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Fluorescent marker for direct detection of specific dsDNA sequences

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Abstract

We have created a fluorescent marker using a mutant EcoRI restriction endonuclease (K249C) that enables prolonged, direct visualization of specific sequences on genomic lengths of double-stranded (ds) DNA. The marker consists of a biotinylated enzyme, attached through the biotin-avidin interaction to a fluorescent nanosphere. Control over biotin position with respect to the enzyme's binding pocket is achieved by biotinylating the mutant EcoRI at the mutation site. Biotinylated enzyme is incubated with dsDNA and NeutrAvidin-coated, fluorescent nanospheres under conditions that allow enzyme binding but prevent cleavage. Marker-laden DNA is then fluorescently stained and stretched on polylysine-coated glass slides so that the positions of the bound markers along individual DNA molecules can be measured. We demonstrate the marker's ability to bind specifically to its target sequence using both bulk gel-shift assays and single-molecule methods.

Introduction

A common feature of single-molecule genotyping assays, which seek to identify patterns of widely separated target sequences across a genome, is the need for markers that permit site-specific detection in double-stranded (ds) DNA. Sequence-specific markers are comprised of various pairings of a sequence-detecting molecule and an optical beacon, and only a few such markers for single-molecule dsDNA studies have thus far been demonstrated. For example, synthetic triplex-formers, including peptide nucleic acids (PNAs)¹ and triplex-forming oligos (TFOs)² labeled with single or multiple fluorophores, have been used to detect particular sequences on dsDNA. Several proteins have also been exploited as marker components for single-molecule dsDNA sequence detection.^{3–6}

Type II restriction endonucleases (REases) are appealing marker candidates due to their extraordinary specificity for their targets^{7,8}, and commercially available Type II REases provide the opportunity to mark over 260 distinct sites⁹. Type II REases have been used successfully for single-molecule optical restriction mapping, a technique in which cleavage sites are detected as breaks in fluorescence on surface-immobilized dsDNA.¹⁰ Most REases, however, exhibit high affinity and specificity for their cognate sites in the absence of magnesium, the natural cofactor for phosphodiester cleavage, so that they are excellent candidate markers for target sites in dsDNA.^{11,12} The widely studied Type II REase EcoRI has previously been incorporated into optical markers by fluorescently labeling the wild type enzyme through random, nonselective modification of its 22 lysine (K) groups.^{13,14} EcoRI

binds to dsDNA at the base sequence 5' GAATTC 3', and it has been shown to bind to its recognition site up to 90,000 times better than sites that have a single incorrect base pair.^{8,15}

Despite the enzyme's selectivity, however, EcoRI-based markers have failed to gain wide usage for single-molecule studies. Chemical modification of certain lysine groups within the binding pocket has been shown to reduce EcoRI binding affinity.¹⁶ Further, significant lysine modification can lead to total inactivation of the enzyme, even if the binding pocket is protected.¹⁷ Selectively modifying the enzyme at a single or small number of nonessential sites may limit reduction in binding affinity. Bircakova *et al.* attached EcoRI to a solid support by its single, native cysteine (C218), located outside the enzyme's binding pocket, and demonstrated activity by digestion assay.¹⁸ More recent work has reported that C218 is buried, and therefore not amenable to modification at high yield.¹⁹

Here, we present a prototype fluorescent marker based on a mutant EcoRI (K249C), in which the nonessential, solvent-accessible lysine (K249) has been mutated into a cysteine in each monomer of the homodimeric EcoRI. We biotinylate the mutated sites, enabling coupling to a fluorescent bead using a biotin-avidin interaction. We show by both gel-shift assay and single-molecule experiments using λ -bacteriophage DNA, which contains 5 copies of the EcoRI target sequence, that we achieve functional, site-specific markers amenable to prolonged, direct detection in single-molecule studies. This technique has the potential to be extended to other Type II REases, which would permit the detection of additional sequences.

