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The DNA-protein cross: a method for detecting specific DNA-protein complexes in crude mixtures

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A method using crude cellular mixtures is described which permits identification of polypeptides and DNA fragments forming specific complexes. Our procedure incorporates elements of both 'Southern' and 'protein' blotting and combines, in two dimensions, the resolving power of a denaturing protein gel with that of an agarose DNA gel. Conditions for 'crossing' have been established using the λ repressor-operator system: the specific complex can be detected by crossing total protein from bacteria overproducing the repressor with a mixture of total genomic fragments from a lysogen.

Key words: cross/DNA-binding proteins/method/λ repressor/λ operator

Introduction

An important fraction of the genome probably contains sequences which are recognised by specific proteins: on binding, these proteins influence the function of neighbouring genes. Even though many thousands of kilobases of various genomes have been isolated, few such DNA-binding proteins and their target sites have been identified (e.g., see Bourgeois and Pfahl, 1976). This is largely because it has proved difficult to resolve one specific protein-DNA complex from the many found in cells. The most successful strategy to date has been to purify a DNA sequence and to use it to select from a mixture any protein that binds to it. The λ and *lac* repressors were isolated from cellular mixtures by their affinity for DNA purified from the appropriate virions (Gilbert and Müller-Hill, 1966; Ptashne, 1967); more recently, DNA sequences purified by molecular cloning have been used (e.g., Hsieh and Brutlag, 1979; Weideli *et al.*, 1980; Sakonju *et al.*, 1981). A reverse strategy is to use purified protein to select a specific DNA sequence. In this way, the SV40 sequence that bound to the T-antigen was isolated (Tijian, 1978). The obvious drawback of both these approaches is the requirement for prior purification of the DNA or protein used as the selective agent. Also high affinity binding of a rare protein or DNA fragment in the mixture – usually the one sought – might be masked by lower affinity binding of species present in greater abundance.

'Protein blotting' (Bowen *et al.*, 1980) was an important technical advance in that it permits detection of DNA-binding proteins in complex mixtures. Proteins resolved by gel electrophoresis are blotted on to a nitrocellulose filter and DNA-binding proteins are detected after incubation of the filter with labelled DNA. Protein blotting has been used to detect DNA-binding proteins from nuclear scaffolds (Lebkowski and Laemmli, 1982) and a larval protein that selectively binds to one DNA sequence in a simple mixture of cloned DNA fragments from a heat-shock locus in *Drosophila* (Jack *et al.*, 1981). Despite these successes, it seems unlikely that the method can be used successfully to detect specific DNA-protein interactions in more complex mixtures.

Principle of the method

Our procedure combines elements of 'Southern' (1975) and 'protein' blotting and permits identification by their sizes of proteins and DNA fragments forming specific complexes (Figure 1). Two gels, both with wide sample wells, are prepared: a mixture of polypeptides is applied to one and labelled DNA fragments to the other. Both mixtures are fractionated according to size by electrophoresis. Next, the polypeptides are diffusion blotted from their gel on to a nitrocellulose filter and this is placed flat on the other gel. It is oriented so that the bands of resolved polypeptides are at right angles to the bands of resolved DNA fragments. (We adopt the convention that proteins run from top to bottom and DNA from left to right.) The native DNA is then transferred out of its gel through the filter, under conditions which enable binding of proteins to DNA (e.g., 50–200 mM NaCl). Only DNA fragments which bind to proteins are retained on the filter, others pass through. Finally, the retained fragments are detected by autoradiography. If a specific protein binds a specific DNA fragment, a spot is obtained where the protein band crosses the original DNA band. The position of this spot is mapped relative to marker DNA fragments and polypeptides.

Results

The interactions of the λ repressor with its two operators (O_L and O_R) are well characterized (see Johnson *et al.*, 1979, 1980, 1981 for recent reviews) and we established the conditions for crossing using this system. We used a strain of *Escherichia coli* (i.e., 294/pKB280) carrying a plasmid that over-expresses the repressor to ~1% of the soluble cell protein (Backman and Ptashne, 1978). Operator fragments were obtained by cutting λ virion DNA with the restriction endonuclease, *Hind*III. The eight resulting fragments were end-labelled with ^{32}P and crossed with total cell protein. Figure 2 is the resulting autoradiograph of the filter. The majority of protein bands present in the total cell extract fail to bind any DNA – most of the autoradiograph is blank. Only two rows of spots are visible, one at 27 kd, the other at ~15 kd. The bottom row reflects the non-specific binding of all λ/*Hind*III fragments to a group of low mol. wt. DNA-binding proteins. [These include HU, H1, H2 and the unwinding protein (Rouviere-Yaniv and Gros, 1975; Cukier-Kahn *et al.*, 1972; Sigal *et al.*, 1972). They migrate anomolously in gels and are not resolved in this autoradiograph (but see later figures).] Only six spots are obtained from the eight DNA fragments since one pair of fragments is insufficiently resolved and the smallest fragment has run off the gel (the spot at 0.6 kb is very faint). The other row contains only two spots, reflecting

