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Brian Bowen[+], Jay Steinberg[+], U.K.Laemmli[+] and Harold Weintraub[++]

[+]Department of Biochemical Sciences, Princeton University, Princeton, NJ 08544, and [++]Department of Genetics, Hutchinson Cancer Center, Seattle, WA 98104, USA

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ABSTRACT

A method, called "protein blotting," for the detection of DNA-binding proteins is described. Proteins are separated on an SDS-polyacrylamide gel. The gel is sandwiched between 2 nitrocellulose filters and the proteins allowed to diffuse out of the gel and onto the filters. The proteins are tightly bound to each filter, producing a replica of the original gel pattern. The replica is used to detect DNA-binding proteins, RNA-binding proteins or histone-binding proteins by incubation of the filter with [^{32}P]DNA, [^{125}I]RNA, or [^{125}I] histone. Evidence is also presented that specific protein-DNA interactions may be detected by this technique; under appropriate conditions, the lac repressor binds only to DNA containing the lac operator. Strategies for the detection of specific protein-DNA interactions are discussed.

INTRODUCTION

As pointed out by Alberts and his colleagues (1), many of the DNA-associated proteins in the cell will have to be individually isolated and characterized before gene function can be defined. The method of DNA-cellulose chromatography (2) has proved to be a powerful technique in purifying such proteins, but often it must be combined with other techniques such as gel electrophoresis and the nitrocellulose filter binding assay to identify and characterize the proteins. In this paper we describe a method which may simplify the procedure of identifying DNA-binding, and other ligand-binding, proteins.

The method involves three steps: separation of proteins by gel electrophoresis, transfer of the separated proteins to a nitrocellulose filter, and analysis of the adsorbed proteins by incubating the filter with a probe, such as radioactively-labelled DNA. The transfer step produces two filter replicas of the protein distribution in the gel. One filter may be used directly to visualize the protein distribution by staining or fluorography (or autoradiography). The other filter is incubated with a probe such as

^{32}P-labelled DNA, ^{32}P- or ^{125}I-labelled RNA, or ^{125}I-labelled histones, and the binding of the probe to adsorbed proteins is then detected by autoradiography.

The method is schematically analagous to the procedure devised by Southern (3), for detecting specific DNA sequences among a distribution of DNA fragments. As with his technique, the protein transfer method makes use of the high resolving power of gel electrophoresis and requires small amounts of the labelled probe. The predominant separation procedure used in this work is electrophoresis in slab gels containing sodium dodecylsulfate, SDS (4). Polypeptides transferred from these gels must be renatured following electrophoresis. We find that many proteins do renature, as judged by their ability to bind DNA under conditions which minimize non-specific binding.

MATERIALS AND METHODS

Isolation of HeLa Nuclear Proteins: Nuclei were prepared from HeLa S3 cells by the method of Wray and Stubblefield (5) with minor modifications. The procedures for nuclease digestion and high salt (2M NaCl) extraction of nuclei were similar to those used in previous experiments on HeLa chromosomes (6).

Gel electrophoresis: SDS-polyacrylamide gel electrophoresis was carried out as described by Laemmli (7). The stacking gel and the separating gel each contained 0.1% SDS (Polysciences) and 4M urea (freshly prepared; Ultra Pure grade, Beckton Dickinson Immunodiagnostics). Acrylamide, obtained from Eastman, was purified by passage through activated charcoal before use. Protein standards for molecular weight determination were prepared by diluting a stock solution (SDS-PAGE Standards, Bio-Rad) 1:40 in sample buffer (.125M Tris HCl, ph 6.8, 2% SDS, 20% glycerol, 0.001% bromophenol blue). Pellets of nuclear proteins were resuspended in sample buffer (with or without 4M urea) and sonicated. Samples were made 10% in 2-mercaptoethanol and heated to 100°C for 90 sec before application to the gel; the heating step was omitted in experiments with the lac repressor. Electrophoresis was performed on the same day that the gel was prepared. All slab gels were 0.8mm thick.

Method of transfer: After electrophoresis, a gel strip (for example, 12 x 4 cm^2) is immersed in 200ml of urea-containing buffer (50mM NaCl, 2mm Na-EDTA, 4M urea, 0.1mM dithiothreitol, 10mM Tris HCl, pH 7.0) and gently agitated for 3 hrs. During this time, the strip increases in length and width by about 20%. The purpose of this step is to remove most of the

SDS from the proteins and gel (8) and to facilitate subsequent renaturation of the proteins (9).

The transfer of proteins from the gel to nitrocellulose filters (BA85, Schleicher and Schuell) occurs by diffusion. The gel is sandwiched between two strips of nitrocellulose, and as proteins diffuse out of the gel, they are adsorbed onto the nitrocellulose. For efficient transfer, the gel and filters are pressed together in a "sandwich" apparatus (Figure 1). Transfer proceeds while the apparatus is submerged in a solution of transfer buffer (0.05M NaCl, 2mM Na-EDTA, 0.1mM dithiothreitol, 10mM Tris Hcl, pH 7.0).

The sandwich is assembled one layer at a time, starting with the screen at the bottom. Prior to assembly, the foam pads and the nitrocellulose strips must be wetted with transfer buffer. Commercially-available foam pads often contain detergents which must be removed by thoroughly washing the pads in water. Nitrocellulose filters are conveniently wetted by first floating them on a solution of transfer buffer and then submerging them. During assembly, the gel strip is removed from solution, laid on the lower nitrocellulose filter, and then covered with the upper filter. This step is repeated if a second gel strip is included in the sandwich. After the upper filter is in place, the ramaining pad and screen are added. Care should be taken to prevent the nitrocellulose filters from drying out during assembly.

The sandwich is submerged in 2 liters of transfer buffer for 36 to 48 hrs. The initial solution is replaced after 12 hrs with fresh buffer. All of the steps described are carried out at room temperature.