Experimental Section

Generation of EcoRI K249C mutant

The EcoRI K249C mutation was constructed in pPS12¹⁵ using the two-stage site-directed mutagenesis method²⁰. The mutant EcoRI gene was then subcloned into pMAL-c4x (New England Biolabs) to produce a maltose-binding protein-EcoRI (MBP-EcoRI) fusion gene. The fusion was designed such that digestion of the fusion protein with Factor Xa protease would produce the complete EcoRI protein with no additional amino acids. This plasmid was transformed into *E. coli* strain K12 TB1 (F⁻ ara Δ (lac-proAB) [ϕ 80dlac Δ (lacZ)M15] rpsL (StrR) thi hsdR) already containing a plasmid (pAXU22-8, New England Biolabs) that directs expression of the EcoRI methylase.

Enzyme expression, purification, and characterization

Cell cultures were grown at 37°C in rich medium. The fusion protein was isolated by affinity chromatography via elution with 10 mM maltose from an amylose column. The MBP-EcoRI fusion was digested with factor Xa protease, and pure EcoRI protein was isolated by salt gradient elution from a heparin column. Protein was flash-frozen in storage buffer (20 mM sodium phosphate pH 7.3, 0.6 M NaCl, 1 mM EDTA, 1 mM NaN₃, 5% v/v DMSO, and 10% w/v glycerol). Protein concentration was determined by optical density scanning of SDS-PAGE gels containing varying amounts of the test sample and a standard curve of known amounts of EcoRI. The concentration of this standard was determined by direct amino acid analysis using norleucine as an internal standard.²¹ Equilibrium binding constants were determined at 22°C in nitrocellulose filter assays as previously described²¹ except that binding buffer was 20mM cacodylate, 0.22 M KCl, 100 mM dithiothreitol, 1 mM EDTA, 100 mg/ml bovine serum albumin, pH 7.3.

EcoRI (K249C) biotinylation

Mutant EcoRI (K249C) was biotinylated in 50- μ l batches using EZ-Link Maleimide-PEO₂-Biotin (Pierce) biotinylation reagent, which forms a thioether bond with the enzyme's cysteines. A 20 mM solution of biotinylation reagent was prepared immediately before use by

dissolving it in storage buffer (30 mM sodium phosphate pH 7.3, 0.5 M NaCl, 1 mM EDTA, 0.01% w/w NaN_3 , 5% v/v DMSO, and 10% w/w glycerol). Biotinylation reagent was added at 0.8 mM to a 50 μl volume of mutant enzyme (4 μM mutant EcoRI in 20 mM sodium phosphate pH 7.3, 0.6 M NaCl, 1 mM EDTA, 1 mM NaN_3 , 5% v/v DMSO, and 10% w/v glycerol), providing 100x excess over solvent-accessible cysteines. The reaction was incubated overnight on ice. Excess biotinylation reagent was subsequently removed from the reaction using a 7-ml capacity 9K MWCO iCON concentrator (Pierce). The reaction contents and 4.4 ml storage buffer were combined in the upper iCON chamber, with 2.4 ml storage buffer in the iCON collection tube to achieve a dead stop volume of 100 μl . The sample was centrifuged using a fixed-angle rotor at 6000x g at 4°C for 70 min. Biotinylated EcoRI was aliquoted, flash frozen, and stored at -80°C. The final biotinylated EcoRI concentration was estimated by noting the change in volume associated with the concentration step, assuming zero loss of enzyme.

Bulk binding experiments

Substrate oligos used in gel-shift assays were prepared by PCR using λ -DNA as template. Primer sequences and information on the products generated are provided in Table S-1. Substrate oligos were diluted to 10 nM in binding buffer (20 mM sodium phosphate pH 7.3, 100 mM NaCl, 1 mM EDTA, and 1 M triethylene glycol (TEG)) supplemented with 40 $\mu\text{g}/\text{ml}$ BSA. Substrate oligos were then combined in 20- μl reactions with 40 nM enzyme and, where indicated, a 2.3-fold excess by mass of non-cognate DNA and incubated for 30 min at 4°C. Reactions were subsequently incubated at room temperature for 30 min. Where indicated, 82.5 nM streptavidin was added and all reactions were incubated an additional 30 min at room temperature. Just prior to loading, a 5x glycerol-based loading buffer (0.05% xylene cyanol, 0.05% bromophenol blue, 2 mM EDTA, 50% v/v glycerol) was added to 1x. Samples were immediately loaded into a 4-15% gradient polyacrylamide gel (BioRad ReadyGel) and run in TBE buffer (89 mM tris base, 89 mM boric acid, 2 mM EDTA) using a quick start at 220 V followed by 45 min at 100 V. Gels were post-stained by submersing in 0.375 $\mu\text{g}/\text{ml}$ ethidium bromide in TBE buffer. DNA bands were visualized using a BioRad VersaDoc.

