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Use of Asymmetric PCR to Generate Long Primers and Single-stranded DNA for Incorporating Cross-linking Analogs into Specific Sites in a DNA Probe

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Photoactivatable DNA analogs have been incorporated enzymatically into DNA and used to map the locations of polypeptides in protein complexes bound to DNA. We have developed a procedure for generating long primers from short oligodeoxyribonucleotides (oligos) to incorporate DNA cross-linkers at specific sites within either strand of DNA probes of ≤206 bp. Single-stranded DNA molecules of 52–206 nucleotides in length were generated by asymmetric polymerase chain reactions (aPCR), using an excess of one short sense-strand primer to be extended and a limiting amount of each short antisense primer that is complementary to and defines the 3′ end of the long primer to be generated. The noncross-linking strand of the DNA probe was also generated by aPCR from the DNA sequence of interest. The long primers were annealed to the full-length noncross-linking DNA strand to form a partially double-stranded DNA. Cross-linking analogs and radioactive deoxyribonucleotides (dNTPs), followed by normal dNTPs, were enzymatically incorporated onto the long primers to form the double-stranded DNA cross-linking probes. This method is reproducible and avoids many of the difficulties encountered by other published methods.

The long-term goal of our study is to map, along the DNA, the locations of eukaryotic RNA polymerase II (RNAP) subunits that are in close proximity to the template DNA in a stalled transcription-elongation complex. We require DNA cross-linkers and adjacent radioactively labeled nucleotides to be incorporated into specific sites in either strand of a DNA template long enough to stall a transcribing RNAP at a site in the DNA that is ≥60 bp from either end of the DNA fragment. Therefore, the cross-linker-containing DNA probes must be ≥120 bp, and have cross-linkers in specific positions within the 50 bp at the center of the DNA fragment. When proteins are assembled onto such analog-containing DNA probes and exposed to ultraviolet (UV) light, the polypeptides close to the site of analog incorporation in the DNA become cross-linked and thus radioactively labeled. We have developed a novel method to enzymatically incorporate cross-linking DNA analogs into such sites in DNA probes. We are incorporating the photoactivatable thymidine analog 5-[N-(*p*-azidobenzoyl)-3-aminoallyl]dUTP (N_3RdUTP; Bartholomew et al. 1990).

Figure 1 summarizes some methods used to incorporate labeled dNTPs and cross-linkers into specific regions of a DNA probe. The procedure shown in Figure 1A (Bartholomew et al. 1990, 1991, 1993) leaves a gap in the cross-linker-containing DNA strand, just 5′ of the downstream oligo. This gap is closed by DNA ligase, with the efficiency of the ligation generally 70–95%, and monitored by measuring the release of a ^{32}P-γ phosphate on the 5′ end of the downstream oligo. Because RNAP will bind nonspecifically to a nick in the DNA, the efficiency of the ligation has to be evaluated when the cross-linking DNA is used as a transcription template. The Gilmour et al. (1990) and Sypes and Gilmour (1994) approaches leave no gap in the DNA, but suffer from three limitations. Although synthesized long primers allow exact placement of cross-linkers in the DNA, they are very costly to make when many are required, and they are limited to ~100 nucleotides in length. When using a strand of a restriction fragment as the primer, the sites into which cross-linkers can be positioned

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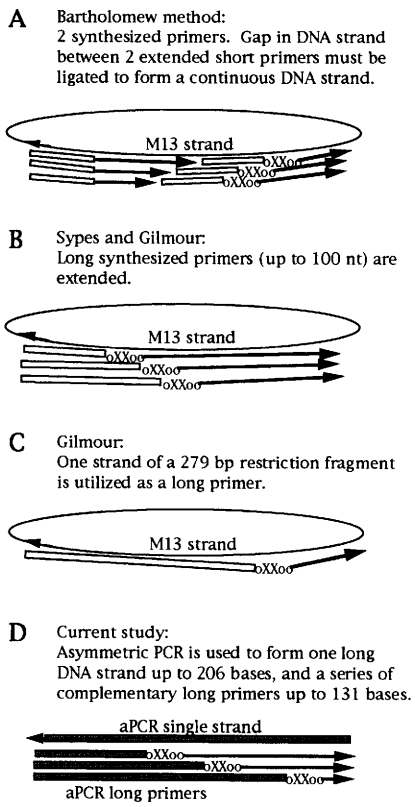

