Ordered deletions for DNA sequencing and *in vitro* mutagenesis by polymerase extension and exonuclease III gapping of circular templates

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ABSTRACT

A simple method is described for generating nested deletions from any fixed point in a cloned insert. Starting with a single-stranded phagemid template, T_4 DNA polymerase is used to extend an annealed primer. This leads to a fully double-stranded circular molecule with a nick or small gap just 5′ to the primer. Exonuclease III initiates progressive digestion from the resulting 3′ end. Removal of timed aliquots and digestion with a single-strand specific endonuclease leads to a series of linear nested fragments having a common end corresponding to the 5′ end of the primer. These molecules are circularized and used to transform cells, providing large numbers of deletion clones with targeted breakpoints. The 6-step procedure involves successive additions to tubes, beginning with a single-stranded template and ending with transformation; no extractions, precipitations or centrifugations are needed. Results are comparable to those obtained with standard Exonuclease III-generated deletion protocols, but there is no requirement for restriction endonuclease digestion or for highly purified double-stranded DNA starting material. This procedure provides a strategy for obtaining nested deletions in either direction both for DNA sequencing and for functional analysis.

INTRODUCTION

Several different strategies are available for the generation of ordered deletions. Some involve the random generation of deletions, followed by size selection (1–5). Others use exonucleolytic degradation to generate deletions progressively, so that size selection is not essential to obtain targeted deletion breakpoints throughout a cloned segment (6–11). Unfortunately, each of these methods has limitations. For example, the nested deletions generated by exonucleolytic methods begin at a restriction site. This limits the general utility of these procedures for constructing deletions to be used in functional analyses, since appropriate sites rarely coincide precisely with ends of functional regions.

Here I describe a new method for the generation of ordered deletions that has advantages over current procedures. It uses single-stranded circular phagemid DNA (12) as starting material and does not require restriction endonuclease digestions. Full extension of an oligonucleotide primer around the circle using T_4 DNA polymerase provides a double-stranded circular molecule with a nick adjacent to the 5′ end of the primer. This nick is attacked by *E. coli* Exonuclease III (Exo III), leading to the generation of targeted deletions. In addition to its usefulness in DNA sequencing, the method makes it possible to generate a nested set of targeted deletions for *in vitro* mutagenesis from any point in a cloned insert using a single custom primer.

MATERIALS AND METHODS

The method is outlined in Figure 1. Starting with single-stranded DNA obtained from helper-infected cells harboring a ColE1-derived phagemid, such as pKUN19 (13), pUC119 (14) or pVZ1 (15), a fully double-stranded molecule is generated by polymerase extension of a primer. The primer is complementary to a sequence within the vector adjacent to the insert, such that extension proceeds through the entire vector first. This leaves a nick or very small gap adjacent to the 5′ end of the primer. Addition of Exo III leads to degradation of the synthesized strand starting at its 3′ end. At regular intervals, the uniformly digested gapped molecules are transferred to a solution containing a single-strand specific endonuclease, such as S1 nuclease, and incubated to remove the single strand opposite to the gap. The ends are repaired for ligation using T_4 DNA polymerase and T_4 polynucleotide kinase (for the non-phosphorylated primer used in the extension) followed by ligation. Transformation of competent *recA*$^-$ *E. coli* cells leads to large numbers of clones with targeted breakpoints, most of which correspond to the extent of Exo III digestion, as judged by agarose gel analysis of treated molecules. Transformants can be used to prepare templates for dideoxy sequencing or for introduction into a host organism for functional analysis.

Details for carrying out this procedure are provided in Tables 1 and 2, which respectively describe the preparation of solutions and a step-by-step procedure.

