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Control of partial digestion combining the enzymes *dam* methylase and *Mbo*I

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ABSTRACT

A method is described which allows the preparation of reproducible partial digests without previous establishment of the incubation conditions. It is based on a combined application of *dam* methylase and the restriction endonuclease *Mbo*I, both recognizing the sequence 5′-GATC-3′ but *Mbo*I unable to cut the methylated site. Due to their competition for the same substrate the DNA is partially digested, with the size of the resulting fragments strongly dependent on the ratio of enzymes. The K_m of the *dam* methylase was determined to be 115 ng DNA/μl indicating a variance in fragment sizes generated at low DNA-concentrations. This effect is minimized above 150 ng/μl. Any influence of digestion time is avoided, because the reaction runs until complete modification of all sites. The dependence on enzyme concentration and presence of agarose was checked. Knowledge of these parameters allows an accurate prediction of fragment sizes generated at different conditions. The technique was successfully used to construct libraries from different sources, in particular chromosome-specific libraries from small amounts of flow-sorted material.

INTRODUCTION

Partial digestion of DNA is a basic prerequisite for the construction of genomic libraries (e.g., 1) as well as restriction mapping of recombinant clones (2). To get fragments of an appropriate size range, the degree of digestion has to be carefully controlled. As carried out up to now (1), partial digestion strongly depends on the DNA's concentration and purity, the amount of restriction enzyme used, as well as the incubation time. Therefore, using either dilutions of the restriction enzyme or different time-intervals of incubation to achieve partial cleavage of the DNA, conditions have to be checked empirically for each DNA. A way of generating partial digests in a time and enzyme amount independent manner (3) uses ultraviolet irradiation to make sites unaccessable for restriction enzymes. Such DNA, however, can not be used for cloning purposes.

As alternative we describe here a method developed to produce accurate partial digestions of any size. The partially cut DNA-fragments can be produced quite easily and digestion is easily controlled. This is particularly valuable, if the DNA-amount is limited, as for instance in the construction of genomic libraries from flow-sorted chromosomes or microdissected DNA. Our approach is based on the combined use of a DNA-methylase and a restriction endonuclease, both acting on identical recognition sites, with the latter enzyme not able to cut the methylated form. The competition for the substrate between both enzymes should result in a partial digestion of the DNA, with the frequency of cleavage dependent on the ratio of methylase to restriction nuclease (4).

Since partial *Mbo*I-digestion is extensively used for cloning purposes, we concentrated

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on this enzyme, using *dam* methylase as antagonist. The *dam* gene of *Escherichia coli* specifies a DNA adenine methyltransferase (*dam* methylase), which modifies adenine residues at the N^6-atom in 5′-GATC-3′ sequences (5, 6). *Mbo*I, as opposed to *Dpn*I, exclusively cleaves unmethylated sites (7, 8). Conditions were determined to prepare partial digests of any size range. The method was successfully applied to DNA from several sources, and libraries representing the human chromosomes X and 21 (Nizetic et al., in prep.), Herpes Simplex Virus Type I (HSVI; Craig et al., submitted), and Drosophila were constructed with such DNA.

MATERIAL AND METHODS

DNA from four different sources was used: unmethylated lambda-DNA was from Promega Biotec (Lot D219B). A preparation of HSVI-DNA was a kind gift of Dr. D. McGeoch (University of Glasgow). DNA of the Drosophila wild type strain Canton S was purified accordingly to a quick protocol of Dr. K. O'Hare (Imperial College of Science, London; personal communication), who also provided the flies. DNA of the cell-line GM1416B containing four human X-chromosomes (4X-DNA) was isolated as described by Herrmann and Frischauf (9). No DNA showed unspecific digestion during overnight incubations in restriction buffer. DNA-concentrations were determined in a pH 12 buffer (20 mM K_3PO_4, 0.5 mM EDTA, 1 μg ethidium bromide/ml) by measuring the increased fluorescence of DNA-intercalated ethidium (10, 11). The lambda-DNA, according to the supplier of a concentration of 387 ng/μl, was determined in three independent measurements with this assay to contain 361 ng/μl, 382 ng/μl, or 395 ng/μl, respectively.

*Mbo*I was from New England Biolabs (NEB; Lots 38 and 49) and Bethesda Research Laboratories (BRL; Lot 71131), *dam* methylase from NEB (Lots 1b and 2). Their activities were checked, in comparison to each other. Unit definitions are as given by NEB. All other restriction enzymes were from BRL.