Single-molecule binding experiments

Marker-tagged DNA was prepared for single-molecule studies as follows. First, 8 nM biotinylated EcoRI, 0.33 nM λ -DNA (New England Biolabs), and 100 $\mu\text{g}/\text{ml}$ BSA in binding buffer were incubated in either 20- μl or 200- μl reactions at 4°C for 1 hr. NeutrAvidin-coated, fluorescent (580/605), 40-nm FluoSpheres (Molecular Probes) were added at a ratio of either 5:1 or 50:1 (spheres:DNA) and incubated at 4°C for 1 hr. The marker-DNA mixture was diluted to 1.25 pM DNA using visualization buffer (10 mM sodium phosphate pH 7.3, 10 mM NaCl, 1 mM EDTA, and 1 M TEG). DNA molecules were stained by adding 50 μM YOYO-1 (Invitrogen) in visualization buffer to the DNA solution to reach 250 nM dye and incubating in the dark for 1 hr at room temperature, yielding a staining ratio of 4:1 (bp:YOYO-1).

To promote stretching and immobilization of the DNA, 5 μl of 0.1 mg/ml poly-L-lysine hydrobromide (Fluka) in visualization buffer was deposited between a glass slide and cover slip. After 1 min, the slide and cover slip were pulled apart to allow the solution to evaporate. A 5- μl aliquot of the stained marker-DNA mixture was then deposited and the cover slip replaced. After 20 min, marker-tagged DNA was visualized using a 100x, 1.4-NA, oil-immersion objective on a fluorescence microscope. Separate red and green images were captured using N2.1 Cy3/TRITC (Vashaw Scientific) and 41001 FITC (Chroma) filter sets in combination with a monochrome image-intensified, cooled CCD camera (Photometrics 512b) and SimplePCI software. Red and green images were superimposed, DNA lengths were measured, and intensity profiles were generated using the software ImageJ. Total lengths of fully extended DNA molecules (Fig. S-1) were approximately 22 μm , consistent with intact,

stained λ -DNA as measured by others.²² Bead locations were assigned for molecules based on positions of the fluorescence intensity peaks normalized by the total length of the DNA molecule. Normalized locations were then converted to base pair positions by multiplying by 48,502 kilobase pairs (kb), the total length of λ -DNA.

Results & Discussion

Fig. 1a shows a schematic representation of our fluorescent marker for single-molecule sequence detection. Biotinylated K249C mutant EcoRI (62,000 Da) binds to its target sequence GAATTC on dsDNA. The enzyme's position is signaled by the presence of a NeutrAvidin-coated, 40-nm, fluorescent polystyrene bead, which couples to the enzyme via a biotin-avidin interaction. The use of fluorescent beads, which contain 100-200 co-localized fluorophores, permits prolonged observation of the marker. DNA is stained with YOYO-1 intercalating dye so that it is also visible in single-molecule experiments. Biotin is selectively attached to the EcoRI mutant via a thioether bond with cysteine, separated by a 29-Å PEG-2 spacer arm. The mutant monomer contains two cysteines: the native C218, as well the K249C mutation. Both are located on the main domain, outside of the enzyme's binding pocket (Fig. 1b).

