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Cellular gels

Purifying and mapping long DNA molecules

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Long DNA molecules of $> 10^5$ bp (0.1 Mbp) are easily broken by pipetting. Therefore, chromosomal DNA is generally isolated after embedding cells in a protective coat of agarose. The embedded DNA can then be cut into long pieces and fractionated on gels using pulsed fields, but these pieces are again easily broken if the resolved DNA molecules are recovered from the gels. We now describe a novel gel matrix, a 'cellular' gel, that permits the recovery of resolved fragments from gels in a form that enables facile manipulation without shear. This facilitates purification and restriction mapping of fragments of 0.1–1.0 Mbp. We illustrate the utility of the method by mapping chromosome III of baker's yeast, which has a length of ~0.36 Mbp. This method should facilitate purification and restriction mapping of yeast artificial chromosomes.

INTRODUCTION

DNA molecules of longer than about 100000 bp (0.1 Mbp) are easily broken by pipetting (Levinthal & Davison, 1961). As a result, long DNA is generally fragmented on isolation. Cells, and the DNA within them, can be protected from shearing forces by embedding them in agarose blocks (Schwartz & Cantor, 1984) or microbeads (Cook, 1984). Then treatment with a strong ionic detergent, such as SDS, and proteolytic enzymes leaves essentially intact DNA embedded in the agarose (Schwartz & Cantor, 1984; Cook, 1984). The use of microbeads of about 50 μm in diameter, rather than the larger blocks, permits rapid equilibration of the bead interior with external buffers and proteins, so allowing shorter incubations and multiple manipulations. The encapsulated DNA can be cut with restriction enzymes that cut infrequently (e.g. *Not*I) into large fragments (e.g. 1 Mbp) and fractionated on gels using pulsed fields (Schwartz & Cantor, 1984; Carle & Olson, 1984; Chu *et al.*, 1986), but these pieces will again be broken if extracted from the gel. The DNA can be recovered and manipulated in blocks cut from the gel, but these are awkward to handle and often impede digestion of the trapped DNA (see, for example, Gardiner *et al.*, 1986). We now describe a novel gel matrix that permits the recovery of such resolved fragments from the gel in a form that enables their continued and facile manipulation without shear. We illustrate the utility of the method by isolating chromosome III of baker's yeast, which has a length of 0.36 Mbp, and then restriction mapping it. We foresee that this method should facilitate the purification and restriction mapping of yeast artificial chromosomes (YACs) (Burke *et al.*, 1987).

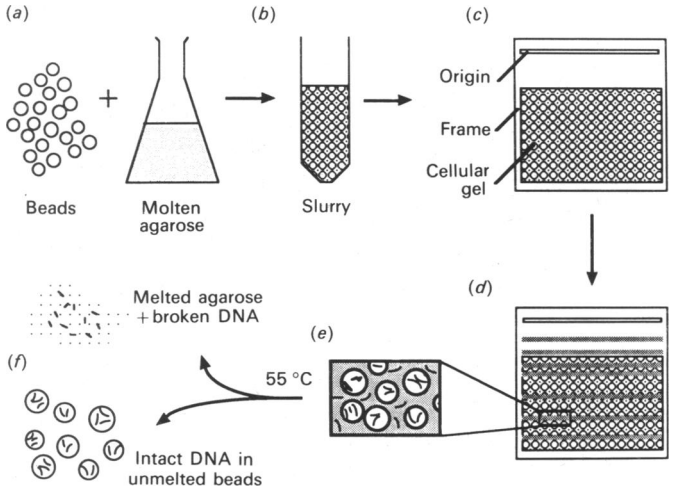

Fig. 1. Cellular gels

(*a*) Agarose microbeads (with a high melting temperature) are mixed with molten agarose (low melting temperature) to give a slurry (*b*) which is (*c*) poured into an agarose frame and allowed to set. (*d*) DNA fragments are resolved electrophoretically in this cellular gel, (*e*) the region of interest excised and heated to (*f*) release beads containing encapsulated DNA.

The gel matrix, or 'cellular' gel, is a composite containing agarose microbeads (with a high melting temperature) suspended in, and permeated by, an interstitial phase of easily melted agarose (Fig. 1). Cellular gels are cast in a 'frame' of homogeneous agarose which both supports the weaker cellular gel and partially resolves DNA fragments before they enter it. After mixing, the slurry of microbeads suspended in the interstitial phase of molten agarose is cast in the 'frame'. Long DNA fragments can then be resolved electrophoretically in this matrix and the part containing the fragment of interest is excised and heated to melt the interstitial agarose, releasing unmelted microbeads. These contain fragments protected from shear yet accessible to enzymes. No special precautions need to be taken to preserve DNA integrity during manipulation; beads can be repeatedly vortex-mixed and pelleted.

