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Target Detection Assay (TDA): a versatile procedure to determine DNA binding sites as demonstrated on SP1 protein

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ABSTRACT

We developed a rapid method designated Target Detection Assay (TDA) to determine DNA binding sites for putative DNA binding proteins. A purified, functionally active DNA binding protein and a pool of random double-stranded oligonucleotides harbouring PCR primer sites at each end are included the TDA cycle which consists of four separate steps: a DNA protein incubation step, a protein DNA complex separation step , a DNA elution step and a polymerase chain reaction (PCR) DNA amplification step. The stringency of selection can be increased in consecutive TDA cycles. Since tiny amounts of retained DNA can be rescued by PCR, buffer systems, salt concentrations and competitor DNA contents can be varied in order to determine high affinity binding sites for the protein of choice. To test the efficiency of the TDA procedure potential DNA binding sites were selected by the DNA binding protein SP1 from a pool of oligonucleotides with random nucleotides at 12 positions. Target sites selected by recombinant SP1 closely matched the SP1 consensus site. If DNA recognition sites have to be determined for known, mutated or putative DNA binding proteins, the Target Detection Assay (TDA) is a versatile and rapid technique for consideration.

INTRODUCTION

Differentiation is thought to be a process determined by genetic programming of individual cells through the differential regulation of transcription initiation. With the analysis of DNA binding proteins it has become clear that the transcription of genes is regulated by DNA-protein interactions and depends on the interplay of common and cell type/ lineage specific DNA binding proteins. In recent years a large number of DNA binding proteins have been cloned based on evolutionary conserved homologies in their DNA binding domains, e.g. homeo domains(1−6), pou-boxes (7−9), paired-boxes (10−12) and zinc (II) finger regions (13−19). However, the DNA sequences to which the various domains bind have been determined for only a few proteins (20−26). Recently, we cloned more than thirty genes encoding zinc finger domains from human T cell cDNA libraries. Some of these genes named Kox 1-30 are thought to be involved in regulating tissue specific gene expression (27).

We designed the Target Detection Assay (TDA) in order to determine DNA recognition sites for these zinc finger proteins. The method requires a pool of randomized oligonucleotides and a functionally active DNA binding protein. To test the applicability of the TDA procedure, we used FPLC purified, recombinant SP1 as a model protein. Template oligonucleotides were chemically synthesized harbouring random nucleotides at N positions surrounded by specific sequences with desired restriction sites on each end. Two primers (5′, primer A and 3′, primer B) complementary to sequences at the ends of the template oligonucleotides were used for PCR amplification (28).

Here, we describe how these double-stranded oligonucleotides containing random nucleotides can be used for determining binding sites of human transcription factor SP1.

MATERIAL AND METHODS

Expression and purification of SP1 protein

The Bam H1−Bgl II fragment derived from $pP_{ac}SP1$-516c (29) encoding three zinc fingers of the human transcription factor SP1 (30) were cloned into the Bam H1 site of pAR 3039 (31) to yield pAR Sp1-516c. Expression of SP1 was induced by the addition of IPTG (0.125mg/ml) to exponentially growing cells followed by a 3 hrs incubation shaking at 37°C. The SP1 E.coli pellet obtained from 800 ml cultures was resuspended in 50 ml buffer I (50 mM NaCl, 20 mM Tris-HCl pH 8.0, 1% Trition X-100), sonicated for 10 min, pelleted (10000 RPM/10min/4°C) and washed consecutively with 20ml buffer II (1 M NaCl, 20 mM Tris-HCl), with 20 ml buffer III (3 M NaCl, 20 mM Tris-HCl) and with 20 ml H_2O. The remaining pellet was dissolved in buffer IV(8M Urea, 0.5 M NaCl, 20 mM Tris-HCl pH 8.0), centrifuged (10000 RPM/10 min/4°C) and the supernatant frozen at −70°C degrees (20ml of the supernatant was analysed on 15% polyacrylamide-SDS gel). Supernatant (5 ml aliquots) was filtered (0.22 micron), diluted 1:10 in buffer A (8 M Urea, 20 mM MES pH 6.0, 3 mM DTT, 2 mM EDTA) and applied to FPLC Mono S. Bound proteins were eluted by a linear gradient (0% −40%) of buffer B (1 M NaCL, 8 M Urea, 20 mM MES pH 6.0, 3 mM DTT, 2 mM EDTA). Peak fractions were collected and

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analysed on 15% polyacrylamide-SDS gel. Fractions containing Spl protein were pooled and the protein concentration was determined to be 500 ng/μl by the method of Lowry.