The extent of protein transfer to a filter can be determined by autoradiography, or fluorography, of the filter if the proteins are radioactively

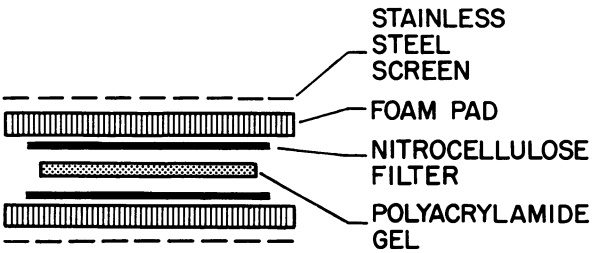

Figure 1. "Sandwich" apparatus. The apparatus is compressed by placing large plastic clips over the wire-mesh screens. This pressure is transmitted to the nitrocellulose filters by foam pads (5mm thick), which also allow buffer to diffuse into and out of the sandwich. The pads and screens are each 18 x 18 cm^2.

labelled (see Results). For unlabelled proteins, the filter is stained for 15 min with 0.1% aniline blue black in 43% methanol, 10% glacial acetic acid, 45% H_2O, and then destained for 1 hr in 3 changes of 90% methanol, 2% acetic acid, 8% H_2O.

DNA Binding: For DNA-binding studies, HeLa or chicken nuclear DNA was labelled to high specific activity (10^7 - 10^8 cpm/ μg) by nick translation synthesis (10). Precursors in the reaction were [^{32}P]dCTP and [^{32}P]TTP (Amersham). Upon termination of the reaction, in some experiments unlabelled E. coli DNA was added as carried (10 μg E. coli DNA per 1 μg HeLa DNA). The mixture was extracted twice with an equal volume of phenol, and then separated from unincorporated nucleotides by passage over Sepadex G-50 (Pharmacia). The DNA was eluted with 0.1M NaCl, 1mM Na-EDTA, 10mM Tris-HCl, pH 7.4, and stored in this buffer.

After protein transfer, a nitrocellulose filter is removed from the sandwich and immersed for 15 to 30 min in 200 ml of binding buffer (1mM Na-EDTA, 10mM Tris HCl, pH 7.0, 0.02% BSA, 0.02% Ficoll, 0.02% polyvinyl pyrollidone) containing 0.05M NaCl, called standard binding buffer. (This step is not absolutely necessary, but may reduce background binding slightly.) The gel strip can also be removed, and stained with Coomassie blue in order to estimate the extent of transfer. The filter is then placed in a plastic pouch (Seal-a-Meal cooking pouches, Dazey), and 10 ml of 10^5cpm/ml [^{32}P]DNA in standard binding buffer is added. Before the pouch is sealed, care should be taken to remove air bubbles in the solution. The strip is incubated with the DNA probe for 60 min at room temperature to permit DNA binding. During incubation, the pouch is occassionally agitated. (Alternatively, DNA binding can be done in a baking dish placed on top of a rotating platform.). BSA, Ficoll, and polyvinyl pyrolidone have been included in the binding buffer, because they reduce non-specific sticking of DNA to nitrocellulose filters (11). The binding reaction is terminated by removing the nitrocellulose strip from the pouch, and washing it in 150 ml of standard binding buffer in a polypropylene dish. If desired, the strip can be washed in binding buffer containing a higher NaCl concentration (see Results). The strip is washed for 60 min with three to four changes of buffer, and afterwards placed on a Kimwipe to dry.

Variations Upon the Basic Method: We have experimented with several modifications of the basic methodology described above and for convenience we briefly describe these variations: (1) For more effective removal of SDS from the gel we have often included 1% Triton X-100 in our gel washing

buffers. This was followed by several rinses and an incubation for several hours in buffer lacking Triton X-100. (2) Instead of the sandwich method, we have also blotted the gel exactly according to the method of Southern using 10 x SSC as a buffer. It is our impression that transfer using this method is slower and slightly less efficient than the sandwich method. (3) "Native" acrylamide gels (the SDS is omitted) have been used to quantitatively transfer the lac repressor and in preliminary experiments total cellular soluble proteins are also efficiently transferred by this method.

RESULTS

Transfer of proteins from a polyacrylamide gel to nitrocellulose filters: To demonstrate the fidelity of protein transfer, an autoradiograph (panel I, Figure 2) of the gel is compared to an autoradiograph (panel II, Figure 2) of a nitrocellulose filter to which proteins were transferred. Marker proteins are shown in lane a, HeLa nuclear proteins in lane b, and nuclear matrix proteins (12) in lane c. Note that the two autoradiographs are nearly indistinguishable. All the major and minor bands are faithfully transferred, with their relative intensities unchanged. Some broadening of the bands occurs as a result of the transfer. Thus, closely-spaced bands in the original gel (panel I) may not be resolved in the pattern on the filter. In general, though, the protein pattern produced on the nitrocellulose filter is a faithful replica of the pattern in the original gel.

The nitrocellulose filter used for panel II was washed in standard (0.05M NaCl) binding buffer following protein transfer. Since DNA-binding studies required that nitrocellulose filters be washed in buffers containing higher NaCl concentrations, the effect of NaCl on retention of proteins by filters was checked. The autoradiograph in panel III demonstrates that proteins adsorbed to a nitrocellulose filter are not removed by washing the filter in binding buffer containing 0.5M NaCl.

In a series of experiments (not shown) the time dependence and efficiency of the protein transfer were determined. The results indicated that an incubation period of about 36 hrs was sufficient to obtain a faithful replica of the gel pattern. After this period, about 75% of the total radioactively-labelled protein applied to the gel was recovered on the two nitrocellulose filters in the "sandwich" (Figure 1). This is the overall efficiency of transfer. Of course, the higher molecular weight proteins in the gel will be transferred more slowly in a diffusion-limited process.

