Use of Gel Retardation To Analyze Protein-Nucleic Acid Interactions

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INTRODUCTION

Specific interactions between proteins and nucleic acids are of fundamental importance in the management of cell growth and behavior. Analysis of these interactions is essential to understanding a multitude of elementary processes, from the control of gene expression to site-specific recombina-
tion. Additional information can be obtained by using less technically demanding methods. Footprinting techniques reveal the location of a protein-binding site on DNA and the structural conformation of protein binding, at single-base-pair resolution, through the pattern of protection afforded by bound protein to a battery of enzymatic and chemical probes (100).

The purpose of this review is to provide an overview of another method, technically the simplest and perhaps the most widely used, for investigating protein-nucleic acid interactions, the gel retardation or band shift assay. The technique has its origins in early work on rRNA-protein interactions (29, 132), but its widespread use dates from its development for studies on transcriptional regulation in bacteria (41, 47). It is based on the observation that binding of a protein to DNA fragments usually leads to a reduction in the electrophoretic mobility of the fragment in nondenaturing polyacrylamide or agarose gels. The assay typically involves the addition of protein to linear double-stranded DNA fragments, separation of complexed and naked DNA by gel electrophoresis, and visualization either by autoradiography, when labeled DNA is used, or by staining with ethidium bromide. The widespread use of the technique is due not only to its ease of execution but also to its requirement for very small quantities of material (in the subpicomole range), its particular suitability to studies of simultaneous binding of several proteins to a single nucleic acid molecule, and the amount of detailed information it can provide. Probably because the early reports explored the thermodynamic and kinetic parameters of protein-DNA interactions, using purified components and carefully established experimental conditions, it was not immediately realized that the technique could be exploited for detecting and identifying DNA-binding proteins in less well-defined systems, including crude extracts, and for analyzing protein-RNA interactions.

In this review we give an account of the physical basis of electrophoretic mobility shifts and survey the types of application in which the technique has been found useful. We have included only an elementary account of the derivation of equilibrium and kinetic parameters. More detailed treatments of this topic may be found in recent articles by Fried (40) and Senear and Brenowitz (130).

**HOW DNA MIGRATES IN GELS**

The effects of conformation on the electrophoretic properties of DNA are important for understanding many aspects of the behavior of protein-DNA complexes in gels. We shall therefore begin by giving a brief and simplified account of the characteristics of electrophoretic migration of DNA itself.

Migration of DNA in polyacrylamide gels can be described to a first approximation by the equation:

\[ \nu = h^2 \cdot Q \cdot E / (L^2 \cdot f) \]

where \( \nu \) is the migration velocity, \( Q \) is the effective charge, \( E \) is the electric field, \( L \) is the contour length, \( f \) is the frictional coefficient, and \( h \) is the end-to-end distance of the DNA molecule (90).

Migration of relatively small linear DNA molecules (in the kilobase range) in agarose and polyacrylamide gels is thought to occur in a wormlike fashion known as reptation (30, 31, 89, 90). Direct visualization of very long molecules (in the 100-kb range) in agarose gels generally supports this model while suggesting that the formation of large "hook" conformations is also an important determinant in impeding migration (21).

Although empirically it is well established that linear DNA molecules migrate as a monotonic function of their length, several notable exceptions have been described for migration in polyacrylamide gels. The earliest example analyzed was that of a 490-bp fragment isolated from a trypansome kinetoplast (k-DNA [92]) which migrates according to its actual length on 1% agarose gels but migrates with an apparent length of 1,380 bp on 12% polyacrylamide gels. Biophysical and electron-microscopic studies of this and other similar fragments indicated that the molecules assume a curved structure (53, 54, 92). An example of the relative migration rates of curved and uncurved DNAs of similar length is shown in Fig. 1. As with k-DNA the curved fragment migrates normally in agarose but is highly retarded in a polyacrylamide gel.

Further analysis demonstrated that curvature is usually due to the cumulative effect of periodically spaced runs of A residues, each contributing a bend component (Fig. 2a) (55, 153). The magnitude of the resulting mobility change appears to be determined mainly by the average distance between the ends of the molecule such that a reduction in this distance, by curvature, impedes reptation of the DNA molecule through the gel matrix (153). Similar changes in gel mobility can result from single-base substitutions, deletions, and insertions, which lead to loss of duplex structure (103) or the formation of bulge-bend structures (9, 117).

Aberrant migration or retardation has been expressed as either relative mobility (i.e., observed distance migrated divided by expected distance, \( X_o/X_e \)) or its reciprocal (\( X_e/X_o \), \( k \) value). We shall use relative mobility, designated \( R_e \), in several articles, throughout. Aberrant migration is governed by several factors, all of which are expected to affect the end-to-end distance.

(i) The angle of curvature is determined by the number and magnitude of individual sequence-directed bends (Fig. 2a). Its effect can be visualized by a simple cylinder representation of the type shown in Fig. 2b. The magnitude of individual bends depends on the length of the A tract. Koo et al. (78) demonstrated that it is maximal for a run of six A residues (\( A_o \)) and estimated the angle to be about 20°.

**FIG. 1. Influence of bends on the electrophoretic mobility of DNA fragments.** A pBR322-derived vector and two derivatives carrying 90-bp fragments inserted in the middle of the smallest (824-bp) vector fragment were subjected to AseI digestion and gel electrophoresis. The insert in the plasmid in lanes 2 carries seven runs of AATAA spaced at 10-bp intervals, while that in lanes 3 carries seven equivalent runs of AAAAA. The positions and sizes (in base pairs) of the relevant fragments are shown in the margins. The fragments migrate according to their size on an agarose gel (left panel), but on a polyacrylamide gel (right panel) the mobility of the bent fragment is anomalously low. (Figure kindly supplied by C. Murtin.)
Although increasing the overall curvature leads to a progressive reduction in relative mobility \((R_L)\), the extent of retardation eventually approaches a limit. Koo and Crothers (77) showed that, as the number of A\(_6\) tracts increases to six, the relative mobility declines as a quadratic function of curvature to reach a minimum at a bend angle of about 120°: additional A tracts have little further effect on mobility. The finding that relative mobility does not change significantly for angles greater than about 120° suggests that different forces determine the migration of DNAs with low and high curvature. Zinkel and Crothers (160) noted that this transition corresponds to a reorientation of the long axis of the molecule (Fig. 2c) and proposed that it brings about a change from reptation to a "gel filtration" mode of migration, which separates simply according to size.

(iii) Aberrant migration is more marked the closer the bend is to the middle of the molecule, where it would generate the smallest end-to-end distance (153). This position effect, shown in Fig. 3a and c, is the basis of a widely used method for localizing protein-induced bends (see the section on permutation, below).

**FIG. 2.** Bending angle. (a) The cumulative effect of 1-, 3-, 4-, and 6-A tracts on overall bending angle is shown (left to right). Each individual bend of approximately 20°, indicated for clarity by dotted lines, is shown as occurring in the same direction in a single plane. (b) Effect of bend angle on end-to-end distance. The DNA molecules are included within a cylinder whose diameter increases with bend angles up to 120°. The figure is designed to underline the notion that increasing bend angle leads to increasing congestion of the gel pores. Migration is occurring in the direction of the long axis of the molecule. (c) Change in long axis as a function of bend angle. The long axis of molecules with a centered bend up to 120° corresponds to a line between the average positions of the ends. Molecules with a centered bend of 120° would appear as an equilateral triangle in which all axes are equivalent. For molecules with a bend greater than 120°, the longest axis becomes parallel to one of the two arms.

**FIG. 3.** Effect of bend position on gel mobility. (a) Effect of bend position on end-to-end distance. A bend of fixed angle is shown at increasing distance from one end of the molecule. The largest cross-sectional area is generated by a bend located at the center of the fragment. (b) The permuted set of DNA fragments whose analysis is represented in panels c and d below, showing the position of the bend (solid circles) relative to the ends. (c) Migration of the collection of DNA fragments shown in panel b, in which a bend of fixed angle is located at various positions relative to an end. Migration is from top to bottom. Open rectangles represent the unbound DNA fragment, and closed rectangles show retarded migration of the fragments with bound protein. (d) Curve of the relative mobilities \((R_L)\) of the fragments.