RESULTS AND DISCUSSION

Application of the method to a typical insert

The procedure was carried out on a 2748 bp *Drosophila* cDNA insert cloned into phagemid pVZ1. This vector has the

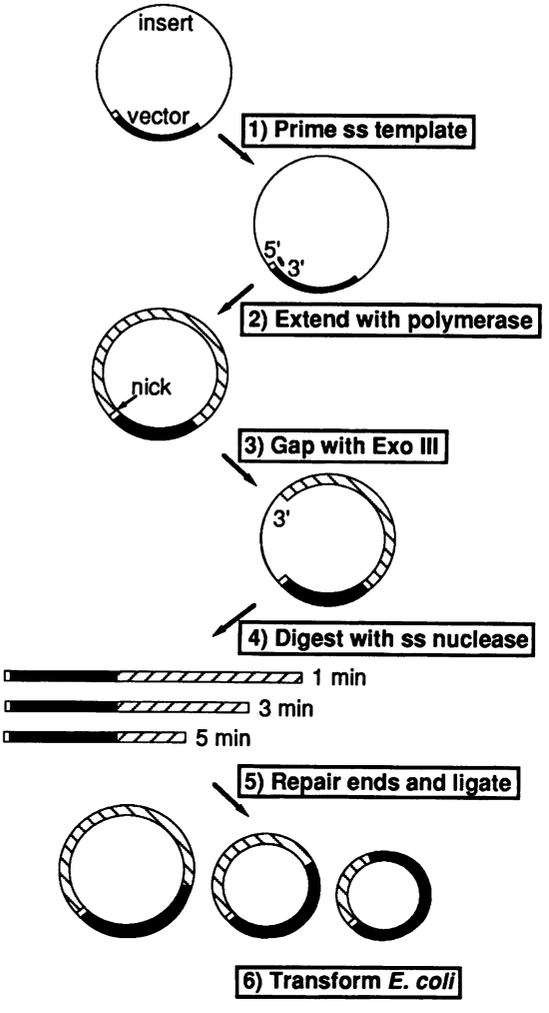

Figure 1 Outline of the method. The open box represents the primer binding site used for extension, also the fixed breakpoint for nested deletions in the resulting clones.

bacteriophage T_3 RNA polymerase promoter adjacent to the polylinker into which the cDNA was inserted. Phagemid particles were produced by infection with M13KO7 under kanamycin and ampicillin selection (14), and the resulting supernatant was used to prepare single-stranded circles by phenol-chloroform extractions of polyethylene glycol precipitated particles (16). A 20-mer complementary to the region of the T_3 promoter ('α-T_3': 5'-CCCTTTAGTGAGGGTTAATT-3') was annealed in 2-fold molar excess to the template (Figure 2 lane 1) and T_4 DNA polymerase was used to extend the primer. A sample electrophoresed on a 0.7% agarose gel after 30 minutes shows only partial extension (lane 2), whereas after 4 hours, nearly all of the material migrates as expected for nicked circles (lane 3). Exo III was added to this fully extended sample, and aliquots were removed at 30 second intervals. Alternate samples were treated with either S1 (lanes 5–10) or mung bean nuclease (lanes 12–16) and portions were electrophoresed. Exo III digestion proceeded synchronously at a rate of about 400 bases per minute, as evidenced by the uniform size range of molecules at each time point, with similar results for both S1 and mung bean nuclease treated samples.

In a similar experiment using this template, single-stranded circles were partially digested with MspI (GGCC) in order to fragment supercoiled circles that occasionally contaminate single-stranded template preparations, presumably due to cell lysis during infection. This was done in the annealing mixture and was followed by heat treatment, necessary both to inactivate the restriction endonuclease and to start the annealing reaction. After extension, Exo III and S1 nuclease treatments, samples were mixed with ligation cocktail, incubated overnight, and 1/4 of the ligation mix was used to transform competent cells. This resulted in 200–400 ampicillin-resistant colonies from each time point. Five colonies from each time point were inoculated into 1.5 ml cultures. These were harvested for supercoil minipreps using a standard alkaline lysis procedure with a single phenol-chloroform extraction and ethanol precipitation (17). Supercoils from 70 clones were partially sequenced ('G-tracked') (16) by the dideoxy method (18) using a 'reverse hybridization primer', which primes 38 bases upstream of the T_3 primer binding site. Sequencing was carried out using Sequenase (US Biochemical), ^{35}S dATP and Mn^{++} substituted for Mg^{++} (to favor sequence close to the primer). The breakpoints for 59 clones from which sequence adequate for precise mapping was obtained are shown as a function of the time of Exo III digestion (Figure 3). The partial sequences of 6 clones were not sufficient for mapping either to the insert or the vector. Five clones did not yield any sequence, even though plasmid was present, suggesting loss of the primer binding site. Except for these 5 clones, the 5'-most base of the α-T_3 primer was found adjacent to the breakpoint in each case.

