Sequence-Enabled Reassembly of β-Lactamase (SEER-LAC): A Sensitive Method for the Detection of Double-Stranded DNA[†]

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ABSTRACT: This work describes the development of a new methodology for the detection of specific double-stranded DNA sequences. We previously showed that two inactive fragments of green fluorescent protein, each coupled to engineered zinc finger DNA-binding proteins, were able to reassemble an active reporter complex in the presence of a predefined DNA sequence. This system, designated sequence-enabled reassembly (SEER), was demonstrated in vitro to produce a DNA-concentration-dependent signal. Here we endow the SEER system with catalytic capability using the reporter enzyme TEM-1 β-lacatamase. This system could distinguish target DNA from nontarget DNA in less than 5 min, representing a more than 1000-fold improvement over our previous SEER design. A single base-pair substitution in the DNA binding sequence reduced the signal to nearly background levels. Substitution of a different custom zinc finger DNA-binding domain produced a signal only on the new cognate target. Signal intensity was not affected by genomic DNA when present in equal mass to the target DNA. These results present SEER as a rapid and sensitive method for the detection of double-stranded DNA sequences.

Virtually all scientific methods for reading the sequence information of DNA rely on the hybridization properties of complementary nucleic acid molecules. Such methods, including PCR, Sanger sequencing, DNA microarray, Southern and Northern blotting, and in situ hybridization, all consequently require denaturation of the native DNA double helix into single strands and subsequent renaturation with specific primers or probes under carefully controlled conditions. In contrast, nature frequently relies on sequence-specific DNA-binding proteins to read the sequence information of DNA during the processes of transcription initiation, intron homing, and defense against invasive DNA by restriction endonucleases. In the human genome, DNA-binding transcription factors comprise one of the largest classes of known genes, with approximately 2000 members (*1*). The most common type of DNA-binding domain is the Cys2-His2 class of zinc fingers.

Here we describe the development of a new methodology that may be readily applicable for the detection of specific double-stranded (ds) DNA sequences. This system, designated SEER (sequence-enabled reassembly), consists of split-protein systems that are able to reassemble an active complex only in the presence of a cognate DNA sequence. This approach merges two rational protein design approaches, the ability to dissect proteins for complementation assays (PCA),

and the ability to design custom zinc fingers (ZF)[11] to most DNA targets in a rule-based manner.

PCA is a methodology initially described for the detection of protein−protein interactions (*2*, *3*). A functional protein, typically a reporter molecule, is dissected into two nonfunctional fragments. Functionality is restored when the fragments are reassembled by attached protein−protein interaction domains such as leucine zippers. Several such systems have been recently reported, including reassemblies of β-galactosidase (*3*), dihydrofolate reductase (DHFR) (*4*), green fluorescent protein (GFP) (*5*), TEM-1 β-lactamase (*6*), and firefly luciferase (*7*, *8*).

Custom DNA-binding proteins can be constructed from modified Cys2-His2 ZF DNA-binding domains. Each ZF domain contains 30 amino acids that form a ββα fold, stabilized by hydrophobic interactions and the chelation of a zinc ion between two histidines and two cysteines. Each domain typically recognizes three to four nucleotides of DNA. The domains can be found in covalent tandem arrays, facilitating the recognition of extended DNA sequences. A protein containing 6 zinc fingers should have the capacity to recognize 18 base pairs of DNA, sufficiently large to specify a unique site in the human genome (*9*). A variety of combinatorial and rational design approaches have been used to modify the binding specificity of naturally occurring ZFs (*10−13*). In particular, Barbas and co-workers have produced a lexicon of interchangeable domains with the ability to recognize unique 3−4-base pair DNA sequences (*14*). Using these predefined recognition modules, DNA-binding proteins can be rapidly assembled to bind virtually any DNA sequence

