strain AB1380 [2]. We compared the efficiency of ligation of DNAs isolated by the agarose bead and the agarose film methods and found these to be almost identical as judged by CHEF gels (data not shown). Under optimal conditions for transformation, we obtained approximately 5×10^6 clones per μg DNA of pYAC55 vector with the insert. Total genomic DNAs of the selected YAC clones were fractionated on CHEF gels, blotted on nylon membranes and hybridized with [^{32}P]dCTP-radiolabeled pBR322 probe. One example of such an analysis is shown in Fig. 3. The inserted DNA in these YAC clones ranged from 30 to 450 kb in length and the average size of inserted DNA was approximately 155–165 kb in the YAC recombinant clones prepared by the agarose bead and agarose film method (Fig. 4). Although the exact reasons for cloning the smaller inserts are not clear, one of the reasons might be the higher molar concentration of the small insert DNA (155–165 kb) than of the larger-sized DNA inserts (see Figure 2B). Sucrose gradient centrifugation to select larger DNAs more than 250 kb may improve the overall average size of the YAC insert DNAs, as mentioned (average size of DNA inserts in YAC clones is about 350-450 kb, transformation efficiency is about 2-4 $\times$ 10^6 clones/μg DNA) (Imai, T. and Olson, M.V., in press). The size fractionation method by pulsed field gel electrophoresis (PFGE) reported by Anand et al. [14] may also be useful in obtaining larger inserts of YAC clones.

Although the average distribution of sizes of the insert DNAs prepared by the agarose film method was not significantly different from that prepared by the agarose bead method, we found differences in the transformation efficiency (Fig. 5). The agarose film method generated about 3 times more transformants than the agarose bead method. One possible reason for this difference may be the mineral oil that is still present in the final preparation of the beads, which may affect the viability of yeast spheroplasts and cause a decreased efficiency of transformation (data not shown). Another

