RecA-AC: single-site cleavage of plasmids and chromosomes at any predetermined restriction site

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ABSTRACT

We have developed a novel version of the Achilles' Cleavage (AC) reaction in which virtually any restriction site on DNA of any size can be converted to a unique cleavage site. We first polymerized RecA protein on a synthetic oligodeoxyribonucleotide (oligo) in the presence of a nonhydrolyzable ATP analogue to generate oligo:RecA nucleoprotein filaments. These filaments were then incubated with plasmid or intact chromosomal DNA from *Saccharomyces cerevisiae* to form stable complexes in the yeast *LEU2* gene at the target sequence identical (or complementary) to that of the oligo. When *HhaI* (*HinfI*) methyltransferase (*M-HhaI*) was added, all of the recognition sites for *HhaI* with the exception of the one protected by the RecA filament were methylated and thus no longer cleaved by the cognate restriction endonuclease (*HinfI*). After inactivation of the RecA and the *M-HhaI*, *HinfI* was used to efficiently cleave the plasmid or chromosome specifically at the targeted restriction site. Since oligos specific for any sequence can be easily synthesized and the other reagents necessary to perform RecA-mediated AC (RecA-AC) reactions on both plasmids and intact chromosomes are readily available, this procedure can be applied immediately to the precise dissection and analysis of genomic DNA from any source and to any other research problem requiring efficient, highly specific cleavage of DNA at predetermined sites.

INTRODUCTION

Achilles' Cleavage (AC) is a simple, general method for combining the cleavage specificities of restriction endonucleases with the binding specificities of other DNA-binding molecules (1,2) (Fig. 1A). With this technique, any restriction site that can be selectively protected from methylation can be made unique. Previous studies have demonstrated that AC allows both plasmid and intact chromosomal DNA to be efficiently cleaved at only those restriction sites that are protected from methylation by repressor proteins (1–4), eukaryotic transcription factors (e.g., GCN4) (5), *E. coli* integration host factor (6), and synthetic oligodeoxyribonucleotides (oligos) capable of forming a triple-

helix structure (7–10). Each of these methyltransferase (MTase)-blocking agents, however, has only a limited range of DNA-binding specificities. In this report we describe a RecA-mediated AC (RecA-AC) reaction. Since the binding specificity of the MTase-blocking agent used in this procedure is derived entirely from a synthetic single-stranded DNA (ssDNA) moiety, essentially any restriction site for which the corresponding MTase is available can be made unique.

The RecA protein from *Escherichia coli* promotes the strand-exchange of ssDNA fragments with homologous duplex DNA (11,12). This process has three major steps: (i) RecA polymerizes on the ssDNA to form presynaptic nucleoprotein filaments, (ii) in the presence of ATP, the ssDNA:RecA filaments bind to the double-stranded DNA (dsDNA) and come into alignment with the homologous sequences (i.e., synapsis), and (iii) the strands are exchanged and a new heteroduplex is formed. If the nonhydrolyzable ATP analogue adenosine 5'-[γ -thio]triphosphate (ATP[γ S]) is used in place of ATP, the nucleoprotein filament remains in a stable complex with the homologous dsDNA (13–15) (Fig. 1B). The ability to form these stable complexes using ATP[γ S] has been exploited both as a means of dissecting the molecular mechanism of RecA-mediated homologous recombination (13–16) and as a tool for screening recombinant libraries (17,18). Here we report that RecA presynaptic filaments can protect restriction sites from methylation in an AC reaction and describe reaction conditions that optimize this protection on both plasmid and chromosomal DNA.

While we were preparing this report for publication, we became aware of the fact that another group was independently developing and have since published (19) an Achilles' Cleavage protocol utilizing RecA nucleoprotein complexes as the methylase-blocking reagent. Their work, which differs from that presented here in detail but not in principle, convincingly demonstrated that RecA-AC can be used to selectively and efficiently cleave complex genomes at predetermined sites.