Because EcoRI is a dimer, each of the monomer's two cysteines (K249C and C218) could potentially be modified during the biotinylation reaction, resulting in a maximum loading of four biotins per bound enzyme. However, a Connolly surface of mutant EcoRI (Fig. S-2) suggests that while K249C is solvent accessible, C218 is buried. Consequently, we expect the majority of our biotinylated K249C mutant EcoRI to have incorporated only two biotins per dimer. This is further supported by data from Stone *et al.*, who observed <10% conversion of the buried C218 in spin-labeling studies.¹⁹

K249C mutant EcoRI retains full binding activity relative to the wild-type enzyme as determined by equilibrium binding assays ($K_A^{\text{Wild-type}} 9.3 \pm 1.4 \times 10^9$, $K_A^{\text{K249C}} 8.5 \pm 0.7 \times 10^9$). Biotinylated K249C mutant EcoRI activity was probed by a gel-shift assay (Fig. 2). The mutant and biotinylated-mutant enzymes were incubated, in the absence of Mg^{2+} , with a 391-bp oligonucleotide (oligo) containing a single copy of the target sequence GAATTC. Oligo-enzyme complexes move more slowly through the gel than the oligo alone, causing a shifted, lagging band to appear when enzyme binds to its substrate. The shift caused by the biotinylated mutant (Lane 5) is similar to that caused by the mutant (Lane 3), indicating the biotinylated mutant retains binding activity. The presence of only one lagging band suggests that a single EcoRI dimer is binding to each substrate DNA. Binding activity has been confirmed for three batches of biotinylated mutant EcoRI, which remains active for at least five months under the storage conditions described in the Experimental Section.

In addition, the oligo shift (Fig. 2, Lane 6) observed for biotinylated mutant in the presence of the protein streptavidin (52,800 Da) is much larger than that observed for the oligo incubated with unmodified mutant and streptavidin (Lane 4). This result confirms that the mutant EcoRI has been biotinylated with high yield. Further, the degree of the shift is consistent with the oligo hosting a biotinylated mutant EcoRI plus two bound streptavidins, which suggests that two biotins have been incorporated per dimer. The data also establishes that the biotinylated enzyme remains bound to its target oligo, even when other bulky molecules, such as streptavidin, are attached to it. Therefore, we have demonstrated a *critical* aspect of marker formation for single-molecule studies, where the enzyme must remain attached to the DNA even with a fluorescent nanosphere attached.

Gel-shift assays also confirm that the EcoRI-based marker binds with high specificity to its target sequence (Fig. 3). The biotinylated enzyme binds to the 391-bp target oligo even when incubated in the presence of an excess of 112-bp, competitor oligo that does not contain the

recognition sequence. The absence of a lagging band for the competitor oligo indicates the binding is specific to the target oligo. Further, no shift is observed when the biotinylated mutant enzyme is incubated with a 376-bp “star” oligo containing the sequence 5' GAATCC 3', which is a single base mismatch (underlined) from the enzyme's target sequence. This demonstrates that the marker is capable of *single-base pair discrimination* for its target sequence.

To confirm its viability for single-molecule sequence detection, the mutant EcoRI-based marker was tested on single molecules of λ -DNA, which is 48.5-kb long and contains five EcoRI target sites at 21.2 kb, 26.1 kb, 31.7 kb, 39.2 kb, and 45.0 kb. We denote these as target sites 1 through 5, respectively. Biotinylated enzyme and DNA were first incubated together in solution, followed by the addition of NeutrAvidin-coated beads at a ratio of either 5:1 or 50:1 (beads:DNA). DNA was fluorescently stained after these incubations. The solution containing bound complexes was then deposited onto polylysine-coated slides, enabling surface adsorption and stretching of the DNA into an extended, linear conformation^{26,27}, which was required for confirmation of site-specific binding. By examining the slides using fluorescence microscopy, we have observed binding to all five EcoRI target sites on λ -DNA (Fig. 4). The differences in bead intensities present in these images can be attributed, in part, to the manufacturer's reported coefficient of variation in bead diameter (>10%), which can lead to significant variation in fluorophore loading per bead. In addition, since potentially both K249C residues are biotinylated in the homodimeric EcoRI, some variability in brightness of signal may derive from the fact that one or two beads might be present.

