TECHNIQUES IN CELL AND MOLECULAR BIOLOGY

Cell Culture

I. Why use cultured cells in research? – study cells & cell function in simplified, controlled, *in vitro* system; can remove cells from influences they are normally subject to within a complex multicellular organism
   A. Cultured cells can be obtained in large quantity
   B. Most cultures contain only a single cell type; a wide variety of different cells can be grown in culture
   C. Many different cellular activities can be studied in cell culture, including endocytosis, cell movement, cell division, membrane trafficking, macromolecular synthesis
   D. Cells can differentiate in culture (conversion of embryonic cells to highly specialized cells)
   E. Cultured cells respond to treatment with drugs, hormones, growth factors & other active substances

II. First vertebrate cell culture (1907) - over time, optimal conditions were determined & contamination by microorganisms eliminated
   A. Early culture media included a great variety unknown substances; even today, most media contain serum
      1. Cell growth was accomplished by adding fluids from living systems to culture (lymph, blood serum, embryo homogenates)
      2. Cells were found to need a variety of nutrients, hormones, growth factors & cofactors to remain healthy & proliferate; even today, most culture media contain large amounts if serum
   B. Cell culturists are trying to develop defined, serum-free media that support cell growth
      1. They have tested combinations of various ingredients for ability to support cell growth & proliferation
      2. A growing number of cell types have been successfully cultured in artificial media that lack serum or other natural fluids
      3. Composition of these chemically defined media is a relatively complex mix of nutrients, vitamins, a variety of purified proteins (insulin, epidermal growth factor, transferrin [provides cells with iron])
   C. Since they are so rich in nutrients, tissue culture media invite the growth of microorganisms
      1. Culturists must go to extreme lengths to maintain sterile conditions within their working space
      2. They use sterile gloves, sterilize all supplies & instruments, employ low antibiotic levels in media and conduct activities beneath a sterile hood

III. Process of cell culture: **secondary culture** - get cells (often previously cultured cells frozen in liquid N_2), thaw them & place them in medium; called secondary culture since cells are derived from a previous culture

IV. Process of cell culture: **primary culture** – get cells directly from organism, most animal cell primary cultures are from embryos
   A. Embryo tissues are more readily dissociated into single cells than those of adults
   B. Take tissue from embryos & treat with proteolytic enzyme, like trypsin - digests extracellular domains of proteins that mediate cell adhesion

C. Wash tissue free of enzyme & usually suspend cells in saline solution lacking Ca^{2+} ions & containing a substance like ethylenediamine tetraacetate (EDTA), a chelator that binds (chelates) Ca^{2+} ions
   1. Ca^{2+} ion removal greatly facilitates cell separation since it plays a key role in cell-cell adhesion

D. Once cells are in single-cell suspension, two kinds of primary cultures can be started: mass culture & clonal culture

V. Types of primary cell culture – mass & clonal culture
   A. **Mass culture** – a relatively large number of cells is added to culture dish
      1. Cells settle & attach to dish bottom & form relatively uniform cell layer; surviving cells grow & divide
      2. After a number of generations the cells form cell monolayer that covers the bottom of dish
   B. **Clonal culture** – a relatively small number of cells is added to a dish so that each cell resides at some distance from its neighbors
      1. Under these conditions, each surviving cell proliferates to form a separate colony or clone whose members are all derived from the same original cell

VI. Normal (nonmalignant) cells can only divide a limited number of times (~50-100) before they senesce & die
   A. Thus, many cells commonly used in tissue culture studies have undergone genetic modifications
      1. These modifications allow them to grow indefinitely (such cells are called a **cell line**)
      2. They typically can grow into malignant tumors, if injected into susceptible laboratory animals
   B. Frequency with which normal cells are transformed into cell line depends on organism from which they are derived —> mouse cells transform at relatively high frequency; human cells only rarely if ever
      1. Human cell lines (e.g., HeLa cells) are typically derived from human tumors or from cells treated with cancer-causing viruses or chemicals

VII. Many different types of plant cells can also be grown in culture
   A. In one approach, culturists can treat cells with cellulase, which digests away surrounding cell wall, releasing naked cell (**protoplast**)
      1. Grow protoplasts in special, chemically defined, medium that promotes their growth & division
      2. Under suitable conditions, cells can grow into an undifferentiated clump of cells (**callus**)
      3. Callus can be induced to develop shoots from which a new plant can regenerate
   B. In alternate approach, leaf tissue cells can be induced by hormone treatment to lose their differentiated properties & develop into callus material —> transfer callus to liquid media & start cell culture

Isolation, Purification and Fractionation of Proteins: Background Information

I. To study a protein's fine structure, it must be isolated in a relatively pure state – most cells contain thousands of proteins, some at low concentration, so this is a challenging task

II. Purification is generally accomplished by the stepwise removal of contaminants
   A. Two proteins may be very similar in one property, like overall charge, but very different in others, like molecular size or shape
   B. Successive techniques that take advantage of different properties of proteins being separated are used
   C. Start with methods that work best with high protein concentrations, then move to more

sensitive ones

III. Measure purification as an increase in **specific activity** (ratio of amount of protein of interest to the total amount of protein present in the sample)

Liquid Column Chromatography

I. Chromatography – term for a variety of techniques in which a mixture of dissolved components is fractionated as it moves through a porous matrix
   A. In liquid chromatography, components in mixture can associate with 1 of 2 alternative phases:
      1. Mobile phase, consisting of a moving solvent; liquid chromatography is distinguished from gas chromatography in which the mobile phase is represented by an inert gas
      2. Immobile phase, consisting of the matrix through which solvent is moving (often packed into column)
   B. Proteins to be fractionated are dissolved in solvent, passed through column & interact with matrix to varying extents
      1. Materials making up immobile phase contain sites to which the proteins in solution can bind
      2. As individual proteins interact with matrix —> their progress through the column is retarded
      3. If a particular protein has a greater affinity for matrix —> its passage through column is slower
      4. Different proteins attracted to matrix with different affinities, so they are retarded to different degrees & thus separated

   C. As solvent passes through column, it is collected as it drips out bottom into series of tubes (**fractions**)
      1. Proteins in mixture with least affinity for matrix appear in first fractions emerging from column
      2. Those with more affinity for matrix come out later or may bind more permanently to column

II. ***H***igh - ***P***erformance ***L***iquid ***C***hromatography (HPLC) - gives very high resolution, much more than traditional chromatographic procedures
   A. Long, narrow columns are used
   B. Mobile phase is forced through a tightly packed noncompressible matrix under high pressure

III. Ion-exchange chromatography – it is unlikely that many proteins in partially purified prep have the same overall charge, since they are large, polyvalent electrolytes; ionic charge is basis of purification
   A. Overall charge of a protein is the sum of the individual charges of its component amino acids
      1. Depends on pH of medium, since the charge of each amino acid R group depends on medium pH
      2. Lower pH & negatively charged groups are neutralized, positively charged groups get more abundant
      3. Increase pH & the opposite happens
   B. For each protein, there is a pH (**isoelectric point**) at which negative & positive charges are equal - most proteins have isoelectric point below 7; they are neutral at that pH
   C. In ion exchange chromatography, charged groups are covalently attached to inert matrix material (cellulose; tiny beads)
      1. ***D***i***e***thyl***a***mino***e***thyl (DEAE)-cellulose - positively charged; binds negatively charged

molecules; an anion exchanger

2. ***C***arboxy***m***ethyl (CM)-cellulose - negatively charged; binds positively charged molecules; a cation exchanger

