UN2005/UN2401 '18 -- Lecture 19 -- Last Edited: 11/19/18 – reformatted slightly 11/19

Handouts: Sticky Ends & Joining. = Left panel = Becker Fig 21-6. See 21A-2 for insertion into a plasmid (right panel). This HO is labeled '18A'
Restriction Enzymes (Table and Examples) – labeled '18B'
For a similar table of restriction enzymes, See Becker table 21-1 or go to Table 3.2 at http://www.biocyclopedia.com/index/biotechnology/genes_genetic_engineering/tools_of_genetic_engineering/biotech_enzymes.php

Reminder: Selected problems are indicated in **red bold**. We suggest doing these problems as soon as possible to consolidate your understanding of the material covered in each lecture. (However, most people need to look over the relevant section of the notes before trying the problems.) Use these problems to learn from; do not use them to test yourself.
Important Reminder: These are NOT the only problems you should do -- you should do ALL of them. For a complete list of problems corresponding to each lecture, see the problems to do page.

I. Viral Genetics – Viral Crosses

Complementation & Recombination can occur with viruses as well as with bacteria. If a cell is simultaneously infected with two variants (mutants) of the same virus, then crossing over and/or complementation can occur between the two viruses during the course of infection. See notes of Lecture 18 & handout 18B for more details on complementation (& how to distinguish complementation and recombination).

For Example:

Suppose 2 viral mutants have the same phenotype, such as inability to grow on a particular strain of bacteria (say, strain X). Neither mutant alone will grow on strain X, but both mutants can grow on strain Y. What if you do a double infection – infect strain X or Y with both mutants at the same time? Can you get recombinants? Can you get the two mutants to complement each other? In either case, how will you know? How will you detect recombination or complementation?

For an example of crosses with viruses, see problem 11-8. (For more problems involving complementation & recombination in viruses, see 11-10 to 11-13.)

II. Restriction Enzymes

A. Introduction: The idea/problem: The existence of plasmids and bacteriophages carrying bacterial genes inspired dreams of genetic engineering. Why not make new combinations to order? That way we could make plasmids with useful genes and add them to bacteria (or even human cells)! But how do you make recombinant plasmids in a laboratory? DNA is very, very long. How can you cut it into useful sized pieces, find the right pieces, stick them together, etc.?? The solution was discovered by pursuing a phenomenon known as restriction (described below), which seemed at the time to be of no practical consequence whatsoever.

B. Modification and Restriction Enzymes. How do they work?

1. The phenomenon: (This is included for historical background; it will not be covered in class.) Some phages grow well on certain bacteria but not others. This property is inherited. For example, suppose virus V can infect bacteria of type A successfully, but does not replicate well on type B, as in (a) below.

a. Restriction: Virus V infects bacteria of type B → very few progeny.

The growth of the phages (viruses) is said to be "restricted" on bacteria of type B.

b. Are the 'few progeny' restricted?'

An occasional virus particle does manage to complete an infection and lyse a type B bacterium in spite of restriction. What happens if you use one of these 'very few progeny' who 'escaped restriction' to infect type B?

c. Loss of restriction: If you take one of the few progeny from (a) and infect bacteria of type B → lots of progeny.

In other words, the progeny phage from the lysed type B cell grow just fine on type B -- they are no longer restricted, and the changed property is inherited.

d. Have the 'few progeny' reverted or mutated? Is the DNA sequence the same or different? It's the same! So what's going on? See below.

What is surprising is that the sequence of bases in the viral DNA is the same before and after restriction! In other words, all the progeny of Virus V have the same DNA sequence, whether you examine the original virus, the progeny from (a) or the progeny from (c). What's going on here? The solution holds the key to all genetic engineering and recombinant DNA technology, and the scientist who discovered it (Arber) received the Nobel Prize in 1978 along with two other scientists (Smith & Nathans) who extended Arber's work.

2. Restriction enzymes – What do they do?

a. What are restriction enzymes? Bacteria have endonucleases ('DNases') that cut DNA molecules at specific sequences. (All previously known endonucleases cut at random with respect to sequence.) These enzymes are called restriction enzymes or restriction endonucleases.

- ***Exo***nucleases remove nucleotides one at a time from the end.
- ***Endo***nucleases break specific phosphodiester bonds in the middle of the molecule.

b. What's the substrate? The enzymes work only on double stranded DNA. They don't cut RNA or single stranded DNA.

c. Where do they cut? Each restriction enzyme catalyzes hydrolysis of a phosphodiester bond between two nucleotides. (Both strands of the DNA are cut at equivalent points.)

d. Specificity: Each different restriction enzyme recognizes (binds to) and cuts the DNA at a different particular sequence. (See handout 18B for some examples.)

e. What is the normal function of these enzymes? To destroy the DNA of infectious phage.

f. Why do we care about these enzymes? They are essential tools for genetic engineering, forensics, genetic diagnoses, etc. More details to come.