Most of the breakpoints map very close to the line representing a rate of 400 nucleotides per minute. With one exception, the other breakpoints are above the line, indicating inserts smaller than expected. These might have resulted from the occasional failure of the polymerase to fully extend, coupled with preferential ligation of the smaller circles. Alternatively, non-specific priming within the insert region would lead to similar artifacts. For this reason, only a 2- to 4-fold excess of primer over template is annealed prior to extension. Four clones were not deleted at all. Interestingly, three of these were from the 1 minute time point (Figure 3). Although this clustering could have been coincidental, a more likely explanation is that during transfer of this aliquot to the S1 mix, the pipettor touched the side of the tube above the solution, leaving a small portion which escaped S1 digestion but not the subsequent end repair and ligation. To avoid this potential problem, it might be worthwhile to centrifuge the aliquots briefly after removal from ice for S1 digestion.

Application of the method to a large insert

Figure 4 shows a time course for T_4 DNA polymerase extension using a phagemid clone that has an insert of 6.2 kb. Examination of aliquots electrophoresed on a 0.6% agarose gel reveals that extension proceeded slowly, and was not complete even after 6 hours (lanes 3–6). However, overnight extension was sufficient to complete the reaction, with better results when supplemented with increased polymerase and deoxynucleoside triphosphates (lanes 7–8). The appearance and eventual disappearance of bands during the reaction reflects pausing of the polymerase at specific sites during synthesis (19, 20). A minor product which co-migrated with full-length linear molecules is seen in each case (see also Figure 2 lane 4). This might have resulted from extension of full-length single-stranded linear molecules from 'looped-back' 3' ends (21). Most of the completely extended molecules were resistant to brief S1 nuclease treatment, as expected for nearly fully double-stranded molecules (Figure 4 lane 9). However, when S1 nuclease treatment was preceded by

Table 1. Preparation of solutions and reagents.

1. Stock solutions:

Template: single-stranded phagemid DNA at 0.2−2 μg/μL H_2O or a low EDTA Tris buffer.
Primer: α-T3 20mer (5′ CCCTTTAGTGAGGGTTAATT 3′), reverse hybridization 17mer (5′ GAAACAGCTATGACCAT 3′) or the equivalent at 4 pmol/μl.
10× TM: 0.66M Tris-HCl pH8, 30mM $MgCl_2$.
DTT: 0.1M dithiothreitol.
dNTPs: 2.5mM each of the 4 deoxynucleoside triphosphates.
S1 buffer concentrate: 2.5M NaCl, 0.3M potassium acetate pH 4.6, 10mM $ZnSO_4$, 50% glycerol.
10× ligation buffer: 0.5M Tris-HCl pH7.6, 0.1M $MgCl_2$, 10mM ATP.
PEG: 50% (w/v) PEG (polyethylene glycol 6000−8000).

2. Enzyme reagents:

Restriction enzyme: (optional) frequent cutter that can be heat-inactivated and that leaves Exo III sensitive ends (such as MspI), tested for the absence of single-stranded nuclease activity.
BSA: 1mg/ml Bovine serum albumen (nuclease-free).
T_4 DNA polymerase: 1−10 units/μL either cloned or from T_4-infected cells.
Exonuclease III: ~150−200 units/μL.
T_4 polynucleotide kinase: 3−10 units/μL.
T_4 DNA ligase: 2−10 units/μL.

3. Working solutions:

S1 mix: 27μL S1 buffer concentrate, 173μL H_2O, 60 units S1 or mung bean nuclease, where 1 unit causes 1μg of nucleic acid to become perchloric acid soluble in 1 minute at 37°C. Prepare fresh and store on ice until use.
S1 stop: 150mM Trizma Base (no HCl), 25mM EDTA.
Ligation cocktail: 290μL H_2O, 50μL 10×ligation buffer, 50μL PEG, 5 μL DTT, 5μL dNTPs, 1 unit T_4 DNA polymerase, 10 units T_4 polynucleotide kinase, 20 units T_4 DNA ligase. Add enzymes just before use.
Host cells: Frozen aliquot (0.5−1ml) of Ca^{++}-treated $recA^-$ cells such as HB101 (35).