D. Procedure - starts by applying protein solution to column packed with resin

1. Allow protein solution to pass through column in buffer whose composition promotes binding of all or some proteins to the resin
2. Proteins are bound to resin reversibly & can be displaced in stepwise manner by sequential addition of different buffers or a continuous gradient in pH or ionic strength
3. Generally displace proteins by increasing ionic strength of buffer (adds small ions to compete with the charged groups of the macromolecules for sites on the resin) and/or changing its pH
4. Proteins are eluted from column in order from least strongly bound to most strongly bound

IV. Gel filtration chromatography - separates proteins (or nucleic acids) primarily on the basis of their effective size (**hydrodynamic radius**)

A. Matrix made of tiny beads packed into column; the solution containing proteins passes through slowly

1. Beads are composed of cross-linked polysaccharides (dextrans, agarose) of different porosity
2. Varying porosity allows proteins to diffuse in & out of the beads differentially

B. Example: protein being purified (with 125 kD molecular mass) is mixed with contaminating proteins (250 & 75 kD) of similar shape, one much larger and one much smaller

1. Pass mix through column with beads that allow entry of globular proteins of <~200 kD into their interiors (Sephadex G-150)
2. As it passes through column bed, 250 kD protein cannot enter beads, stays dissolved in moving solvent phase & elutes as soon as preexisting solvent in column (**bed volume**) has dripped out
3. The other 2 proteins diffuse into interstices within beads & are retarded in passage through column
4. As more & more solvent moves through column, the proteins move down its length & out bottom
5. When they eventually move out of column, they do so at different rates —> smaller species retarded more than larger ones; 125 kD protein is pure since it elutes, while 75 kD protein still in column

V. Affinity chromatography - takes advantage of unique structural properties of desired protein; contrasts with previous techniques, which used the bulk properties of a protein to effect purification or fractionation

A. Desired protein can be specifically withdrawn from solution, while all others stay behind in solution

1. Proteins interact with specific substances: enzymes interact with substrates, receptors with ligands, antibodies with antigens
2. Covalently attach such an interacting molecule to column's inert, immobilized material (matrix) & pass protein mixture through column —> protein that binds column-linked molecule is retained

B. Example: attach insulin to agarose beads, pack them into column & pass a crude insulin receptor prep through the column

1. If conditions favor interaction, receptor binds to insulin on column; contaminants go through
2. After contaminants have passed through the column & out the bottom, insulin receptor can then be displaced from matrix binding sites by changing ionic composition and/or pH of column solvent

C. Can achieve near total purification of desired protein in a single step, unlike other

procedures

Determining Protein-Protein Interactions

I. One way to learn about a protein's function is to identify the proteins with which it interacts; several techniques have been used to do this, including affinity chromatography

II. Another technique uses ABs – consider protein A, which has been identified & purified, that is part of a complex with 2 other proteins in the cytoplasm, proteins B & C
   A. After A is purified, prepare an AB against it & use it as a probe to bind to & remove A from solution
   B. If cell extract is prepared containing A-B-C complex & then incubated with anti-A AB, then binding of antibody to the A protein will usually result in coprecipitation of other proteins bound to A
   C. In this case, proteins B & C should coprecipitate; they can then be identified by other means

III. Yeast two-hybrid system – technique most widely used to search for protein-protein interactions; invented in 1989 by Stanley Fields & Ok-kyu Song (SUNY-Stony Brook)

   A. Depends on expression of reporter gene, like β-galactosidase (*lacZ*), whose activity is readily monitored by a test that detects a color change when the enzyme is present in a yeast cell population
      1. *lacZ* gene expression in this system is activated by a particular protein, a TF) that contains 2 domains, a DNA-binding domain & an activation domain
      2. DNA-binding domain mediates binding to promoter & activation domain mediates interaction with other proteins involved in gene expression activation; both domains needed for transcription to occur
   B. 2 different types of recombinant DNA molecules are prepared:
      1. One has DNA segment encoding TF DNA-binding domain linked to DNA segment encoding a "bait" protein X (a protein already characterized & for which potential binding partners are sought)
      2. The other DNA has a DNA segment encoding TF activation domain linked to DNA encoding an unknown protein Y; assume Y is a protein capable of binding the "bait" protein
   C. Such DNAs (or cDNAs) are prepared from mRNAs by reverse transcriptase; both recombinant DNAs, if expressed in yeast cell, lead to production of hybrid proteins
   D. If produced in cell alone, neither the X- nor Y-containing hybrid proteins activate *lacZ* transcription
      1. If yeast cell is transfected with both of these recombinant DNAs, the X & Y proteins interact with one another to reconstitute a functional TF that transcribes the *lacZ* gene
      2. The transcription of *lacZ* that results can be detected by cell's ability to make β-galactosidase
   E. Allows researchers to "fish" for proteins encoded by unknown genes that are able to interact with the "bait" protein

Polyacrylamide Gel Electrophoresis

I. **Electrophoresis** - techniques depending on the ability of charged molecules to migrate if placed in electric field; a powerful technique widely used to fractionate proteins
   A. Electrophoretic separation of proteins is usually accomplished using **polyacrylamide gel electrophoresis** (**PAGE**)
      1. Proteins are driven by applied current through a gelated matrix made of polymers of small organic molecules (acrylamide)
      2. Polymers are cross-linked to form molecular sieve (may be in form of thin slab formed between 2 glass plates or a cylinder formed in a glass tube)
   B. Once polymerized, the gel (slab or tube) is suspended between 2 compartments containing

buffer into which opposing electrodes have been dipped

1. In slab gel, concentrated, protein-containing samples are applied in slots at top of gel (prepared in glycerol or sucrose solution, whose density prevents mixing with buffer in upper compartment)
2. Apply voltage between buffer compartments & current flows across slab —> proteins move toward electrode of opposite charge
3. Usually buffers are alkaline, so proteins have "-" charge & move toward "+" electrode, the anode, at the opposite end of the gel
4. After electrophoresis, the slab is removed from the glass plates & stained

C. Relative protein movement in gel depends on size, shape & charge density (charge/unit of mass)

1. High charge density moves protein through gel more forcefully —> migrates at more rapid rate

2. Polyacrylamide forms a cross-linked molecular sieve that entangles proteins passing through gel

3. The larger the protein the more it is entangled in molecular sieve & held back (retarded; migrates more slowly)
4. Shape is also a factor: compact globular proteins move faster than long fibrous proteins of comparable molecular mass

D. The acrylamide & cross-linking agent concentrations are also important factors affecting movement

1. If acrylamide is less concentrated (down to ~2%) —> less cross-linking —> protein migrates faster
2. Gel containing 5% acrylamide might be useful for separating proteins of 60 – 250 kDa, while a gel of 15% acrylamide might be useful for separating proteins of 10 – 50 kDa