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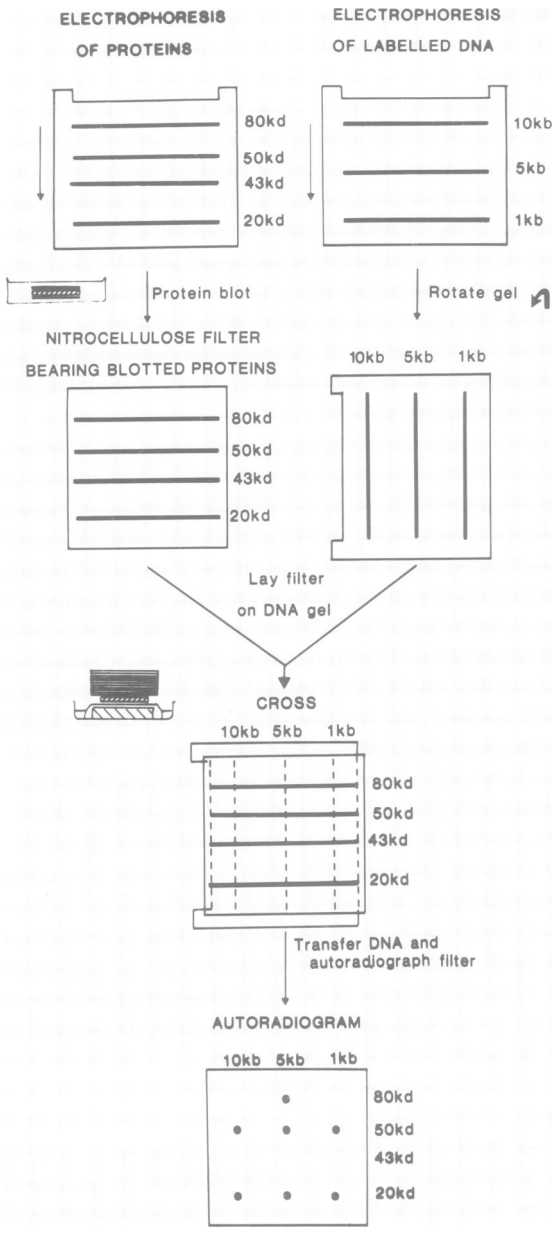

Fig. 1. The 'crossing' procedure. A mixture of four proteins is resolved by electrophoresis in an acrylamide gel and transferred to a nitrocellulose filter. Transfer is effected by sandwiching the acrylamide gel between two sheets of nitrocellulose and immersing in 'transfer buffer': the proteins diffuse out of the gel and stick to the filter, yielding two replicas. During the protein transfer, a mixture of three labelled DNA fragments is resolved by electrophoresis in a second gel. This gel is then rotated through 90° and laid on blotting paper soaked in 'crossing buffer': it is then overlaid with one nitrocellulose filter bearing the adherent proteins as shown. Finally, dry blotting paper is laid on the filter. Fluid flows by capillarity from the wet blotting paper underneath to the dry paper on top. Labelled DNA fragments, carried by this flow, pass through the filter unless bound by protein. Bound DNA is detected by autoradiography of the filter. In the example illustrated, the 43-kd protein has no affinity for any DNA fragment, the 50-kd and 20-kd proteins show affinity for all three fragments, whilst the 80-kd protein specifically binds only the 5-kb fragment.

the binding of the two operator fragments of 9.5 and 6.7 kb to the 27-kd repressor. Controls with total cell proteins from *E. coli* not containing the plasmid show no such spots. Furthermore, only the operator-containing frgments are bound specifically when the λ DNA is cut with different restriction enzymes (see later).