PCR oligonucleotides

Template oligonucleotides and corresponding primers were synthesized on 380A Applied Biosystems DNA Synthesizer. Two template oligonucleotides were designed and synthesized: template A that contains two consensus binding sites for human transcription factor SP1 and template B that contains N randomized oligonucleotides at 12 positions flanked by specific sequences of 22 nucleotides at each end. In addition, two primer of 22 nucleotides (5′ primer and 3′primer) were synthesized for generating and rescuing double-stranded oligonucleotide mixes by PCR amplification. PCR amplification was done using amplification kit (Perkin Elmer Cetus) with denaturation 93°C, 30 sec; annealing 45°C, 2 min; temperature shift 45°C−67°C, 1min; extension 3 min, 67°C; cycles 25×. The oligonucleotide mix was extracted by phenol/chloroform , purified from a 6% polyacrylamide gel using ElutipD (Schleicher&Schüll). Oligonucleotides were dissolved in 30 μl T.E. (10 μl are used in one filter binding assay). Filter eluted oligonucleotides were amplified by using 1 μl of eluted material (200 μl).

Filterbinding

Double stranded oligonucleotide mixes (10 μl) were incubated with recombinant SP1 (1mg) in Tris-HCl binding buffer (200 μl, 9mM Tris-HCl pH 8.0, 90 mM KCl, 90 μM $ZnSO_4$) for 30 min. Nitrocellulose filters (BA85, Schleicher&Schüll) were inserted in a suction chamber (32) and prewet with 600 ml Tris-HCl binding buffer. SP1 protein-oligonucleotide and albumin-oligonucleotide mixes were applied to nitrocellulose filters with gentle suction and the filters washed with 4 ml Tris-HCl binding buffer. In the first three TDA cycles, randomized oligonucleotides retained on the filter were eluted with 200 μl Tris-HCl binding buffer substituted with 1 mM 1−10-o-phenanthroline. In the fourth TDA cycle the DNA was eluted as before, then with 1mM 1−10-o-phenanthroline and 500 mM KCl and finally with 10 mM phenanthroline and 500 mM KCl. The DNA of the last fraction was subjected to a fifth TDA cycle in the presence of 500 mM KCl and eluted with 10 mM phenanthroline and 500 mM KCl.

Band shift electrophoresis

Bandshift gels containing 0.25% TBE and 6% polyacrylamide (30% acrylamide/0.8% bisacrylamide) were prerun for 30 min. Optimal protein concentrations were determined titrating SP1 protein. Good gel-shift activities were obtained when the SP1 protein was diluted in MES buffer, substituted with 200 mM NaCl in the absence of urea. In a standard band shift, 10ng protein were added to the band shift mix (30 μl) containing 100 mM NaCl, 10 mM Tris-HCl pH 8.0, 1 mM EDTA, 100 μM $ZnSO_4$, 10% glycerol, labelled oligonucleotides (40000−100000 cpm Cerenkoff) and competitor DNA (poly dA/dT or poly dI/dC). The gels were run at 10 V/cm for 3 hrs, dried onto Whatman paper and exposed to Kodak XAR between 3hrs and 24hrs. Oligonucleotides derived from the fifth TDA cycle were labelled and subjected to band shift electrophoresis. Retarded oligonucleotides were rescued by PCR and cloned.

Cloning

The oligonucleotides derived from nitrocellulose filter binding or from band shift electrophoresis gels were eluted, amplified, phosphorylated with T4 polynucleotide kinase and cloned into the dephosphorylated Eco RV site of Bluescript plasmid. The transformed host strain JM-103 was plated on LB/ampicilline plates containing IPTG and x-gal. White colonies were picked and inserted oligonucleotides obtained using PCR conditions from above. The oligonucleotides were isolated from 6% polyacrylamide gels and concentrated by Elutip D. The nucleotides were resuspended in 20 μl T.E. For band shift assays, 1 μl of the oligonucleotides were kinased with 100 μCi $\gamma^{32}P$ ATP or internally labelled using 20 μCi $\alpha^{32}P$ dCTP during one PCR cycle with an extension time of 10 min.