Table 2. Constructing nested deletions

1. All operations are carried out in 0.5ml microfuge tubes. Mix in a volume of 22μL: 2−4μg single-stranded phagemid DNA, 4μL 10× TM, 4 pmole α-T3 (or equivalent) oligonucleotide. [Optional: add 1 unit of a heat-inactivatable 4-cutter restriction enzyme (such as MspI) to partially digest contaminating double-stranded plasmid, and incubate 10 minutes at 37°C.] Heat to 75°C 5 minutes, then allow to cool slowly to 37°C over a period of 30−60 minutes. Evaporation and condensation can be minimized by placing a piece of insulating styrofoam over the tube in an aluminum tube-heating block. Remove a 2 μL aliquot for subsequent gel analyses.

2. Mix in a volume of 20μL: 2μL DTT, 4μL dNTPs, 4μL BSA, 5 units T_4 DNA polymerase. Prewarm to 37°C and add to the primed DNA at 37°. Incubate 2−8 hours; extension can be monitored by removing 0.5−1μL aliquots and electrophoresing on an agarose gel. Inactivate polymerase by heating for 10 minutes at 70°.

3. Prepare tubes with 3μL S1 mix on ice, one for each desired time point. (At 37°, the rate of exonuclease III digestion is 400−500 bases/min, increasing by about 10% per 1°C in the range of 30−40°C (30). Choose a temperature and a time interval to give the desired range of deletions. (It is very important to maintain a uniform temperature within the reaction solution during the incubation to obtain tightly clustered deletions; it helps to use a tube-heating block as a preheated dispenser for yellow tips.) Warm to the desired temperature a tube containing enough polymerase-extended circle to provide 1μL per time point. Add 1/10 volume exonuclease III, mixing thoroughly and rapidly with the pipettor. Remove successive timed 1μL aliquots to the S1 mix tubes, pipetting up and down to mix. Hold on ice until all aliquots are taken.

4. Remove tubes to room temperature and incubate 15−30 minutes. Add 1μL S1 stop, removing a 1μL aliquot for gel analysis at the same time. Heat tubes to 70°C for 10 minutes.

5. Add 16μL ligation cocktail. Incubate the resulting ligation mix at room temperature 1 hour to overnight. (The number of transformants obtained increases with long incubations.)

6. Transform by combining 5 μL ligation mix with 15 μL freshly thawed Ca^{++}-treated cells on ice. After 30 minutes, heat-shock 1.5 minutes at 42°C, add 100 μL SOC (36) or Luria broth and allow to recover about 30 minutes at 37°C before plating. Yields typically range from 10 to 1000 transformants depending on the ligation time and the competence of Ca^{++}-treated cells. The remaining ligation mixtures can be stored frozen for later use after overnight incubation.

digestion with Exo III, all of the molecules become susceptible to S1 nuclease attack (lane 10). In this case, 1.3 kb was removed, yielding molecules of about 8 kb.

A portion of the sample shown in Figure 4 lane 10 was used for ligation and transformation. The distribution of deletion breakpoints for 36 of the resulting clones is shown in Figure 5, except for 3 plasmids which were not interpretable by restriction mapping. Three plasmids were not detectably deleted (the optional restriction digestion of step 1 was omitted), 12 were of the desired size with breakpoints at about 1.3 kb into the insert, 13 were smaller with breakpoints within the 6.2 kb insert, and 5 were smaller with breakpoints within the vector. This result indicates that the method can be used effectively for targeting breakpoints in relatively large inserts.

Use of T_4 DNA polymerase for extension

Whereas the strong 3′-5′ exonuclease activity of T_4 DNA polymerase might seem to be a disadvantage in the extension reaction, since it greatly reduces the rate of polymerization, it appears that this activity is essential to prevent strand-displacement which occurs with other polymerases, such as PolIK (Klenow) and modified T_7 DNA polymerase (22; data not shown). On the one hand, strand displacement activity allows an enzyme to melt out hairpins during polymerization. T_4 DNA