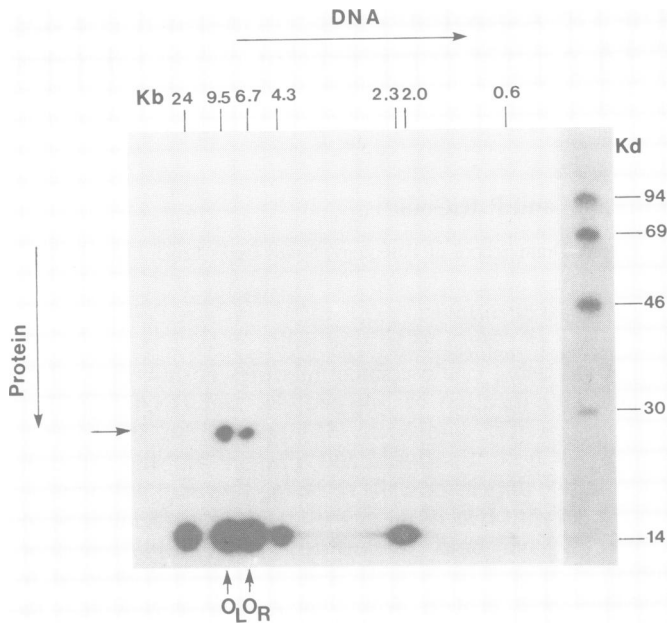

Fig. 2. Autoradiogram of the filter after crossing total cell proteins (1 mg/gel) from the λ repressor-overproducer with end-labelled λ DNA cut with *Hind*III (1 μg). The acrylamide gel included 4 M urea, renaturation was for 1.5 h in transfer buffer, crossing and autoradiography for 6 h each. The positions (determined by autoradiography) of the end-labelled DNA fragments which migrated from left to right are shown at the top. Proteins run from top to bottom: detection of the size markers (at the right) required a longer exposure. (Note the 14-kd marker has almost completely diffused out of the gel during renaturation and the 200-kd marker has not transferred during protein blotting). Proteins of ~15 kd bind all fragments whereas the repressor (27 kd) binds only two (i.e., O_L and O_R, arrowed).

Crossing time

The relative intensities of the six spots at ~15 kd are different but probably do not reflect any differences in affinity. Rather, they reflect the different extents of transfer of the various DNA fragments. During the 6 h of transfer, the smaller fragments have completely leached out of the gel, bound to these proteins, then dissociated as the DNA-free crossing buffer flowed past and so were removed. At the other extreme, only about half of the 24-kb fragments have left the gel and had a chance to bind. For the fragments of intermediate size, the balance between association and dissociation leaves maximal amounts bound. (The 4.3-kb spot is less intense than adjacent spots: the marker track indicated that it was present in less than an equimolar quantity. Presumably some cohesive ends had reannealed and so were not end-labelled.) As the crossing time affects the relative intensities of the spots – and so is an important factor in detecting novel DNA-binding proteins using this method – we investigated it further.

The results of two shorter crossing times are shown in Figure 3. After 1.5 h (Figure 3a) the 15-kd/24-kb spot is invisible and the 15-kd/9.5-kb spot is faint but becomes stronger after 3 h (Figure 3b). By 6 h (Figure 2) it is as strong as the 15-kd/6.7-kb spot. In contrast, the smaller DNA fragments yield the most intense spots at the shorter time (cf., Figures 3a and 2). These results are consistent with explanations given above for the variability in intensities.

However, at all times, only two spots are obtained at 27 kd, and at all times the 27-kd/9.5-kb spot is more intense than the 27-kd/6.7-kb spot, reflecting a highly specific inter-

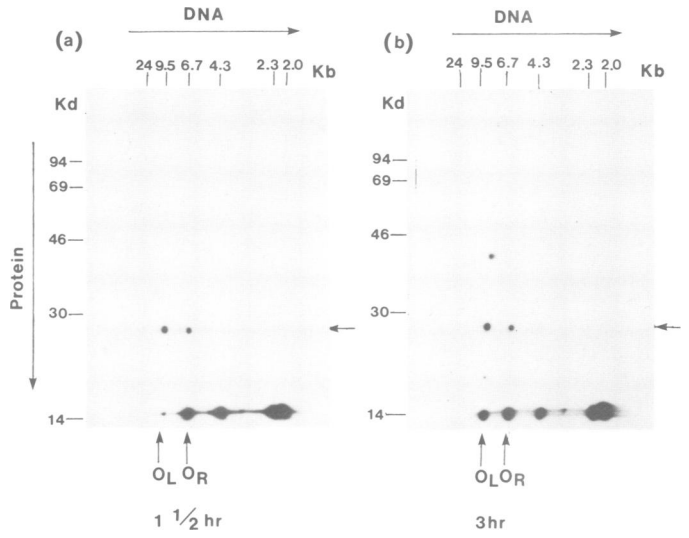

Fig. 3. The effect of crossing time on spot intensity, **(a)** 1.5 h, **(b)** 3 h. Total cell protein (700 μg per gel) from the overproducer was crossed with end-labelled λ DNA cut with *Hind*III (1 μg). All other conditions were identical in **(a)** and **(b)**. Specific complexes of repressor with the O_L and O_R are arrowed. Note that the spot intensities of the larger fragments bound to the ~15-kd proteins increases with time as more are transferred out of the gel. However, the 27-kd/9.5-kb spot intensity is greater than that of the 27-kd/6.7-kb spot at both times indicating preferential binding to the former. Note the spurious spot at ~40-kd/9.5-kb: these occur occasionally (see also Figure 7) but do not occur on the duplicate.