E. Follow electrophoresis progress by charged tracking dye migration moving just ahead of fastest proteins

1. When tracking dye has moved to desired location —> current is turned off & gel removed from its container —> stained to see proteins (Coomassie Blue or silver stain)
2. If proteins are radiolabeled, can locate them with autoradiography (press gel against X-ray film) **or**
3. Slice gel up into fractions to isolate proteins, which retain their biological activity

F. Alternatively, can transfer proteins with current to nitrocellulose filter placed against gel

1. Proteins are absorbed onto membrane (filter) surface in same relative positions they occupied in gel
2. Proteins in individual bands are identified by their interaction with specific antibodies (Western blot)

II. SDS-PAGE - PAGE usually done in presence of "-" charged detergent, sodium dodecyl sulfate (SDS)

A. SDS molecules bind in large numbers to all types of proteins —> denature proteins —> lose activity

1. Electrostatic repulsion between SDS molecules unfolds proteins into similar rod shape; differences in shape are no longer a factor in separation
2. # of protein-bound SDS molecules is roughly proportional to molecular mass (~1.4 g SDS/g protein)
3. Thus, all proteins have same charge density & are driven through gel with same force, regardless of size
4. Large proteins are retarded more by cross-linking than are smaller proteins

B. Thus, proteins are separated by SDS-PAGE on the basis of a single property - molecular mass

1. SDS-PAGE can also be used to determine the molecular mass of various proteins
2. Can compare band positions of proteins of unknown MW to those of known standards to

find MW

III. Two-dimensional gel electrophoresis (1975) - 2-D separation based on 2 different properties; developed by Patrick O'Farrell at Univ. of Calif., San Francisco; used to fractionate complex mixtures of proteins
   A. First separate proteins by isoelectric point with isoelectric focusing within tube gel
   B. Tube gel is removed & placed on top of SDS-saturated polyacrylamide slab gel & subjected to SDS-PAGE —> proteins move into slab & are separated according to molecular mass
   C. Once separated, individual proteins can be removed from gel & digested into peptide fragments —> fragments can be analyzed by mass spectrometry
   D. Resolution is sufficiently high that virtually all proteins in cell can be distinguished (several thousand)

   E. Due to resolving power, 2D-gel electrophoresis is ideally suited to detect changes in proteins found in cell under different conditions, at different developmental or cell cycle stages or in different organisms
      1. Not suitable for distinguishing among proteins that have high molecular mass (MW), that are highly hydrophobic, or that are present at very low copy numbers per cell

Mass Spectrometry

I. Proteomics depends heavily on analysis of proteins by mass spectrometry
   A. Mass spectrometers are analytical instruments used primarily to measure molecular masses, determine chemical formulas & molecular structure, & identify unknown substances
   B. Mass spectrometers accomplish these goals as follows:
      1. They convert substances in a sample into positively charged, gaseous ions, which are accelerated through a curved tube toward a negatively charged plate
      2. As ions pass through tube, they are subjected to a magnetic field causing them to separate from one another according to molecular mass (or, more correctly, according to mass-to-charge [m/z] ratio)
      3. The ions strike an electronic detector located at the end of the tube
      4. Smaller ions travel faster & strike detector more rapidly than larger ions, which travel more slowly
      5. The input to the detector is converted into a series of peaks of ascending m/z ratio

II. Mass spectrometers have been a favorite of chemists for a while, but only in the last decade have biologists discovered their usefulness - protein biochemists can identify unknown proteins in a matter of hours

III. Procedure - proteins are generally digested with trypsin & the resulting peptides are gently ionized & made gaseous by one of two procedures – MALDI & ESI
   A. In matrix-assisted laser desorption ionization (MALDI), protein sample applied as part of crystalline matrix; it is irradiated by laser pulse; laser energy excites matrix & converts peptides into gaseous ions
   B. In electrospray ionization (ESI), an electric potential is applied to peptide solution, causing peptides to ionize & the liquid to spray as a fine mist of charged particles that enter the spectrometer
      1. Because it acts on molecules in solution, ESI is well suited to ionize peptides prepared from proteins fractionated by a widely employed liquid chromatography technique

IV. Once molecular masses of peptides in sample are determined, the complete protein can be identified by a database search
   A. If protein is not identified unambiguously, one or more of peptides generated by tryptic digestion can be fragmented in a second step & subjected to another round of mass spectrometry
      1. Fragmentation is accomplished in mass spectrometer by collision of peptides with inert gas

like argon

2. The energy of collision breaks peptide bonds to produce a random collection of fragments of the original peptide
3. Each fragment's amino acid sequence, & hence that of original peptide, can be determined by searching a database
4. The database contains the masses of theoretical fragments having every possible sequence of amino acids that can be formed from the proteins encoded by the genome

B. This two-step procedure (called **tandem MS** or **MS/MS**) yields the amino acid sequence of the peptide(s) & hence the unmistakable identification of the protein

1. MS/MS is so powerful that complex mixtures of hundreds of unknown proteins can be digested & subjected to mass spectrometry; the identity of each protein in mixture can be determined all at once

molecular structures (ex. – structure of ribosome); quite often the biggest challenge is getting usable crystals

Purification and Fractionation of Nucleic Acids

I. DNA purification – very different from steps used to purify proteins due to differences in structure; first step is usually homogenization of cells & isolation of nuclei from which DNA is extracted

A. Extraction medium is buffered salt solution + detergent, like SDS, that lyses nuclei, releasing DNA; solution viscosity rises as DNA is released; detergent also inhibits nuclease activity found in preparation

B. The goal of the rest of the procedure is to separate DNA from contaminants, like RNA & protein

1. Deproteinization usually accomplished by shaking extract with phenol or phenol/chloroform mix

precipitate

3. Phenol & buffered saline are immiscible, so one centrifuges to separate the phases: DNA (& RNA) are in solution's upper aqueous phase; protein present as precipitate at boundary between phases
4. Aqueous phase is taken from tube & procedure repeated until no more protein is at phase boundary
5. Precipitate DNA/RNA with cold ethanol layered on top of aqueous phase (spool DNA out at saline/ethanol interface; in contrast, RNA goes to vessel bottom, settling as flocculant precipitate)
6. Redissolve DNA & RNase treat it to remove contaminating RNA; then destroy RNase with a protease
7. Then deproteinize with phenol to remove protease & reprecipitate DNA with ethanol

II. RNA can be isolated in similar way, but treat with DNase, instead of RNase, in final purification steps; an alternative procedure for isolating RNA in a single step was published in 1987 (see below)

A. Homogenize tissues in a solution containing 4 M guanidine thiocyanate

B. Mix RNA extract with phenol & shake with chloroform (or bromochloropropane)

C. Centrifuge the suspension, leaving RNA in the upper aqueous phase & both DNA & protein at the interface between the 2 phases

III. Separation of DNAs by gel electrophoresis - separate nucleic acids by MW (nucleotide length)

A. Small RNAs or DNAs (a few hundred nucleotides or less) can be separated by PAGE

B. Larger nucleotide molecules are separated on agarose gels because of this gel's greater porosity; they have trouble making their way through the cross-linked polyacrylamide

1. Agarose is a polysaccharide extracted from sea weed
2. It is dissolved in hot buffer, poured into a mold & gelated by lowering temperature
3. Lower agarose concentrations (as low as 0.3%) are used to separate larger DNA fragments

