SYLICA 2013
Bowater lectures

Biophysical Methods to Study Molecular Interactions

Bowater Lectures in Brno, Feb. 2013

4 lectures on linked topics will be delivered during the coming week:

- *Contemporary DNA Sequencing Technologies* – 26/2/2013 @ 10:00
- *Using 'Omic Technologies to Investigate Gene Function* – 26/2/2013 @ 14:00
- *Biophysical Methods to Study Molecular Interactions* – 27/2/2013 @ 10:00
- *Synthetic Biology & Nanotechnology: Tomorrow's Molecular Biology?* – 28/2/2013 @ 10:00

Molecular Interactions

- For biological systems to function, interactions occur between many different types of molecules: DNA, RNA, Protein, Lipids, etc.
- To ensure that biological systems function appropriately, such interactions must be carefully regulated
- Wide range of Biophysical Chemistry approaches are useful for studying these interactions

Bonds & Molecular Interactions

- Interactions between molecules are central to how cells detect and respond to signals and affect:
  - Gene expression (transcription & translation)
  - DNA replication, repair and recombination
  - Signalling
  - And many other processes....
- Interactions are (mainly) mediated by many weak chemical bonds (van der Waals forces, hydrogen bonds, hydrophobic interactions)
- Accumulation of many bonds influences affinity and specificity of interactions

Biophysical Chemistry Approaches for Studies of Molecular Interactions

- Wide range of Biophysical Chemistry approaches are useful for studying molecular interactions:
  - NMR
  - X-ray crystallography
  - SPR
  - ITC
  - CD
  - Gel electrophoresis
  - EPR
  - Mass spectrometry
  - Fluorescence

In vitro

In vitro and *in vivo* (?)

Will also discuss other types of *in vivo* studies

Biophysical Chemistry Approaches for Studies of Molecular Interactions

- Wide range of Biophysical Chemistry approaches are useful for studying molecular interactions:
  - NMR
  - X-ray crystallography
  - SPR
  - ITC
  - CD
  - Gel electrophoresis
  - EPR
  - Mass spectrometry
  - Fluorescence

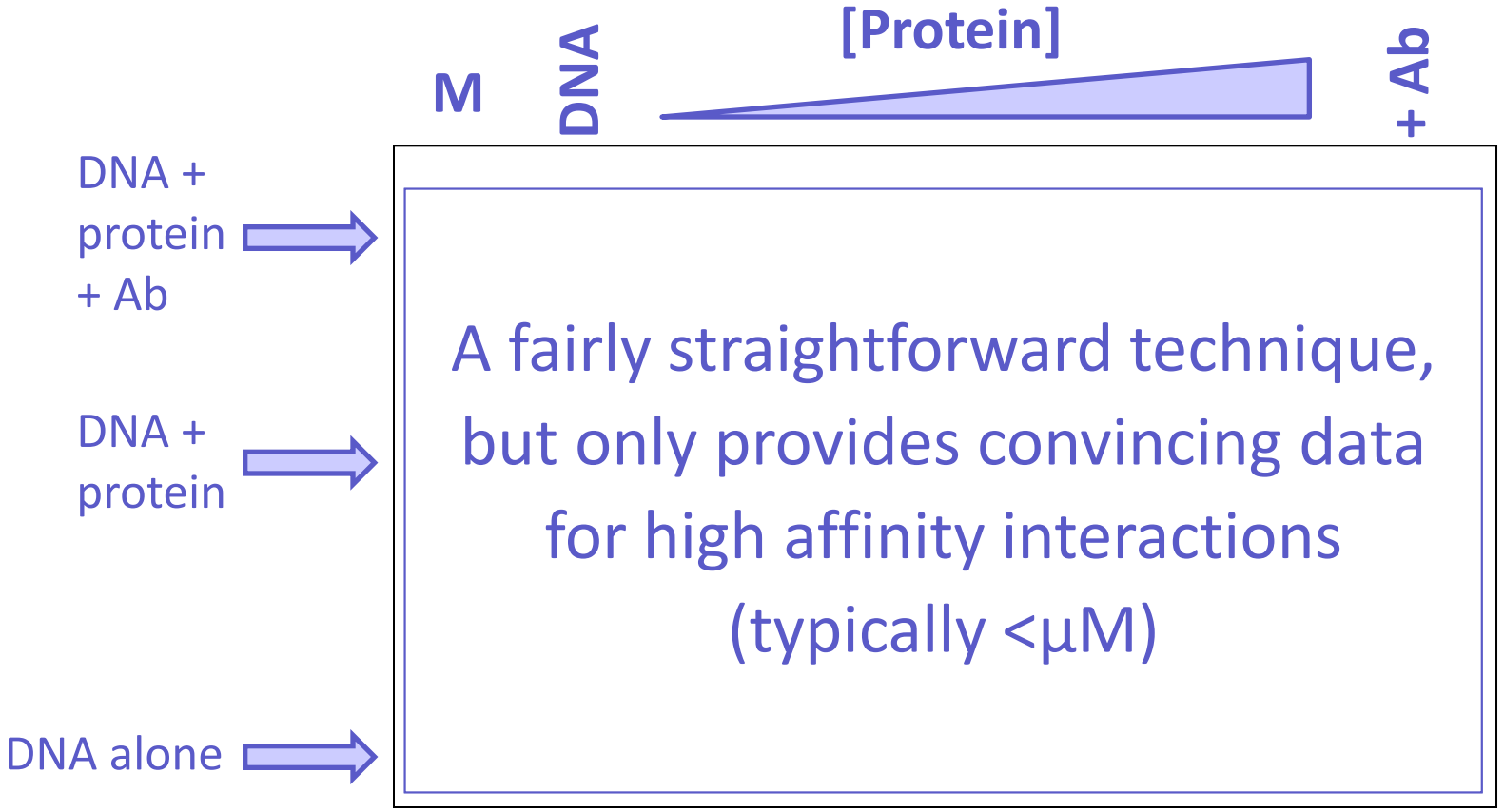
Many of these techniques are particularly useful for determining the strength (affinity) of interactions

Protein-Nucleic Acid Interactions

- A wide range of Biophysical Chemistry methods have been used to study interactions between proteins and nucleic acids
- Particularly good for determining the strength (affinity) of the interactions
  - High affinity, µM – nM: tend to involve sequence-specific interactions, e.g. restriction enzymes
  - Low affinity, mM – µM: proteins tend to recognise aspects of "overall" structure i.e. not sequence-dependent

EMSA ("Gel Shift" Assay)

- Electrophoretic Mobility Shift Assay (EMSA) or "gel shift" can provide information about protein-NA interactions