MATERIALS AND METHODS

Materials

Oligodeoxyribonucleotides (Oligos). Oligos were synthesized by the UW-Madison Biotechnology Center and were resuspended in double-distilled water (ddH₂O). The sequences of the oligos

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used in the experiments described were derived from the *LEU2* gene located on chromosome III of *Saccharomyces cerevisiae*. The 70-mer had the sequence 5'-CTAGGATCATGGCGGCA-GAATCAATCAATTGATGTTGAACCTTCAATGTAGG-GAATTCGTTCTTGATGGT-3' and contained recognition sites (underlined) for the restriction enzymes *MboI* (GATC), *HhaII* (HinfI, GATC), and *EcoRI* (GAATTC). The shorter oligos, all of which extend to the same point at their 3' ends, had the sequence 5'-³⁶GGGTTC ³⁰TAACT ²⁴AGGATC ¹⁸ATG ¹⁵GCG ¹²GCAGAATCAATC-3', with the 5' ends of the respective oligos (36-mer to 12-mer) identified by superscripts.

RecA and ATP[γ S]. RecA (6.25 μ g/ μ l) was purchased from Pharmacia. A 10 mM solution of ATP[γ S] (Sigma) was made in sterile ddH₂O, stored in small aliquots at -70°C , and was diluted to 0.4 mM just before use.

Methyltransferases (MTases) and S-adenosylmethionine (SAM). Nuclease-free M-*HhaII* was provided by Dr. S.Chandrasegaran (20) and 1 μ l of a 1:50 dilution was used for each methylation reaction (this was the amount that was found empirically to fully methylate 30 μ l of microbead-embedded yeast chromosomes in a one hour reaction). M-*EcoRI* was purchased from New England Biolabs (NEB) and 100 units (U) was used for each methylation reaction. Due to the exquisite susceptibility of intact genomic DNA to nuclease degradation in the presence of Mg^{2+} , which is required for RecA binding, the MTases used in this procedure must be free from contaminating nucleases. To confirm that the MTases used were nuclease-free, yeast chromosomes (below) were incubated with the MTase preparations in 1 $\times$ RecA buffer for 1 hr at 37°C and then analyzed for degradation by PFGE. SAM was obtained from NEB, stored in 1 μ l portions at -20°C , and diluted to either 1.6 mM (20 $\times$) or 2.4 mM (30 $\times$) with ice-cold 5 mM H₂SO₄ just before use.

Plasmids and genomic DNA. HindIII-linearized pRS305 (21), a yeast shuttle-vector containing the *LEU2* gene, was used as target DNA for the plasmid experiments. Intact chromosomal DNA from *S.cerevisiae* strain YNN295 (22) was prepared in agarose microbeads as described (4, 23) and served as the genomic target for the RecA-AC experiments. To equilibrate the microbeads in the RecA reaction buffer, the microbead pellet was washed once with 200 μ l of TEX [10 mM Tris·HCl (pH 8.0/25 $^{\circ}\text{C}$), 1 mM EDTA, 0.01% Triton X-100] and twice with 200 μ l of the reaction buffer. After inactivation by detergent, an additional wash with 200 μ l of TE [10 mM Tris·HCl (pH 8.0/25 $^{\circ}\text{C}$), 1 mM EDTA] was performed prior to the wash with TEX and equilibration in the digestion buffer.

Titration of the RecA stock

It is vital to form the oligo:RecA complex with the proper molar ratio of RecA monomers to nucleotides in order to eliminate the nonspecific protection that results from RecA monomers interacting with the dsDNA target. Although a nt:RecA monomer ratio of about 3:1 seems to work best (e.g., ~ 0.165 μ g oligo and ~ 6.25 μ g RecA), it was always necessary to experimentally determine the working ratio for each lot of reagents with a titration reaction. A series of dilutions of the oligo was made in ddH₂O and the RecA-AC reaction was performed on linearized plasmid DNA, as described below, using 1 μ l of the oligo dilution and 1 μ l of the RecA stock. The oligo concentration that optimized

protection of the targeted restriction site and eliminated nonspecific protection of the other sites was used as the working concentration for subsequent RecA-AC experiments.