(iii) Altering the spacing of A tracts was shown to influence relative mobility (78). The greatest reduction in relative mobility was found to occur when the A-tract sequence repeats and DNA helix repeats were in phase. Under these conditions, sequence-directed conformational changes occur in the same direction and would yield a planar molecule with a minimal end-to-end distance (Fig. 4, cis configuration). This property can be used to determine bend direction (see the section on phasing, below).
As might be expected on theoretical grounds, the magnitude of the conformation-induced migration effect increases with increasing gel concentration (i.e., decreased pore size and hence increased frictional coefficient [92]) and decreasing temperature (32, 33). It can also be enhanced by the addition of Mg$^{2+}$ (32) and other divalent metal cations (e.g., Ba$^{2+}$ [84]) and can be reduced or eliminated by several low-molecular-weight DNA-binding ligands (e.g., distamycin [153] and ditercalinium [7]).

Many proteins induce conformation changes of the same kind as those caused by sequence elements described above, with similar consequences for relative gel mobility.

**RETENTION OF NUCLEIC ACID MOLECULES BY PROTEINS**

Detection of protein-DNA complexes within a gel depends critically on two factors: the resolution of complexes from uncomplexed nucleic acid and the stability of complexes within the gel matrix. In addition, the significance of detectable complexes depends largely on the specificity of the interactions involved.

### Resolution of Complexes

The properties of a protein-nucleic acid complex which alter its migration relative to that of the naked nucleic acid are the ratio of mass of protein to that of nucleic acid, the alteration of charge, and changes in DNA conformation. It is often difficult to evaluate the individual contributions of these components. External factors such as the composition of the gel matrix and the temperature of electrophoresis also influence the separation of naked and complexed DNA fragments.

**Effect of mass.** The mass of DNA fragments used in gel retardation experiments (60 to 500 bp; 40 to 330 kDa) is of the same order as that of typical DNA-binding proteins (10 to 100 kDa). The increased mass of a DNA fragment resulting from binding of a protein would, on its own, be expected to decrease relative mobility. In early studies of Lac repressor binding to a fragment containing the operator, it was observed that the relative mobilities of the retarded bands obtained by specific and nonspecific binding were those expected on the basis of successive addition of molecules having the molecular weight of the Lac repressor tetramer (40, 41) (Fig. 5a). In this case, since the Lac repressor does not induce an appreciable bend on binding (153, 162), the conformational contribution (see the sections on conformation effects and analysis of conformation, below) to the decrease in relative mobility is small. Furthermore, this relationship between relative mobilities and bound Lac repressor was maintained even when the fragment was complexed with a second protein species (in this case the catabolite activator protein [CAP]) (5, 40).

The influence of protein mass on relative mobility is also apparent from the behavior of sets of truncated proteins and is unaffected by protein-induced conformational changes as long as the DNA-binding properties of the set are unaltered. The relative mobility of complexes between the truncated proteins and their cognate binding sites decreases with increasing protein size (mass) as illustrated by the *Saccharomyces cerevisiae* transcriptional regulator protein GCN4 (65) and the phase Mu transposase (12) (Fig. 5b).

Whereas increasing protein mass results in a reduction in relative mobility, an increase in the mass of the DNA component tends to have the opposite effect. For example, for Lac repressor binding to operator DNA fragments of between 80 and 500 bp, the $R_L$ value increases from 0.5 to 0.68 (40).

These results indicate that it is the ratio of protein and nucleic acid masses rather than their absolute mass which is important in the resolution of complexed from naked nucleic acid.

Interestingly, several authors have noted that protein binding can sometimes provoke an acceleration in mobility. This has been observed for the migration in agarose gels of relatively large linear DNA fragments complexed with a DNA-binding protein from the hyperthermophilic bacterium *Methanothermus fervidus*, where it was shown to result from protein-induced DNA condensation (123). Similar behavior is also observed on binding of the nonhistone proteins HMG14 and HMG17 (122). Moreover, acceleration, presumably due to protein-induced condensation, is frequently encountered in studies of protein binding to supercoiled DNA (79, 161; see the section on supercoil binding, below).

**Effect of charge.** Little information concerning the effect of
protein charge on migration properties is available. In one study in which this question was explicitly addressed, Carey (24) demonstrated that although the relatively acidic Trp repressor efficiently binds its operator over an extensive pH range, significant retardation was detected only at lower pH values. At pH values above the isoelectric point (pI = 6) of the Trp repressor, the negative charge on the protein accelerates the complex and tends to compensate for the mobility reduction due to mass or DNA conformational changes.

Conformation effects. Binding of many proteins to their cognate sites results in bending of the target molecule. The “bent” complex exhibits a lower relative mobility that cannot be explained simply by the additional mass of the protein. This degree of aberrant migration depends both on the bending angle and on the position of the bend with respect to the ends of the DNA molecule (see the section on how DNA migrates in gels, above) (Fig. 3). Conformational changes can therefore play a major role in resolution of complexes from naked DNA. In view of the importance of these conformational changes in generating specific three-dimensional protein-DNA structures necessary for a variety of biological processes, we examine these effects in detail later (see the section on analysis of conformation).

Gel concentration and composition. In general, optimum resolution is obtained at the smallest average pore diameter compatible with migration of the naked nucleic acid through the gel, and therefore polyacrylamide gels usually offer the best conditions for resolving complexes (40). The average pore size of polyacrylamide gels has been estimated to be between 50 and 200 Å (5 and 20 nm) for acrylamide concentrations between 10 and 4% (depending on the degree of cross-linking [57, 146]) and would therefore be expected to impose significant frictional drag on macromolecular assemblies with comparable dimensions such as a complex of Lac repressor tetramer (35 by 30 by 130 Å [3.5 by 3 by 13 nm]) with Lac operator (60 by 13 Å [6 by 1.3 nm]) (96) or on bent structures such as CAP-Lac operator complexes. Typically, 4 to 5% polyacrylamide is used for complexes formed with DNA fragments of a few hundred base pairs and 10 to 20% polyacrylamide is used for oligonucleotides or small RNAs. As expected from these considerations, the separation of protein-DNA complexes from naked DNA increases with polyacrylamide concentration. For example, Prentk et al. (111) found that the $R_L$ of Escherichia coli integration host factor (IHF)-DNA complexes varied linearly with the gel concentration in the range of 4 to 15% polyacrylamide.

In contrast, agarose gels, whose average pore diameters are in the range of 700 to 7,000 Å (70 to 700 nm) (131), do not resolve DNA on the basis of conformational changes (Fig. 1) and are less effective unless the relative mass of protein is large (but see reference 22). For example, CAP-Lac operator complexes which are bent and strongly retarded in 4% polyacrylamide gels are barely resolved from the naked operator fragment in 1% agarose, whereas a complex of the same DNA fragment with RNA polymerase (480 kDa) shows a strong reduction in relative mobility (115). Nevertheless, agarose gels have been used for analysis of larger DNA fragments (>1 kb) (11) as well as for protein-RNA complexes (6). Even for molecules of less than 1 kb, agarose gels may be satisfactory provided loss of resolution can be tolerated (38).

Complex Stability within the Gel

Gel matrix. Early studies (41, 47) showed that protein-DNA complexes appear more stable during electrophoresis than indicated by their kinetic stability in free solution. This was interpreted to indicate a specific gel stabilization effect termed “caging” (41), in which the gel matrix impedes diffusion of dissociated components. The effect of this would be to maintain the concentrations of protein, DNA, and complex at levels at least as high as those in the original equilibrium binding reaction.

There has been some disagreement as to whether the gel matrix influences the dissociation constant. The finding of reduced transfer of Lac repressor from a preformed complex to a naked operator fragment within the gel (41) provided support for a gel-mediated change in dissociation constant. This interpretation was, however, compromised by the large reduction in salt concentration experienced by the complexes on entering the gel (from 40 to 0 mM), which may have increased complex stability (see the section on electrophoresis buffer composition, below). Indeed, measurements of the CAP-lac promoter dissociation constant (116), in which identical salt concentrations were used in solution and in the gel, failed to reveal changes in binding constant.

A detailed model has recently been proposed which can account for the apparent increase in kinetic stability without the necessity of invoking gel-induced changes in dissociation
constants. In this model, dissociation of a complex is postu-
lated to occur in a two-step manner. The first step involves
a reversible dissociation in which the DNA and protein
components remain trapped within the same microscopic
"cell" of the gel, and the second involves an irreversible
macroscopic dissociation when the protein component es-
capes from the cell (22).

Electrophoresis buffer composition. Since protein-nucleic
acid interactions involve an ionic component, the ionic
strength and pH of the buffer can play a major role in
complex stability (see, for example, reference 114). Al-
though to our knowledge no systematic analysis of these
factors has been published, some general features have
emerged.

