1068–1074 Nucleic Acids Research, 1994, Vol. 22, No. 6

Direct selection of binding proficient/catalytic deficient variants of *Bam*HI endonuclease

Lydia F.Dorner and Ira Schildkraut*
New England Biolabs, 32 Tozer Road, Beverly, MA 01915, USA

Received November 10, 1993; Revised and Accepted February 1, 1994

ABSTRACT

Variants of *Bam*HI endonuclease in which the glutamate 113 residue has been changed to lysine or the aspartate 94 to asparagine were shown to behave as repressor molecules *in vivo*. This was demonstrated by placing a *Bam*HI recognition sequence, GGATCC, positioned as an operator sequence in an antisense promoter for the *aad*A gene (spectinomycin resistance). Repression of this promoter relieved the inhibition of expression of spectinomycin resistance. This system was then used to select new binding proficient/cleavage deficient *Bam*HI variants. The *Bam*HI endonuclease gene was mutagenized either by exposure to hydroxylamine or by PCR. The mutagenized DNA was reintroduced into *E.coli* carrying the *aad*A gene construct, and transformants that conferred spectinomycin resistance were selected. Twenty Sp^r transformants were sequenced. Thirteen of these were newly isolated variants of the previously identified D94 and E113 residues which are known to be involved in catalysis. The remaining seven variants were all located at residue 111 and the glutamate 111 residue was shown to be involved with catalysis.

INTRODUCTION

Despite the impact type II restriction endonucleases have had on modern biology only a few restriction endonucleases themselves have been the target of both genetic and protein structural analyses. The best studied are *Eco*RI and *Eco*RV both of whose structures are known (1–3). Unlike their cognate methylases, little or no protein sequence similarity has been observed among the majority of type II restriction endonucleases (4,5). The *Eco*RI and *Eco*RV structures have little in common except for their homodimeric symmetry. However the structure determinations, along with mutational analyses, have led to an understanding of which residues in *Eco*RI and *Eco*RV are involved in catalysis and DNA recognition. Both site-directed mutational (6–8) and crystallographic (3) studies of *Eco*RV have allowed assignment of catalytic function to D74, D90, and K92. In the case of *Eco*RI, King *et al.* (9) isolated an *Eco*RI variant, E111G, based on the property of non-lethality in a host where the *Eco*RI methylase gene was absent. Over 200 isolates were screened to identify the one variant which bound to DNA but could not cleave it. The revised crystallographic structure of *Eco*RI has shown E111 to be positioned together with D91 and K113 directly across from the scissile phosphodiester bond. It has been proposed that these three residues in *Eco*RI are analogous to D74, D90, and K92 in *Eco*RV (8,10).

We have chosen to study the *Bam*HI restriction endonuclease (recognition sequence, GGATCC) by mutational analysis. The gene encoding the *Bam*HI endonuclease has previously been cloned (11), sequenced (12), and its product has been overexpressed and crystallized (13). We are pursuing the genetic determination of the residues in *Bam*HI that are involved in catalysis and recognition, while the three dimensional structure is being determined (14). In an earlier report, 137 *Bam*HI mutants, that lacked or had greatly reduced activity were isolated by selecting transformants in the absence of expression of the *Bam*HI methylase gene (15). Three of these *Bam*HI variants (E77K, D94N, E113K) retained their ability to bind to the DNA sequence GGATCC *in vitro*, but had lost the ability to cleave DNA. These three *Bam*HI variants exhibited properties similar to the *Eco*RI and *Eco*RV variants discussed above, and the altered residues should therefore be involved in catalysis. The *Bam*HI apoenzyme crystal structure has been determined at 1.95 Å (14), and bears out this relationship among *Bam*HI, *Eco*RI and *Eco*RV. The DNA cocrystal structure will be forthcoming shortly (A.K.Aggarwal, personal communication).

In the present study we analyze the two *Bam*HI binding proficient/cleavage deficient (*cat*$^-$) variants D94N and E113K by using the *in vivo* transcriptional interference assay of Elledge and Davis (16). The assay requires that a protein bind to a specific DNA recognition sequence and repress the transcription of an antisense RNA that would otherwise interfere with expression of the spectinomycin resistance gene, *aad*A (aminoglycoside adenylyltransferase). This transcriptional interference system consists of a plasmid pNN388 containing the *aad*A gene, the product of which confers spectinomycin resistance to the cell, and a derivative of conII, a strong constitutive *E.coli* promoter, which is located downstream of and in an antisense orientation to the *aad*A gene. Transcription from conII interferes with transcription of the *aad*A gene product, resulting in the cell becoming sensitive to spectinomycin. By inserting recognition sequences of specific DNA binding proteins into specific regions

*To whom correspondence should be addressed

of the promoter transcription from conII can be regulated. Binding of the protein to this region of conII prevents transcription, thereby relieving the transcriptional interference that impedes the synthesis of the *aad*A gene product (Figure 1). This results in a change of phenotype to spectinomycin resistance.

