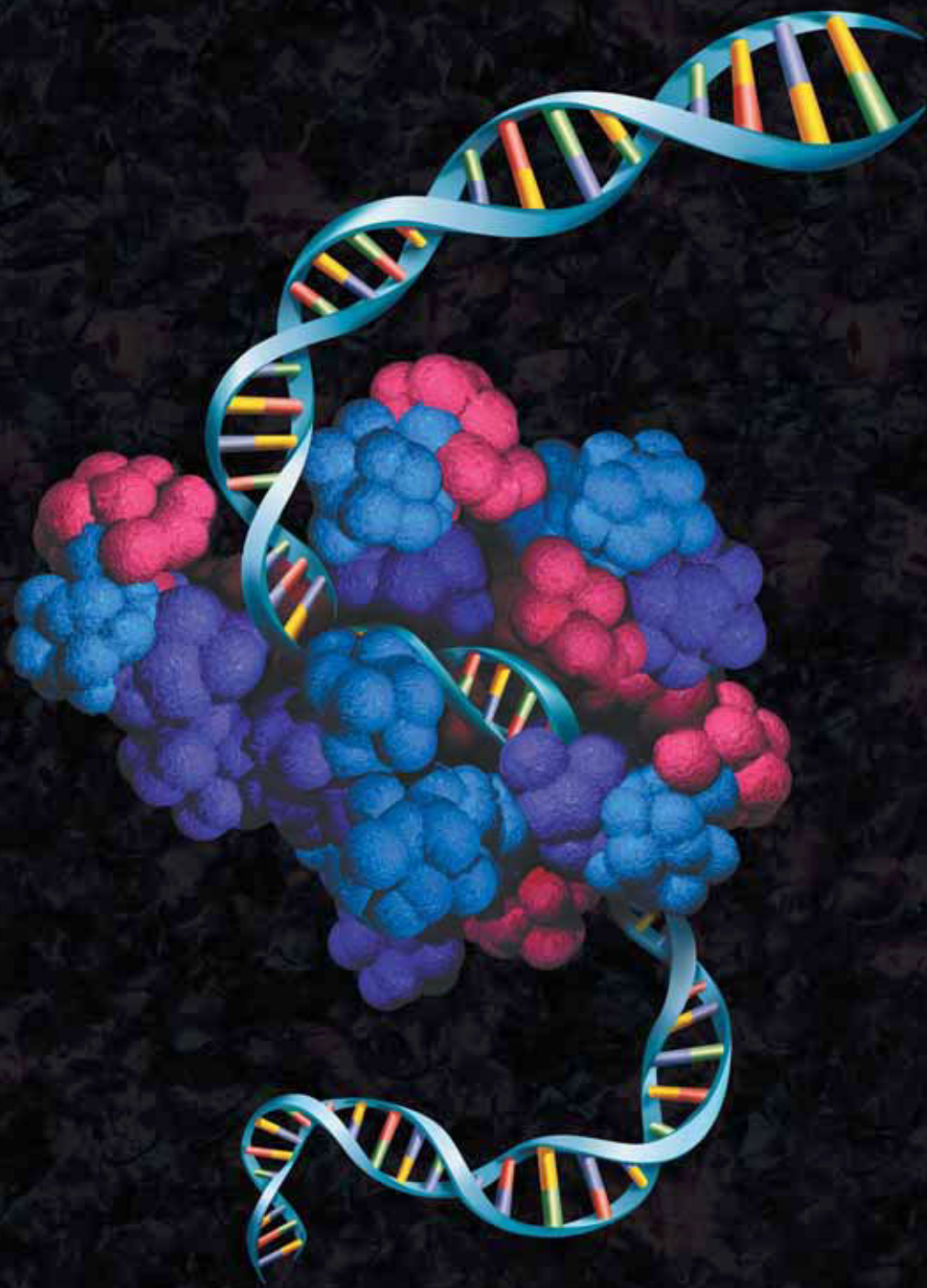


CHAPTER

3

About the Image:

In this illustration protein-DNA binding is shown. One example of such an interaction is the binding of transcription factors to DNA, which results in regulation of DNA synthesis.



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Chapter Three: Protein-Nucleic Acid Interactions

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Introduction

Cell-free systems have become popular tools for in vitro production of proteins, examined for their activity in protein-nucleic acid interactions. Putative DNA binding proteins, such as transcription factors, can be analyzed for their ability to bind specific sequences on radiolabeled DNA or RNA. Generally binding of proteins to nucleic acids is detected by electrophoretic mobility shift assay (EMSA). Using this assay, protein-nucleic acid complexes demonstrate retarded migration compared to their non-nucleic acid-binding counterparts.

