

DNA: Properties and Replication

DNA: Structure, Organization, Characteristics, Enzymes

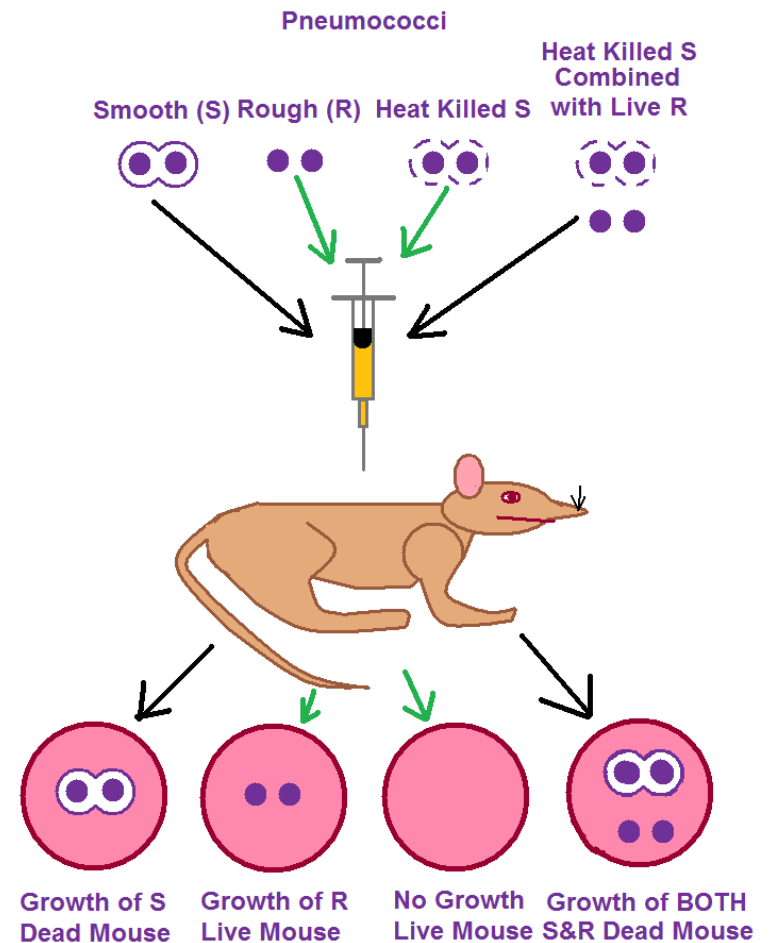
How DNA, RNA, Enzymes and
Ribosomes Work as An Intracellular
Team

Techniques for Isolating, Identifying and Sequencing
Nucleic Acids Included

Griffith's Transformation

- The first evidence that DNA was responsible for transmitting genetic information was shown by Griffith in 1928, LONG before Watson and Crick pilfered Pauling's data and showed the secondary structure of DNA to be a double strand of DNA in an α -helix.
- Griffith's transformation, as his experiment has become known, demonstrated conclusively that DNA was "the stuff of heredity".

- Griffith began his experiment by astutely observing that when some strains of *Streptococcus pneumoniae* were injected into mice, they didn't die, while other strains of the same bacterium caused the mice to die.
- As Griffith delved further into this mystery, he noticed that there was a big difference between the two bacterial strains: one had a capsule around itself (he called this the smooth or "S" strain) and the other did not (he called this the rough or "R" strain).
- He then took some of the smooth bacterium (this was the one that had previously killed the mouse) and heat-killed it.
- This dead bacterium was injected into a mouse and the mouse lived.
- Up to this point, whenever Griffith had injected bacteria into the mouse, he had always been able to culture it.
- After injecting the heat-killed bacteria, he was unable to re-isolate any bacteria.
- This was a positive thing, for it conclusively demonstrated that the bacteria was, indeed, dead.
- It was the next step in Griffith's experiments that turned the heredity world on its head: Griffith took BOTH heat-killed smooth bacteria and live rough bacteria and injected them simultaneously into another mouse.
- The mouse died and when Griffith isolated bacteria from this animal, he observed the growth of both S and R strains of the bacteria in culture.
- Since the S strains were previously heat-killed, the only other answer to explain this phenomenon was that the R strains of the bacterium had taken up [some of] the genetic material and begun synthesizing and releasing a capsule based upon that genetic information.



4 Mechanisms to Take DNA Apart

- 1) fracturing the strands with enzymes,
- 2) during replication,
- 3) during transcription and
- 4) in DNA repair

First: Fracturing the Strands of DNA

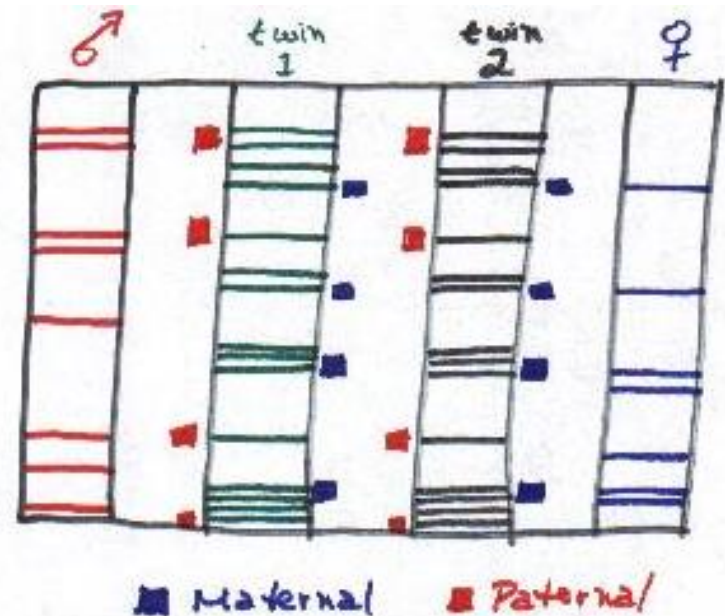
There are four classes of enzymes necessary to accomplish this task:

| | | | |
|---|--|---|---|
| <u>Exonucleases:</u> remove a single nucleotide from the end of the nucleotide chain | <u>Endonucleases:</u> "cuts" strands from within leaving 3'-OH and 5' phosphate ends; very specific | <u>Restriction enzymes:</u> A special class of enzymes -- recognizes a target sequence in the DNA; used to detect paternity, criminal presence, | <u>Topoisomerases:</u> remove supercoiling as replication progresses (think overwound rubber band), e.g., DNA gyrase |
|---|--|---|---|

Two Examples in The Use of Restriction Enzymes:

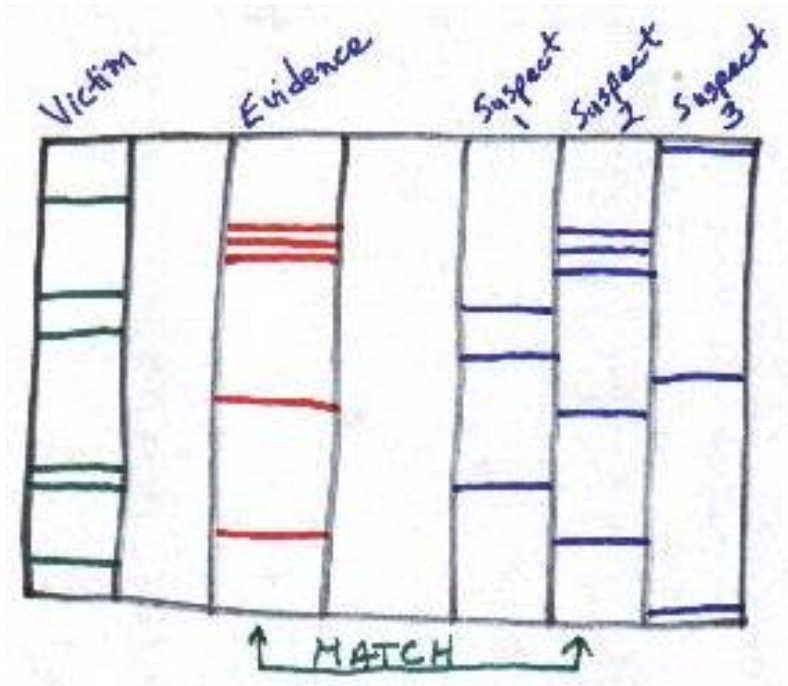
Paternity and Criminal Presence

- A representation of the electrophoretic patterns of the DNA for 4 people:
 - a male,
 - a female and
 - 2 identical twin children.
- While this is an over-simplification, it nevertheless illustrates how paternity may be determined using restriction enzymes.
- The first "column" (or "lane", as they're called) in red represents the lane in which the putative father's DNA was run out.
- Lanes 2 and 3 (twin 1 and 2, respectively) represent the DNA of each twin.
- The last lane shows the DNA from the mother.

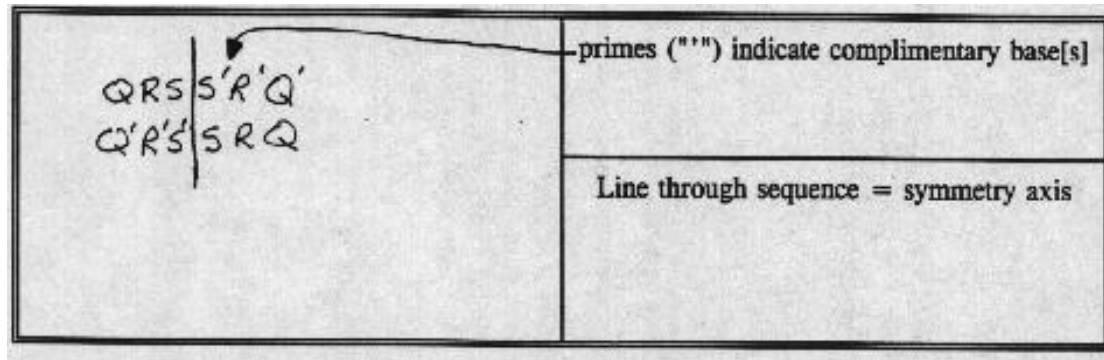


- Identical twins are not entirely identical: they have unique fingerprints and their retinal maps are unique, as well.
- Their DNA, however, is close enough that without special techniques it's difficult to tell them apart.
- Each twin inherited half of his or her genetic information from each parent.
- While each twin will have his or her own unique DNA, each will also have sequences identical to each parent.
- Note that there are red boxes and blue boxes next to the representative DNA bands.
- These boxes show which bands (lines) came from which parent, demonstrating paternity.