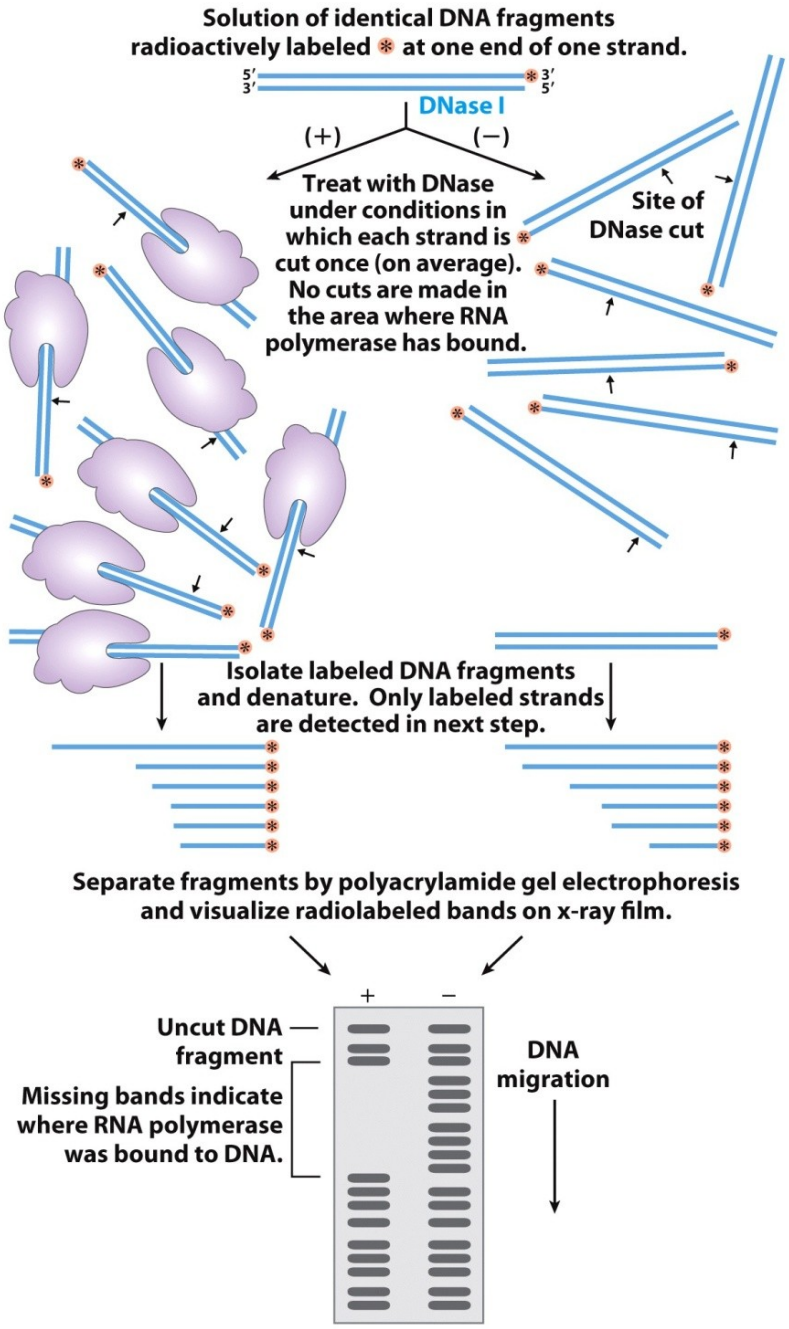


"Footprinting" is a Technique to Identify a DNA-binding site

Premise: DNA bound by protein will be protected from chemical cleavage at its binding site

1) Isolate a DNA fragment thought to contain a binding site and "label" it
2) Bind protein to DNA in one tube; keep another as a "naked DNA" control
3) Treat both samples with chemical or enzymatic agent to cleave the DNA
4) Separate the fragments by gel electrophoresis and visualize bands on X-ray film or imager plate

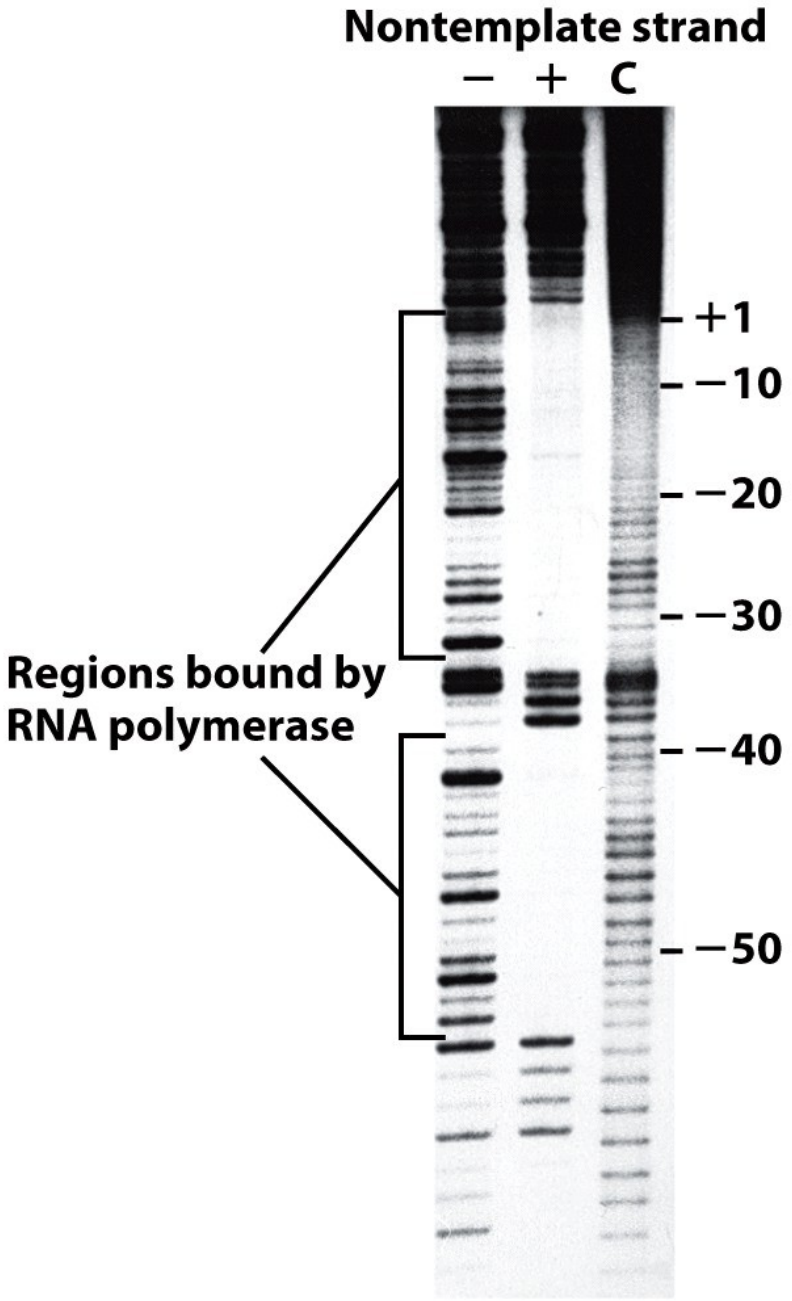
Protein-DNA Footprinting



Box 26-1 Figure 1

Lehninger Principles of Biochemistry, Sixth Edition

Footprinting Results of RNA Polymerase Bound to Promoter



Box 26-1 Figure 2
Lehninger Principles of Biochemistry, Sixth Edition

Binding of Proteins to DNA Often Involves Hydrogen Bonding

- Gln/Asn can form specific H-bond with Adenine's N-6 and H-7 H's
- Arg can form specific H-bonds with Cytosine-Guanine base pair

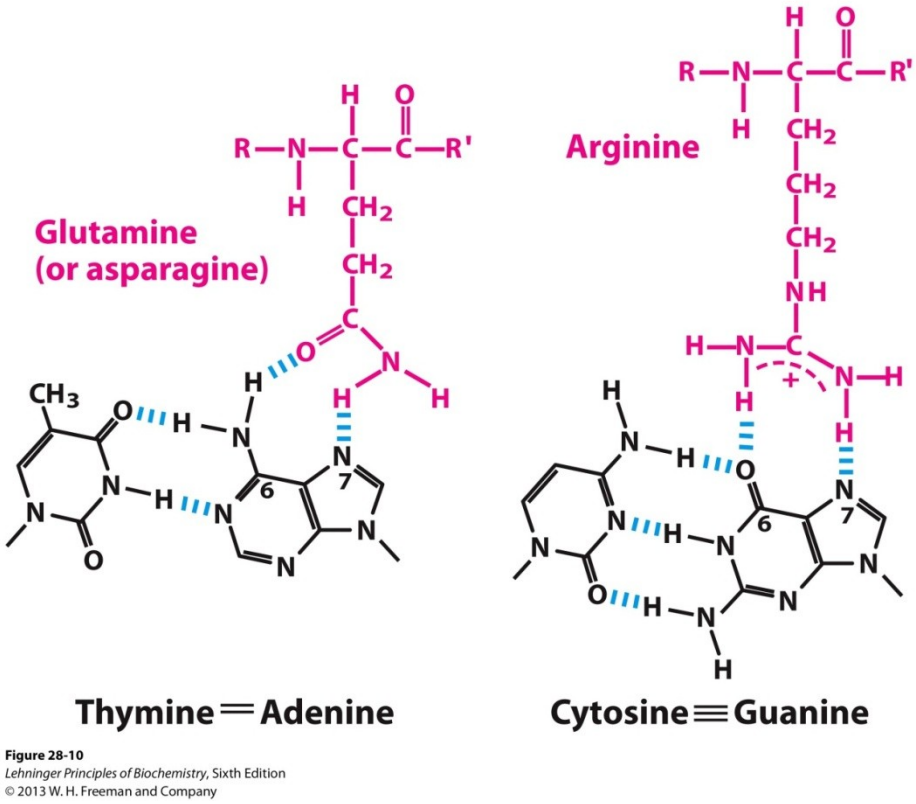


Figure 28-10
Lehninger Principles of Biochemistry, Sixth Edition

- Major groove is right size for α-helix and has exposed H-bonding groups

DNA-binding domains

- Proteins generally recognise aspects of nucleic acid sequence, or variations in structure and/or flexibility
- High-resolution structures of many protein-DNA complexes have now been solved
- Similar structural domains occur in different proteins:
  - Helix-turn-helix
  - Zinc-finger
  - Zinc-binding domain
  - Basic region-leucine zipper (bZIP)
  - β-sheet recognition