C. Separation of DNA molecules > ~25 kb is generally done by technique of pulsed-field electrophoresis in which the direction of the electric field in the gel is periodically changed

1. Change in field direction causes DNA molecules to reorient themselves during their migration

D. Principles for DNA/RNA electrophoresis are similar to those for protein SDS-PAGE - all nucleic acid molecules have similar charge density (the number of negative charges per unit of mass)

1. All nucleic acids, regardless of length, have an equivalent potential for migrating in electric field with agarose providing resistance
2. The greater the molecular weight, the more slowly a DNA/RNA fragment travels through the gel

3. Technique is sensitive; can separate on basis of single nucleotide (important in DNA sequencing)

E. See specific DNA fragment in gel by binding it to labeled probe with complementary sequence – identify DNA fragments containing insulin gene sequence by binding DNA probe containing that sequence

F. See all DNA fragments in gel by immersing gel in ethidium bromide that intercalates into double helix

1. Ethidium bromide causes DNA bands to appear fluorescent when viewed under UV light
2. Ethidium bromide intercalates into the double helix

Ultracentrifugation

I. Solution or suspension stability depends on its components; some things float, some sediment & some things stay in solution (remain stable) indefinitely - why do substances sediment through a liquid medium?

A. Factors, which determine whether or not a given component will settle through a liquid medium

1. Size, shape & density of component
2. Density & viscosity of the medium

B. Component in solution or suspension will sediment through centrifugal field if it has greater density than surrounding medium —> it is concentrated toward bottom of centrifuge tube

1. Larger particles sediment more rapidly than smaller particles of similar shape & density
2. Tendency of sedimentation process to concentrate molecules is counteracted by the effects of diffusion, which causes molecules to be redistributed in a more uniform or random arrangement
3. Sedimentation depends on rate of diffusion compared to opposing centrifugal force applied
4. Larger proteins & nucleic acids diffuse slower than smaller ones

which is high enough to overcome the effects of diffusion & cause macromolecules to move to bottom of tube

1. Centrifugation proceeds in a near vacuum to minimize frictional resistance

II. Various macromolecules & their complexes are referred to as having a particular S value – the unit S (or Svedberg, after the inventor of the ultracentrifuge) is equivalent to a sedimentation coefficient of 10^{-13} sec

A. S value alone does not provide molecular mass because the velocity at which a particle moves through a liquid column depends on a number of factors, including shape

B. It is a good measure of relative size as long as one deals with the same type of molecule: the 3 *E. coli* rRNAs (5S, 16S & 23S) have lengths of 120, 1600 & 3200 nucleotides, respectively

III. There are two basic types of rotors (centrifuge heads)

A. Some are constructed to allow centrifuge tubes to swing out, causing particles to move in a direction parallel to tube walls - **swinging bucket rotors**; they are useful for separating different-sized molecules

B. Fixed-angle rotor - tubes fit into fixed slots at oblique (14 – 40°) angle & are thus maintained at that specific angle during centrifugation; useful for sedimenting particles to the bottom of the tube

IV. Sedimentation behavior of nucleic acids - DNA & RNA are extensively analyzed by using ultracentrifuge

A. Velocity (or rate-zonal) sedimentation - nucleotide molecules separated according to nucleotide length

1. Sample layered over solution with increasing sucrose concentration (or some other

substance)
2. The preformed gradient increases in density & viscosity from top to bottom
3. Molecules move through gradient at rate determined by their sedimentation coefficient when subjected to high centrifugal forces
4. If sedimentation coefficient is larger, molecule moves farther in given time period of centrifugation
5. Nucleic acids are denser than medium so they continue to sediment as long as centrifuge is on; (never reach equilibrium); densest sucrose is ~1.2 g/ml density, while DNA has density of 1.7 g/ml
6. After prescribed period, tube is removed from centrifuge & its contents are fractionated; the relative positions of the various molecules are determined
7. Presence of viscous sucrose prevents mixing of tube contents (due to convection or handling) allowing molecules of the same S value to form a band & stay together
8. Find S values by comparing studied molecule positions to standards with known S values (**markers**)

B. Equilibrium (isopycnic) sedimentation - separates nucleic acids on the basis of their buoyant density
1. Mix highly concentrated heavy metal salt solution (CsCl or Cs_2SO_4) with DNA
2. Centrifuge with high force (speed) for extended period (2 - 3 days) to make continuous gradient
3. Heavy cesium ions are slowly driven to bottom of tube, forming a continuous density gradient through the liquid column
the opposing tendency of diffusion to redistribute the cesium ions —> the gradient is stabilized
5. DNA molecules place themselves at the point in the gradient equaling their buoyant density & are then no longer subject to further movement
6. Molecules equivalent density form narrow bands in tube; very sensitive; can separate on basis of base composition or different isotopes of nitrogen (^{15}N vs. ^{14}N)

Nucleic Acid Hybridization

I. **Nucleic acid hybridization** includes a variety of related techniques that are based on the observation that 2 single-stranded nucleic acid molecules of complementary base sequence can form a double-stranded hybrid
A. Mix many DNAs, some of them are complementary —> complementary DNAs form hybrid; mix usually has 100s of DNA fragments of identical length & overall base composition; differ only in sequence
1. Assume one DNA fragment among them was a portion of the β-globin gene, while all the other fragments contained unrelated gene sequences
2. There is only one way to distinguish between the β-globin gene & the others —> hybridization
B. For example, mix many single-stranded DNA fragments (the β-globin gene & many unrelated genes) & excess β-globin mRNAs —> form DNA-RNA hybrids; leaving other DNA fragments single-stranded
1. Separate hybrids & single-stranded DNA by passing mix through hydroxylapatite under ionic conditions allowing hybrids to bind Ca phosphate salts in column
2. Nonhybridized DNA molecules pass through unbound
3. Release hybrids from column by increasing elution buffer concentration
C. Hybridize complementary single-stranded nucleic acids under conditions (temperature, ionic strength, etc.) favoring formation of hybrids or double-stranded molecules
1. Done with both hybridizing nucleic acids in solution or one immobilized by adsorption on filter (nylon or nitrocellulose) or as part of chromosome

II. Typical procedure for hybridization - the Southern blot (named after its developer Edward

Southern)

A. One population of single-stranded nucleic acids in hybridization experiment is often immobilized in electrophoretic gel – example: electrophorese mixture of genomic DNA restriction fragments down gel
B. To make hybrids, denature DNA & transfer single-stranded DNA from gel to nitrocellulose membrane (**blotting**); DNA is fixed onto membrane by heating it to 80°C in vacuum
   1. Once DNA is attached, membrane is incubated with radioactively labeled, single-stranded DNA or RNA probe capable of hybridizing to complementary group of fragments
   2. Unbound radioactivity then washed away & bound probe location determined by autoradiography
C. Method called Southern blot; one or a few restriction fragments containing a particular sequence can be identified in Southern blot; this is true even if there are 1000s of unrelated fragments in gel
D. RNA molecules separated by electrophoresis can also be identified with labeled DNA probe after blotting onto a membrane – Northern blot

amounts of the enzyme DNase I, which introduces single-stranded nicks in double-stranded molecules