To achieve partial digestion, usually 40 ng DNA/μl were incubated at 37°C in 20 μl of 33 mM Tris-acetate (pH 7.9), 65 mM potassium acetate, 10 mM magnesium acetate, 0.5 mM dithiothreitol (TAK-buffer; 12, 13), supplemented with 80 μM S-adenosylmethionine (AdoMet). Cleavage was carried out with 0.5 unit *Mbo*I and an appropriate amount of *dam* methylase, dependent on the ratio used. The reaction was terminated by addition of EDTA to a final concentration of 25 mM and heat-inactivation at 68°C for 10 minutes. In several experiments shown in the RESULT section one parameter of these standard conditions was varied as indicated.

Electrophoresis was in 0.3% or 0.5% agarose gels submerged in 40 mM Tris-acetate (pH 8.2), 1 mM EDTA, 0.5 μg ethidium bromide/ml at 2.5 to 5 V/cm. The gels were destained in water overnight, UV-illuminated (peak wavelength 254 nm) and photographed. UV-excitation was kept brief to avoid light induced bleaching of the bands. In order to determine either relative DNA-amounts of distinct bands or the fragment distribution of partially cleaved DNA, each lane was scanned (LKB Ultroscan XL). By comparison to marker molecules the size-distribution could be determined. The maximum of absorption is not identical with the mean of the distribution originating from partial digestion, because more ethidium intercalates into larger fragments. This results in an uneven signal, its intensity proportionally increasing with size. Therefore, to obtain normalized values, each signal was divided by the relevant fragment length. In addition some digestions were end-labeled by Klenow-enzyme and autoradiographed.

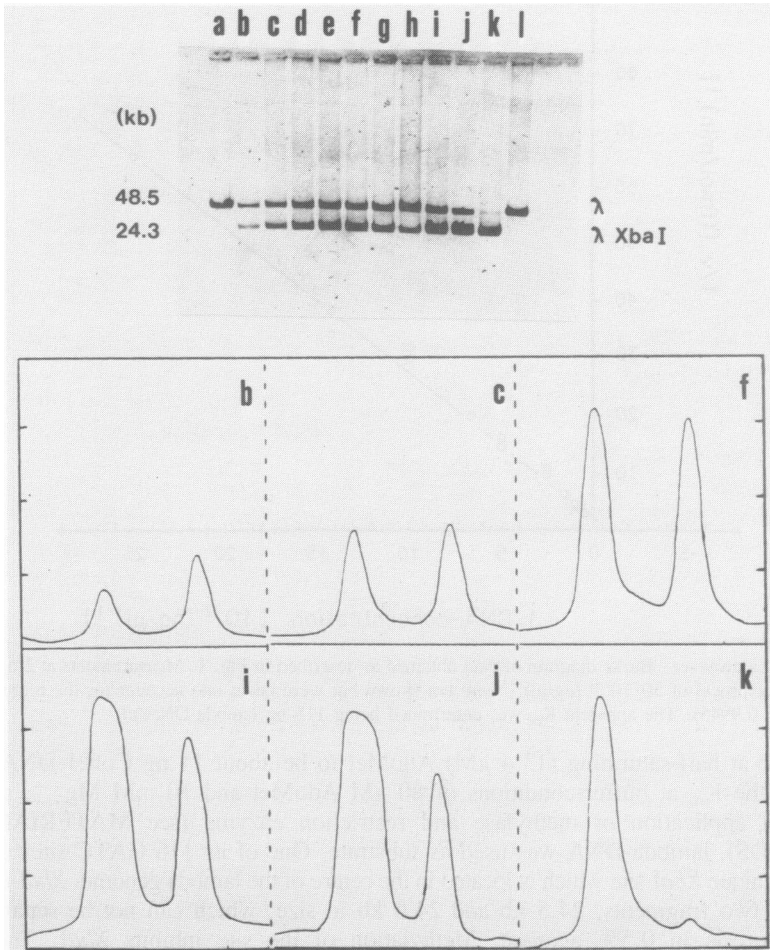

Figure 1. Determination of the apparent K_m of *dam* methylase. Reactions of 10 μl contained 33 mM Tris-acetate (pH 7.9), 65 mM potassium acetate, 10 mM magnesium acetate, 0.5 mM dithiothreitol, 80 μM AdoMet, and 0.1 unit *dam* methylase. 20 ng (b), 40 ng (c), 70 ng (d), 100 ng (e), 150 ng (f), 200 ng (g), 400 ng (h), 1000 ng (i), and 2000 ng (j) of lambda-DNA were incubated at 37°C for 60 min, followed by heat-inactivation of the enzyme and subsequent cleavage with *Xba*I. **Upper panel**, electrophoresis was in 0.5% low-melting-point agarose, stained with 0.5 μg ethidium bromide/ml. Fragment-sizes (in kb) are indicated on the margin. Lane a is an untreated control; in lanes k and l, buffer had been substituted for *dam* methylase or *Xba*I, respectively. **Lower panel**, scans of some lanes (b, c, f, i, j, k) are shown, the direction of electrophoresis from right to left.