Figure 1 Methods used to incorporate cross-linkers into double-stranded DNA fragments. Cross-linkers (X's) and radioactive dNTPs (o's) are incorporated onto the 3′ ends of oligodeoxyribonucleotide primers. The source of the primers varies, and primers are denoted by narrow rectangles. Procedures *A–C* require a desired sequence to be subcloned into single-stranded phage DNA to form the noncross-linking strand of the probe. For our study (*D*) we generated the noncross-linking strand by asymmetric PCR instead of using phage DNA. (*A*) Bartholomew and coworkers incorporated a cross-linker and label onto the 3′ end of a primer annealed to M13 DNA, then annealed a second short primer upstream of the first. Both primers were extended with normal dNTPs. A gap resulted in the cross-linker-containing strand. The DNA probe was released from the rest of the M13 DNA by cutting the double-stranded DNA with restriction enzymes. (*B*) Sypes and Gilmour incorporated label onto the 3′ ends of synthesized primers ≤100 bases long, and then extended with normal dNTPs. (*C*) One of the strands of a 279-bp restriction fragment served as a long primer. (*D*) For our current study we generated both the long strand for cross-linking probes and long primers by aPCR. Cross-linkers and label were incorporated onto the ends of the primers which were then extended with normal dNTPs.

are limited by the availability and positions of restriction sites.

The procedure we developed utilizes asymmetric polymerase chain reaction (aPCR) to generate single-stranded DNA (ssDNA) ≤206 nucleotides long, and we see no reason why this method could not be used to generate longer single-stranded DNAs. The noncross-linking full-length strand of the DNA probe and the long primers onto which the cross-linker is added are both derived from aPCR (Fig. 1D). The cross-linking strand is generated first by annealing the long primer from aPCR to a full-length complementary strand. Then, using a modified T7 DNA polymerase (Sequenase; Tabor and Richardson 1987), the DNA analog (in place of thymidine) and one adjacent radioactive dNTP are incorporated onto the 3′ end of this primer. Sequenase is much more processive than the Klenow fragment of DNA polymerase I. Finally, the generation of a double-stranded DNA (dsDNA) probe is completed by adding an excess of all four normal dNTPs to the DNA polymerase reaction. By generating the primer using aPCR, we are able to incorporate the cross-linker into a specific site, and further downstream into the DNA fragment than if we used a shorter synthesized primer, without having to ligate two shorter strands of DNA, without using chemically synthesized long primers, and without using a restriction fragment as a primer.

Before developing the aPCR alternative for incorporating cross-linkers, we subcloned our desired sequence into an M13 phage vector to follow the cross-linking protocol described by Bartholomew et al. (1990); thus we used the phage construct M13TT18 as the source of template DNA for the PCRs shown in this study. The aPCR procedure worked equally well using single-stranded M13 DNA or a dsDNA fragment as template, but did not work with supercoiled plasmid DNA (data not shown). The hybridization of a short primer to one strand of a supercoiled plasmid is energetically much less favorable than the reannealing of the two plasmid strands. In typical PCR, the much greater number of primers (to template) compete favorably for hybridization, and the initial PCR products serve as templates for the later products. In aPCR we use a limiting amount of one of the primers. We think it is possible that a small amount of single-stranded product may be made in an aPCR with supercoiled plasmid, but that the reaction is very inefficient under these conditions. Thus, our proce-

dure eliminates the need to subclone the DNA sequence being studied into a single-stranded phage vector to generate the template DNA for cross-linking probes.