The Helix-turn-helix Motif is Common in DNA-binding Proteins

- Each "helix-turn-helix" covers ~ 20 aa
  - One α-helix for DNA recognition, then β-turn, then another α-helix
  - Sequence-specific binding due to contacts between the recognition helix and the major groove
- Four DNA-binding helix-turn-helix motifs in the Lac repressor

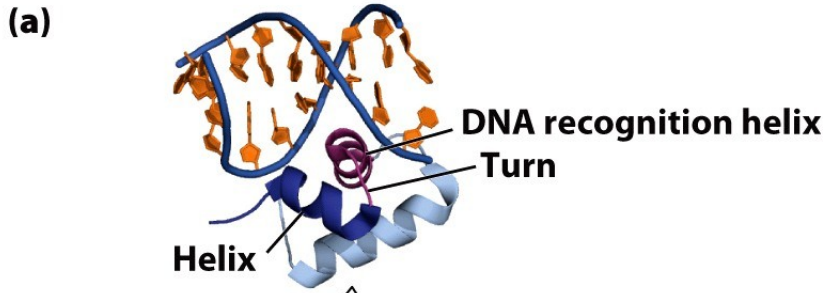


Figure 28-11
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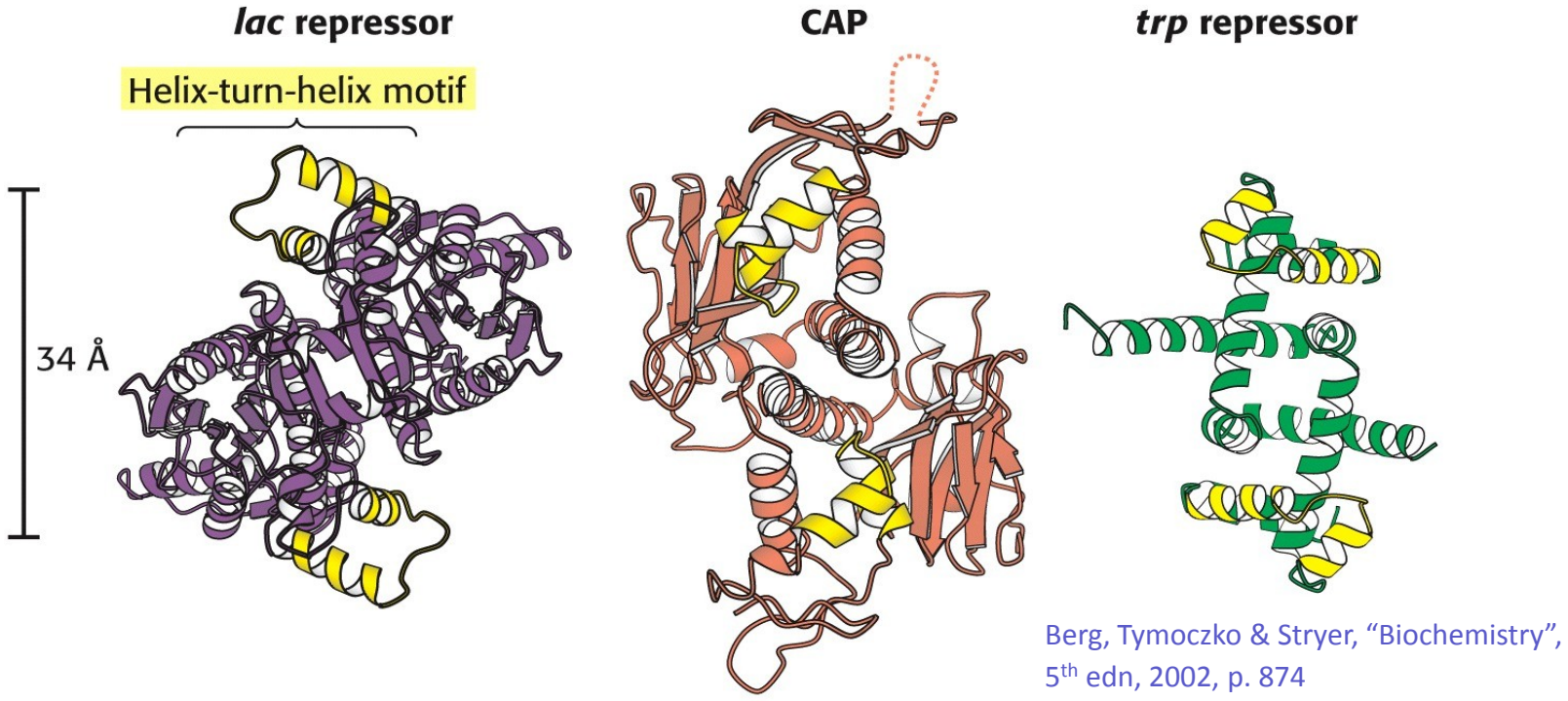
Helix-turn-helix

- Helix-turn-helix is most common observed DNA-binding unit in prokaryotes

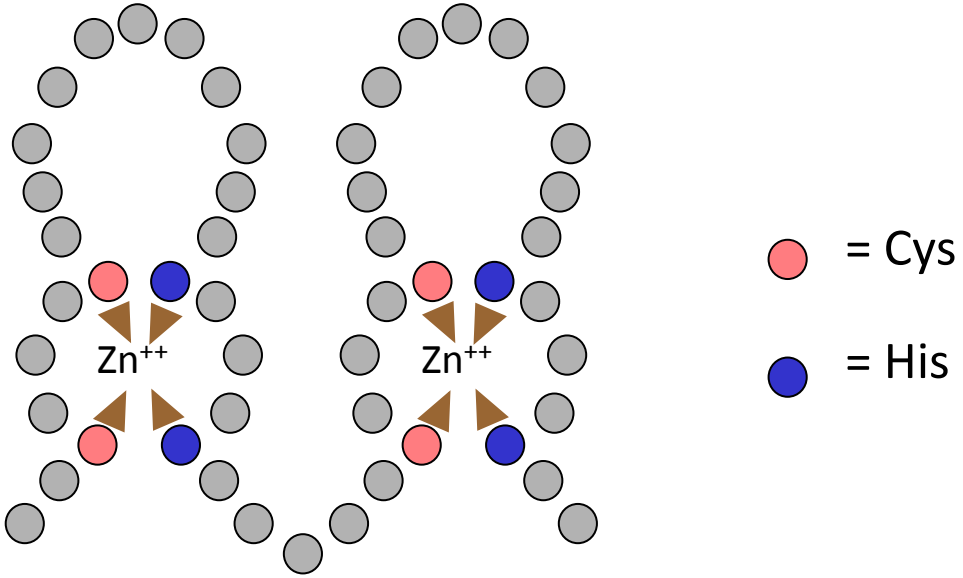


Berg, Tymoczko & Stryer, "Biochemistry", 5th edn, 2002, p. 874

- Note that 34 Å corresponds to 1 turn of DNA

Zinc-finger

- One of best-studied examples of DNA binding domain, but also binds RNA
- Each covers ~30 aa
- Binding is relatively weak, so typically there are a series of zinc fingers