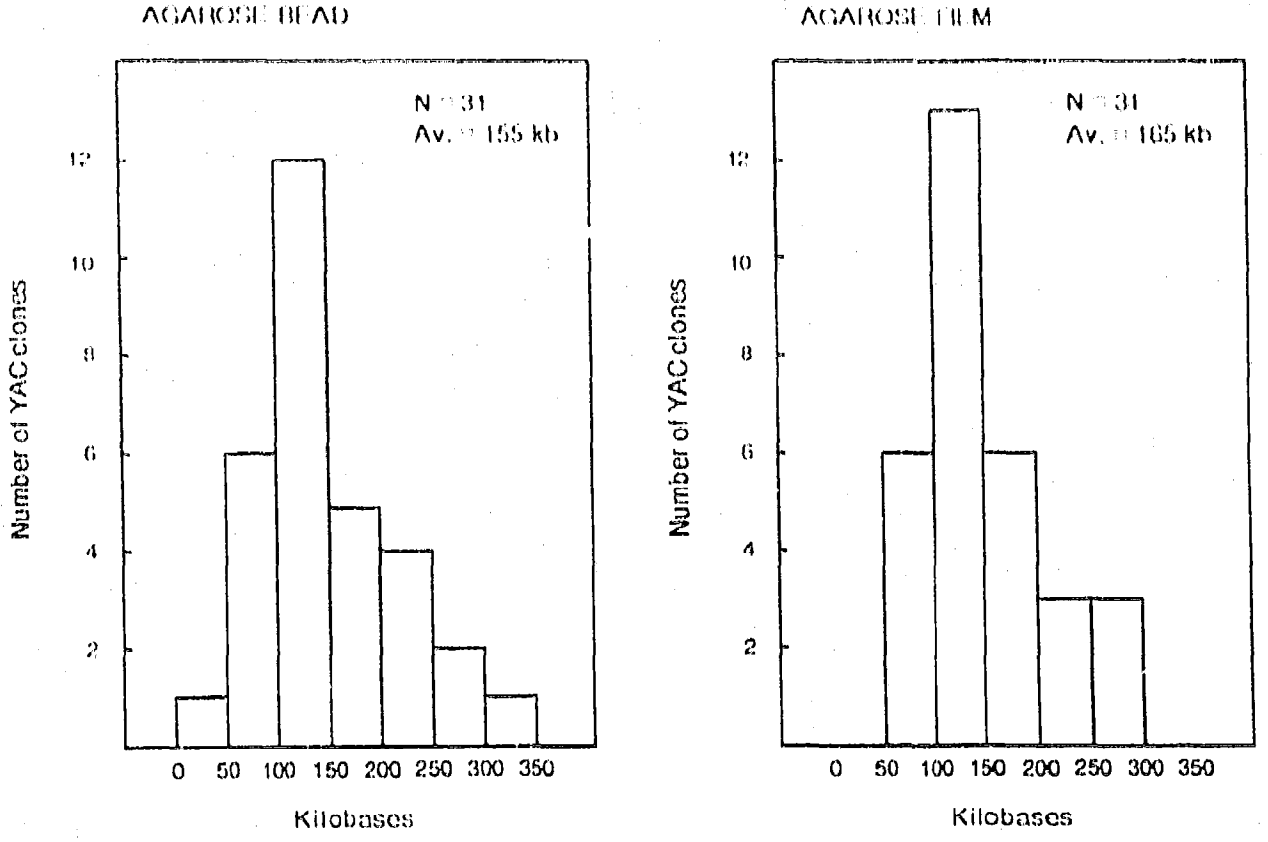

Fig. 4. Histograms of the distribution by size of DNA from the hybrid cell (153E9a3) chromosome pool derived by the agarose bead method and the agarose film method. Random transformants from each of the ligations were fractionated by size on pulsed-field gels, transferred to nylon membrane, and hybridized with radiolabeled pBR322 probe. Sizes of artificial chromosomes were determined relative to the HY-1 chromosome as standard. The sizes are plotted in terms of numbers of clones.

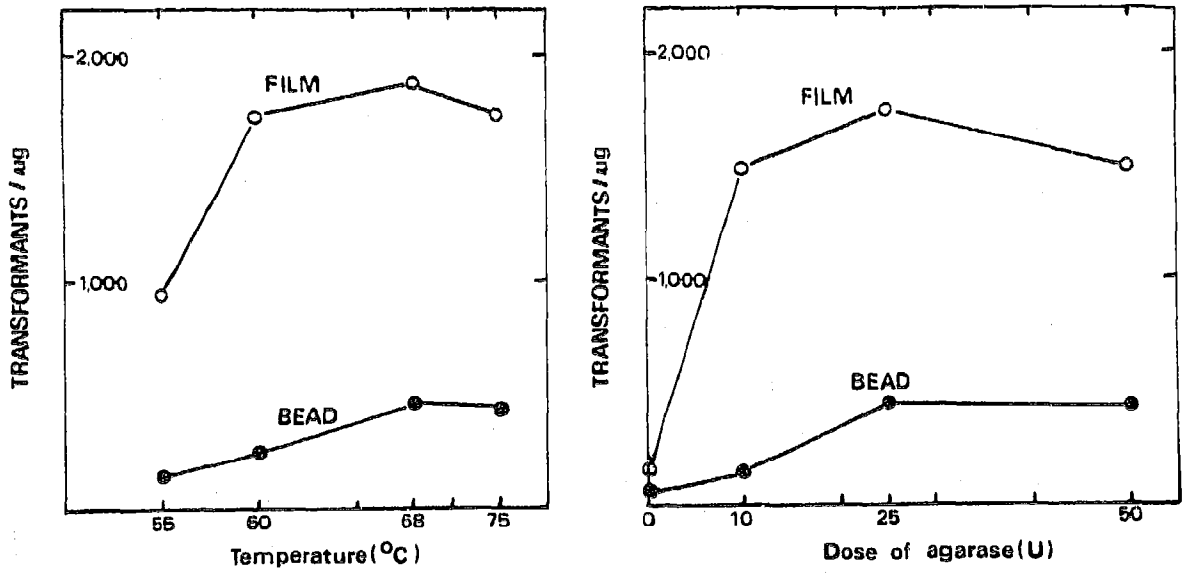

Fig. 5. Comparative study of frequency of transformation between the agarose bead and agarose film methods. Effects of temperature (left lane) and concentration of agarase (right lane) used to dissolve the ligated human DNA-pYAC55 before transformation. The mean efficiencies of transformation are compared.

possible reason is the poor melting of agarose beads during the heat treatment that precedes digestion with agarase of the agarose matrix to release the ligated DNAs before transformation. Both agarose beads and films were heated at various temperatures or digested at various doses of agarase, to compare transformation frequencies between the two methods (Fig. 5). The agarose films were easily melted by heating at 60°C for 15 min, but agarose beads were not melted under these conditions, indicating the lower melting capacity of agarose beads as compared to agarose films. Heating at 68°C for 15 min at least was required for the melting of agarose beads. Sometimes, even under these conditions, residual incompletely melted agarose beads still remained in the transformation mixture (data not shown). A similar result was obtained at the higher concentration of agarase. It is clear that the DNA in agarose films is much more susceptible to liberation by agarase than that in beads (approximately 3–4-fold higher amounts of DNA were released). The low melting capacity of agarose beads may possibly be reflected as a more rigid structure of the agarose matrix in agarose beads than in the agarose films. Undigested agarose matrix may entrap DNA fragments, disturbing their introduction into yeast spheroplasts, and cause a significant subsequent decrease in the efficiency of transformation. Our new rapid cloning method using agarose films has several advantages as described above when larger insert DNAs in YAC recombinant clones are required. In combination with sucrose gradient centrifugation (Imai, T. and Olson, M.V., in press) or size fractionation by PFGE [14], this agarose film method can be easily used to isolate the large fragments of DNA necessary for construction of a human DNA-YAC clone library.

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