RESULTS

Generation of the Noncross-linking Full-length DNA Strands for Probe Synthesis

To form a 143-bp cross-linking probe with cross-linkers incorporated into either strand, we used aPCR to generate either one strand or its complement from a DNA sequence subcloned into an M13 vector (Fig. 2). The ratio of the sense and antisense primers At3Sma and AccB determined whether the top strand or bottom strand was generated. We used 100-fold more of the primer that formed the 5′ end of the strand desired. The top strand t143 has primer At3Sma at its 5′ end and was consistently generated at least twice as efficiently as the "bottom" strand b143, which has primer AccB at its 5′ end (data not shown). The efficient generation of single-stranded b143 DNA required starting the amplification reaction at an elevated temperature (a modified hotstart). We obtained equal yields when the aPCR was started by adding the Mg^{++} at 94°C, by adding the *Taq* DNA polymerase at 94°C, by using HotStart100 wax bead tubes (Molecular BioProducts), or by placing the reaction immediately into a thermal cycler preheated to 94°C after pipeting in all components (data not shown).

Asymmetric PCR to Generate Long Primers

With aPCR we were able to generate long primers of 52 to 131 bases (data shown only for primers with top strand sequence and up to 112 bases). We annealed those long primers with the same 5′ ends, but of differing lengths, to strand b143 to place cross-linkers in different positions within the top strand of the DNA probe. Because *Taq* DNA polymerase has been reported to add primarily nontemplate-directed deoxyadenosine (dATP) to the 3′ ends of amplified products (Clark, 1988), antisense primers (such as b107) were designed to account for the adenosine addition to resulting long primers (such as t107). Adenosine is the next base to be added onto each of the annealed primer–templates to form the double-stranded probe (see Fig. 2), thus an

Figure 2 Template DNA M13TT18 and oligodeoxyribonucleotides. Oligos used to construct M13TT18 are shown in bold. Sma-At and Hindsac-Bb were used as PCR primers. Oligos Sacsty-Bt (in bold on top strand) and Stysac-Bb (in bold on bottom strand) were annealed to form Region B. DNA sequences upstream of Region A and downstream of Region B are from the vector. Primers used for aPCR are shown as arrows. Cross-linking probes had the sequence shown when primers At3Sma and AccB were used to generate the noncross-linking full-length bottom strand (b143). When primer M13 Forward was used instead of AccB, the resulting product was 206 bp, the b143 sequence plus an additional 63 bp of M13 vector sequence. Primers b52, b74, b80, b94, b107, and b112 (shown as 5′ to 3′ arrows) have the orientation of and sequences from the bottom strand. These primers were used to define the 3′ ends of resulting long primers of the designated length and allow cross-linker (X) and radioactive dNTP (o) placement into the sites shown above the sequence; that is, b74 was used to generate a 74-nucleotide primer t74, which allows cross-linker placement at position 76 and radioactive dA at positions 75 and 77, in the resulting probe.

adenosine added by the *Taq* DNA polymerase onto the long primer would not alter the resulting DNA sequence of the probe. We found that ~75% of primers had a nontemplate-directed 3′ dNTP added. For probes t94 and t112, which require the addition of a dATP at the 3′ end of the primer to be able to incorporate the dTTP cross-linking analog and labeled dCTP, we were able to generate labeled probes from the presumed 75% of primer–templates to which the 3′ dATP had been added.

The aPCRs generate both single-stranded and double-stranded products (Fig. 3). On a nondenaturing 15% polyacrylamide gel, the single-stranded product migrates with double-stranded DNA markers of ~70% the length of its corresponding double-stranded product. Thus, the single-stranded 107-base primer has the mobility of an ~70-bp DNA fragment.

When the full-length complementary strand b143 was added to the DNA from long primer aPCR reactions under annealing conditions, the single-stranded primers annealed to b143, forming a partially double-stranded product. The annealed primer–templates were detected on nondenaturing 15% polyacrylamide gels by their mobility, which was distinct from the mobilities of all of the reactants (Fig. 4).

