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# Identification of protein binding sites in genomic DNA by two-dimensional gel electrophoresis

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## ABSTRACT

We describe a simple two-dimensional electrophoresis procedure to identify the recognition sites of DNA-binding proteins within large DNA molecules. Using this approach, we have mapped *E. coli* IHF (Integration Host Factor) binding sites within phage Lambda (48 kb) and phage Mu (39 kb) DNA. We are also able to visualize IHF binding sites in *E. coli* chromosomal DNA (4,700 kb). We present an extension of this technique using direct amplification by PCR of the isolated restriction fragments, which should permit the cloning of a collection of recognition sequences for DNA binding proteins in complex genomes.

## INTRODUCTION

The isolation of the target sequence of a DNA binding protein is an important step to characterize its functional role. Using available techniques (i.e. footprinting, gel shift assay), a systematic search for protein binding sites can only be performed on small DNA molecules, or on cloned DNA fragments. We were interested in finding an approach which would allow the rapid isolation of binding sites for the protein IHF within a large set of DNA fragments. IHF is a small histone-like protein of *E. coli* consisting of two subunits which are the products of the *himA* and *himD* (*hip*) genes. It was first identified genetically by its function in the integration of bacteriophage  $\lambda$  both *in vivo* (1, 2) and *in vitro* (3). The IHF protein appears to have a role in many cellular processes (see 4 for a review). It is involved in plasmid replication and segregation, site-specific recombination, transposition, transcription, and perhaps even translation of several prokaryotic genetic units. The pleiotropic effects of IHF are a direct consequence of its remarkable DNA-binding properties: unlike most histone-like proteins, IHF binds preferentially to specific sites in DNA (5), it makes most of its sequence-specific contacts in the minor groove of DNA (5, 6), it is among the strongest DNA-bending proteins (7, 8).

Little is known about the number and the position of IHF sites in the *E. coli* chromosome. Most IHF binding sites have been found in extrachromosomal elements such as phage, plasmids or transposons. From footprinting analysis, several related consensus sequences have been proposed. The common motif is the sequence: YAANNNTTGATW (5, 9–11). Binding sites

can, however, vary significantly from the consensus and several studies have shown that the context can also play a role in binding efficiency.

We describe here a simple procedure which allows the visualization and cloning of IHF binding sites within large collections of DNA fragments. This procedure consists of a simple two-dimensional band-shift assay, performed at two different temperatures. The combination of this approach with PCR offers a powerful way to recover rare protein binding sites from populations of DNA molecules (12). Individual binding sites can be amplified by PCR directly from the dried gel (13), and subsequently cloned in a suitable vector. We have begun to use this approach with several genomes over a broad range of complexity: small plasmids (5 kb), bacteriophages Lambda (48 kb) and Mu (39 kb), or the *E. coli* chromosome (4,700 kb). It should be applicable to the study of other DNA-binding proteins as well.

## MATERIALS AND METHODS

### DNA Techniques

To avoid partial digestion with methylation sensitive restriction enzymes, unmethylated Lambda DNA was purchased from Promega. Plasmid pBR322::IS1 # 11 is a pBR322 derivative with an IS1 insertion at coordinate 3291 (14). Mu DNA was a gift of R. Alazard (CNRS, Toulouse). *E. coli* chromosomal DNA (strain CSH5), was obtained by lysis of the cells with lysozyme and sarkosyl (0.6%) followed by an extensive treatment with RNAase A and proteinase K. It was then purified by standard protocols (15, 16).