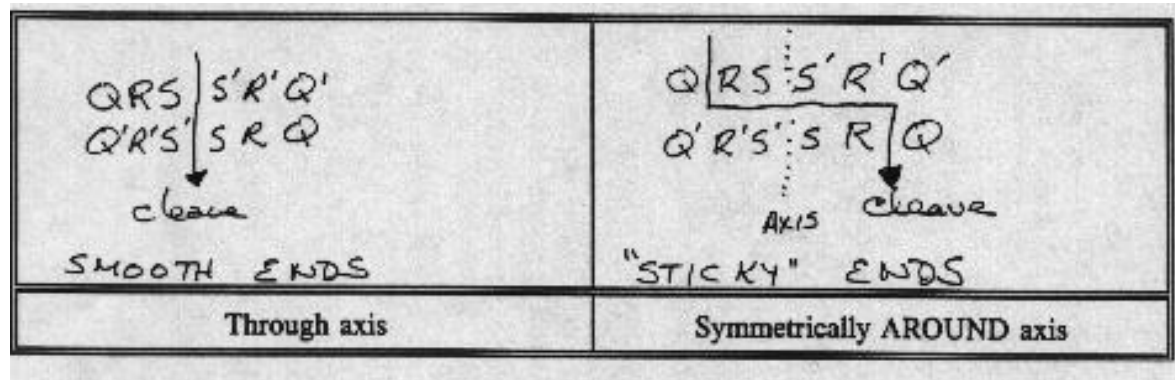
- Restriction enzymes to include or exclude three individuals from having been present at a crime.
- The far-left lane is the victim's DNA, the 2d lane is the DNA from the evidence and the last three lanes are DNA from each of three suspects.
- Note that the patterns of the evidence and the DNA from Suspect 2 match.
- Suspects 1 and 3 may go home and Suspect 2 will be convicted.



- The number of DNA fragments depend upon at least three features:
 - 1) the DNA itself (how was the sample cared for; how old is the sample; is there a mixture of DNA's),
 - 2) the restriction enzymes used for the separation (ya hafta use the same restriction enzymes for every technique in the "batch" you are running) and
 - 3) the technique used by the individuals running the gels (good technique gives good results; bad technique gives bad results; ya gotta run quality control with your samples).



- Restriction enzymes are endonucleases that recognize target sequences in the DS DNA.
- Most sequences of recognition are palindromes (read the same way front to back and *vice versa*, e.g., Madam, I'm Adam).
- Above illustrates a palindromic sequence along with its axis of symmetry.
- It is about this axis of symmetry that the restriction enzyme will "clip".
- At this time, there are no known sequences less than 4 bp in length, hence, there are generally 4 or more bp's per recognition site for each specific restriction enzyme.



- Restriction enzymes may cleave 2 different ways:
 - They may cleave right through the axis of symmetry leaving blunt or smooth ends or
 - they may cleave around the axis leaving sticky or overlapping ends.
- As a general rule, the biological function of restriction enzymes is to cause the destruction of foreign DNA, e.g., viral DNA.
- Since it's possible that the cells' own DNA may contain sequences identical to binding sites for restriction enzymes, these sites are methylated (capped) to render them resistant to the restriction enzymes.

| Source of Enzyme | Name of Enzyme | Recognition/Binding Site |
|-------------------------------------|----------------|---|
| <i>E. coli</i> | EcoRI | $ \begin{array}{c} 5' G \text{A A T T C} \\ 3' C T T A A G \end{array} $ |
| <i>B. amyloliquefaciens</i> H | BamHI | $ \begin{array}{c} G \text{G A T C C} \\ C C A T G G \end{array} $ |
| <i>H. influenzae</i> R _d | HindIII | $ \begin{array}{c} A A G C T T \\ T T C G A A \end{array} $ |
| | NotI | $ \begin{array}{c} G C C G G C C G C \\ C G C C G G C G \end{array} $ |

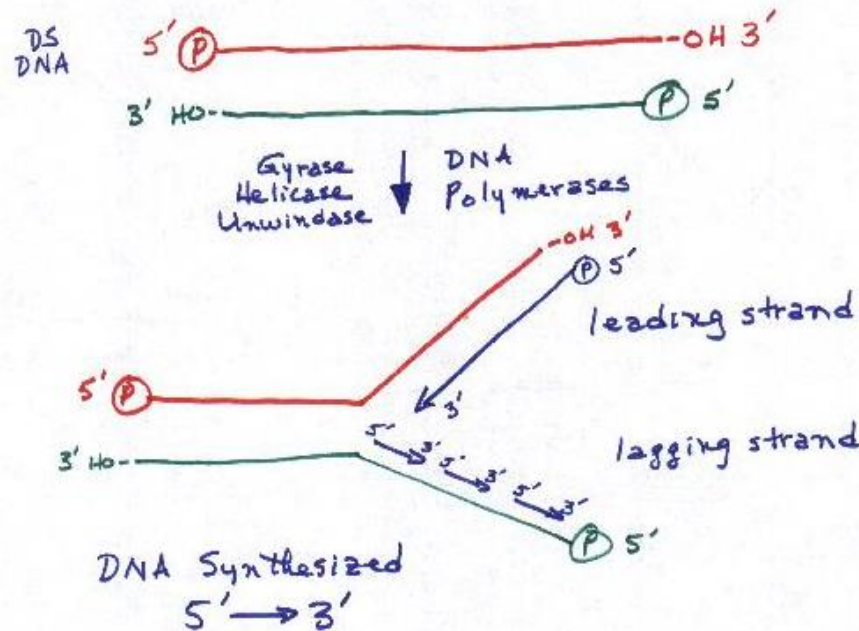
4 examples of restrictions enzymes and their bacterial sources.

Note that all of the examples in this figure are for leaving sticky or over-lapping ends and that each site is very specific for cleavage.

| Source of Enzyme | Name of Enzyme | Recognition/Binding Site |
|---|----------------|--------------------------|
| <i>H. aegyptius</i> | HaeI* | |
| <i>S. marcescens</i> | SmaI | |
| | HindII | |
| | AluI | |
| <p>* A and T orientation appears to not make much difference to this enzyme, AS LONG AS the organization of the two bases is symmetrical.</p> | | |

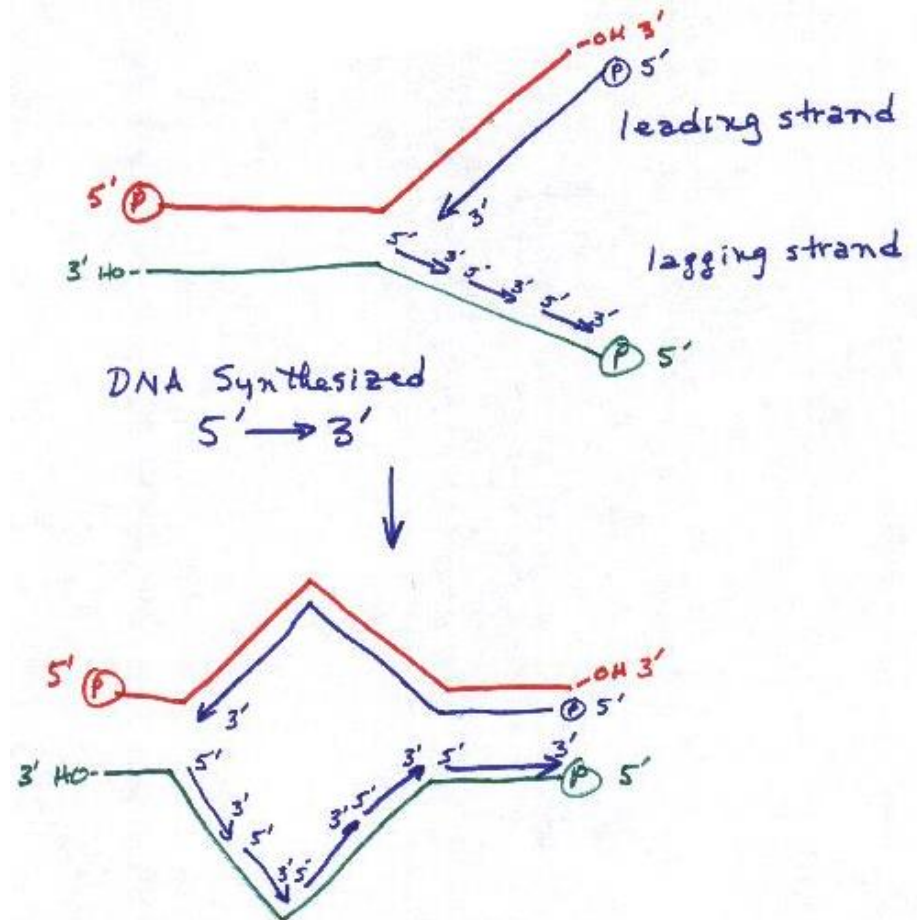
- 4 examples of restriction enzymes and their bacterial sources.
- Note that all of the examples in this figure are for leaving blunt, smooth or flush ends and each site is not as specific for cleavage as were those in the previous slide.
- The recognition site for HaeI relies upon the location of dA and dT as long as they are symmetrically arranged about the axis of symmetry -- note the "*"s" in the figure.
- Note, also, HindII's site: the axis of symmetry is through a pyrimidine-purine center that is complementarily repeated.