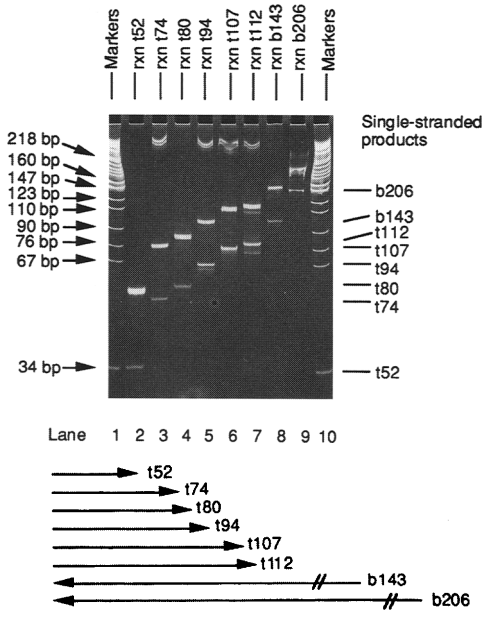

Figure 3 Nondenaturing PAGE shows that the asymmetric PCRs generate both single-stranded and double-stranded products. Long primers t52 to t112 (lanes *2–7*) are generated by *Taq* DNA polymerase extending the 28 nucleotide primer At3Sma to result in primers with the indicated length (52 to 112 nucleotides). To form cross-linking probes, long primers were hybridized to either strand b143 or b206 (lanes *8* and *9*).

Photoactivatable Cross-linkers Incorporated Enzymatically into DNA Probes

We use radioactive nucleotides adjacent to the photoactivatable cross-linker N_3RdUTP in our DNA probes to radioactively tag associated pro-

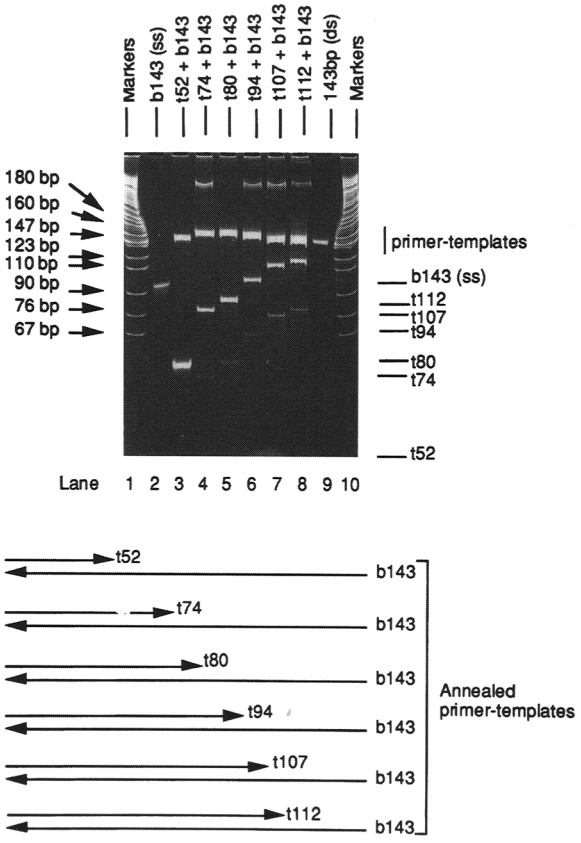

Figure 4 Long primers anneal to template strand b143. (Lane *1*) 2 pmole of gel-purified single-stranded b143 (ss); (lanes *2–8*) products from annealing 1.5 pmole b143 (ss) with aPCR reactions containing long primers t52 to t112. Both single- and double-stranded (ss and ds) long primers of the designated sizes (52 to 112 bp) are in the reaction, but their sizes are all less than the ds b143 (lane *9*). Numbers on the right of the panel indicate primers remaining ss. All of the annealed primer–templates have mobilities similar to 143 bp, but distinct from their ss and ds long primers.

teins when the cross-linker's azido group is activated by ultraviolet light. DNase I and micrococcal nuclease are used to digest the DNA following UV irradiation, leaving a small radioactive DNA tag on the cross-linked proteins.