Back-titration with oligo(dT)

As an alternative to working at precisely the proper ratio of oligo to RecA, nucleoprotein filaments can be formed with an excess of RecA monomer provided that any unpolymerized RecA is functionally removed from the reaction before the target DNA is added. In this case, the targeting oligo was incubated with a slight excess of RecA (determined by the titration reaction described above), ATP[γ S] was added to stabilize the complex, and then a 'nonspecific' oligo [e.g., oligo(dT) 60 nt in length] was added at approximately the same concentration as the targeting oligo to absorb the RecA monomers still in solution. The filaments formed with the nonspecific oligo cannot protect any restriction sites from methylation and therefore did not interfere with the RecA-AC reaction.

RecA-AC of plasmid DNA in solution

To form oligo:RecA nucleoprotein filaments, 1 μ l of oligo (at the proper concentration, see above) and 1 μ l of RecA stock were incubated at 37°C in a buffer consisting of 25 mM Tris·acetate (pH 7.5/25 $^{\circ}\text{C}$) and 1 mM magnesium acetate. After 30–60 sec, 1 μ l of 0.4 mM ATP[γ S] was added (bringing the final volume to 10 μ l) and the reaction was incubated at 37°C for another 10 min [oligo(dT) was added at this point when used]. Linearized plasmid DNA (0.5 μ g) was added, the buffer was adjusted to 25 mM Tris·acetate (pH 7.5/25 $^{\circ}\text{C}$), 4 mM magnesium·acetate, 10 mM dithiothreitol, and 0.1 mg/ml BSA (27 μ l reaction volume), and incubation was continued at 37°C for 30 min. The magnesium acetate concentration was then adjusted to 8 mM, MTase (1 ml M-*HhaII* or 100 U of M-*EcoRI*) and SAM (to 80 nM) were added (30 μ l final reaction volume) and the methylation was carried out at 37°C for 30 min. The MTase and RecA were inactivated and removed from the methylated DNA by adding 100 μ l of the DNA Purification Resin from Magic Minipreps (Promega, Madison WI) to the reaction and following the procedure described by the manufacturer. The deproteinized plasmid was eluted with 20 μ l of TE [10 mM Tris·HCl (pH 8.0/25 $^{\circ}\text{C}$), 1 mM EDTA] and digested (30 min, 37°C) with either 5 U *HinfI* (NEB) or 10 U *EcoRI* (NEB) in the buffer recommended by the supplier.

RecA-AC of genomic DNA embedded in agarose microbeads

Microbeads (25 μ l) containing embedded yeast chromosomes were equilibrated with the standard RecA binding buffer [25 mM Tris·acetate (pH 7.5/25 $^{\circ}\text{C}$), 4 mM magnesium acetate and 10 mM DTT] as described (see above, 4, 23) and 3 μ l of BSA (1 mg/ml) was added to the microbead pellet. The oligo:RecA filaments were formed in a separate tube as described above for the 'RecA-AC of plasmid DNA', but with 2 ml each of the oligo and the RecA stock in the 10 μ l reaction. After the 10 min incubation that followed the addition of the ATP[γ S], 5–10 μ l of the oligo:RecA filament mixture was added to the microbead pellet (prewarmed to 37°C) and sufficient magnesium acetate was added to maintain the magnesium acetate concentration at 4 mM. The sample was placed on ice overnight to allow the filaments to diffuse through the agarose matrix. After diffusion, the sample was warmed to 37°C and incubated for 30 min. The magnesium acetate concentration was adjusted to 8 mM, fresh DTT was

Figure 1. The principle of RecA-mediated Achilles' Cleavage (RecA-AC). (A) Achilles' Cleavage (AC). Any restriction site that can be specifically protected from modification by a methyltransferase (MTase) can be converted into a functionally unique restriction site with the AC procedure. (B) Oligo:RecA nucleoprotein filaments can be made in the presence of ATP[γ S] that will form stable complexes with dsDNA at the sequence identical to that of the oligo used. Since ssDNA of any sequence will interact with RecA, these filaments can be used in the AC procedure to make essentially any restriction site unique.