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[1] [1]Abbreviations: ZF, zinc finger; GFP, green fluorescent protein; MBP, maltose-binding protein; HS-DNA, herring sperm DNA; ZBA, zinc buffer A.
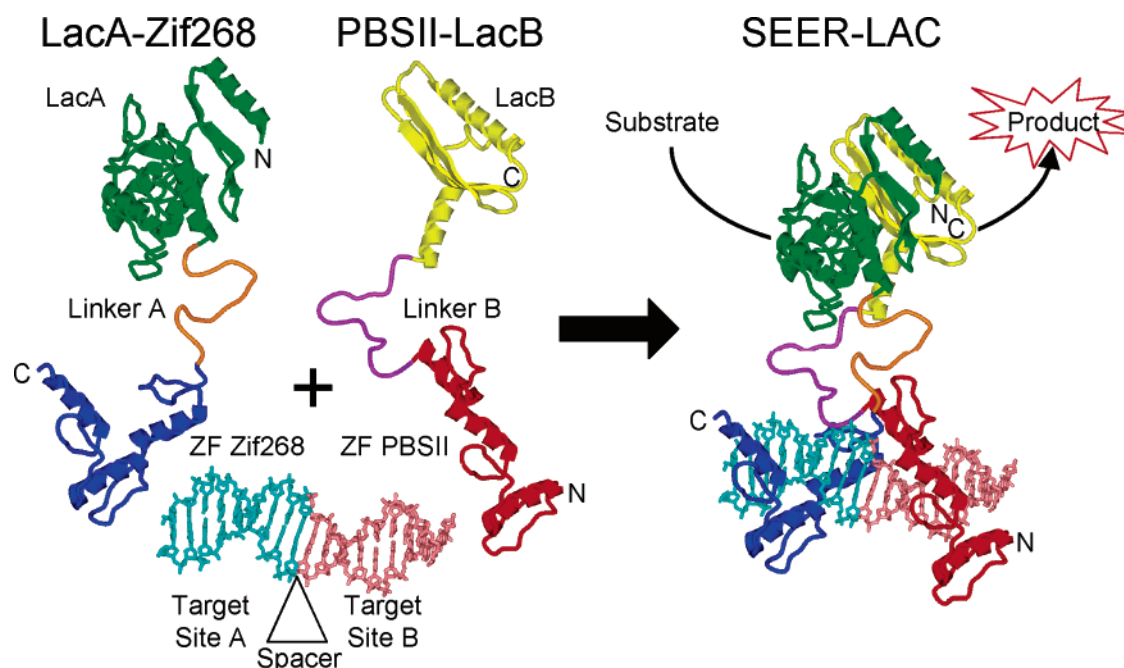
FIGURE 1: SEER-LAC strategy. LacA-Zif268 comprises residues $26-196$ of β-lactamase (green) fused by a 15-aa linker (orange) to the DNA binding ZF Zif268 (blue). PBSII-LacB comprises the ZF PBSII (red) fused by a 15-aa linker (magenta) to residues $198-290$ of β-lactamase (yellow). In the presence of target DNA-containing binding sites for Zif268 (cyan) and PBSII (pink) with an appropriate spacer (0-bp spacer is shown), SEER fragments reassemble to form an active reporter enzyme.

or gene in the human genome (*15*). The three-finger proteins typically have affinities in the $1-50$ nM range and are highly specific for their target site (*9*). These custom-made ZF proteins can be linked to functional domains to generate novel chimeric proteins that produce the desired activity at specific DNA sequences. This approach has been employed in designing targeted transcription factors (*14, 16*), targeted endonucleases (*17*), and targeted integrases (*18*).

Previously, we demonstrated the chromophore regeneration of dissected ZF-linked fragments of GFP upon forming a ternary complex in the presence of a specific DNA sequence (SEER-GFP) (*19*). One advantage of the SEER-GFP approach was an extremely low background due to the inability of the two unfolded GFP fragments to spontaneously reassemble. The signal produced by SEER-GFP was expected to be one GFP molecule for every copy of the target site. Although this may be desirable in some instances, for example, to have a signal that would be linearly proportional to the target copy number, this feature may limit the use of SEER-GFP for the detection of low copy number sequences. In addition, poor protein solubility coupled with a slow rate of chromophore formation required assays times of $72-96$ h for maximal SEER-GFP signal intensity.

In the current study, we extend the SEER concept to the sequence-specific reassembly of *E. coli* TEM-1 β-lactamase (SEER-LAC) enzymatic activity. β-Lactamase is a promising candidate for SEER. This enzyme has previously been shown in a PCA approach (*6*). Its structure and function are well characterized. It is small (29 kDa), can be easily expressed, and is nontoxic to both prokaryotic and eukaryotic cells. Eukaryotic cells do not contain endogenous β-lactamase activity. Its activity can be easily assayed by the hydrolysis of nitrocefin, which changes from yellow to red (peak absorbance at 486 nm) when the β-lactam ring is hydrolyzed by β-lactamase. We demonstrate that a SEER system can be generated by dissecting TEM-1 β-lactamase into two-

halves with each fragment linked to a ZF domain. These protein fragments can be functionally reassembled when brought into proximity in the presence of target DNA sequences for the ZFs (Figure 1). The enzymatic signal amplification enables the sensitive detection of even a single-base substitution within an 18-bp site in under 5 min, representing a greater than 1000-fold rate improvement over our previous design. These results show the possibility of utilizing an enzymatic reporter protein to create a SEER system that has improved the potential for further development into a useful detection tool.