The incorporation of cross-linkers and radiolabeled dNTPs onto the 3′ ends of the primers t74 to t112 is monitored by running the products of the extension reactions onto denaturing polyacrylamide gels and subsequent autoradiography (Fig. 5). The resulting lengths of the primers plus label and analog depend on the DNA sequence and are indicated in the figure. Cross-linker incorporation reactions show a small amount of readthrough, presumably attributable to free dNTPs that copurify with the single-stranded DNAs.

Double-stranded DNA probes are formed by further extending the cross-linker-containing primers with all four normal dNTPs (data not shown). After this extension, the full-length cross-linker-containing DNA strands from all of our primer–templates are the same length, as expected (data not shown). The small percentage of primer–templates that are not fully extended in the Sequenase reaction are not a problem in our system. RNAP binds and transcribes from only single-stranded 3′ DNA ends, not the 5′ ends that result from incomplete extension. The chance of having a cross-linker beside a labeled deoxyribonucleotide outside the desired site of incorporation (resulting from the extension with 100 μl of 250 μM normal dNTPs added to 2 μM labeled dNTP and 20 μM cross-linker in a 20-μl reaction) is less than one in 30,000 in this system.

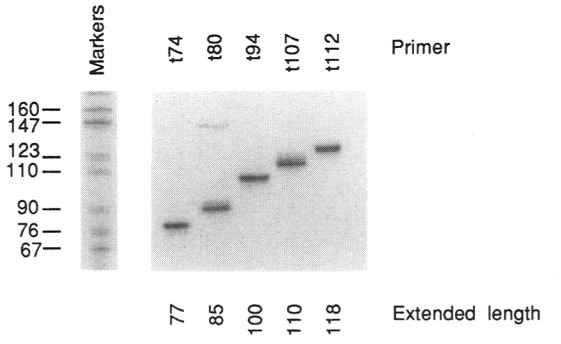

Figure 5 Incorporation of the cross-linkers and radioactive dNTPs onto the long primers. Synthesis of the cross-linker-containing DNA strand was visualized upon denaturing PAGE. Cross-linking analogs and labeled dNTPs are incorporated onto the primers indicated at the top of the panel, extending the primers to the expected lengths indicated below the panel. DNA markers are *Msp*I-digested pBR322.

DISCUSSION

Cross-linkers have been previously incorporated into DNA probes with DNA polymerase, incorporating the cross-linking analog and radioactive dNTPs onto an oligodeoxyribonucleotide primer complementary to a DNA sequence of interest expressed in M13 phage single-stranded DNA (Bartholomew et al. 1990, 1991, 1993). Such a procedure requires researchers to subclone their DNA of interest into a phage vector with an f1 origin to obtain the single-stranded DNA. Incorporating the cross-linker into either one strand or its complement requires subcloning the desired DNA sequence into such vectors in both orientations. We were able to generate either one of the full-length strands by aPCR from an M13 clone in only one orientation. The aPCR method to generate a long primer worked as well using a double-stranded DNA fragment as with the single-stranded phage DNA as template (data not shown).

The procedure we developed using aPCR to generate long primers from short oligodeoxyribonucleotides allows us to incorporate cross-linkers into specific sequences along the length of a 143 or 206 bp cross-linking probe, and in either strand, without having a gap in the cross-linking DNA strand, and without having to ligate such a gap. Using these techniques, we generated a series of probes using the same short sense primer and a series of short and inexpensive antisense primers. The single-stranded amplified products have as their 5′ end the primer that was in excess during the aPCR, and at their 3′ end the DNA sequence complementary to the antisense primer that was limiting.

Quantitative evaluation of single-stranded products by EtBr-staining may be deceptive. Because EtBr intercalates into double-stranded DNA, single-stranded DNA stains much less well. Although the long primers stained faintly, each 100-μl reaction typically generated 3 pmole of single-stranded product. A typical cross-linking experiment requires ≤50 fmole of probe DNA.