Low-ionic-strength buffers are usually preferred because
they contribute to detection of complexes in a number of
ways. Compared with high-ionic-strength buffers, they gen-
erate less heat and increase the speed of migration by
increasing the fraction of the current carried by the mac-
molecules. Moreover, they reduce disruption of ionic bonds
and so may have an important effect on stabilization of
complexes following their passage from the salt-containing
binding buffer into the gel (73). Standard electrophoresis
buffers such as TBE (90 mM Tris-borate, 2 mM EDTA [pH
8.3]) and TAE (40 mM Tris-acetate, 1 mM EDTA [pH 7.9])
commonly prove satisfactory, but others such as TE (10 mM
Tris, 1 mM EDTA) have been used (41).

One illustration of electrophoresis buffer-dependent sta-
lility is the case of complexes formed between phage Mu
repressor and its operators. Specific complexes cannot be
detected by using the standard TBE buffer but are readily
detected by using Tris glycine (50 mM Tris-glycine [pH 9.4])
(2).

Buffer components, such as salts of divalent ions (139),
dithiothreitol, and polyamines such as spermine (134), may
also contribute to stability of certain complexes.

Cofactors. For certain proteins, complex formation and sta-
lility may require the inclusion of specific cofactors both
in the initial binding reaction and in the gel buffer. This is
illustrated by proteins such as CAP and the arginine (ArgR)
and tryptophan (TrpR) repressors, which depend on cyclic
AMP (cAMP), arginine, and tryptophan, respectively, for
efficient site-specific binding (24, 39, 138).

Elimination of nonspecific binding. All nucleic acid-binding
proteins that recognize specific sites exhibit some degree of
nonspecific affinity. Nonspecific binding can confuse the
interpretation of gel retardation studies by provoking smearing
of the retarded band or by generating multiple bands.
Discrimination can be enhanced in several ways. Addition of
salt to the binding mixture can increase selectivity by
disrupting nonspecific ionic bonds while leaving other, more
specific, types of interaction unimpaired. A more general
approach is to add excess "nonspecific" competitor nucleic
acid. Although bulk chromosomal DNA from E. coli, salmon
sperm, or calf thymus is often used, the possibility that these
carry specific binding sites has led to the use of synthetic
copolymers such as da-daT and dl-dC or the polysulfated
carbohydrate heparin (see, for example, reference 26). The
amounts added to the reactions must be determined empir-
ically since excess competitor may sequester sufficient pro-
tein to eliminate specific complex formation. It should be
noted that kinetic data have shown that competitor nucleic
acid may act not merely as a sink for spontaneously liberated
protein but also as an active inducer of complex dissociation,
variably by transitory formation of unstable ternary
complexes with the competitor DNA (42, 44).

APPLICATIONS

Characterization of Complex Formation

Binding constants. One of the earliest applications of gel
retardation was the measurement of kinetic and thermody-
namic parameters, in particular association, dissociation,
and equilibrium-binding constants of protein-DNA inter-
actions. Association rates are determined by mixing reactants
of known concentration and adding samples at intervals to a
running gel, whereas for dissociation rates the time course is
begun by addition of competitor nucleic acid to performed
complexes. Binding constants can be determined by measur-
ing the amount of complex formed either as a function of
protein concentration at equilibrium or as the ratio of the
association and dissociation rates. After electrophore-
sis, the concentrations of reaction products are measured by
densitometry of the stained or autoradiographed gel or by
direct measurement of radioactivity in the bands. The valid-
ity of equilibrium-binding constants calculated from such
data depends critically on knowledge of the proportion of
active protein in the initial preparation (see, for example,
reference 42). The association rate for a bimolecular reac-
tion, $k_a$, is the slope of

$$\frac{1}{(P - N)} \ln \left[ \frac{N(P - P/N)(P - N)}{P(N - P/N)} \right]$$

versus time. The dissociation rate for a first-order reaction,
$k_d$, is the slope of $-\ln P/N_0$ versus time. $P$, $N$, and $P/N$ are
molar concentrations of protein, nucleic acid, and complex,
respectively, and $P(N)$ is complex concentration at time zero.
The equilibrium constant, $K_{eq}$, can be derived either as the
ratio $k_{eq}/k_d$ or measured directly as the concentration of
protein at which half of the target nucleic acid is complexed
at equilibrium.

Whereas standard gel retardation is suitable for measuring
dissociation constants of relatively stable complexes (subna-
molar range), quantitation of complexes of low stability is
more problematic. A modified gel procedure, zone interfer-
eence electrophoresis, has been applied to the study of
interactions of the translational elongation factor, EF-
Tu-GTP, with tRNA, which exhibits a dissociation constant
in the range of $10^{-6}$ to $10^{-4}$ M (1). EF-Tu-GTP was coelec-
trophoresed with increasing concentrations of tRNA in a gel
which allowed sufficient diffusion to maintain equilibri-
unbinding conditions throughout the run. Migration of EF-
Tu-GTP was accelerated on interaction with the tRNA, and
the distance migrated (determined by staining of the protein
with Coomassie blue) was a function of the time spent as
complex and hence of tRNA concentration. A Scatchard-
like plot of the distance migrated by the complex as a
function of the tRNA concentration provided a measure of
the dissociation constant.

Cooperativity. Biological processes often involve the bind-
ing of several proteins to a short DNA segment. One feature
of such multiprotein complexes is that the protein compo-
nents frequently bind in a cooperative manner. This may be
the consequence of direct protein-protein interactions at
neighboring sites or may result from protein-induced defor-
mations of the DNA backbone which either facilitate adja-
cent binding of a second protein or bring together molecules
bound at separated sites (see the section on loops and
sandwiches, below).

In general, cooperativity in the gel retardation assay can be
inferred from the underrepresentation of complexes in-
termediate between the unbound and fully saturated states.
This arises by their conversion into the fully saturated state.
within a smaller protein concentration range than would be expected on the basis of successive, independent filling of the binding sites (130).

Multiprotein complexes can be composed of a single protein species or of several different proteins. Situations which involve different proteins binding to distinct sequences are more directly amenable to analysis by gel retardation because the stability of complexes with one protein, in the presence or absence of the other, can be assessed by the sensitivity of complexes to specific competitor DNA (140) (see the section on footprinting, below).

For situations in which a single protein binds to several sites, analysis can be more difficult since the absence of intermediate bands does not necessarily demonstrate that binding is cooperative. An elegant study by Kleinschmidt et al. (73) has shown that for weak interactions of Tet repressor with a fragment carrying two operator sites, underrepresentation of the complex with a single occupied site is due to its preferential loss during electrophoresis. Computer simulations as well as experimental evidence suggested that this phenomenon is a reflection of the different fates of singly and doubly occupied complexes during electrophoresis. If dissociation occurs within the gel, reassociation in the double-occupancy band can only regenerate the original complex. In the single-occupancy band, however, a "disproportionation" reaction can occur in which dissociation of a singly occupied complex in the presence of another sometimes results in the formation of a doubly occupied complex accompanied by the release of a free DNA fragment (and therefore loss of the original complex). High dissociation rates increase the likelihood of this disproportionation and result in a gel mobility pattern which can be misinterpreted as cooperative binding. Not unexpectedly, the effect is exacerbated by long gel running times and by the presence of salt in the gel buffer.

The utility of gel retardation in detecting cooperative binding of a single protein to several sites is illustrated by the interaction of transposon y6 site-specific recombinase (resolvase) with a DNA fragment carrying three neighboring sites in the rea region (69). As the concentration of wild-type resolvase was increased over a 10-fold range, moderately retarded complexes with one occupied site were transformed into a highly retarded complex in which all three sites were occupied. In contrast, over the same concentration range, certain mutant resolvases generated an intermediate complex in which two sites were occupied, suggesting that the mutation affects protein-protein interactions involved in cooperative binding.

An additional manner in which cooperative interactions can be explored is based on phasing (see the section on phasing, below). Addition of DNA in steps of 1 bp between the participating binding sites changes the relative disposition of the two sites and therefore disturbs the protein-protein interactions necessary for cooperative binding. This type of approach can be used to distinguish between cooperative interactions (see, for example, references 61 and 140) and independent binding to closely spaced sites (see, for example, reference 71).