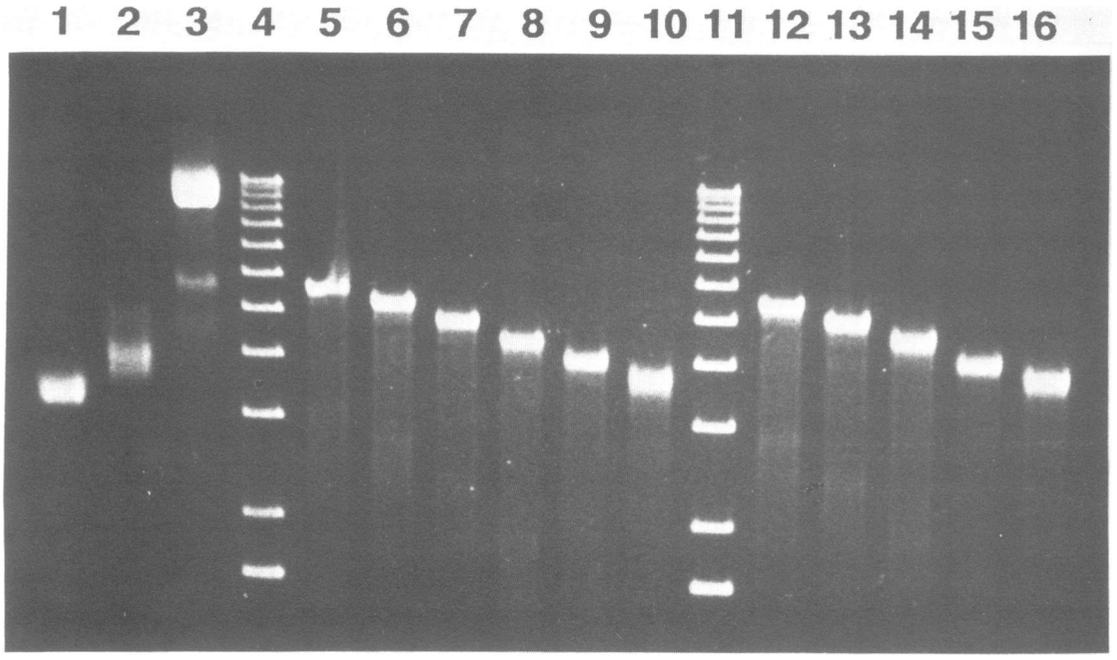

Figure 2 Agarose gel analysis of a deletion series (Table 2, steps 1–4) carried out on a cDNA insert from the *D. melanogaster bw* locus (37) cloned into pVZ1. Annealing to α-T_3 primer followed partial digestion of 4 μg single-stranded phagemid DNA with EcoRI (lane 1). Extension with T_4 DNA polymerase (Boehringer Mannheim, 1 unit/μL) was carried out according to the procedure (Table 2), except that dNTPs were at a concentration of 0.15 mM. Aliquots were taken at 30 min (lane 2) and 4 hours (lane 3). Shortly thereafter the reaction was terminated by incubation at 70° C and placed at 37° C for digestion with Exo III (Boehringer Mannheim, 175 units/uL). One microliter aliquots were removed at 30 second intervals and placed alternately into 3 μL S1 nuclease (Promega 50 units/μL) or mung bean nuclease (Bethesda Research Labs 50 units/μL) and digested 30 minutes at room temperature. S1 stop was added and the aliquots were loaded onto the gel. Each sample represents 1/40 of the starting material. Samples are (lanes 5–10) 0′, 1′, 2′, 3′, 4′, 5′ Exo III digestion followed by S1 nuclease treatment and (lanes 12–16) 0.5′, 1.5′, 2.5′, 3.5′, 4.5′ Exo III digestion followed by mung bean nuclease treatment. Markers (lanes 4 and 11) are 1 kb ladder fragments with sizes (in bp from top to bottom) 12216, 11198, 10180, 9162, 8144, 7126, 6108, 5090, 4072, 3054, 2036, 1636 (Bethesda Research Labs). The 0.7% agarose gel in TBE buffer (34) contained 0.2 μg/ml ethidium bromide.

polymerase is known to be impeded by such regions in substrate molecules (19, 20, see Figure 4). Although this might be a problem in some cases, it appears that given enough time, this enzyme can extend through most sequences, stopping when it reaches the 5′ end of the primer. On the other hand, a polymerase that can cause strand displacement will continue beyond the 5′ end of the primer by displacing this end, synthesizing a rolling circle. Such molecules are unsuitable as substrates for this method (data not shown). Conceivably, displacement of the primer end caused by an enzyme such as modified T_7 DNA polymerase might be prevented by by cross-linking the primer to the template (R. Garber, personal communication). The use of this latter enzyme or *E. coli* DNA polymerase III might reduce extension times from hours to minutes.