MATERIALS AND METHODS

Yeast DNA

Saccharomyces cerevisiae (strain X2180–1B; mid-exponential phase; 10^7/ml) were washed in 1 M-sorbitol and treated (10^9/ml; 10 min; 25 °C) with 0.001 vol. of 2-mercaptoethanol, re-washed and encapsulated (4×10^9 cells/ml) in 0.5% agarose microbeads (Cook, 1984) in 1 M-sorbitol. Spheroplasts (> 99% by microscopy) were prepared by incubating beads with 400 μg of Zymolyase 20T (Miles)/ml at 37 °C for 2 h in 1 M-sorbitol and lysed using 1% lithium dodecyl sulphate/25 mM-Na_2EDTA/10 mM-Tris (pH 8.0). Beads were washed immediately five times

Abbreviation used: YAC, yeast artificial chromosome; OFAGE, orthogonal field-alternation gel electrophoresis.
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in this, then five times at hourly intervals, then in 0.5 × TBE (1 × TBE is 89 mM-Tris/89 mM-boric acid/2 mM-EDTA) and finally resuspended in an equal volume of 0.5 × TBE; 20 μl of beads were used per well as markers. Chromosomes remain intact at 20 °C for over 1 year.

Cellular gels

Bio-Gel A150m (Bio-Rad; ~1% agarose; 100 μm diameter; 100–200 mesh; 37 °C) is mixed with an equal volume of 2% ultra-low-gelling-temperature agarose (Sigma type IX, dissolved in 1 × TBE by boiling and cooled to 37 °C). The slurry is agitated (> 16 h; 37 °C) on a bottle roller (1 rev./min; magnetic stirrers damage beads), spun (5000 *g*; 3 min) and the supernatant removed to leave a bead paste with ~40% original volume. This is cooled to 30 °C to prevent cracking on setting, spun briefly to remove trapped air bubbles and poured into the frame (at 4 °C to avoid cracking) by holding the base of the centrifuge tube against a vortex mixer, enabling the thixotropic paste to flow. The paste is smoothed flush with the frame, air bubbles removed with a syringe, re-smoothed and left (4 °C; 3 h) to set. The frame is cut from a 0.8% agarose slab (Sigma type VII in 0.5 × TBE). A low percentage of agarose and long pathlength through the frame increase resolution.

Electrophoresis

Yeast chromosomes were generally purified using cellular gels and orthogonal field-alternation gel electrophoresis (OFAGE) (Carle & Olson, 1985): 10V/cm; 0.5 × TBE; 16–18 h; switching intervals of 25 and 50 s, for fragments of 200–500 kbp and > 500 kbp, respectively), using a single well (8 cm × 0.5 cm × 0.2 cm) loaded with 1 ml of encapsulated yeast DNA. DNA moves about as fast through a cellular gel as through a homogeneous 1.5% gel. After staining with ethidium (0.1 μg/ml) for 1 h the required band (about 6 cm × 0.5 cm × 0.4 cm) was excised under u.v. light, heated (10 ml of 0.5 × TBE; 55 °C; 15 min with vortex mixing at intervals) and the released beads washed (0.5 × TBE; 55 °C), chilled (4 °C, 30 min), re-washed thrice (3000 *g*; 60 s) and stored at 4 °C. Chromosomal DNA was recovered (30–50% efficiency) at 0.5–2 μg/ml of packed beads.

Restriction mapping

The following enzymes gave too many or too few fragments for analysis: *Apa*I (~60, 65 and 75 kbp fragments plus many of < 30 kbp), *Bam*HI (> 15 fragments of < 50 kbp), *Bgl*I (unresolved 'smear' below 50 kbp), *Not*I (did not cut), *Rsr*II (non-specific degradation), *Sal*I (one fragment of ~50 kbp and an unresolved smear below 30 kbp), *Sfi*I (~8 and 355 kbp fragments) and *Xho*I (> 11 fragments of < 30 kbp). *Sma*I and *Sst*II were used for mapping. DNA in beads was cut with *Sma*I [20 units in 60 μl containing 30 μl (packed volume) of beads; 10 min; 30 °C; manufacturer's buffer) or *Sst*II (100 units/60 μl; 60 min; 37 °C; manufacturer's buffer) and labelled by translation of pre-existing nicks using DNA polymerase I (4 units with 30 μl of beads in 60 μl of 50 mM-Tris (pH 8.0)/5 mM-$MgCl_2$/3 mM-dithiothreitol/3 μM-dATP + dGTP + dTTP/[α-^{32}P]dCTP (15 μCi; 5000 Ci/mmol) for 15 min at 17 °C; unincorporated label was removed by washing beads twice). Marker DNA of λ (strain cIndIts857Sam7; 48.5 kbp) and T5 (121.3 kbp) phages was cut with various restriction enzymes or ligated into multimers and labelled with ^{32}P using the Klenow fragment of DNA polymerase (Maniatis *et al.*, 1982).