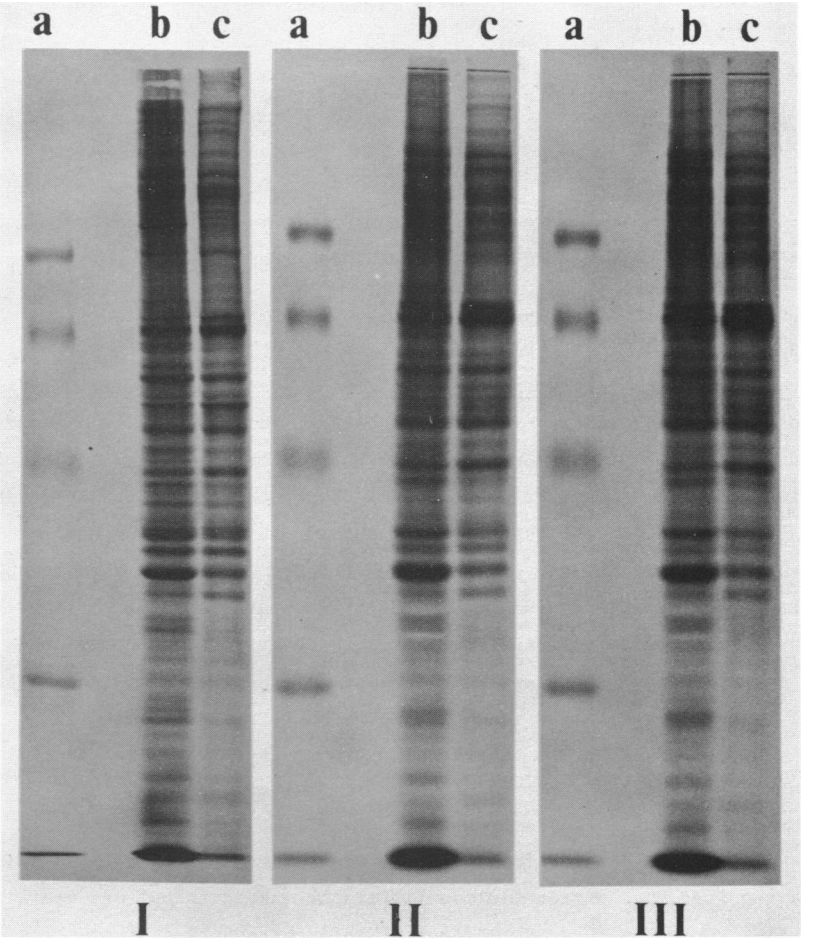

Figure 2. Protein transfer. ^{35}S-labelled proteins were separated by electrophoresis on a 10% acrylamide gel. Samples were loaded in triplicate, and after electrophoresis, the gel was cut into three identical strips. One strip was stained for visual examination. The remaining two strips were placed in a "sandwich" apparatus to permit transfer of the proteins to nitrocellulose filters.

Panel I. Autoradiograph of the SDS-polyacrylamide gel. The stained gel strip was dried and then exposed to Kodak XR-5 film for 4 days. (a) Marker proteins, from top to bottom: phosphorylase B (M = 94,000) bovine serum albumin (M = 68,000), ovalbumin (M = 43,000) carbonic anhydrase (M = 30,000). Soybean trypsin inhibitor (M = 21,000) and lysozyme (M = 14,300) co-migrated with the dye front. (b) HeLa nuclear proteins. (c) HeLa nuclear matrix proteins. Purified nuclei were suspended in 10mM NaCl, 5mM $MgCl_2$, 1mM $CaCl_2$, 0.5mM PMSF, 10mM Tris-HCl, pH 7.4. Half of these were used in (b). The other half were incubated with 40 µg/ml micrococcal nuclease (18,500 U/mg, Worthington) at 0°C for 90 min, and then

diluted with an equal volume of 4M NaCl 20mM Na-EDTA, 0.2% ammonyx AO (Onyx Chemical Co.), 1mM PMSF, 20 mM Tris-HCl, pH 9. The mixture was layered onto a 10% sucrose cushion containing high-salt buffer (2M NaCl, 10mM Na-EDTA, 0.1% ammonyx AO, 0.5mM PMSF, 10mM Tris-HCl, pH9) and centrifuged at 12,000 rpm for 45 min in the Beckman SW50.1 rotor. The pellet containing nuclear matrix proteins was washed once with water, and then resuspended in electrophoresis sample buffer.

Panel II. Autoradiograph of a nitrocellulose filter washed in binding buffer containing 0.05M NaCl. ^{35}S-labelled proteins were transferred to a filter, which was then treated as described in Materials and Methods ("DNA binding" section). ^{32}P-labelled DNA was omitted from the buffer in the pouch. After washing, the filter was dried and exposed to XR-5 film for 4 days.

Panel III. Autoradiograph of a nitrocellulose filter washed in binding buffer containing 0.5M NaCl. The filter received the same treatment as in II, except that after removal from the pouch, the filter was washed in binding buffer containing 0.5M NaCl. A second filter washed in binding buffer containing 0.2M NaCl gave identical results.

Identification of DNA-binding proteins: Proteins transferred to a nitrocellulose filter are tightly, but non-covalently, bound to the filter and should be available for ligand binding, assuming renaturation of the polypeptides has occurred. To explore the potential of the protein transfer method, we have identified several DNA-binding proteins from the nuclear matrix of HeLa cells. The matrix retains some of the structural features of the nucleus and is obtained following treatment (see Figure 2) of isolated nuclei with nucleases and high salt (see, for example, reference 12). Since this structure is an aggregate of a large number of different proteins, identification of the DNA-binding proteins would be difficult by conventional DNA-cellulose chromatography, for which soluble proteins are required.