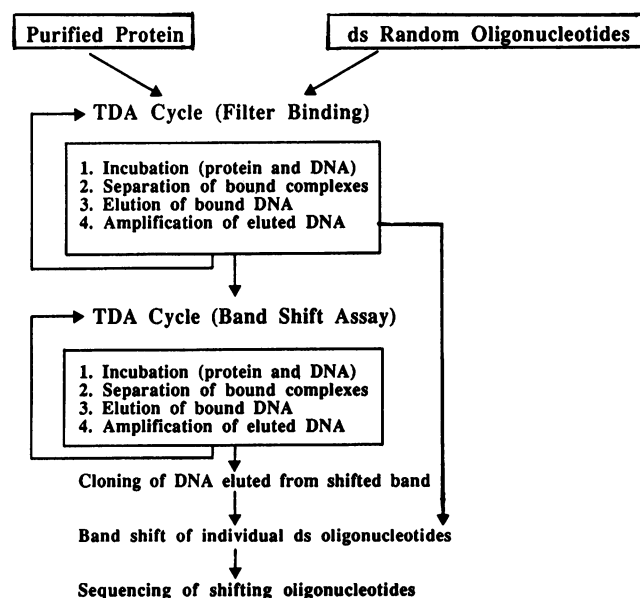

Figure 1. Target Detection Assay (TDA): The target detection assay (TDA) requires a purified functionally active DNA binding protein and a pool of random oligonucleotides. The TDA cycle describes the core procedure of this technique. Filter binding is performed during the first TDA cycles. Selected oligonucleotides can be cloned or subjected to further TDA cycles using band shift electrophoresis. Finally, the TDA selected oligonucleotides are cloned, analysed and sequenced.

Sequencing

Oligonucleotides which were selected by positive band shift were sequenced using double-stranded miniprep DNA according the manufacturer's protocol (deaza-Kit, Pharmacia).

RESULTS

Double stranded randomized oligonucleotides generated by PCR

Recently, we demonstrated that mutagenesis cassettes can be generated by synthesis of a template oligonucleotide harbouring random nucleotides at N positions surrounded by specific nucleotides for PCR amplification (28) We indicated that these oligonucleotides could be used in studying protein DNA interactions. In order to evaluate the TDA procedure outlined in Figure 1, we designed template oligonucleotides containing two consensus SP1 binding sites (Figure 2, template A) or random nucleotides at 12 positions (Figure 2, template B) The template oligonucleotides with specific nucleotides at each end (Figure 2) were made double-stranded by PCR and gel purified.