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Protein-DNA Interactions

One of the most commonly used procedures for detecting DNA-binding proteins or protein complexes (such as transcription factors) is the electrophoretic mobility shift assay (EMSA) or gel shift assay. This popular technique uses *in vitro* expression to synthesize putative DNA binding proteins, which are then incubated with an oligonucleotide containing a target consensus sequence site. Binding to the DNA is detected by gel shift assay (Figures 1 and 2). Either partner can be radiolabeled (usually 5'-end-labeled [³²P]DNA), and with proper controls, the translation extract containing the synthesized candidate binding factor can be used directly in the assay (1).

When c-Rel was cotranslated with a small amount of p98, very little inhibition of c-Rel binding to DNA was observed (Figure 2, lane 3). However, when a 4-fold higher ratio of p98:c-Rel DNA was used in the TNT[®] System co-expression, and the binding reaction was then performed using an amount of c-Rel equivalent to that in lane 3, no c-Rel-DNA complex was observed (Figure 2, lane 4). Thus, p98 acts as

an I-κB *in vitro* by preventing c-Rel from binding DNA. This *in vitro* interaction is similar to the ability of p98 to retain c-Rel in the cytoplasm *in vivo* (2).

Many researchers investigating transcription factor binding use the coupled wheat germ translation systems rather than rabbit reticulocyte systems, as the wheat germ extract does not contain endogenous mammalian transcription factors, such as NF-κB (3). If the reticulocyte system is used, a rapid method to remove endogenous DNA-binding proteins from the reticulocyte system has been developed using biotinylated DNA and streptavidin magnetic beads (4).

Protein-RNA Interactions

Many of the techniques using *in vitro* translated proteins to detect DNA-binding proteins can be used to study protein-RNA interactions as well. However, one of the ways in which the techniques differ is in the ability to produce a number of modified RNAs using *in vitro* transcription. In one example, the direct interaction of the U1 snRNP-A protein with SV40 late

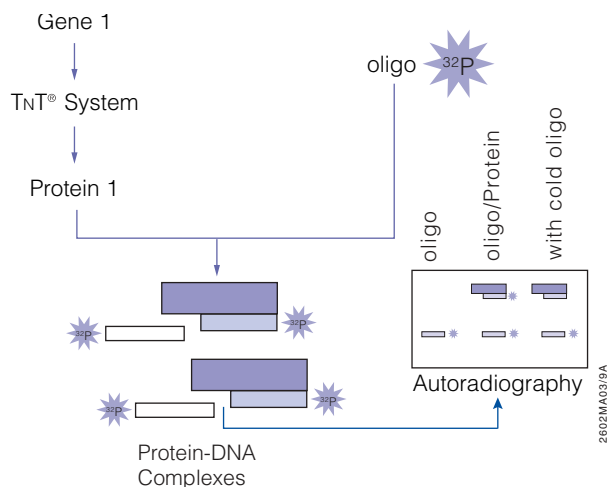


Figure 1. Use of the TNT[®] Systems for studying protein-DNA and protein-RNA interactions. An unlabeled (non-radioactive) protein is produced in a TNT[®] System reaction and then mixed with a ³²P-labeled oligonucleotide containing a consensus sequence for the suspected DNA binding protein (e.g., a transcription factor target sequence). The protein-DNA complexes are analyzed by migration on polyacrylamide gels. Unbound oligonucleotide will migrate to a position near the bottom of the gel. The oligonucleotide-protein complex will show retarded mobility and will migrate to a position near the top of the gel. The mobility shift can be measured quantitatively. DiDonato and Karin demonstrated that p98 sequesters c-Rel in the cytoplasm, thereby acting as an I-κB (2). In the same experiment as described in Chapter 2 (5), the functional significance of this interaction *in vitro* was examined by gel shift assays to determine the effect of p98 binding on the DNA-binding capacity of c-Rel. Figure 2 demonstrates that gel shift assays can be performed using single or multiple proteins expressed in a TNT[®] reaction and added directly to the assay.

