low-affinity proteins might be significantly enhanced by using DNA probes containing multiple binding sites that are spaced such that the probe can simultaneously bind two or more immobilized protein molecules. Enhanced sensitivity with a multisite probe has been impressively demonstrated in the molecular cloning of a mammalian regulatory protein (Staudt et al., 1988). Finally, since the binding constants of sequence-specific proteins are dependent on ionic strength, temperature, and pH, manipulation of these factors might also enhance detection.

The DNA-binding domains of sequence-specific proteins need to be overexpressed in E. coli to permit detection with radiolabeled recognition-site probes. These proteins, when expressed to a level of ~1% of the total cellular protein, can be readily detected (Singh et al., 1988; Staudt et al., 1988). This level of recombinant protein expression is typical of lgt11.

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 transcription and translation. As described in UNIT 10.17, protein-coding sequences are cloned into a vector containing a promoter for SP6 or T7 RNA polymerase (UNIT 1.5), messenger RNA is produced by transcribing the DNA template (UNIT 3.8), and the desired protein is synthesized as a 35S-labeled species by in vitro translation. Such in vitro synthesized proteins are extremely useful for determining whether a cloned gene encodes a specific DNA-binding protein and for analyzing DNA-protein interactions. To detect DNA binding activity, the labeled protein is incubated with specific DNA fragments, and protein-DNA complexes are separated from free protein by electrophoresis in native acrylamide gels (UNIT 12.2). Unlike the more conventional mobility shift assay which utilizes 32P-labeled DNA and unlabeled protein, the assay described here generally utilizes 35S-labeled protein and unlabeled DNA. Major advantages of this method are that any desired mutant protein can be tested for its DNA-binding properties simply by altering the DNA template, and the subunit structure (e.g., dimer, tetramer) can be determined.

COMMENTARY

The procedure described here for analyzing DNA-protein interactions was first utilized in studies on the yeast GCN4 transcriptional activator protein (Hope and Struhl, 1985). It differs from conventional biochemical approaches in that the protein of interest is not obtained from cells, but rather is synthesized by in vitro transcription and translation of a cloned gene. However, once the protein is synthesized, many standard procedures for studying specific DNA-protein interactions (e.g., UNITS 12.2, 12.3, & 12.4) can be performed with only minor modifications. In addition, the method is extremely useful for analyzing the properties of mutant proteins and for determining subunit structure, issues that are much more difficult to investigate by more classical biochemical techniques.

The protocol for synthesizing [35S]protein by in vitro transcription and translation is detailed in UNIT 10.17. DNA-binding activity is detected by incubating the labeled protein with appropriate DNA fragments, and separating the protein-DNA complexes from free protein by electrophoresis in native acrylamide gels. This DNA-binding assay is essentially the reverse of the standard mobility shift assay

(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 glutaraldehyde 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