Another feature of T_4 DNA polymerase that makes it suitable for this method is the exceptionally high fidelity with which it replicates DNA. Its error frequency has been estimated to be no more than 10^{-7}, or about 10–100-fold lower than that for DNA polymerase I (23). This is a particularly important consideration for the generation of clones to be used in functional analyses and in DNA sequencing. The enzyme also completely lacks a 5′-3′ exonuclease activity, so it will not nick-translate, a problem for this method similar to strand-displacement. Furthermore, both the cloned enzyme and that from T_4-infected cells have yielded excellent results, even after very long incubations, indicating very good stability and a lack of detectable endonuclease activity. The enzyme also is widely available and relatively inexpensive, as are the other components of this method.

One should be aware, however, that the highly active 3′-5′

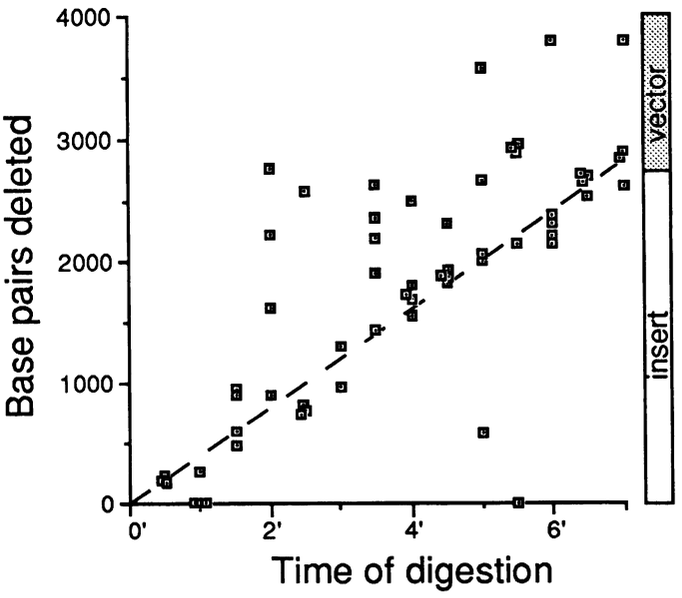

Figure 3 Results of an Exo III digestion series carried out as described in the legend to Figure 2, except that 2 μg single-stranded template was used, predigestion was done with MspI (Bethesda Research Labs), extension was carried out using cloned T_4 DNA polymerase (United States Biochemical) and single-stranded nuclease digestion using S1 nuclease.

exonuclease activity of T_4 DNA polymerase will cause a 'stuttering' after completion of extension; this might eventually deplete the reaction of deoxynucleoside triphosphates (dNTPs)