**Stoichiometry.** Stoichiometry is an important parameter in the description of protein-DNA complexes and one which is often tedious to determine accurately. The use of apparent mass changes is not generally applicable to this problem in view of the potential complications of charge and conformation effects on migration (see the sections on resolution of complexes, above, and analysis of conformation, below). However, mass increments can often be exploited in a different way. Truncated or extended derivative proteins which exhibit wild-type binding (and multimerization) properties will, when mixed with the wild-type protein, give rise to additional bands reflecting the number of protein monomers bound. Thus, for the yeast transcription control protein, GCN4, a mixture of full-length and truncated protein added to a DNA fragment carrying a single binding site gave rise to an intermediate third band in addition to those formed by each of the proteins separately. Another example of this approach is shown in Fig. 6. The additional band presumably represents a complex containing a single molecule of each protein species and strongly suggests that GCN4 normally binds as a dimer (66). Similarly, the appearance of three extra bands upon binding by a mixture of wild-type phage P22 Arc repressor and a longer repressor derivative suggested that this protein binds as a tetramer (17). An extension of this approach is to use monoclonal antibody Fab fragments specific for epitopes of the DNA-binding protein which are accessible in the complex. Addition of these to the preformed complexes can result in an additional retardation. The number of increments in retardation can provide an estimate of the number of protein monomers bound (98).

Apart from such specific cases, determination of absolute stoichiometry requires precise measurement of the molar ratios of protein and DNA in a given complex. This is generally accomplished by labeling protein and DNA to known specific activities with different isotopes and measuring the ratio of isotopes in a given band (39, 48, 58). Alternatively the amount of protein in a complex can be determined by quantitative Western immunoblotting of the retardation gel (51) or, less accurately, by Coomassie blue staining (41).

**Defining protein composition.** Separation of protein-DNA complexes by gel retardation provides a convenient means of identifying protein components which is particularly useful when analyzing complexes generated by using crude or partially purified extracts (see the section on cell extracts, below).
Participation of candidate proteins in a complex can be confirmed by using unlabeled probe DNA and crude or partially fractionated cell extracts in which the protein has been selectively labeled. A powerful method for selective labeling involves the use of the rifampin-resistant phase T7 RNA polymerase to transcribe a cloned gene under the control of a phage T7 promoter (143). Comparison of retarded bands consisting of unlabeled extract and labeled probe DNA with those containing selectively labeled extract and unlabeled probe can be used to determine whether the cloned gene product binds specifically to the target DNA (157). Extracts of uniformly labeled cells can also be used for isolating sufficient labeled protein in a retarded band to determine its size (13, 120). Such an approach may allow identification of the protein in the retarded complex by microsequencing.

Another approach to defining protein composition is the use of specific monoclonal antibodies. The antibody can be added prior to electrophoresis to give rise to a supplementary reduction in complex mobility (81), or it can be used to probe Western blots of the retardation gel (51) (see the section on stoichiometry, above).

When extracts from various related sources produce retarded complexes with a given DNA probe, the question of whether the binding proteins responsible are identical to each other may be resolved by partial proteolysis of the complexes before electrophoresis (the proteolytic band-clipping assay). The pattern of bands formed by species ranging from the whole protein to the minimal peptide capable of binding and retardation can be used as a specific signature (127). Using this approach, Scholer et al. (126) were able to distinguish a number of different mouse proteins which bind to a cis-acting octamer motif common to several promoters but generate complexes with similar relative mobilities.

**Protein domains.** The DNA-binding properties of a protein can often be ascribed to a relatively small domain. Defining a DNA-binding domain within a protein has been greatly facilitated by gel retardation techniques. Two principal approaches have been used. Partial deletion (see, for example, references 64 and 155), or fusion of parts of the gene with a reporter gene, provides sets of truncated proteins whose binding activity can be monitored directly. Using the partial-deletion approach, Hope and Struhl (64, 65) developed an efficient system for domain mapping of the yeast transcriptional regulator, GCN4. Truncated proteins, produced from a set of deleted genes by in vitro transcription and translation, were added directly to probe DNA for mobility shift assays. The smallest peptide capable of specific binding consisted of the C-terminal 60 amino acids of the protein.

An alternative approach is to use partial proteolytic cleavage of the purified protein, followed by gel retardation, isolation of the bound peptide, and localization on the cleavage map of the protein (27).

**Thermal stability.** Measurement of differential thermal stability of complexes by using various mutant proteins can provide information on protein structure and binding functions. This can be directly assessed by using temperature gradient gel electrophoresis devices which were originally developed for the study of structural transitions in nucleic acids (119). Such devices generate a temperature gradient perpendicular to the direction of migration, and structural or conformational transitions are visualized as changes in migration rates across the gel. Although not yet in general use, these devices have proved useful in comparing the thermal stabilities of complexes formed with wild-type and mutant Tet repressors and in characterizing denaturation intermediates (119, 149).

**Defining Binding Sites**

A major part of the study of protein-DNA interactions is the definition of the site bound by a given protein. The first step is generally the localization of the binding site by footprinting in solution. This can also provide some information concerning specific protein-DNA contacts and the length of DNA covered by the protein, but it does not provide a detailed picture of all the individual base pairs involved in binding. Gel retardation can be used to characterize essential components of a binding site such as a consensus binding sequence or more subtle interactions with flanking DNA (see the section on bending and binding affinity, below).

**Footprinting.** Although nuclease protection of unfracti-onated DNA-protein mixtures effectively identifies a binding site by providing access to interactions at the nucleotide sequence level, removal of uncomplexed DNA by fragmentation, using gel retardation, can sometimes increase the sensitivity of footprinting analysis (115). Chemical or enzymatic attack of complexes can be carried out either before gel separation or within the gel after electrophoresis.

One of the first procedures to be used in this way was that of interference, in which target DNA is partially methylated (N-7 of guanines in the major groove and N-3 of adenines in the minor groove) or ethylated (backbone phosphates) before complex formation and separation on the gel (58, 155). Alternatively, DNA can be partially depurinated or depyrimidinated (19). Retarded complexes and uncomplexed DNA are purified, chemically cleaved at the modified positions, and compared on a sequencing gel. Modification at positions involved in interaction with the protein will interfere with binding and result in their underrepresentation in the gel-resolved complex. In situations in which a DNA fragment carries multiple binding sites for a protein and thus gives rise to multiple retarded bands, this technique can be used to correlate site occupation with a given complex (see, for example, references 18 and 118).

Nuclease attack of preformed complexes before gel separation has also been used in certain cases (115, 135). The approach suffers from the drawback that exchange of protein molecules between alternative binding sites on the DNA fragment could occur in the time between treatment and separation on the gel by "microdisassociation" or sliding (see the sections on cooperativity, above, and sliding and protein redistribution, below). This would "average" protection over all sites located on the fragment. The severity of this effect depends critically on the strength of the particular protein-DNA interactions and the time required for the complex to enter the gel. A relatively new method which may overcome these problems is UV-laser cross-linking of the protein to its site before electrophoresis (62). It has been applied with some success in the analysis of CAP and RNA polymerase interactions with the lac promoter (20).

An alternative approach is direct footprinting within the gel, using 1,10-phenanthroline-copper ion, an effective in situ footprinting reagent because of its small size and the ease with which it penetrates the gel matrix (83). The entire gel or isolated gel slices can be simply incubated in the reagent. DNA is then isolated from the gel, deproteinized, and analyzed on a sequencing gel. Some success has also been reported with DNase I (140). It must be kept in mind, however, that, like nuclease attack before gel separation,
chemical attack in situ may provide misleading results when intermediate occupation of several sites by a single protein molecule is being analyzed.

**Binding requirements.** Because of its sensitivity, gel retardation can be used to detect specific binding to low-affinity binding sites, thereby allowing the establishment of a hierarchy of affinities among a collection of mutant sequences. An obvious limitation lies in the number of different DNA sequences which can be examined separately. Use of the polymerase chain reaction can circumvent this problem. Conclusive sequences for various proteins have been defined by examining protein binding to a heterogeneous population of molecules (14, 52, 94, 109, 144, 148). Starting with a population of double-stranded oligonucleotides carrying random variations within the binding site, rare molecules recognized by the protein are recovered by gel retardation and amplified by the polymerase chain reaction. In principle, sequencing this population to determine the base frequency at each position could generate the consensus binding sequence (50). In most cases, however, the authors have first fractionated the molecules by cloning and then characterized them individually.

Nucleic acid sequence requirements for protein binding can also be assessed by using double-stranded oligonucleotides with various sequence differences as competitors in the binding reaction. Examples of this approach are presented in the following two sections.

An elegant technique for investigating the more subtle influence of flanking DNA and for defining a minimum size of a protein-binding site has been developed by Liu-Johnson et al. (85) with CAP-DNA interactions as a model. Partial primer extension was carried out on complementary DNA strands containing a CAP-binding site to generate two populations of double-stranded oligonucleotides in which members differed in length by 1 bp. Each population was subjected to gel retardation in the presence of CAP. The complexes were isolated, and the length distribution of the complex DNA was determined by electrophoresis on a denaturing gel (Fig. 7). This indicated that a 28- to 30-bp region, the "thermodynamically defined" binding site which extends approximately 15 bp on either side of the center of dyad symmetry of the consensus site, is necessary for strong protein retention. Such an approach should be applicable to analysis of other protein-binding sites.