Fragments of > 20 kbp were resolved by field-inversion gel electrophoresis (Carle *et al.*, 1986) and those of < 20 kbp in conventional gels and sized from autoradiographs of dried gels using an LKB Ultroscan-XL laser densitometer. Repeated measurements of the same fragment gave sizes to within 3%. For two-dimensional gels, the strip cut from the gel used in the first dimension was equilibrated (2 × 20 min; 4 °C) successively with an Mg^{2+}-free buffer and then an Mg^{2+}-containing buffer, then cut with *Sma*I (1 unit/μl; 3 h; 30 °C).

RESULTS

Cellular gels

Fig. 2(*a*) compares the resolution obtained with a conventional homogeneous gel matrix (left) with that using a cellular gel (right). Yeast were encapsulated, lysed, and the beads containing total chromosomal DNA applied to the wide slot at the top of the gel and subjected to pulsed-field electrophoresis. Although some resolution is lost at the frame/cellular-gel interface (dependent on agarose batch) and migration is slower through the cellular gel, yeast chromosomes of 0.24–1.0 Mbp (i.e. bands 1–10) are well resolved in the cellular gel (right).

Fig. 2(*b*) illustrates a typical preparative cellular gel in which total yeast DNA was resolved using a hexagonal electrode array which gives straight bands. Bands from such gels were excised, and the interstitial phase of agarose was melted to release the microbeads containing the resolved DNA molecules. These were shown to be pure and intact by re-running them on a conventional slab gel using pulsed fields (Fig. 2*c*). In contrast to these encapsulated DNA molecules, naked DNA is easily sheared by pipetting or vortexing (Fig. 2*d*). Clearly, the recovered yeast chromosomes are excellently protected from breakage. Note

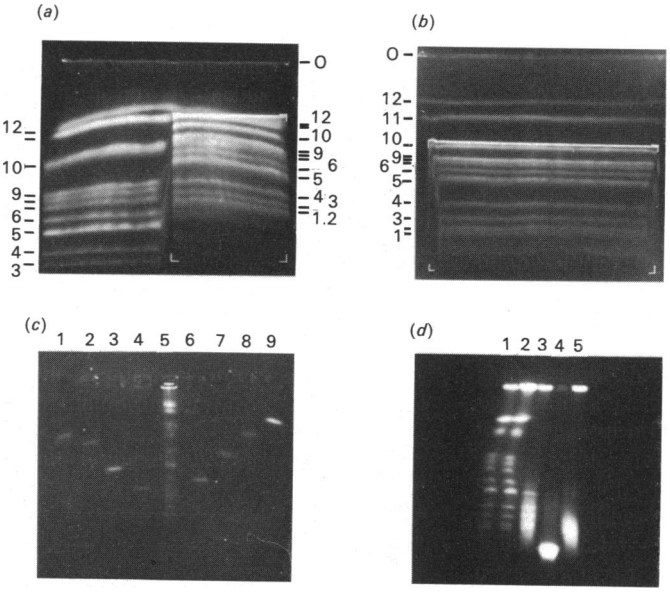

Fig. 2. Purifying yeast chromosomes in cellular gels

(*a*) Ethidium-stained yeast chromosomes (numbered from the smallest upward) resolved by orthogonal field-alternation gel electrophoresis using a conventional 0.8% gel (left) and cellular gel (right). White angles show the corners of cellular gel. O is the origin. Lanes are typically bowed in such gels. (*b*) A preparative cellular gel using a hexagonal electrode array giving straight tracks. (*c*) Chromosomes purified from cellular gels were re-run on a conventional gel (OFAGE; 1.5% agarose; 10V/cm; 50 s switching time; 15 h). Tracks 1–4 and 6–9, pure DNA molecules (i.e. bands 9, 7, 5, 3 and 4, 6, 8, 10, respectively). Track 5, unfractionated yeast chromosomes. (*d*) Yeast were encapsulated, lysed and beads containing total DNA treated variously; then samples, loaded using a wide-bore pipette, were resolved by OFAGE as in (*c*). 1, untreated; 2, heated (60 °C; 20 min) to melt beads, releasing unprotected DNA; 3, heated as 2 and pipetted ten times (tip diameter 1.5 mm); 4, heated as 2 and pipetted four times through a yellow tip (diameter 0.5 mm); 5, heated as 2, then vortex-mixed for 10s.