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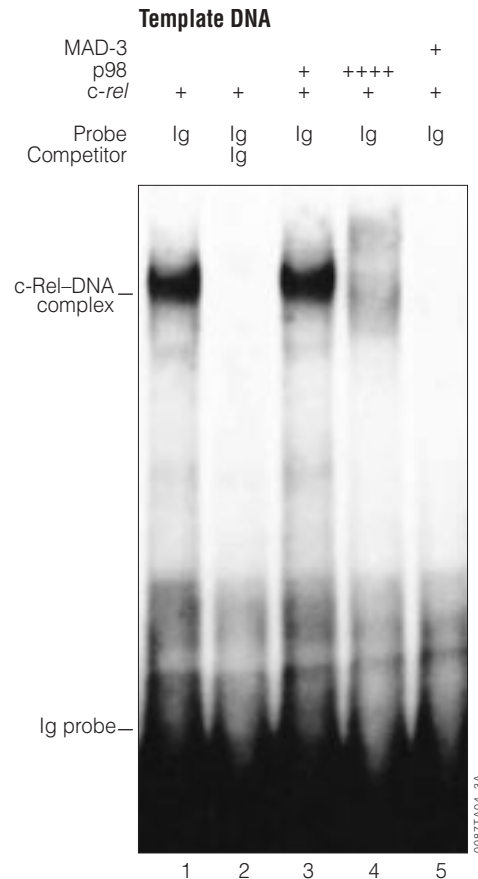
mRNAs was demonstrated by mixing [³⁵S]Met U1 snRNP-A fusion protein containing a g10 epitope tag synthesized in vitro and ³²P-labeled viral RNA transcribed in vitro. The complexes were then immunoprecipitated, and coprecipitating RNAs were extracted and analyzed by electrophoresis (6).

Denman (7) described another technique that can be applied to binding studies of both DNA and RNA. This technique, outlined in Figure 3, uses a three-step scheme for isolating targets of DNA- or RNA-binding proteins. In this example, RNA was used. First, a biotinylated binding protein or protein binding domain is synthesized by incorporating biotinylated lysines (e.g., Transcend™ Biotinylated tRNA, Cat.# L5061) in a coupled transcription/translation reaction using the TNT® System programmed with plasmid DNA. At least one lysine residue is required for biotinylated protein synthesis. In the unlikely event of no naturally occurring lysine residues, a lysine tag can be engineered at the end of the protein or peptide fragment.

Next, the biotinylated binding protein is coupled to a SoftLink™ Soft Release Avidin^(h) solid support (8) making an affinity column. Total RNA or poly(A)⁺ RNA (9) is then incubated with the affinity resin in TBS buffer, allowing the protein/target RNA interaction to occur. Unbound RNA is removed by centrifugation, and after extensive washing with TBS buffer, the bound RNA is eluted from the resin under strong denaturing conditions. This RNA, which contains the entire subpopulation of RNA specifically bound to the biotinylated protein, can then be fractionated and analyzed using differential display RT-PCR⁽ⁱ⁾ (DDRT-PCR⁽ⁱ⁾), subtractive hybridization or reverse Northern blotting (Figure 2).

Many of the in vitro protein modifications used for detection of protein-protein interactions can be used for investigation of purported nucleic acid-binding proteins. When combined with the variety of standard DNA and RNA analysis procedures, the options for identification and characterization of these interactions increase dramatically.

Figure 2. Gel shift assays using proteins produced in the TNT® Wheat Germ System. c-Rel, p98 and MAD-3 proteins were produced in unlabeled 25µl TNT® Wheat Germ Extract^(a,b,c,e) reactions containing 1.0µg of *c-rel* template DNA (lanes 1–5), 0.25µg or 1.0µg of p98 template DNA (lanes 3 and 4, respectively), and 1.0µg of MAD-3 template DNA (lane 5). The relative expression levels of each protein were estimated using a parallel set of TNT® reactions containing [³⁵S]methionine. Gel shift assays were performed as described in reference 10. Based on the data from the ³⁵S-labeled reactions, the volume of unlabeled TNT® reaction products added to each gel shift reaction was adjusted to contain a constant amount of c-Rel (from 2–8µl). Each reaction also contained 40pg of ³²P-labeled Ig enhancer probe DNA (10). In lane 2, the unlabeled competitor Ig probe was added in 50-fold excess (2ng). The gel shift reactions were analyzed by electrophoresis on a 5% polyacrylamide gel and detected by autoradiography for 24 hours at –70°C. c-Rel bound specifically to the immunoglobulin kappa light chain enhancer probe (lanes 1 and 2) (10). As expected, this binding could be inhibited by adding in vitro-translated MAD-3 (I-κB-α, a 37kDa member of the I-κB family) (11) to the *c-rel* binding reaction (lane 5).