3. Modification enzymes. Why are bacteria "immune" to their own restriction enzymes? Why don't the restriction enzymes cut up the bacterium's own DNA?

a. Modification enzymes: Bacteria have a second set of enzymes that modify DNA -- these enzymes add methyl groups to specific sequences on the DNA -- the same sequences cut by the restriction enzymes.

b. Role of modifications: These modifications make the DNA (at restriction sites) resistant to the restriction enzymes. That's why bacteria don't destroy their own DNA.

c. Modifications of DNA can be inherited through binary fission -- the state of DNA (methylated or not) is maintained generation after generation, once it is set up, as follows:

- The modification enzymes normally add methyl groups only to DNA that is hemi-methylated -- methylated on one strand, but not the other.
- When methylated DNA is replicated, the product is hemi-methylated -- the new strand has no methyl groups. The new strand is methylated by modification enzymes soon after it is made.
- When un-methylated DNA is replicated, no methyl groups are added.

4. How do rare virus particles escape restriction? If DNA is accidentally methylated, then it will be methylated every time it is replicated thereafter. The rare particles have DNA that was methylated accidentally. Therefore the DNA of the rare virus particles and their progeny are resistant to the restriction enzymes.

C. Why do we care about Modification Enzymes?

1. Modification of DNA can block action of restriction enzymes. That's why bacteria don't destroy their own DNA, and how some viruses escape restriction.

2. Modification of DNA &/or associated Proteins can be inherited during asexual reproduction

a. Inheritance: The state of methylation is heritable through binary fission, as explained above.

b. Terminology -- epigenetics.

(1.) Definition: All inherited changes in the state of the DNA that don't change the base sequence are called 'epigenetic.'

Note: 'inherited' here means passed on during asexual reproduction of individual cells. It does not necessarily mean passed on from generation to generation in multicellular organisms. See below.

(2). Modification (by methylation of DNA) is an epigenetic phenomenon. The state of modification (or the pattern of methylation) is said to be 'epigenetic.' The DNA sequence isn't changed, but the DNA is modified. The modified state of the DNA is stable, and the state is inherited (during binary fission or mitosis).

(3). Not all epigenetic changes in DNA involve methylation. Other groups can be added and/or modifications in packing can occur. (Modifications of associated proteins are important in eukaryotes.)

c. How important are epigenetic changes? The study of epigenetic changes is currently a very hot topic of research.

(1) Role in Asexual Reproduction. There is good evidence for epigenetic inheritance during asexual reproduction of individual cells (binary fission in prokaryotes, mitosis in eukaryotes).

(2) Role in Development. Epigenetic changes are very important in directing development (growth & specialization) of cells in multicellular organisms, and maintaining differences between specialized cells in the adult.

(3) How significant are epigenetic factors during sexual reproduction in multicellular eukaryotes? Are epigenetic changes inherited from generation to generation in mice, plants and humans? The importance of epigenetic factors in determining the phenotype of the next generation is not yet clear. For an opinion article from the *NY Times* go to http://www.nytimes.com/2012/09/09/opinion/sunday/why-fathers-really-matter.html

3. Modification is wide spread: Modification of macromolecules by enzymatically adding or removing a small group or two is very wide spread, especially in eukaryotes.

a. Function: Modification is a common method of regulating and/or fine tuning macromolecular function permanently or temporarily (many modifications are reversible).

b. Examples so far (irreversible): removal of met from amino end of proteins; conversion of the base A in tRNA into the base I.

c. Examples to be discussed next term (reversible): many enzymes are activated or inhibited by addition of phosphate groups; some sections of DNA may be kept "off" by addition of methyl groups to the DNA itself or to associated proteins (histones). Details of these examples (and many others) will follow next semester.

D. Examples & Properties of Restriction Enzymes (Details on Handout 18B & Becker fig. 21-2 & table 21-1 (Box 18B)

1. *Restriction Sites are often palindromes* (= read the same forwards and backwards)

English examples:	"Madam I'm Adam"
	"Able was I ere I saw Elba."
	"A man, a plan, a canal, Panama."
DNA example:	5' GAATTC 3' 3' CTTAAG 5'

What do we mean by "reads the same backwards and forwards" for the DNA example? There are several ways to explain this :

a. By base sequences. The sequences on the two individual strands are the same if both sequences are read 5' to 3'. (This becomes very clear if the bottom strand is written with the letters upside down.)

b. By base pairs. The base pairs are the same, right to left and left to right as long as the "top" strand is always 5' to 3'.

c. By rotation. If you rotate the DNA 180 degrees, it looks exactly the same.