RESULTS

Determination of the K_m and V of dam methylase

To achieve reproducible partial digestion with relatively small amounts of enzyme, it would be of advantage to work well above the K_m of both the methylase and the restriction enzyme, since then the result of a combined reaction would be almost independent of the DNA-concentration. Herman and Modrich (14) determined the apparent K_m of *dam*

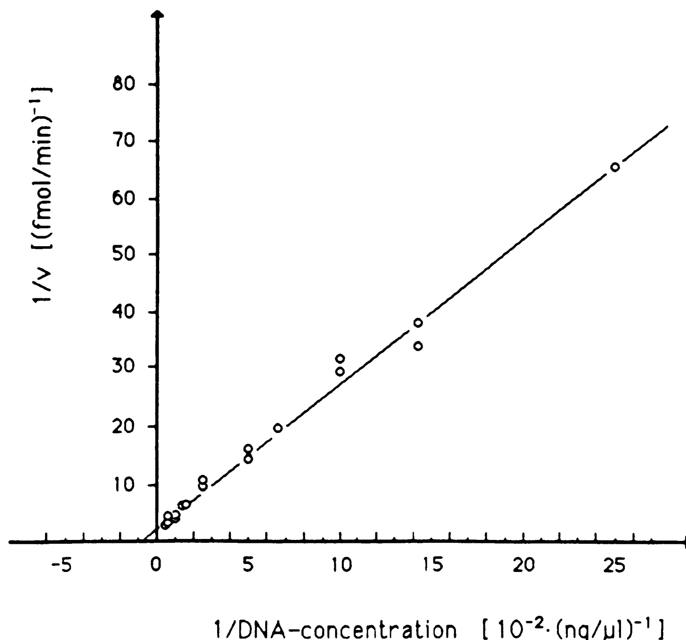

Figure 2. Lineweaver – Burke diagram of data obtained as described in Fig. 1. Measurements at 2 ng DNA/µl (giving a reciprocal of $50 \cdot 10^{-2}$ $(ng/\mu l)^{-1}$) are not shown but were taken into account for the regression line (correlation 0.9945). The apparent K_m was determined being 115 ng lambda-DNA/µl.

methylase at half-saturating (13.4 µM) AdoMet to be about 11 ng ColE1-DNA/µl. To measure the K_m at buffer-conditions of 80 µM AdoMet and 10 mM Mg^{++}, used for combined application of methylase and restriction enzyme (see MATERIAL AND METHODS), lambda-DNA was used as substrate. One of its 116 GATC-sites overlaps with the unique *Xba*I-site which is located in the centre of the lambda genome. *Xba*I-cleavage produces two fragments, 24.5 kb and 24.0 kb in size, which can not be separated by electrophoresis in 0.5% agarose. Methylation of the site inhibits *Xba*I. Therefore, electrophoresis of a complete digest of partially methylated DNA shows the degree of methylation on account of the relative band intensities of cut to uncut DNA (Fig. 1). In the experiment, lambda-DNA of a concentration between 2 ng/µl and 200 ng/µl was incubated with 0.1 unit *dam* methylase at 37°C for 60 minutes. The enzyme was heat-inactivated at 68°C for 10 minutes, and subsequently the DNA was cut with *Xba*I. Fig. 1 shows a few digestions as well as resulting scans. Using this approach the maximal velocity V of the *dam* methylase was found to be 52 fmol sites/min (Fig. 2), under the assumption that all 116 sites in lambda behave like the one overlapping with the *Xba*I-site. The apparent K_m was derived from the Lineweaver-Burke plot to be 115 ng lambda-DNA/µl. Therefore an effect of the DNA-concentration on the activity of the *dam* methylase has to be expected at low DNA-concentrations, for example in library-preparations from flow-sorted, chromosomal DNA, in which only restricted amounts of material are available.

Influence of the DNA-concentration on combined dam/MboI digestion.