Elledge *et al.* (17) exploited the spectinomycin transcriptional interference assay described above as a genetic selection for cloning genes expressing sequence-specific DNA binding proteins. Specific DNA sequences positioned as an operator sequence near the near the start of transcription of the strong synthetic antisense constitutive promoter, created promoters that could be repressed by the corresponding sequence-specific DNA-binding protein. Each protein was shown to repress the appropriate promoter and alleviate transcriptional interference of *aad*A, resulting in drug resistance in cells expressing the appropriate DNA-binding protein. Here we demonstrate that *Bam*HI E113K and D94N can confer Sp^r by functioning as *in vivo* repressors of transcriptional interference and that transcriptional interference can be used as a direct selection for catalytic mutants of *Bam*HI.

MATERIALS AND METHODS

Media, strains and plasmids

Luria–Bertani (LB) medium and LB agar were as described (18). LB agar plates were supplemented with chloramphenicol (Cm, 40 μg/ml), spectinomycin (Sp, 80 μg/ml), ampicillin (Ap, 100μg/ml) or isopropylthiogalactoside (IPTG, 10 μM) as required. *Escherichia coli* strain ER2267 [*Δ(argF-lac)U169 supE44 recA1 endA1 thi-1 Δ(mcrC-mrr)114::IS10/F' proA*$^+$*B*$^+$ *lacI*^{q} *Δ(lacZ)M15 zzf::mini-Tn10 (Kan*^{r}*)*] and the isogenic strain ER2263 that lacks the F' were from Elisabeth Raleigh (New England Biolabs). *E.coli* strain ADK21 is a derivative of K802 (*lac3 galK2 galT22 metB1 hsdR2 supE44 mcrB1 mcrA1*) which expresses the *Bam*HI endonuclease gene constitutively (13). pNN388 was a gift from R.Davis. The pAEK14 plasmid that carries the *Bam*HI endonuclease gene was from W.E.Jack (New England Biolabs) and the *Bam*HI mutant derivatives E113K, D94N, and E77K were described in (15).

Construction of *Bam*HI methylase host

The Ind$^-$ lambda prophage that constitutively expresses *Bam*HI methylase (13) was induced from ADK21 by superinfection with wild type lambda (19). This phage lysate was used to infect ER2263 and establish a new lysogenic strain carrying *Bam*HI methylase, LD2263. LD2263 was mated with ER2208 [F' *proAB lacI*^{q} Δ(lacZ M15 zzf:: Tn10) from Elisabeth Raleigh, New England Biolabs] and a prototrophic Tet^r transconjugant was designated, LD2264. LD2264 was then transformed with pLD102 (see below) to form LD2265.

Construction of plasmids pLD102 thru pLD105

pNN388 (16) was modified to remove the unique *Bam*HI site by digesting the plasmid with *Bam*HI, filling in with DNA polymerase I Klenow fragment and ligating with T4 DNA ligase. This derivative of pNN388, pSX388, was used in all of the subsequently described plasmid constructions (Table I). The reporter plasmids containing a derivative of the regulated promoter con II (16) were constructed by annealing one of four oligonucleotide sequences listed in Table I with an 18mer oligonucleotide 5'-GTTGGCAAGCTTACCTGC-3'. The DNA was made fully double stranded by using DNA polymerase I Klenow fragment. The DNA was then digested with *Not*I and *Hin*dIII and cloned into *Not*I/*Hin*dIII-cleaved pSX388. The four unique plasmid constructs were sequenced through the promoter region, two of the inserts in pLD102 and pLD103 were found to have a single base deletion at position +4 (Table I).

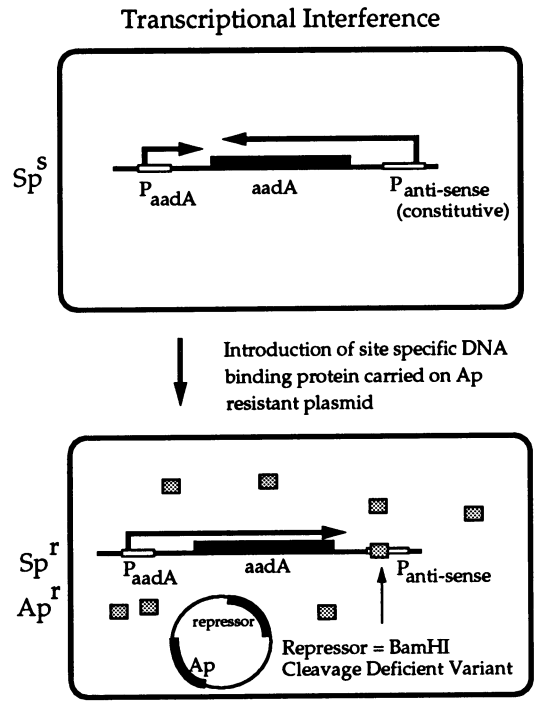

Figure 1. Schematic representation of transcriptional interference. Shaded boxes represent the BamHI binding proficient–catalytic deficient mutant protein which acts as a repressor by binding to the antisense promoter.