2. State of the ends. Cuts made by restriction enzymes can be staggered (generating so called "sticky ends") or blunt (see handout 18B or Becker fig. 21-2 & table 21-1 (Box 18B in 8th ed) for examples)

3. There are a wide variety of restriction enzymes made by different bacteria. (See handout 18B for some examples; see texts or Google for more.) Therefore there are many different options for cutting up any given DNA.

a. Different enzymes recognize sequences of different lengths.

(1). Some recognize relatively short sequences. For example, an enzyme may be a "4 cutter" = enzyme that recognizes a 4 base pair site. (See handout.) Short sites (sequences) are found more often, and enzymes that cut them produce many relatively short fragments.

(2). Some enzymes recognize longer sequences. Longer sites are found less often, and enzymes that cut them produce a smaller number of relatively long fragments.

b. Different enzymes recognize different sequences, even if the sequences they recognize are the same length.

c. Different enzymes cut differently -- some give blunt ends and some give staggered "sticky ends."

4. Sites can sometimes be methylated

a. Methylation makes some sites resistant to cutting.

b. Inheritance: The state of methylation is heritable through binary fission, as explained above.

c. Consequence

(1). For viruses: Viruses whose DNA gets methylated are immune to the corresponding restriction enzyme, and can replicate in bacteria making the restriction enzyme.

(2). For Bacteria: Bacteria don't destroy their own DNA.

E. Why do we care about Restriction Enzymes? Essential tools for Recombinant DNA Analysis & Forensics

1. Allows you to cut up DNA into manageable size pieces for manipulation and analysis.

a. Most DNA is very long. Without some sort of breakage, most DNA molecules are too big to handle.

b. Before this, all known DNases cut at random → big mess (random collection of different sized pieces).

c. Restriction enzymes cut DNA into fixed size pieces. Pieces resulting from restriction enzyme digestion can be separated by size using gel electrophoresis. (Sadava fig. 15.12 (15.13)) or Becker fig. 21-1 (18-12.) Principle is similar to SDS gel electrophoresis, except no SDS is used -- all nucleic acids are negatively charged and migrate to the positive pole -- smaller fragments travel farther. (Same procedure as used for analysis of PCR products.)

What can you do with discrete pieces you get from cutting with restriction enzymes?

2. Joining.

Allows you to join, not just cut, DNA's readily, using overlapping ends and ligase. This allows you to make new, recombinant molecules in a test tube. This in turn allows molecular cloning, as shown on handout 18A.. (Note: Double stranded DNA molecules with so called 'blunt ends' can also be ligated, but it is much easier if the molecules have 'sticky ends.')

3. Forensics/ IDs & RFLPs

a. Inherited variations in base sequence lead to differences in places where DNA is cut. For example, if a sequence is GAATTC, EcoR1 will cut the DNA. If the sequence is changed to GGATTC, EcoR1 will not cut the DNA. So a change of A to G can "remove" a restriction site while a change of G to A can "add" a restriction site. (See handout 18B.) Note "addition" or "loss" of a restriction site does not necessarily mean the insertion or deletion of bases. An "addition" means a change in base sequence so a particular stretch of DNA is now recognized by a particular restriction enzyme; a loss means a change so that the DNA is no longer recognized by the restriction enzyme.

b. Detection. Differences in restriction sites (or variation in the lengths of the sequences between sites) are detected by cutting the DNA with restriction enzymes and running gels of pieces (to compare sizes). How to locate the pieces from a particular region of the DNA will be discussed below or next time.

c. RFLPs. When DNA from different individuals gives a different pattern of pieces, this is known as a RFLP = restriction fragment length polymorphism. (See handout 18B or Becker fig. 21-15 (fig. 18C-1), or Sadava fig. 15.12 (15.13) for examples.)

d. How does this compare to PCR fingerprinting?

- PCR method for DNA fingerprinting (described previously) picks up differences in the numbers of repeats in a particular region (between primers). See Sadava 15.13. (15.14)..
- To use PCR, you have to know enough about the DNA to make the correct primers. No primers are needed for this method.
- This method picks up differences in length -- for any reason -- between restriction sites. Variations in length can be due to differences in the restriction sites themselves (as in **problem 13-3**) or to differences in the length of the sequences between the restriction sites.
- Additional uses of RFLPs will be explained in detail later, after human genetics.

