HASTEN: A technique to identify the primary structure of terminal DNA hairpins

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Terminal DNA hairpins are made by a covalent linkage between two anti-parallel strands of DNA. They can be found at the end of a viral DNA genome (1), during the creation of cDNA by retrovirus reverse transcriptase (2), or at the end of a DNA recombination intermediate, such as V(D)J recombination coding end intermediates (3) or as postulated following transposition in plants (4). In an attempt to determine the structure of V(D)J coding end intermediates, we have developed a variation of the ligation mediated PCR technique (5), called **HASTEN** for **H**Airpin **S**equencing using **T**7 gene 6 **E**xo**N**uclease, to determine the sequence of a DNA hairpin. While it may be theoretically possible to amplify DNA hairpins using a single PCR primer (the same primer will bind to one strand of the original DNA hairpin and the opposite strand of the newly generated hairpin), we were unsuccessful at doing so under various PCR conditions (unpublished data).

HASTEN provides a novel approach to determine the structure of terminal DNA hairpins, within a known sequence context. This approach does not require purification of the DNA hairpin from non-hairpinned DNA with the same sequence, as the hairpin DNA will be enriched while the non-hairpinned DNA will be eliminated. In addition, HASTEN has the potential to detect any sequence variability between hairpin loops. The basis for this approach is to use T7 gene 6 exonuclease, which acts on double stranded DNA to remove nucleotides in a 5' to 3' direction from a strand break (6). This results in removal of one strand of the hairpin stem to release the hairpin loop while maintaining the nucleotides within the loop. Removal of one strand of the hairpin stem will also render the PCR primer binding sites accessible for PCR amplification of the DNA loop. The overall approach for this technique is as follows (and outlined in Figure 1): 1) Hairpin DNA is restricted to provide a starting point for T7 gene 6 exonuclease. The restriction site is chosen such that the primer binding site will be eliminated from non-hairpinned DNA following exonuclease treatment. This can be accomplished if the primer binding site is found proximal to the 5' end of the strand on which it is located within non-hairpinned DNA. The hairpin loop should be located within the restriction fragment containing the primer binding site. 2) The restricted DNA is treated with T7 gene 6 exonuclease. 3) The treated DNA is subjected to primer extension using the gene specific PCR primer. 4) The extended DNA is ligated to a blunt, double stranded linker and subjected to multiple rounds of PCR amplification. 5) The PCR amplified products can then be cloned and sequenced.

In order to test the utility of this approach in enriching and amplifying DNA hairpins for sequence determination, we created a model hairpin on pUC19 and subjected it, in parallel with a non-hairpinned control, to the treatment outlined above. pUC19 was restricted with PstI (all enzymes were obtained from New England Biolabs unless otherwise noted) and ligated to a hairpin oligomer using T4 DNA ligase (Figure 1). Hairpin ligated pUC19 (50 ng) and untreated pUC19 (50 ng) were restricted with DraII (Boehringer Mannheim) and HindIII. The HindIII site was selected because of its proximity to the primer binding site, which allows for the elimination of the primer binding site by the exonuclease on non-hairpinned DNA molecules, thus enriching the hairpins. Restricted pUC19 and hairpin pUC19 were treated with 75 units of T7 gene 6 exonuclease (USB) for varying times. The treated DNAs were subjected to multiple rounds of primer extension using 1 μ M of a pUC19 specific primer located 298 bp upstream of the hairpin loop, 100 nM each dATP, dCTP, dGTP, and dTTP, 5 mM KCl, 1 mM Tris pH 8.3, 1.5 mM MgCl₂, 0.01% gelatin, 1 unit Taq polymerase (Perkin Elmer Cetus), and 2.3 μ Ci dCT³²P (NEN DuPont). The cycles of primer extension were as follows: 1 cycle of 94°C for 3 minutes, 63°C for 1 minute, 72°C for 1 minute and 34 cycles of 94°C for 1 minute, 63°C for 1 minute, and 72°C for 1 minute. The primer extension products were run on Long Ranger (AT Biochem) sequencing gels and exposed to film (Figure 2A). Treatment of hairpin pUC19 with 75 units of T7 gene 6 exonuclease for 30 minutes was required in order to generate a primer extension product. Longer treatment (60 minutes) did not enhance the amount of product generated. In contrast, non-hairpinned DNA does not contain a specific primer extension product indicating that the exonuclease was able to fully degrade this template.