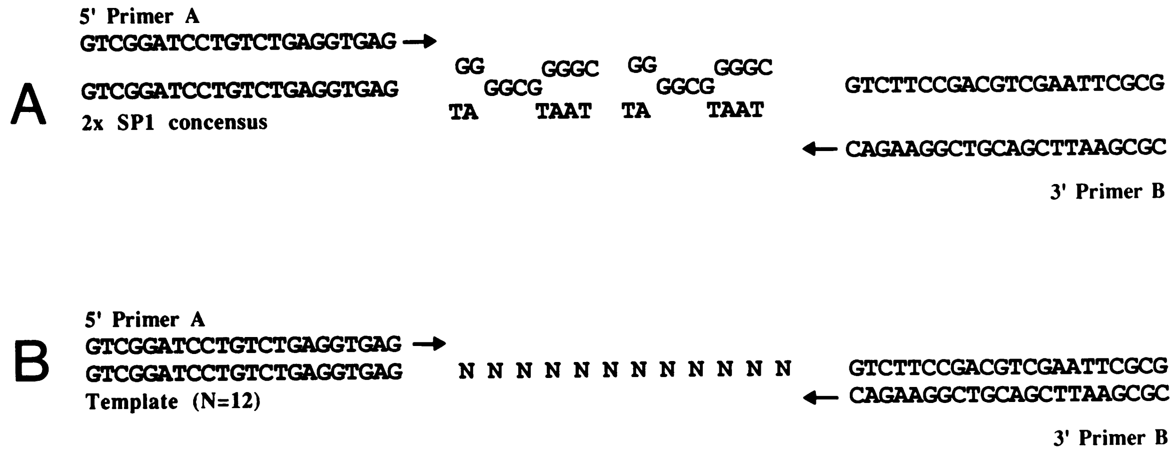

Figure 2. PCR oligonucleotides: Four oligonucleotides, two template oligonucleotide and two primers, were synthesized on Applied Biosystems DNA Synthesizer. The template A oligonucleotide was designed to contain two consecutive SP1 consensus binding sites and template B to contain random oligonucleotides at 12 positions both flanked by specific sequences of 22 nucleotides at each end. Primers of 22 nucleotides (5′ primer A and 3′primer B) complementary to these ends are used for generation of double-stranded oligonucleotide mixes by PCR amplification.

DNA binding of FPLC purified recombinant SP1 protein

We cloned and expressed SP1 protein using the expression plasmid pAR SP1-516c in order to use its sequence-specific DNA binding in the Target Detection Assay (TDA). Recombinant SP1 was finally purified using Mono S cation-exchange chromatography (Pharmacia). Double-stranded oligonucleotides harbouring two SP1 binding sites (Figure 2, template A) were used to assess the DNA binding activity of recombinant SP1 by filter binding and band shift electrophoresis. The SP1 consensus oligonucleotides incubated respectively with SP1 protein and albumin were selectively retained on a nitrocellulose filter (Figure 3). SP1 protein from the pooled MonoS fractions regained its biological activity when diluted in the filter binding buffer. Sequence analysis of oligonucleotides that were retarded by band shift electrophoresis demonstrated that individual oligonucleotides derived from the Sp1 consensus mixture were recognized by recombinant Sp1, could be rescued by PCR amplification and subsequently cloned (data not shown).

Selection of SP1 binding sites by TDA

We generated double-stranded oligonucleotides harbouring random nucleotides at 12 positions (Figure 2, template B) to test whether SP1 binding sites can be selected from a pool of random oligonucleotides. The procedure of the TDA cycle and its processing steps are illustrated in Figure 1. The DNA protein complex was retained by nitrocellulose filter binding. To enrich for DNA binding that depends on the presence of zinc in the SP1 finger domains, DNA was recovered using 1 mM 1-10-o-phenanthroline, a zinc-chelating reagent. During the fourth TDA cycle, the bound DNA was eluted a second time with 500 mM KCl in the presence of 1 mM phenanthroline. Of fifteen inserts screened, only three gave a reasonable band shift (data not shown). In order to increase the efficiency of cloning high affinity binding oligonucleotides, we washed the nitrocellulose filter a third time with 10 mM phenanthroline and 500 mM KCl and did a fifth TDA cycle in presence of 500 mM KCl. After adding one band shift electrophoresis step the retarded oligonucleotides were cloned. This modification resulted in seventeen out of twenty cloned oligonucleotide fragments giving reasonable band shifts in comparison to three out of fifteen. With increasing exposure

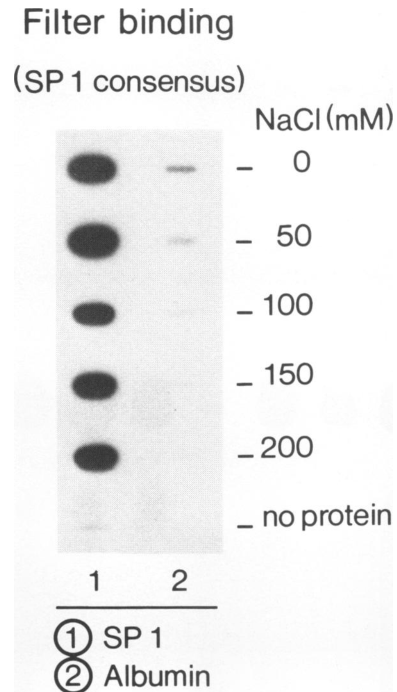

Figure 3. Nitrocellulose filter binding using recombinant SP1 protein. Double-stranded oligonucleotides harbouring two SP1 binding sites were labelled by T4 polynucleotide kinase and incubated with recombinant SP1 (500 ng) or albumin (500 ng). Filter binding studies were performed in presence of increasing salt concentrations.

time even nineteen out of twenty double-stranded oligonucleotides showed binding to SP1 protein (data not shown).