Digestions with restriction enzymes were performed according to the manufacturer's (Boehringer Mannheim; New England Biolabs; Biofinex) recommendations. The restriction fragments were end-labeled by filling-in, using Klenow polymerase (Biofinex or Boehringer-Mannheim). In a standard labeling reaction (27  $\mu$ l), 1  $\mu$ g of DNA was resuspended in 10 $\times$ MSB (medium salt buffer) containing 200  $\mu$ M of each dCTP, dGTP, dTTP, 20  $\mu$ Ci of [ $\alpha$ -<sup>32</sup>P]-dATP (>3000 Ci/mmol; Amersham) and 1  $\mu$ l Klenow polymerase (1U/ $\mu$ l). Modified conditions (500  $\mu$ M of dGTP, dTTP and dATP, 15  $\mu$ Ci of [ $\alpha$ -<sup>32</sup>P]-dCTP diluted in 1 $\times$ Klenow buffer (50 mM Tris, 10 mM MgCl<sub>2</sub>, 1 mM DTT, 50 mg/ml BSA)) were used to label a second set

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of *HinfI* and *DdeI* restriction digestions, because the recognition sequences of those two restriction enzymes (G/ANTC and C/TNAG respectively) allow a selective labeling at the N position.

### Binding Reactions

Binding reactions were carried out by incubating several concentrations of labeled DNA (0.02 nM to 3 nM) with IHF (25 nM) for 30 min at 25°C in 1× binding buffer (Tris-HCl 50 mM pH 7.5, KCl 70 mM, MgCl<sub>2</sub> 7 mM, CaCl<sub>2</sub> 3 mM, EDTA 1.1 mM, glycerol 10%, BSA 200 µg/ml, β-mercaptoethanol 1 mM). The stock solution of IHF (5 µM) was sonicated twice for 10 seconds before use to disrupt protein aggregates. The reactions were placed on ice, and the samples loaded on a polyacrylamide gel at 4°C without loading dye.

### Two Dimensional Polyacrylamide Gel Electrophoresis

Standard vertical slab polyacrylamide (6% or 7.5% acrylamide; acryl/bis ratio 29:1) gels were used for electrophoresis in the first dimension. The sizes of glass plates were 16×14.5×0.15 cm (narrow gels) or 30×15.5×0.15 cm (wide gels). The wide gels were run on an adjustable sequencing gel apparatus in which the top tank can be lowered. Electrophoresis was performed in a cold room (4°C) at 14 V/cm for approximately 2 hours. Running buffer was TBE 1×(15). The gels were prerun for several hours to permit the stabilization of conductivity.

For the second dimension, the lateral spacers were removed, leaving the gel between the glass plates. The gel was rotated by 90°, and placed in a horizontal electrophoresis apparatus. It was then submerged in buffer (1 cm above the gel) prewarmed to 70°C to disrupt the protein-DNA complexes, and the temperature throughout electrophoresis was maintained at 60°C by recirculating the buffer with a peristaltic pump through a closed tank placed in a 75°C waterbath. Taking into account the heat losses during circulation, the flow rate of the pump (approximately 200 ml/min) can be used to adjust the temperature of electrophoresis to 60°C in the second dimension. The gel was run for two hours at 7 V/cm, then dried and autoradiographed. When high DNA concentrations were loaded on the gel, staining with ethidium bromide was used to visualize the bands.

### Addition of linkers to genomic DNA

3 µg (450 pmoles) of complementary 'catch' (12) linkers (5'-TTCTGTACTCGAGATGAA-3' and 5'-TTCATCTCGAGTGTACAGAA-3') containing a *XhoI* internal cut site and a *EcoRI* hemisite at each end, were labeled and phosphorylated at the same time, using 2.5 units of T4 polynucleotide kinase (Boehringer), 100 µCi of [ $\gamma$ -<sup>32</sup>P]-ATP (3000 Ci/mole; Amersham) diluted in suitable buffer. After 15 min at 37°C, the reaction volume was diluted two-fold with buffer; nonradioactive ATP (final concentration 1 mM) and kinase (10 units) were added and the incubation extended for 45 min. The reaction was inactivated 10 min at 70°C, and transferred to a large beaker of water which was allowed to cool slowly from 70°C to room temperature to promote annealing of the two oligonucleotides. These were then purified from unincorporated nucleotides using a Sephadex G-25 spin column.