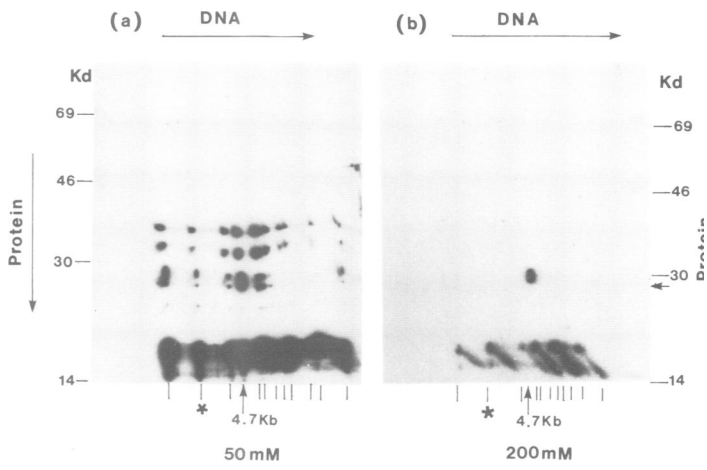

Fig. 4. The effect of salt concentration on binding. Total cell protein (1.35 mg/gel) from the overproducer was crossed in **(a)** 50 mM NaCl, **(b)** 200 mM NaCl with 1.5 μg λ DNA cut with *Bam*HI and *Eco*RI. Crossing time was 18 h and autoradiography for 4 h. Raising the salt concentration abolishes much of the non-specific binding but not the binding of the single operator fragment of 4.7 kb (arrowed). The positions of the DNA fragments are indicated at the bottom: their sizes are 16.3, 9.0, 5.5, 4.7, 3.8, 3.8, 3.5, 3.2, 2.8, 2.6, 1.7, 1.1 kb. The band of 9 kb arises from reannealing of the cohesive ends and is starred.

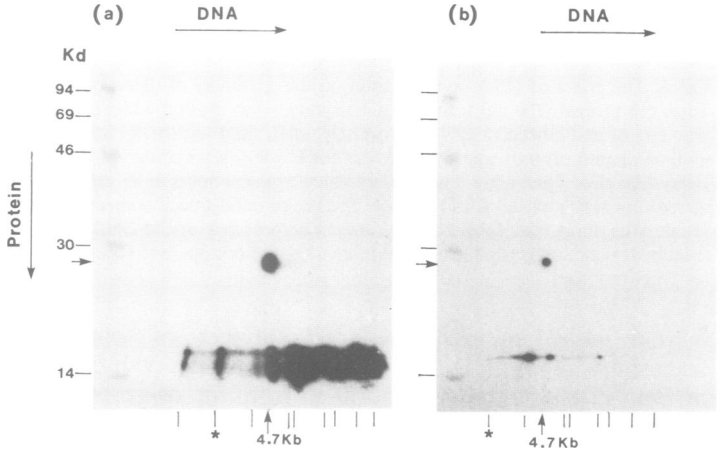

Fig. 5. The effect of protein concentration on binding. Various amounts of total cell protein from the overproducer were crossed with 1.5 μg end-labelled λ DNA cut with *Bam*HI and *Eco*RI. **(a)** 1.35 mg, **(b)** 0.27 mg. Crossing time 18 h, autoradiography 24 h. The arrows mark the position of the repressor-operator spot. The positions of the DNA framents (see Figure 4 for sizes) are indicated at the bottom.

action and the greater affinity of the larger operator fragment (which contains O_L) for the repressor (Maniatis and Ptashne, 1973). The binding of the operator to the repressor is so tight that the complex remains even after 18 h crossing (see Figure 4).

When investigating novel DNA-protein interactions it is convenient to have proteins such as the ~15-kd proteins or histones within the protein mixture. As they bind all the DNA fragments non-specifically, they serve as internal checks on the degree of transfer of fragments of various sizes.

The effect of salt concentration during crossing

Figure 4 shows the profound effect that salt has on the cross. When DNA is cut with *Bam*HI and *Eco*RI, one fragment contains both O_L and O_R. If this DNA is crossed in 50 mM NaCl with total cell protein a number of proteins are detected which bind all the DNA fragments, i.e., a number of different DNA-binding proteins are detected (Figure 4a). This binding is of a low-affinity since it is abolished by raising the salt to 200 mM, leaving only the non-specific binding to the ~15-kd proteins and the specific binding to the repressor (Figure 4b).

When searching for novel DNA-protein interactions the choice of salt concentration is important. We generally use 50 mM and 200 mM in pilot experiments.

The effect of protein concentration

Transfer of protein from gel to nitrocellulose filter falls off dramatically as the concentration of protein applied to the gel is reduced. Best results are obtained using the highest loading of protein compatible with efficient resolution of the proteins. This limitation in protein blotting is reflected after crossing and is shown in Figure 5. Application of 20% of the protein leads to <20% of the spot intensity. This effect is magnified for low mol. wt. proteins (e.g., HU proteins), which are preferentially lost from the gel on soaking before blotting (cf. Figure 5a and 5b).