Matrix proteins were separated by SDS-gel electrophoresis, and then transferred to nitrocellulose filters. The filters were incubated with ^{32}P-labelled HeLa DNA and subsequently washed in binding buffer containing either .05M NaCl, 0.2M NaCl, or 0.5M NaCl.

Figure 3 shows the staining pattern of the nuclear matrix proteins in lane a and the marker proteins in lane b. The autoradiograph (lane c), identifies those proteins of the nuclear matrix which bind DNA at a salt concentration of 0.05M NaCl. It is clear from a comparison of the stained pattern (lane a) to that of the autoradiograph (lane c), that some proteins (indicated by arrows) bind DNA, while many do not. Thus, DNA does not bind indiscriminately. No binding was observed for the marker proteins, phosphorylase B, bovine serum albumin, ovalbumin, carbonic anhydrase, and soybean

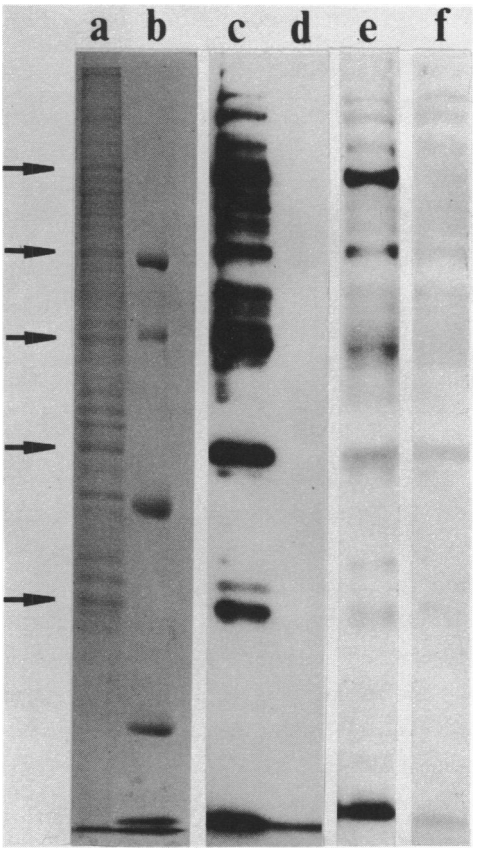

Figure 3. DNA binding to HeLa nuclear matrix proteins. Nuclear matrix proteins were isolated as described in Figure 2, and separated by electrophoresis on a 10% acrylamide gel. Samples were loaded as in Figure 2, and after electrophoresis, the gel was cut into identical strips. One strip was stained with Coomassie blue, showing (a) nuclear matrix proteins and (b) marker proteins (as in lane a, Figure 2). Proteins in the remaining strips were transferred to nitrocellulose filters. Each filter was incubated with 10^5cpm/ml ^{32}P-labelled HeLa DNA and then washed. (c) and (d) Autoradiograph of filter washed in binding buffer containing 0.05M NaCl; (c) and (d) received the same samples as (a) and (b), respectively. (e) and (f) Autoradiographs of filters washed in binding buffer containing 0.2M NaCl and 0.5M NaCl, respectively; (e) and (f) received the same sample as (a). Arrows indicate the positions of several proteins which bind DNA. Autoradiographs were exposed to XR-5 film for 24 hrs.

trypsin inhibitor, as expected (compare lanes b and d). Some binding was detected at the dye frone in lane d. This was due to lysozyme which is known to bind DNA (13). For most proteins, doubling the concentration of [^{32}P]DNA in the binding reaction usually produced a doubling of the band intensity (data not shown); thus, it is likely that the DNA is limiting in this type of assay.

When filters were washed following DNA binding in buffer containing 0.2M NaCl, DNA was dissociated from many of the proteins (lane e). Interestingly, one high molecular weight protein continued to bind DNA strongly. If filters were washed in buffer containing 0.5M NaCl, the binding of DNA to all proteins was dramatically reduced (lane f). A small amount of tenaciously bound DNA (about 1% - 2% of the DNA bound in 0.05M NaCl) remained following the high salt wash.

Figure 4 shows the DNA-binding proteins of intact, isolated nuclei. Many nuclear proteins formed complexes with DNA in standard binding buffer (lane a). Most of these complexes were dissociated following a 0.2M NaCl wash, but several tightly binding proteins are observed (lane b). As in Figure 3, washing the filter with buffer containing 0.5M NaCl reduced the binding to a low background level. In preliminary experiments (data not shown), we have routinely observed strong DNA binding to HeLa histones, separated on 12.5% or 15% acrylamide gels. In these gels, the core histones, H2A, H2B, H3 and H4, migrated more slowly than the dye front and thus could be identified by their large abundance in nuclei. The results were similar to those in Figure 7A, lane b, where the core histones from chicken erythroblast nuclei are easily identified as the two darkly-stained regions near the bottom of the gel. DNA binding to these regions is shown in panel C, lane h. However, in Figure 4 the HeLa nuclear proteins were applied to a 10% acrylamide gel, which did not separate the core histones from the dye front. Consequently, the heavy, broad band at the dye front in Figure 4 primarily represents DNA binding to the core histones. This binding is reduced, but not eliminated, following a 0.5M NaCl wash (lane c).

As with the core histones, we have routinely observed DNA binding to the HeLa H1 histones. Binding of DNA to the H1 histones is shown in Figure 4 (indicated by a bracket). We note that the proteins within the bracket bind DNA following a 0.2M NaCl wash, but not after a 0.5M NaCl wash. Evidence for the binding of chicken H1 histones to DNA is also shown in Figures 5 and 7.

In addition to proteins of the nuclear matrix, a second class of well-defined non-histone nuclear proteins has been analyzed by these techniques.