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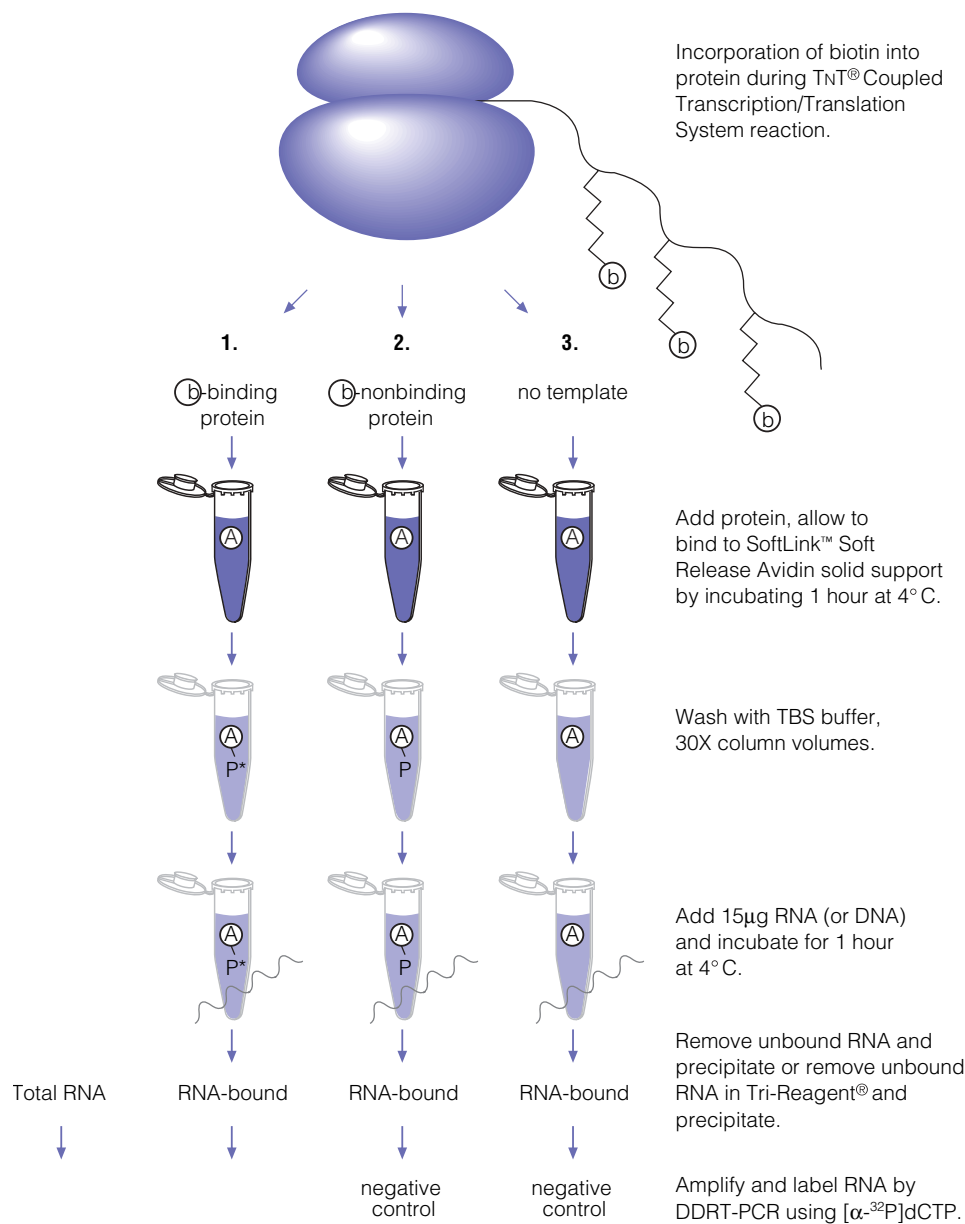


Figure 3. Isolating targets of RNA- and DNA-binding proteins. Biotinylated RNA- or DNA-binding proteins were produced in a 25µl TNT® Coupled Transcription/Translation System^(a,b,c,e) reaction in the presence of 2µl Transcend™ tRNA (12) and were bound to 100µl of SoftLink™ Soft Release Avidin Resin^(h) (Cat.# V2011, V2012) at 4°C. Fifteen micrograms of total RNA or genomic DNA were added and allowed to bind for 1 hour. Unbound nucleic acid was then eluted in TriReagent® (Molecular Research Center; 12) and purified by ethanol precipitation. One-third of the purified products were converted to complementary ssDNA using one of the 3'-DDRT-PCR^(l) primers. A fraction (1/25) of the complementary ssDNA sample was then amplified by DDRT-PCR in the presence of 2µCi [α-³²P]dCTP (13). The products were sized on denaturing polyacrylamide gels and select bands were cloned (14) and sequenced. To demonstrate specificity, two control reactions were run in parallel with the protein of interest, an unrelated control protein (column 2) and transcription/translation reaction without plasmid DNA (column 3).

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