Zinc Finger Motif is Common in Eukaryotic Transcription Factors

- Regulatory protein Zif268, complexed with DNA

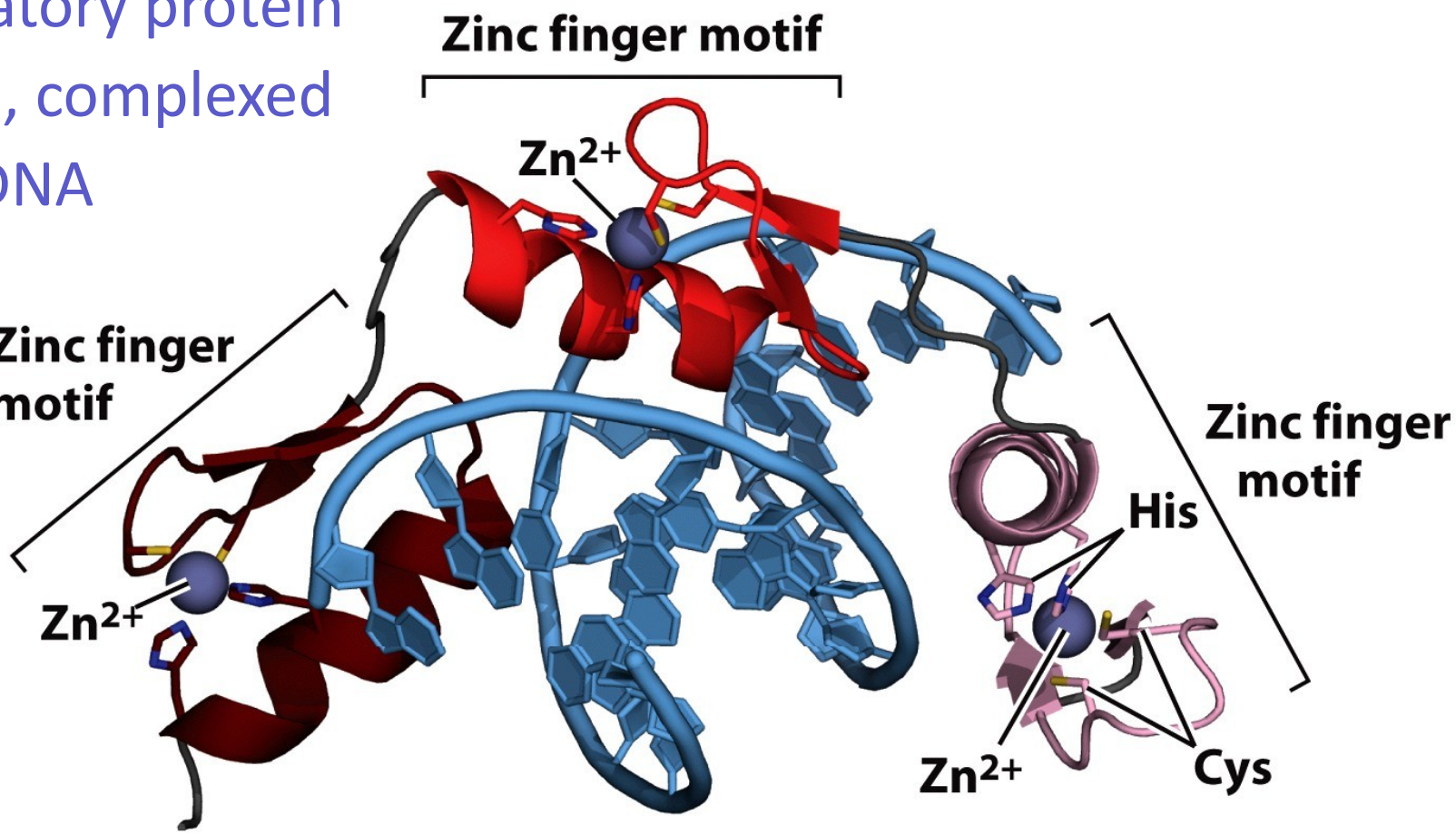


Figure 28-12
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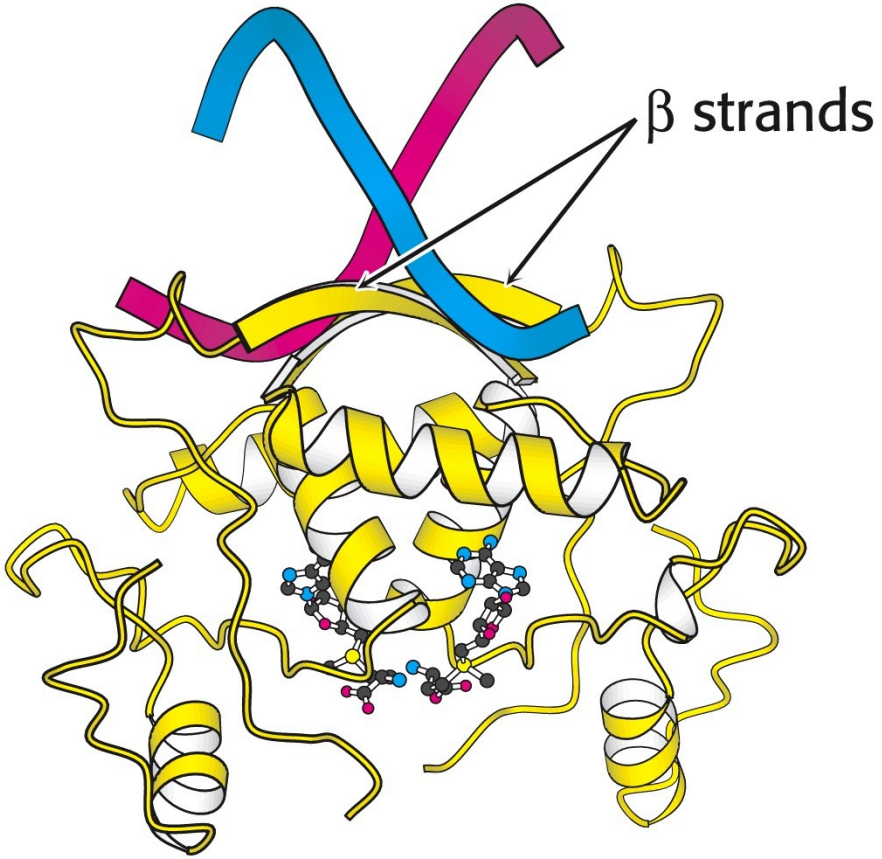
β-recognition motif

- In some prokaryotic regulatory proteins, this is an alternative DNA-binding motif

- *E. coli* methionine repressor binds DNA through insertion of pair of β-strands into major groove



Berg, Tymoczko & Stryer, "Biochemistry", 5th edn, 2002, p. 874

Protein-protein Interactions

- Various techniques are used to investigate protein-protein interactions, including:
- Biochemical/biophysical
  - Isothermal calorimetry
  - Surface plasmon resonance (e.g. BIACore)
  - Mass spectrometry e.g. from protein complexes
  - "Pull-down" assays – one protein can be bound by an antibody (immunoprecipitation) or via a "tag"
- Molecular/cellular biological
  - Two-hybrid experiments
  - Fluorescent proteins

Identifying Protein–Protein Interactions

- Protein complex isolation
  - Epitope tag one protein in the complex
  - Gentle isolation of epitope-tagged protein will also isolate stably interacting proteins
  - All proteins isolated can be separated and identified