A. Preparation is then incubated with DNA polymerase I, an enzyme that possesses both polymerase & endonuclease activities, in the presence of radioactively labeled DNA precursors
B. Polymerase molecules attach to nicks in duplex & move down nicked strand toward its 3' end – it removes existing nucleotides & replaces them with radioactively labeled versions
C. Technique is called **nick translation** because nick moves down DNA molecule as nucleotides are removed & replaced

IV. Hybridization can also give a measure of the similarity in sequence between 2 DNA samples, for example, from two different organisms

A. The more distant the evolutionary relationship between 2 species, the greater is the divergence of their DNA sequences
B. Mix purified DNA from 2 different organisms, A & B, denature it & allow it to reanneal, a percentage of the DNA duplexes are formed by DNA strands from the 2 species
   1. Since they contain mismatched base pairs, such duplexes are less stable than those formed from DNA strands of same species; instability is reflected in lower temperatures at which
      they melt
   2. When DNAs from different species are allowed to reanneal in different combinations, the melting temperature of hybrid duplexes provides a measure of evolutionary distance between organisms

V. Hybridization is also important in *in situ* hybridization & hybridization to cDNA microarrays (discussed in earlier chapters)

Recombinant DNA Technology: Cloning – Background Information

I. What is recombinant DNA? - a piece of DNA containing sequences derived from >1 source

A. Over last 25 years, it has allowed advances in analysis of eukaryotic genome
B. Can be used in myriad ways

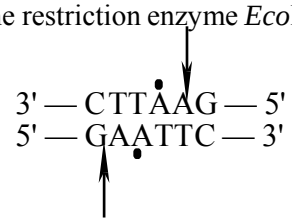
II. Before discussing the uses of recombinant DNA, it is important to discuss the major tool that allowed the production of recombinant DNAs – **restriction enzymes** (**restriction endonucleases**)

A. Restriction enzymes were discovered in 1970s in bacteria; they are nucleases that recognize short nucleotide sequences in duplex DNA & cleave DNA backbone at specific sites on both duplex strands
   1. They were called this because they function in bacteria to destroy viral DNAs that

might enter the cell, thereby restricting the potential growth of the viruses

2. The bacterium protects its own DNA from nucleolytic attack by methylating the bases at susceptible sites, a chemical modification that blocks the action of the enzyme
3. Enzymes from several hundred different prokaryotic organisms have been isolated; together, they recognize >100 different nucleotide sequences
4. Most restriction enzymes recognize restriction sites that are 4 - 6 nucleotides long & make cuts at specific sites on both duplex strands; they are characterized by particular type of internal symmetry

B. A sequence recognized by the restriction enzyme *Eco*R1 is:



1. Segment has **twofold rotational symmetry**; it can be rotated 180° without change in base sequence; if one reads sequence in same direction (3' to 5' or 5' to 3') on either strand, base order is same
2. A sequence with this type of symmetry is called a **palindrome**
3. When *Eco*R1 attacks this palindrome, it breaks each strand at the same site in the sequence, indicated by arrows between the A & G residues
4. The dots indicate the methylated bases in this in this sequence that protect the host DNA from enzymatic attack
5. Some restriction enzymes cleave bonds directly opposite one another on the 2 strands producing blunt ends; others, like *Eco*R1, make staggered cuts

C. Since a particular sequence of 4 to 6 nucleotides occurs quite frequently simply by chance, any type of DNA is susceptible to fragmentation by these enzymes

1. The use of restriction enzymes allows DNA of human genome or that of any other organism to be dissected into a precisely defined set of specific fragments
2. Once DNA from a particular individual is digested with a restriction enzyme, the fragments generated can be fractionated on the basis of length by gel electrophoresis
3. Different enzymes cleave the same DNA prep into different sets of fragments and the sites within the genome that are cleaved by various enzymes can be identified & ordered into a restriction map

III. Formation of recombinant DNAs – can be formed in a variety of ways

A. Example method – DNA molecules from 2 different sources are treated with a restriction enzyme that makes staggered cuts in DNA duplex

1. Staggered cuts leave short, single-stranded tails that act as sticky ends that can bind to a complementary single-stranded tail on another DNA molecule to restore a double-stranded molecule
2. Often one DNA fragment is bacterial plasmid (a small, circular, double-stranded DNA molecule that is separate from main bacterial chromosome)
3. The other DNA fragment comes from human cells after treatment with the same restriction enzyme used to open the plasmid
4. When human DNA fragments & plasmid are incubated together + DNA ligase, the 2 types of DNAs are H bonded to one another by sticky ends & then ligated to form circular DNA recombinants
5. Recombinant DNAs also formed from DNA fragments generated by restriction enzymes that make blunt ends; plasmid & restriction fragment ends are later altered to allow them to stick to one another

6. First recombinant DNAs made by basic method above (1973) – Paul Berg, Herbert Boyer, Annie Chang & Stanley Cohen (Stanford & U. of Ca., San Fran,); birth of modern genetic engineering

C. Once this is done, you have produced a large number of different recombinant molecules, each of which has a bacterial plasmid & a piece of human DNA incorporated into a DNA circle
   1. If you want to isolate a single human gene like the one that encodes insulin, you have to separate this specific fragment from all of the others
   2. This is done by **DNA cloning**

IV. DNA cloning - technique for producing large quantities of a specific DNA sequence; one can make millions of copies of recombinant DNA in a short period of time from one or a few initial copies
   A. DNA to be cloned is first linked to **vector DNA**, a vehicle for carrying foreign DNA into a suitable host cell, like *E. coli*; the vector contains DNA sequences that allow it to replicate in host cell
   B. 2 types of vectors are usually used to clone DNAs within bacterial hosts after incorporating the desired gene within them
      1. DNA segment to be cloned is joined to a plasmid; then investigators make bacteria pick up plasmid from medium; as bacteria reproduce so does plasmid containing the desired gene
      2. Foreign DNA is inserted into a portion of λ (lambda) bacteriophage viral genome; altered virus is allowed to infect bacteria —> many viral progeny containing foreign DNA are produced
   C. Whichever type of vector is used, once the DNA segment is inside a bacterium, it is replicated along with the bacterial or viral DNA & partitioned into the daughter cells or progeny viral particles
      1. The number of recombinant DNA molecules increases in proportion to the number of bacterial cells or viral progeny formed
      2. From a single recombinant plasmid or viral genome inside a single bacterial cell, millions of copies of the DNA can be formed in a short period of time
   D. Once the amount of DNA has been sufficiently amplified, recombinant DNA can be purified & used in other procedures; thus, cloning can be used to amplify a particular DNA sequence & also to purify it
      1. One can isolate a pure form of any specific DNA fragment among a large, heterogeneous DNA molecule population

Recombinant DNA Technology: Cloning Eukaryotic DNA in Bacterial Plasmids

I. Foreign DNA is inserted into plasmid (recombinant DNA) & bacteria are transformed & grown in culture
   A. Plasmids used for cloning are modified versions of those found in bacterial cells; like natural counterparts they contain:
      1. An origin of replication
      2. One or more genes that make the recipient cell resistant to one or more antibiotics; antibiotic resistance allows researchers to select for those cells that contain a recombinant plasmid
   B. Recombinant DNAs with different foreign DNAs are added to bacterial culture where they can be taken up by bacterial transformation as demonstrated by Avery, MacLeod & McCarty
      1. In most common technique, they pretreat bacteria with Ca^{2+} ions, then briefly heat shock them —> after treatment, they are stimulated to take up DNA from surrounding medium
      2. Transformation - recombinant DNA gets into bacteria; usually, only a small percentage of bacterial cells is competent to pick up & retain one of the recombinant plasmids