Hydroxylamine mutagenesis

5 μg of pAEK14 was incubated for 5.5 hours with freshly prepared NH_2OH as described (15). Plasmid DNA was ethanol precipitated, washed twice with 70% ethanol, dissolved in TE buffer and transformed into the appropriate host.

PCR mutagenesis

Two oligonucleotide primers, one of the following sequence 5'-C-AATTTCACAGGAAACAGCCC-3', which primes just upstream of the *Nco*I site at the 5' end of the *Bam*HI endonuclease gene, and the other of the following sequence 5'-GGCGCG-AGGCAGCCAAGCTTTGCCATT-3', which contains a HindIII site beyond the 3' end of the gene, were incubated with pAEK14 DNA, which contains the complete nucleotide sequence of the *Bam*HI endonuclease gene, and subjected to 30 cycles of PCR using *Taq* DNA polymerase (Perkin-Elmer-Cetus) as described (20). The phenol-extracted, isopropanol precipitated amplified DNA was digested with *Nco*I and *Hin*dIII. Following another round of phenol extraction and isopropanol precipitation, the DNA was dissolved in water and ligated into *Not*I–*Hin*dIII digested pAEK14.

DNA sequencing

DNA for sequencing was prepared using Qiagen plasmid mini kits (Qiagen) and sequenced using the Circumvent Phototope™ Kit (New England Biolabs) which is based on thermal cycle sequencing and employs biotinylated primers for chemilumine-

Table I. Anti-sense promoter sequences

		-35	-10	+1	
pLD102	5'	CGGTAGCGGCCGCTGTTGACAATTAATCATCGGCTCGTATAATGGATCCGCAG.TAAGCTTGCCAAC			3'
pLD103	5'	CGGTAGCGGCCGCTGTTGACAATTAATCATCGGCTCGTATAATAGATCCGCAG.TAAGCTTGCCAAC			3'
pLD104	5'	CGGTAGCGGCCGCTGTTGACAATTAATCATCGGCTCGTATAATGGATCTGCAGGTAAGCTTGCCAAC			3'
pLD105	5'	CGGTAGCGGCCGCTGTTGACAATTAATCATCGGCTCGTATAATAGATCTGCAGGTAAGCTTGCCAAC			3'

scent detection. Two of the variants were sequenced using the Applied Biosystems DNA sequencer. The sequences of the biotinylated primers and unlabeled primers were:

5' TATAATGTGTGGAATTGTGAGCGGAT 3'
5' CACCTATTTGGCCAGCAACCTCA 3'
5' AAAAAAGGTGGTCCGATTGATG 3'
5' AAATTGATTTGGCTATTATC 3'
5' ATTGGATTTAATGCTGAGGC 3'

Selection of spectinomycin resistant mutants

Mutagenized DNA (100 ng) was electroporated into electrocompetent ER2267 cells (40 μl) which contained pLD102. A 0.1 cm cuvette was used with a single pulse at 25 μF, 1.6 kV and 200 ohms (time constant = 4.6–4.7) using a Bio-Rad Gene Pulser® apparatus with a Pulse Controller. Cells were immediately diluted to 1 ml with LB at room temperature. The cells were incubated with aeration at 37°C in the absence of antibiotics or IPTG for one hour. Various dilutions of the outgrown cells were added to 3 ml of top agar and plated directly onto LB plates supplemented Ap, Sp and IPTG. Plates were incubated at 37°C for 24 to 48 hours. When the *Bam*HI variant E113K plasmid was used as a control and electroporated as above the efficiency was 2×10^8 transformants per μg of plasmid DNA.

Purification of E111K variant protein

ER2267 cells carrying the reporter pDL102 and the pAEK14 vector encoding the gene for the *Bam*HI variant E111K were grown and the variant protein was induced as described (15). E111K was purified to >90% homogeneity essentially as described (13) with the omission of the final Q-sepharose chromatographic step.

Efficiency of plating assays

Cells were grown in LB supplemented with Cm and Ap to approximately 70 Klett units. Cells were diluted into LB and 0.1 ml of various dilutions were added to top agar and poured onto LB plates supplemented with Sp, Ap and IPTG or Ap and Cm. Plates were incubated at 37°C and colonies counted after 24 hours in the presence of Ap and Cm, or 48 hours in the presence of Sp and IPTG. Plating by embedding cells in top agar was used throughout since spreading the various dilutions directly on plates led to significantly lower plating efficiencies.

End-labeling DNA with biotinylated dATP

A 333 bp pBR322 DNA fragment containing a single *Bam*HI recognition sequence was prepared from pBR322 by digestion with *Nde*I and *Sph*I and isolated by agarose gel electrophoresis. The 333 bp DNA fragment (2.5 μg) was end-labeled in a 50 μl reaction in NEB buffer #2 (New England Biolabs) supplemented with 50 μM dCTP, dGTP, dTTP, 20 μM biotin-14-dATP and 10 units *E.coli* DNA polymerase Klenow fragment (New England Biolabs). The reaction was incubated at room temperature for one hour. The reaction was stopped and the DNA isolated by phenol extraction and alcohol precipitation. A complete *Sau*3AI digest of pUC19 was end-labeled as described above and the 341 bp fragment containing no *Bam*HI recognition sequences was purified by gel electrophoresis.