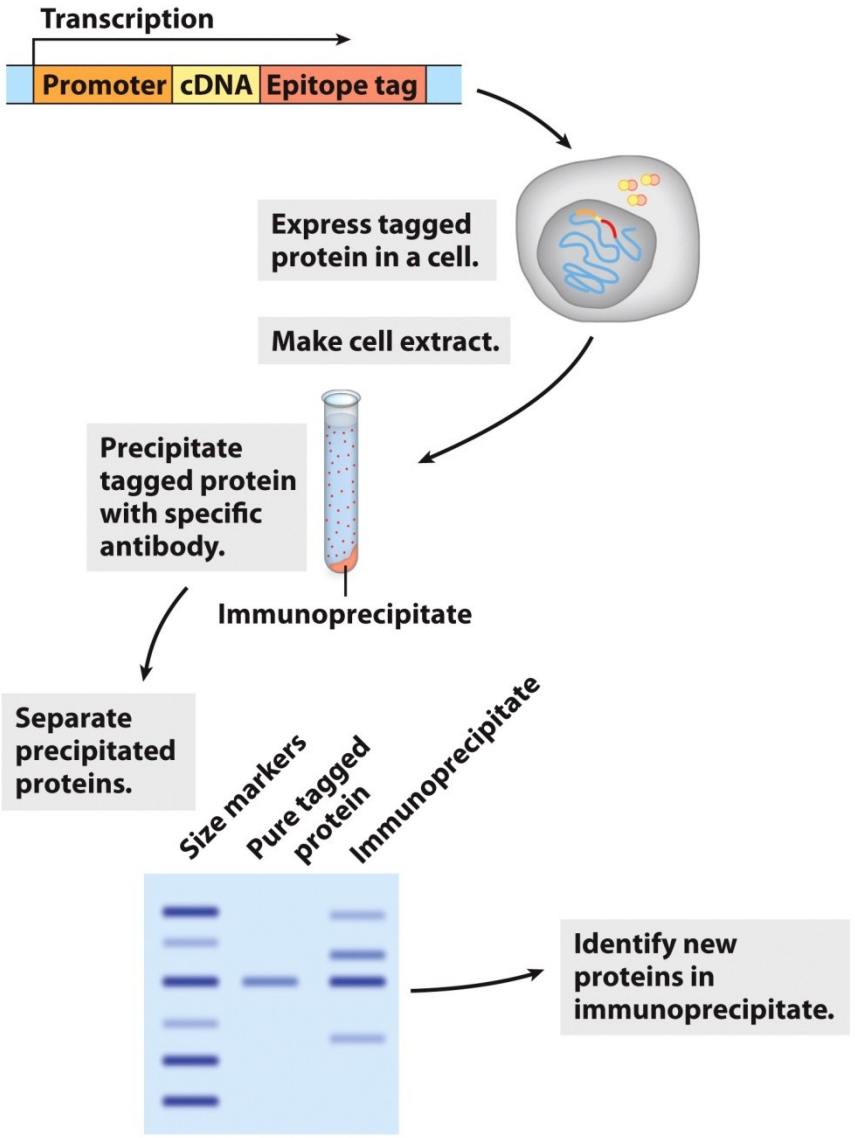


Figure 9-19
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Procedure for TAP–Tagged Proteins

- Use of Tandem Affinity Purification (TAP) tags has enhanced the procedure
- Allows two purification steps eliminating loosely associated proteins, and minimizing non-specific binding

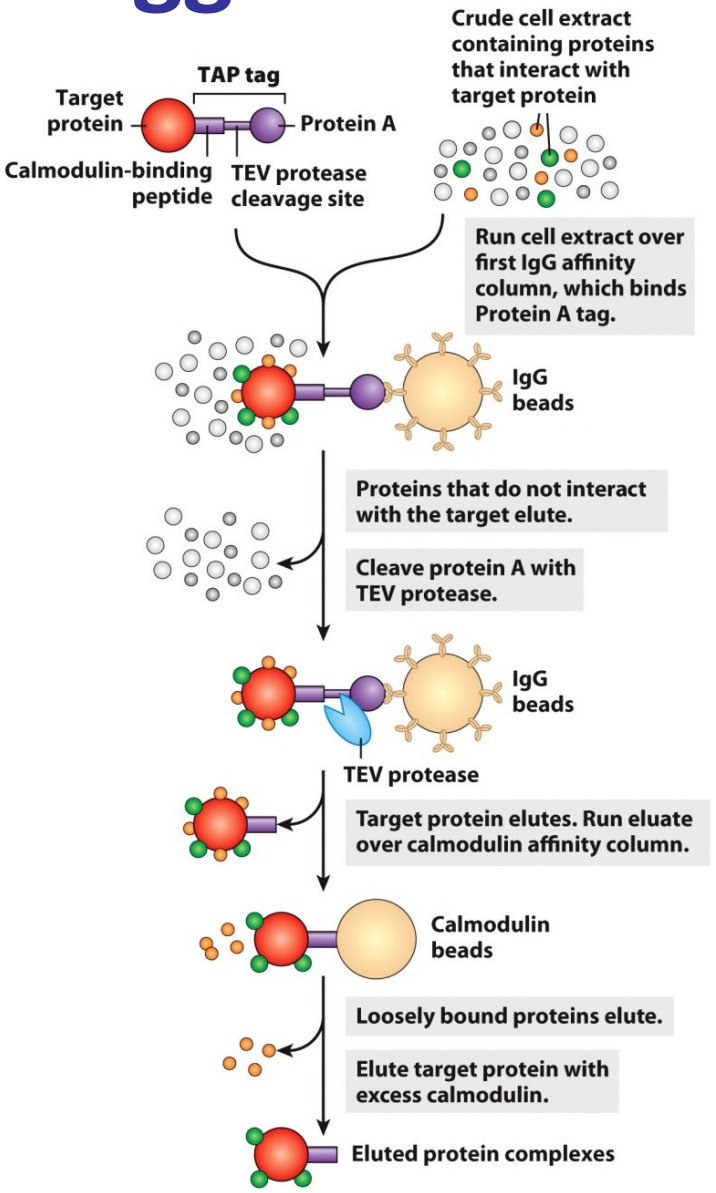

Figure 9-20
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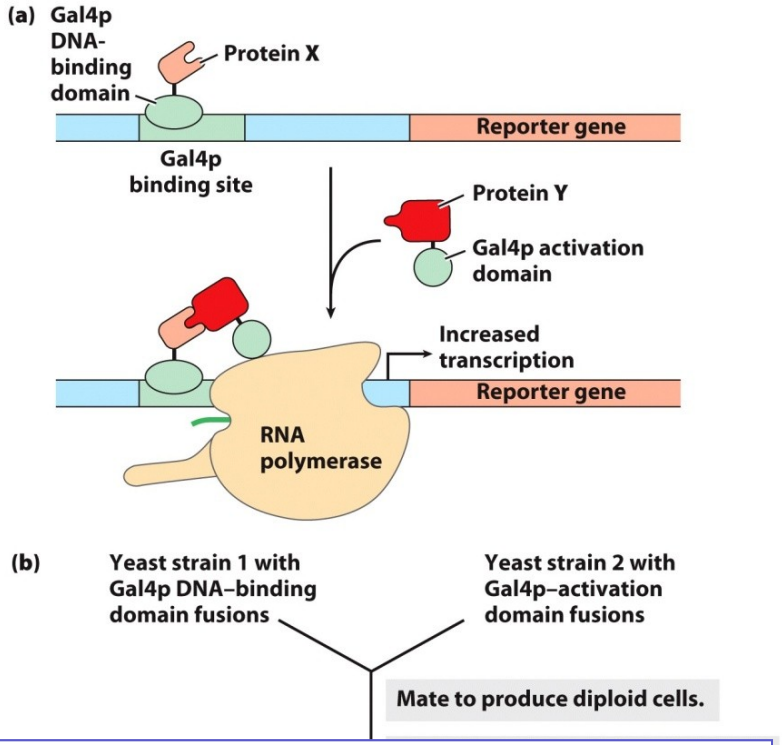
Yeast-Two Hybrid System

- Protein of interest tagged with the GAL4-activation domain
- DNA library with all yeast genes tagged with Gal4-binding domain
- Reporter gene under the control of Gal4
- Differentially tagged proteins must interact in order to get expression of the reporter gene



Similar techniques developed to use with bacterial and mammalian cells

Figure 9-21
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Assessment of Protein-protein Interaction Data

- Currently believed that yeast has >30,000 different interactions (for ~6,000 proteins)
- Variety of studies using yeast (see von Mering *et al.* (2002) *Nature*, **417**, 399-403)
- Overall conclusion is: different techniques identify different complexes!
- Results from protein-protein interaction studies should be confirmed by more than one experimental technique
- Especially important for considering if *in vitro* observations are relevant for *in vivo* situations