EXPERIMENTAL PROCEDURES

Cloning, Expression, and Purification of the Proteins. *E.coli* TEM-1 β-lactamase DNA was obtained by PCR using the bacterial expression vector pMAL-c2X (New England Biolabs) as the template. LacA (aa26-aa196) and LacB (aa198-aa290) were cloned into separate pMAL-c2X vectors using standard cloning procedures (Supporting Information). LacA contained an M182T mutation to enhance the stability of the protein (*6*). ZF proteins were constructed by PCR using overlapping primers. Zif268 was cloned *C*-terminal to LacA, whereas PBSII and PE1A were cloned *N*-terminal to LacB. The ZF and Lac domains were separated by a 15-aa linker, $(GGGGS)_3$. The protein was expressed in BL-21 star cells (Invitrogen). To 100 mL of LB growth media, 100 μM of $ZnCl_2$ was added. At $OD_{600} = 0.6-0.8$, protein expression was induced with 1 mM isopropyl β-D-thiogalactoside (IPTG) for 5 h at 37 °C. The cells were pelleted and resuspended in zinc buffer A (ZBA: 100 mM Tris base, 90 mM KCl, 1 mM $MgCl_2$, and 100 μM $ZnCl_2$ at pH 7.5)/5 mM DTT. The MBP-tagged proteins were purified over amylose columns and eluted in ZBA/5 mM DTT/10 mM maltose, following the methodology of the protein fusion and purification system (New England Biolabs).
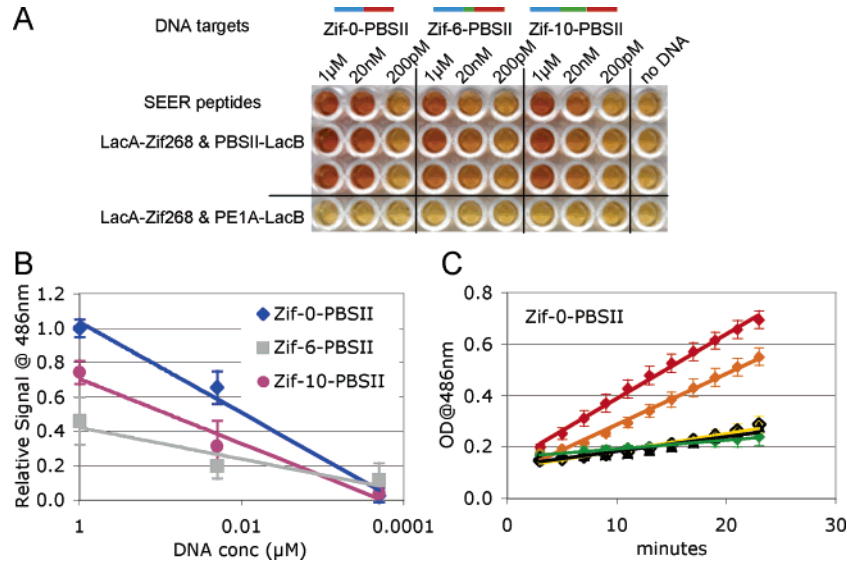
FIGURE 2: DNA concentration-dependent SEER signal. (A) Digital image of triplicate nitrocefin assays after 30 min of incubation. DNA target oligonucleotides (with target site spacings of 0, 6, and 10 bp) and their concentrations are indicated above the image; SEER fragments (0.5 μM each) are indicated on the left. (B) Relative signal vs DNA concentration plot for the assay shown in A. The relative signal (normalized and background subtracted) after 20 min of incubation was calculated on *Zif-0-PBSII* DNA (blue diamonds), *Zif-6-PBSII* DNA (gray squares), and *Zif-10-PBSII* DNA (purple circles) as described in the Experimental Procedures. (C) Kinetic data for the reactions with *Zif-0-PBSII* DNA. Absorbance at 486 nm was measured at 3 min and every 2 min after that. Raw data were plotted for LacA-Zif268 and PBSII-LacB with 1 μM DNA (red diamonds), 20 nM DNA (orange diamonds), 200 pM DNA (yellow diamonds), and no DNA (black triangles) as well as the noncognate LacA-Zif268 and PE1A-LacB (green diamonds) with 1 μM *Zif-0-PBSII* DNA. Hydrolysis rates were calculated as the slope of the linear fit of the kinetic data (solid lines).
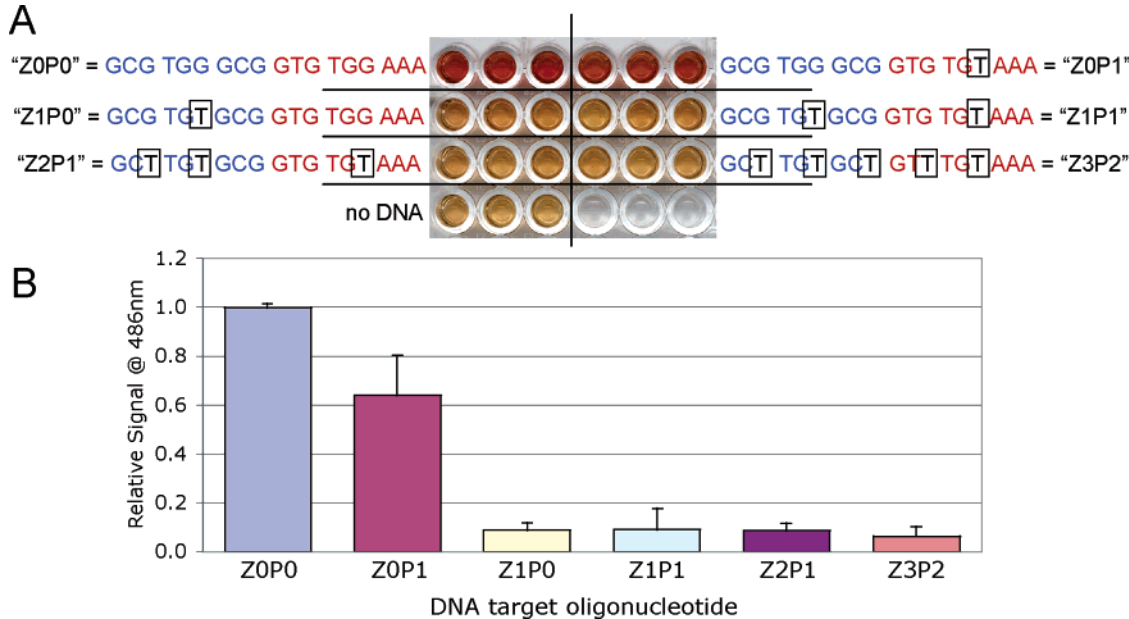