We estimate that 10-20% of observed λ -DNA molecules were tagged by EcoRI-based markers. Most marker-DNA complexes observed had only a single marker bound, although occasional DNA molecules contained multiple probes. The presence of fluorescent dye in the DNA backbone is not expected to greatly impact marker binding in these experiments because enzymes were bound to DNA prior to staining. While we have no quantitative measure of the difference in binding efficiency of stained versus unstained DNA, we have confirmed that the enzyme binds to YOYO-stained DNA in gel-shift assays (data not shown). Current experimental work is underway to understand the paucity of binding of multiple probes. Markers were also observed bound to free-flowing stained DNA molecules, suggesting the mutant EcoRI-DNA complexes were robust to flow.

Position data were compiled for 204 beads bound to 170 molecules stretched to at least 16.5 μ m, which is 75% of the reported contour length²² of stained λ -DNA (Fig. 5). Without additional labeling, the ends of the 48.5-kb λ -DNA are optically indistinguishable in our staining protocol. Measurements were made based on the end-assignment that yielded the best overall match between the marker positions and the known target sites on λ -DNA, as our gel-shift assays have demonstrated that binding occurs only at the target sequences.

We can distinguish five distinct peaks in our histogram of binding position, and all five peaks align well with expected EcoRI target sites. A fit to the data using a linear combination of Gaussians gives peaks at 21.1 kb, 25.6 kb, 31.9 kb, 39.7 kb, and 46.1 kb, with standard deviations ranging from 1-2 kb. Peak positions predict the target sites to within 0.6 kb for target sites 1-4 and within 1.2 kb for target site 5, which is consistent with errors of 1-2 kb that have been reported for other protein-based markers.^{4,6,14} Error in target site determination and the large standard deviation of the peaks is unlikely the result of indiscriminant marker binding, as the gel-shift experiments indicated that the biotinylated mutant was not susceptible to nonspecific binding. Instead, a portion of the data spread arises because much of the measured DNA was not fully extended. For example, position errors totaling up to 5 kb can be expected for a λ -DNA molecule stretched to only 90% of its contour length. In addition, DNA stretching and adsorption onto the slide is not necessarily uniform along the molecule. Here, error in predicted target location is systematically larger at the ends of the molecule than near the center.

Peaks 1 and 2 are shifted to the left of the known target sites, giving low estimates of the expected locations, while peaks 3-5 are shifted to the right of the target sites. Work showing that DNA relaxation from an extensional flow proceeds inward from the ends of the molecule supports these observations.^{28,29} Markers may also appear bound at non-target positions due to small amounts of photocleavage of the DNA or coincidental bead-DNA co-location on slides. Finally, we note that the relative symmetry of sites 1 and 2 about the DNA's center makes assignment of a single marker bound to one of these sites difficult and may contribute to incorrect assignment of position at these sites.

Though the marker might be expected to bind each site with equal probability, this was not observed in the single-molecule studies. Previous studies have used cleavage as the metric for studying the relative activity at different sites. For example, Thomas and Davis reported that EcoRI cleaves λ -DNA with lowest frequency at the innermost target (site 1) and greatest frequency at the endmost target.³⁰ Like Thomas and Davis, we also observed a greater probability of mutant EcoRI-based marker binding at the target sites nearer the end of the molecule, but we found this tendency could be influenced by enhanced mass transfer (Fig. S-3). Without continuous mixing during incubation, roughly 40% of the marker positions measured were located at the target site closest to the end (site 5). In contrast, gently mixing the samples during incubation simultaneously increased the number of molecules having a bead bound and more evenly distributed the binding over the five positions, with only about 25% of binding occurring at the endmost site. We hypothesize this may be due to a slight extension of the DNA that occurs as the molecules are mixed, potentially making the interior sites more accessible to either bead or enzyme binding. As an alternative to mixing, a more equitable distribution of binding among the sites might also be achieved by altering the salt concentration to favor a more distributed target search by the enzyme.