Study of Protein-protein Interactions *In Vivo*

- Popular technique is “Two-hybrid” screen (yeast, mammalian or bacterial)
- Various fluorescent techniques are also in use:
  - FRET – fluorescence resonance energy transfer; reports on distance between 2 fluorophores
  - Fluorescent reporters – expressed proteins emit fluorescence at specific wavelength
  - FRAP (FLIP) – fluorescence recovery after photobleaching (fluorescence loss in photobleaching); allow movement of reporters to be monitored

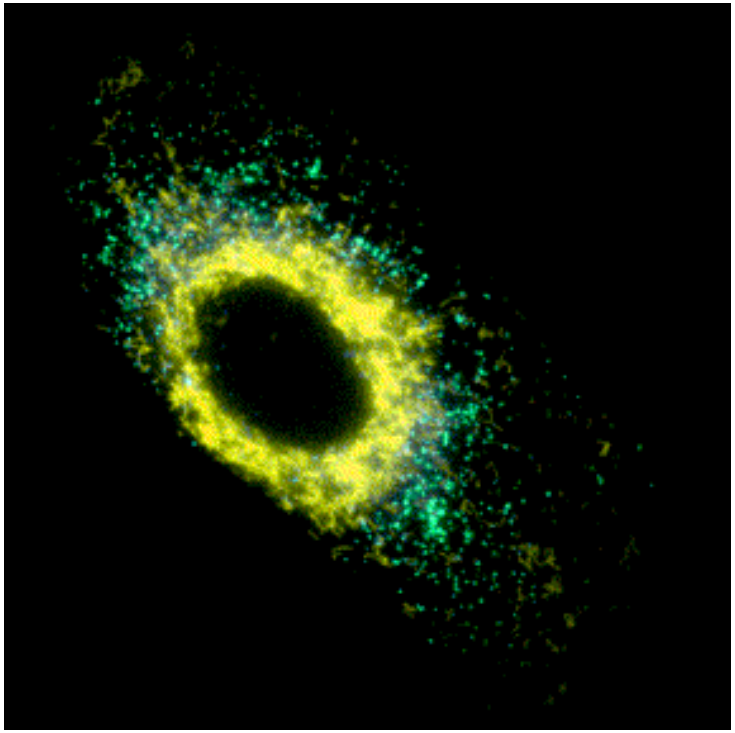
Fluorescence can be used to Determine Protein Location *In Vivo*

- Use recombinant DNA technologies to attach Fluorescent Proteins to protein of interest
  - ➢ Visualize with a fluorescent microscope
- Immunofluorescence
  - ➢ Tag protein with primary antibody and detect with secondary antibody containing fluorescent tag
  - ➢ Protein can also be fused to a short epitope and the primary antibody detecting the epitope can be fluorescently labeled

Fluorescently-tagged Proteins

- Combination of molecular and cell biological studies analyse *in vivo* localisation of proteins expressed with a fluorescent "tag"
- Important that "tag" does not interfere with protein activity

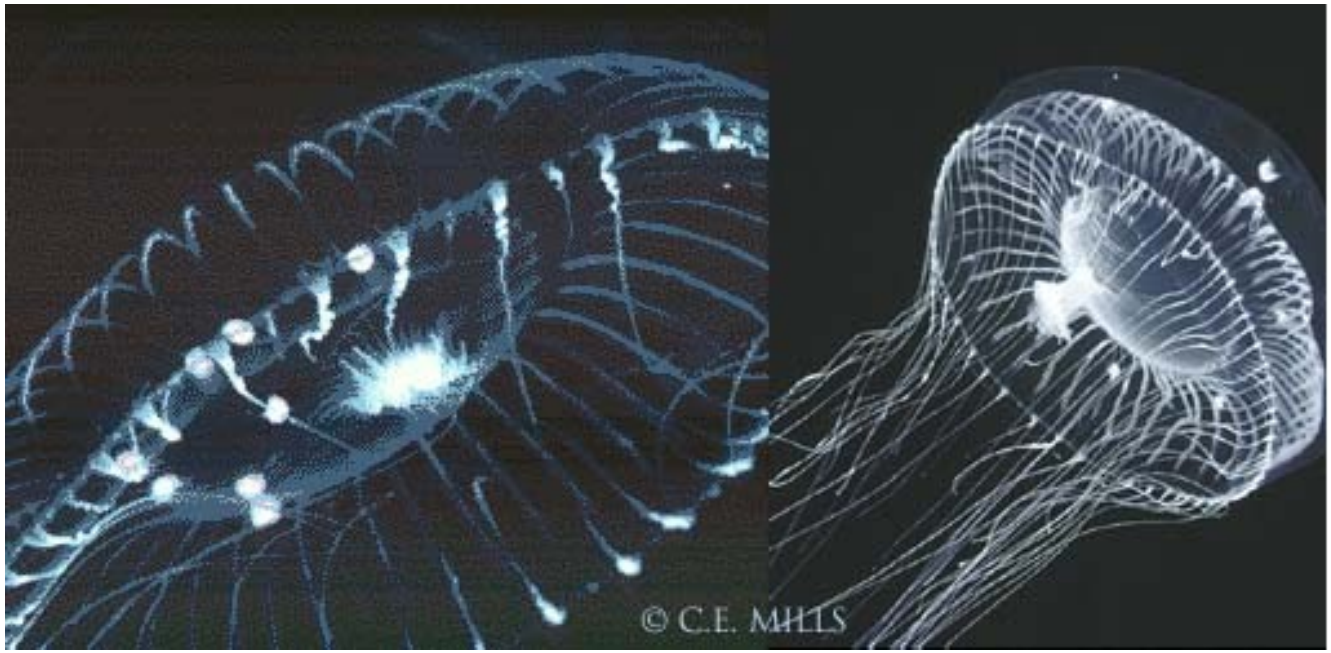
- Can examine localisation of proteins containing different fluorophores



Bastiaens & Pepperkok (2000) *TiBS,* **25**, 631-637

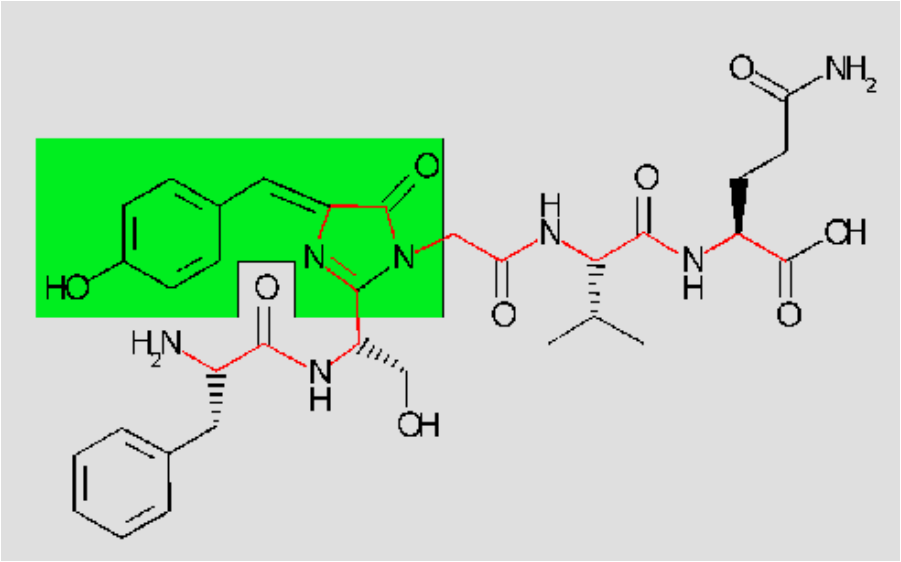
Green Fluorescent Protein Tags

- Widely used tag is “Green fluorescent protein” (GFP)
- GFP was first discovered as a companion protein to aequorin, the chemiluminescent protein from *Aequoria victoria*



