(UNIT 12.2) in which unlabeled proteins are examined for their ability to retard the mobility of a
32P-labeled DNA fragment. By incubating the protein with a variety of DNA fragments, it is possible
to examine its specific and nonspecific DNA binding properties and to precisely localize the
DNA-binding sequences. This "reverse mobility shift assay" is very convenient, and it has the
advantage that the fate of the 35S-labeled protein is followed directly. However, in vitro synthesized
proteins can be tested for their DNA-binding properties by the conventional mobility shift assay using
32P-labeled DNA (in which case the protein does not have to be radiolabeled) or by
immunoprecipitation of protein-DNA complexes (Johnson and Herskowitz, 1985).

In interpreting the results of such reverse mobility shift assays it is necessary to consider the
parameters governing gel mobilities. The mobility of a given free protein in nondenaturing gels
depends upon its charge:mass ratio, a property that varies greatly among proteins and is strongly
affected by pH. This means that the band corresponding to free protein can appear anywhere on the
gel and its location can be strongly affected by the precise gel conditions. Thus, in order to distinguish
between bands corresponding to free protein and those corresponding to protein-DNA complexes, it is
crucial to perform parallel control reactions that lack DNA (since the DNA is unlabeled, its mobility
in the absence of protein is irrelevant). With respect to the specific protein-DNA complex, the
mobility is affected by the amount of nonspecific bulk DNA in the reaction. In the absence of carrier
DNA, the complex migrates very slowly because it contains nonspecific DNA-binding proteins from
the translation extract in addition to the 35S-labeled protein. As the amount of bulk DNA is increased,
these nonspecific DNA-binding proteins are competed off the target DNA; hence the protein-DNA
complex migrates further in the gel. In addition, it is important to note that because the protein is
radiolabeled, nonspecific DNA-binding complexes can be observed; for this reason, it is inadvisable
to use very high concentrations of bulk carrier DNA. Finally, the mobility of a protein-DNA complex
is strongly influenced by the molecular weight of the protein component; complexes with larger
proteins migrate more slowly.

Perhaps the most significant advantage of this approach is that any desired mutant protein can be
created simply by altering the DNA template, and then tested for its DNA-binding properties. For
example, by creating a set of N- or C-terminal deletions of the protein, the DNA-binding domain can
be localized (Hope and Struhl, 1986). The availability of truncated but functional proteins can be
useful for determining the subunit structure of a protein (Hope and Struhl, 1987). Specifically, the
target DNA is incubated with a cosynthesized mixture of two proteins of different size obtained by
carrying out the protocol on an equimolar mixture of DNA templates. As mentioned above,
protein-DNA complexes involving each of the individual proteins will have a different electrophoretic
mobility. If, for example, the protein binds as a dimer, the cosynthesized mixture will generate three
protein-DNA complexes in a 1:2:1 molar ratio, with the complex of intermediate mobility
representing a heterodimer containing the two different protein species. Proteins binding as monomers
will yield two equimolar complexes, whereas proteins binding as tetramers will yield five complexes
in a 1:4:6:4:1 molar ratio. The principle of using different sized proteins to determine stoichiometry is
not constrained to the specific DNA-binding assay. Other methods, such as gluteraldehyde
crosslinking followed by SDS-PAGE can be used to examine the protein species.

Rapid Separation of Protein-Bound DNA from Free DNA Using Nitrocellulose Filters
This protocol relies on the ability of nitrocellulose to bind proteins but not double-stranded DNA. Use of radioactively labeled double-stranded DNA fragments allows quantitation of DNA bound to the protein at various times and under various conditions, permitting kinetic and equilibrium studies of DNA-binding interactions. Purified protein is mixed with double-stranded DNA in an appropriate buffer to allow interaction. After incubation, the mixture is suction filtered through nitrocellulose, allowing unbound DNA to pass through the filter while the protein (and any DNA interacting with it) is retained.

ALTERNATE PROTOCOL: DETECTION OF SPECIFICITY IN DNA BINDING

When the binding site of a protein is unknown, the pure protein can be added to a mixture of fragments to select those fragments of DNA for which it has the greatest affinity. Specificity of binding can be influenced by the buffer conditions and filtering regimen. This alternate protocol creates conditions that disrupt weaker, presumably nonspecific binding interactions, while retaining the stronger binding interactions. Differences from the basic protocol include using more labeled DNA and more extensive washing. The goal is to recover enough of a single input fragment to visualize by subsequent autoradiography. This protocol uses the same binding buffer as the basic protocol, however increasing the KCl to 150 mM (final concentration in the 1x binding buffer) could further improve detection of specific binding in some cases.

COMMENTARY

Filter binding is used predominantly to characterize interactions between purified DNA-binding proteins and their specific sites as well as various nonspecific DNAs. This assay is ideal for kinetic and equilibrium studies because bound DNA can be rapidly separated from free DNA. However, slower methods such as the mobility shift (UNIT 12.2) or immunoprecipitation (UNIT 10.16) assays, have the advantage that they can be used with cruder protein preparations.

DNA retained on the filters can be recovered for gel electrophoresis. This has allowed selection of fragments containing a specific binding site from a pool of several fragments in the initial reaction mix (Strauss et al., 1981). Filter binding has also been used to separate bound DNA from free DNA for methylation interference experiments (UNIT 12.3), although the mobility shift assay is more popular for this purpose.

The use of the filter-binding technique for kinetic and equilibrium analyses is described briefly below. For more detailed discussion of the validity of these approaches, as well as alternative approaches, consult Riggs et al. (1970) and Hinkle and Chamberlin (1972b).

Analysis of DNA-Protein Interactions Using Proteins Synthesized In Vitro from Cloned Genes

The availability of a cloned gene makes it possible to synthesize the encoded protein by in vitro