Samples of various DNA-concentrations were incubated with different ratios of *dam*/*Mbo*I. Figure 3 shows the average size of the fragments generated. In a double-logarithmic plot

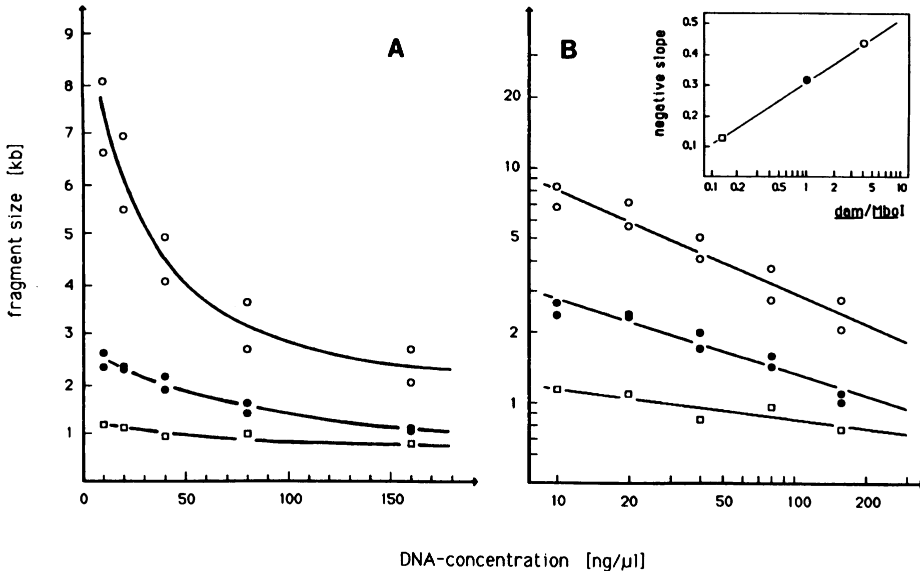

Figure 3. Linear (**A**) and double-logarithmic (**B**) plots of the average fragment-sizes generated at various DNA-concentrations. *dam*/*Mbo*I ratios of 4/1 (○), 1/1 (●), and 0.125/1 (□) were used. In the inserted graphic the slope of the regression lines from **B** is plotted versus the *dam*/*Mbo*I ratio at a logarithmic scale.

(Fig. 3 B) the decrease of size with the increase of DNA-concentration is described by a straight line, its slope related to the *dam*/*Mbo*I ratio. This relationship is linear in a semi-logarithmic plot (Fig. 3 B, inset), allowing an extrapolation for any *dam*/*Mbo*I ratio. Results of single experiments performed at other ratios fitted to predictions made from these data (not shown).

For most practical applications, e.g. partial digestion for cloning purposes, the influence of the DNA-concentration can be neglected above 150–200 ng DNA/µl, since only relatively minor changes occur. But even below that value the effect is smaller in combined *dam*/*Mbo*I cleavage than in an ordinary partial digestion. The latter depends linearly on the amount of DNA—a tenfold increase causes a tenfold lower frequency of cleavage. The former shows different dependences at different ratios; i.e., at a ratio of 4/1 a shift from 10 ng/µl to 100 ng/µl changes the resulting fragment length by a factor of 2.6 only (Fig. 3 A).

Size generation controlled by enzyme-ratio.

Due to the competition of *dam* and *Mbo*I for the same substrate the DNA is partially cleaved by a combined incubation, with the size of the resulting fragments dependent upon the ratio of the enzymes. In Figure 4 several partial digestions are shown which were prepared at ratios ranging from 40/1 down to 0.62/1. In a double-logarithmic plot, the average size of the generated fragments increased linearly with the ratio used (Fig. 5). Ratios of up to 120/1 were used, but it was not possible to determine the average fragment size of those high-ratio digestions. Nevertheless, inspection of the smallest DNA-fragments generated at such ratios, which create a smear below the main band of limited mobility, showed a progressive reduction in the frequency of cleavage with increasing ratios.

enzyme-
Δ ratio dilution

27491
23130
13437
9416
6557
4361
3912
2898
2322
2027
1639
854
(bp)

40/1 → 0.62/1 4/1 ratio

Figure 4. **Left**, partial digestion of DNA controlled by the ratio of *dam*/*Mbo*I. Genomic 4X-DNA was digested overnight using *dam*/*Mbo*I ratios of 40, 20, 10, 5, 2.5, 1.25, and 0.62. The left and right most lanes show controls of uncut and completely *Mbo*I-digested DNA, respectively. **Right**, the effect of enzyme dilution is shown. 4X-DNA was incubated with 2 units *dam* and 0.5 unit *Mbo*I (ratio 4/1) or progressing 1:2 dilutions of the identical enzyme mixture (left to right) for 17 hours. Marker sizes are given on the margin.