that, in the experiments to follow, the encapsulated chromosomes are repeatedly pelleted, vortex-mixed and pipetted to allow transfer from one buffer to another during labelling, cutting with restriction enzymes etc.

The integrity of DNA of 0.24–1 Mbp recovered from cellular gels is consistently high (Fig. 2*c*; P. H. Dear, unpublished work), presumably because bead diameters ($\sim 100\ \mu m$) is large relative to DNA length. Experience shows that DNA of 0.24–1 Mbp is recovered from our cellular gels with yields of 30–50 %; recoveries of > 70 % cannot be expected, as this is the proportion of the gel occupied by microbeads (P. H. Dear, unpublished, work). (Note that the densest packing of rigid uniform spheres is also about 70 %.)

Restriction-mapping of chromosome III

As we foresee that this method will be used to map human inserts in YACs (Burke *et al.*, 1987; Coulson *et al.*, 1988; Garza *et al.*, 1989), we tested the feasibility of doing so by restriction-mapping yeast chromosome III (band 3). In principle, maps can be built after completely digesting DNA with the two restriction enzymes (i.e. from fragment sizes in 'single' and 'double' digests). However, this approach is limited to simple cases with less than ten fragments in the double digest, even when implemented by fast computers; the number of possible maps that must be tested increases factorially with fragment number. (It is largely for this reason that so few inserts in YACs have been mapped.) Therefore preliminary experiments were directed at selecting a pair of restriction enzymes that cut the DNA efficiently and without degradation into about five fragments with dispersed sizes. None was found, but *Sst*II and *Sma*I (which give 18 fragments in the double digest) came closest to meeting the criteria (see the Materials and methods section). Although cutting by *Sma*I is impeded by methylation of the internal cytosine (CC^{5}mCGGG), DNA of *S. cerevisiae*, unlike mammalian DNA, is not methylated.

Before sizing the fragments in single and double digests, we sized the chromosome accurately. It was purified using a cellular gel, labelled and re-run in a slab gel using field-inversion gel electrophoresis next to oligomers of λ-phage DNA (Fig. 3*a*). The average size from four tracks in two separate gels was 363.7 kbp (range 362.5–365.0).

Next, fragment sizes in single and double digests were obtained. The purified chromosome was labelled, cut with the two restriction enzymes, either singly or in succession, and the fragments re-run on conventional or field-inversion slab gels and sized (Fig. 3*b*; Table 1).

The 18 fragments resulting from complete digestion with both enzymes can be arranged in $18! = 6.4 \times 10^{15}$ different orders. Even assuming an unrealistically low error of 1 % in fragment sizes, no unique solution to the map could be found using a computer implementation of the 'branch and bound' approach (Fitch *et al.*, 1983; Nolan *et al.*, 1984; Bellon, 1988; Krawczak, 1988; Tuffery *et al.*, 1988). Therefore we had to include data from partial digestion with *Sma*I (Table 1) and of sequential digestion by *Sst*II followed by *Sma*I (with sizing on two-dimensional gels; Fig. 3*c*) to obtain a unique *Sma*I map (Fig. 4). Digestion by *Sma*I then *Sst*II could not be performed, as *Sst*II digestion was impeded in the gel slice; therefore the *Sst*II map could not be derived. Finally, we confirmed the *Sma*I map and oriented it with respect to the genetic map (Mortimer & Schild, 1985) by Southern