SP1 binding studies to individual oligonucleotides:

The affinity of several target sites was studied in the presence of competitor DNA in band shift electrophoresis. Binding of three oligonucleotides with different affinities to SP1 protein is demonstrated in Figure 4, 5 and 6. The binding of oligonucleotides 5q and S3 to SP1 protein is still detectable with poly dA/dT concentrations of more than 2 μg. In the case of oligonucleotide S2 a ten fold stronger competition is observed. Poly dI/dC concentrations of 20 ng already show total competition in binding to oligonucleotides 5q and S2 while retarded S3 can still be detected. The higher sensitivity to competition with polydI/dC indicates that the selected oligonucleotides probably resemble binding sites with higher similarity to poly dI/dC than to poly dA/dT. In addition, these competition experiments reveal that oligonucleotide S3 has a higher affinity to SP1 protein than S2 oligonucleotide. Oligonucleotide 5 q has an intermediate affinity to SP1 protein in comparison to S2 and S3. Oligonucleotides 12t and 13q showed lower affinities than 5q to SP1 protein (data not shown). Though the recombinant SP1 is truncated at the N-terminal end, full length SP1 and truncated SP1 proteins produce similar band shifts (data not shown).

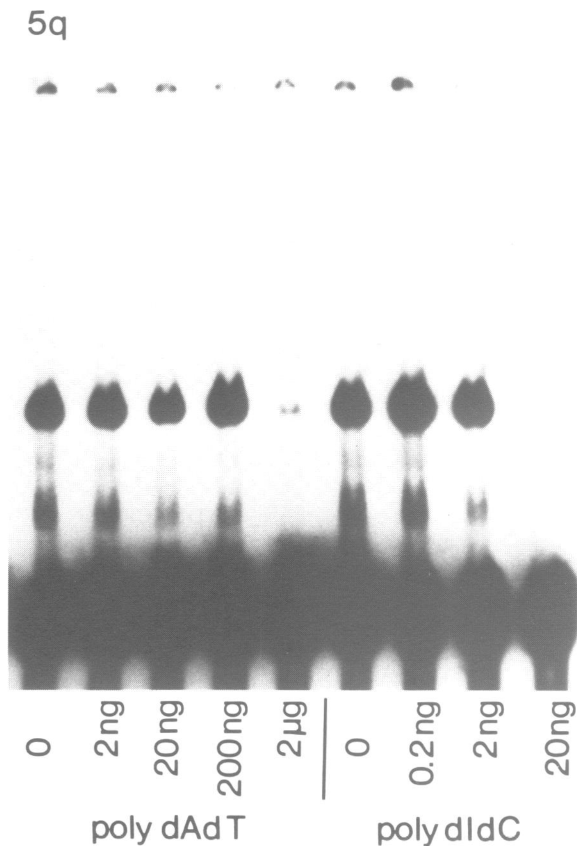

Figure 4. Binding to oligonucleotide 5q was tested in presence of competitior poly dA/dT and poly dI/dC. Gel from band shift electrophoresis was exposed for 12 hrs. Standard band shift conditions were used with or without competitor as indicated.