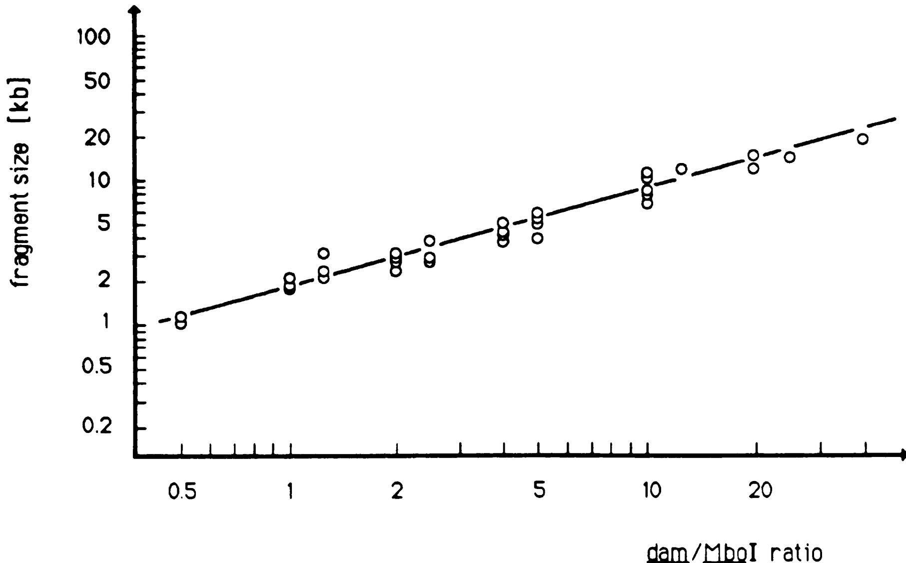

Figure 5. Dependence of the average fragment size produced at particular enzyme ratios. In independent experiments, 40 ng DNA/μl were digested overnight. The average DNA length was plotted versus the enzyme ratio used, both at logarithmic scale. In such a plot, the increase in size is linearly dependent on the ratio, with a correlation coefficient of 0.967.

Although the samples shown here were digested overnight, such long incubation is not necessary at higher ratios of methylase to *Mbo*I. From kinetic studies, using the *Xba*I-site of lambda as an indicator of the degree of methylation, it was found that at 40 ng DNA/μl *dam* methylase achieved 50% of methylation of 1 μg lambda with about 15 unit-minutes, the equivalent of e.g. 0.5 unit *dam* reacting for 30 minutes (data not shown). At higher ratios, results identical to overnight incubations were generated in a few hours. For ratios above about 10/1 incubation of less than one hour is possible.

Influence of enzyme concentration.

0.025 unit *Mbo*I/μl (0.65 unit/μg DNA at standard conditions) was chosen as the usual amount of restriction endonuclease, because it completely cut the DNA in overnight digestions (Fig. 6). So, even at low ratios the degree of cleavage is entirely determined by the ratio of the two enzymes. In addition, the fragment sizes generated by a combined reaction changed only slightly at higher concentrations of both enzymes, if the ratio was kept constant. At lower *Mbo*I concentrations the DNA length declines rapidly (Fig. 4; Fig. 6) with dilution, until there is little or no influence of the *dam* methylase remaining but partial digestion takes place due to the low concentration of the *Mbo*I. To avoid any major effect of enzyme dilution on the degree of cleavage, a concentration of 0.025 unit *Mbo*I/μl (and an appropriate amount of *dam*) was used in combined reactions.

Size prediction

Based on the data shown above it was possible to derive the following empirical equation predicting the average fragment size (*Mbo*I concentration 0.025 unit/μl).

$$\text{length} = c_{(\text{DNA})}^{[(-0.089 \cdot \ln(dam/MboI)) - 0.313]} \cdot [dam/MboI]^{0.97} \cdot 5760 \text{ bp}$$