To review restriction enzymes, try Problems 13-1 & 13-2 parts B & C, 13-3, & 13-8 A. To review RFLPs try 13R-4, parts A & B. (Homozygous means both copies of the gene in a diploid, such as a human, are the same.)

III. CRISPR-Cas9 -- Another Useful Bacterial Endonuclease System

A. Basic Idea.

1. Cas9 is a endonuclease.

2. Site of Cutting: Where Cas9 cuts the DNA depends on a 20 base long guide RNA (this is the CRISPR part). The RNA hybridizes to the complimentary DNA, and triggers cutting by Cas9.

3. Targeting the Cutting site: By varying the RNA guide sequence used, almost any site in the DNA can be targeted.

4. 'Editing' the Genome: After the DNA is cut, various types of DNA repair enzymes can be used to make mutations, insert new sequences, etc.

B. Function of the CRISPR-Cas9 System

1. In bacteria: CRISPR-Cas9 is a defense mechanism used by the bacteria to destroy infecting bacteriophage.

2. In the lab: CRISPR-Cas 9 can be used in virtually any organism, to do 'genome editing' -- altering specific sequences in the DNA.

- There are other ways to do genome editing, but they are generally harder to use.
- The CRISPR system can be used to target different enzymes (or variants of Cas9) to a specific site in the DNA.

C. More Background on CRISPR -- discovery, how it's used, ethical issues, etc. See op ed from the *NYTimes*

http://www.nytimes.com/2015/11/10/opinion/the-risks-of-assisting-evolution.html
For a longer article, see
http://www.nytimes.com/2015/11/15/magazine/the-crispr-quandary.html

For recent results with humans, see
http://www.nature.com/news/crispr-fixes-disease-gene-in-viable-human-embryos-1.22382

More science background at:
http://www.nature.com/nprot/journal/v8/n11/full/nprot.2013.143.html
http://www.the-scientist.com/?articles.view/articleNo/44448/title/Engineering-Virus-Resistant-Plants/
http://www.sciencemag.org/content/346/6213/1258096.full

More general info at http://www.newyorker.com/magazine/2015/11/16/the-gene-hackers

IV. Probes

A. What is a probe for?

To find the right piece of DNA -- to find the piece from a particular part of the genome. If you cut up genomic DNA, you will have many pieces of DNA, and you want to find all the ones carrying a particular sequence. For example, to detect an RFLP (such as the one shown on handout 18B), you need to look at DNA from that particular part of the genome. But if you cut up the total DNA of an organism, you get many, many pieces. How will you find the right pieces, that is, the ones carrying a particular gene or section of the DNA where the RFLP is? (How will you know if your DNA is type 1, 2, or 3?)

B. What's a probe? It's a nucleic acid that's complementary to the target sequence you are looking for, and it is usually labeled or tagged in some way -- with radioactivity, fluorescence, or something else that's relatively easy to detect. Probe may consist of single stranded DNA or double stranded DNA. (Double stranded DNA must be denatured before it will hybridize to the target DNA.)

C. How do you get a probe that is complementary to a particular gene? Will be discussed next time. Stay tuned!

D. Probe and target do not have to match perfectly

1. Probe can be shorter or longer than target sequence or fragment you are looking for. Probe and target need not be the same length, but there must be overlap, so some region of target hybridizes to probe. Sample must "capture" probe. (Think Velcro.)

2. . The probe and target must have complementary sequences of 10-20 bases to form a stable hybrid. A restriction site alone is not long enough to form a stable hybrid to a probe.

3. Permissive vs stringent conditions: Conditions for detecting the formation of hybrida can be varied to pick up either perfect sequence matches (stringent conditions) or to detect less perfect matches formed between related, but not identical sequences (permissive conditions).

E. Blots -- This is a method for using probes to detect DNAs that are immobilized on a solid support. DNA does not need to be purified first. Can be released from colonies in place (*in situ*) or "blotted" from a gel. Once DNA is stuck to a support, it can be denatured while still attached to the support. Then you can add a solution of probe (the complementary, labeled, DNA) and see if probe hybridizes to the denatured DNA. You wash off unattached (unhybridized) probe and see what is left. That allows you to identify band, colony, etc. containing the nucleic acid of interest (= nucleic acid that hybridizes to and/or traps probe.) Blots allow you to test hybridization of probe to many DNA samples at once. (More details of these procedures will be discussed next time.)

Then: After we wrap up genetic engineering, we'll start the mitotic chromosome cycle (Lect 20), and the actual steps of mitosis; then meiosis and some implications.