The linkers were ligated to genomic DNA which had been successively restriction digested, treated with Klenow polymerase (12, 15) to fill-in the ends (when needed) and *EcoRI*-methylase (Promega Biotech) to avoid digestion at internal *EcoRI* sites. The ligation mixture was then cleaved with *EcoRI* to eliminate linker multimers.

### Amplification and Cloning of Shifted Bands

The DNA obtained in the previous experimental step was incubated with IHF and run on a 2D-gel as described above. The superimposition of the autoradiogram and the gel dried on Whatman 3MM paper allows one to cut out with precision a retarded band from the dried gel (13). The piece of gel was incubated in 200 µl PCR buffer (Tris pH 8.3 10 mM, KCl 50 mM, MgCl<sub>2</sub> 2.5 mM, gelatin 100 µg/ml), containing 1 µM of each linker, 200 µM each dNTP, and 10 units of Taq polymerase (Perkin-Elmer). A standard amplification cycle of 1 min at 94°C, 1 min at 54°C and 2 min at 72°C was repeated 25 times (17). The last cycle was followed by 10 min at 72°C to allow a complete extension of the PCR products. The major PCR product was purified on a 6% PAGE gel (16), digested with *XhoI*, and cloned into *XhoI* digested plasmid Bluescript SK<sup>-</sup> or KS<sup>-</sup> (Stratagene) as described (15). The ligation mixture was transformed in a DH5α *E. coli* strain and selected on LB with Ap (100 µg/ml), X-gal and IPTG.

### Sequencing

The plasmids with PCR inserts were directly sequenced using the dideoxy chain termination method as described (18), after a rapid miniscale purification (19).

### Computer Search

The simulation of different restriction digestions of Lambda DNA was carried out with a sequence database manipulation program (Seqman) of the IDEAS package (M. Kanehisa, NIH), and managed by a VAX operating system.

## RESULTS

### Two Dimensional Band-Shift Assay

To identify DNA fragments that bind to a protein, we have developed a simple and rapid procedure which is illustrated schematically in Figure 1. Polyacrylamide gels are run in two dimensions, successively. In the first dimension, a standard gel shift electrophoresis is performed (20, 21). In the second, electrophoresis is performed perpendicularly, and at high temperature to destabilize the DNA-protein complex. The previously retarded fragments now migrate according to their real size, and are thus displaced from the diagonal formed by

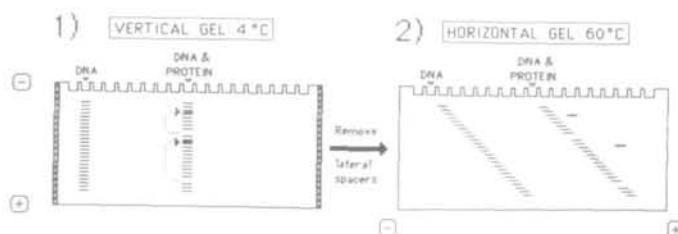


Figure 1. Schematic representation of the two-dimensional electrophoresis of protein-DNA complexes. The first dimension (A) is performed on a standard vertical polyacrylamide slab gel at 4°C. The retardation of the DNA-protein complex during the run is indicated by dotted arrows. The lateral spacers are then removed, and the gel, still between its glass plates, is placed in a horizontal gel apparatus (B). It is then submerged in buffer prewarmed to 70°C, and the temperature throughout electrophoresis is maintained at 60°C. DNA fragments that were complexed with protein run ahead of the diagonal in the second dimension. The size of the 'off the diagonal' bands can be estimated by projection on the diagonal (see text).

the fragments which were never bound by the protein. This system thus allows the identification of DNA fragments containing one or more specific binding sites for the protein by their migration 'off the diagonal'. Projection of the middle of each shifted band onto the diagonal then defines the size of the retarded band. DNA fragments carrying sequence-induced bends may also be slightly displaced from the diagonal (22, see below), irrespectively of the presence of the protein.