**Non-sequence-specific DNA binding.** The protein-DNA complexes mentioned so far to illustrate gel retardation analysis have been characterized by highly sequence-specific interactions. Many proteins show limited or no sequence specificity in binding. Non-specific binding can, however, give rise to defined retarded bands under certain conditions, enabling the method to be extended to the analysis of this type of interaction. Examples of such protein-DNA interactions are found with CAP, the small prokaryotic "histonelike" proteins (IHF, HU, and TF1), proteins involved in nucleosome assembly, and the E. coli RecA protein which catalyzes homologous recombination.

Non-specific binding of CAP has been studied either by omitting the required binding cofactor, cAMP, or by presenting the protein with a DNA target which lacks specific binding sites. The first approach results in the appearance of discrete bands which do not appear to exhibit the characteristic CAP-induced bend (68) (see the section on analysis of conformation, below). In the second case, CAP binding is highly cooperative (see the section on cooperativity, above) and results in the appearance of very low mobility species which hardly enter a polyacrylamide gel (42).

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**FIG. 7.** Defining a binding site by primer extension. The consensus sequence for protein binding is shown as solid circles. Partially double-stranded molecules generated by primer extension are retained by the protein as a function of their binding affinities. Maximum binding affinity is obtained for molecules in which synthesis has extended slightly 3' to the consensus (shaded circles). The "thermodynamic" binding sequence (indicated by the cross-hatched area in the middle of the figure) is defined by comparison of binding of molecules in which synthesis was on the top strand (left; solid line) with those in which synthesis was on the bottom strand (right; dot-dash line) (85). Some of the closely related prokaryotic histonelike proteins, such as IHF and TF1, bind with relatively high specificity, whereas others, such as HU, bind DNA with no apparent sequence specificity. It has recently been shown, by using small double-stranded oligonucleotides, that HU can generate defined retarded bands (16) whose number depends on the size of the target DNA (at least up to 42 bp). A systematic study of the binding of these proteins (125) shows that defined retarded bands can be observed in all three cases and suggests that the proteins may bend the target DNA in a similar manner (see the section on sliding and protein redistribution, below).

The assembly of protein-DNA structures such as nucleosomes is also characterized by non-sequence-specific protein-DNA interactions. Individual eukaryotic histones do not retard DNA in a sequence-specific manner, although it was observed some time ago that nucleosome band shifts occur in the presence of the nonhistone chromosomal proteins HMG-14 and HMG-17 (91) and that their affinity for core particles is higher than for histone-free DNA (122).
Retardation studies have revealed that sequence-independent loading of core DNA with histones H2A and H2B results in the appearance of five defined retarded bands, corresponding to the successive binding of protein dimers (3). Moreover, binding of histone H1 to larger DNA fragments, like nonspecific binding of CAP, is highly cooperative and gives rise to complexes which do not enter the gel, although small amounts of high-mobility complexes can be observed. These correspond to successive loading of DNA fragments with histone H1 monomers (71a).

The RecA protein binds to single- or double-stranded DNA. Binding can be monitored on agarose gels through the disappearance of the naked DNA band to form a heterogeneous mixture of retarded complexes (88, 136). In this case, individual complexes can be visualized as a more compact band by prior fixation of the complexes with glutaraldehyde (136).

With these examples in mind, it is clear that although gel retardation is useful in exploring non-sequence-specific DNA binding, in general the conditions required depend on the particular proteins involved.

**Mismatches, four-way junctions, and single-stranded DNA and RNA.** Specific interaction of a protein with a nucleic acid molecule can result from recognition of structural motifs rather than nucleotide sequence and can also occur with single-stranded nucleic acids. Such motifs include bends, distortions of the DNA helix introduced by mismatches, hairpins, and four-way DNA junctions such as Holliday structures (63). Although secondary structure plays an important role in determining the migration properties of the naked nucleic acid molecule, gel retardation can nevertheless be effectively exploited in exploring these types of interaction.

For example, binding of the E. coli H-NS protein was investigated by using band shift assays involving competition between double-stranded oligonucleotides with and without A-tract-directed bends. The protein was shown to exhibit a strong preference for bent DNA (154). A second example, which has permitted evaluation of the contribution of structural parameters that can influence binding, is the methyl-directed mismatch repair system of E. coli. This system recognizes and corrects both dG/dT and dA/dC mismatches at comparable efficiencies in vivo. Gel retardation demonstrated that a mismatch recognition protein, HU, is capable of generating specific large DNA hairpins and short double-stranded oligonucleotides carrying either the dG/dT or dA/dC mismatches. Moreover, oligonucleotide competition was used to determine a hierarchy of binding affinities for different combinations of mismatches. The use of oligonucleotides containing base analogs coupled with variation in the pH of the binding reaction allowed specific proposals as to the nature of the structural change which might be recognized (70). Confirmation of the molecular basis of these interactions at this level, however, requires additional and more powerful approaches such as nuclear magnetic resonance analysis.

Many proteins exhibit preferences for single-stranded DNA or RNA and show sequence or secondary structure specificity. As with double-stranded DNA targets, gel retardation can provide an effective means of analyzing and defining important recognition determinants and, moreover, of distinguishing between structural and sequence components.

Its use in this context is illustrated by the following examples. (i) For single-stranded DNA, a specific nuclear protein, H16, from cultured monkey cells was inferred to bind in a sequence-specific manner in the control region of the late coding strand of simian virus 40 (45), since the sequence of the bound single strand showed no obvious potential for the formation of secondary structure and no complexes could be detected by gel retardation with the corresponding RNA, complementary single-stranded DNA or double-stranded DNA (44).

(ii) A striking example of structural recognition is that found with Holliday junctions. These structures are intermediates in homologous recombination (63), in which two duplex DNA molecules are linked at a single crossover point. Such linked intermediates must be resolved by further cleavage to generate the final two recombined products. Gel retardation has proved incisive in identifying enzymes from bacteriophages T4 and T7 and from E. coli which specifically recognize (and cleave) these four-way junctions with little or no preference for nucleotide sequence (36, 105, 106). Another common type of structure which can provide specific recognition properties consists of hairpins/loops generated in various mRNAs. Binding of a translational inhibitor to the iron-responsive element in rat transferrin mRNA was shown to depend primarily on the presence of a stem-loop structure and was little affected by base sequence (8).

(iii) Sequence and structural elements can together play important roles in recognition. Thus binding of the human immunodeficiency virus trans-acting transcriptional activator, Tat, to the 5' end of the long terminal repeat transcript, TAR, was shown to require a bulge-bend within a duplex region as well as a uracil residue in the bulge and two specific base pairs in the adjacent duplex (34, 151).

**Genome scanning.** The ability of gel retardation to select rare molecules from a large population has been applied to localizing specific binding sites within genomes. One major problem with this type of analysis is that the complexity of the DNA molecules could prevent the resolution of some retarded fragments since they might migrate with larger noncomplexed fragments. To overcome this problem, a simple two-dimensional gel electrophoresis method has been developed (15). Restriction digests of complete genomes are first subjected to a normal gel retardation assay, and the retarded fragments are then resolved by electrophoresis in a second dimension under conditions (e.g., high temperature) which disrupt the complexes and allow the fragment to migrate according to its real size, ahead of the diagonal (Fig. 8).

Core DNA has been used to localize IHF-binding sites on bacteriophage Mu and λ genomes and on the E. coli chromosome.

**Analysis of Conformation**

Protein-induced bending was first proposed to explain the observation that the "eccentricity" of the binding site influences the migration of complexes between the activator protein CAP and the lac, mal, and gal operators (74, 153) (Fig. 3a and c). Similar conclusions were also reached by modeling CAP-DNA interactions on the basis of CAP crystal structure (150). The proposal has been confirmed independently for complexes in solution by the demonstration of enhanced ligase-catalyzed circularization (72) and by circular dichroism measurements (110).

**Permutation.** The effect of eccentricity on relative migration offers a simple and general method of detecting protein-induced bending and of localizing the binding/binding site. This property was first analyzed systematically for CAP. A permuted set of DNA fragments was generated from a tandem dimer of a lac operator fragment by using a set of
restriction sites found once in each monomer (Fig. 3b). The CAP-binding site was therefore located at various distances from the ends of the molecules (153). Measurements of the relative mobilities of complexes formed between molecules and CAP demonstrated that the greatest migration effect occurred when the binding site was in the middle (Fig. 3c and d). The point of minimum relative mobility obtained by extrapolation yielded the position of the bend center, which could be resolved to within a few base pairs. Specific vector plasmids have since been developed to facilitate this type of permutation analysis. They permit cloning of a single target sequence between tandem dimers of a series of defined and suitably spaced restriction sites (72, 111, 112), thus obviating the dependence on restriction sites within the cloned fragment.