C. Once taken up, the plasmid replicates autonomously & is passed on to progeny during cell division

D. Select for bacteria with plasmid by growing bacteria on antibiotic whose resistance gene is on plasmid
1. This procedure eliminates bacteria that have not taken up plasmids
2. Next, those bacteria with plasmids carrying the desired DNA fragment, if any, must be identified

II. Identification of bacteria containing desired piece of DNA (e.g., insulin) in their plasmids
A. Plate out plasmid-bearing cells at low density on petri dishes so that all of the progeny of each cell (a clone of cells) remain physically separate from the progeny of other cells
1. Each cell contains a different piece of foreign DNA, since you started out with lots of recombinant plasmids; once they have grown into separate colonies, search for those with desired gene (insulin)

B. Look for colonies or phage plaques with insulin gene using replica plating & then *in situ* hybridization
1. Replica plating - make plates with representatives of all bacterial colonies in precisely the same position on each dish, one of which is then used to localize the DNA sequence being sought
2. Replica plating requires that the cells be lysed & the DNA fixed onto the surface of a nylon or nitrocellulose membrane
3. Once DNA fixed in place, it is denatured to prepare it for *in situ* hybridization
4. The membrane is incubated with labeled, single-stranded DNA probe containing the sequence complementary to that being sought
5. After incubation, wash off unhybridized probe & locate hybrids by autoradiography
6. Culture live representatives of identified clones from corresponding sites on original plates & grow into large colonies, which serves to amplify the recombinant DNA plasmid
7. Extract DNA from cultured cells that have been harvested & readily separate recombinant plasmid from larger chromosome by various techniques, including equilibrium centrifugation
8. Treat isolated recombinant plasmids with same restriction enzyme used to form it —> cloned DNA segment is released from rest of vector DNA & isolated from the plasmid by centrifugation

C. Not practical to search for single human gene since it would require cloning tens of thousands of DNA fragments & hundreds of separate petri dishes —> better to use viral vector for this

Recombinant DNA Technology: Background Information and Formation of a DNA Library

I. DNA libraries - collections of cloned DNA fragments; 2 basic types: genomic & cDNA libraries A. Genomic library – made from total DNA extracted from nuclei; contain all DNA sequences of species
1. Once the genomic library of a species is available, researchers can use the collection to isolate specific DNA sequences, like those containing the human insulin gene

B. cDNA library – derived from DNA copies of an RNA population; typically made from mRNAs present in a particular cell type & thus correspond to genes that are active in that type of cell

II. Production of a genomic library - treat genomic DNA at low enzyme concentration with 1 or 2 restriction enzymes that recognize very short sequences (*Hae*III - recognizing GGCC; *Sau*3A - recognizing GATC)
A. Low enzyme concentration prevents cleavage of all susceptible sites; only a small percentage

cleaved

1. A given tetranucleotide is expected to occur by chance with such a high frequency that any sizable DNA segment will be sensitive to fragmentation
2. DNA is randomly fragmented since the DNA is treated with enzymes under conditions in which most susceptible sites are not cleaved

B. The partially digested genome is fractionated by gel electrophoresis or density gradient centrifugation

1. Fragments of suitable size (e.g., 20 kb in length) are incorporated into lambda phage particles
2. These phage are used to generate the million or so plaques needed to ensure that every single segment of the mammalian genome is represented

C. Store recombinants for later use; it is a permanent collection of all sequences in the genome of the species (**library**)

1. If one wants a particular sequence, s/he grows phage on bacteria & screens for presence of that sequence, using *in situ* hybridization
2. Randomly cleaved DNA has advantage – it generates overlapping fragments; useful in chromosome walking (analysis of chromosome regions extending out in both directions from particular sequence)

D. Chromosome walking - depends on such overlapping fragments

1. Isolate fragment containing globin gene coding region, label that fragment & use it as a probe to screen genomic library & isolate fragments with which it overlaps
2. Repeat with new fragments used as labeled probes in successive screening steps, moving along DNA; one gradually isolates a longer & longer part of the original DNA molecule
3. Using this approach, one can study organization of linked sequences in extended chromosome region

4. DNA sequence evolution (duplication, rearrangement) by comparing DNA of different species
5. Interspersion of transposable genetic elements

Recombinant DNA Technology: Formation of a cDNA Library

I. Cloning of cDNAs has also been important in the analysis of gene structure & gene expression; allows you to do things that cloning the entire genome does not

A. cDNA cloning allows identification of sequences active at given time in a particular cell type

II. To produce a cDNA library:

A. Isolate mRNA population & use reverse transcriptase to form a population of DNA-RNA hybrids —> then convert DNA-RNA hybrid to double-stranded cDNA population

1. Nick the RNA of DNA-RNA hybrid with RNase H & replacing the RNA with DNA by using DNA polymerase I

B. The double-stranded cDNA is then combined with the desired vector (e.g., a plasmid) & cloned

III. mRNA populations typically contain thousands of different messages, but individual species may be present in markedly different numbers (they exhibit different abundance)

A. Thus, a cDNA library has to contain a million or so different cDNA clones to be certain that all of the rarer mRNAs will be represented

B. Also reverse transcriptase is not a very efficient enzyme; it tends to fall off its template mRNA before the copying job is completed; thus, it can be difficult to obtain a population of full-length cDNAs

C. Clones must be screened to isolate one particular sequence from a heterogeneous population of recombinant molecules

IV. Analysis of cloned cDNAs serves several functions – it is generally easier to study a diverse set of cDNAs than the corresponding population of mRNAs, since the cDNAs are more stable
A. One can use the cDNAs to learn about the variety of mRNAs present in a cell
B. One can determine the percentage of mRNAs shared by two different types of cells
C. One can determine the number of copies of different mRNAs present in a cell

V. A single, cloned & amplified DNA molecule is also useful
A. The cDNA contains only that information present in the mRNA
B. Thus, comparison between a cDNA & its corresponding genomic locus can provide information on the precise locations of the noncoding intervening sequences (introns) within the DNA
C. The purified cDNA can be readily sequenced to determine the polypeptide amino acid sequence; a shortcut & very practical
D. Labeled cDNAs can be used as probes to screen for complementary sequences among recombinant clones
E. cDNAs also lack introns & thus have an advantage over genomic fragments when one is trying to synthesize eukaryotic proteins in bacterial cell cultures