Several reaction conditions affect the yield of single-stranded DNA. The concentration of the limiting primer in an aPCR must be empirically determined (McCabe 1990). As observed by other researchers doing double-stranded PCR, we

found PCR primers with annealing temperatures of ≥55°C to function better in our aPCR than primers requiring lower annealing temperatures (Innis and Gelfand 1990). Using a modified hotstart procedure as described was important for generating single-stranded product. The elapse of more than a couple of minutes between the addition of all the components and the start of the reaction appeared to have a detrimental effect on the yield of some single-stranded products.

In conclusion, we expect the use of asymmetric PCR for generating long primers to be a useful approach to incorporate DNA analogs at desired sequences throughout a DNA fragment up to at least 200 base pairs long. We have used DNA probes made by the procedures described to detect the yeast RNA polymerase II subunits close to cross-linker-containing DNA templates that were being transcribed. The results of our protein–DNA cross-linking experiments will be described elsewhere.

METHODS

Oligonucleotides and Template DNA

All oligonucleotides used were synthesized by the University of Wisconsin Biotechnology Center and are shown in Figure 2. Enzymes were purchased from Promega, New England Biolabs, or GIBCO-BRL, unless otherwise noted. Plasmid p(C_2AT) was a gift from Dr. Robert Roeder (Sawadogo and Roeder 1985).

Standard cloning techniques were used (Sambrook et al. 1989) to construct an M13 phage with a segment of 76 bases that lack deoxycytidine in the coding strand after the *Sma*I site. This feature serves as a mechanism to stall transcribing RNAP molecules along the DNA template where they will be cross-linked. We constructed M13TT18 by inserting a Region A–Region B fragment into the *Sma*I site of vector M13mp18 (Pharmacia, see Fig. 2). Region A is a 76 bp segment of DNA obtained by standard PCR on the G-less cassette plasmid p(C_2AT) using the primers Sma-At and Hindsac-Bb. This PCR product was treated with the Klenow fragment of DNA polymerase I to remove any overhanging 3′ bases and cut with *Sac*I to remove the downstream end. The resulting Region A fragment was ligated to a complementary sticky end of the 30-bp Region B, formed by annealing phosphorylated oligo Sacsty-Bt and unphosphorylated oligo Stysac-Bb (Fig. 2). Following ligation, the reaction mixture was digested with *Sty*I and filled in for blunt-end ligation. The resulting 108-bp Region A-Region B fragment was ligated into the *Sma*I-digested, double-stranded M13mp18 vector.

Generation of Single-stranded DNA

The 143 nucleotide noncross-linking strand of the DNA probe has the sequence of the bottom strand shown in Figure 2 and is designated b143. When we used M13/pUC forward primer instead of AccB, we generated an alternative bottom strand b206 that results in a 206-bp cross-linking probe. Single-stranded b143 or b206 were generated by aPCR as follows: A 100-μl aPCR reaction typically generates 0.75–4 pmoles of ssDNA. Each cross-linking experiment requires only 50 fmoles of the resulting probe. Batches of 10 tubes of 100-μl reactions were prepared in 1× Thermophilic buffer (Promega), with 1.5 mM $MgCl_2$, and 50 μM dNTPs. Each 100-μl reaction included 50 pmoles of either primer AccB (to generate strand b143) or Universal M13/pUC (−47) forward primer (to generate strand b206), 0.5 pmole of primer At3Sma, 20 ng of single-stranded M13TT18, and 2.5 units of *Taq* DNA polymerase. Reaction components were mixed, adding *Taq* DNA polymerase last, and rapidly aliquoted into reaction tubes. The reactions were started by immediately setting them into the thermal cycler preheated to 94°C (a modified hotstart). Reactions were heated for 30 sec at 94°C, and then run for 30 cycles of denaturing for 30 sec at 94°C, annealing 30 sec at 65°C and extending for 1 min at 72°C. Extensions were completed by incubating at 72°C for 10 min. Reactions were phenol-extracted and the DNA precipitated with 2.5 M ammonium acetate (NH_4OAc), 10 mM $Mg(OAc)_2$, and 2–3 volumes of ethanol. Pellets were washed with 70% ethanol, dried, and resuspended in 0.5× TE (pH 7.9) (5 mM Tris-HCl at pH 7.9, 0.05 mM EDTA). The ssDNA was then gel-purified as described below.