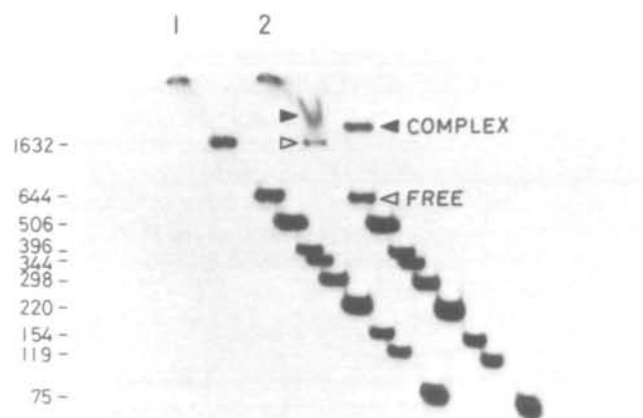
### IHF Binding Sites on pBR322 Derivatives

To demonstrate the feasibility of this approach, we have used a plasmid carrying several IHF binding sites. This plasmid is a pBR322 derivative, pBR322::IS1 # 11 (5.1 kb; 14), which contains a copy of insertion sequence IS1. IHF binds to a single site at each end of IS1 (7, 9), but a point mutation in the right end of IS1 in pBR322::IS1 # 11 abolishes binding by IHF. Additional IHF binding sites are located on the sequence of pBR322, near the beginning of the ampicillin resistance gene (9).

Plasmid pBR322::IS1 # 11 (5 ng) was digested with *Hin*I and radioactively labeled. This generates 13 fragments, two of which should bind IHF: the 1632 bp fragment, carrying the pBR322 sites, and the 644 bp fragment carrying the left end of IS1. The DNA was incubated with IHF (25 nM) and subjected to two-dimensional electrophoresis. As expected, electrophoresis of end-labeled DNA yields a diagonal pattern (Figure 2, lane 1). Prior incubation of DNA with IHF (lane 2) results in the appearance of two detached bands corresponding to the 644 bp and the 1632 bp fragments. IHF binding to the pBR322 sites was clearly detected on smaller fragments after a two-dimensional analysis of *Dde*I digests of pBR322::IS1 # 11 or pBR322 (data not shown).

### IHF Sites in Lambda DNA

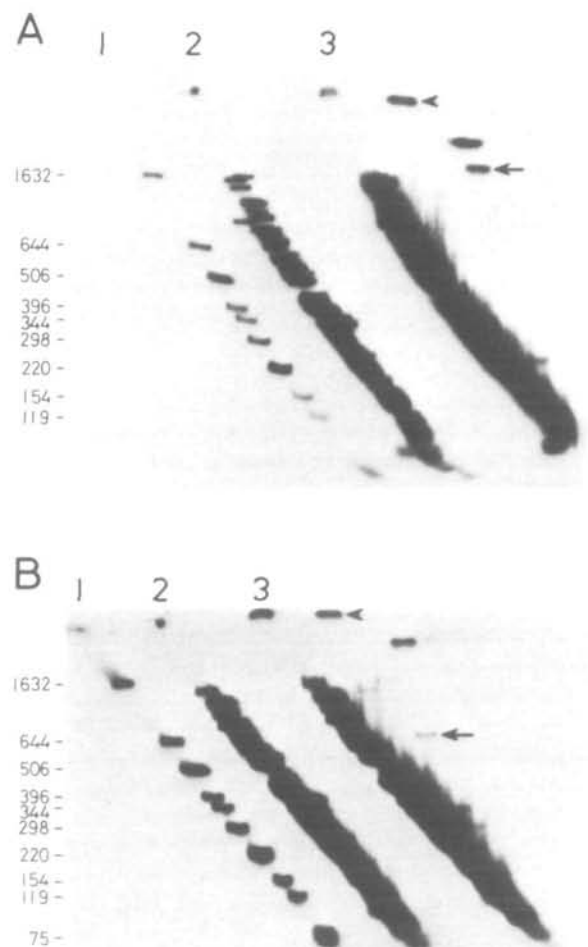
The phage  $\lambda$  genome (48,502 bp; 23) is an order of magnitude larger than pBR322 and several binding sites for IHF have been previously identified. They are located in the *att* locus (attachment site; 5) in the *cos* region (cohesive ends; 24, 25), upstream of the *cro-cll* genes (5, 26), *p'*<sub>R</sub>, *p*<sub>bL</sub> (10), and *p*<sub>L</sub> promoter (27).