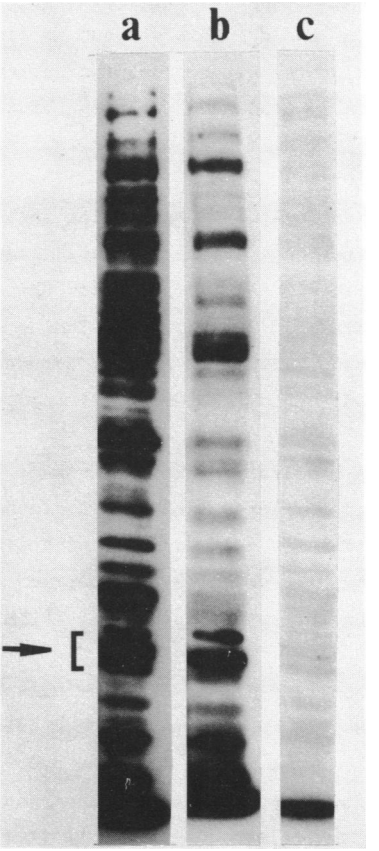

Figure 4. DNA binding to HeLa nuclear proteins. Nuclear proteins were also applied to the gel as in Figure 3. Autoradiographs were made from filters washed in binding buffer containing (a) 0.05M NaCl, (b) 0.2M NaCl, and (c) 0.5M NaCl. The arrow points to the region (bracket) of H1 histones.

These are the high mobility group (HMG) proteins (14). HMG 1 and 2 are easily removed from chromatin by mild digestion with nuclease; in contrast, HMG 14 and 17 remain bound to nucleosomes even after extensive digestion (15). The latter proteins have been shown to be responsible in part for the DNase I sensitivity of actively-transcribed nucleosome monomer particles (16). Preparations enriched in these two sets of proteins were analyzed for their DNA-binding properties using the methods previously described. H1 histone was added to each preparation as an internal control for DNA binding. While HMG 1 and 2 bound little or no DNA (not shown) compared to H1, HMG 14 and 17 demon-

strated a significant affinity for DNA (Fig. 5). Although the exact interpretation of these results is ambiguous because the two sets of HMG proteins may not renature equivalently, the experiment clearly shows that two proteins (HMG 14 and 17) known to bind to specific regions of the chromosome can renature and bind DNA in this assay. Preliminary results using a solution binding assay also suggest that HMG 14 and 17 bind more tightly to chromatin than HMG 1 and 2 (H. Weintraub, unpublished observations).

In conclusion, the experiments reported here demonstrate that the protein transfer method can be used to detect DNA binding proteins. Many proteins, denatured and separated in an SDS-polyacrylamide gel appear to be renatured during the transfer procedure since they regain their DNA-binding capacity. The background binding of the DNA to the filter is low, allowing the detection of minor DNA-binding proteins.

DNA Binding to Protein Fragments: The protein blotting method can also be used to assay regions of proteins with particular biochemical characteristics. Figure 6A shows the electrophoretic pattern of the major peptide products from digestion of histones H1a and H1b with Staphylococcus aureus V8 protease (17). The peptides were visualized by staining a filter replica of the gel electrophoretic pattern, as described in Materials and Methods. The peptides from a parallel gel were also transferred to nitrocellulose and assayed for DNA binding (Figure 6B). While the small peptides from both H1a and H1b appear to bind DNA (poor resolution of the spots in the small peptide region precludes an unequivocal statement), the large peptide from H1a does not. This appears to be in agreement with the reduced binding to DNA of H1a versus H1b, which was observed in Figure 7.

RNA-Binding Proteins: RNA-binding proteins can be identified following protein transfer. Figure 7 shows the stained filter (panel A) and the [^{125}I]

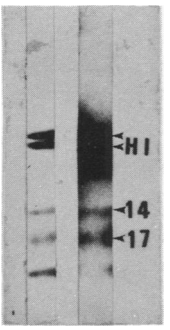

Figure 5. DNA binding to HMG proteins. Fractions of chicken nuclear proteins enriched in HMG 14 and 17 were separated on 15% SDS gels, transferred to nitrocellulose, and challenged in the [^{32}P]DNA binding assay. The stained gel is shown on the left and the DNA-binding proteins on the right. Histone H1 has been added as an internal standard for DNA binding.

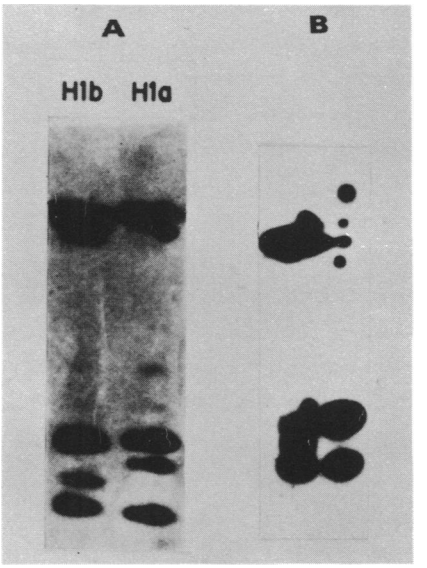

Figure 6. Binding of DNA to different proteolytic segments of H1: H1a and H1b were partially digested in situ as described by Cleveland et al. (16) and separated on a 15% SDS getl.
A. Stained filter from a parallel experiment.
B. DNA-binding proteins and gragments.

RNA binding proteins present in preparations from RSV virions (lane a), whole nuclei (lane b), and a partially purified hnRNP preparation (lane c). For RSV, only p12, the internal core protein, binds RNA, the remaining virion proteins do not (panel B). This protein also binds some DNA (panel C). P19 is known to bind RNA (18); however, this binding is not reproducibly observed using our methods. The major proteins observed in the hnRNP preparation bind RNA (lane f) and a reduced amount of DNA (lane c). Interestingly, a class of high molecular weight protein is observed in whole nuclei which bind RNA (lane e) but little or no DNA (lane h). Note that the histones seen in the pattern of whole nuclei bind both RNA and DNA as expected.