Table II. Expression of anti-sense promoters of pLD102 and its derivatives

	Cm	Cm+Sp
pLD102(GGATCC)	1	0.0005
pLD103(AGATCC)	1	0.0007
pLD104(GGATCT)	1	0.0003
pLD105(AGATCT)	1	0.0005
pSX388	1	1

Electrophoretic mobility shift assay

Electrophoresis of DNA–*Bam*HI complex in native polyacrylamide gel was performed a described (21), except that the DNA was end-labeled with biotinylated-dATP. Gels were run 2 hours prior to sample loading. Electrophoresis was carried out at 150 constant volts at room temperature with 84×90×1.0 mm gels. DNA was immediately tranferred to Immobilon™ S (Millipore) membranes by first exposing the gel to 0.5 M NaOH for 5 minutes followed by exposure to 1.5 M Tris–HCl for 5 minutes. The DNA was then blotted to Immobilon™ S membrane for 20 minutes as described in Phototope™ detection kit manual (New England Biolabs). The DNA was visualized with Phototope™ detection kit.

RESULTS

Spectinomycin sensitivity of reporter plasmids

To test the *Bam*HI *cat*⁻ variants E113K and D94N for repressor activity *in vivo*, it was first necessary to establish, in the absence of the *Bam*HI variant proteins, that constructs in Table I containing our putative operator sequences in the anti-sense promoter for the *aad*A gene conferred spectinomycin sensitivity. Elledge and Davis found that 95% of tested binding sites placed between positions −5 to +5 in con II resulted in con II becoming a regulated promoter. We positioned the *Bam*HI recognition sequence and its derivatives at position −4 (Table I). The four reporter plasmids were transformed into *E.coli* ER2267. Cm^r transformants were selected and tested for Sp^s as described in Methods section. The data in Table II show that the four reporter plasmids contained antisense promoters of sufficient strength to interfere with transcription of the aadA gene, conferring Sp sensitivity. The plating efficiency on medium with Sp was less than 10^{-3}. In contrast, the parent plasmid pSX388 which does not contain an anti-sense promoter is resistant to spectinomycin and has a plating efficiency on medium with Sp of 1.

In vivo repressor activity of E113K

The plasmid encoding the *Bam*HI variant E113K was tested for regulation of Sp^r with the reporter plasmid pLD102. E113K had been previously shown to lack cleavage activity but retained its binding specificity *in vitro* (15). The IPTG inducible E113K

Table III. Efficiency of plating E113K (*Bam*HI variant) with pLD102

	Cm+Ap	Cm+Ap+Sp
E113K	1	0.5
E113K/no IPTG	1	0.002
E113K/m.*Bam*	1	0.001
E113K(4 base insertion)	1	0.003
E113K(deletion)	1	0.003

Table IV. Efficiency of plating *Bam*HI variants

	Cm+Ap	Cm+Ap+Sp
E113K and pLD102(GGATCC)	1	0.6
E113K and pLD103(AGATCC)	1	0.001
E113K and pLD104(GGATCT)	1	0.001
E113K and pLD105(AGATCT)	1	0.001
D94N and pLD102(GGATCC)	1	0.6
D94N and pLD103(AGATCC)	1	0.026
D94N and pLD104(GGATCT)	1	0.014
D94N and pLD105(AGATCT)	1	0.011
E111K and pLD102(GGATCC)	1	0.7
E111K and pLD103(AGATCC)	1	0.001
E111K and pLD104(GGATCT)	1	0.001
E111K and pLD105(AGATCT)	1	0.001

Table V. *Bam*HI variants obtained by PCR mutagenesis: base substitutions and corresponding amino acid changes

Variant #	Codon Change	aa change
1	GAA>AAA	E113K
2	GAT>GGT	D94G
3	GAT>GGT	D94G
	ATT>GTT	I22V
	AAA>AAG	K132K
4	GAT>GGT	D94G
5	GAA>GTA	E111V
6	GAA>GTA	E111V
7	GAA>AAA	E113K
8	GAT>AAT	D94N
	TTC>TTT	F45F
9	GAA>AAA	E113K
	ACA>ACG	T153T
10	GAT>GGT	D94G
11	GAT>AAT	D94N
12	GAA>AAT	E111K
13	GAA>GTA	E111V
	TTT>TTC	F112F

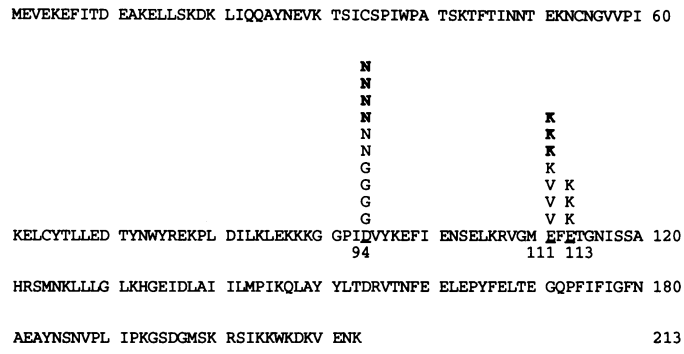

Figure 2. Histogram of *Bam*HI mutations which confer spectinomycin resistance. Residues in normal fonts are derived from PCR mutagenesis. Bold residues are derived from hydroxylamine mutagenesis.