The effect of DNA concentration

The method detects DNA fragments with great sensitivity. Figure 6 shows the effects on spot intensity of crossing 10-fold reductions in DNA. Crossing 1 μg λ DNA cut with *Hind*III yields two repressor-operator spots (27 kd/9.5 kb and 27 kd/6.7 kb) which can be readily detected after autoradiography for 1 day (Figure 6a). 0.01 μg DNA can be detected after autoradiography for 7 days (Figure 6d). This sensitivity suggests that the method could be used to detect an operator fragment present in a mixture of total genomic fragments obtained from a lysogenic bacterium (i.e., present

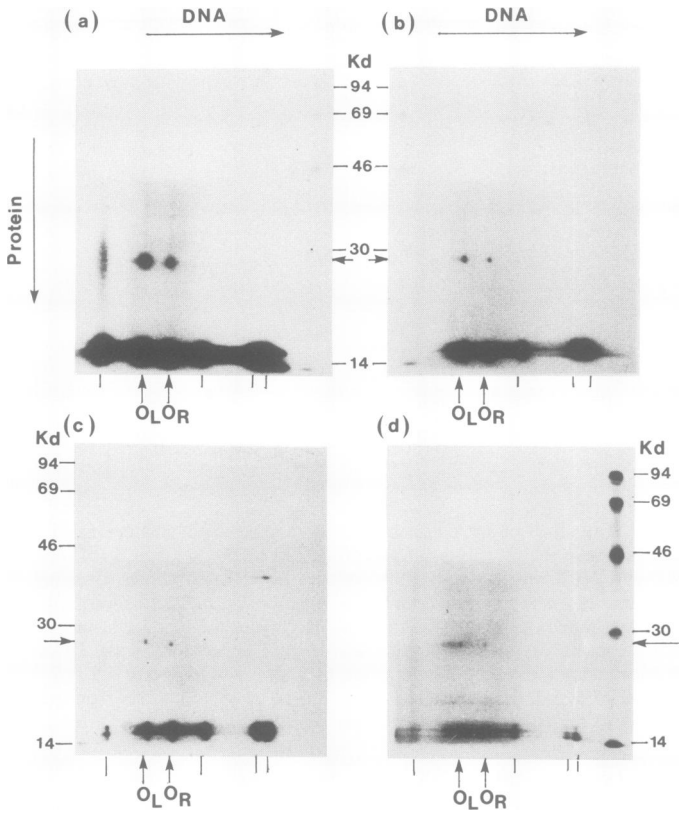

Fig. 6. The effect of DNA concentration on the sensitivity of detection. Total cell protein (700 μg) from the overproducer was crossed with various amounts of end-labelled λ DNA cut with *Hind*III. **(a)** 1 μg; **(b)** 0.1 μg; **(c)** 0.05 μg and **(d)** 0.01 μg. Crossing was for 6 h and autoradiography for 1 day **(a,b,c)** or 7 days **(d)**. The two repressor-operator spots at 27 kd (arrowed) are visible at all dilutions. Note the background that is sometimes visible after short (i.e., 6 h) crossing times. The positions of the DNA fragments containing O_R and O_L are marked at the bottom (see Figure 2 for fragment sizes).

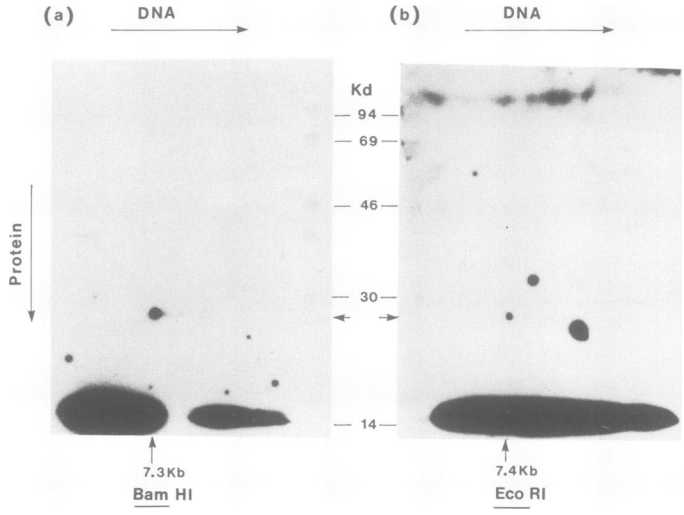

Fig. 7. Detection of a single fragment in a mixture of total genomic fragments. Total DNA was isolated from a lysogenic bacterium and cut with **(a)** *Bam*HI or **(b)** *Eco*RI and end-labelled. 1 mg of total cell protein from the overproducer was blotted to yield two filters. Each filter was then crossed with 16 μg of DNA. Autoradiography was for 1 day. The arrows indicate the expected positions of the 27-kd repressor, the 7.4-kb *Eco*RI fragment the 7.3-kb *Bam*HI fragment which each contain the two operators. These autoradiograms illustrate two drawbacks of the method. Other spots (in both **a** and **b**) are sometimes obtained, but occur in different positions in duplicates and so are probably artefacts. In **(a)** some imperfection during blotting has generated the discontinuous band at ~15 kd.

in one copy amongst the total number of genomic fragments). DNA from such a bacterium was cut with *Eco*RI or *Bam*HI and crossed with protein from the overproducer (Figure 7). The total range of DNA fragments binds to the ~15-kd proteins generating the broad band across the bottom of the autoradiograph. Spots were also found at the positions expected for specific repressor-operator complexes [i.e., at 27 kd/7.3 kb in (a) and 27 kd/7.4 kb in (b)]. Other spots are also evident. Some of these are artefacts (see legend to Figure 7), but others may reflect unknown DNA-protein interactions.