Detection of Protein-Protein Interactions: The protein blotting method may also be used to detect protein-protein interactions. As an example, we use the well known interaction between histones. Histones H2b and H2a are known to bind to histones H3 and H4 (19). H2b and H2a were iodinated as previously described (20) and the iodinated protein allowed to bind

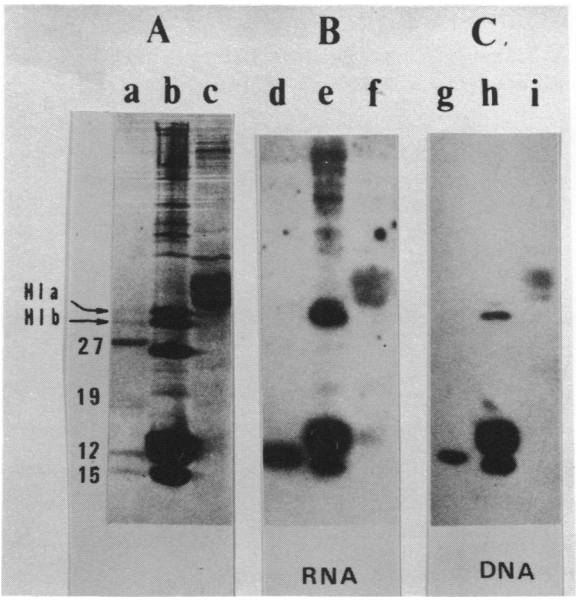

Figure 7. [^{125}I]RNA-binding proteins.
A. Stained filter showing left to right: (a) RSV-virion proteins; (b) erythroblast nuclear proteins; (c) proteins from a partially purified hnRNP preparation. Proteins were resolved on 15% acrylamide gels.
B. Autoradiograph after incubation of filters with 10^6 cpm of RSV [^{125}I]RNA. Lanes (d), (e), and (f) correspond to (a), (b), and (c) in panel A.
C. Autoradiograph after incubation of filters with 10^6cpm of [^{32}P]DNA. Lanes (g), (h), and (i) correspond to (a), (b), and (c).
The bands in lane (b) corresponding to histones H1a and H1b are indicated by arrows to left of panel A. The numbers to the left of panel A identify the RSV-virion proteins in lane (a).

to blotted H3 and H4. Figure 8 shows that binding of ^{125}I-labelled H2a and H2b to unlabelled H3 and H4 is readily observed, however separate controls show that neither lysozyme, BSA, trypsinogen, nor ovalbumin bind [^{125}I] H2a and H2b. While the data in Figure 8 show that a reasonably specific interaction can be detected by these methods, high background radioactivity has been a persistent problem. The use of 10x Denhardt's solution as well as the addition of 1mg/ml lysozyme to the binding buffer reduces, but does not eliminate, the background radioactivity. While this work was in progress, Renart et al. (21) showed that proteins covalently coupled to cellulose can be detected by specific antibody binding (see Discussion). It is likely that

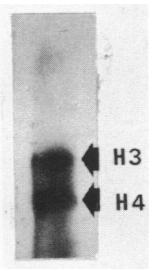

Figure 8. Binding of H2a-H2b to H3 and H4. Procedures for binding [^{125}I]H2a-H2b were as described in Methods except that 10X Denhardt's was used with 1mgm/ml of lysozyme. Input radioactively was 10^6cpm of [^{125}I]H2a-H2b.

the cellulose paper used by these workers gives a much lower background for monitoring protein-protein interactions.

Specific Binding of Protein to DNA: The lac repressor (5 µg per slot) was separated in 10% SDS acrylamide gels and transferred onto nitrocellulose filters. Two adjacent lanes were then challenged for DNA binding: one lane, to nick-translated ^{32}P-lamdba plac DNA (this DNA contains the lac operator sequence); the other lane to ^{32}P-lambda DNA (this DNA does not contain the lac operator sequence). Both DNA preparations were at the same specific activity and both bound equally well to a blot of total nuclear protein. Figure 9 shows that the blotted lac repressor prefers to bind DNA containing the lac operator; however, in 2 out of 6 independent experiments with the lac repressor no binding of the lac repressor to either λ or λ plac DNA could be obtained. We assume that this variability results from a failure of the repressor to renature properly. Since specific conditions for renaturation are likely to depend on the particular protein being studied, we have made no attempts to develop specific conditions for maximizing lac repressor renaturation.

Interpretation of the data shown in Figure 9 is somewhat more complicated for the following reason: before washing the filters it is possible using a Geiger counter to obtain a rough estimate of the amount of bound radioactive DNA for λ and λ plac. For the experiment shown in Figure 9 the initial binding of the two types of DNA was about the same. With increased washing, however, the λ-DNA dissociated much more rapidly than λ-plac DNA. Thus, by extensively washing the bound DNA from the blotted protein, a specific interaction can be detected based presumably upon differences in the dissociation rate constant for the DNA-protein complex. Controls show that with extensive washing (3 hours) even the binding of λ-plac DNA is greatly decreased (to about 10% of the level shown in Figure 9). Thus, we interpret the data in Figure 9 to show that in the case of the lac repressor, specific binding to lac operator can be detected by the protein blotting technique. The speci-