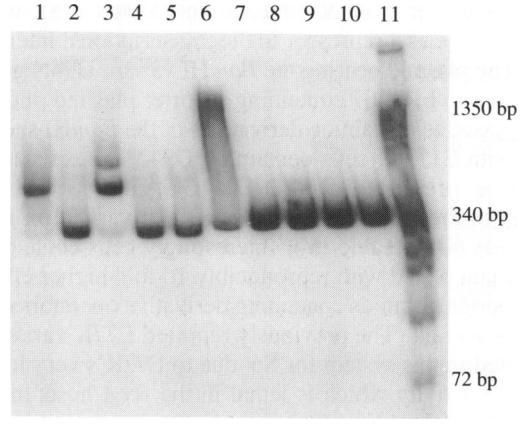

Figure 3. Electrophoretic mobility shift: 10 nM end-labeled DNA was incubated with purified E111K or wild type *Bam*HI protein in 10 μl of 100 mM NaCl, 10 mM Tris−HCl, pH 7.8, 1 mM EDTA, 10 mM β-mercaptoethanol, 100 μg/ml bovine serum albumin. After incubation at 37 °C for 30 min, glycerol was added to 10% and the protein−DNA complex was loaded directly onto a 6% polyacrylamide gel cast in TBE (45 mM Tris, 45 mM borate, 2 mM EDTA). The DNA was visualized as described in the methods section. Lanes 1−5 contained the 333 bp sequence with a GGATCC site. Lanes 6−10 contained the 341 bp fragment which did not contain a *Bam*HI recognition sequence. Lane 11 contained prebiotinylated DNA size markers (New England Biolabs). DNA in lanes 1 and 6 were incubated with 100 nM wild type *Bam*HI protein; DNA lanes 2 and 7 with 10 nM wild type *Bam*HI protein. DNA in lanes 3 and 8 were incubated with 100 nM E111K protein; DNA lanes 4 and 9 with 10 nM E111K protein. Lanes 5 and 10 contained no added protein.

which was carried on an Ap^r plasmid was transformed into ER2267 containing the Cm^r spectinomycin reporter plasmid pLD102. The plating efficiency of the Ap^r and Cm^r transformants on plates with and without Sp was then determined. As can be seen in Table III, E113K conferred the Sp^r phenotype to the host containing pLD102. The plating efficiency of E113K on spectinomycin was 0.5 in the presence of 10μM IPTG. The level of IPTG was critical, and concentrations much above 20 μM reduced the efficiency of plating (data not shown). Furthermore, without induction of E113K transcription, plating in the absence of IPTG, the cells were sensitive to spectinomycin.

If E113K were acting as a repressor at the *Bam*HI site, then site-specific methylation of GGATCC by *Bam*HI methylase should interfere with binding of E113K and thus repression. To test this we constructed a strain containing a chromosomal copy of the *Bam*HI methylase gene; the reporter plasmid, pLD102; and the Ap^r plasmid harboring E113K. This strain was then tested for IPTG inducible Sp^r. As expected the strain remained spectinomycin sensitive (Table III). Therefore methylation abolished the repressor-like behavior of E113K. To confirm that the E113K protein and not other products of the Ap resistant plasmid was acting as a repressor, two modified versions of E113K protein were introduced into the host containing the reporter plasmid pLD102 (Table III). One, an *Eco*RI−*Hin*dIII deletion that eliminates > 95% of the E113K coding sequence, and the other, a 4-base insertion at the *Eco*RI site that shifts the

coding sequence out of frame, were tested for their ability to act as repressors. Both of the interrupted versions of the E113K protein failed to confer resistance to spectinomycin, indicating that E113K was responsible for the observed phenotypic change.

Action of E113K protein required the presence of an authentic *Bam*HI site. This was determined by transforming plasmid bearing E113K into cells containing reporter plasmids with a *Bam*HI site or containing derivative reporter plasmids which differed by one and two bases at the position of the *Bam*HI site. Cells became resistant to spectinomycin only in the presence of pLD102 (the GGATCC operator) plating with an efficiency of 60%. Cells containing reporter plasmids, pLD103, pLD104 and pLD105 with derivative sites (AGATCC, GGATCT or AGATCT respectively) into which plasmids bearing E113K were transformed, plated with efficiencies approximately 1000-fold lower on spectinomycin (Table IV).