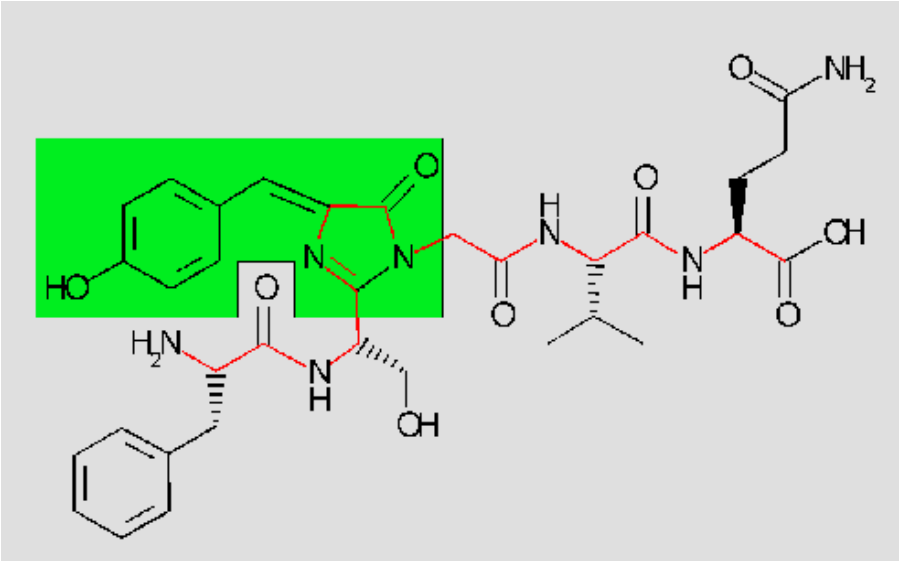
Green Fluorescent Protein Tags

- For GFP, the chromophore is a p-hydroxybenzylidene-imidazolidone (green background)
- Consists of residues 65-67 (Ser - *dehydro*Tyr - Gly) of protein and their cyclized backbone forms the imidazolidone ring
- Peptide backbone is shown in red



Green Fluorescent Protein Tags

- Amino acid sequence SYG can be found in a number of other non-fluorescent proteins, but it is usually not cyclized, and Tyr is not oxidized
- Implies that this tripeptide does not have intrinsic tendency to form such a chromophore



Development of Fluorescent Tags

- Mutagenesis studies yielded GFP variants with improved folding and expression properties
- Changes help:
  - accelerate speed and intensity of fluorophore formation
  - help the molecule fold correctly at 37 °C
  - overcome dimerization
  - improve expression by converting codons to those used by the organisms of interest
- These characteristics are combined in the GFP variant known as enhanced GFP (EGFP)

GFP–Tagged Protein Localization

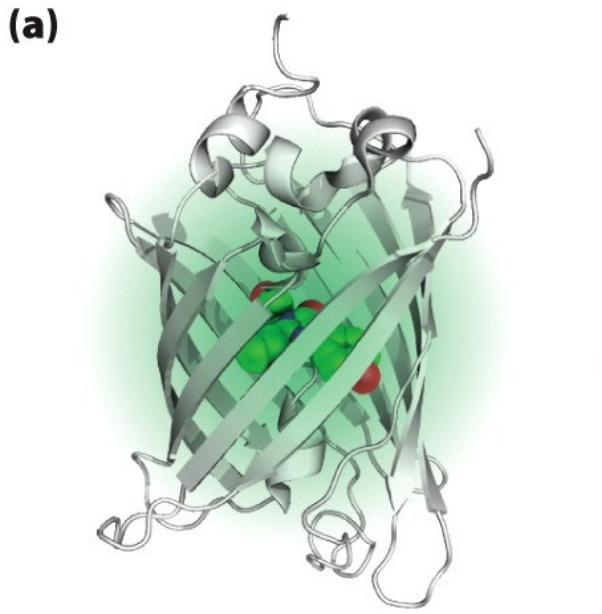


Figure 9-16
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Further Development of Tags

- Continued efforts to engineer (or isolate) new fluorophores and reporter classes:
  - ➢brighter and more red-shifted proteins useful for multi-spectral imaging and FRET-based methods
  - ➢increased brightness will help track single molecules
  - ➢more pH resistance useful in acidic environments
- Advances in imaging systems are also important:
  - ➢more sensitive and quicker camera systems
  - ➢filter systems for detecting different fluorophores
  - ➢software for discriminating fluorescent signals
- Understanding complex protein interactions and dynamics also requires kinetic modeling and analysis

Lippincott-Schwartz & Patterson (2003) *Science,* **300**, 87-91

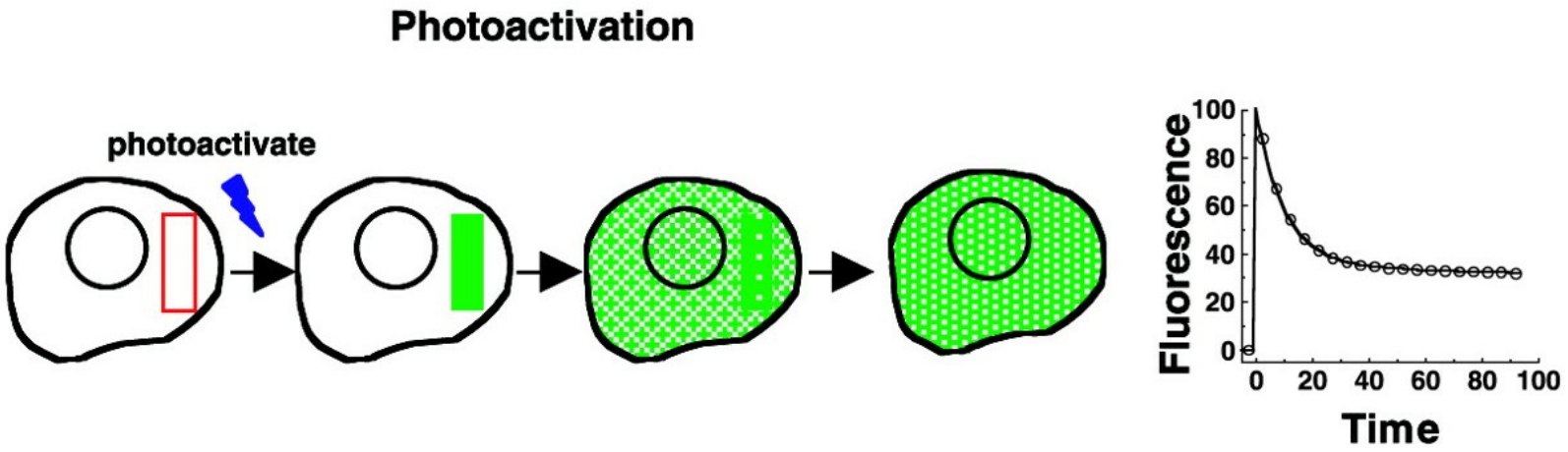
GFP Turnover

- Analysis of protein turnover or temporal expression pattern and behavior is difficult with conventional GFP because the GFP chimeras are continuously being synthesized, folded, and degraded within cells
- Thus, at any particular time, proteins at different stages of their lifetime are being observed
- Several promising approaches have used FPs which have different fluorescent properties over time
- Another promising approach to studying protein lifetimes and turnover rates is the use of photoactivable fluorescent proteins

Lippincott-Schwartz & Patterson (2003) *Science,* **300**, 87-91

GFPs in Action!