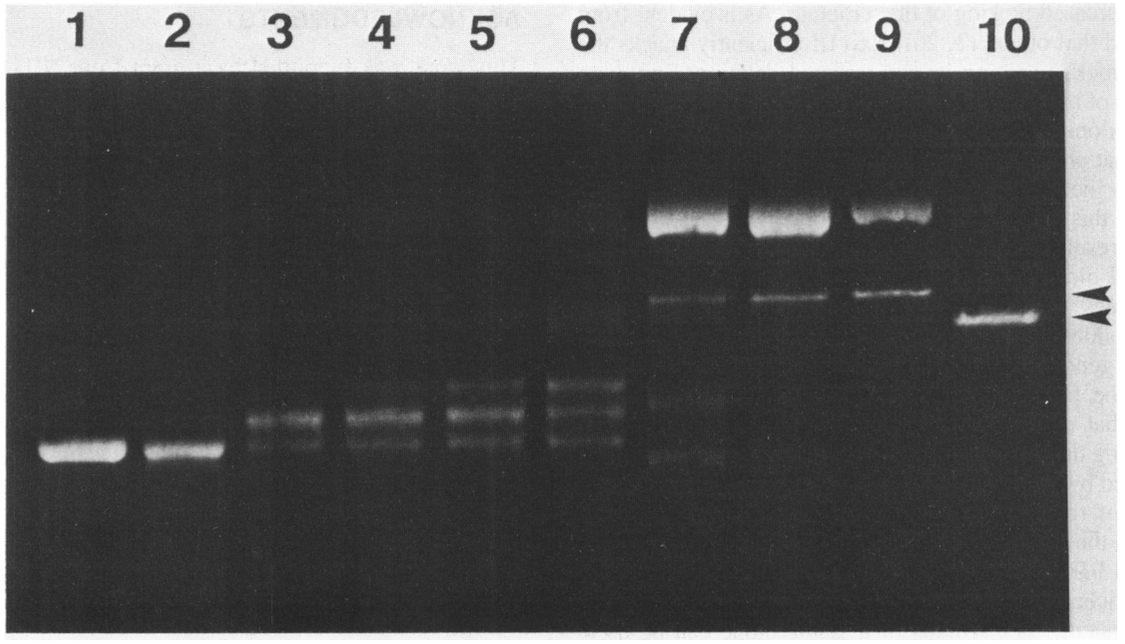

Figure 4 Agarose gel analysis of a T_4 DNA polymerase extension and Exo III digestion of 4 μg single-stranded phagemid DNA generated from a 6201 bp SalI genomic fragment from the *Drosophila pseudoobscura Gart* locus (15) cloned into pVZ1. Lane 1: starting single-stranded material; lane 2: annealed to α-T_3 primer; lanes 3–7: T_4 DNA polymerase extension for 1, 2, 3, 6 and 20 hours, respectively; lane 8: same as in lane 7 except that after 6 hours the reaction was supplemented by addition of 0.1 units/μL polymerase and 0.4 mM dNTPs; lane 9: S1 nuclease treatment of the sample electrophoresed in lane 8; lane 10: Same, except after a 6 minute Exo III digestion. Markers run in a nearby lane are indicated by arrows representing 9162 and 8194 bp. Aliquots represent 1/40 of the starting material. The 0.6% agarose gel in TBE buffer contained 0.2% ethidium bromide.

leading to exonucleolytic degradation of the synthesized strand (20, data not shown). The ability of S1 nuclease to fully linearize the polymerase extended sample shown in Figure 2 (compare lanes 3 and 5) but not the sample shown in Figure 4 (compare lanes 8 and 9) is attributable to the lower dNTP concentration during the extension reaction in the former case, but not in the latter. It appears that successive depletion of as many as 3 of the 4 dNTPs will lead to a very small gap, which will have little consequence for this method. However, when all 4 dNTPs are depleted, the 3′-5′ exonucleolytic activity can progressively degrade the synthesized strand. For this reason, when incubating for extended periods, the reaction should be supplemented with more dNTPs. Concentrations as high as 0.5 mM in each dNTP have been used without apparent ill effects. Since some chelation of Mg^{++} by NTPs occur, an equimolar amount of $MgCl_2$ also should be added if higher levels of dNTPs are found to be necessary.

Comparison to other strategies for generation of deletions

The method described here uses polymerase extension of a primed single-stranded circle to prepare a substrate for Exo III digestion, avoiding the need for highly purified plasmid DNA or for the presence of unique restriction sites. The deletion strategy of Dale *et al.* (8) shares these features. However, their method results in a broad distribution of breakpoints, rather than a tight clustering, and is reported to be hampered by certain sequences (24). A different strategy is to use polymerase extension of a primer annealed to a single-stranded template followed by single-strand specific endonuclease treatment to generate fragments which are cloned into a double-stranded vector (24, 25). However, this strategy requires at least one unique restriction site and is not very effective at targeting deletion breakpoints for larger inserts (24).