FIGURE 3: Sensitivity of SEER to mutations in the target DNA. (A) Digital image of triplicate nitrocefin assays after 30 min of incubation. A series of modified *Zif-0-PBSII* target oligonucleotides were used at 1 μM, containing 1, 2, 3, or 5 G to T substitutions (boxed) in the Zif268 (blue) or PBSII (red) binding sites, as indicated. SEER fragments LacA-Zif268 and PBSII-LacB were used with each at 0.5 μM concentration. (B) Graphical representation of the relative signal intensities after 20 min of incubation for the assay shown in A.

Nitrocefin Assay. Hairpin oligonucleotide DNA target *Zif268-0-PBSII* had the sequence, 5′-GGC TTT CCA CAC CGC CCA CGC GGG TTTT CCC <u>GCG TGG GCG</u> <u>GTG TGG AAA</u> GCC-3′, and *Zif268-0-PE1A* had the sequence, 5′-GGC GTT ATT TAT CGC CCA CGC GGG TTTT CCC <u>GCG TGG GCG</u> <u>ATA AAT AAC</u> GCC-3′, where 5′-GCG TGG GCG-3′, 5′-GTG TGG AAA-3′, and 5′-ATA AAT AAC-3′ are the target sites for ZFs Zif268, PBSII, and PE1A, respectively. All oligonucleotides were heated to 95 °C for 10 min at 10 μM in ZBA, then slowly cooled to room temperature to form hairpins with a four-thymidine loop. A

more complete description of the DNA targets use in this study is offered in Supporting Information.

In a 96-well plate, 120 μL of ZBA was added to the wells followed by 20 μL of 10 μM, 200 nM, 2 nM, or no hairpin oligonucleotide DNA targets (1 μM, 20 nM, 200 pM, 0 final concentration, Operon). For the experiment in Figure 5, the volume of ZBA was reduced 20 μL, and 20 μL of 160 μg/mL sheared, double-stranded herring sperm DNA (HS-DNA, Invitogen) was added. Protein fragments (20 μL, 5 μM) LacA-Zif268 and PBSII−LacB/PE1A-LacB (0.5 μM final concentration of each fragment) was added to the wells
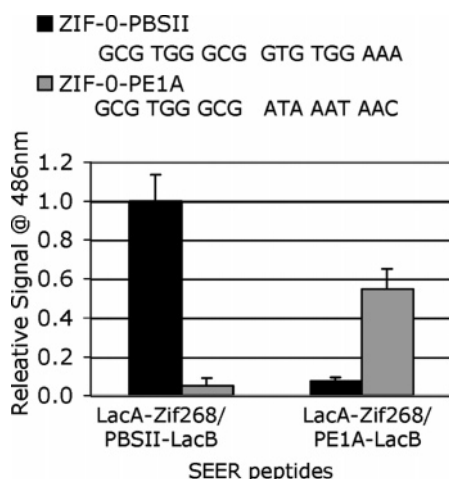
FIGURE 4: SEER activity using various combinations of ZF binding domains and DNA targets. The relative signal intensities after 20 min of incubation for triplicate nitrocefin assays are shown. Target oligonucleotides at 1 μM are indicated above the graph (black and gray bars); SEER fragments at 0.5 μM each are indicated below.
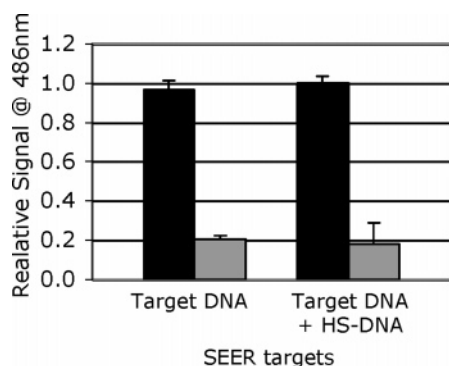