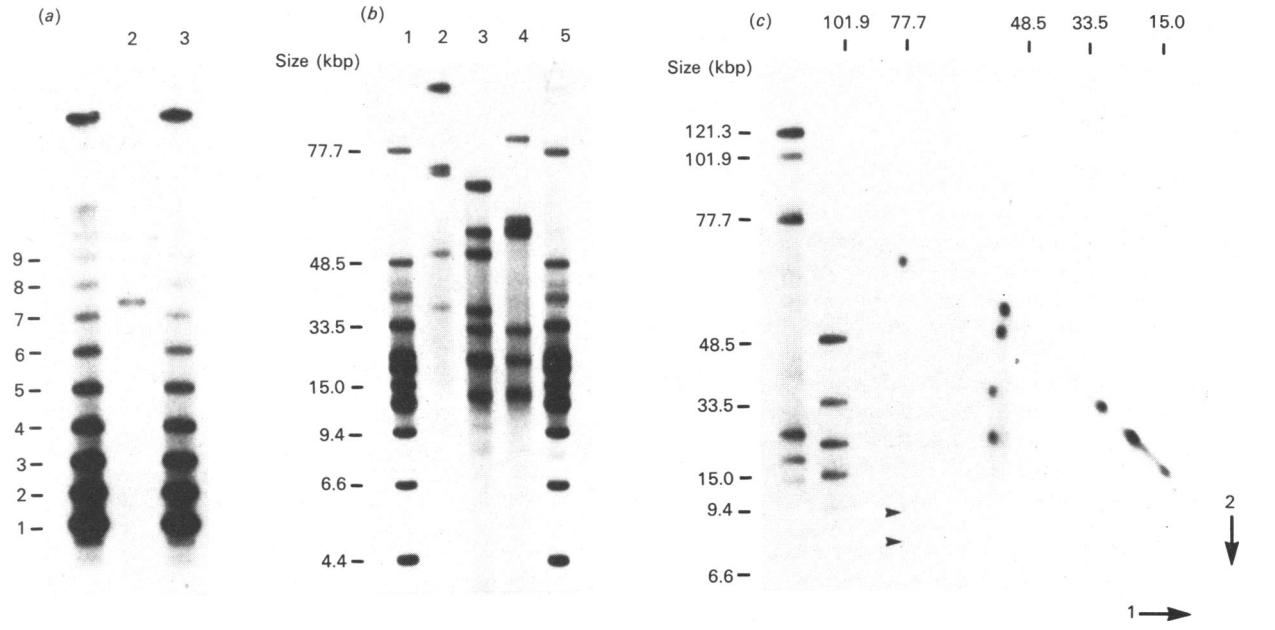

Fig. 3. Mapping of chromosome III

(*a*) The chromosome, purified using cellular gels, was labelled with ^{32}P, and sized (lane 2) on a gel by comparison with λ-phage DNA multimers (lanes 1 and 3; multimers of 48.5 kbp indicated). The autoradiograph of the dried gel is shown. (*b*) Sizes of *Sma*I and *Sst*II fragments. Purified and encapsulated chromosome III was completely digested with *Sma*I or *Sst*II or with *Sma*I then *Sst*II. After labelling with ^{32}P, fragments were subjected to field-inversion gel electrophoresis (10 V/cm; 14 °C; forward 0.3–3 s ramped over 18 h; reverse was 1/3 forward throughout) and an autoradiograph was prepared. Lanes 1 and 5, markers (kbp); lanes 2, 3 and 4, *Sma*I (lane 2), *Sma*I and *Sst*II (lane 3) and *Sst*II (lane 4) fragments. (*c*) Analysis in two-dimensional gels of *Sst*II/*Sma*I fragments. Chromosome III was labelled, cut completely with *Sst*II and fragments resolved as in (*b*). The gel strip containing the fragments was excised, incubated with *Sma*I, set into another gel, the fragments resolved by field-inversion gel electrophoresis in the second dimension and, after drying, an autoradiograph was prepared. Arrows show direction of migration in the two dimensions; arrowheads indicate spots visible after a longer exposure. Markers (two lanes on left and along the top; in kbp) are shown. Only the parts of the autoradiographs (*b* and *c*) containing resolved fragments are shown.

Table 1. Sizes (kbp) of *Sma*I and *Sst*II fragments

The lettering of *Sma*I fragments (mA–mH) and *Sma*I partials (pA–pH) in order of increasing size corresponds to that in Fig. 4. Lines indicate the relation of fragments to one another established from two-dimensional gels.