Discussion

We have developed a method for detecting specific DNA-protein interactions in complex mixtures. It combines, in two dimensions, the resolving power of a denaturing protein gel with that of an agarose DNA gel. We have established conditions for this 'crossing' procedure using the λ repressor-operator system. We can readily and reproducibly detect one DNA fragment, in a mixture of ~10, bound specifically to a protein present as 1% of a crude mixture (i.e., proteins of an overproducer of the λ repressor crossed with λ DNA cut with a restriction enzyme). With care, the same protein mixture can be used to select one DNA fragment amongst ~10^3 others (i.e., the operator fragment in a mixture of total genomic fragments from a lysogen). We have tried unsuccessfully to detect the repressor-operator complex by crossing total protein from a lysogenic bacterium with total DNA from that lysogen [such a lysogen contains only 200 molecules/cell (Backman and Ptashne, 1978) so that the repressor constitutes ~0.07% of the total protein]. However, we calculate that this should just be possible using the best conditions described here. Sensitivity can in theory be improved further by labelling to higher specific activities, increasing the loading of DNA and protein on the gel and careful choice of renaturation and crossing conditions.

We believe that the cross has one major theoretical advantage over other methods for detecting DNA-binding proteins. Crude cellular mixtures contain many proteins and DNA sequences able to form complexes with varying affinities. A low affinity binding by either one abundant protein or a multiplicity of different rare proteins (or DNA sequences) could well mask the specific binding sought. Therefore, tight binding by rare species might only be detected by prior purification of both DNA and protein. In our method, the interacting species – although initially present in crude mixtures – are first resolved by electrophoresis and so are relatively pure at the centre of the cross. Subsequently they can complex relatively free of competition from other molecules.

Application to eukaryotic systems

The method has proved very sensitive when applied to the extremely tight binding of a bacterial repressor to its operator. However, we wish to apply the method to eukaryotes: is it feasible to do so? Although a great number of variables will in theory influence sensitivity, probably the major factor affecting the utility of the method will be the affinity of the protein to be studied for its binding site. The affinities of the λ and *lac* repressors and a viral T-antigen for their target sequences is ~10^{-12} M (Johnson *et al.*, 1980;

Linn and Riggs, 1975; McKay, 1981). However, it is not the absolute value of the affinity that is important, but its value relative to that for non-specific binding. The ratio of specific to non-specific affinities of the λ and *lac* repressors is $\sim 10^{-8}$ (Johnson *et al.*, 1981; Linn and Riggs, 1975). We will assume for the sake of discussion that a typical human cell expresses roughly the same number of polypeptides as a bacterium (i.e., $\sim 1-5 \times 10^3$; see O'Farrell, 1975; O'Farrell *et al.*, 1977) but contains 10^3 times more DNA (i.e., when cut with *Eco*RI it contains $\sim 10^6$ fragments of ~ 3 kb). Then if the gel systems used each resolve 100 polypeptides and 100 DNA fragments, at any point ~ 10 proteins will cross $\sim 10^4$ DNA fragments. Therefore, a multiplicity of low-affinity interactions may still obscure the higher-affinity interaction sought.

Is it worth enriching 10-fold the protein sought prior to gel electrophoresis? If each polypeptide were present in equimolar amounts, there would be little need to do so, since only a small fraction of cellular proteins are basic (O'Farrell *et al.*, 1977) or bind DNA (Bowen *et al.*, 1980). However, as the proteins sought are unlikely to be very abundant, we need to maximise the protein loading (see Linn and Riggs, 1975 for a discussion of the abundance of eukaryotic 'repressors'). Therefore, it would seem sensible to select DNA-binding proteins by column chromatography or by using nuclei or their salt extracts [HeLa nuclei and a 0.4 M salt extract contain 20% and 10% of the total cell proteins (Colman and Cook, 1977 and unpublished observations)].

Is it worth enriching the DNA fragment sought prior to gel electrophoresis? We think it is essential with unique sequences because practice has shown that a specific target sequence must represent between 1 and 10% of a DNA fragment before it can be detected in a competitive binding assay. This is true of the target sequences of the *lac* repressor (Linn and Riggs, 1972, 1975), the *E. coli* CAP protein (Riggs *et al.*, 1971; Nissley *et al.*, 1972) and a progesterone receptor (Mulvihill *et al.*, 1982). Assuming a typical target site of ~ 20 bp, or $1-10\%$ of the length of the average restriction fragment, this means we require only one fragment type at the centre of the cross, i.e., an enrichment of 10^4 times. Obviously this is most conveniently achieved by molecular cloning and it is in the crossing of nuclear extracts with recombinant plasmids or cosmids that we foresee the widest application of the method.

Disadvantages

Some of the major disadvantages of the method are listed below and are mainly those of protein blotting. (1) Large proteins (>100 kd) are transferred poorly. 'Electroblotting' (Towbin *et al.*, 1979) may improve this. (2) The DNA may bind only to heteropolymeric proteins. Such binding will go undetected as we use denaturing gels which disrupt the polymers. The use of non-denaturing gels may solve this problem. (3) Different proteins may require different conditions for renaturation and binding, so necessitating lengthy trials. (4) A number of the practical difficulties with the method are mentioned in the legends to the Figures and can generally be overcome by duplicating the cross (one protein blot yields duplicate filters).