FIGURE 5: SEER binding in the presence of genomic DNA. LacA-Zif268 and PBSII-LacB, each at 0.5 μM concentration, were incubated with 1 μM Zif-0-PBSII (black bars) or 1 μM Zif-0-PE1A (gray bars) for 20 min in the presence or absence (as indicated) of 3.2 μg of sheared, double-stranded herring sperm DNA. This concentration is equal in moles of base pairs (5.2 nmol bp) to 1μM concentraion of the target oligonucleotide.

before adding 20 μL of 1 mM nitrocefin (0.1 mM final concentration, Calbiochem). All steps were carried out at room temperature. Absorbance at 486 nm was monitored over 20 min with a SpectraMax M2 (Molecular Devices). The relative signal was determined by subtracting the background (samples containing no DNA) from triplicate measurements and then normalizing to the highest signal value. Error bars in Figure 5 indicate the standard deviation of triplicate samples. To ensure that trace contamination of the pMAL-C2X-expressed full-length β-lactamase was not contributing to background hydrolysis, the samples containing individual SEER fragments were incubated with the substrate. No detectable hydrolysis was observed over the assay interval (Supporting Information).

RESULTS

Protein Design. SEER-LAC proteins consisted of two inactive fragments of β-lactamase fused to zinc finger proteins with the ability to recognize specific DNA sequences. The two fragments were designed to bind near each other at adjacent sites in the presence of a user-defined DNA target site to generate a signal. Two 3-finger ZF proteins

binding in this way would have the collective capacity to recognize 18 bp of DNA, a target site sufficiently large to be unique in the human genome (9). However, because biologically relevant target sites could not be chosen until the optimal spacer and orientation parameters were established, the initial experiments employed designed target sites that were recognized by existing, well-characterized ZFs. Zif268 is a naturally occurring 3-finger ZF that has been extensively studied structurally and biochemically (20, 21). It binds the 9 bp sequence 5′-GCG TGG GCG-3′. PBSII and PE1A are designed 3-finger ZFs assembled from predefined modified ZF domains (14, 15) and recognize the sequences 5′-GTG TGG AAA-3′ and 5′-ATA AAT AAC-3′, respectively.

Two inactive fragments of the 290-amino acid TEM-1 β-lactamase protein can be generated by splitting the protein between residues 196 and 198 (6). To maintain the correct polarity of the protein fragments, Zif268 was appended to the *C*-terminus of β-lactamase residues 26−196 (LacA-Zif268; lacking the *N*-terminal secretory signal sequence), and PBSII or PE1A was appended to the *N*-terminus of residues 198−290 (PBSII-LacB or PE1A-LacB). The ZF and β-lactamase domains were separated by a 15-aa linker, (GGGGS)₃ (Figure 1). All proteins were expressed from the vector pMAL-c2X, which additionally appended the 392-amino acid maltose-binding protein (MBP) to the *N*-terminus of all three protein fragments. All experiments were performed with the MBP domain attached.

DNA-Dependent Enzyme Activity. β-Lactamase activity assays were conducted using the colorimetric substrate nitrocefin, which changes from yellow to red (486 nm) upon hydrolysis. On the basis of similar studies with chimeric ZF endonucleases (Discussion), we expected the spacing between the two ZF sites (spacer) on the target DNA to be crucial for efficient enzyme reassembly. To examine this relationship, nitrocefin assays were performed in triplicate with 0.5 μM concentration each of LacA-Zif268 and PBSII-LacB proteins in the presence of hairpin oligonucleotides containing the two target sites at spacer lengths of 0, 6, and 10 bp (labeled *Zif-0-PBSII*, *Zif-6-PBSII*, and *Zif-10-PBSII* in Figure 2) at concentrations of 1 μM, 20 nM, 200 pM, and no DNA as a control. DNA-assisted enzyme reassembly is shown with all three spacer lengths (Figure 2A). The relative signal intensity after 20 min of incubation was highest for the 0 bp spacer, followed by 10 and 6 bp (Figure 2B). The signal intensity was generally proportional to the DNA concentration for all spacings, although a slightly higher correlation was observed for the 0 bp spacer, followed by 10 and 6 bp (R^2 correlation coefficients of 0.986, 0.971, and 0.887, respectively).