Second: During Replication

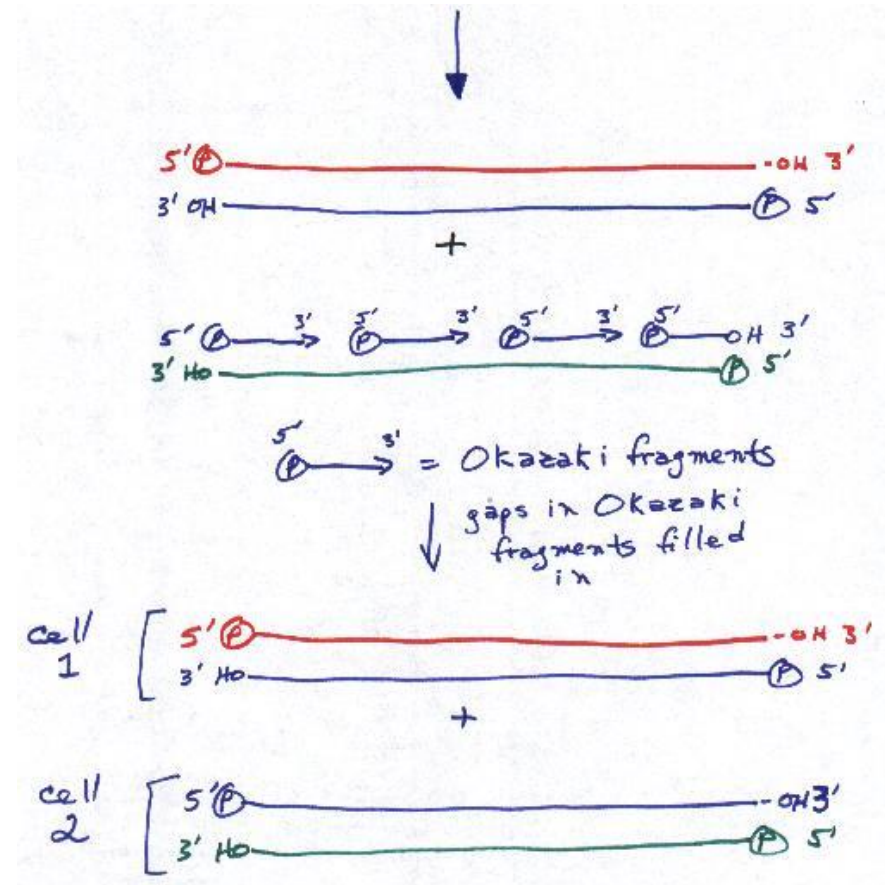


- The process of replication occurs during cell division and consists of the DNA making a copy of itself.
- The first thing DS, anti-parallel DNA must do is open a portion of its structure so that the necessary enzymes may "read" the DNA a strand at a time and in the opposite directions.
- Note that the 5' end has a phosphate and the 3' end has the -OH.
- A series of enzymes, gyrase, helicase, unwindase and DNA polymerases, must unwind the DNA, then read the DNA, then transport in the complementary nitrogenous bases to accommodate
- DNA replication process. As the DS DNA opens up, it forms a replication fork.
- This replication fork allows both strands of the DNA to be replicated simultaneously, albeit in two different manners.

- Note that in the previous slide that there is a strand of DNA called the leading strand.
- This strand is so-called as it is continuously replicated along its complementary strand and is always ahead of the other strand being replicated.
- The latter strand is called the lagging strand because its synthesis is always lagging behind the lead strands.
- It is also incompletely replicated.
- The reason for this is that the synthesis of the lagging strand depends on the opening of the replication fork and the space that the opening permits to accommodate the enzymes, hence, its synthesis starts with a spit and a sputter, leaving small fragments as replication continues.
- These fragments are called Okazaki fragments after their discoverer.
- Note, also, that regardless of the direction, DNA is synthesized from the 5' to the 3' direction, i.e., in a complementary fashion.
- As replication progresses, the replication fork forms a replication bubble in the DNA until, at the very end of the strand of DNA, it forms the last replication fork.



- Before the cell may continue on towards division and before G_2 phase is complete, the Okazaki fragments must be annealed.
- This requires energy (ATP).
- Once the gaps are filled in, the cell may leave G_2 and move into mitosis to make two identical cells to the parent cell.
- Also note that 50% of the old DNA goes to the daughter cells and 50% of the new goes to the daughter cells, i.e., an old strand and a new strand go to the new cells.



Semi-Conservative Replication

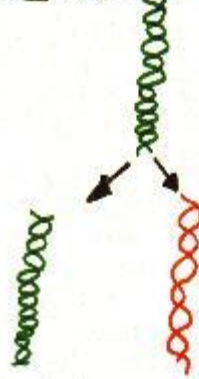
- This whole process is called semi-conservative replication.
- How, though, was it determined that DNA replicates in a semi-conservative manner?
- This came about from the elegant Meselson-Stahl experiments.
- They felt that there were three options available to explain how DNA was replicated.

| Semi-conservatively | Conservatively | Dispersively |
|---|--|---|
| Each strand of the original was used as a template for the synthesis of a complimentary strand, with which it is combined in the daughter cell. | Each strand of the DNA is used as a template strand for the synthesis of new strands that form identical DNA, but via old-old and new-new strand combinations. | The DNA is replicated willy-nilly and aligns however it wishes. |

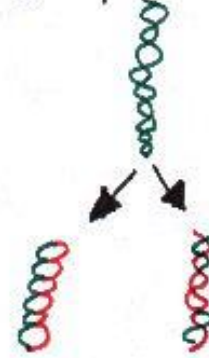
Semi Conservative

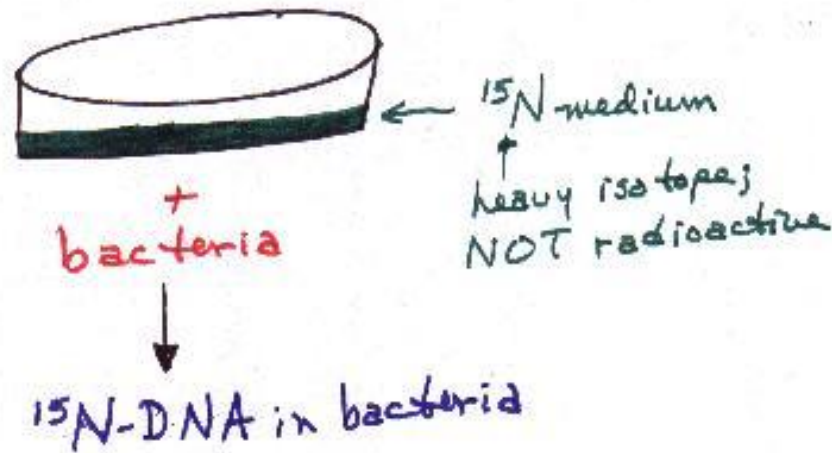


Conservative



Dispersive

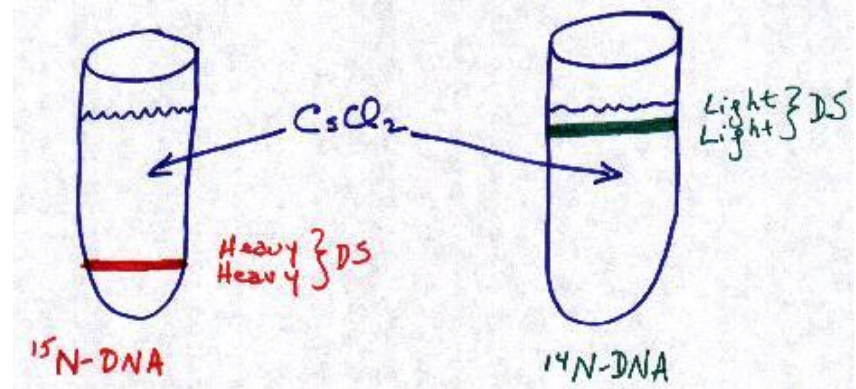


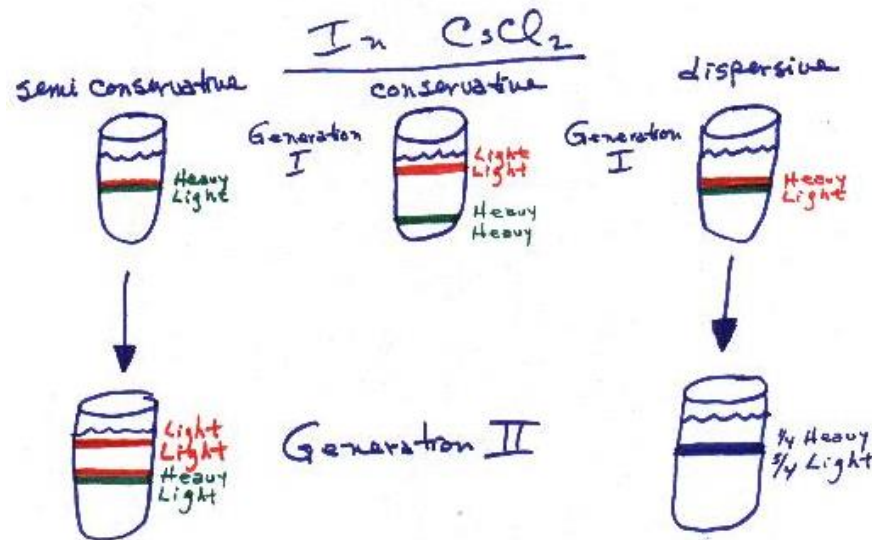


- The question was "Which one is correct?"
- Meselson and Stahl came up with a slick way to determine the answer to this.
- They made a medium for bacterial growth that contained ^{15}N (a heavy isotope of nitrogen, NOT radioactive) and grew their bacteria on it.
- The idea was that the bacteria would incorporate the ^{15}N into their DNA, making their DNA heavier than other DNA.
- Then the bacteria were removed from this heavy medium and re-grown on medium that contained ^{14}N (the "regular" (lighter) isotope of nitrogen) for one or more generations.