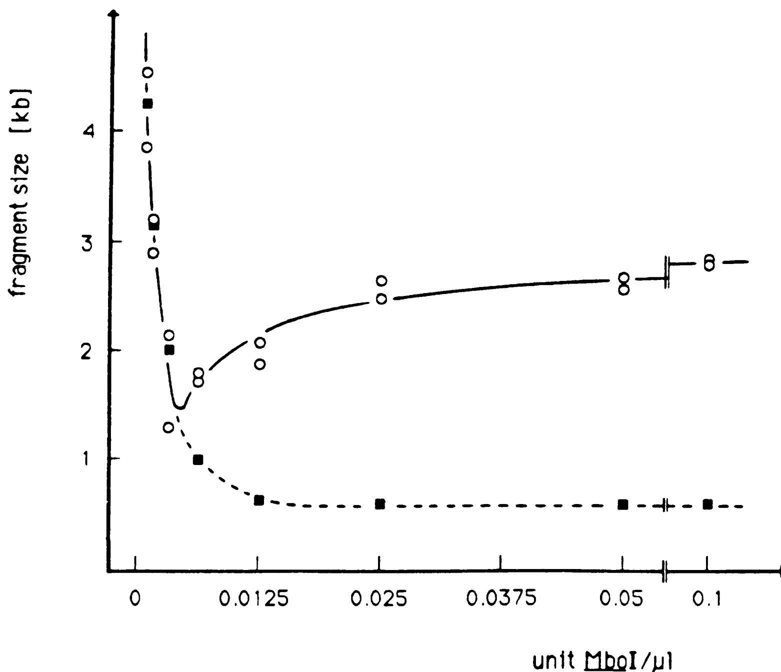

Figure 6. Effect of enzyme dilution in combined *dam*/*Mbo*I digestions. A *dam*/*Mbo*I ratio of 2/1 was used (○). The enzyme mixture was progressively diluted 1:2, indicated by the amount of *Mbo*I, and was added to identical samples of 20 µl containing 800 ng DNA. Cleavage was for 19 hours at 37°C. A size minimum was reached with about 0.004 unit *Mbo*I/µl. Further dilution caused an increase in fragment length due to incomplete *Mbo*I-digestion, as visible from parallel dilutions of *Mbo*I alone (■).

where $c_{(DNA)}$ is the DNA-concentration in ng/µl. For example, a digestion at 100 ng DNA/µl and a *dam*/*Mbo*I ratio of 50/1 produces fragments of an average size of 12.2 kb.

Library preparation.

To check the influence of the quality of the DNA-preparation on the results of partial cleavage, DNA was used which had been prepared from four different sources by four protocols of different stringency—from high purity DNA in case of commercial lambda-DNA to a 2.5 hours quick preparation in case of Drosophila-DNA (see MATERIAL AND METHODS). Figure 7 shows an example of individual partial digestions carried out with those DNAs. Since enzyme volumes of 0.8 to 1.5 µl were used, the deviation in DNA-length is within the range of pipetting inaccuracy. No significant difference was found on account of the DNA used.

tRNA is quite often used as a carrier to enhance DNA-precipitation. To checked its influence, lambda was cleaved at a ratio of 0.25/1 in presence of different amounts of tRNA and subsequently run on a gel. The generated fragment pattern was scanned to detect even small changes of intensity. Up to a concentration of 0.4 µg/µl of tRNA no shift in the band intensities was found, but higher concentrations slightly increased the cleavage frequency.

The method of combined *dam*/*Mbo*I incubation was used to prepare both cosmid and lambda libraries from HSVI, flow-sorted human chromosomes, DNA from cell-lines, and