Phasing. Since migration of DNA is determined by end-to-end distance, the apparent location of the bend center determined by permutation analysis will be sensitive to the presence of neighboring bends (85). Rotation of one bend with respect to the other, by addition of short DNA segments, results in a sinusoidal variation of end-to-end distance with a periodicity of one helical turn (Fig. 4). If two bends lie in the same plane and have the same direction (cis isomer) the end-to-end distance should be minimal and the effect on migration should be maximal. Curvature in opposing directions (trans isomer) increases the end-to-end distance, and the migration effect should be minimal.

The dependence of end-to-end distance, and therefore migration, on overall curvature can be exploited to determine the direction of bending at a given site with respect to a reference bend carried by the same DNA fragment. Two studies have addressed the A-tract-directed bend of k-DNA (see the section on how DNA migrates in gels, above). In one of these (159) the reference used was the CAP-induced bend in the lac operator, which had been inferred from extensive footprinting and modeling to occur into the minor groove toward the protein in the middle of its binding site. In the other, Salvo and Grindley (121) used the y6 resolvase-induced bend at the res site, which, on the basis of similar types of evidence, occurs into the major groove. Despite the different geometry induced by protein binding at these two sites, both sets of phasing results predicted that the k-DNA bends into the minor groove at the center of each A tract.

A recent analysis of the phasing phenomenon has exposed a potential problem in interpretation of this kind of result but has provided some clues to the way in which DNA may migrate within the gel matrix. Using a set of fragments containing two A6 tract-directed bends, Drak and Crothers (35) observed that the relative mobilities of bands phased at +2 and −2 bp from the trans isomer (Fig. 4a) were, respectively, higher and lower than expected. This difference increased with increasing gel concentration. As shown in Fig. 4a, the cis and trans isomers are both planar. When the two bends occur out of the plane, they create an overall twist on the molecule and generate either a left-handed (+2 bp) or a right-handed (−2 bp) superhelix. It was suggested that this superhelical twist on the molecule facilitates migration when it is in the same sense as the DNA helix (right handed) and impedes migration when it is in the opposite sense (left handed). This behavior may reflect a screwlike component to the motion of DNA through the gel. Since it is not known whether DNA carrying a bend-inducing protein has sufficient freedom to respond in the same way, the impact of this phenomenon on the determination of bend geometry derived from phasing experiments is not yet clear.

Angle. Many other proteins have been shown to induce curvature as judged by the permutation effect (147). The amplitude of the permutation curve differs in each case, suggesting that each protein bends its cognate DNA site to a different extent.

To estimate bending angles, Thompson and Landy (145) constructed a set of DNA fragments with increasing degrees of curvature to generate a standard plot of relative mobility versus bending angle from which the angle of a protein-induced bend could be derived by interpolation. The set of curved standards consisted of fragments carrying various numbers of A6 tracts. By using an A6-directed bend angle of 18°, estimated previously by electric dichroism and ligase-mediated circularization, the overall curvature of each frag-
ment was calculated as the sum of the bend angles contributed by each \( A_b \) tract.

Provided that precautions were taken to correct for the contributions of protein mass and intrinsic curvature to the mobility of the DNA carrying the binding site, and provided that the test fragments were of similar length to the standards and were within the range of 300 to 500 bp, a value of 50 to 56° was obtained for the \( E_{coRI} \)-induced bend angle. This result is consistent with predictions based on the structure of \( E_{coRI} \)-DNA cocrystals (95).

An alternative way of analyzing these data arose from the observation that the relative mobility of curved DNA fragments, in which the bend occurs in the middle, can be described by the equation

\[
R_e = \cos(\alpha/2) \quad \text{(where } \alpha \text{ is the bend angle)}
\]

for 8% polyacrylamide gels. The more general case in which the bend occurs at various distances from the end can be described by the equation

\[
h^2 = 1 - 2x(1 - \cos \alpha) + 2x^2(1 - \cos \alpha)
\]

where \( h \) is the end-to-end distance and \( x \) is the fractional distance of the bend from one end. The bend angle can thus, in principle, be calculated directly. This approach is useful for moderate angles but yielded a value for \( CAP \)-induced bending of about 140°, in poor agreement with that later obtained from crystallographic data (90° (129)).

A related method for determining protein-induced bend angles has been developed by Zinkel and Crothers (160). In this case, a standard curve was generated by plotting the relative mobility against the square of the number of \( A_b \) tracts. This treatment was based on the quadratic relationship previously observed between these parameters (77) (see the section on how DNA migrates in gels, above). The relative mobility of the \( CAP \)-DNA complex was found to be equivalent to 5.6 \( A_b \) tracts and gave a \( CAP \)-induced bend angle of 101°, in better agreement with the angle obtained by Schultz et al. (129).

The disparity between the two gel mobility-based determinations of the \( CAP \) bending angle was probably due to differences in phasing of the \( A_b \) tracts within the fragments used to generate the standard curves. Inappropriately phased \( A_b \) tracts result in nonplanar molecules whose end-to-end distance is not a minimum. Consequently, relative mobilities deviate from those of equivalent planar molecules. This deviation increases with the number of \( A_b \) tracts and results in a higher mobility (higher \( R_e \) values) than expected for a given angle of curvature. The angle of protein-induced bends determined from their \( R_e \) values would thus be progressively overestimated with increasing angle. Bend angles estimated from standard fragments with phasing at 10.5 bp (160), close to the helix repeat, are expected to be more accurate than those estimated from fragments with phasing of 10 bp (145).

In view of the apparent change in the mode of migration of strongly bent (>120°) DNA (see the section 1) it seems unlikely that gel retardation can be used to accurately measure large protein-induced bends such as those induced by IHF.

**Bending and binding affinity.** DNA bending must be associated with unstacking of neighboring base pairs. The affinity for a given binding protein which induces bending is therefore likely to depend not only on sequence-specific recognition but also on the ability of a given site to be deformed. The binding sites of several proteins are known to carry sequence-directed bends. For phage \( \lambda \), gel retardation was used to demonstrate that a sequence-directed bend found in the replication origin is essential for binding of the replication initiation protein (156).

For \( CAP \), whose binding site is shown below,

\[
\ldots \text{AAGGCAATTAAATGGTGGATTGCTCATTACCGACAC} \ldots \ P_{\text{Ro}}
\]

| consensus | thermodynamic |
|-----------|---------------|

the magnitude of the induced bend, as judged by the amplitude of the retardation effect, can be varied by changes in sequence outside the consensus binding sequence (doubly underlined) but within the thermodynamically defined binding sequence ( singly underlined) (85) (see the section on binding requirements, above). The data show that increasing G and C substitution for bases within the sequence AAT TAA (bold letters), which directly flanks the consensus nucleotides, reduces bending, as does A and T substitution within the sequence CGC located approximately half a helix turn further upstream (49). The changes in the degree of bending, as judged by the results of permutation experiments, are accompanied by concomitant changes in \( CAP \)-binding affinity. Since model building indicates that bound \( CAP \) contacts the minor groove of the A+T-rich region and the major groove of the G+C-rich region, this result implies that the DNA external to the consensus nucleotides bends round the protein by compression of the minor groove at the A+T-rich sequence and of the major groove at the correctly phased G+C-rich region. A similar A+T-rich sequence flanking the consensus nucleotides required for binding of the LexA repressor in the colicin A (\( caa \)) promoter is thought to facilitate LexA-mediated DNA bending (86), since LexA binding to the \( recA \) promoter, which does not carry an equivalent sequence, does not induce significant bending.

Another example concerns the Xenopus transcription factor TFIIIA, which has been shown to bind to its cognate site in low-salt buffers but does not induce significant bending (as judged by a permutation assay) under these conditions. Addition of salt (10 mM NaCl) to the electrophoresis buffer, however, uncovers a pronounced TFIIIA-induced bend (128). This behavior has been attributed to the effect of salt on the flexibility of DNA (56) and to the relatively large recognition site of this zinc finger motif binding protein. These and other results of gel retardation studies are generally consistent with the notion that, for proteins which bend DNA, the binding affinity and degree of bending are often closely linked. However, other explanations have been proposed to account for the relationship between binding affinity and apparent degree of bending (see below).