Recombinant DNA Technology: Chemical Synthesis and Site-Directed

I. Development of chemical techniques devised to synthesize polynucleotides having a specific base sequence was begun in early 1960s as part of an attempt to decipher the genetic code by H. Gobind Khorana
A. Khorana et al. (1970s) – synthesized a complete bacterial tyrosine tRNA gene (126 bp), including the nontranscribed promoter
1. Put together from >20 segments: each was synthesized individually & later joined enzymatically
2. Artificial gene was placed in bacteria having mutations for this tRNA —> replaced deficient function
B. Keiichi Itakura et al. (City of Hope Med. Ctr., 1977) - gene for somatostatin (a small, 14 amino acid residue hypothalamic peptide hormone) was synthesized
1. Inserted gene into specially constructed plasmid downstream from bacterial regulatory sequences
2. Introduced into *E. coli*, where it was transcribed & translated
C. 1981 – gene for the first average-sized protein, human interferon, was synthesized; required synthesis & assembly of 67 different fragments to produce a single duplex of 514 bp
1. Made whole gene with initiation & termination signals recognized by bacterial RNA polymerase
D. Now have automated DNA-synthesizing machines - can make polynucleotides of any desired sequence (or a randomly generated sequence) ~75 - 100 nucleotides in length
1. Each fragment is assembled one nucleotide at a time from the 3' to the 5' end of the segment
2. Once synthesized, such fragments can be joined covalently to make much longer synthetic DNAs

II. **S**ite-**d**irected **m**utagenesis (SDM) - DNA-synthesis machines can alter sequences of natural DNA
A. Natural mutations are rare events & it is not feasible to use them to study the role of particular amino acid residues in the function of a particular protein
1. No longer need to wait for interesting phenotypes to appear naturally & then identify the responsible mutation
2. Researchers can now mutate a specific gene or its associated regulatory regions in a desired way & observe the resulting phenotypic change

3. Using *in vitro* techniques, small sections of a DNA sequence can be deleted, additional nucleotides inserted or one specific base can be changed to another one

B. This procedure was developed by Michael Smith (Univ. of British Columbia) & is called SDM

1. Usually accomplished by first synthesizing DNA oligonucleotide containing the desired change
2. Then allow the oligonucleotide to hybridize to a single-stranded preparation of the normal DNA & use the synthesized oligonucleotide as a primer for DNA polymerase
3. DNA polymerase elongates the primer by adding nucleotides complementary to the normal DNA
4. Next, clone the modified DNA & determine the effect of the modification by introducing the modified DNA into an appropriate host cell
5. If site is part of regulatory region —> it may affect gene expression rate, which can be monitored
6. If site is part of coding region, it gives insight into site's role in overall protein structure &

Recombinant DNA Technology: Determining Gene Function by Gene Elimination – Knockout Mice and RNA Interference

I. Until recently, investigators discovered new genes & their function by screening for mutants that exhibited abnormal phenotypes

A. The process of learning about genotypes by studying mutant phenotypes is called **forward genetics**

B. Since the development of gene cloning & DNA sequencing techniques, researchers have been able to identify & study genes directly without knowing anything about the function of the encoded protein

C. Over last 15 years, researchers have developed means to carry out **reverse genetics**, which is a process of determining phenotype (i.e., function) based on knowledge of genotype – 2 types widespread

1. Knockout mice
2. RNA interference

< II. Knockout< < < < < mice - < scientists

< < < < < < < < < < < < < < < < < < < < < < < < < produced mice that lack a functional copy of a particular gene to see the phenotype & determine the significance of the missing gene product

A. For example, *p53* knockout mice (lacking functional *p53* gene) – invariably develop malignant tumors

B. Knockout mice can give a unique insight into a human disease's genetic basis & are also a mechanism for studying the various cellular activities in which a particular gene product might be engaged

III. The various procedures used to generate knockout mice were developed in the late 1980s by Mario Capecchi (Univ. of Utah), Oliver Smithies (Univ. of Wisconsin) & Martin Evans (Cambridge Univ.)

A. Isolate embryonic stem (ES) cells (have virtually unlimited powers of differentiation) from mammalian blastocyst

1. Mammalian blastocyst is early embryonic development stage comparable to blastula stage in other animals; it is composed of 2 distinct parts: **trophectoderm** & **inner cell mass** (**ICM**)
2. Outer blastocyst layer is trophectoderm; it gives rise to most of extraembryonic membranes characteristic of mammalian embryos
3. Trophectoderm's inner surface contacts cell cluster (ICM), projecting into spacious cavity (blastocoel)
4. ICM gives rise to cells that make up embryo & contains ES cells, which differentiate into all of the various tissues of which a mammal is composed

V. RNA interference – RNAi is a process in which a specific mRNA is degraded due to the presence of a small, double-stranded RNA (dsRNA) whose sequence is contained within the mRNA sequence
   A. Plant, nematode or fruit fly gene function is studied by simply injecting a dsRNA into organism & examining the phenotypes of the organism that result from degradation of the corresponding mRNA
      1. Using this approach, information about the functions of large numbers of genes can be gathered in a relatively short period of time
      2. Less laborious & costly than generating knockout animals & yields essentially same information
   B. RNAi can be used to study mammalian cell gene function by incubating the cells with small dsRNAs or by genetically engineering the cells to produce the dsRNAs
      1. Once inside the cells, the dsRNA leads to degradation of target mRNA, leaving the cell unable to produce additional protein encoded by that gene
      2. Any deficiencies in the cell phenotype can be attributed to a marked reduction in the level of the protein being investigated

Recombinant DNA Technology: Enzymatic Amplification of DNA by PCR

I. ***P***olymerase ***C***hain ***R***eaction (PCR) - developed by Kary Mullis (Cetus Corporation, 1983); widely used to amplify, cheaply & readily specific DNA regions without the need for bacterial cells
   A. Many different PCR protocols have been developed for a multitude of different applications in which anywhere from one to a large population of related DNAs can be amplified
   B. Readily adapted to RNA templates by first converting them to complementary DNAs using reverse transcriptase

II. Simplest protocol – employs a heat-stable DNA polymerase (**Taq polymerase**), originally isolated from ***T****hermus* ***aq****uaticus*, a bacterium that lives in hot springs at temperatures >90°C (higher than normal)
   A. Mix DNA sample with 4 deoxyribonucleotides & an aliquot of Taq polymerase
      1. Also add a large excess of 2 short synthetic DNA fragments (oligonucleotides) that are complementary to DNA sequences at the 3' ends of the DNA region to be amplified
      2. These short oligonucleotides serve as primers to which nucleotides are added during the following replication steps
   B. Then heat mixture to ~93°C, hot enough to melt (denature) DNA in mix & separate DNA molecules into their 2 component strands
   C. The mix is then cooled to ~60°C —> allows primers to bind both target DNA strands
   D. Raise the temperature to ~72°C —> allows the thermophilic polymerase to add complementary nucleotides to the 3' end of the primers
      1. As the polymerase extends the primers, it selectively copies the target DNA
      2. Forms new complementary DNA strands
   E. Raise temperature again causing newly formed & original strands to separate from each

other

F. The sample is then cooled to allow synthetic primers in mixture to bind once again to the target DNA, which is now present at twice the original amount

G. Repeat cycle over & over again, doubling the amount of the specific DNA region flanked by the bound primers with each cycle

H. Generates billions of copies of this one specific DNA region from minute amounts in just a few hours using a **thermal cycler** - used in criminal cases

1. Thermal cycler automatically changes the temperature of reaction mixture, allowing each step in the cycle to take place

III. PCR can generate large amounts of DNA from miniscule starting samples like that in a single cell

A. PCR has been used in criminal investigations to generate DNA quantities from a spot of dried blood left on crime suspect's clothing or from DNA present as part of single hair follicle left at crime scene