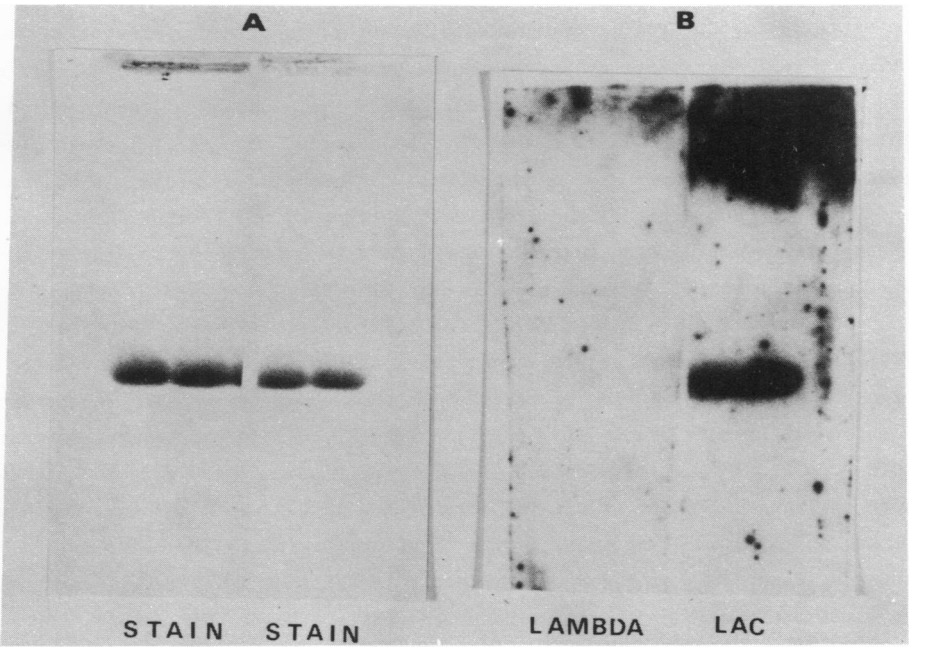

Figure 9. Specific binding of lac repressor to lac operator. Lac repressor was separated on 10% SDS-polyacrylamide gels.

A. Stained upper filters.

B. Binding of lower filters to 10^6cpm of λ-DNA or 10^6cpm of λ-plac DNA. Filters were extensively washed for 2 hours using 7 changes of washing buffer (10mM Tris-HCl, pH 7.4; 50mM NaCl). The standard binding buffer also contained 10mM $MgCl_2$ and 50mM KCl. Binding was monitored with the use of a hand-held Geiger counter.

ficity of the binding requires washing and therefore is probably based upon differences in dissociation rate constant between the two challenging DNA species. Finally, the efficiency of lac repressor protein renaturation is certainly variable under these conditions and it is likely that for any single protein, particular conditions for renaturation will have to be worked out independently.

DISCUSSION

Proteins separated by electrophoresis in polyacrylamide gels have been accurately transferred to nitrocellulose filters. These proteins, adsorbed to filters, were then assayed for their ability to bind other molecules such as

nucleic acids. The transfer method is designed to allow renaturation of proteins following SDS-gel electrophoresis; however, we have not presented direct evidence that most proteins are renatured by this method. Several experiments are currently being performed which will treat the question of renaturation in more detail (B. Bowen and U. K. Laemmli, unpublished results). The experiments presented in this paper are concerned with the problems of protein transfer and DNA (or other ligand) binding. In this respect, the results demonstrate that proteins may be transferred from SDS gels to nitrocellulose filters and that, after transfer, many of the proteins bind DNA.

We have tried other methods of transferring proteins from SDS gels to filters. Neither electrophoresis nor the Southern (3) method of convective transfer shortened the time of transfer or produced patterns with as much resolution as those in panels II and III, Figure 2. During the progress of our work, two alternative methods for transferring proteins from gels to filters were published. Renart et al. (21) proposed a method which involves cleavage of the polyacrylamide gel matrix with alkali or periodate, transfer of proteins by Southern's procedure (3) to activated diazobenzyloxymethyl-paper and covalent attachment of the proteins to the paper. Using this method, it was demonstrated that specific proteins could be identified among a mixture of proteins by incubating the paper first with antisera raised against the proteins of interest and then with ^{125}I-labelled protein A from Staphlococcus aureus. A second method, proposed by Towbin, et al. (22), involves electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets. As with the first method, specific proteins were detected by immunological procedures. To our knowledge, neither method has been applied to the detection of DNA-binding proteins.

Immediately after transfer, filters with adsorbed proteins are washed in binding buffer. We found that the presence of BSA, Ficoll, and polyvinyl pyrollidone (11) in the buffer reduced non-specific binding of DNA to nitrocellulose, and thus, we included them in our DNA-binding and washing solutions. Denhardt (11) has reported that BSA alone is quite effective and we have also observed this. One important variable of the washing conditions which we studied was the NaCl concentration. The standard binding buffer contained 0.05M NaCl. When this was increased to 0.5M NaCl, the same protein distribution (panel III, Figure 2) was still obtained qualitatively. This result is in agreement with the findings of Yarus and Berg (23) who observed little change in the efficiency of retention of ile-tRNA synthetase (ile-tRNA) complexes on nitrocellulose filters between 0.025M-0.5M potassium phosphate.

Both Yarus and Berg (14) and Hinkle and Chamberlin (15) studied the effect of buffer and pH on retention efficiency. Yarus and Berg found that Tris buffer was less efficient than phosphate and that retention efficiency depended strongly on pH, rising from a minimum at pH 7 to a maximum at pH 5.5. Hinkle and Chamberlin, investigating the retention of RNA polymerase-T7 DNA complexes, made the same observation regarding Tris buffer; however, the complexes were relatively insensitive to variation in pH between 6.0 and 9.0. We have varied the pH of the transfer and binding buffer between pH 7.0 and pH 7.5 only and have not detected any qualitative differences in the protein patterns on the filters (B. Bowen, unpublished results).