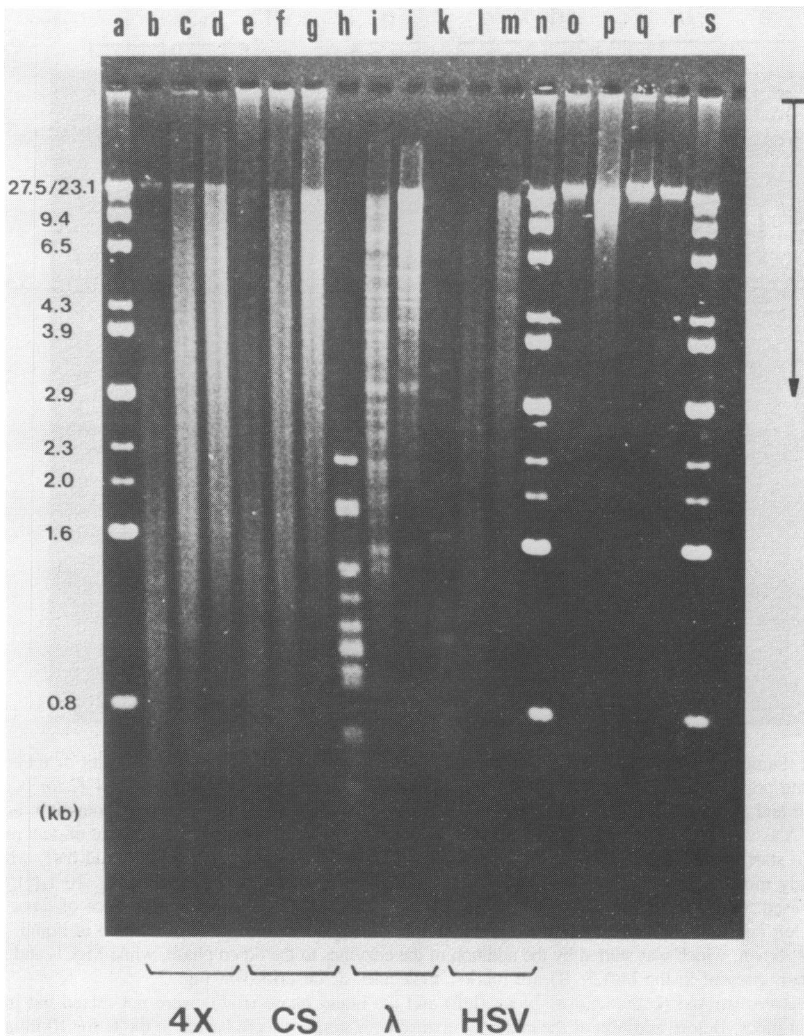

Figure 7. Reproducibility of partial digestion by combined *dam*/*Mbo*I incubation. Cleavage was carried out with DNA from four different organisms [GM1416B-cells (uncut: o; digested: b–d), Drosophila strain Canton S (p; e–g), lambda (q; h–j), and HSVI (r; k–m)] which had been purified by different protocols (see MATERIALS AND METHODS). For each reaction the enzyme mixture was individually prepared. Cleavage was overnight with *Mbo*I (b, e, h, k) or *dam*/*Mbo*I ratios of 1.25/1 (c, f, i, l) and 5/1 (d, g, j, m), respectively. The size of standard molecules (lanes a, n, s) is indicated.

Drosophila-DNA (Craig et al., submitted; Nizetic et al., in preparation; Volinia, personal communication; Hoheisel, unpublished data). At 40 ng DNA/μl a ratio of 150/1 was found to create fragments of an appropriate size for cosmid-cloning, while for lambda libraries ratios of 60/1 to 80/1 were used. Addition of EDTA and heat-inactivation of the enzymes were essential, if the DNA was phenol-extracted, because otherwise the DNA was lost at high enzyme concentrations. In comparison to ordinary partially digested DNA of similar

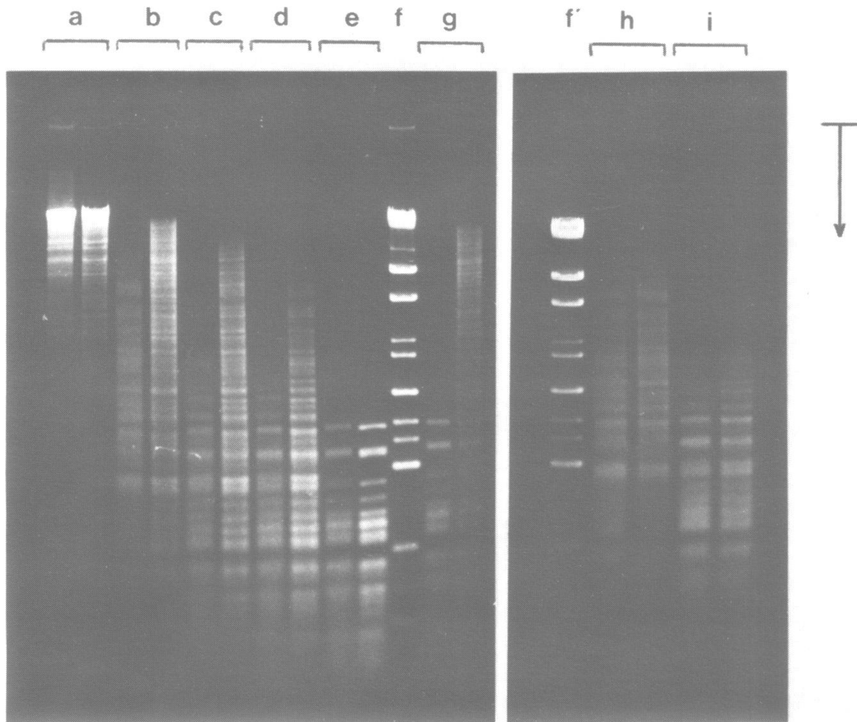

Figure 8. Samples of 50 μl were split into halves and mixed with one volume of either water or melted 1.5% low-melting-point agarose, which had been cooled to 37°C. The agarose was hardened at 4°C for two hours. The liquid and the agarose-block, both contained 33 mM Tris-acetate (pH 7.9), 65 mM potassium acetate, 1 mM EDTA as well as 2 μg lambda-DNA (40 ng/μl), 1.3 unit *Mbo*I, and an appropriate amount of *dam* methylase (**a**−**e**). To start the reaction, 20 mM $MgCl_2$ and 160 μM AdoMet were added to the liquid half, which was immediately mixed with the block. Incubation was overnight at 37°C. Ratios of 20/1 (**a**, **b**), 10/1 (**c**), and 5/1 (**d**) were used. (**a**) shows incubations without $MgCl_2$ and AdoMet, (**e**) digestions in absence of *dam*. In each case, the left lane shows the DNA of the agarose-block, the right one the DNA as cleaved in liquid. In (**g**) a reaction is shown, which was started by the addition of the enzymes to the liquid phase, while $MgCl_2$ and AdoMet were already present in the buffer. (**f**) are marker molecules as described in Fig. 7.