**Sliding and protein redistribution.** Intramolecular transfer of proteins from site to site on DNA is thought to play an important role in vivo. One way in which this may occur is by sliding along the DNA molecule (10). Indeed, gel retardation has been used to demonstrate protein sliding in solution in the case of simian Ku protein (104). Although this protein appears to require DNA ends for initial binding, subsequent cleavage of bound DNA fragments with a restriction enzyme indicated that internal DNA fragments are also retarded. This observation implies that the protein rapidly slides along the DNA with no preferential binding to specific sequences.

In principle, redistribution of protein molecules on a DNA fragment could occur within the gel during electrophoresis. Microdissociation and reassociation have already been invoked to explain disproportionation with the accompanying loss of complexes during electrophoresis (see the section on
FIG. 9. Protein (re)distribution during electrophoresis. A DNA fragment with three binding sites for IHF (a, b, and c) is shown at the bottom. The diameters of the circles indicate relative binding affinities. The affinity of one end site (c) decreases from left to right. The top section illustrates the behavior of the fragment during electrophoresis. It gives rise to three retarded complexes (complexes 1, 2, and 3) involving one, two, and three IHF molecules, respectively. A hypothetical distribution of a single protein molecule between these sites in band 1 is shown as shaded peaks. It is proposed that the protein is partitioned among the sites, as a function of their relative affinities, either by disproportionation or by sliding. As the affinity of the end site is reduced (by mutation), the protein is increasingly partitioned into sites a and b (left to right). Since IHF induces a pronounced bend on binding, increased occupancy of the central site (whose contribution to retardation is large) results in a reduction in the overall relative mobility of the complex. Similar behavior would be expected for band 2, in which two protein molecules are complexed with the fragment (111).

cooperativity, above), but sliding of the protein from one site to another on a DNA fragment may also occur. Both types of protein redistribution would be expected to have an impact on electrophoretic mobilities, which could offer an alternative explanation for the relationship often observed between the degree of bending (retardation) and binding affinities described above.

In many cases it is observed that DNA fragments carrying multiple (n) sites for a DNA-binding protein yield only n specifically retarded complexes, each presumably representing the progressive loading of the DNA fragment with 1 to n promoters of the protein (see, for example, reference 111). Independent occupancy of each site would be expected to lead to the formation of $2^n - 1$ (and not n) complexes. For example, a fragment with three specific binding sites should generate a total of seven complexes (three for the separate occupancy of each site, three for the simultaneous occupancy of two sites, and one for the occupancy of all three sites).

In the case of such a fragment carrying three IHF-binding sites, only three complexes are observed (Fig. 9). Such behavior could be explained by a hierarchy in binding affinities between the sites such that one site is always occupied before the others. An alternative explanation, however, is that the protein "sees" and is continuously partitioned among all the sites on the DNA fragment during electrophoresis. The average mobility would therefore be a reflection of the average distribution among sites. IHF appears to behave in just this way. Mutations which progressively decrease the affinity of a site situated near an end (111) led to a concomitant gradual reduction in the mobility of the lower retarded bands (Fig. 9). The interpretation of this behavior was based on the known effects of binding-site position on mobility: protein binding at a central site causes a relatively large reduction in mobility, whereas binding at terminal sites has a small effect (see the sections on how DNA migrates in gels and permutation, above). Hence, as the affinity of the terminal site is decreased, the protein spends a correspondingly higher fraction of its time at the higher-affinity central site, a position which, owing to induced bending, would contribute in a major way to overall retardation.

Additional evidence suggesting that protein is partitioned among sites during electrophoresis has been obtained from the analysis of mobilities of complexes formed between the closely related proteins IHF, HU, and TF1 (all of which induce target bending [111, 125, 137, 152]) and various circularly permuted target molecules. Flattening of the permutation curve (reduced apparent bending) was observed in certain cases of nonspecific binding of these proteins. As shown in Fig. 10, this is still compatible with sharp protein-induced bending and was interpreted as reflecting the (re) distribution of promoters among the many (nonspecific) "sites" on the fragment (125). If redistribution were to occur rapidly compared with the migration rate, the effect of protein-induced bending would be averaged out over the population of complexes and thus would reduce the amplitude obtained in permutation experiments.

Although all these results are consistent with protein redistribution during electrophoresis, they do not distinguish between sliding and microdissociation/reassociation. A critical test of these hypotheses has yet to be devised.

**Loops and sandwiches.** Several operons carry multiple binding sites for regulatory proteins within their control regions. This arrangement has suggested that certain proteins might be capable of interacting with pairs of sites to form a DNA loop. Such structures were proposed, on the
basis of genetic evidence, to be involved in the control of expression of the gal, lac, deo, and ara operons (for reviews, see references 28 and 93).

The use of gel retardation in confirming and physically analyzing these types of interaction is exemplified by the lac operon. Interaction of the tetrameric Lac repressor at low concentrations with a linear DNA fragment containing two operators was shown to generate a single highly retarded complex (80). This resulted from simultaneous binding of a single repressor tetramer to both operators to form a loop, visible by electron microscopy (Fig. 11C). Raising the repressor concentration chased the loop complex into a faster-migrating species in which each operator was filled by a separate repressor molecule (Fig. 11E). The importance of the tetrameric form of the Lac repressor in this behavior was demonstrated by the finding that such looped structures are not generated with mutant repressor unable to form tetramers (102).

Because the formation of loops involves similar interactions on both sides of the repressor tetramer, it should be sensitive to helix phasing between the operators. When the spacing between the two operators was varied in 1-bp steps, the loop band appeared cyclically with a periodicity of one helical turn (about 10.5 bp) (80) (Fig. 11B and C).

The potential of a protein to form loops can also be tested by determining whether it can act as an intermolecular bridge to link two fragments in a sandwich structure. Such structures are formed by Lac repressor with two operator-carrying fragments (80) (Fig. 11D). By using two DNA fragments of unequal size, sandwich structures were readily identified in gel retardation experiments as bands intermedi-}

ate in mobility between the bands formed with either fragment alone.

The biological effect of loop formation is to provide a greater degree of control by promoting cooperative transcriptional repression in vivo and is reflected in vitro in the formation of more stable repressor-operator complexes.

Looping between two sites by a protein can be promoted by a second protein binding to an intervening site. This behavior can also be analyzed by gel retardation. In the case of bacteriophage Mu repressor, two binding sites, 01 and 02, in the phage early operator region are separated by an IHF-binding site (60). Although purified Mu repressor protects these sites from nuclease attack in solution, repressor-mediated retardation cannot be observed in standard TBE buffers (see the section on electrophoresis buffer composition, above). On the other hand, IHF generates a slowly migrating complex with this DNA, consistent with the characteristic IHF-induced bend. Addition of repressor to such complexes results in the appearance of bands which are further retarded and implies that the bend introduced by IHF stabilizes the binding of repressor to 01 and 02, presumably by promoting a repressor-dependent bridge between the two sites (2, 46).

**Supercoil Binding**

Intracellular DNA is in general topologically constrained into negative supercoils, a state which might be expected to influence protein-DNA interactions. Study of protein binding to supercoiled DNA should therefore yield information more relevant to in vivo conditions. Gel retardation is, however, more laborious with supercoiled than with linear DNA because of the restricted size range (under 600 bp) of closed-circular molecules which can enter a polyacrylamide gel and be resolved (67, 133). Although attempts have been made to use mixed polyacrylamide-agarose gels to resolve protein complexes with larger molecules (plasmids of over 3 kb [60]), this has met with only limited success. Circles of ideal size for detailed study are too small to be isolated as replicating plasmids and must be prepared either in vitro by ligation of linear fragments or in vivo by using vectors which permit circle formation by defined site-specific recombination reactions.

The first experiments reporting the use of closed-circular DNA in gel retardation assays were designed to detect binding of an antibody raised against left-handed Z-DNA (101). DNA circles carrying different numbers of supercoils were prepared by ligation of approximately 350-bp 5'-end-labeled fragments in the presence of various concentrations of ethidium bromide. Anti-Z-DNA monoclonal antibody was observed to retard specifically those topoisomer circles which had sufficient torsion to generate a region of Z-DNA. Moreover, the relative affinity for different topoisomers could be quantitated and provided an estimate of the effect of the level of supercoiling on the formation of Z-DNA.

The supercoil retardation technique has been applied to the study of the lac operon, and the results have underlined the importance of supercoiling in modulating protein-DNA interactions. Repressor-binding affinities for individual gel-purified topoisomers of a 452-bp fragment containing one or two ideal operators were found to increase with increasing negative supercoil density. Binding to circles containing one operator gave rise to a retarded complex. In contrast, circles containing two operators, and thus having the potential for loop formation, gave rise to two complexes of different mobilities. Some topoisomers generated a retarded complex,
whereas others gave rise to an accelerated complex. The acceleration of the complex was ascribed to a reduction in hydrodynamic volume resulting from supercoiled density-dependent loop formation. The relative migration of such complexes could be varied by inclusion of \( \text{Mg}^{2+} \) in the gel buffer. This increases the helix twist and can permit finer resolution. Negative supercoiling was found not only to stabilize the looped complex but also to change the optimal spacing required for loop formation between the two ideal lac operators from that observed for linear DNA fragments (79).