A kinetic analysis of the reaction using the 0 bp spacer (Figure 2C) revealed hydrolysis rates of 25 mU/min at 1 μM DNA (red), 20 mU/min at 20 nM (orange), 7 mU/min at 200 pM (yellow), and a background rate of 6 mU/min with no DNA (black). A hydrolysis rate of 3 mU/min was observed for the negative control of LacA-Zif268 paired with PE1A-LacB on the above target DNA (green). A difference in the relative signal intensity between the 1 μM DNA sample and the background was distinguishable by the earliest time point in our assay (3 min) and became more pronounced over time. All hydrolysis rates were linear over the 23 min

assay interval, with correlation coefficients generally greater than 0.99.

Effects of Target Site Mutations on SEER Signal Intensity. To determine the sensitivity of SEER to mutations, nitrocefin assays were performed using oligonucleotide targets carrying different mutations on either one or both of the ZF binding sites (Figure 3). At 1 μM DNA concentration and 0.5 μM concentration of each protein, a single mutation in the Zif268 target site reduced the relative signal to nearly background levels. A single base-pair mutation in the PBSII target site resulted in a 36% reduction in relative signal intensity. Target sites carrying two or more mutations also lowered the relative signal levels by at least 90%.

SEER Binding Domains Are Interchangeable. To demonstrate the generality of SEER-LAC to target significantly different binding sites, a nitrocefin assay was performed with two different DNA target sequences (Figure 4). One target contained Zif268 and PBSII binding sites with no spacer (*Zif-0-PBSII*, black bars), and the other contained Zif268 and PE1A sites with no spacer (*Zif-0-PE1A*, gray bars). Both SEER combinations reassembled in the presence of their cognate DNA sequences. Inappropriate target DNA produced a signal similar to the background signal of no target DNA.

SEER Binding in the Presence of Genomic DNA. The previous experiments were conducted with purified DNA targets. However, some applications of this technology might require it to recognize its target in the presence of complex DNA such as a genome, which might contain multiple alternative sites for the individual SEER proteins. To investigate if the presence of complex double-stranded DNA would interfere with this assay, a nitrocefin assay was performed in the presence or absence of herring sperm DNA (HS-DNA). The concentration of HS-DNA used was equimolar in base pairs (i.e., equal in mass) to 1 μM oligonucleotide target DNA. Under these conditions, there was no difference in relative signal intensity when 0.5 μM concentration each of LacA-Zif268 and PBSII-LacB proteins were incubated with 1μM of *Zif-0-PBSII* target DNA in the presence or absence of HS-DNA (Figure 5, black bars). As a negative control, the proteins were also incubated with the *Zif-0-PE1A* target DNA (gray bars). Although the relative signal generated using this target was somewhat higher than in previous assays, there was essentially no change in the signal intensity in the presence of HS-DNA.

DISCUSSION

In this study, the SEER-LAC system produced a DNA-concentration-dependent signal. Signal amplification remained linear over the assay time, and target DNA could be distinguished from nontarget DNA in less than 5 min. The proteins were expressed from the plasmid pMAL-C2X, which appends an MBP domain to the *N*-terminus. On the basis of previous experience (*9, 22, 23*), the presence of the MBP domain was not expected to interfere with SEER binding or activity. In fact, the MBP domain may have contributed to the solubility of these protein fragments, facilitating their use in in vitro assays. In our previous studies with a SEER-GFP system (*19*), GFP-containing fragments were insoluble, requiring an extensive (48 h) renaturation process, followed by a lengthy (24–48 h) incubation period for chromophore formation. The use of soluble protein

fragments, coupled with the enzymatic signal amplification provided by β-lactamase, enabled the detection of DNA in less than 5 min. This enhancement represents a greater than 1000-fold time improvement over our previous design.

