Selection of sequences recognized by a DNA binding protein using a preparative Southwestern blot

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ABSTRACT

We have tested the feasibility of using a preparative scale Southwestern blot to select DNA sequences specifically recognized by a DNA binding protein. We constructed a library of random 15-mer oligonucleotides and screened it using a preparative Southwestern blot with the transcriptional repressor, PRDI-BF1. Several sequences specifically recognized by PRDI-BF1 were isolated. All of these sequences are similar to PRDI, a regulatory element previously demonstrated to bind PRDI-BF1.

INTRODUCTION

Several methods have been reported for the determination of DNA sequences recognized by a DNA-binding protein. These procedures use immunoprecipitation (1–3), protein affinity columns (4), and gel mobility shifts (5) to select specifically bound sequences from a library of genomic or chemically synthesized DNA. These methods each have limitations. The use of immunoprecipitation requires an antibody that can precipitate the protein of interest when it is bound to DNA, and do so without disrupting the complex. The use of protein affinity columns requires that the protein of interest bind to DNA after it is crosslinked to Sepharose. The use of gel mobility shifts requires advance knowledge of the mobility of the protein-DNA complex during electrophoresis. Furthermore, immunoprecipitation and protein affinity columns do not provide an assay for specific binding between the protein of interest and the selected sequences. Specific DNA binding can be detected using DNase I protection (6), gel mobility shifts (7–8), or Southwestern blotting (9–10).

Southwestern blots have been used to determine the binding specificity of many proteins (11). The nitrocellulose-bound proteins often exhibit a wide range of binding affinities to probes containing different DNA sequences. For example, the transcriptional repressor PRDI-BF1 has been shown by DNase I protection and methylation interference to bind specifically to the PRDI element in the interferon- β gene promoter (12). On a Southwestern blot, PRDI-BF1 binds sequences containing PRDI much more avidly than other sequences (13). We tested the feasibility of using a preparative scale Southwestern blot with

PRDI-BF1 protein to isolate DNA sequences specifically recognized by PRDI-BF1 from a library of random oligonucleotides. We constructed a random 15-mer oligonucleotide library and screened it with PRDI-BF1 using the preparative Southwestern blot method. The same method used for selection was used as a DNA-binding assay to follow the progress of the selection. We isolated several DNA sequences specifically recognized by PRDI-BF1, each of which contained a PRDI-like consensus sequence.

MATERIALS AND METHODS

Bacterial production of PRDI-BF1 protein

A partial cDNA encoding the C-terminal 364 amino acids of PRDI-BF1 was cloned into the phage T7 expression vector (14). Protein was purified by the isolation of inclusion bodies, denaturation, and dialysis according to Gaul et al. (15). The protein was estimated to be ~50% pure as judged by SDS PAGE and staining with Coomassie Brilliant Blue.

Preparative Southwestern blot

Bacterially produced PRDI-BF1 (100 μ g) was electrophoresed on a 10% SDS polyacrylamide gel in a 4cm $\times$ 0.15cm lane and transferred to nitrocellulose in buffer containing 25mM Tris(8.3), 192mM glycine, and 20% methanol at 30 Volts overnight at 4°C. The blots were trimmed and subjected to denaturation/renaturation and incubation with DNA probes according to Vinson et al. (16). A buffer containing 12mM Tris(7.9), 40mM KCl, 0.12mM EDTA, 30 μ M ZnSO₄, and 400 μ M β mercaptoethanol was used for the denaturation, binding, and washing steps. BSA (fraction V) rather than dried milk was used as a blocking agent prior to and during the binding reaction. All binding reactions were carried out at 4°C for 24 hours. The sequences of the PRDI and PRDII oligos are listed below:

PRDI GATCCAAGTGAAAGTGAAAGTGAAAGTGAGATC
PRDII GATCTGTGGGAAATTCCTGGGAAATTCGGATC

For the library screen, 2 μ g of gel purified ζ library inserts were applied to the blots. Washes were performed at room temperature for 30 minutes with several changes of buffer.

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Colony Hybridization

Bacterial colonies were plated on nitrocellulose paper. After growth of colonies, filters were denatured, renatured, and baked according to Maniatis et al. (17). They were then soaked 3 times in $3\times\text{SSC}$, 0.5% SDS for 20' at 65°C to remove excess baked-on bacteria. Filters were then placed in prehybridization solution at 60°C for 2 hours and hybridized overnight with probe at 60°C. The filters were then washed in $2\times\text{SSC}$ for 30' at 37°C. The prehybridization and hybridization buffer contained $6\times\text{SSC}$, $5\times\text{Denhardt's}$, 2 $\mu\text{g/ml}$ salmon sperm DNA, 0.2% SDS, 10mM phosphate buffer pH7, 1mM EDTA, and 6×10^6 counts/ml probe. PRDI and PRDII probes were made from PRDI and PRDII oligonucleotides (sequences given above) which were ligated to 200–500bp lengths and nick-translated. The two probes were initially tested to ensure that they did not cross react.