added to 10 mM (1 ml of 0.6 M DTT; this is critical for good methylation), and 1 μ l of M-*Hha*II and SAM (to 80 mM) were added. The methylation was carried out at 37°C for 1 hr. To inactivate the RecA and MTase, 25 μ l ES [1% N-lauroylsarcosine (w/v) in 0.5 M EDTA] was added and the sample was incubated at 52°C for 30 min. The digestion with *Hin*fl (5 U, NEB, 1 hr, 37°C) was performed as described (4, 23) in the recommended buffer.

As an alternative to first forming the RecA filament and then diffusing it into the agarose, in some experiments the oligo and RecA were added separately to the microbeads and the filament was formed within the agarose matrix. In this procedure, 1–2 μ l of oligo were added to the microbead pellet and allowed to diffuse at 37°C for 15 min. The appropriate amount of RecA stock (1 or 2 μ l) was added and the incubation was continued for another 10 min. ATP[γ S] (1 μ l of 0.4 mM stock) was then added and the reaction was incubated at 37°C for 30 min. The magnesium acetate concentration was adjusted to 8 mM, fresh DTT was added to 10 mM, M-*Hha*II and SAM (to 80 mM) were added and methylation was carried out as described above.

RESULTS

Optimizing RecA-AC of plasmid DNA in solution

Experimentally, the challenge in developing RecA-AC was to optimize the specific protection afforded by oligo:RecA nucleoprotein filaments and to eliminate the nonspecific protection caused by RecA monomers interacting with dsDNA. To do this, we found that it was essential to polymerize the RecA on just enough ssDNA to completely titrate the RecA. In this way, all of the RecA monomers were incorporated into complete nucleoprotein filaments and were no longer free to interact with the dsDNA, which was added in the next step. A titration reaction in which the RecA stock was titrated with an oligo of 70

nucleotides (nt) in length (70-mer) is shown in Fig. 2. At an optimal RecA:nucleotide molar ratio of about 1:3, the *Hin*fl site in the targeted sequence was protected from methylation and all other *Hin*fl sites were completely methylated (Fig. 2, lane 7). When less than the optimal amount of 70-mer was used, other *Hin*fl sites on the plasmid were protected nonspecifically by the excess RecA (Fig. 2, lanes 4–6). When excess 70-mer was used, the efficiency of protection at the RecA-AC site was significantly reduced (Fig. 2, lanes 8–10), most likely as a result of incomplete filament formation.

The conditions for RecA-mediated protection used for the experiment shown in Fig. 2 are those described for optimized complex formation in the presence of ATP[γ S] (16). However, as shown in Fig. 3, we found it necessary to adjust these conditions in order to optimize RecA-AC. We obtained consistently better results if we adjusted the concentration of Mg^{2+} at each step of the process to that optimal for filament formation (1mM–4mM Mg^{2+}), synapsis (4mM Mg^{2+}) and protection during the methylation reaction (8 mM or higher Mg^{2+}) (Fig. 3, compare lanes 4–6 to lane 3 and Fig. 2). In addition, increasing the incubation time for synapsis (i.e., after the addition of dsDNA but before the MTase was added) from 10 min to 30 min significantly improved the protection from methylation (Fig. 3, compare lane 6 to lane 4). Doubling the amount of the complex used slightly improved the protection (Fig. 3, compare lane 5 to lane 4), but this also resulted in a small degree of nonspecific protection and cleavage. Protection of the *Eco*RI site from M-*Eco*RI using the optimized RecA-AC conditions was slightly more efficient than the protection of the *Hin*fl site from M-*Hha*II. However, unlike *Hin*fl, *Eco*RI has only a single recognition site in the target plasmid DNA and so the degree of cleavage due to specific RecA-AC and that due to partial methylation cannot be accurately determined in this experiment.