Repressor activity of other *Bam*HI variants

Similarly, another *cat*$^-$ *Bam*HI variant, D94N (15), was also found to behave as a repressor in the transcriptional interference system. The plasmid bearing the *Bam*HI variant D94N gene was introduced into ER2267 containing reporter plasmid pLD102 or reporter plasmids containing derivatives of the *Bam*HI site (Table IV). As with E113K, cells containing D94N are resistant to Sp only in the presence of pLD102 (GGATCC). The plating efficiency of the cells containing the D94N variant on medium with Sp was 60% (Table IV). Interestingly cells containing the D94N variant plated with reproducibly 10 fold higher efficiency on the reporter plasmids containing derivative operators than did the E113K variant. The previously reported E77K variant could not be tested in this system for Sp^r due to E77K's very low level of cleavage activity which is lethal in the *recA* hosts that were used (data not shown).

Selection for *de novo Bam*HI mutants using reporter plasmid pLD102

Because of our interest in defining the catalytic residues of *Bam*HI endonuclease, we used spectinomycin resistance as a direct selection for mutations in *Bam*HI endonuclease gene. We took advantage of the lethal nature of the wild type *Bam*HI endonuclease gene when introduced into a host in the absence of the *Bam*HI methylase gene and reasoned that selection for viability and spectinomycin resistance would yield only *cat*$^-$ variants. Any variants retaining low levels of endonucleolytic activity, such as E77K, should be lethal in the *recA* hosts used here. We mutagenized the wild type *Bam*HI endonuclease gene by two different methods, exposure of plasmid DNA to hydroxylamine (15) and PCR mutagenesis (20).

Hydroxylamine treated pAEK14 was introduced into *E.coli* ER2267/pLD102 by electroporation (see Methods) and the cells were plated directly onto LB plates containing Ap, Sp and IPTG. This resulted in 100 Sp^r transformants. Three of these were picked and the plasmids from each of the three was isolated and retransformed into ER2267/pLD102. The plating efficiency in the presence and absence of spectinomycin was determined to be about 50%. The *Bam*HI endonuclease gene of each was sequenced. All three had the same single amino acid substitution, glutamate 111 to lysine, E111K.

The other method used to mutagenize wild type *Bam*HI endonuclease gene was PCR. The DNA was prepared as described in Methods and electroporated into ER2267 cells containing pLD102. These cells were then plated directly onto LB plates containing Ap, Sp and IPTG. From a single electroporation of 350 ng of PCR mutagenized DNA 1300 Sp resistant transformants were observed. Thirteen of these were picked and the plasmid isolated and retransformed into ER2267/pLD102. All plated with about 50 % the efficiency on medium with Sp. Nine carried a single amino acid substitution encoded for in the *Bam*HI endonuclease gene. One plasmid carried three substitutions in the *Bam*HI endonuclease gene; one substitution was D94G, the second was I22V, and the third was a silent K132K codon change. We assume that the I22V mutation has no large effect since the D94G mutation accounts for the phenotype. The other isolates with multiple substitutions were D94N, E111K, and E111V and each carried respectively single silent codon changes in F45F, T153T, and F112F. The codon and amino acid residue changes are listed in Table V.

In summary 16 variants generated by two completely different methods of mutagenesis, identified only three amino acid residues. Figure 2 is a histogram of all the residue changes accounting for Sp resistance that were obtained by either PCR or hydroxylamine mutagenesis. Two of these, D94N and E113K, had been identified in the earlier mutagenesis study. These two variants were known to be binding proficient and cleavage deficient.

Residue E111 had not before been implicated in catalysis. Therefore, the purified variant E111K protein was assayed for cleavage activity on λ DNA. No detectable cleavage was seen by agarose gel electrophoresis after incubation overnight of 0.75 μg of E111K protein with λ DNA at 37°C (data not shown). Furthermore in Figure 3 it can be seen that E111K causes a site specific gel mobility shift and appears to behave more similarly to D94N in gel mobility shift than E77K or E113K. E111K does not demonstrate appreciable non-specific binding in the gel shift, nor did D94N, whereas E77K and E113K are shown to enhance binding to non-specific DNA (15). The small amount of a slower moving complex found with E111K is most likely due to the absence of any non-competing DNA which is unlike the conditions used in the previous study (15). We conclude that E111K is a cleavage deficient and binding proficient variant.

The ability of E111K to specifically bind to the *Bam*HI site was also demonstrated using the transcriptional interference assay. The efficiency of plating for cells containing both E111K and pLD102 or its derivative operators (pLD103, pLD104, pLD105) was determined. E111K acted as a site specific repressor molecule by conferring Sp^r at a high efficiency only in the presence of pLD102, as can be seen from Table IV. As with E113K, cells containing E111K plated on medium with Sp 600-fold less efficiently with pLD103, pLD104, or pLD105 than with pLD102. Whereas D94N plated only about 30-fold less well on the derivative plasmids. Therefore in this assay E111K behaved more like E113K, than D94N.