The sensitivity of HASTEN was tested in a titration experiment. Hairpin pUC19 was titrated in unmodified pUC19 to keep the total amount of DNA constant at 50 ng. The DNA was then DraII/HindIII restricted and treated with 75 units T7 gene 6 exonuclease for 30 minutes. The products of that reaction were either subjected to multiple rounds of primer extension as described above or to one round of primer extension as described above without including the radiolabeled nucleotide. The unlabeled primer extension products were ligated to 5 pmol of blunt, double stranded linker (7) that contained a 6 bp overhang at one end to prevent ligation of the linkers. PCR was performed as described for the primer extension reaction with the addition

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Figure 1. Outline of HASTEN as performed on pUC19. pUC19 (not to scale) was linearized with PstI (P) and ligated to a 37 base hairpin oligo that contained a 14 bp stem, a 5 base loop, and a 4 base 3' PstI extension. Unmodified pUC19 and hairpin ligated pUC19 were restricted with DraII (D) and HindIII (H). Only the fragments that contain the primer binding sites (above the small arrow) are shown (hatched). Treatment of the restricted pUC19 with T7 gene 6 exonuclease (T7 g 6 exo) will degrade each strand of the duplex from the 5' end until the double stranded template is eliminated. Treatment of the hairpin modified pUC19 will remove the stem from the 5' end until the double stranded template is removed (when the loop is released). Exonuclease treated products were primer extended using a pUC19 specific primer (small arrow). T7 g 6 exo treatment of the unmodified pUC19 sample would remove the primer binding site resulting in no extension product. A product of approximately 298 bp would be expected for the hairpin modified pUC19. PCR amplification of the linker ligated, primer extension products would generate a specific product of approximately 318 bp from the hairpin modified pUC19. No product is expected from the exonuclease treated, unmodified pUC19.

of 1 μ M linker specific primer. The products of the primer extension and the PCR reactions were run on Long Ranger (AT Biochem) sequencing gels. As seen in Figure 2B, DNA hairpin ends can be detected by primer extension following a ten fold dilution in non-hairpinned DNA or at 0.1 pmol. By PCR (Figure 2c), 5.9 fmol of hairpin ends could be detected, which corresponded to a 200 fold dilution, in non-hairpinned DNA.

To determine the extent to which T7 gene 6 exonuclease removes nucleotides from the hairpin stem and loop, 50 ng hairpin ligated pUC19 was DraII/HindIII restricted, T7 gene 6 exonuclease treated, primer extended (one round without label incorporation), linker ligated, PCR amplified, cloned (TA cloning kit, Invitrogen), and sequenced (Sequenase, USB). Sequence analysis of the amplified products indicated the presence of the hairpin loop and part of the palindromic stem in 30% of the clones (11/31 clones) (data not shown). Approximately 50% (14/31 clones) of the clones had deletions of up to 40 bases from the hairpin end. The remaining 20% (6/31 clones) contained over 150 bases deleted from the hairpin end. These sequences presumably result from T7 gene 6 exonuclease operating at a nick in the DNA that results from incomplete ligation of the hairpin oligomer to pUC19.

Figure 2. Time course and titration of HASTEN treated hairpin ligated pUC19. **A.** Time course of T7 gene 6 exonuclease treated hairpin modified pUC19. Hairpin modified and unmodified pUC19 were treated as described (see text) for the times indicated above each lane. Primer extension products for these samples and untreated pUC19 are shown. The specific bands are shown (arrow). **B.** and **C.** Primer extension and PCR amplification, respectively, of exonuclease treated, titrated hairpin ligated pUC19. Hairpin modified pUC19 was titrated and treated as described (see text). The amount (in ng) of hairpin pUC19 included in each sample is indicated above each lane. The results of primer extension (**B**) and PCR (**C**) are shown. The arrows indicate the specific bands.

HASTEN is a technique that can be used to enrich and determine the sequence of a terminal DNA hairpin loop. We demonstrate that this approach can detect 5.9 fmol of DNA hairpin ends. In addition, approximately 30% of the clones obtained with this technique contained palindromic stem loop structures by sequence analysis. We are currently using this approach to determine the structure of DNA hairpins associated with putative coding end intermediates found in V(D)J recombination.

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