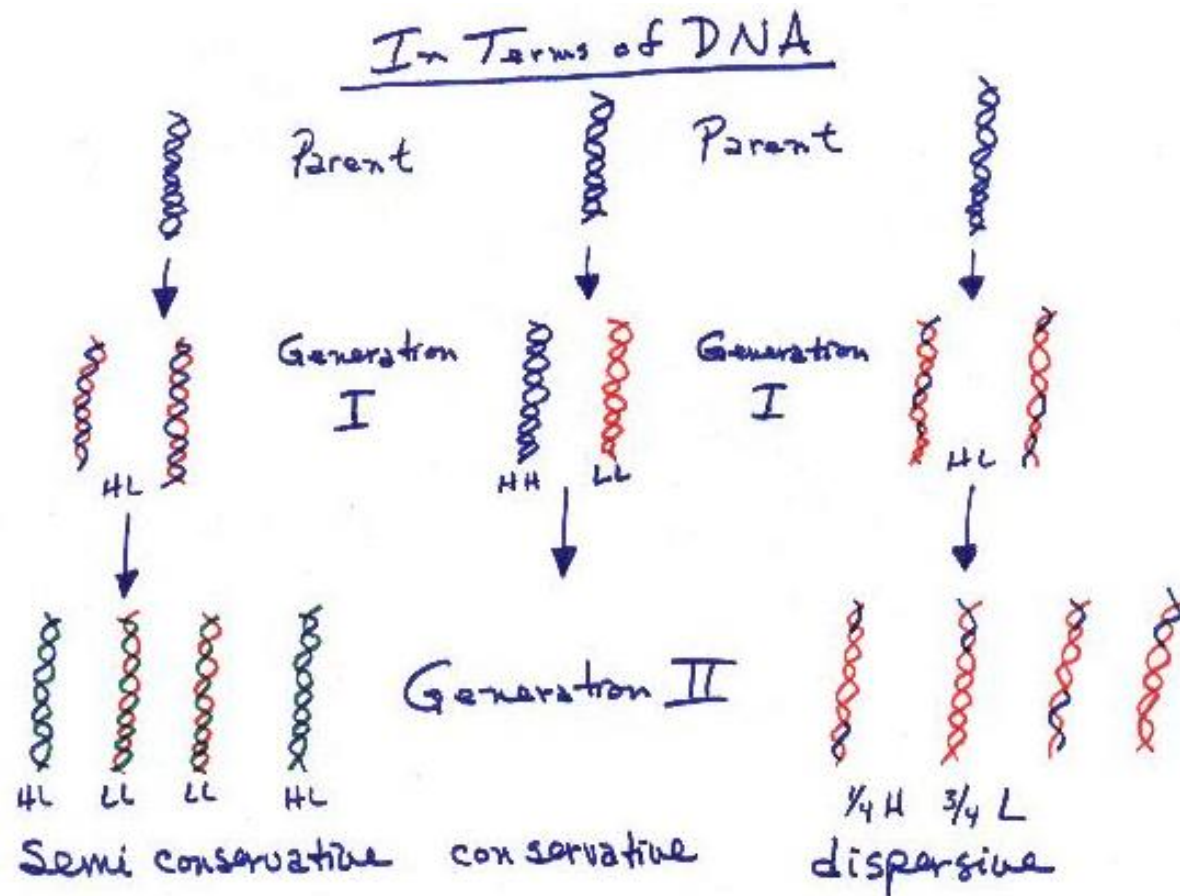
- They then harvested the bacterial DNA and studied it in CsCl_2 via ultra-centrifugation.
- In a nutshell, ultra-centrifugation is a process whereby tubes are spun at pre-determined speeds that permit forces on the samples in the tubes to go far beyond gravitational pull.
- By using CsCl_2 , Meselson and Stahl were pretty clever. Cesium chloride solutions have a density about the same as DNA.
- When the cesium chloride solutions are spun, though, in a centrifuge, a density gradient (think about concentration gradients you learned about earlier) is set up in this salt solution.
- The DNA then floats, as it were, to the region of density that most closely approximates its own density in the cesium chloride as it's spun down.
- Typically, this is near the -- more or less -- middle of the tube.

- For the purposes of illustration, though, we'll use artistic license as shown below to visualize the differences between the heavy and light DNA in the cesium chloride solutions.





- Meselson and Stahl predicted DNA patterns for each of the three models of DNA replication, above, in cesium chloride.
- For semi-conservative replication, they predicted that the first generation's band in the density gradient would consist of a strand of heavy and a strand of light DNA.
- For conservative replication, they predicted that the first generation would have two bands: one with two strands of light DNA and one with two strands of heavy DNA.
- Since this didn't happen, they moved on from there to the dispersive model.
- They predicted that the dispersive replication would give one band in cesium chloride that was part heavy DNA and part light DNA.
- Meselson and Stahl predicted that the second generation's DNA following semi-conservative replication would produce two bands: one that consisted of 2 strands of light DNA and the other that consisted of one light and one heavy strand of DNA.
- They, likewise, predicted that if the DNA was replicated in a dispersive manner that the second generation's DNA would be in one band: 25% heavy DNA and 75% light DNA.



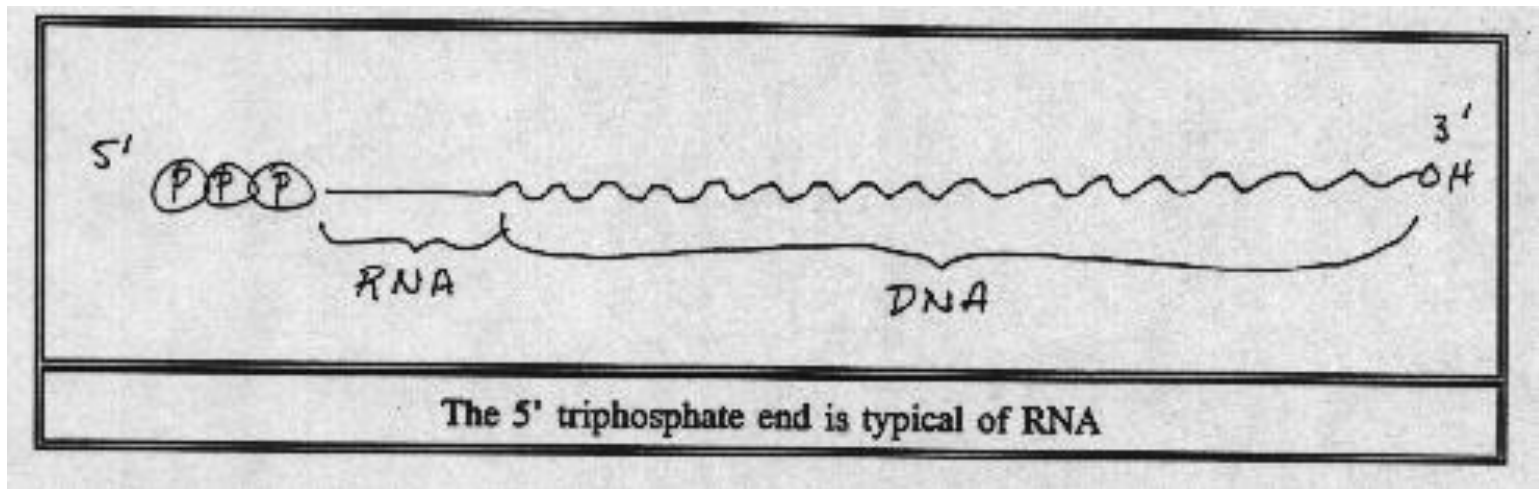
- Above illustrates how this would look with the DNA, itself.
- The bottom line is that it turned out that the semi-conservative model of DNA replication was born out by the Meselson-Stahl experiments.
- DNA continues to be studied on cesium chloride gradients, as well as on sucrose.

There are some fine points to keep in mind when considering DNA replication

ONLY 5' triphosphates are appropriate substrates for DNA polymerase (DNA Pol)

1.

- A primer is required for DNA replication.
- This primer is an RNA oligonucleotide, 4-60 bp in length.
- The primer requires RNA Pol and Primase that copies the DNA sequence from 1 DNA strand.
- This primer is different from classical RNA as this RNA remains H-bonded to the DNA template.
- Illustrates the appearance of the RNA primer/DNA strand.
- Note that on the 5' end there is a triphosphate. This is typical of RNA.



2. Nick translation is very important in DNA ligation.
- It occurs in the lagging strand to force the Okazaki fragments to join together at the expense of ATP (the enzyme, here is a ligase).
 - When DNA Pol makes enough DNA to run into the next chain of RNA primer (each Okazaki fragment has its own RNA primer), its exonuclease activity removes the RNA and replaces it with DNA (DNA Pol is a multi-functional enzyme).
 - The 2 Okazaki fragments are then joined together with ligase.

3. The helix is unwound with helicases.
 - The exact mechanism is not understood.
 - It is known that helicases require ATP.
4. After the DNA is unwound to form two SS DNA, it must be stabilized or it will "re-fuse" or form H-bonds with itself.
 - The DNA remains stable with SS DNA binding proteins (SSDBP).
 - Although the mechanism for the removal of the SSDBP's is unknown, they are replaced by the advancing polymerases.

5. The rate of polymerization (formation of many nucleotides into a strand) in eukaryotes in DNA synthesis is about 0.5-5 kbp (kilo base pairs) per minute!
6. When DNA is replicated, the histones are dissociated from the replicating fork in the immediate area, then by unknown mechanisms, octamers and DNA reassociate.
 - Makes one wonder about the relationship between the histones and the SSDBP's, doesn't it?

7. Once DNA has been replicated there is still one little problem: there will be an RNA sequence at the end.
- It's easy to remove, but it seems to be replaced, instead.
 - The replacement mechanism is unknown although we DO know that the synthesis of DNA necessarily depends on the presence of this RNA primer.

We also know the following:

1. The RNA can not remain, because its properties would alter the properties of the DNA.
2. It doesn't seem reasonable to expect that the ends would remain SS after the RNA was removed, as this would alter the properties of the DNA, as well. It would also effect the function of telomerase.

All the above suggests that the following may play a role in the RNA replacement:

1. Perhaps a "terminase", a DNA Pol that remains to be identified, is involved in altering the RNA end into a DNA end.
2. Perhaps a 2'-hydroxylase, heretofore unknown, simply strips the 2' - OH group from the ribonucleotides to change them into deoxyribonucleotides -- challenge: how would this take care of the T vs. U characteristics between DNA and RNA? (DNA Pols are not capable of distinguishing between dUTP and dTTP since H-bonding occurs between the two and A in DNA: hence dUTP becomes incorporated into the DNA -- therefore, no major problem, since the dUMP will be removed from the newly synthesized strand of DNA and replaced under the influence of ligase.)
3. Although unlikely, perhaps the enzyme ligase is a multifunctional enzyme, i.e., has ligase activity and hydrolase activity or has ligase activity and transferase activity, and simply has not been elucidated with current technology.