Preparation of ζ 15-mer oligonucleotide library

(See also text and Figure 1.) Two oligos were synthesized and hybridized according to Maniatis et al. (17). One of the oligos, the template, has 15 positions which contain 25% of each of the four nucleotides. The other oligo was made to serve as a primer. The two oligos were then hybridized, made completely double stranded by primer extension with Klenow, and cleaved with BamHI and EcoRI. These inserts were ligated to a pUC vector and electroporated into HB101 bacteria. The electroporation was performed using the Gene Pulser apparatus (BioRad) with settings of 25 μF , 2.5kV, and 200 Ω . The bacteria were grown to log phase, pelleted, and washed twice with water prior to electroporation (Bio-Rad Bacterial Electro-Transformation and Pulse Controller Instruction Manual, Version 1.0).

Southwestern blot binding assay

When the Southwestern blot was used as an assay for sequence specific binding, only 1–10 μg of bacterially produced PRDI-BF1 was used. Probes were prepared from the isolated ζ inserts by filling in the ends of $\sim 50\text{ng}$ of gel-purified fragment with Klenow using ^{32}P -labeled nucleotides. Equal counts of each probe were used.

Methylation interference

End-labeled fragments were treated lightly with DMS and incubated with $\sim 75\text{ng}$ of bacterially produced PRDI-BF1 for 20' at room temperature in a buffer containing 13mM Tris(7.9), 60mM KCl, 12.5mM NaCl, 12% glycerol, and 75 $\mu\text{g/ml}$ polyd(I)-polyd(C). Bound complexes were separated from unbound probes by electrophoresis on a low ionic strength 4.5% nondenaturing gel at 10V/cm at room temperature (7–8, 18). Bands were cut out of the gel, the DNA eluted from each band in TE, phenol

extracted, and ethanol precipitated. DNA was then cleaved at the methylated guanosine residues by treatment with piperidine and resolved on a sequencing gel (19).

Calculations

$$\begin{aligned}\text{Complexity of random n-mer} &= 4^n/2 \\ \text{complexity of 14-mer} &= 1.3 \times 10^8 \\ \text{complexity of 15-mer} &= 5.4 \times 10^8 \\ \text{Enrichment (e) over q rounds} &= e^q \\ 60\text{-fold enrichment over 5 rounds} &= (60)^5 = 7.8 \times 10^8\end{aligned}$$

RESULTS

The protein PRDI-BF1 has five zinc finger domains and binds specifically to the PRDI element of the interferon- β gene promoter. A bacterially produced partial PRDI-BF1 peptide which contains all five PRDI-BF1 zinc fingers, protects the PRDI element from cleavage by DNase I (12). The partial PRDI-BF1 peptide also exhibits sequence specific binding on a Southwestern blot (10, 16). For example, nitrocellulose-immobilized PRDI-BF1 binds to probes containing the PRDI motif, GAGAAGTG-AAAGTG, with ~ 100 -fold higher affinity than to probes containing the adjacent PRDII motif in the interferon- β promoter, GTGGGAAATTCC (13). We took advantage of the differential binding affinity of PRDI-BF1 to PRDI and PRDII to test the feasibility of using a preparative Southwestern blot to select specifically recognized DNA sequences.

The Southwestern blot with PRDI-BF1 was scaled up to a preparative level. One hundred micrograms of bacterially produced partial PRDI-BF1 protein ($\sim 50\%$ pure) was loaded onto each of two 4cm $\times$ 0.15cm lanes of an SDS polyacrylamide gel, electrophoresed alongside molecular weight markers, and transferred to nitrocellulose paper. The resulting nitrocellulose blots were trimmed of regions not containing PRDI-BF1 protein. One of these blots was incubated with 1.5ng of a 'specific' PRDI oligo, and the other was incubated with 1.5ng of a 'non-specific' PRDII oligo. Both the PRDI and PRDII oligos had BamHI/BglII ends to facilitate subsequent cloning. After washing to remove non-specifically bound probe, the bound oligos were removed from the blots by phenol extraction, precipitated, ligated to a pUC vector, and transformed into bacteria. The number of resulting colonies should be roughly proportional to the number of oligos bound to PRDI-BF1 on the blots. As a control, 1.5ng of the PRDI and PRDII oligos were independently ligated to the pUC vector without incubation with PRDI-BF1.