- Photoactivatable fluorescent proteins display little initial fluorescence under excitation at imaging wavelength (λ)
- Fluorescence increases after irradiation at a different λ – highlighting distinct pools of molecules within the cell
- Since only photoactivated molecules exhibit noticeable fluorescence, their behaviour can be studied independently of other newly synthesized proteins



Lippincott-Schwartz & Patterson (2003) *Science,* **300**, 87-91

Immunofluorescence

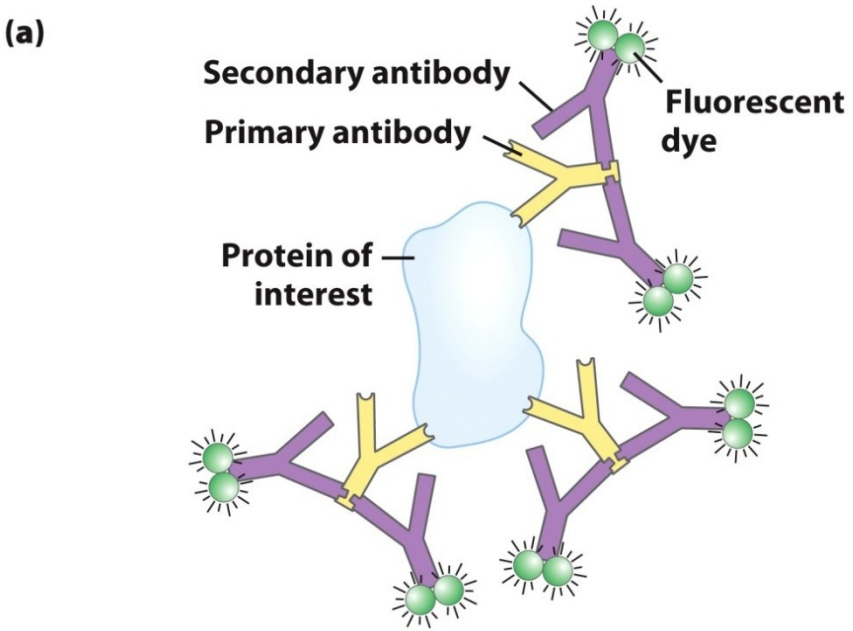


Figure 9-17
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Identifying Regions Involved in Protein-protein Interactions

- Once protein-protein interactions have been identified, it is important to establish how the interactions occur e.g. what regions or specific amino acids are important for the interaction?
- Well-used approach is to prepare different fragments or mutations of proteins and see if there is any effect on the protein-protein interaction
- Results usually confirmed by more than one experimental technique

Yeast-Two Hybrid System

- Protein of interest tagged with the GAL4-activation domain
- DNA library with all yeast genes tagged with Gal4-binding domain
- Reporter gene under the control of Gal4
- Differentially tagged proteins must interact in order to get expression of the reporter gene

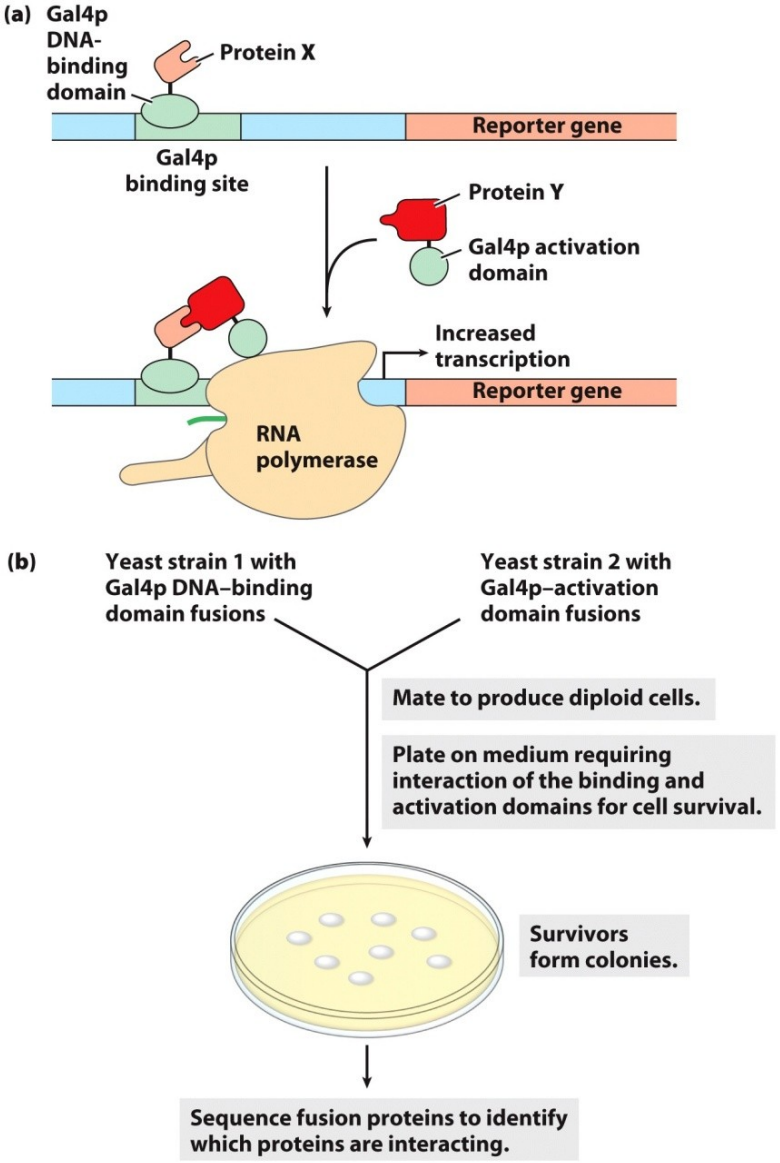

Figure 9-21
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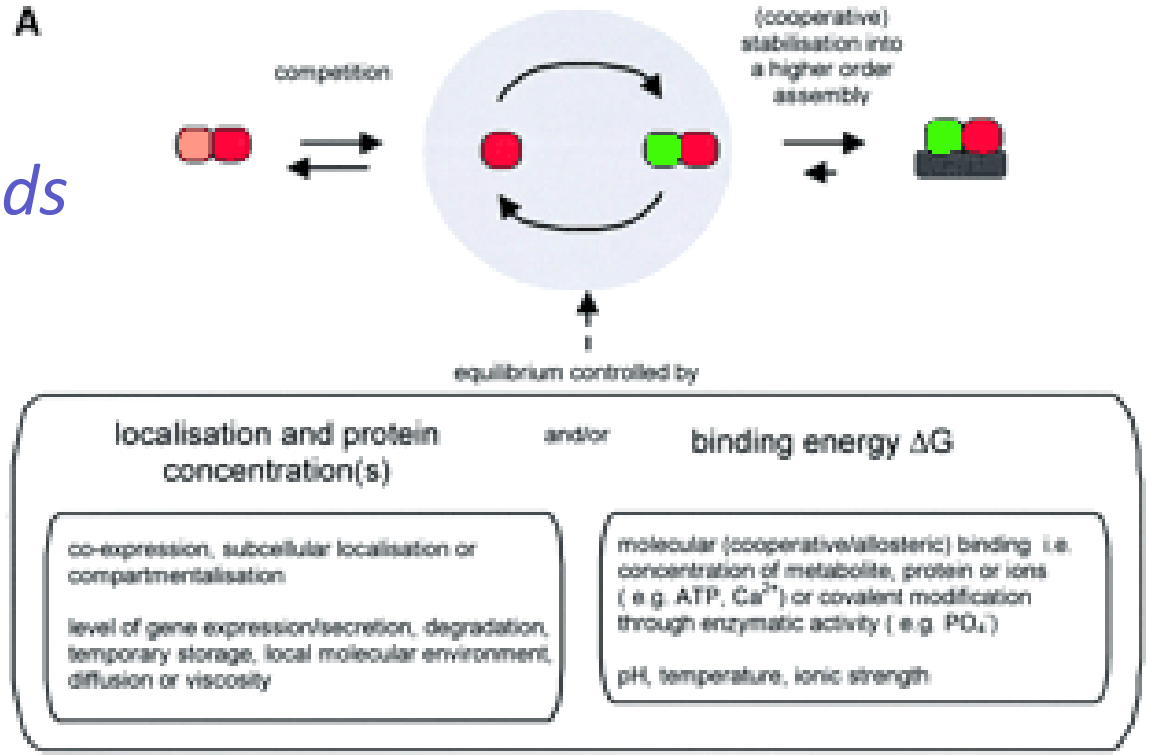
Transient Protein-protein Interactions

- Current proteomics studies have allowed the identification of protein interactions on large scale
- Protein networks underline the multi-specificity and dynamics of complexes involving transient interactions
- *Biophysical methods are very useful to characterise such interactions*



Nooren & Thornton (2003) *EMBO J.*, **22**, 3486-3492

Molecular Interactions Overview

- Biophysical chemistry approaches are good for studies of macromolecular interactions, particularly because they can provide quantitative data
- High-resolution structures have been identified for a wide range of interactions; particularly well-defined for some proteins binding to nucleic acids
- Many techniques developed to study protein-protein interactions *in vivo*
- Applications of fluorescence and fluorescent proteins provide important information about macromolecular interactions