In the samples (**h**) and (**i**) the agarose-block (left) and the liquid phase (right) were not mixed but incubated in different tubes. Before addition of the enzyme mixture both samples were heated to 68°C for 10 minutes and then cooled to 37°C. Incubation was overnight with ratios of 10/1 (**h**) and 5/1 (**i**).

size, ligated into the identical vector preparation, there was no difference in the efficiency of *in vitro*-packaging.

Digestion in agarose.

Although *dam* methylase is comparatively small with a diameter of 2.4 nm (14) and, therefore, should not be prevented from diffusion in an agarose network, its activity is inhibited in agarose. Samples of lambda were digested in a single tube, half the DNA in liquid, the other half embedded in 0.75% low-melting-point agarose (Fig. 8). If the reaction was started by the addition of the enzyme to the liquid phase—Mg^{++} and AdoMet already present in the buffer and the block—the DNA in the agarose block was completely digested (**g**), while the DNA of the liquid phase was appropriately partially cleaved. The same result was produced, if there was DNA present in the agarose block

only (not shown). During such an incubation some of the completely digested DNA diffused from the block into the medium, thereby slightly changing the fragment pattern of this sample (**g**).

In the experiments shown in lanes **a**–**e** both enzymes had been embedded in the agarose together with the DNA. The reaction was started by adding Mg^{++} and AdoMet to the liquid phase. Even without adding Mg^{++}-ions, the DNA was slightly, but specifically, digested by the *Mbo*I during the incubation (**a**), probably due to Mg^{++}-contamination in the agarose. Using different enzyme ratios, it was found that the fragments produced in the blocks were about 35% of the size produced by digestion in solution. Several batches of agarose obtained from different manufactures showed the same effect on the fragment size.

Good partial cleavage in the presence of agarose was achieved, if the blocks were melted at 68°C for 10 minutes, subsequently cooled to 37°C, and kept at that temperature to prevent polymerisation. Lanes **h** and **i** show such digestions, samples with 0.75% liquid low-melting-point agarose (left lane) compared to those without (right). Still, the activity of the *dam* methylase is slightly inhibited, but the difference to comparable digestions in absence of agarose as about 75% instead of the only 35% for solid agarose.

DISCUSSION

A method of creating partial DNA-digests by a combined incubation of a restriction endonuclease and a related methylase is presented. Using *dam* methylase and *Mbo*I, this approach has a number of advantages to other protocols which either use enzyme dilution or limited incubation times to control the frequency of cleavage. The size of the fragments generated is independent of the amount of enzyme, as long as more than 0.025 unit *Mbo*I/µl (plus the appropriate amount of *dam*) is added. There is also no influence of the duration of incubation. All sites get modified, by either methylation or cleavage.

The ratio of *dam*/*Mbo*I makes the extent of cleavage easy to control, all the more because the influence of the DNA-concentration on the frequency of cleavage is relatively small, especially if DNA-concentrations above about 150 ng/µl are used. Therefore, even if the exact DNA-concentration is not known, a quite accurate partial digestion can be carried out. This is of advantage if the amount of DNA is restricted, as in a preparation of flow-sorted chromosomes or microdissected DNA, because no material has to be used to determine the appropriate partial digestion conditions. Since the frequency of cleavage is set by the ratio *dam*/*Mbo*I and does not have to be checked by gel electrophoresis, even partially degraded DNA preparations can be used to generate a library.

If necessary, partially *dam*/*Mbo*I digested DNA can still be cut further with Sau3A, because this restriction enzyme cleaves GATC-sites independent of the methylation status.

The contrary specificity of *Dpn*I, recognizing the sequence 5′-GATC-3′ but specifically cutting the methylated form, should allow the creation of a jumping-library (15, 16), consisting of clones containing only both end-segments of each fragment. After ligation of the fragments of *dam*/*Mbo*I treated DNA into an unmethylated vector, subsequently the insert could be completely cleaved with *Dpn*I, while the vector and the likewise unmethylated cloning sites would not be cut. Religation would produce clones each containing both terminal *Dpn*I segments of one fragment. Such a library could be used as a complement to a genomic library made from the identical *dam*/*Mbo*I digest, for instance, bridging gaps which are due to non-clonable sequences.

The method of combined incubation with *dam* methylase and the restriction enzyme

MboI simplifies and accelerates the task of performing partial *MboI*-digestions. Fragments of any size range can be produced in a well controlled manner. As outlined recently (4) similar approaches can be developed with other methylase/restriction enzyme combinations. Considering the wide application of partial digestion in preparative and analytical techniques, careful and detailed studies on other combinations might be useful.

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