fragment, generating blunt ends that can be ligated with phage T4 DNA ligase. Because *Bal* 31 digestion continues in a nearly linear fashion for up to 10 to 15 minutes, a nested set of deletions can be easily created. The number of nucleotides separating the ends of the deletions can be varied by adjusting the amount of *Bal* 31 used, the time of digestion, and by the use of commercially available preparations of *Bal* 31 which are characterized as "fast" or "slow." A particular advantage of the protocol described here is that the high transformation frequency of MC1061 cells makes it unnecessary to repair blunt ends resulting from digestion with *Bal* 31.

Literature Review

In addition to the exo III and *Bal* 31 methods of generating nested sets of deletions, several other procedures have been developed for large-scale sequencing projects. Shotgun cloning of sonicated or DNase I-treated DNA followed by sequencing of clones chosen at random has been used to sequence large stretches of DNA (Anderson, 1981; Sanger et al., 1982; Bankier and Barrel, 1983). The disadvantage of the random approach is that the sequencing of large fragments can become extremely tedious when only small gaps of the target DNA remain to be sequenced (Anderson, 1981; Sanger et al., 1982). A common problem associated with sonication to generate random fragments is the creation of frayed ends and the subsequent difficulty of end repair.

Another approach to sequencing large regions of DNA is the extension of a sequence by primer-directed dideoxy sequencing; thus an oligodeoxynucleotide is synthesized with the sequence of the 3′ end of a region already sequenced. The oligodeoxynucleotide is then used as a primer to sequence into the unknown region. The widespread introduction of automated DNA synthesizers is making this approach widely accepted.

Finally, Dale et al. (1985) have developed an elegant strategy for generating a set of overlapping deletions starting with single-stranded DNA cloned in M13. One important advantage of this method is its independence of the restriction sites occurring in the insert.

Critical Parameters

When preparing phage from M13mp clones, vigorous aeration is required to obtain high titers. If low phage titers are a problem, try adding 100 mM MOPS buffer, pH 6.5, to the 2× TY medium used for growing the phage.

When examining M13mp phage for inserts, considerably better resolution can be obtained if large gels are run for 10 to 15 hr at low voltage. Following precipitation of the phage with PEG, it is very important to remove as much PEG as possible before resuspending the phage in TE buffer. Contaminating PEG will result in high background in the sequencing gels. Phage pellets can be resuspended en masse in TE buffer in the phenol extraction step if the tubes are vigorously shaken by hand in a suitable rack. Phenol remaining in the aqueous phase is readily removed by washing the ethanol precipitates with 100% ethanol. This also hastens drying and reveals the single-stranded DNA as an arched film.

Exonuclease III. It is important to start with a highly purified DNA preparation that has been subjected to two rounds of CsCl/ethidium bromide centrifugation. Preexisting nicks in the DNA will create a substrate for exo III and S1 nuclease, leading to unspecific degradation. During the double digestion step, it is very important that the DNA is completely digested. Choosing restriction sites that are as far apart as possible increases the probability of obtaining a complete digestion.

If the yield of DNA from longer digestion times turns out to be disproportionately low, the exo III is probably contaminated with other nucleases. A high background of randomly deleted clones is another sign of nuclease contamination in the exo III, the restriction enzymes, or the S1 nuclease. However, even these clones may be useful in obtaining sequence information.

Bal 31 exonuclease. It is important to start with a highly purified DNA preparation which has been subjected to two rounds of CsCl/ethidium bromide centrifugation since *Bal* 31 is very sensitive to RNA contamination. Complete linearization of the plasmid DNA by excessive treatment with the appropriate restriction endonuclease to the extent that the ends are damaged is not a problem since *Bal* 31 will convert a damaged end into a blunt end, suitable for ligation.

If insufficient digestion occurs with *Bal* 31, repeat the procedure using a higher concentration of enzyme. Although it is not necessary for this protocol because of the high transformation frequency of MC1061, some researchers improve the quality of the *Bal* 31-generated ends by filling out with Klenow fragment (UNIT 3.5) or by digestion with S1 nuclease or mung bean nuclease (UNIT 3.12).

EGTA very effectively stops *Bal* 31 diges-

tion by chelating Ca^{++}; however, it also inhibits subsequent digestion with a variety of restriction enzymes. Therefore, it is very important to carefully ethanol precipitate samples after the addition of EGTA. This will allow digestion with minimal amounts of restriction enzymes, thus providing high quality ends for subsequent ligation reactions.