B. For this purpose, one selects regions of genome for amplification that are highly polymorphic (i.e., vary at high frequency within population) - thus, no 2 individuals will have same-sized DNA fragments

C. Same procedure can be used to study DNA fragments from well-preserved fossil remains that may be millions of years old

The Use of Antibodies

I. What are antibodies (ABs; also known as immunoglobulins)? - proteins made by lymphoid tissue after antigen (Ag; foreign materials) exposure; they exhibit a high degree of specificity (makes them useful)

A. AB preparation binds only those select molecules in cell having a small part that fits into Ag-binding site of AB; can select a few proteins out of the thousands in a cell

B. Can distinguish between two polypeptides that differ by as little as one amino acid

C. There are basically 2 approaches to the preparation of ABs that interact with a given Ag – traditional & monoclonal AB approaches

II. Traditional approach for the production of ABs

A. Repeatedly inject animal (typically a rabbit or goat) with Ag

1. After a period of several weeks, blood is drawn that contains the desired ABs
2. Whole blood is treated to remove cells & clotting factors —> produces an antiserum
3. Test antiserum for AB titer & purify immunoglobulins (Igs) from it

B. Traditional approach still used, but it has certain inherent disadvantages:

1. Due to AB synthesis mechanism, animal invariably makes a variety of different species of Igs (with different V regions in their polypeptide chains; **polyclonal**) even if the Ag was highly purified
2. Cannot get a pure preparation of a single AB species with this technique, since the ABs are too similar to be fractionated; the ABs are polyvalent

III. Preliminary discoveries leading to production of monoclonal (univalent) ABs

A. ABs made by a clone of AB-producing cells (derived from single B lymphocyte) have identical Ag-combining sites

1. Polyvalent AB heterogeneity against a single purified Ag is due to activation of many B cells
2. Each of the B cells has membrane-bound ABs with an affinity for a different part of the Ag

B. Theoretically, one could obtain a pure preparation of a single monoclonal AB if 1 AB-

producing plasma cell were isolated by a procedure something like the one that follows:

1. Inject an animal with a purified Ag & wait a period of weeks for ABs to be produced
2. Then remove the spleen or other lymphoid organs and prepare a suspension of single cells
3. Isolate those cells producing the desired ABs from the suspension & grow these particular cells as separate colonies so as to obtain large quantities of this particular Ig

C. This procedure should yield an AB molecule prep made by a single colony or clone of cells, a **monoclonal antibody**, but……. antibody-producing cells do not grow & divide in culture

1. To make monoclonal ABs in this way, an additional manipulation is required

D. Malignant myeloma cells are a type of cancer cell that grows rapidly in culture & makes lots of AB

1. ABs made by myeloma cells were valuable in AB structure study, but of little use as analytic tools since the ABs produced by them are not formed in response to a specific Ag
2. Instead, myeloma cells develop from random conversion of a normal lymphocyte to a malignant state; they make AB that was being made by the particular lymphocyte before it became malignant

IV. Monoclonal antibody production - Cesar Milstein & Georges Köhler (Med. Research Counc., Cambridge, Engl., 1975) – did experiments leading to development of univalent AB preps directed against specific Ags

A. They combined the properties of normal AB-producing lymphocytes & the immortal myeloma cell

B. They fused malignant myeloma cells & normal lymphocytes to make hybrid cells —> **hybridomas**

1. They grow & proliferate indefinitely, producing large amounts of a single, monoclonal AB
2. The AB made is the one being made by normal lymphocyte before fusion with myeloma cell

C. Procedure – do not even need purified Ag; can use soluble Ag or one that is part of cell

1. Ag was injected into mouse to cause proliferation of specific AB-producing cells
2. After several weeks, spleen is removed & dissociated into single cells
3. AB-producing lymphocytes were fused with a population of malignant myeloma cells, making the hybrids immortal (capable of unlimited cell division)
4. Hybrids selected from unfused cells by ability to grow in medium in which only they can survive
5. Hybridomas are then grown clonally in separate wells & individually screened for production of AB against Ag being studied
6. Clone hybrid cells containing appropriate AB in culture (*in vitro*) or as tumor cells in recipient animal (*in vivo*) —> get lots of monoclonal AB, in essentially unlimited amounts
7. Once produced, they can be stored indefinitely in frozen state & aliquots can be made available to researchers around the world

C. Do not need to begin procedure with purified Ag; it may even be minor component of entire mixture

D. Monoclonal ABs are useful in research, but also useful in diagnostic medicine to determine concentration of specific proteins in blood or urine

1. Monoclonal ABs form the basis of certain home-pregnancy tests that monitor the presence of a protein (chorionic gonadotrophin) that appears in the urine a few days after conception

V. No matter how they are obtained, one can use ABs as highly specific probes in a variety of analytic techniques, e.g., localizing proteins in cells

A. ABs can be used as highly specific probes in protein purification - add purified AB to crude protein mixture —> specific protein being sought selectively combines with AB & precipitates from solution

B. ABs can also be used along with various types of fractionation procedures to identify a

particular protein (Ag) among a mixture of proteins

1. In Western blot, a mixture of proteins is first fractionated by 2D gel electrophoresis
2. The fractionated proteins are then transferred to a sheet of nitrocellulose filter
3. Filter is then incubated with an AB prep that has been labeled either radioactively or fluorescently
4. The location on the filter of a specific protein bound by the AB can be determined from the location of the bound radioactivity or fluorescence

C. Monoclonal ABs have also been useful as therapeutic agents in humans – however, efforts to develop human hybridomas that produce human ABs have been unsuccessful, **so……**

1. Mice have been genetically engineered so that ABs they produce are increasingly human in amino acid sequence
2. Some of these humanized monoclonal ABs have been approved for the treatment of several diseases
3. More recently, mice have been engineered so that their immune system is essentially human in nature —> they produce ABs that are fully human in structure

D. Immunolocalization in cells – visualizing specific molecules in cells using labeled antibodies (see below)

VI. Immuolocalization in cells depends on the use of ABs made specifically against a particular protein; the ABs are then conjugated to a substance that makes them visible under the microscope (LM or EM)

A. The binding of the substance making the AB visible does not interfere with the specificity of AB-Ag interactions

1. Often label ABs with small fluorescent molecules (fluorescein, rhodamine) for light microscopes —> forms derivatives that are incubated with cells or sections of cells
2. Visualize the AB-binding sites in the fluorescence microscope (**direct immunofluorescence**)

B. Types of immunolocalization – direct or indirect immunofluorescence

1. Direct immunofluorescence - labeled AB is attached directly to Ag in cell
2. Indirect immunofluorescence – incubate cells first with unlabeled AB (complexes with corresponding Ag); then, expose cell to fluorescent AB directed against AB used in first step; often preferable

C. Advantages of indirect immunofluorescence

1. Brighter image than direct method because numerous secondary ABs can bind to single primary AB
2. Also has practical advantage – conjugated (fluorescent) secondary AB is readily bought from vendors

D. Immunofluorescence (direct or indirect) provides remarkable clarity since only the proteins bound by the AB are revealed to the eye; all of the unlabeled materials remain invisible

E. For EM, tag ABs with electron dense materials (iron-containing protein ferritin or gold particles)