Figure 2. Titration of a RecA stock solution with an oligo to find the optimal ratio of RecA to oligo (indicated by a * above lane 7). RecA nucleoprotein filaments were formed with a constant amount of RecA (6.25 μ g) and an increasing amount of a synthetic 70-mer (40 ng, 80 ng, 120 ng, 160 ng, 200 ng, 240 ng, and 280 ng in lanes 4–10, respectively, as measured by absorbance at 260 nm). RecA was also added to a control reaction (lane 3) without 70-mer. The titration experiment was performed essentially as described in Materials and Methods. However, the buffers for filament formation, synopsis and methylation all contained 4 mM magnesium acetate and the synopsis incubation step was 10 min rather than 30 min. For the size control (lane 1), linearized plasmid DNA was digested with *EcoRI* to generate DNA fragments of approximately the size expected from RecA-AC at the targeted *HhaII* (*HinfI*) site. In the methylation control reaction (lane 2), the linearized plasmid was methylated and digested as described for lanes 3–10 but in the absence of either the 70-mer or RecA. The samples were analyzed by agarose gel electrophoresis on a 1.2% agarose gel containing ethidium bromide.

Since the expense of synthesizing an oligo is proportional to its length, we were interested in determining the minimal oligo length needed for the maximum protection from methylation. We synthesized a series of six oligos ranging in size from 12 nt to 36 nt in length that were designed to protect the same *HhaII* site as did the 70-mer used in our previous experiments (see Materials). However, based on published reports indicating that the most reactive region of the ssDNA molecule in a RecA strand-exchange reaction is the 3' end (24), these shorter oligos, unlike the 70-mer, all had the *HhaII* site located near their 3' end. The results of RecA-AC of linearized pRS305 using this series of oligos is shown in Fig. 4. RecA-AC of the plasmid was nearly quantitative with the 36 nt oligo (Fig. 4., lane 8) and only slightly less efficient with the 30-mer (Fig. 4., lane 7). The efficiency of the RecA-AC reaction with the 24-mer, which was the shortest oligo to result in strictly specific protection of the targeted site, was essentially identical to that of the RecA-AC reaction using the 70-mer from our earlier experiments (Fig. 4, compare lane 6 to lane 9). Although the 18-mer showed significant protection of the targeted *HhaII* site, some nonspecific protection of other *HhaII* sites was also apparent (Fig. 4, lane 5). RecA-AC reactions using oligos shorter than 18 nt (the 12-mer and the 15-mer) resulted in nonspecific MTase protection (Fig. 4, lanes 3 and 4) similar to that seen when free RecA monomers are incubated with the dsDNA target.

Although the RecA-AC protocol described in Fig. 4 can result in quantitative or near quantitative cleavage of plasmid DNA with 36-mers, the process is not highly robust. In particular, the delicate balance between RecA and oligo concentrations is fairly susceptible to small pipetting errors. This weakness can be

Figure 3. Optimizing the RecA-AC reaction conditions to maximize specific cleavage at the AC site by changing the Mg^{2+} concentration and incubation times. The size and completeness-of-methylation controls (lanes 1 and 2, respectively) and the first RecA-AC reaction (lane 3) were performed precisely as described for the identical reactions in Fig. 2 (lanes 1, 2, and 7, respectively). In lane 4, the reaction buffer initially contained 1 mM magnesium acetate and was adjusted to 4 mM after the addition of dsDNA and to 12.5 mM just before the addition of 1 μ l of *M·HhaII*, but was otherwise treated as the reaction shown in lane 3. The samples in lanes 5 and 6 were treated essentially as that in lane 4, but in lane 5 twice the amount of RecA and 70-mer was used and in lane 6 the synopsis incubation step was extended from 10 min to 30 min (i.e., as described in Materials and Methods). The DNA samples in lanes 7 and 8 were treated exactly as that in lanes 6 and 2, respectively, but were methylated with *M·EcoRI* (100 U) rather than *M·HhaII* and digested with *EcoRI* instead of *HinfI*.