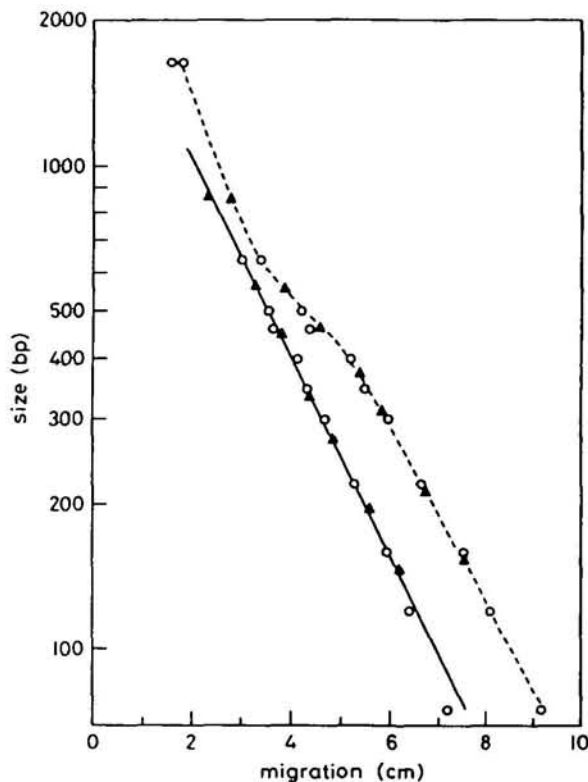
**Figure 2.** Identification of IHF binding sites in plasmid pBR322::IS1 # 11. Two-dimensional pattern of end-labeled plasmid DNA (*Hin*I digest, 5 ng) loaded without (lane 1) or with (lane 2) prior incubation with IHF (25 nM). The position of the free DNA and of the IHF-DNA complex is indicated by an open or closed arrowhead respectively.

In preliminary experiments, we have varied both IHF and DNA concentrations and found that the best results are obtained at low (0.02 nM) DNA concentration with saturating but low (25 nM) concentrations of IHF. At an IHF concentration of 10 nM, the intensity of the signal of shifted bands was significantly reduced (data not show); in contrast, for a concentration > 50 nM, a 'smearing' effect is observed. This is probably due to non-specific binding of IHF. The addition of carrier DNA has a deleterious effect during the migration in the second dimension: an increased background interferes with the visualization of the shifted bands.

Examples of two-dimensional analysis of phage  $\lambda$  DNA are shown in Figure 3. Phage  $\lambda$  was digested first with *Hin*I, which generates 148 fragments ranging in size from 1800 bp to a few bp, and the end-labeled fragments subjected to two dimensional gel electrophoresis (Figure 3, panel A). Again, naked DNA (Figure 3A, lane 2) gives a diagonal pattern in which individual fragments are, however, too close to each other to be resolved. Several restriction fragments, especially when A + T-rich, migrate slightly 'off the diagonal' because of sequence induced bending.