Selection of *Bam*HI variants using reporter plasmid pLD105 (AGATCT)

Because of the ability to select directly for *cat*$^-$ *Bam*HI variants using spectinomycin resistance we attempted to select for a *Bam*HI variant that could bind to a a non-cognate recognition sequence. 300 ng of hydroxylamine mutagenesized pAEK14 DNA resulted in only 13 transformants when introduced into *E.coli* ER2267/pLD105. This strain harbors the AGATCT reporter plasmid. Seven of these transformants were picked, the plasmid isolated and reintroduced into ER2267/pLD105. Four of these plasmids plated on spectinomycin with efficiencies of

about 2−4% when compared to plating without spectinomycin, the other three plated with plating efficiencies of less than 0.1% (background). All four plasmids that plated with efficiencies of 2−4% on pLD105 were sequenced. All four isolates contained the D94N substitution.

CONCLUSION

The transcriptional interference system of Elledge and Davis has proven to be a useful method for selecting *Bam*HI variants that have defects specifically in their catalytic residues. The direct selection of catalytic mutants of *Bam*HI has genetically defined the catalytic residues as D94, E111 and E113. The *cat*$^-$ variants selected in this study together with the earlier *Bam*HI variants which total 26 variants all map to only these three residues, plus two E77 representatives. Upon inspection of the *Bam*HI apoenzyme three dimensional structure, it appears that residues D94 and E111 correspond directly with D74 and D90 in the *Eco*RV structure and D91 and E111 in the *Eco*RI structure. These two sets of residues have been implicated in coordination of the magnesium ion, which suggests that D94 and E111 of *Bam*HI also coordinate the Mg ion. The E113K variant loss of catalytic activity defines the 113 glutamate as a catalytic residue, and it is interesting to note that this position is equivalent phenotypically and structurally to the lysine residues at positions 113 and 92 in *Eco*RI and *Eco*RV, respectively, however the lysine residues are not chemically equivalent to glutamate.

This system should allow rapid identification of catalytic residues for other type II endonucleases. The placement of the operator in the anti-sense promoter at position −4 has proven successful for selecting *Bam*HI variants, and currently a number of Sp^r conferring mutants of *Bgl*II (AGATCT) are being isolated using the pLD105 (AGATCT) reporter plasmid (unpublished, Bitinaite and Schildkraut).

It has been reported by Taylor *et al.* (22); and Zebala *et al.* (23) that *Eco*RV and *Taq*I, respectively, unlike *Eco*RI and *Bam*HI do not demonstrate site specific binding *in vitro* in the absence of Mg^{++}. This difference for *Eco*RV and *Taq*I may typify a class of restriction endonucleases which may not be amenable to selection for repressor-like activities. Mutations that result in loss of Mg binding may also abolish site specific binding. However, it would be of interest to determine if repressor-like behavior can be selected with endonucleases which are thought to require Mg for site specific binding. In this case, repressor-like behavior would suggest selection of mutations at residues other than those which are involved in Mg coordination or DNA recognition, yet do not allow cleavage.

The identification of catalytic residues among the type II restriction endonucleases should assist in sequence alignments. To date there is an apparent lack of sequence similarities among this highly diverse class of proteins. Possibly these catalytic residues may reveal necessary reference points where comparisons of these molecules can be made.

One of the *Bam*HI catalytic mutants, E113K, has been shown to have utility in manipulating DNA *in vitro*. The E113K protein has protected GGATCC sites *in vitro* against Bal31 nuclease digestion to facilitate unidirectional deletions and has been used to specifically block a GGATCC sequence from Sau3A digestion (24). Pavco and Steege (25) and Herendeen *et al.* (26) have shown that *Eco*RI catalytic variants of E111 can block transcription *in vitro*.

Catalytic variants may also lead the way to selecting restriction endonucleases with altered recognition specifity. Altering the specificity of an active endonuclease without concomitant alteration of its cognate methylase is lethal. However, selection of altered binding specificity should not be lethal when cleavage deficient variants are used. Our results suggest that the transcriptional interference of spectinomycin resistance might prove a useful method to select for altered specificities. We have also shown here that a mutation that leads to only 2% plating efficiency (D94N) may be isolated through the Sp^r selection on a non-cognate reporter plasmid (AGATCT). This is an encouraging result for further studies where we plan to try to alter DNA specificity using cassette mutagenesis at the residues responsible for DNA recognition as they are identified by three dimensional protein/DNA cocrystal structure.

Our results suggest that a general genetic approach involving three steps will permit the construction of a mutant type II endonuclease with a new specificity of cleavage. The first step is the isolation of a *cat*$^-$ variant as described in this paper. The second step uses the *cat*$^-$ variant to isolate variants with altered binding specifities by selection using spectinomycin reporter plasmids containing altered operator sequences in their anti-sense promoters. These altered specificity binding variants should not be lethal because they are defective for catalysis. Gel shift analysis of these variants on various DNA fragments should define the full spectrum of specificity for the variant and thus enable construction of a suitable host expressing the appropriate methylase(s) which will protect its DNA from cleavage. The third step is the introduction into the protected host the endonuclease gene where the catalytic residue was restored to wild type. This should yield a fully functional restriction endonuclease, which could then be expressed without lethality.

