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.

Purification of Sequence-Specific DNA-Binding Proteins by Affinity Chromatography

Many biological processes, such as recombination, replication, and transcription, involve the action of sequence-specific DNA-binding proteins. Analysis and purification of these proteins by conventional chromatographic methods is often difficult because DNA-binding proteins typically make up <0.01% of the total cellular protein. Various methods have been developed for purifying such proteins. Affinity chromatography is a very effective means of purifying a protein based on its sequence-specific DNA-binding properties and is relatively straightforward if proper care is taken. The affinity chromatography procedure described in this unit uses DNA containing specific recognition sites for the desired protein that has been covalently linked to a solid support. Researchers in the past have purified proteins based on the proteins' ability to bind DNA, only to be disappointed to find that the DNA-binding activity of their highly purified products was not sequence-specific. This situation can be avoided by carefully performing all of the preliminary steps and experimental controls.

The first basic protocol describes preparation of a DNA affinity resin, including cyanogen bromide (CNBr) activation of the agarose support. The alternate protocol provides a method to couple DNA to commercially available CNBr-activated Sepharose. The first support protocol describes how to purify crude synthetic oligonucleotides by gel electrophoresis prior to preparation of the affinity resin. The second basic protocol outlines the affinity chromatography procedure. The second support protocol describes determination of the appropriate type and quantity of nonspecific competitor DNA that

should be used in the procedure and its preparation. Parameters essential to the success of an affinity chromatography experiment are discussed in detail in the Commentary. Figure 12.10.1 provides a summary diagram of the entire chromatography procedure.

BASIC PROTOCOL: PREPARATION OF DNA AFFINITY RESIN

Correct choice of oligonucleotide sequence (discussed in detail in the Commentary) and preparation of the affinity resin are probably the most important parts of the affinity chromatography procedure. Preparation of affinity resin can be broken down into four steps: (1) preparing oligonucleotides; (2) activating Sepharose; (3) coupling DNA to resin; and (4) blocking unreacted CNBr. The first step requires highly purified oligonucleotides that contain the recognition sequence for the desired protein. Once purified, the complementary oligonucleotides are annealed, phosphorylated with T4 polynucleotide kinase, and ligated into long, multimeric chains (averaging >=10-mers) with T4 DNA ligase. Sepharose CL-2B is activated with CNBr, the ligated DNA added, and the coupling reaction carried out overnight. Because CNBr is very toxic, researchers may prefer to employ the alternate protocol, which uses commercially available CNBr-activated Sepharose and therefore avoids direct handling of CNBr. After the coupling reaction, the remaining reactive groups are blocked with ethanolamine. This protocol is designed to prepare 10 ml of affinity resin.

ALTERNATE PROTOCOL: COUPLING THE DNA TO COMMERCIALLY AVAILABLE CNBr-ACTIVATED SEPHAROSE

The major advantage of this alternate procedure is that it begins with commercially available CNBr-activated chromatography resin, avoiding the need for preparation of CNBr-activated resin (first basic protocol). However, commercial CNBr-Sepharose is considerably more expensive than the homemade variety; moreover, the resulting column tends to run more slowly than one prepared as described above. Both resins are effective, leaving it up to the researcher's discretion which to use.

BASIC PROTOCOL: DNA AFFINITY CHROMATOGRAPHY

Affinity chromatography is performed by combining a partially purified protein sample with appropriate competitor DNA, pelleting the insoluble protein-DNA complexes by centrifugation, and loading the resulting soluble material by gravity flow onto the affinity resin. Nonspecific DNA-binding proteins flow through the column while the specific protein is retained by the column. The protein is then eluted by gradually increasing the salt concentration of the buffer and individual fractions are tested for DNA-binding activity and purity. Fractions containing appropriate DNA-binding activity can be reapplied to the affinity resin if further purification is desired. By using two sequential affinity chromatography steps, a typical protein can be purified 500- to 1000-fold with ~30% yield. The method described below is performed with buffer Z, but many other buffers will work as well. Buffer choice is addressed in the Commentary.

COMMENTARY

Purification of sequence-specific DNA-binding proteins has historically been a difficult task, mainly because such proteins are a small fraction of the total cellular protein. However, it is now possible to purify these factors quickly, simply, and effectively by using multimerized synthetic oligonucleotides that contain the recognition sequence for a particular DNA-binding protein. Early efforts with DNA-affinity-chromatography involved adsorption or coupling of nonspecific DNA (such as calf thymus DNA) to either cellulose (Alberts and Herrick, 1971) or agarose (Arndt-Jovin et al., 1975)

supports. These methods paved the way for the development of a variety of sequence-specific DNA affinity chromatography techniques, including the procedures described in this unit.

Other methods have been described to purify sequence-specific DNA-binding proteins, including chromatography using biotinylated DNA fragments attached to various supports by biotin-avidin or biotin-streptavidin coupling (UNIT 10.6; Chodosh et al., 1986; Kasher et al., 1986; Leblond-Francillard et al., 1987), oligonucleotides synthesized onto Teflon-based beads (Duncan and Cavalier, 1988), or synthetic oligonucleotide monomers attached to agarose supports (Wu et al., 1987; Blanks and McLaughlin, 1988; Hoey et al., 1993); and preparative gel mobility shifts (Gander et al., 1988). Although more than 50 sequence-specific DNA-binding proteins have been purified by the method described in this unit (Kadonaga, 1991, and references therein), it is likely that many of the techniques listed above are also effective for purifying sequence-specific DNA-binding proteins.

There are a variety of reasons that CNBr activation is commonly used in the preparation of affinity resins: it is simple, works well with agarose matrices, and is mild enough to bind ligands such as DNA. Briefly, the chemistry of the CNBr activation reaction is as follows. At high pH, the hydroxyl groups on the agarose resin react with CNBr. The majority of the CNBr added to the reaction reacts with water to yield inert cyanate ions, which is part of the reason such a large amount of CNBr is required. Additionally, the majority of the cyanate esters that are formed on the agarose either are hydrolyzed to form inert carbamate or react with the matrix hydroxyls to form imidocarbonates. The imidocarbonates that form can act effectively as chemical cross-links, thus stabilizing the matrix (which is in most cases beneficial, particularly if the agarose resin chosen is not covalently cross-linked). The remaining active cyanate esters are coupled to the amino-containing ligands (in this case, oligonucleotides) at physiological pH. Finally, the unreacted cyanate esters are blocked with an excess of a suitable reagent, such as ethanolamine, to prevent coupling of the protein sample to the matrix (Janson and Ryden, 1989).