**Figure 3.** Two-dimensional pattern of  $\lambda$  DNA. A. *Hin*I digest without (lane 2) and with (lane 3) IHF (25 nM). Lane 1: size standard (pBR322::IS1 # 11, *Hin*I digest). The shifted bands containing the IHF binding sites located in the *cos*B region are indicated by an arrow or an arrowhead. The other strong band corresponds to the *att*B sites. B. Same as in A, but with *Hin*I + *Xmn*I digestion. The change in position and strength of one of the *cos*B bands (arrowhead) is caused by the *Xmn*I cut, which removes 33 bp and inactivates one of the four binding sites present.

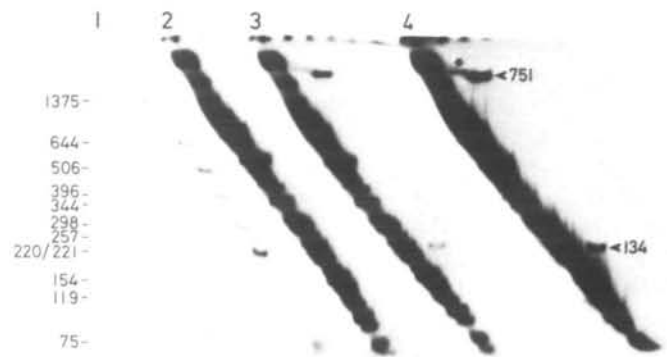


**Figure 4.** Calibration plot of the 2-D gel shown in Figure 3B: the hyphenated line corresponds to the migration in the first dimension (4°C), the solid line to the second dimension (60°C). The size of the standard fragments corresponding to plasmid pBR322::IS1 # 11 digested with *HinfI* (o), and the estimate of the bands carrying IHF binding sites (triangles) are shown.

(22). Prior incubation of the DNA with IHF (lane 3) results in the appearance of 6 shifted bands. Using a standard (Figure 3A, lane 1), their sizes can readily be estimated from calibration plots such as that shown in Figure 4. The distance migrated in either dimension can be used for calibration, yielding approximately the same result. Strikingly, in the case of the second dimension (temperature run 60°C) these sequence-induced effects are abolished (28) and a straight line is obtained.

The fragments containing the known strong IHF binding sites, located in the *cos* (24) and *attP* region (5) were easily identified on the autoradiograms shown in Figures 3A and 3B. Note that  $\lambda$  DNA was heated for 20 min at 67°C before labeling to denature the cohesive ends. Two shifted *HinfI* fragments of 314 and 723 bp are obtained for the *cos* region (24), containing the binding sites located on the right side of *cosB*, and on the left side of *cosB* respectively (24; 25). *CosB* comprises a region of about 40 bp to the left of *cosN* and a region of about 170 bp between *cosN* and *Nu1*. Each of the *cos* fragments is significantly retarded, as is the 317 bp fragment containing *attP* (5; Figure 3A).

To confirm the assignment of these bands, we have analyzed the two-dimensional pattern obtained after an additional digestion with *XmnI* (Figure 3B). *XmnI* has 25 cleavage sites on  $\lambda$  DNA, one of which is located in the *cosB* region, within the 314 bp *HinfI* fragment. There are no *XmnI* sites within the 723 bp *HinfI* fragment carrying *cosB*, nor in the 317 bp *attP* fragment. In Figure 3B, we can see that, as expected, the 317 bp (*attP*) and 723 bp (*cosB*) fragments are not affected by cutting with *XmnI*, whereas the 323 bp (*cosB*) fragment is replaced with a 281 bp



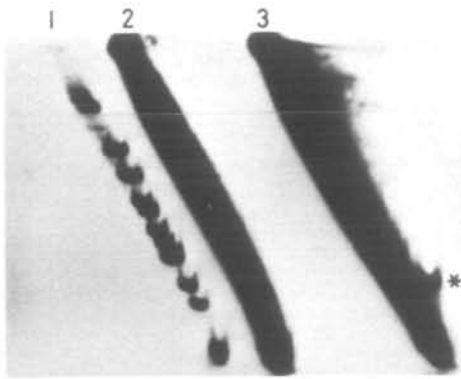
**Figure 5.** Phage Mu DNA after two-dimensional electrophoresis. Lane 1: size standard (end-labeled plasmid pBR322::IS1 # 11, *HinfI*+*Bam*HI digest). Lane 2: Mu DNA (*Sau*3AI digest, 6ng). Lanes 3 and 4: Mu DNA (6 ng and 15 ng respectively) incubated with 25 nM IHF. Shifted fragments containing the two known IHF binding sites are indicated by arrows, the unknown by a \*.