Size (kbp)			
*Sst*II	*Sma*I + *Sst*II	*Sma*I	*Sma*I partials
9.22	1.3	mA 3.44	pA 18.3
	7.87	mC 9.28	pB 79.7
9.55	9.60	mF 71.1	pC 89.3
11.7	11.8	mG 72.0	pD 121.9
12.3	12.3	mH 112.6	pE 130.2
14.9	14.6		pF 204.3
	0.5		pG 223.7
23.8	24.1		pH 308.7
32.0	32.2		
56.7	56.1		
57.2	50.6	mE 50.5	
	3.52		
	2.75		
59.4	24.1		
	37.8	mD 38.2	
82.2	6.89		
	8.85	mB 8.92	
	68.4		

blotting (Maniatis *et al.*, 1982) and hybridizing total yeast DNA (cut with *Sst*II or *Sma*I) with four probes (i.e. *leu2*, *his4*, *cdc39* and *pgk1*) from different parts of the chromosome (Fig. 4).

The *Sma*I map is incomplete in one respect: the smallest fragment has not been placed. Unfortunately, this had a size close to that of the error in measuring the largest fragment, there was no *Sst*II site within it and the partial digests were uninformative (Table 1). However, this omission represents < 1 % of chromosomal length.

DISCUSSION

Cellular gels

These experiments show that DNA molecules of up to 1.0 Mbp can be resolved in cellular gels and then recovered intact from them in a form that allows their facile manipulation without further breakage. Note that, during mapping, the encapsulated DNA was repeatedly pelleted and resuspended as it was transferred from one buffer to another. Equilibration between the interior of the microbeads and the buffer was rapid, allowing short incubations. Moreover, digestion of DNA purified in this way was efficient, in contrast with that of DNA in blocks cut from conventional gels. Yields and integrity of the largest yeast chromosomes were similar to those of smaller ones, presumably because the diameter of the beads (100 μm) is large relative to the folded length of the DNA in the gel. The folded length of 1 Mbp in a gel is $\leqslant 10$ μm, a fraction of its extended length of 340 μm (Smith *et al.*, 1989). As cellular gels can be made from larger beads (P. R. Cook, unpublished work), which can be made simply (Cook, 1984) or are available commercially (e.g. Bio-Gel A150m, diameter 200–300 μm; 50–100 mesh), perhaps even larger molecules might be purified unbroken in good yield.

Mapping inserts in YACs

Of the data needed to solve the map, those derived from complete digestion of chromosome III were the easiest to obtain; conditions for partial digestion were only obtained by trial and error. It would be a great advantage, then, if computer programs were available which could solve the map solely using this easily obtained data. However, even if the formidable computational problems can be overcome (Stefik, 1978; Pearson, 1982; Fitch *et al.*, 1983; Durand & Bregegere, 1984; Nolan *et al.*, 1984; Polner *et al.*, 1984; Allison & Yee, 1988; Bellon, 1988; Krawczak, 1988; Tuffery *et al.*, 1988), a unique solution seldom exists for maps of > 10 fragments (Durand & Bregegere, 1984; Nolan *et al.*, 1984; Allison & Yee, 1988; Krawczak, 1988). More complex data are needed to resolve inevitable ambiguities. In our case we used the partial digests and two-dimensional gels shown in Fig. 3. When mapping inserts in YAC vectors, identification of the end fragments by hybridization should simplify mapping.

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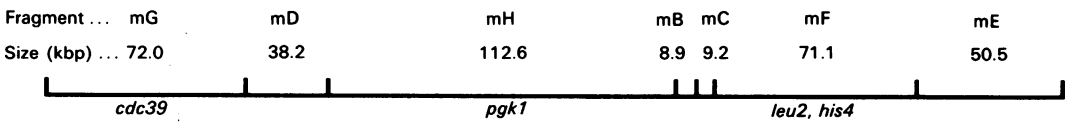

Fig. 4. *Sma*I map of chromosome III

The map was deduced from the data in Table 1 as follows. *Sma*I partials pA, pB, pC, pD and pE give an incomplete map with the *Sma*I fragment order mH–mB/C–mC/B–mF/G. Relationships between *Sma*I, *Sst*II and double-digest fragments (established using two-dimensional gels; solid lines in Table 1) resolve and extend this incomplete map to mH–mB–mC–mF–mE, leaving only mA, mD and mG unplaced. Disregarding mA, only six possible *Sma*I maps remain; of these, only that shown can yield all of the *Sma*I partials in Table 1 (pA = mB + mC; pB = mC + mF; pC = mB + mC + mF; pD = mH + mB; pE = mH + mB + mC; pF = mH + mB + mC + mF; pG = mG + mD + mH; pH = mG + mD + mH + mB + mC + mF). The four loci were assigned to *Sma*I fragments by Southern blotting and hybridizing total yeast DNA (cut with *Sma*I and resolved using field-inversion gel electrophoresis) with appropriate probes (results not shown).

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