A 15-aa linker between the zinc fingers and the β-lactamase domains was originally chosen to avoid possible steric clashes. A linker of this length may be useful for applications in which the target sites are separated by 1–10 bp. However, future SEER variations may benefit from the use of shorter linkers. Further insight can be gained from studies of chimeric ZF endonucleases (*24*). In this analogous system, two monomers of the dimeric endonuclease FokI are brought together by DNA-binding ZF domains to create an active cleavage enzyme. The optimal DNA spacing between the target sites was found to be related to the length of the protein linker. Long linkers (15 aa) allowed greater flexibility in the spacer size between the ZF binding sites. However, reducing the linker length to 0 aa improved the specificity of interaction (allowing reassembly only at 6 bp) and improved cleavage efficiency. In the current study, hydrolysis rates were highest in the targets with 0 and 10 bp spacers, with 0 bp providing the maximal signal. Thus, maximum efficiency was achieved with the current SEER design when the β-lactamase fragments were positioned close together on the same face of the DNA helix. As in the endonuclease studies, shorter linkers might provide a more rigid positioning of the β-lactamase fragments, resulting in more efficient reassembly and less tolerance to variations in target site spacing.

A single base-pair substitution in the Zif268 DNA-binding sequence reduced the relative signal 10-fold to background levels, demonstrating the sensitivity of SEER to detect point mutations. However, a similar substitution in the PBSII site reduced the relative signal by only 36%. This discrepancy likely results from differences in the binding properties of the two ZF proteins. Interestingly, the mutant site was recognized by the same ZF domain in both fragments. However, other differences, such as inter-domain amino acid interactions and sequence-dependent DNA deformation, may influence specificity. These results identify the quality of the ZF-DNA interaction as an important determinant of SEER function. Generally, the specificity of designed zinc finger proteins has been shown to be excellent (*9, 11, 25*).

Substitution of a different custom zinc finger DNA-binding domain generated a new SEER that produced a signal only on the new cognate target. These results clearly demonstrate that the observed effects were not due to special properties of ZFs Zif268 and PBSII but could be achieved using other custom ZFs such as PE1A. This feature suggests that SEERs can be rapidly designed to detect virtually any desired DNA sequence using custom ZF DNA-binding technology (*14, 15*).

In summary, we have demonstrated the use of TEM-1 β-lactamase to improve the sequence-enabled enzyme reactivation method for the detection of double-stranded DNA. The assay can detect its target sequence rapidly and with high specificity. The technology could be advantageous for certain applications, particularly those for which purified DNA is not limiting. For example, SEER-LAC might be useful for the rapid detection of specific sequences in amplified DNA. The linear relationship between signal and DNA target concentrations suggests that SEER could be developed for the quantitative determination of target copy numbers, or for measuring the length of repeated sequences

such as telomeres. In principle, the ability to directly detect double-stranded DNA could provide some novel capabilities. However, further studies on the range and utility of the SEER-LAC system will be necessary before its general applicability can be assessed.

SUPPORTING INFORMATION AVAILABLE

Details of the SEER-LAC protein fragment design, construction, and an annotated sequence as well a full description of the oligonucleotide DNA targets used in this study, and additional information on the assay background. This material is available free of charge via the Internet at http://pubs.acs.org.