The filter-binding assay for DNA-protein complex formation (24, 25) involves mixing protein and radioactively-labelled DNA together in solution, filtering the mixture, and determining the amount of radioactivity retained on the filter. In our protein transfer method, renatured proteins are adsorbed on the filter initially and then the filter is incubated in a DNA solution. It is our hypothesis that, in general, proteins in solution and adsorbed proteins will bind DNA similarly. Our experiments provide initial, qualitative support for this hypothesis. Of the marker proteins (Figure 3), only lysozyme binds [^{32}P]DNA in 0.05M binding buffer. Non-specific binding between DNA and acidic proteins (phosphorylase B, pI $\sim$5.8; BSA, pI $\sim$4.7, ovalbumin, pI $\sim$4.6; soybean trypsin inhibitor, pI $\sim$4.5) does not occur at pH 7. Lysozyme, which is very basic (pI $\sim$11.0) does bind to DNA. This result is in agreement with data of Cattan and Bourgoin (13), who found that lysozyme is maximally associated with DNA at an ionic strength of 0.05. Lysozyme has also been shown to bind to DNA-cellulose at 0.05M NaCl (26).

As demonstrated by DNA-cellulose chromatography (1) and the filter binding assay (24), DNA-protein complexes are usually dissociated by increasing the salt concentration in aqueous solutions. This proprerty is used to fractionate DNA-binding proteins on DNA-cellulose. For this reason, the effect of increasing NaCl concentration on complexes between DNA and adsorbed proteins was investigated. In standard binding buffer many complexes between nuclear matrix proteins and DNA were detected (lane c, Figure 3). When the NaCl concentration was raised to 0.2M, a few complexes remained (lane e, Figure 3); in particular, those involving a high molecular weight protein and those at the dye front. The lysozyme-DNA complex (lane d, Figure 3) was dissociated in 0.2M NaCl (not shown). In Figure 4, H1 histones (bracket) remained associated with DNA at 0.05M NaCl (lane a) and 0.2M NaCl (lane b). When filters were washed in binding buffer containing 0.5M NaCl, only com-

plexes between DNA and core histones (at the dye front) survived (lane c, Figure 4).

The effect of NaCl concentration on the stability of DNA-protein complexes was studied by varying the composition of the washing solution. Other aspects of the washing procedure should be investigated further. We observed that filters washed (3 changes of buffer) for 10 min produced autoradiographic patterns similar to filters washed for 60 min; however, overall background binding was higher on the filters washed for 10 min (B. Bowen, unpublished results). Some of this background could have been contributed by DNA, strongly bound to minor protein bands, which was washed off. In this respect, Riggs, et al. (27) found that lac repressor-DNA complexes are slowly washed off nitrocellulose filters. Also, Hinkle and Chamberlin (24) showed that excessive washing of RNA-polymerase-T7 DNA-complexes retained on filters (28) can cause significant losses of T7 DNA from the filter-bound complex.

At this time, it appears that the protein blotting method, by combining the resolving power of gel electrophoresis with a nitrocellulose filter binding assay, will provide a valuable analytical tool. The technique may be used in parallel with DNA-cellulose chromatography to screen crude lysates for DNA-binding proteins, particularly proteins from the "insoluble" fraction of extracts which can be applied to SDS gels. In addition, numerous variations in the filter binding assay are possible; we have, for example, recently found that the prominent high-molecular weight, DNA-binding protein in lane e, Figure 3 binds HeLa DNA tightly in the presence of a 10^3-fold excess of E. coli DNA or dextran sulfate (unpublished results) in the binding buffer. We can envision a number of additional applications of these techniques. For example, using radioactive hormone, hormone-binding proteins could be detectable by this method or for that matter, a number of other types of protein-ligand interactions could be identified as well as specific enzymes, assuming an appropriate assay were available and the enzyme was renaturable.

One theoretical limitation of the technique involves proteins whose activity depends on the interaction of several different subunits. Native acrylamide gels might be used in such a case. Alternatively, it may be possible to restore activity by sequentially transferring 2 identical SDS-gel separations onto the same filter but at a 90° angle in much the same way as described by Sato et al., (29) for DNA fragments.

Clearly, the major advantage of protein blotting would be the detection of specific protein-DNA interactions using cloned DNA and protein extracts. Our preliminary results with the lac-repressor suggest that such interactions

should be detected by this method. One of the main problems is the ability to renature the protein. Procedures for renaturation will have to be worked out for each protein, although the general methods described here seem to be a reasonable starting point. A rough calculation suggests that renaturation need not be complete for adequate detection: If there is 1 µgm of protein present in a band and if only 1 part in 10^5 renatures, then by probing with 10^6 cpm of a cloned DNA fragment (at 10^8 cpm/µgm) and assuming the kinetic problems of the interaction are adjusted accordingly, about 10^3 cpm could be bound to a specific protein band. This can easily be detected after an exposure of about 3 hours. A more important problem is the non-specific binding of DNA to other proteins. Here it is important to distinguish two types of non-specific interactions: those that are reversible and those (like the binding of DNA to histones) that appear irreversible. We have found that the latter can be suppressed by prior incubation of the filters with cold calf thymus DNA (50 µgm/ml). Upon extensive washing of these filters, reversibly bound DNA is removed and now the filters are capable of being reincubated with labeled, specific sequence DNA. The detection of a particular protein at this point is complicated by the presence of other proteins that bind non-specifically, though reversibly to DNA. As described in the text, it is, in principle, possible to maximize specific interactions in this situation by taking advantage of the fact that most specificity in DNA-protein interactions seems to be related to differences in the off-rate of the protein from the DNA (27). Thus, by washing the filters extensively, non-specific binding is preferentially lost and since the decay is exponential (for both specific and non-specific binding) an enormous degree of specificity (as defined by the ratio (not the absolute amount) of specific to non-specific cpm bound) can, in theory, be achieved by washing for longer and longer times.

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