Modifications of the basic method

Unpublished experiments show that the basic method can readily be modified to detect binding of RNA, single-stranded or modified DNA (e.g., methylated or DNA damaged by u.v. light). Unlabelled DNA can be used during crossing if it can be detected by subsequently hybridizing it with an appropriate probe. For example, we have successfully detected operator-repressor complexes by crossing unlabelled λ DNA fragments, denaturing and fixing any bound DNA to the filter as described in Materials and methods and then detecting it by hybridization with a 'nick-translated' λ probe. This procedure is at least as sensitive as using end-labelled DNA. More importantly it can also be applied to molecules that cannot be end-labelled (e.g., superhelical DNA, unpublished). Another modification is to elute bound DNA from the filter following autoradiography and then to clone it.

Materials and methods

Bacterial strains

E. coli 294/pKB280, a strain which overproduces the λ-repressor was obtained from B.Meyer and grown as described (Backman and Ptashne, 1978). *E. coli* strain W3110 is a laboratory strain lysogenic for λ. Control genomic blots showed that it contained only one λ genome per bacterial genome.

Protein samples

Total proteins were routinely prepared from 100 ml cultures of *E. coli* in late log phase. The cells were washed in ice-cold phosphate-buffered saline and suspended in 2.0 ml 10 mM Tris (pH 7.5), 0.1 mM EDTA containing 100 μg/ml lysozyme and incubated on ice for 30 min. An equal volume of 2 x sample buffer (Laemmli, 1970) was added and mixed thoroughly, the mixture heated at 100°C for 5 min and then spun at 100 000 *g* for 2 h to pellet the DNA. The supernatant contained protein at a concentration of ~ 10 mg/ml, determined using the dye-binding kit from Biorad Laboratories. This can be used with samples containing SDS and mercaptoethanol (Bradford, 1976; Rubin and Warrell, 1977). 100 μl of this supernatant was applied to a 57 x 0.75 mm well in the acrylamide gel.

Electrophoresis of proteins

Proteins were resolved by electrophoresis on a level surface in 0.75 mm thick SDS-acrylamide slab gels (Laemmli, 1970). In some cases the gels contained 4 M urea. The width of the sample wells, gel type and electrophoresis conditions varied from experiment to experiment. The width of the sample well in the acrylamide gel was determined by the width of the sample well to be used subsequently with the agarose gel. For initial experiments we generally use 5.7 cm wide wells in a 10% acrylamide gel and run the tracking dye, bromo-phenol blue, 5.7 cm. The dye facilitates orientation of the gel and monitoring the efficacy of soaking prior to blotting. Greater resolution can be obtained by using wider wells, running the polypeptides further and varying the acrylamide concentration in the separating gel. (If high mol. wt. polypeptides are being studied, lower acrylamide concentrations should be used to facilitate resolution and transfer during 'blotting'.)

Mixtures of ^{14}C-labelled proteins (Amersham International) are applied to a narrow and adjacent channel in the gel as markers: these are blotted on to the nitrocellulose and, after crossing, yield bands on autoradiography. They are myosin (200 kd), phosphorylase b (94 kd), bovine serum albumin (69 kd), ovalbumin (46 kd), carbonic anhydrase (30 kd) and lysozyme (14.3 kd).

Protein blotting

The method used is essentially as described by Bowen *et al.* (1980). After electrophoresis, notches are cut in the gel to enable orientation and the gel soaked in transfer buffer for 0.5–4 h (four changes) at room temperature to remove SDS and allow the polypeptides to renature. Transfer buffer is 10 mM Tris (pH 7.5), 50 mM NaCl, 2 mM EDTA, 0.1 mM dithiothreitol. 4 M urea and/or 1% Triton X-100 may be included during the initial changes to aid renaturation. We find soaking for >0.5 h leads to considerable loss of polypeptides of <30 kd. During this soaking, the gel increases in size by $\sim 10\%$. Polypeptides were blotted on to nitrocellulose filters over 36–48 h, with replacement of transfer buffer every 24 h, in the blotting apparatus on a level surface. Blotting may be carried out for longer to allow polypeptides >110 kd to transfer. It is important to mark the dry nitrocellulose filters prior to assembly of the apparatus to enable orientation during the cross. After blotting, the replicas are kept wet and used immediately for the cross or stored in transfer buffer in sealed plastic bags at 4°C. [The λ repressor retains all its operator binding capacity for at least a week when stored in this way (unpublished).]

DNA samples

λ DNA and restriction enzymes were obtained from Bethesda Research Laboratories. Bacterial DNA was prepared from lysed bacterial cells by the

Gross-Bellard *et al.* (1973) method and banded on CsCl to purify it.