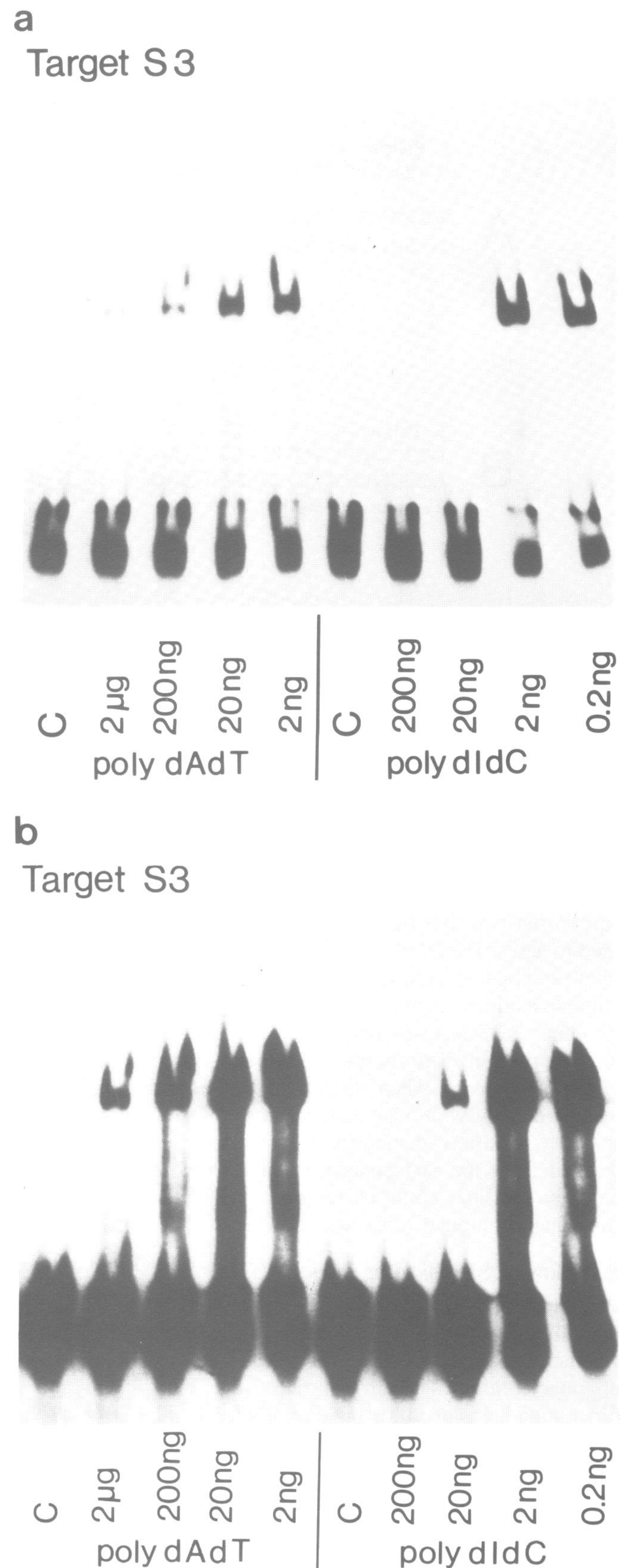

Figure 5a and 5b. Oligonucleotide S3 was tested in the presence of competitior poly dA/dT and poly dI/dC. Gel from band shift electrophoresis of S3 was exposed for 90 min (Figure 5a) and for 12 hours (Figure 5b). Standard band shift assay conditions were used; poly dA/dT and poly dI/dC (Pharmacia) were added as competitors. Reaction mixes without protein are in lane C.

Panel of SP1 binding sites

Several SP1 binding sites were sequenced and aligned (Figure 7). This analysis indicated that the sequences are quite homologous to each other. Furthermore, all binding sites have a high homology to the SP1 concensus binding sites determined previously (33,34). The consensus derived from 11 putative SP1 binding sites reflects the common SP1 consensus GGGGCGGGGC except for the last residue (Figure 7). Some of these binding sites only differ at one position such as 12T, S10 and S16 in comparison to the general SP1 consensus. Applying the TDA method to SP1 protein we generated a panel of DNA binding sites which can now be studied and compared by further analysis. It seems worth asking what nucleotide changes within the consensus site have considerable and what nucleotide changes have less dramatic effects on the affinity of SP1 protein binding to their putative target sites. An analysis of how well a particular nucleotide is conserved will give a measure of its importance for protein binding. For example, the higher affinities of 5q and S3 in comparison to 12t and 13q might be due to the base change from G to C in the SP1 core element GGCG characterized by Letovsky and Dynan (35).