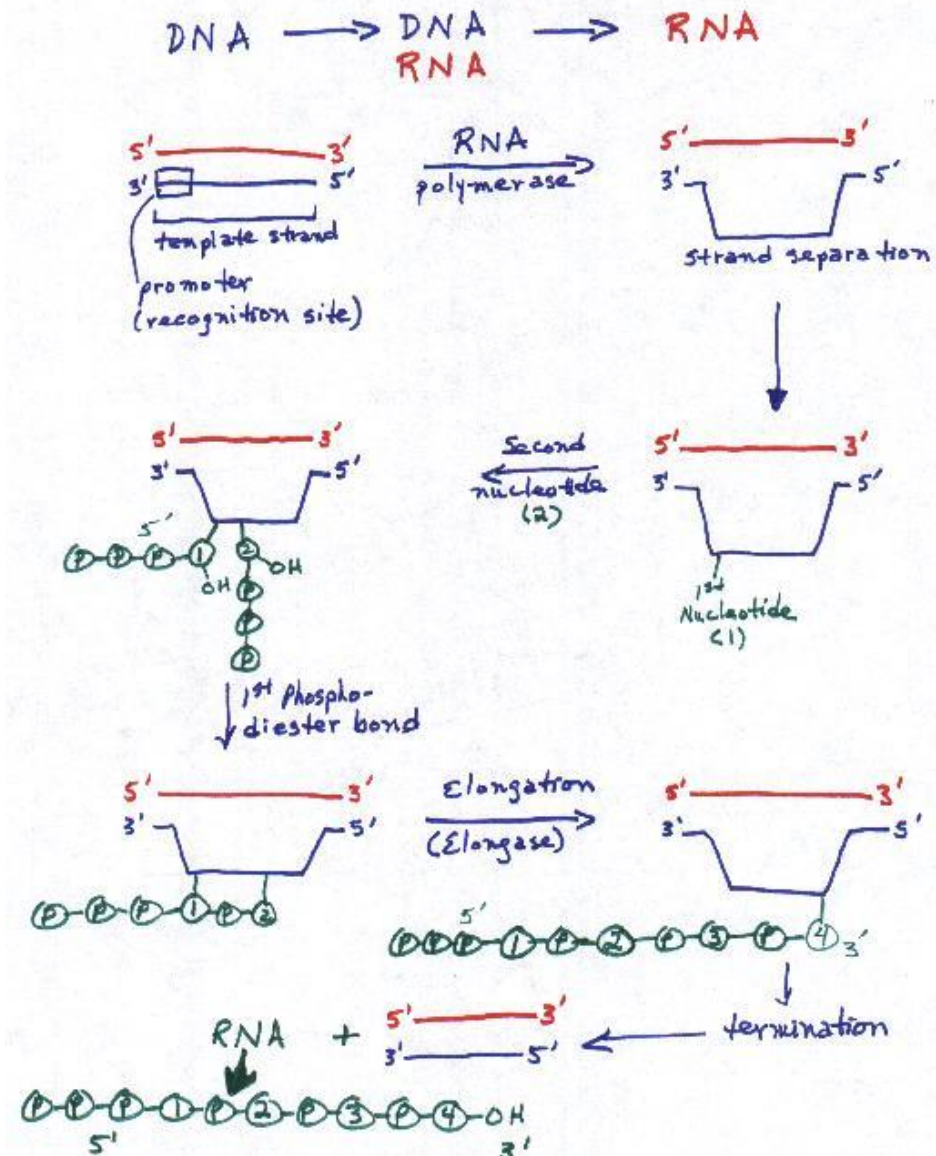
- DNA Synthesis may be inhibited by numerous chemicals and/or drugs.
- The following table summarizes 3 classes of compounds used to inhibit DNA synthesis:

| <u>Precursor Synthesis Inhibitors</u> | <u>Template Altering Drugs/Inhibitors of Strand Priming</u> | <u>Polymerase/Other Enzyme Inhibitors</u> |
|--|---|---|
| Covered in Future Courses; examples listed below are NOT inclusive | Intercalating agents (review ethidium bromide or acridine); Daunorubicin, doxorubicin and plicamycin | Chain terminators: 2',3'-dideoxyribonucleotides, cordycepin Acyclovir in Herpes Simplex; Aphidicolon on SOME DNA Pol in some viruses |
| DHFR* inhibitors: Methotrexate in bacteria and protozoans; Pyrimethamine in protozoans; Trimethoprim in bacteria and protozoans | Chain breakers: Bleomycin, Zinostatin | |
| Inhibitors of purine biosynthesis: sulfonamides in bacteria; Azaserine in humans (inhibits inosinic acid synthesis); 6-diazo-5-oxo-L-norleucine (DON), also | Chain cross linkers: Alkyl sulfonates, anthramycin, nitrogen mustards, mitomycin C | Bacterial DNA Gyrase inhibitors: novobiocin, oxolinic acid, nalidixic acid |
| Inhibitors of AMP and GMP formation: Hadacidin (from fungi) in bacteria, plants and tumors -- AMP inhibition; Ribavirin monophosphate -- GMP inhibition | Template activity inhibitors: platinum coordination compounds (review PI-PUFA's) | |

*DHFR inhibitors are those inhibitors that block dihydrofolate reductase from reducing dihydrofolate to tetrahydrofolate so that our bodies (or bacterial cells or protozoans) won't be able to adequately utilize folic acid to its utmost.

The Third manner in which DNA may be taken apart is through the process of transcription, i.e., RNA synthesis

- During transcription, only ONE strand of the DNA is actively read and transcribed. The strand to be transcribed contains a promoter (recognition site) that is recognized by RNA Pol. This enzyme causes the DS DNA to separate and it adds on the first of a sequence of nucleotide triphosphates (NTP's) to initiate transcription.
- Note that the DNA is the template for the new strand of RNA. In this instance, the bases in RNA will H-bond with the DNA in a complimentary manner, i.e. AU and/or AT (depending on the nucleic acid and orientation) and GC.
- As the polynucleotide progresses, the enzyme elongase extends the polynucleotide chain until termination occurs with the release of RNA (much more on this in the RNA chapter).



Regulatory Proteins: DNA Binding for Transcriptional Control

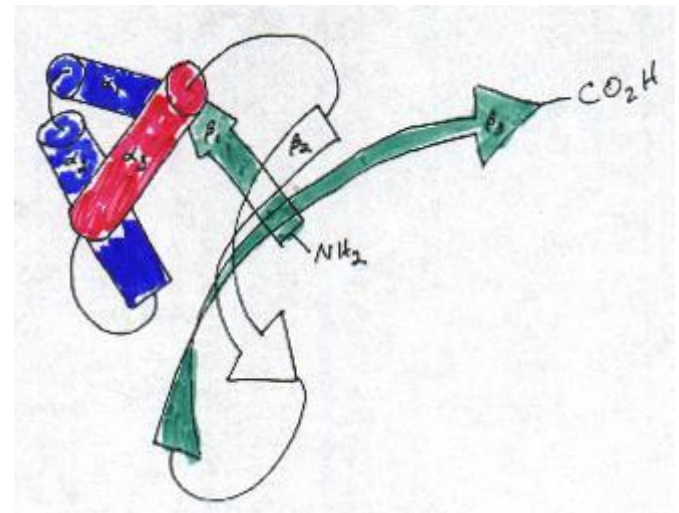
- There are three motifs of DNA binding proteins:
- All three of these bind to DNA
 - Helix-turn-helix,
 - zinc (metallo) fingers and
 - leucine zippers.

There are some general rules that are applicable to these DNA binding proteins:

1. They have a high affinity binding (binds tightly and favorably) to a specific site on the DNA.
2. They have a low affinity for the rest of the DNA.
3. Only a "small bit" of these proteins may directly contact the DNA.
4. The styles of binding sites for all 3 motifs increase cooperative binding and increase the affinity of the protein of/for/with DNA -- analogy: cooperative binding of oxygen onto hemoglobin as you learned previously.

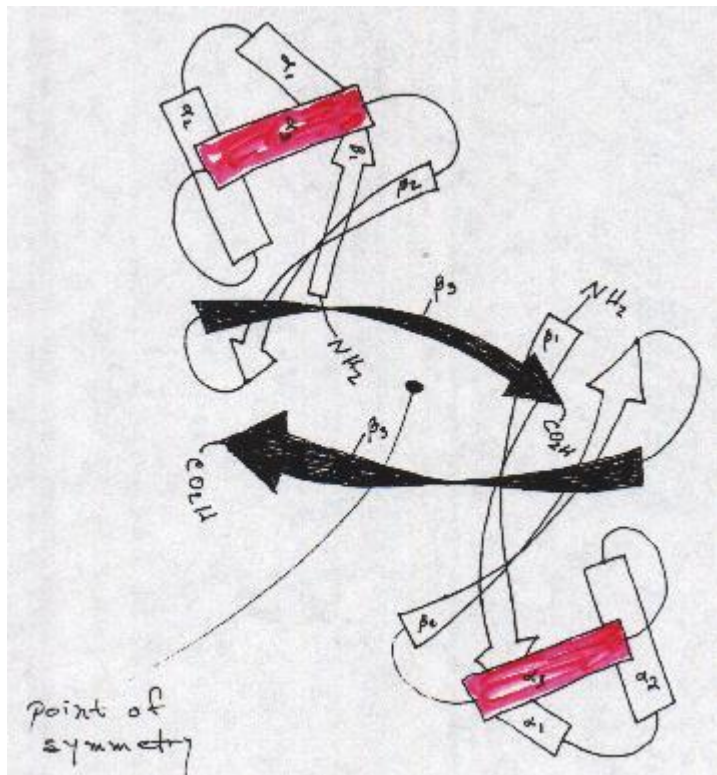
Helix-turn-helix Motif

- Typically this motif has 3 antiparallel β -pleated sheets and 3 α -helices .