ACKNOWLEDGEMENTS

We thank S.-y.Xu for modifying pNN388 to pSX388. E.Schildkraut for assistance with the gel mobility shift experiment; L.Mazzola, L.Friedman, and L.Sears for assistance with DNA sequencing; E.Raleigh for strains, guidance in constructing lambda lysogens and critical review of the manuscript; W.E.Jack for the *Bam*HI methylase lysogenic strain. We also thank P.Riggs and S.-y.Xu for helpful discussions.

REFERENCES

1. Rosenberg, J. M. (1991) Curr. Opinion Struct. Biol., 1, 104−113.
2. Y. Kim, J. C. Grable, R. Love, P. J. Greene and Rosenberg.J. M. (1990) Science, 249, 1307−1309.
3. Winkler, F.K., Banner, D.W., Oefner, C., Tsernoglou,D., Brown, R.S., Heathman, S.P, Bryan, R.K., Martin, P.D., Petratos, K., and Wilson, K.S. (1993) EMBO J. 12: 1781−1795.
4. Heitman, J. (1993) in Genetic Engineering (J.K. Setlow ed), pp57−107, Plenum Press, New York.
5. Roberts, R.J. and Halford, S.E. (in press) in Nucleases, 2ndEd. (R.J. Roberts, S.M. Linn and R.S. Lloyd, eds.) Cold Spring Harbor Press, Cold Spring Harbor, New York.
6. Pingoud, A., Thielking, V., Selent, U., Kohler, E., Liedtke, M., Wolfes, H., Pieper, U., Geiger, R., and Winkler, F. K. (1991) Biomol. Struct. Dyn. 8, A165.
7. Thielking, V., Selent,U., Kohler, E., Wolfes, H., Pieper, U., Geiger, R., Urbanke, C., Winkler, F. K., and A. Pingoud, Biochemistry, (1991) 30, 6416−6422.
8. Selent, U., Ruter, T., Kohler, E., Liedtke, M., Thielking, V., Alves, J., Oelgeschlager, T., Wolfes, H., Peters, F., and Pingoud, A.(1992) Biochemistry 31, 4808−4815.

9. King,K., Benkovic, S. J., and P. Modrich (1989) J. Biol. Chem., 264, 11807–11815.
10. Winkler, F.K. (1992) Curr. Opin. Struct. Biol. 2, 93–99.
11. Brooks, J.E.,Benner, J.S.,Heiter, D.F., Silber, K. R.,Sznyter, L.A., Jager-Quinton, T.,Moran, L.S., Slatko, B. E., Wilson, G. G.and D. O. Nwankwo. (1989)Nucleic Acids Res., 17, 979–997.
12. Brooks, J.E., Nathan, P.D., Landry, D., Sznyter, L.A., Waite-Rees, P.,Ives, C.L., Moran, L.S., Slatko, B.E. and Benner, J. S. (1991)Nucl. Acids Res. 19, 841–850.
13. Jack, W.E., Greenough, L., Dorner, L.F.,Xu, S.-y., Strzelecka, T.,Aggarwal A. K., and Schildkraut, I. (1991) Nucleic. Acids Res., 19, 1825–1829.
14. Newman, M.N., Strzelecka, T., Dorner, L.F., Schildkraut, I., and A.K. Aggarwal (submitted).
15. Xu, S.-y., and Schildkraut,I. (1991) J. Biol. Chem., 266, 4425–4429.
16. Elledge SJ; Davis RW. (1989) Genes Dev., 3 , 185–197.
17. Elledge SJ; Sugiono P; Guarente L; Davis RW.(1989) Proc. Natl. Acad. Sci., 86, 3689–3693.
18. Miller,J.H. (1972). Experiments in Molecular Genetics,Cold Spring Harbor Laboratory, Cold Spring Harbor , NY
19. Jacob, F., Sussman, R., and J. Monod (1962) C.R. de l'Acad. des Sciences 254, 4214–4216.
20. Zhou,Y., Zhang X.,and Ebright, R.E.(1991) Nucl. Acids Res. 19, 6052.
21. Thompson, J. and Landy, A. (1988) Nucl. Acids Res. 16, 9687–9705.
22. Taylor, J.D., Badcoe, I.G., Clarke, A.R., Halford, S.E., (1991), Biochemistry, 30, 8743–8753.
23. Zebala, J., Choi, J., Barany, F. (1992), J. Biol. Chem., 267, 8097–8105,
24. Xu,S.-y., and Schildkraut, I. (1993) Biotechniques, 15, 310–315.
25. Pavco, P.A., and Steege, D.A., (1990) J. Biochem, 265, 9960–9969.
26. Herendeen, D.R., Kassavetis, G.A. and Geiduschek, E.P., (1992) Science, 256, 1298–1303.