Primers of 52–112 nuceotides in length were generated by extending the upstream 28mer At3Sma in aPCR reactions. Limiting amounts of the oligos shown in Figure 2 as b52, b74, b80, b94, b107, and b112 were used as antisense primers. The resulting long primers are designated by their length preceeded with "t" for top-strand orientation and sequence. For example, long single-stranded primer t107 was generated by extension of the short synthetic oligo At3Sma when a limiting amount of synthetic oligo b107 was used as the downstream primer in an aPCR. The aPCR reactions were carried out as described for generating single-stranded b143 and b206, with 50 pmoles of upstream primer (At3Sma for this series of primers), and 0.75–2 pmoles of a downstream primer with the bottom-strand orientation (b52 to b112, Fig. 2). Annealing temperatures were determined experimentally. Reactions were phenol-extracted and the DNA ethanol precipitated, as above, prior to gel purification of the single-stranded long primers.

Gel Purification of Single-stranded DNAs

Single-stranded DNAs from 52 to 206 nucleotides in length were visualized on nondenaturing 15% polyacrylamide gels (19 acrylamide:1 bis-acrylamide) in 0.5× TBE (44.5 mM Tris-Borate, 1 mM EDTA, pH 8.2), and stained in 0.5 μg/ml ethidium bromide (EtBr) for 5 min. Bands were cut out of the gels and the DNA passively eluted in 400 μl (800 μl for large gel slices) of 0.5 M NH_4OAc, 0.1 mM EDTA and 0.1% SDS by shaking at 37°C for 4–17 hr. DNA was precipitated by addition of 0.1 volume of 10 M LiCl and 2 volumes of ethanol. The DNA was resuspended in 100 μl of 0.5× TE. Yields were measured by the absorbance at 260 nm, assuming A_{260} = 1 corresponds to 33 μg ssDNA/ml.

Annealing Primers to the Full-length (Template) Strand

Equimolar amounts (2 pmol each) of gel-purified long primer and template strand b143 were combined in 15 μl, brought to 30 mM NaCl, and annealed by heating at 90°C for 10 min, then placing the tubes into a 60°C heat block and allowing them to cool to 37°C over 1 hr.

Synthesis of Double-stranded Photoactivatable DNA Probes

The DNA analog N_3RdUTP was synthesized by the method of Bartholomew et al. (1990). Cross-linker and radioactive label were incorporated onto annealed primer–templates as follows. ^{32}P-labeled dATP was incorporated into primer–templates t74, t80, and t107. ^{32}P-labeled dCTP provided the label for t94 and t112. In a 20 μl volume, 2 pmole primer–template, 2 μM ^{32}P-dATP or dCTP (sequence dependent), 20 μM N_3RdUTP, 1 mM 2-mercaptoethanol (2-ME), and 0.1 mg/ml BSA were combined in 1× Sequenase buffer (50 mM Tris-HCl at pH 7.9, 50 mM NaCl, 10 mM $MgCl_2$). Additionally, the t80 reaction included 50 μM dGTP to add ATGAG onto primer t80. After equilibrating to 37°C, 2 units of Sequenase Version 2.0 (United States Biochemical) were added and the reaction proceeded for 3 min at 37°C. The volume was then brought to 100 μl in 1× Sequenase buffer, 1 mM 2-ME, 0.1 mg/ml BSA, 250 μM each dATP, dCTP, dGTP, and dTTP, pre-equilibrated to 37°C. Extensions were for 5 min at 37°C after adding 5 units of Sequenase. The reaction was terminated by phenol extraction. The DNA was ethanol precipitated and resuspended in 0.5× TE.

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