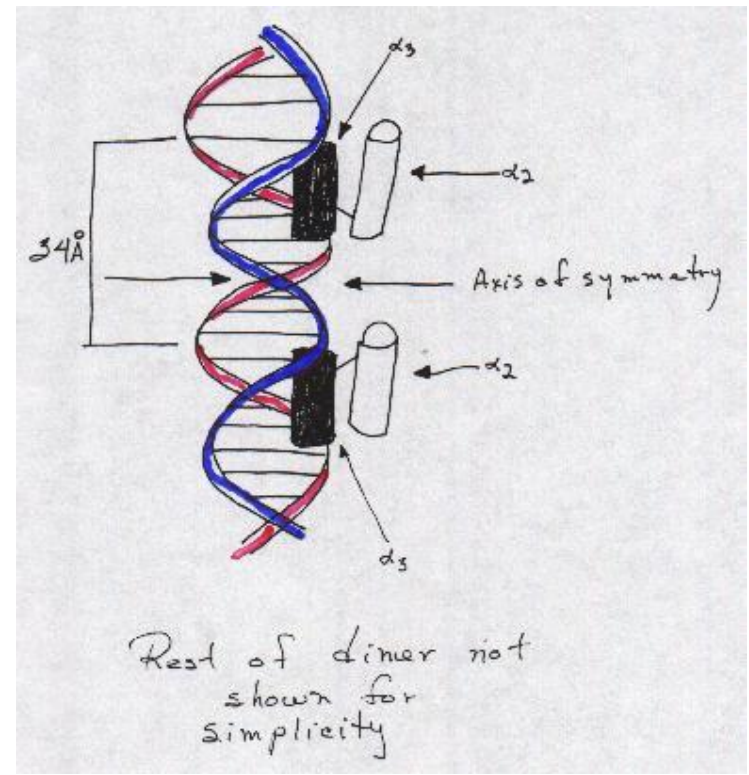


- Each sub-unit plays a different role in the binding of this protein with DNA:
-
- The α_3 subunit interacts with the major groove over about 5 bp's.
- β_3 interacts with another helix-turn-helix protein as it dimerizes.
- Therefore, 2 α_2 subunits then interact with the major groove to regulate this section of DNA.
- α_3 and α_2 sub-units are perpendicular to each other to maintain the position of α_3 .

- The structure of the dimeric form of this motif. Note the point of symmetry between the two β_3 sub-units.

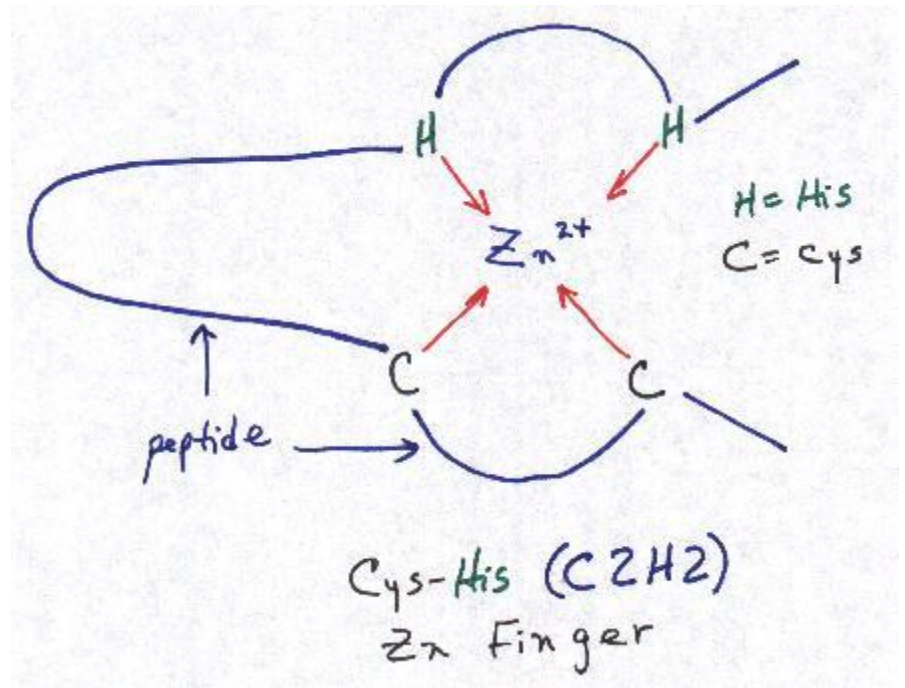


- The binding of two dimers in two regions of DNA.
- Note that the rest of the protein is not shown to simplify this illustration.

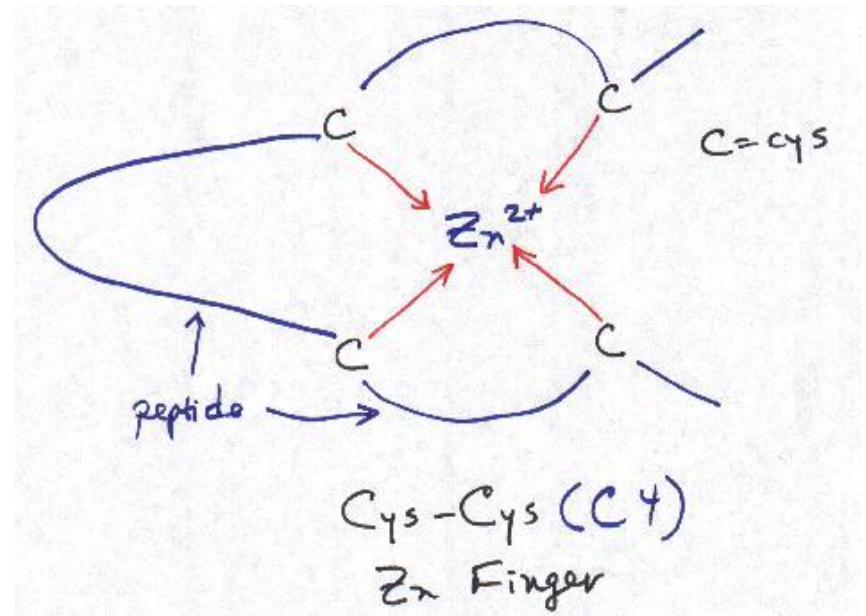


Zinc Finger Motifs

- These were the second DNA binding proteins to be studied.
- Studies revealed in a protein called TFIIIA (Transcription Factor IIIA) that it has 9 Zn^{2+} ions, each of which are complexed by 2 Cys residues close to each other and by 2 His residues 12-13 residues later.
- This protein is known as a Cys-His Zn Finger or a C_2H_2 Zn Finger.
- The "C"s stand for cysteine residues and the "H"s stand for the histidine residues.

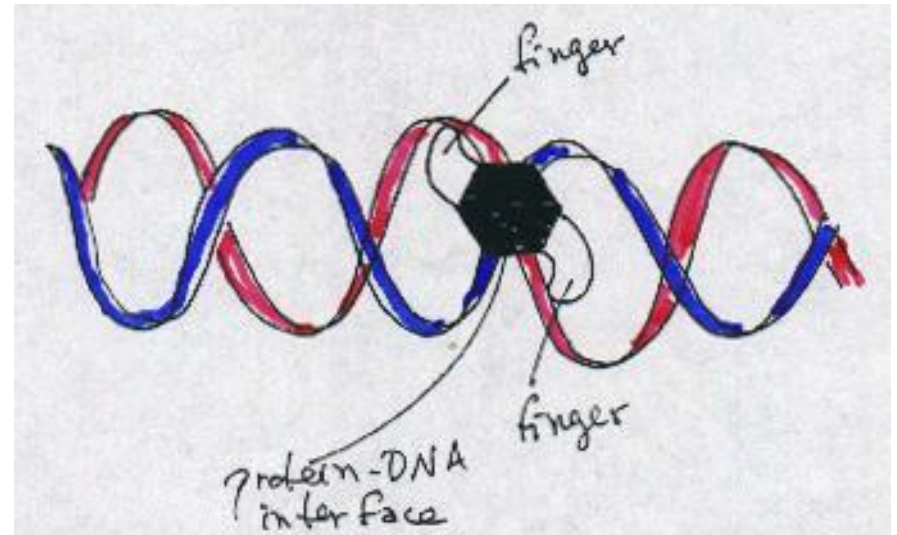


- Of the forms of Zn fingers known, a change occurs in steroid receptors and thyroid hormone receptor families: the 2 His residues are replaced with 2 Cys residues.
- This is a Cys-Cys Zn Finger or C_4 Zn Finger.
- It is important to note that in all known Zn fingers, the Zn^{2+} is in tetrahedral geometry (review bio-organic chem lecture).



- A variation of the C4C4 finger is the C3HC4 RING finger, so called due to its appearance of two fingers turned back on each other forming a ring.
- RING fingers are found in BRCA-1, the gene identified as being the cause in familial breast cancer -- this is under controversial discussion, at this time.
- It is possible that the same RING finger style may be in BRCA-2, a gene implicated more in familial ovarian cancer than breast cancer -- again, this is under discussion at this time.

- Proteins with Zn fingers lie on a face of the helix with its fingers "stuck" into the major groove.
- Each finger spans 5 bp's (some sources suggest 2-5 bp's) as does the helix in the helix-turn-helix binding motif.