As shown in Table 1, many more specific than non-specific oligos were bound by nitrocellulose-immobilized PRDI-BF1. The retained PRDI oligos, when ligated to pUC and transformed

Table 1. Selectivity of PRDI-BF1 in a preparative Southwestern blot.

Oligo	Colonies resulting from selected oligos	Colonies resulting from unselected oligos	% Retention	Sensitivity (fg/colony)
PRDI	20,410	35,450	58	73
PRDII	170	24,450	0.7	8823

PRDI oligo or PRDII oligo (1.5ng) were incubated with nitrocellulose-immobilized PRDI-BF1. After washing, the bound oligos were recovered and cloned into pUC. The number of resulting transformants was determined. The percent retention was calculated by normalizing the number of colonies resulting from cloning the selected oligos to the number resulting from cloning unselected oligos. The sensitivity was calculated as the amount of oligo applied to the blot divided by the number of colonies resulting from the bound oligos.

into bacteria, resulted in 20,410 colonies. In contrast, the retained PRDII oligos resulted in only 170 colonies. Each of these numbers was normalized to the number of colonies resulting from ligating 1.5ng of the unselected oligo to pUC and transforming the products into bacteria. Whereas 58% of the PRDI oligos in the binding reaction were retained by the immobilized PRDI-BF1, only 0.7% of the PRDII oligos were retained, a difference of over 80-fold. Furthermore, these results indicate that 73fg of specific PRDI oligo need be applied to the blot in order that at least one bound oligo can be recovered. This amount could be decreased two orders of magnitude by using electroporation rather than calcium phosphate for transforming the ligation products into bacteria (data not shown)(20–21). Therefore, we calculate that incubation of nitrocellulose-immobilized PRDI-BF1 with only 400ng of a random sequence oligo of similar length to PRDI should ensure the recovery of every possible 15-mer sequence recognized by PRDI-BF1.

As a further test of the feasibility of using a preparative Southwestern blot to screen a random oligonucleotide library, nitrocellulose-immobilized PRDI-BF1 was simultaneously incubated with both specific and non-specific oligos. In particular, 5pg of specific PRDI oligos and 100ng of non-specific PRDII oligos were mixed together, a ratio of 1:20,000, and incubated with nitrocellulose-immobilized PRDI-BF1. Two consecutive rounds of binding and recovery were conducted with the mixed oligos. After each round, a portion of the recovered oligos was ligated to pUC, transformed into bacteria, and assayed by colony hybridization with PRDI and PRDII probes separately. This allows the determination of the ratio of the PRDI and PRDII oligos in the bound population. Table 2 shows that after the first round of selection, PRDI oligos were enriched 40-fold to a representation of 1:500. After the second round of selection, PRDI oligos were enriched an additional 80-fold to a representation of 1:6. Thus an average 60-fold enrichment is achieved with each binding/recovery round. This level of enrichment agrees well with the previously observed 80-fold difference in binding affinity of nitrocellulose-immobilized PRDI-BF1 to the PRDI and PRDII oligos. Therefore, a Southwestern blot with PRDI-BF1 can enrich for specifically bound sequences from a mix containing excess non-specific sequences.

In a random 15-mer oligonucleotide library, each 15-mer sequence is represented at a frequency of $1:5.4 \times 10^8$. Since a ~60-fold enrichment for specific sequences is achieved in each binding/recovery round with nitrocellulose-immobilized PRDI-BF1, five rounds of selection should be sufficient to enrich for any specifically bound 15-mer sequence. However, since the

recovery per round of the PRDI oligo was 58%, five sequential rounds of selection may result in significant recovery loss of specifically bound 15-mer sequences. In order to compensate for the compounded loss of specifically bound sequences during multiple rounds of selection, an increased amount of random 15-mer library oligos must be incubated with the preparative Southwestern blot at the first round.

A random 15-mer oligonucleotide library was constructed from two synthesized single-stranded DNA molecules. The first contained 15 degenerate positions flanked by the restriction sites BamHI and EcoRI. The second synthesized DNA molecule was complementary to the 3' end of the first to function as a primer. Figure 1 shows that the two synthesized DNA molecules were annealed and made completely double-stranded by primer extension. The products were cleaved with BamHI and EcoRI, and cloned into pUC for amplification. Electroporation was used to achieve the highest possible titer of random 15-mer oligos. The ζ library had a titer of 2×10^8 , just short of 5.4×10^8 which is necessary to ensure the representation of every 15-mer. The ζ library thus contains every possible 14-mer sequence.