Interestingly, digestion assays on native DNA are not sufficient to address issues of site-to-site variation, as cleavage of the molecule changes the position of the target with respect to the ends as well as changes the molecule's radius of gyration. Therefore, we note that in addition to sequence detection, use of an REase-based marker may permit detailed study of enzyme binding preferences under various conditions.

Conclusions

We have demonstrated the viability of a novel, mutant EcoRI-based, fluorescent marker for single-molecule DNA sequence detection. Bulk gel-shift assays have provided evidence of the marker's activity and specificity and have confirmed that the biotinylated enzyme binds with single-bp precision to its target site. Single-molecule studies demonstrated binding to all five λ -DNA target sites, though binding to some target sites was observed with higher frequency than others.

The mutant EcoRI marker design we have developed has several key benefits. The biotinylated enzyme itself has a long shelf life, retaining activity after months of storage and thereby eliminating the need to create fresh marker for each experiment. Long observation times are made possible by the use of a fluorescent bead; however, utilizing the demonstrated thioether chemistry, two fluorophores could instead be coupled to the K249C dimer directly to create a less bulky, albeit less bright, marker. The biotin-avidin coupling of enzyme and bead provides flexibility in incubation order, potentially enabling the marker to be assembled prior to incubation with the DNA, which could permit study of enzyme binding dynamics. Further, this marker protocol can be adapted to other Type II REases with solvent-accessible native or mutated cysteines to create additional probes having different target sequences, provided biotinylation does not interfere with binding.

Finally, the marker-DNA complex is robust, eliminating the need for chemical cross-linking or other DNA modification required by some demonstrated markers⁴⁻⁶, and making the mutant EcoRI-based marker an excellent candidate for use in flow-based assays. Currently, we are employing this marker for sequence-specific detection in flow.³¹ Single-molecule studies using Type II REase-based markers prepared using this method have the potential to reveal interesting behavior about the enzyme binding preferences either in solution or on surfaces.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

(a) Schematic representation of the mutant EcoRI marker bound to stained ds DNA. (b) X-ray structure of the wild-type EcoRI specific complex. Coordinates are determined from a highly refined version of the protein data base (PDB) entry 1CKQ.^{23,24} DNA is indicated in magenta, residues C218 (yellow) and K249C (red) are shown as spheres. The K249C mutation was modeled into the wild-type structure using Pymol²⁵. C218 and K249C are located in the main domain, away from the DNA-binding cleft.

Figure 2.

Bulk confirmation of mutant enzyme activity and biotinylation by gel-shift assay. Lanes 1 and 2: 391-bp oligo containing a single copy of EcoRI's target sequence GAATTC, with and without streptavidin. Lanes 3 and 4: Bound mutant EcoRI (40 nM) decreases the oligo's mobility through the gel, causing a band shift. Lane 5: Biotinylation does not affect enzyme binding. Lane 6: Streptavidin-EcoRI complex binds to oligo, causing a larger band shift.

Figure 3.

Bulk confirmation of biotinylated mutant enzyme specificity using gel-shift assay. Mutant and biotinylated mutant enzymes cause shifts of the 391-bp target oligo (Lanes 3 and 5) but not of a 112-bp oligo lacking the recognition sequence placed in competition for binding (Lanes 4 and 6). Neither mutant nor biotinylated mutant cause a shift in the 376-bp star site oligo (Lanes 9 and 10), which contains the sequence 5' GAATCC 3', a single bp substitution from the EcoRI target.

Figure 4.

Representative fluorescence images of marker-tagged λ -DNA, stretched and immobilized on slides, with the corresponding fluorescence intensity profiles along the DNA backbone; bead positions are clearly evident as peaks in the profiles. Dashed vertical lines indicate expected EcoRI target positions 1-5 on fully stretched λ -DNA. Scale bar is 5 μ m.

Figure 5.

Histogram of measured binding positions for 204 markers bound to slide-immobilized λ -DNA molecules. The solid line is a Gaussian fit to the data. Dashed vertical lines indicate expected EcoRI target sites 1-5 on fully stretched λ -DNA.