Figure 4. Finding the optimal oligo length for RecA-AC of plasmid DNA. RecA nucleoprotein filaments were formed with oligos ranging from 12 nt to 70 nt in length, as indicated above each lane (lanes 3–9). Each RecA-AC reaction was performed precisely as described in Materials and Methods with the same amount of RecA (6.25 μ g) and oligo (160 ng). *M·HhaII* and *HinfI* were the MTase/restriction enzyme pair used. The oligos from 12 to 36 nt in length, all of which extend to the same point of the targeted sequence at their 3' ends, have the *HinfI* site near their 3' end whereas the 70-mer has the *HinfI* site near the middle of the oligo (see Materials). The size control (lane 1) and methylation control (lane 2) were performed as described for Fig. 2.

corrected by first forming nucleoprotein filaments with the targeting oligo and an excess of RecA monomers, adding ATP[γ S] to stabilize the complexes, and then removing any unpolymerized RecA still in solution by adding a 'nonspecific' oligo such as oligo(dT). In this way the filaments formed with the targeting oligo are complete and efficiently protect the chosen restriction site from methylation whereas any excess RecA is sequestered into partial complexes that do not protect any non-targeted restriction sites. The results from an experiment demonstrating this process are shown in Fig. 5.

Figure 5. Back-titration of excess RecA monomers with oligo(dT). RecA nucleoprotein filaments were formed with the 36 nt oligo (see Materials) and the amount of RecA indicated above each lane ($6.25 \mu\text{g}/\mu\text{l}$). The first three RecA-AC reactions were performed as described in the legend to Fig. 4, whereas oligo(dT) (60 nt in length) was added to the next three reactions (160 ng, 160 ng, and 320 ng, respectively) just before the addition of the linearized plasmid DNA. The samples were analyzed by agarose gel electrophoresis on a 1.2% agarose gel containing ethidium bromide.

Figure 6. RecA-AC of a yeast chromosome. The 370-kb chromosome III was specifically and efficiently cleaved (lane 3) into fragments (IIIa and IIIb) of the sizes expected from cutting at the targeted gene (*LEU2*). The RecA-AC reaction (lane 3) was performed as described in Materials and Methods using $5 \mu\text{l}$ of a 70-mer:RecA 'filament mix' (made with $0.32 \mu\text{g}$ oligo and $12.5 \mu\text{g}$ RecA in a $10 \mu\text{l}$ volume) that was allowed to diffuse into the agarose matrix overnight. *M*-*Hha*II and *H*inI were the MTase/restriction enzyme pair used. The methylation control (lanes 2) was treated exactly as was the RecA-AC sample but without the addition of the RecA filament. Lane 1 is an 'untreated' sample control. The chromosomes were analyzed by pulsed-field gel electrophoresis [5 V/cm, 30–50 s ramped switch time, 1% agarose, 14°C , on a Chef Mapper (Bio-Rad)] as described (23).

Optimizing RecA-AC of genomic DNA embedded in agarose

Although the results with plasmids clearly demonstrate that RecA-AC is a practical tool for selectively digesting small DNA molecules, the most pressing need for this type of procedure is as a tool for the precise dissection of large genomes. For this reason, it was important to test whether RecA-AC could specifically and efficiently cleave targeted restriction sites on

intact chromosomes embedded in agarose. As shown in Fig. 6 (lane 3), we were able to use RecA-AC to efficiently cleave chromosome III of *Saccharomyces cerevisiae* into two fragments of the expected sizes. No other cleavage sites are visible. More efficient digestion at a single site was obtained by increasing the amount of RecA:ssDNA filament added to the agarose-embedded chromosomes (not shown). In addition, we suggest that it may be possible to further improve the overall yield of cleaved chromosome by targeting two restriction sites in close proximity to one another with separate (or in some cases the same) oligo(s).