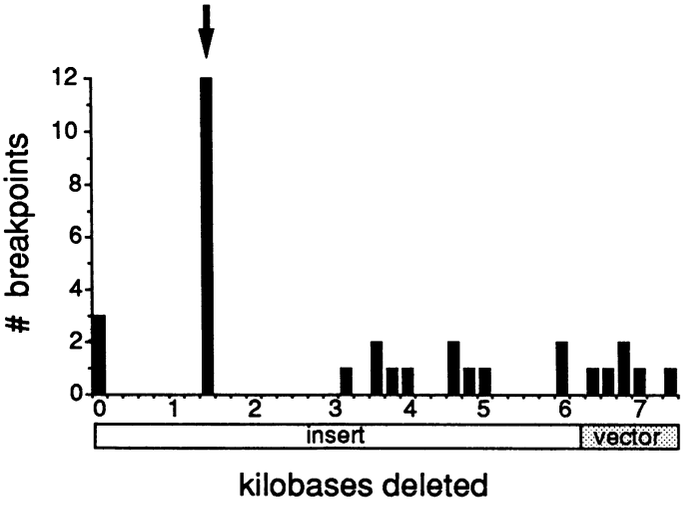

Figure 5 Mapping of 33 deletion breakpoints from a single early time point for a 6.2 kb cloned insert. A portion of the sample shown in Figure 4 lane 10 was incubated for 4 hours after addition of ligation cocktail and used to transform *E. coli*. The arrow indicates the approximate extent of digestion based on the gel analysis shown in Figure 4.

There are other problems with deletion methods that require restriction endonuclease digestions. For example, in a strategy based on Exo III digestion introduced previously (7), two unique restriction sites must be present in the polylinker region of the vector. For this procedure to be effective, digestion by the two restriction endonucleases must be complete. Since the two sites are very close, it is often difficult to determine this by agarose gel analysis. Thus extended digestions are sometimes carried out,

leading to increased nicking of the template. As is evident from this work and that others (2, 26), Exo III efficiently attacks the 3′ end at a nick, resulting in non-targeted deletions and even frequent loss of the primer binding site. Other problems are that restriction endonucleases do not always digest well in a common buffer and that phenol extraction and ethanol precipitation steps are generally necessary after restriction cutting. The method described in this paper avoids these problems, yet still yields comparable results.

A potential disadvantage of this method is that the resulting deletion clones cannot be sequenced using single-stranded templates produced by infection with the same helper phage that was used to generate the parent template. This is because the polarity of the primer used to extend the parent template is opposite to that needed for sequencing through the breakpoint of the resulting deletion template (see Figure 1). However, this can be avoided by the use of pKUN vectors, which have a second viral origin of replication derived from phage IKe oppositely orientated to the origin for phage M13 (13). Infection with a chimeric M13-IKe helper such as *Mike*, yields pure single stranded particles of the correct polarity for DNA sequencing. Using this system, both strands of a single clone can be used for the generation of deletions. Infection with an M13 or f1 helper such as M13K07 to obtain one strand for polymerase extension and with a *Mike* helper to obtain the other strand should allow one to generate deletion series from either end of the insert. Single-stranded templates for sequencing from the resulting clones can be obtained by infection with *Mike* in the first case and with M13K07 in the second.

Possible modifications and extensions of the method

The novel feature of this method is the preparation of a substrate for Exo III. This substrate can be used for other applications, such as 'ExoMeth' sequencing (27). Other modifications described in previous studies can be incorporated. For example, problem templates that fail to extend quantitatively can be gel-purified after S1 or mung bean nuclease treatment as described (28, 29). This might be useful for obtaining targeted deletions within large inserts for early time points, where the presence of even low levels of smaller molecules during the ligation can reduce the frequency with which targeted deletions are obtained (Figures 4 and 5). In addition, the Exo III reaction can be slowed down in a controlled manner by reduction in temperature or enzyme concentration (30) or by addition of NaCl (31). The method should be readily automated, since it requires only successive additions and temperature changes.

Previous methods for generating ordered deletions have been used primarily as subcloning strategies for DNA sequencing. For *in vitro* deletion mutagenesis, these methods have been limited by their requirement for a restriction site at the point from which deletions are generated. Since the current method has no such requirement, it allows for nested deletions to be made from any point in a cloned insert using a custom primer. Unlike current procedures for site-specific generation of deletions (32–34), this method can be used for obtaining many different breakpoints from a single primer. These deletions are generated at nearly 100% efficiency, with a large fraction targeted to a predetermined region, making this method potentially applicable to many situations in which deletions are desirable. In addition, the use of the pKUN vector system (13) should allow such deletions to be made in either direction from a single parent clone, even multiple deletions generated successively.

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37. Dreesen,T.D., Johnson,D.H. and Henikoff,S. (1988) *Mol. and Cell. Biol.*, **8**,5206–5215. **Table 1** Preparation of solutions and reagents