DISCUSSION

The TDA procedure

We describe a rapid and versatile method designated Target Detection Assay (TDA) which can be used to determine target sequences for putative DNA binding proteins. The method can be divided into several sections. The general scheme is outlined in Figure 1. The key to the method is to expose a putative DNA binding protein to an oligonucleotide mix habouring potential DNA binding sites. Depending on the size of the putative DNA binding site, oligonucleotides with an appropriate number of random nucleotides have to be synthesized. Random nucleotides at N positions generate a maximum of 4^N sequences. However, 4^N may be larger than the number of molecules synthesized. If a template has random nucleotides at 23 positions and a length of 100 residues, each template oligonucleotide should be theoretically represented in a conventional oligonucleotide synthesis (36). In turn, the complexity of mixes can be reduced, for example by generating only sequences having a consensus in common (Figure 2, template A).

The TDA cycle is the core technique. Mixtures of randomized oligonucleotides are bound to purified DNA binding proteins, bound complexes are separated, enriched oligonucleotides are subjected to PCR amplifications and reapplied to further rounds of selection. The selectivitiy of each round can be increased by varying the salt concentrations of the binding reaction. It should be noted that optimal binding conditions have to be established empirically for each binding protein by changing salt concentrations and buffer systems to have appropriate pH values. The oligonucleotides can be amplified at any step of the TDA cycle. Since DNA binding proteins have general affinities to DNA, the binding characteristics between non-specific DNA and specific DNA can vary between a few folds and some orders of magnitude. The stringency of binding can be increased in consecutive TDA cycles in order to select for high affinity binding sites. In addition, the DNA retained on the nitrocellulose filter can be eluted stepwise in order to enrich for high affinity binding

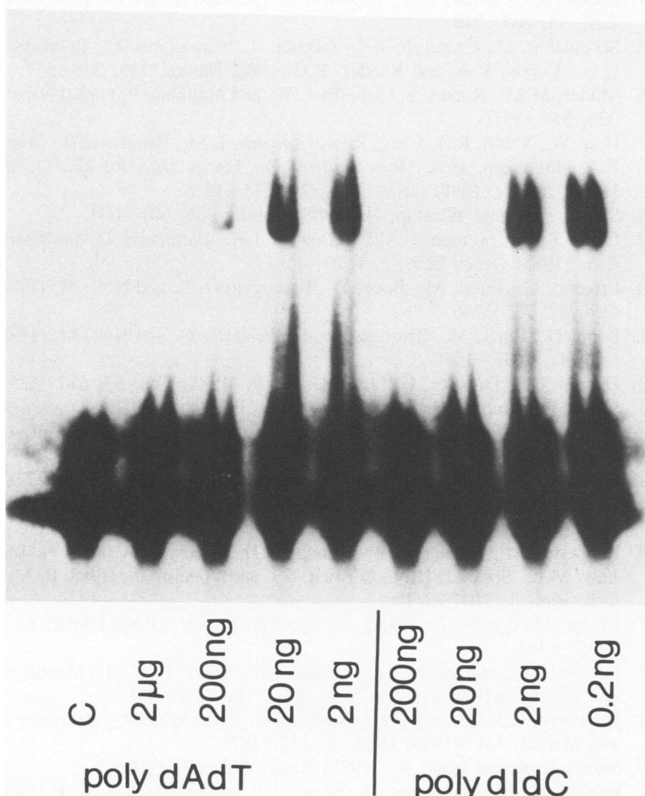

Figure 6. Oligonucleotide S2 was tested in the presence of competitior poly dA/dT and poly dI/dC. Gel of oligonucleotide S2 was exposed for 12 hrs. Standard band shift assay conditions were used , poly dA/dT and poly dI/dC (Pharmacia) were added as competitors. Protein mix without protein is in lane C.