This analysis has been extended to show looping with the natural lac operators, O1, O2, and O3 (37), and has been used to demonstrate the formation and destruction of a repression loop in the ara control region (87).

Measurement of the relative affinities of proteins for supercoiled substrates in these studies was based on titration of the supercoiled complex with added specific linear competitor DNA. In principle the relative affinity of a protein for supercoiled DNA can be determined by inverting the procedure and titrating complexes formed on a radioactively labeled linear DNA fragment by addition of known quantities of unlabeled supercoiled test DNA (see, for example, reference 107). In this case, there is no size limitation on the supercoiled DNA and the binding region of interest can be simply prepared in a suitable plasmid backbone.

### Cell Extracts

Another powerful application of gel retardation is to define DNA-binding proteins from crude cell extracts and to serve as a means of monitoring their purification. Since crude extracts contain many sequence-specific and -nonspecific DNA-binding proteins, the use of sufficient unlabeled carrier DNA (see the section on elimination of nonspecific binding, above) together with the specific labeled target sequence can be critical. Nonspecific binding may also be diminished by reducing the length of the target molecule to the minimum necessary for full stability of the specific complex. The utility of crude extracts for gel retardation analysis was first demonstrated in defining a DNA-binding protein from African green monkey cell nuclei (142), and the technique has been extended to cell-free extracts from yeasts (4) and bacteria (97, 157).

Bacterial extracts may contain significant levels of DNase activity (mostly exonucleases) which can remove the radioactive label from end-labeled DNA probes. The problem can be overcome by using homogeneously labeled fragments generated by the polymerase chain reaction or end-labeled fragments including an \( \alpha \)-phosphothioate moiety which is more resistant to 3',5'-exonuclease activity (82, 113). Alternatively, provided that protein binding, unlike exonuclease activity, can occur in the absence of divalent cations such as \( \text{Mg}^{2+} \), EDTA can be added to the binding reaction. The nucleosome problem can be further alleviated if the protein of interest is overproduced from a cloned gene, since smaller amounts of extract can then be used.

There are several advantages in using cell extracts. Perhaps the most obvious is that, with the use of cloned and overexpressed genes, many mutant derivatives of a protein can be analyzed efficiently. Moreover, by growing cells under different conditions before extract preparation, the dependence of the activity of endogenous proteins on the physiological state of the cells can be assessed directly (4). Crude extracts also allow access to components of multiprotein complexes and, by use of mutants, can uncover a dependence of binding of one protein on the presence of others (see, for example, reference 46).

In addition to determining the protein components in given complexes (see the section on defining protein composition, above), it is possible to determine the protection pattern afforded by binding (see the section on footprinting, above) and to estimate the relative binding constants of a collection of mutated binding sites and hence to define the sequence determinants of binding (158).

### Analysis of Dynamic Processes

The applications of gel retardation described above have concerned relatively simple "static" protein-DNA interactions. The method also lends itself to analysis of sequential reactions which occur during gene expression. This is illustrated by studies on transcription initiation, where it has proved a sensitive method for analysis of ternary transcription complexes and may even discriminate between different polymerase-promoter conformations. It has also been applied to the dissection of elaborate processes such as RNA splicing.

Although such in vitro studies can provide extremely detailed information, it should always be borne in mind that conditions within the gel do not reflect those occurring in vivo, and therefore direct extrapolation to in vivo processes requires caution.

**Ternary transcription complexes and in jello transcription.** Chelm and Geiduschek (25) first used agarose gels to fractionate complexes composed of DNA and nascent \( ^{32} \text{P} \)RNA produced by \( E. \text{coli} \) or \( B. \text{subtilis} \) RNA polymerase from a mixture of restriction fragments, thus providing a simple means of localizing promoters and mapping transcripts. By using polyacrylamide gels, which provide a greater degree of resolution, Straney and Crothers (139) were able to detect five polymerase-lacUV5 promoter complexes with distinct migration properties. When explored by additional analytical techniques such as DNase I footprinting, direct determination of protein content, and in situ transcription within the gel, these gel-fractionated complexes could be defined as the closed binary complex, two types of open complex, and, in the presence of ribonucleoside triphosphates, two initiated complexes.

The closed complex was observed only at low temperatures. The two open forms were observed at higher temperatures, were resistant to challenge by competitor DNA, and were in rapid equilibrium in solution. More significantly, they were both capable of directing transcription in situ within the gel when provided with the relevant substrates and gave rise to different products, in one case to abortive transcripts and in the other to full-length transcripts. This, together with the facts that the relative abundance of the open complexes was determined by temperature, that one species was preferentially eliminated by limited treatment of the DNA with dimethyl sulfate, and that both species exhibited a pattern of DNase I protection consistent with occupation of the promoter by a single polymerase molecule, suggested that they reflect different conformational forms. Gel fractionation was also used to determine the protein species present in each complex and, as expected, indicated that the open complexes contain \( \beta, \beta', \alpha \), and \( \sigma \) whereas initiated complexes have shed the \( \sigma \) subunit. Similar studies with the phage T7 A1 promoter indicated that up to three core enzyme molecules could bind to (and retard) the 130-bp promoter-carrying fragment in a heparin-sensitive manner (59). Addition of \( \sigma \) factor, however, resulted in a single
slowly migrating heparin-resistant (open) complex whose stability decreased with decreasing temperature.

These techniques have also been extensively applied to analyzing the role of CAP binding at the wild-type lac P1 promoter (140) to demonstrate that CAP both strengthens binding in the closed complex and increases isomerization into the open complex by protein-protein interactions. They have also been used to demonstrate that Lac repressor stimulates RNA polymerase binding at the lacUV5 promoter (141), thus endowing the repressor with the property of transient gene activation.

**Splicing.** Splicing reactions can be monitored in vitro by the addition of a nuclear extract to RNA containing an intron. Direct fractionation of such mixtures on nondenaturing gels reveals the assembly of a splicing complex, or spliceosome, as a retarded RNA-protein species. Two early studies in which the reaction pathway was elucidated made use of competitor molecules to arrest the progress of the splicing reaction. Konarska and Sharp (76) added heparin at various intervals to interrupt complex formation on an adenovirus late transcript. Gel electrophoresis and subsequent analysis of the resulting retarded complexes revealed several steps in the reaction pathway. The first heparin-resistant complex(es), formed in the absence of ATP, progressed to a complex carrying intact pre-mRNA specifically protected from RNase T1 attack at the 3' end of the intron and led to a complex containing the 5' exon and the intron-lariat-3' exon splicing intermediates. Pikielny et al. (108) dissected a single retarded band formed by a yeast splicing complex by addition of excess tRNA as a nonspecific competitor nucleic acid before electrophoresis. The resulting removal of weakly and nonspecifically bound protein revealed that at least four complexes with different migration rates, each having a distinct mixture of RNA splicing intermediates, were contained in the original band. Subsequent denaturing gel analysis of complexes isolated from the gel showed that those formed at early times contained mostly pre-mRNA whereas those formed later contained predominantly intermediates and products of the splicing reaction.

**OUTLOOK**

We have attempted to give an outline of the impact of gel retardation on the study of protein-nucleic acid interactions with an emphasis on qualitative aspects. Although much of the discussion has centered on protein-DNA interactions, the technique is equally applicable to studying protein-RNA interactions. In view of the extensive use of the technique, it has proved impossible to cover all applications in detail. It appears to us, however, that the potential applications are far from being exhaustively explored.

One area which has yet to be fully exploited is the use of specific antibodies. Although these have been employed to enhance resolution of complexes and to determine protein composition, they may provide access to protein-protein interactions within multiprotein complexes and to changes in protein conformation which could occur on binding. Another underexplored area is that of binding to supercoiled DNA, a biologically more relevant substrate than linear DNA fragments. The development of vectors based on site-specific recombination systems such as bacteriophage P1 lac/cre (124), phage λ integration and excision (99), or transposable elements (108a) will permit the production of large quantities of small supercoiled molecules and so facilitate such studies. Finally, the development of new high-resolution agarose gel matrices (22) is likely to extend the use of the technique and may uncover structural and conformational changes which are not accessible with a polyacrylamide matrix.

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