The amplified ζ library was screened by nitrocellulose-immobilized PRDI-BF1 using two different methods. In the first method shown in Figure 2A, random 15-mer inserts are excised from the ζ library by cleavage with BamHI and EcoRI, subjected to two rounds of binding and recovery with nitrocellulose-immobilized PRDI-BF1, and cloned back into pUC for amplification. The recovered sequences are then radiolabeled and assayed for specific binding to PRDI-BF1 on a Southwestern blot. If no binding to PRDI-BF1 is detected, the amplified sequences

Table 2. Enrichment of the PRDI oligo in preparative Southwestern blot selection with PRDI-BF1.

	Ratio PRDI oligo : PRDII oligo	PRDI oligo enrichment / round
No Selection	1:20,000	---
Round 1 PRDI-BF1 Selection	1:500	40x
Round 2 PRDI-BF1 Selection	1:6	80x

The ratio of PRDI to PRDII oligos and the enrichment for the PRDI oligo after no round, one round, and two rounds of selection are listed. The ratio of the two oligos was determined by cloning a portion of the recovered sequences after each step of selection and assaying the resulting transformants by colony hybridization with a PRDI and PRDII probe separately.

Figure 1. Construction of the ζ random 15-mer library. (see text for description)

Figure 2. Methods for using a preparative Southwestern blot to screen the ζ random 15-mer library. **A.** Excision of ζ library inserts with BamHI and EcoRI; **B.** Excision of ζ library inserts with PstI and EcoRI. (see text for description)

are re-excised by cleavage with BamHI and EcoRI and subjected to additional rounds of selection with the preparative Southwestern blot. If specific binding is detected, individual clones are isolated, sequenced, and assayed for binding to PRDI-BF1. This method requires as many as three amplification steps to compensate for the recovery loss of specifically bound oligos during each round of selection. Because not all sequences are replicated in bacteria with equal efficiencies, amplification will alter the distribution of sequences in the sample. At worst, some sequences will be lost.

The second method for screening the ζ library avoids amplification until the last step. Random 15-mer inserts are excised by cleavage with PstI and EcoRI so that they contain the BamHI site from the pUC polylinker. This enables the recovery of rare bound sequences. Figure 2B shows that the ζ library inserts are subjected to many successive binding/recovery rounds with nitrocellulose-immobilized PRDI-BF1, and then cloned into a pUC vector whose BamHI site has been destroyed. After amplification, very small amounts of recombinant plasmids containing bound ζ library sequences can be selected above the vector background by excision with BamHI and EcoRI. The excised inserts are then cloned into pUC for assay. This method

requires that the ζ library inserts be cleaved with PstI, as well as with BamHI and EcoRI. Therefore, all sequences containing a PstI site will be lost.

Both methods for screening the ζ library with PRDI-BF1 on a preparative Southwestern blot were successfully employed. For the first method, 2 μ g of ζ BamHI-EcoRI random 15-mer inserts were incubated with nitrocellulose-immobilized PRDI-BF1 through six binding/recovery rounds, with amplification after every other round. Figure 3A(i) shows that when unselected ζ inserts were labeled and incubated with immobilized PRDI-BF1 on a Southwestern blot, little binding was evident. However, when the selected inserts, PRDI-BF1(ζ), were used as probe, strong binding was observed. Fifteen individual clones of the PRDI-BF1(ζ) pool were isolated and sequenced. Several of them were found to be identical to each other. Independent clones were tested for sequence specific binding to PRDI-BF1 on a Southwestern blot. Figure 3A(ii) shows the results for three of the clones. One can see that little of the radiolabelled PRDI-BF1(ζ)₁ probe was retained by the nitrocellulose-immobilized PRDI-BF1. This clone, and 40% of the total isolated, was a 'false positive'. In contrast, the other two sequences, PRDI-BF1(ζ)₂ and PRDI-BF1(ζ)₃, exhibited strong specific binding to PRDI-

Figure 3. Isolation of sequences specifically recognized by PRDI-BF1. **A.** Southwestern blot with PRDI-BF1 probed with (i) unselected inserts, ζ, or inserts selected by PRDI-BF1 on the preparative Southwestern blot, PRDI-BF1(ζ); or probed with (ii) individual isolates from the PRDI-BF1(ζ) mix: PRDI-BF1(ζ)₁, PRDI-BF1(ζ)₂, and PRDI-BF1(ζ)₃. **B.** Sequences of the selected ζ inserts and other known PRDI-BF1 binding sites. Each insert is flanked by a BamHI site at one end and an EcoRI site at the other. The minimal PRDI consensus sequence is underlined. For PRDI-BF1(ζ)₃ and PRDI-BF1(ζ)₄, guanines, which when methylated interfere with binding to PRDI-BF1, are indicated by a dot above them.