fragment. The signal from the shifted bands is strong and sharp, indicating an efficient binding. It should be noted that the intensity of the shifted bands is variable. This presumably reflects the relative binding efficiency of the sites. In addition to the strong *cos* and *attP* IHF binding sites, we have identified three other known binding sites located in the *cro-cII* (5), *p'*<sub>R</sub> and *p*<sub>BL</sub> (10) regions. Additional restriction digestions of Lambda genome confirm the assignment of the known IHF binding sites described above. We have also selectively labeled the *HinfI* and *DdeI* digested fragments with dCTP (see Materials and Methods), which allows an additional discrimination.

#### Unknown Binding Sites of Lambda: Localization by Restriction Analysis and Cloning of PCR Products

In principle, the unknown IHF binding sites in  $\lambda$  DNA can be identified by analyzing the size of shifted fragments with several restriction enzymes. A computer comparison of the size of these fragments and the restriction map of  $\lambda$  suggested their possible location. The sequences obtained were screened for the presence of an IHF consensus sequence. In contrast to the fragments described above, more than one map location is possible for several fragments. To solve this problem, we have ligated labeled double stranded linkers to *HaeIII* digested  $\lambda$  DNA in order to amplify the fragments by PCR (12, 15). The shifted fragments were amplified from the dried gel. The PCR products were incubated with IHF, both before and after cloning to demonstrate the presence of a binding site (data not shown). An amplification of the bands, followed by cloning in plasmid Bluescript, sequencing, and subsequent gel retardation of the insert, shows that several DNA fragments bind IHF with different affinities.

A 527 bp *HaeIII* fragments carries new IHF binding site(s) and is distinctly retarded by IHF. After a search for consensus binding sequences in this fragment, we found several potential sites for IHF. The best match (CAC...TTGATT; coord. 34,254–34,266; 23) is located at the N-terminus of the *ral* gene, which protects  $\lambda$  from the host *EcoB* and *EcoK* restriction systems (29). This site was not detectable with the restriction analysis described above. We have calculated the similarity score of the DNA sequence according to the formula of Goodrich *et al.* (11). The value founds (41.8) is acceptable. For comparison Giladi *et al.* (27) demonstrate the presence of two distinct active IHF binding sites near *p*<sub>L</sub> with 30.9 and 50.1 similarity scores



**Figure 6.** IHF binding sites on *E. coli* chromosomal DNA. DNA from strain CSH5 was purified, digested with *Hin*I, and end labeled with  $^{32}\text{P}$ - $\alpha$ -dCTP (15). Lane 1: size standard (pBR322::ISJ # 11, *Hin*I digest). Lanes 2 and 3: *E. coli* DNA without and with IHF (65 nM) respectively. The strong retarded band (\*) is an internal standard (a 72 bp oligonucleotide with a strong IHF site). At least 30 shifted bands can be seen on the original autoradiogram.

respectively. The low concentration of IHF used in our assays (25–50 nM) implies that the sites reported here have a good affinity for IHF. A few other weaker sites remain to be characterized in  $\lambda$  using this approach.

### Mu DNA

Contrary to  $\lambda$ , the sequence of phage Mu is only partially known (30). This includes the IHF binding site located near the left operator  $O_1$ , whose role in transcription has been demonstrated (31). Another site is located downstream of the *mom* gene (J. Kur, S. Hattman, and W. Szybalski, pers. comm.).