To obtain satisfactory results when applying RecA-AC to genomic DNA, we modified the protocol used for plasmids by either (i) adding a diffusion step to allow the large 70-mer:RecA complex to pass through the agarose (results shown in Fig. 6) or (ii) adding the oligo, RecA and ATP[γ S] sequentially to the agarose-embedded chromosomes so as to form the oligo:RecA filaments within the agarose matrix (results not shown). This latter approach, which is possible because RecA binds rapidly to ssDNA but not to dsDNA in the absence of ATP (12), is faster than the first but tends to result in slightly more nonspecific cleavage products.

Despite our success using oligos significantly shorter than 70 nt in the RecA-AC reaction on DNA in solution, RecA-AC of chromosomes embedded in agarose was much less efficient when the 36-mer rather than the 70-mer was used (not shown). As was the case with the 70-mer, initial experiments indicate that RecA filaments formed on the 36-mer were also apparently too large to diffuse rapidly into the agarose microbeads. However, unlike the 70-mer:RecA filaments, these shorter filaments appeared to be insufficiently stable to remain active after an overnight diffusion. In addition, forming the 36-mer:RecA filaments in the presence of the chromosomal target resulted in excessive nonspecific cleavage, presumably because the shorter oligos did not efficiently compete with the genomic DNA for the binding of RecA. Based on these results, we suggest that oligos used to cleave genomic DNA embedded in agarose be at least 60 nt in length.

DISCUSSION

The experiments shown, together with results published elsewhere (19), demonstrate that RecA nucleoprotein filaments formed with synthetic oligos can be used as efficient and specific methylase-blocking reagents in an Achilles' Cleavage reaction (1) to make any restriction site unique on DNA molecules of any size. We have found that the reaction buffer and the length and timing of the various incubation steps can have a profound effect on the efficiency and specificity of the RecA-AC reaction and describe those conditions that are optimal in our hands. Since the details of the protocol we describe differs from those published elsewhere by other researchers (19) in several aspects, it is perhaps not surprising that some of the parameters found to influence the specificity and efficiency of the RecA-AC reaction differ as well. For instance, we did not find that inclusion of ADP in reaction buffers improved the specificity of the reaction or that the efficiency of protection from methylation had a strict dependence on the location of the restriction site within the oligo. Since we have not made a careful experimental comparison of our protocol and that of Ferrin and Camerini-Otero (19), we are unable to explain these discrepancies at this time.

Restriction enzymes, which efficiently cleave DNA at short but specific sequences, have played a central role in the development of modern molecular genetics. By dramatically

increasing the specificity of these enzymes, RecA-AC may amplify the power of many aspects of conventional recombinant DNA technology. RecA-AC should be particularly useful for the precise dissection and analysis of large genomes. Genetic and physical linkage maps for many organisms are currently being generated in which the basic landmarks are short tracts of unique sequence known as sequence-tagged sites (STSs) (25,26). It is likely that essentially all STSs will have at least one potential RecA-AC site (assuming the availability of eight nuclease-free MTases, each with a different 4-bp cleavage specificity, a potential RecA-AC site would be expected to occur every 32 bp). RecA-AC at adjacent STSs offers a means of both determining the precise physical distance between these landmarks and of specifically isolating and therefore cloning the intervening region of chromosome. This ability to isolate and clone particular DNA fragments using genetic and physical map data will give researchers immediate access to chromosomal regions of interest to them and in many cases obviate the need to construct and order random genomic clone libraries.

The reagents necessary to perform RecA-AC reactions on both plasmids and intact chromosomes are readily available. Oligos of the appropriate size and of any sequence can be easily synthesized and the ATP[γ S], RecA, and a variety of MTases are sold commercially (as a cautionary note, many commercial MTases are not enzymatically pure and should be tested for nucleases before they are used for RecA-AC of genomic DNA). Although both the reagents employed and the protocol described will undoubtedly be improved with time, RecA-AC can be applied immediately to any research problem requiring efficient, highly specific cleavage of even the largest genomic DNA.

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