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5q            G A G G C G C G G A        2
12t           G A G G G G T G G T        1
13q           T C G G G G T G G T        2

S2            A G G G C A G G C T        3
S3            A G G G C G T A T A        3
S5            G T G G C G G T G T        2
S9            T G G G T A G G G A        3
S10           G A G G C G G G C C        1
S11           C G G G C T G G A T        2
S13           G G G G C T G T G T        2
S16           G G G G C A G G G C        1

Cons.(TDA):   g g G G c g g g g t

              T A       T A A T
Cons.(SP1):       G G C G
              G G       G G G C
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Figure 7. Sequences of 11 SP1 binding sites. The alignment of 11 SP1 binding sites generates a TDA consensus (GGGGCGGGGT) which is compared to the general SP1 consensus site used in template A (Figure 2). Underlined nucleotides and numbers on the right site indicate which and how many residues of the TDA derived SP1 binding sites differ from the general SP1 consensus. Oligonucleotides were sequenced using double-stranded miniprep DNA according to the manufacturer's protocol (deaza-Kit, Pharmacia).

sites. Highest affinity binding sites can be rescued by extracting the nitrocellulose filter finally with phenol/chloroform. After having selected a pool of putative DNA binding sites by various TDA cycles using nitrocellulose filter binding, we added one or two steps of band shift electrophoresis. This second TDA procedure ensures that oligonucleotides are cloned that almost exclusively bind to the protein under study. By adding competitor DNA in the band shift electrophoresis, affinities of the selected oligonucleotide mix can be studied and high affinity oligonucleotides can be selected in presence of competitor DNA. However, the use of poly dA/dT or poly dI/dC might introduce a bias due to structural similarities between non-specific DNA and putative binding sites.

Advantages of using randomized oligonucleotides

Recently Oliphant, Brandl & Struhl reported that GCN4 protein selects specific oligonucleotides when subjected to mixture of random oligonucleotides (37). Kinzler and Vogelstein described a model system to determine target sites on genomic DNA using PCR and TFIIIa protein (38). They used their method successfully to determine putative binding sites for GLI, a human zinc finger protein in the human genome (39). Instead of using genomic DNA fragments, we decided to use synthetic oligonucleotides containing random nucleotides. Using recombinant SP1 as a model protein we demonstrated that the Target Detection Assay (TDA) is a rapid and convenient method for determining DNA sequences to which proteins bind. Synthetic DNA confers several advantages. Protein binding can be directed to a specific region of limited size on the DNA. While performing the TDA the stringency of selection can be monitored. Amplified fragments can be used in band shift assays because all putative DNA binding sites are of the same length. The complexity of DNA mixtures can be assessed according to the purposes of the TDA assay.

Potential applications of the target detection assay

In general, the TDA method can be used to search for new binding sites of known and putative DNA binding proteins. In the case of SP1, various putative binding sites that differ from the SP1 consensus in two or even three positons were identified that would probably not have been found easily by conventional methods. By synthesizing only three oligonucleotides, a panel of putative SP1 binding sites has been determined. These putative target sites can further be analysed by DNAse I footprinting, by methylation interference and/or functional assays using reporter constructs. In additon, if binding sites for a protein under study have already been determined, the TDA can also be applied to search for binding sites with higher affinities. One feasible approach would be to consecutively replace two or three nucleotides of a known binding site by random nucleotides starting at one end of the binding site and then always select for binding sites with higher affinities.

In particular, this TDA method can be used to study mutants of the protein which effect the DNA recognition. It should be emphasized that the target detection assay is an excellent method to determine whether proteins mutated in their DNA binding domains have lost their general DNA binding capacity or changed their target specificities. For example, since many zinc finger proteins contain multiple consecutive zinc fingers (16,17,18,19) and each finger can be considered independently folded (40) the application of the TDA method could verify whether a zinc finger specific DNA binding code can be determined by associating specific interactions between specific amino acids and their corresponding base pairs (27). For example, we are currently replacing individual amino acids in the finger region of SP1 to demonstrate that the binding specificity of mutated zinc fingers are changed depending on the amino acids used for substitution. These experiments can not be conducted using genomic DNA fragments. If putative DNA binding proteins are analysed whose binding sites are expected to recognize more than 20 nucleotides, it might be advantageous to use genomic DNA fragments (38) to exclude that DNA binding sites are determined that do not occur in the corresponding genome. The methods using genomic DNA (38) and our Target Detection Assay (TDA) complement each other quite nicely . However, the Target Detection Assay (TDA) seems to be the method of choice for studying binding specificities of mutated DNA binding proteins.

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