DNA samples were cut using restriction endonucleases using the manufacturers's conditions and end-labelled using the large fragment of DNA polymerase (New England Biolabs) and the appropriate [^{32}P]deoxytriphosphates (sp. act. ~ 3000 Ci/mmol) to a specific activity of $1-10 \times 10^6$ d.p.m./nmol (Jacobsen*et al.*, 1974). [DNA is more usually end-labelled using polynucleotide kinase (Richardson, 1971).] Alternatively the DNA was labelled (sp. act. $\sim 5 \times 10^8$ d.p.m./μg) by nick-translation (Maniatis *et al.*, 1975) using the Amersham International kit.

Electrophoresis of DNA

DNA samples were applied to wide sample wells and fractionated by electrophoresis in agarose gel slabs (3 mm thick) on a level surface. Gels routinely contained 0.66% agarose and 40 mM Tris base, 2 mM EDTA, 20 mM sodium actate adjusted to pH 8.3 with glacial acetic acid. The agarose concentration may be varied depending on the sizes of the DNA fragments to be fractionated. Marker DNA fragments were run in narrow and adjacent channels in the gel and, after removing the channel from the body of the gel, visualized by autoradiography or staining with ethidium.

The cross

After electrophoresis, the agarose gel containing DNA fragments is trimmed to the required size, notched for orientation and soaked in 'crossing' buffer (400 ml) for 30 min with intermittent shaking. The choice of 'crossing' buffer depends on the type of DNA-protein interaction being investigated. In studying λ repressor-operator interactions, we use 10 mM Tris (pH 7.5), 200 mM NaCl, 0.1 mM EDTA, 2 mM $CaCl_2$, 1 x Denhardt's solution (1966) and 5% dimethylsulfoxide (DMSO). We have found subsequently that omission of DMSO does not affect the results.

The cross is based on the method described by Southern (1975) with three major differences. First, the DNA fragments in the agarose gel are generally double-stranded and not denatured. Second, the crossing buffer contains a low salt concentration to permit DNA-protein binding, rather than 20 x SSC. Third, the nitrocellulose filter bears the resolved polypeptides blotted from the acrylamide gel and is oriented as shown in Figure 1: the face which contacted the protein gel contacts the DNA gel. Special care must be taken to ensure that the crossing buffer does not flow around the edges and is drawn uniformly from the reservoir beneath, through the agarose gel, filter and into the overlying paper towels. If the DNA is end-labelled with ^{32}P its transfer can be monitored conveniently with a Geiger counter. Buffer flow is ~0.5 ml/h/cm^2. Transfer is usually carried out at room temperature for 6 h but may be conducted at 37°C (nearly all fragments <15 kb are transferred out of a 0.66% agarose gel in 6 h, but considerable amounts of larger fragments remain, i.e., 50% of 24-kb fragments remain after 6 h).

After crossing, the filters are carefully removed, rinsed once for 5 min in 200 ml crossing buffer, dried, and an autoradiograph prepared using preflashed Fuji RX X-ray film and Ilford Fast Tungstate intensifying screens (Laskey and Mills, 1977). Instead of drying the filters, it is sometimes useful to seal them wet in plastic bags and prepare autoradiographs. Whilst this prevents the [^{14}C]marker proteins from being imaged, it enables the filters to be rewashed at higher salt concentrations or incubated subsequently with other probes (e.g., antisera or radioactive lectins).

When using unlabelled DNA for the cross, any bound DNA can be detected using a complementary labelled probe. In this case, filters – after the 5 min rinse – are dipped in 0.4 M NaOH for 20 s, then 0.5 M Tris (pH 7.5), 1.5 M NaCl for 20 s, blotted dry and baked for 2 h at 70°C under vacuum. The baked filters are stored under vacuum, hybridized with appropriate 'nick-translated' probes by conventional methods and autoradiographs prepared.

A number of variables affect this procedure, including protein and DNA loading, the percentage of acrylamide or agarose used in the gels, the conditions used during renaturation, protein blotting and crossing. We obtain best results with the maximal protein loading compatible with resolution into individual protein bands. DNA loadings varied from 0.1 μg to 30 μg DNA/5.7 cm wide well for bacteriophage and *E. coli* DNA, respectively. When investigating novel DNA-protein interactions we generally renature for as long as possible (0.5 – 6 h) bearing in mind that sigificant amounts of low mol. wt. proteins (e.g., λ repressor, 27 kd) are lost from a 10% acrylamide gel after only 0.5 h in transfer buffer. We also use Triton X-100 and 4 M urea in the transfer buffer when soaking the gel prior to protein blotting to aid removal of SDS and renaturation. In addition we cross in 10 mM Tris (pH 7.5) or 10 mM Hepes (pH 6.8), 2 mM $CaCl_2$, 2 mM $MgCl_2$ and 50 or 200 mM NaCl.

We recommend that the procedure should be tested by crossing λ DNA (0.5 μg cut with *Hind*III and end-labelled to a specific activity of $1-5 \times 10^6$ c.p.m./μg, applied to a 5.7 cm wide well) with 1 mg total protein from *E. coli* 294/pKB280. Good spots indicating specific operator-repressor binding are obtained after 3 h autoradiography.

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