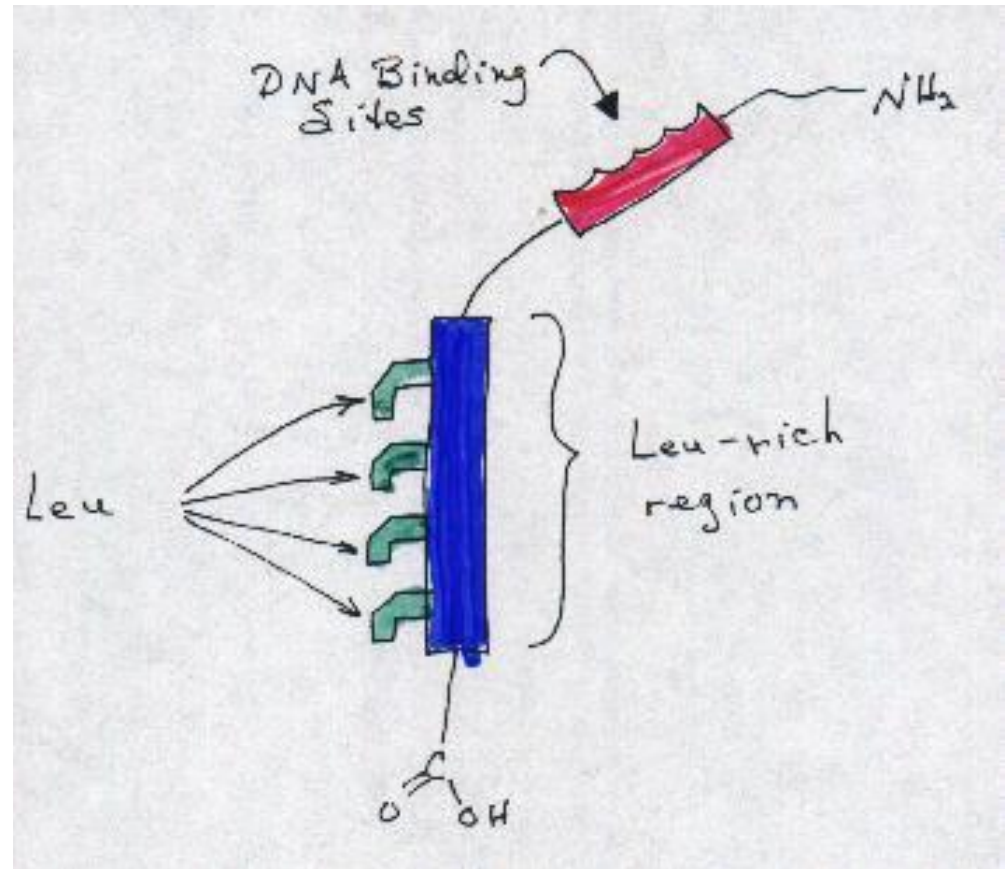


How Important Are Zn Finger Proteins?

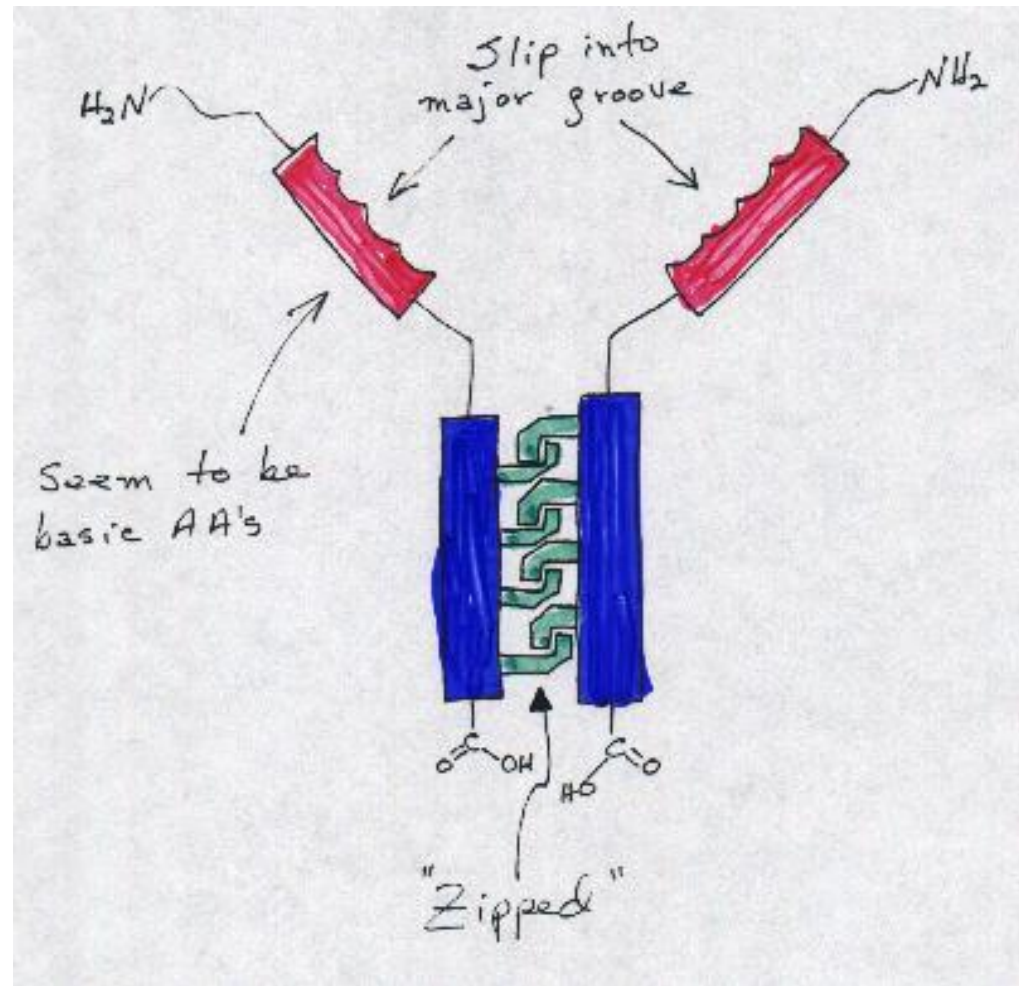
- Vitamin D receptor proteins have 2 Zn fingers.
 - If ONE mutation occurs in either finger, this receptor will NOT bind vitamin D and Vitamin D resistant rickets manifests.
- Zn fingers seem to regulate CNS development.
- Zn fingers may function between DNA-RNA hybrids.
- Zn fingers may be oncoproteins in leukemogenesis (development of leukemias).
 - Under normal conditions/structures, hematopoietic cells develop into blood cells.
 - When abnormal, lead to cancers of the blood (leukemias).
- Zn (RING) fingers may be required for viral "growth", indeed, it now appears that Zn fingers are involved with HIV.
- Zn fingers may prevent inappropriate major histocompatibility class (MHC) II expression (more on MHC in BIOL 224 or 251), immunoregulatory genes which are responsible for causing multiple sclerosis as well as insulin dependent diabetes mellitus (IDDM).

Leu Zipper Motif

- This motif is found in the C-terminus of enhancer binding proteins.
- The leu zipper has α -helical conformation and every 7 residues there is a leu.
- This occurs for 8 turns with 4 repeats, i.e., leu is found at numbers 1, 8, 15 and 22.
- This motif is monomeric until it's needed to bind with/to DNA, next slide.
- The "zipper teeth" in the next slide represent the leu residues.



- When two leu zipper monomers dimerize, these two leu-rich regions zip together into a coiled-coil which appears to increase the association of the DNA binding sites with their DNA target sites, next slide.
- Note that the "zipper" is located on the C-terminus of the proteins and the binding region is in the N-terminus of the proteins.
- The binding region of the leu zipper seems to consist of basic amino acids (positively charged amino acids).
- This works out reasonably well, since the phosphate backbone is negatively charged and affords good binding to basic amino acids.

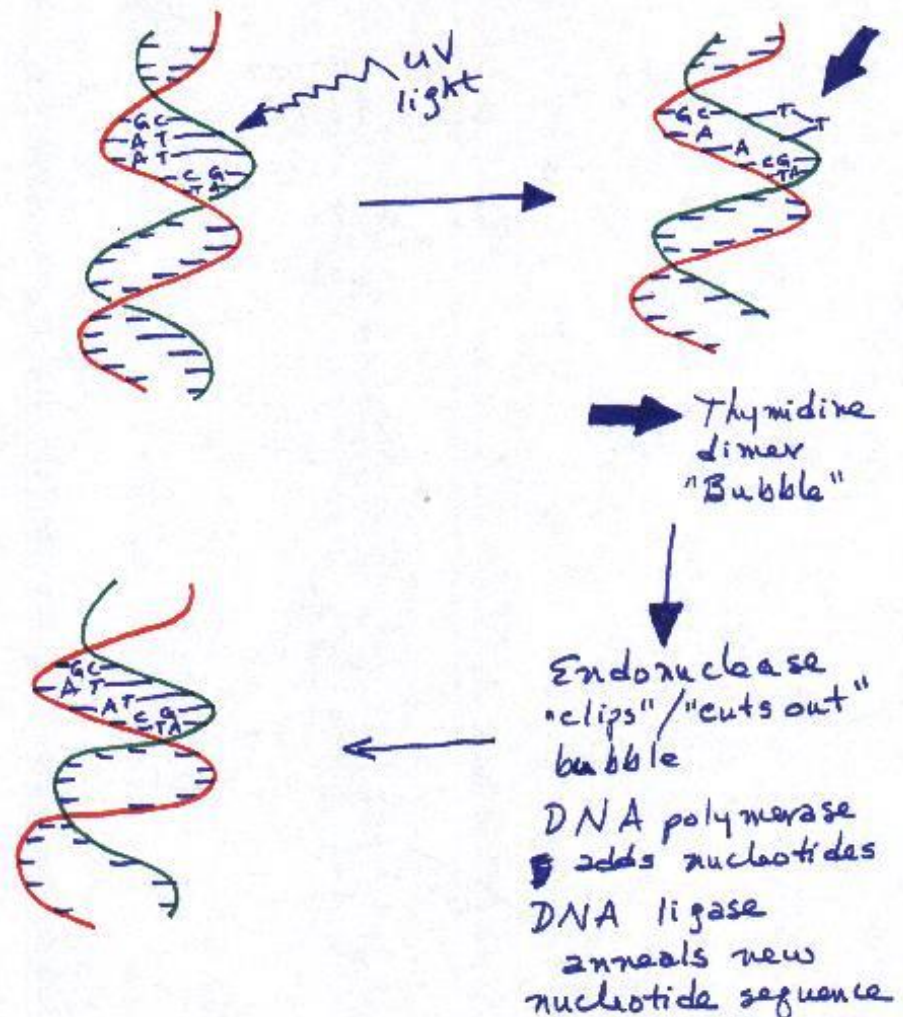


ALL three motifs are capable of positive/negative regulation of transcription.