REFERENCES

1. Venter, J. C., Adams, M. D., Myers, E. W., Li, P. W., Mural, R. J., Sutton, G. G., Smith, H. O., Yandell, M., Evans, C. A., Holt, R. A., and et al. (2001) The sequence of the human genome, *Science 291*, 1304−1351.
2. Johnsson, N., and Varshavsky, A. (1994) Split ubiquitin as a sensor of protein interactions in vivo, *Proc. Natl. Acad. Sci. U.S.A. 91*, 10340−10344.
3. Rossi, F., Charlton, C. A., and Blau, H. M. (1997) Monitoring protein−protein interactions in intact eukaryotic cells by beta-galactosidase complementation, *Proc. Natl. Acad. Sci. U.S.A. 94*, 8405−8410.
4. Pelletier, J. N., Campbell-Valois, F. X., and Michnick, S. W. (1998) Oligomerization domain-directed reassembly of active dihydrofolate reductase from rationally designed fragments, *Proc. Natl. Acad. Sci. U.S.A. 95*, 12141−12146.
5. Ghosh, I., Hamilton, A. D., and Regan, L. (2000) Antiparallel leucine zipper-directed protein reassembly: application to the green fluorescent protein, *J. Am. Chem. Soc. 122*, 5658−5659.
6. Galarneau, A., Primeau, M., Trudeau, L. E., and Michnick, S. W. (2002) beta-Lactamase protein fragment complementation assays as in vivo and in vitro sensors of protein protein interactions, *Nat. Biotechnol. 20*, 619−622.
7. Ray, P., Pimenta, H., Paulmurugan, R., Berger, F., Phelps, M. E., Iyer, M., and Gambhir, S. S. (2002) Noninvasive quantitative imaging of protein−protein interactions in living subjects, *Proc. Natl. Acad. Sci. 99*, 3105−3110.
8. Paulmurugan, R., Umezawa, Y., and Gambhir, S. S. (2002) Noninvasive imaging of protein−protein interactions in living subjects by using reporter protein complementation and reconstitution strategies, *Proc. Natl. Acad. Sci. U.S.A. 99*, 15608−15613.
9. Segal, D. J., Beerli, R. R., Blancafort, P., Dreier, B., Effertz, K., Huber, A., Koksch, B., Lund, C. V., Magnenat, L., Valente, D., and Barbas, C. F., III. (2003) Evaluation of a modular strategy for the construction of novel polydactyl zinc finger DNA-binding proteins, *Biochemistry 42*, 2137−2148.
10. Segal, D., and Barbas, C. F., III. (2001) Custom DNA-binding proteins come of age: polydactyl zinc-finger proteins, *Curr. Opin. Biotechnol. 12*, 632−637.
11. Liu, Q., Xia, Z., Zhong, X., and Case, C. C. (2002) Validated zinc finger protein designs for all 16 GNN DNA triplet targets, *J. Biol. Chem. 277*, 3850−3856.
12. Lee, D. K., Seol, W., and Kim, J. S. (2003) Custom DNA-binding proteins and artificial transcription factors, *Curr. Top. Med. Chem. 3*, 645−657.
13. Klug, A. (2005) Towards therapeutic applications of engineered zinc finger proteins, *FEBS Lett. 579*, 892−894.
14. Blancafort, P., Segal, D. J., and Barbas, C. F., III. (2004) Designing transcription factor architectures for drug discovery, *Mol. Pharmacol. 66*, 1361−1371.
15. Segal, D. J. (2002) The use of zinc finger peptides to study the role of specific factor binding sites in the chromatin environment, *Methods 26*, 76−83.
16. Jamieson, A. C., Miller, J. C., and Pabo, C. O. (2003) Drug discovery with engineered zinc-finger proteins, *Nat. Rev. Drug Discovery 2*, 361−368.
17. Carroll, D. (2004) Using nucleases to stimulate homologous recombination, *Methods Mol. Biol. 262*, 195−207.
18. Tan, W., Zhu, K., Segal, D. J., Barbas, C. F., III, and Chow, S. A. (2004) Fusion proteins consisting of human immunodeficiency virus type 1 integrase and the designed polydactyl zinc finger protein E2C direct integration of viral DNA into specific sites, *J. Virol. 78*, 1301−1313.
19. Stains, C. I., Porter, J. R., Ooi, A. T., Segal, D. J., and Ghosh, I. (2005) DNA sequence-enabled reassembly of the green fluorescent protein, *J. Am. Chem. Soc. 127*, 10782−10783.
20. Elrod-Erickson, M., Rould, M. A., Nekludova, L., and Pabo, C. O. (1996) Zif268 protein-DNA complex refined at 1.6 A: a model system for understanding zinc finger-DNA interactions, *Structure 4*, 1171−1180.
21. Wolfe, S. A., Nekludova, L., and Pabo, C. O. (2000) DNA recognition by Cys2His2 zinc finger proteins, *Annu. Rev. Biophys. Biomol. Struct. 29*, 183−212.
22. Segal, D. J., Dreier, B., Beerli, R. R., and Barbas, C. F., III. (1999) Toward controlling gene expression at will: selection and design of zinc finger domains recognizing each of the 5′-GNN-3′ DNA target sequences, *Proc. Natl. Acad. Sci. U.S.A. 96*, 2758−2763.
23. Beerli, R. R., Segal, D. J., Dreier, B., and Barbas, C. F., III. (1998) Toward controlling gene expression at will: specific regulation of the erbB-2/HER-2 promoter by using polydactyl zinc finger proteins constructed from modular building blocks, *Proc. Natl. Acad. Sci. U.S.A. 95*, 14628−14633.
24. Bibikova, M., Carroll, D., Segal, D. J., Trautman, J. K., Smith, J., Kim, Y. G., and Chandrasegaran, S. (2001) Stimulation of homologous recombination through targeted cleavage by chimeric nucleases, *Mol. Cell Biol. 21*, 289−297.
25. Tan, S., Guschin, D., Davalos, A., Lee, Y. L., Snowden, A. W., Jouvenot, Y., Zhang, H. S., Howes, K., McNamara, A. R., Lai, A., Ullman, C., Reynolds, L., Moore, M., Isalan, M., Berg, L. P., Campos, B., Qi, H., Spratt, S. K., Case, C. C., Pabo, C. O., Campisi, J., and Gregory, P. D. (2003) Zinc-finger protein-targeted gene regulation: genomewide single-gene specificity, *Proc. Natl. Acad. Sci. U.S.A. 100*, 11997−12002.
26. Choo, Y., and Klug, A. (1994) Selection of DNA binding sites for zinc fingers using rationally randomized DNA reveals coded interactions, *Proc. Natl. Acad. Sci. U.S.A. 91*, 11168−11172.