Successful cloning of *Bal* 31-digested fragments into M13mp vectors also depends on using the minimal amount of restriction endonuclease treatment to prepare the M13mp vector for ligation. Unfortunately, because the M13mp vector is first cut with *Sma*I and then with a second enzyme which cuts only a few bases away in the polylinker, it is difficult to determine the extent of cleavage of the second enzyme. M13mp vectors suitable for ligation are best prepared by carefully titrating all restriction enzymes with uncut M13mp vector to determine the minimal amount to completely digest M13mp RF DNA. If necessary, check the ability of ligase to circularize the cut vector following *Sma*I digestion and again following digestion with the second enzyme. There should be a dramatic decrease in the vector's ability to be self-ligated following treatment with the second enzyme. Excess prepared vector should last for months or years if kept frozen.

Time Considerations

Exonuclease III. Starting with CsCl/ethidium bromide–purified plasmid DNA, it should take 3 or 4 days to generate and characterize a set of 30 overlapping fragments of a 3.0-kb target DNA.

Bal 31 exonuclease. Starting with CsCl/ethidium bromide–purified plasmid DNA, it should take 6 days to generate a set of 20 ordered fragments for a 2.0-kb insert of interest.

Literature Cited

Anderson, S. 1981. Shotgun sequencing strategy using cloned DNase I-generated fragments. *Nucl. Acids Res.* 9:3015-3027.

Bankier, A.T. and Barrell, B.G. 1983. Shotgun DNA sequencing. *In* Techniques in Life Sciences, B5: Nucleic Acid Biochemistry (R.A. Flavell, ed.) pp. 1-34. Elsevier/North Holland, Limerick, Ireland.

Chen, E.Y. and Seeburg, P.H. 1985. Supercoil sequencing: A fast and simple method for sequencing plasmid DNA. *DNA* 4:165-170.

Dale, R.M.K., McClure, B.A., and Houchins, J.P. 1985. A rapid single-stranded cloning strategy for producing a sequential series of overlapping clones for use in DNA sequencing: Application to sequencing the corn mitochondrial 18 S rDNA. *Plasmid* 13:31-40.

Guo, L.-H. and Wu, R. 1982. New rapid methods for DNA sequencing based on exonuclease III digestion followed by repair synthesis. *Nucl. Acids Res.* 10:2065-2084.

Henikoff, S. 1984. Unidirectional digestion with exonuclease III creates targeted breakpoints for DNA sequencing. *Gene* 28:351-359.

Ozkaynak, E. and Putney, S.D. 1987. A unidirectional deletion technique for the generation of clones for sequencing. *Biotechniques* 5:770-773.

Hoheisel, J. and Pohl, F.M. 1986. Simplified preparation of unidirectional deletion clones. *Nucl. Acids Res.* 14:3605.

Poncz, M., Solowiejczyk, D., Ballantine, M., Schwartz, E., and Surrey, S. 1982. "Nonrandom" DNA sequence analysis in bacteriophage M13 by the dideoxy chain-termination method. *Proc. Natl. Acad. Sci. U.S.A.* 79:4298-4302.

Putney, S.D., Benkovic, S.J., and Schimmel, P. 1981. A DNA fragment with an α-phosphorothioate nucleotide at one end is asymmetrically blocked from digestion by exonuclease III and can be replicated in vivo. *Proc. Natl. Acad. Sci. U.S.A.* 78:7350-7354.

Sanger, F., Coulson, A.R., Hong, G.F., Hill, D.F., and Peterson, G.B. 1982. DNA sequencing with chain-termination inhibitors. *Proc. Natl. Acad. Sci. U.S.A.* 162:729-773.

Yanisch-Perron, C., Vieira, J., and Messing, J. 1985. Improved M13 phage cloning vectors and host strains: Nucleotide sequences of the M13mp18 and pUC19 vectors. *Gene* 33:103-109.

Key References

Henikoff. 1984. See above.

Hoheisel and Pohl. 1986. See above.

Describe the original procedure from which the exo III protocol is adapted.

Poncz et al. 1982. See above.

Describes the original procedure from which the Bal 31 protocol is adapted.

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