BF1. In the second method for screening the ζ library, 2 μg of ζ PstI-EcoRI random 15-mer inserts were subjected to five rounds of selection. The selected sequences were recovered, amplified, and re-excised by cleavage with BamHI and EcoRI. This led to the isolation of three additional sequences specifically bound by PRDI-BF1, PRDI-BF1(ζ)₄, PRDI-BF1(ζ)₇, and PRDI-BF1(ζ)₉.

Figure 3B shows the five sequences selected by PRDI-BF1 from the ζ library, as well as the sequences of two known PRDI-BF1 binding sites, the PRDI element of the interferon-β gene promoter (22–23), and the hexamer repeat (24). These sequences each contain a close variant of the motif AGTGAAGTG, part of the larger PRDI element previously defined. The PRDI-BF1(ζ)₃ sequence is remarkably similar to the interferon-β gene promoter, identical to it at 15/16 nucleotide positions. This sample

of sequences illustrated in Figure 3B is not large, but nevertheless suggests that the AGTGAAGTG motif is a consensus sequence recognized by PRDI-BF1. Four of the selected ζ sequences are longer than 15 basepairs. It is possible that the single-stranded DNA molecule with 15 degenerate positions used to construct the ζ library had significant length heterogeneity. Alternatively, some of the random 15-mer oligos may have imperfectly hybridized with each other during primer extension.

Methylation interference studies (19) were performed on PRDI-BF1 bound to two of the selected ζ library sequences, PRDI-BF1(ζ)₃ and PRDI-BF1(ζ)₄. The guanines which, when methylated, disrupt binding between PRDI-BF1 and the PRDI element of the interferon-β gene promoter have been previously determined to be at positions –72, –70, and –66 (12). They are indicated as dots above the sequence in Figure 3B. Methylation at the equivalent guanines in the selected ζ library sequences was found to interfere with binding to PRDI-BF1. Interestingly, the PRDI-BF1(ζ)₄ sequence has a guanine instead of an adenine at the equivalent of position –73 in the interferon-β gene promoter. Methylation at this guanine, in addition to the other three, interfered with binding to PRDI-BF1 (see Figure 3B).

DISCUSSION

We have developed a new preparative Southwestern blot method to identify nucleotide sequences specifically recognized by a DNA binding protein. The protein is immobilized on nitrocellulose and incubated with a random sequence oligonucleotide library. After several rounds of binding, washing, and recovery, the selected oligos are sequenced and assayed for specific binding to the protein. The preparative Southwestern blot method should be applicable to any protein that is capable of binding to DNA following denaturation and renaturation. This should include many proteins that are identified in λgt11 expression screens on the basis of their binding to a DNA probe.

The preparative Southwestern blot method was applied to the zinc finger protein, PRDI-BF1. A random oligonucleotide library with sufficient complexity to include every possible 14-mer sequence was screened with nitrocellulose-immobilized protein. Five sequences containing a close variant of the sequence, AGTGAAGTG were isolated. This consensus sequence is part of the previously defined PRDI element (22), GAGAAGTGAAGTG, present in the interferon-β gene promoter and originally used to isolate the PRDI-BF1 cDNA clone from a λgt11 library (12). Methylation interference studies confirmed that two of the selected library sequences are contacted by PRDI-BF1 in a manner similar to the PRDI element.

Since the enrichment per round of selection using the preparative Southwestern blot was ~60-fold and the number of false positives significant, several rounds of selection were required before individual positive clones could be isolated. As a result, this method likely identifies only the highest affinity binding sites. The fact that all five sequences isolated from the unbiased ζ library of random oligonucleotides contain PRDI-like sequences suggests that PRDI is a high affinity target for PRDI-BF1. Particularly striking is the isolation of one sequence having 15/16 identical bases with the interferon-β gene promoter. The identical region extends from promoter positions –77 to –62 and includes all of the PRDI element and five nucleotides at the 5' end of the PRDI element. This coincides remarkably well with the region of the interferon-β gene promoter from –79 to

–61 that is protected from DNase I digestion upon binding to PRDI-BF1 (12). If the selectivity of PRDI-BF1 is the same in vivo as it is in vitro, then the interferon- β gene promoter is likely to be one of the true targets of PRDI-BF1 in the cell.

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