The autoradiogram shown in Figure 5 demonstrates the presence of 3 strong IHF binding sites in phage Mu DNA. The reagents concentrations were 25 nM for IHF and 0.01–0.02 nM for Mu DNA (*Sau*3AI digest). The 751 bp fragment contains the  $O_1$  operator since it carries one of the two *Hind*III sites of Mu (data not shown). The 134 bp fragment has the right size to carry the *mom* distal region. Digestion with other restriction enzymes have confirmed the assignment of these two sites. Finally, a shifted band corresponding to a size of approximately 700 bp carries an unknown binding site whose location can be narrowed down to a 260–280 bp interval after digestion with *Hin*I or *Hin*I + *Hind*III (data not shown). We are in the process of characterizing this band by PCR.

### *E. coli* Chromosome

An interesting use of this technique would be to isolate a collection of IHF binding sites from the *E. coli* genome. The best two-dimensional gels (Figure 6) were obtained by increasing the amount of chromosomal DNA (35 ng per reaction) relative to that used for  $\lambda$  DNA, while increasing the IHF concentration only 2–3 fold (65 nM). After digestion with *Hin*I (recognition site G/A<sub>1</sub>NTC) and labeling with dCTP opposite the N position (see Materials and Methods) at least 30 shifted bands are visible on the original autoradiogram (Figure 6). An oligonucleotide carrying a strong IHF binding site was included in the binding reaction as a control. Since only on average 7/16 of the chromosomal fragments have a C opposite the N position in at least one of their 3' ends and hence get radioactively labeled, we can estimate the number of IHF binding sites in the chromosome to be a minimum of about 70. The known IHF

binding sites in *E. coli* number only 5 or 6 (4). Although many of them may correspond to binding sites at the ends of endogenous ISJ elements (7), or to the sites contributed by the integrated copy of phage  $\lambda$ , a significant number of new sites remain to be isolated and characterized.

### DISCUSSION

We have described a simple method to visualize, clone and characterize protein binding sites from genomic DNA. Using this approach, we have identified most of the known IHF sites within Lambda DNA, and have localized a potential new site. The two-dimensional electrophoresis of protein-DNA complexes has several advantages. It allows the estimation, in one autoradiogram, of the minimal number of binding sites for a protein in a given genome. Since the denaturation is performed simply by increasing the temperature, there is no need for denaturing buffer in the second run. This allows the processing of several samples on the same gel, without tedious additional manipulations, and eliminates any problem with detergents in subsequent experimental steps.

The technique is easily reproducible. We always obtain the same pattern of IHF shifted bands in different assays with a given DNA sample, as well as after distinct labeling reactions. The relative intensity of each shifted band is also constant, which reflects the different binding affinities to various sites. A low concentration of the protein is needed in the assay in order to ensure specific binding. The system is sensitive to the IHF concentration, which, in the case of  $\lambda$  must be within a narrow range between 10 and 25 nM. Exceeding the upper limit of protein concentration can result in the appearance of smears on the gel. The DNA concentration may be varied over a 100 fold range (up to 1  $\mu$ g), without deleterious effects on the binding reaction as seen on the gel. Although IHF is stable for at least 10 min at 100°C, its binding to DNA is abolished at temperatures above 50°C (data not shown). The heat lability of binding is a requirement for a protein to be used in this assay, a condition which should be met with many other DNA-binding proteins. It has recently been possible, using this approach, to visualize binding sites for eukaryotic transcription factors from unfractionated nuclear extracts (J. Wuarin and U. Schibler, pers. comm.).

The combination of two-dimensional electrophoresis with PCR offers a powerful way to isolate rare protein binding sites. By definition, all DNA fragments which are shifted ahead of the diagonal are of interest. They can be eluted and cloned individually, as was done for the  $\lambda$  fragments. But, in the case of a more complex genome, one could simply pool all the fragments which migrate off the diagonal and sort them individually after cloning in a plasmid. We are currently using this approach to generate a library of the IHF sites from the *E. coli* chromosome.

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