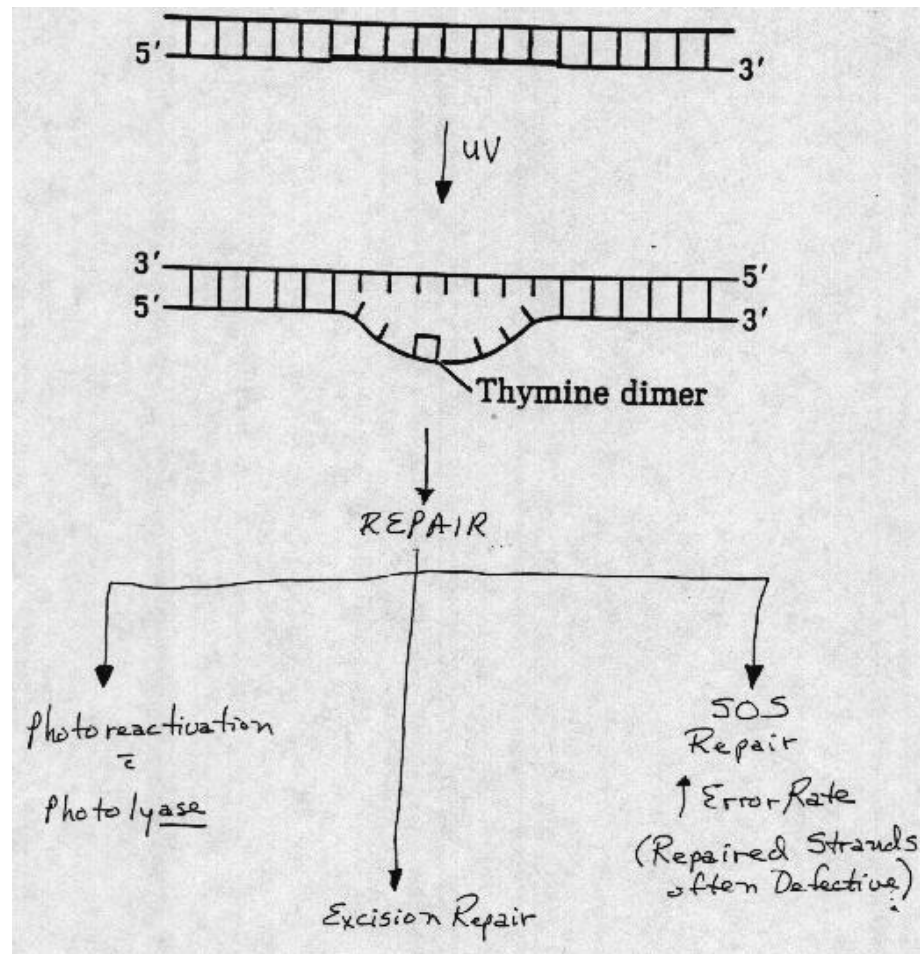
The Fourth and last manner in which we can take DNA apart has
to do with DNA repair.

- The first we want to examine is the effect of UV light and the last is the effect of ionizing radiation.
- UV light is absolutely miserable when it comes to DNA repair.
- This is the same UV light that causes malignant melanoma.
 - The UVA range is wavelengths from 320 to 400 nanometers (used for suntanning; shorter UVA wavelengths are considered possibly cancer-causing; responsible for cutaneous aging changes).
 - UVB refers to wavelengths from 290 to 320 nanometers (These wavelengths are more hazardous than UVA wavelengths, and are largely responsible for sunburn. The ozone layer partially blocks these wavelengths. UVB is largely blamed for malignant melanomas. Important in cutaneous vitamin D metabolism).
 - UVC refers to shorter UV wavelengths, usually 200 to 290 nm (Wavelengths in the UVC range, especially from the low 200's to about 275 nm, are especially damaging to exposed cells. Relatively little reaches our skin; well absorbed by atmospheric ozone).
- It is because of the UVB light-DNA interactions that we eventually develop malignant melanoma following unprotected sun exposure.
- These interactions trigger four (4) types of repair mechanisms: 1) photoreactivation with photolyase, 2) excision repair, 3) endonuclease repair and 4) SOS repair.
- The latter has an incredible error rate, as the repaired strands are often defective.

- The slide illustrates what happens when UV (ultra-violet) light interacts with DNA and how endonuclease activity repairs it.
- UV light "looks" for dTdT pairs on the same strand of DNA.
- When these pairs are hit by UV light, they "naturally" react to form thymine dimers, which are held together by a cyclobutyl bridge.
- These thymine dimers make bubbles in the DNA, much like a speed bump through J.C. Penney's parking lot.
- Just like the speed bump bouncing cars when they drive too fast over the bumps, the dimers knock the enzymes off the DNA, thereby making inappropriate DNA.
- Endonuclease "clips" or "cuts out" the bubble.
- DNA Pol adds nucleotides to the "cut area" and DNA ligase anneals the new nucleotide sequence.

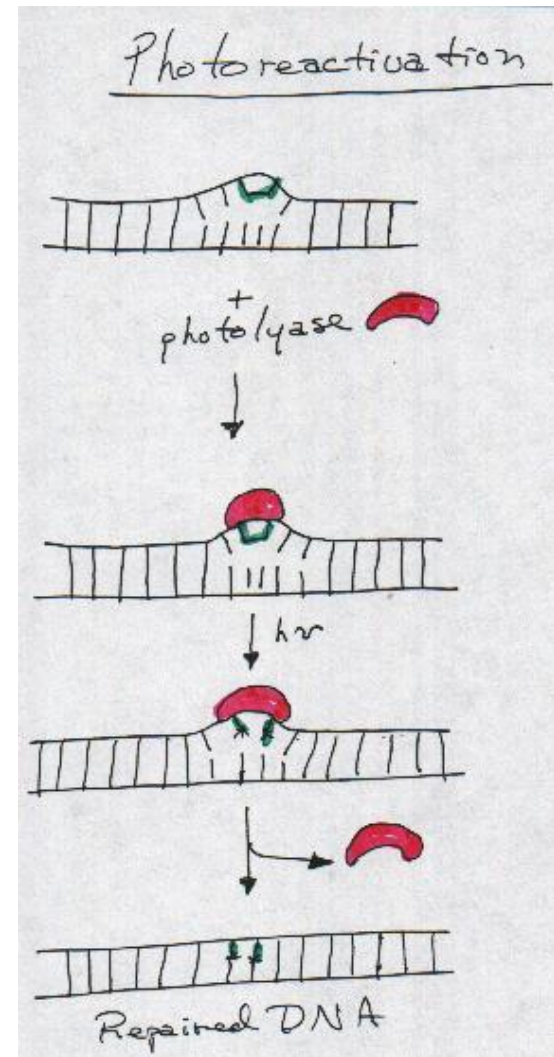


3 More Significant Repair Mechanisms Following UV-induced DNA-damage



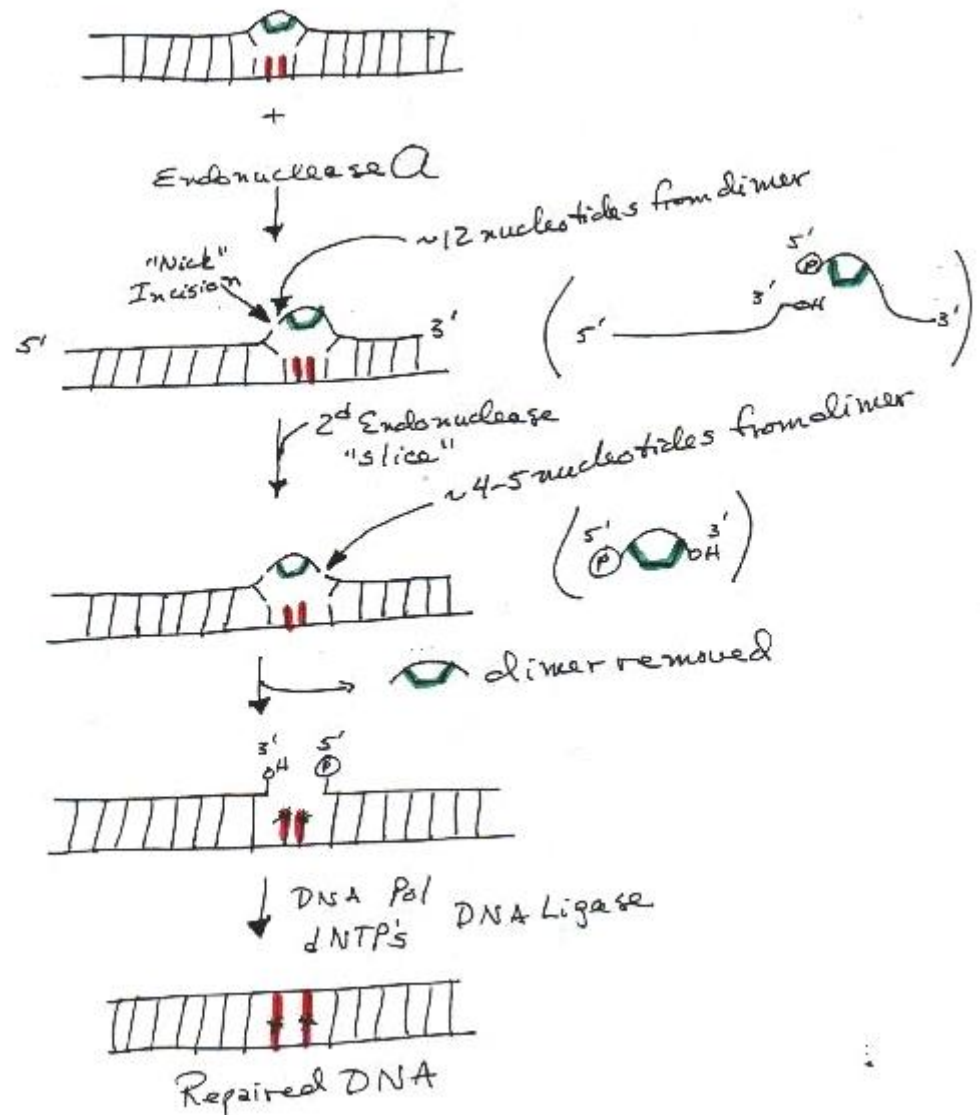
The first mechanism under discussion is photoreactivation.

- This mechanism works by having an enzyme (photolyase) bind to the site of the thymine dimer formation, once dimer formation has occurred.
- Light then activates photolyase to "clip" the bonds holding the cyclobutyl bridge together, permitting the dT's to reanneal with their complementary base[s] (dA).

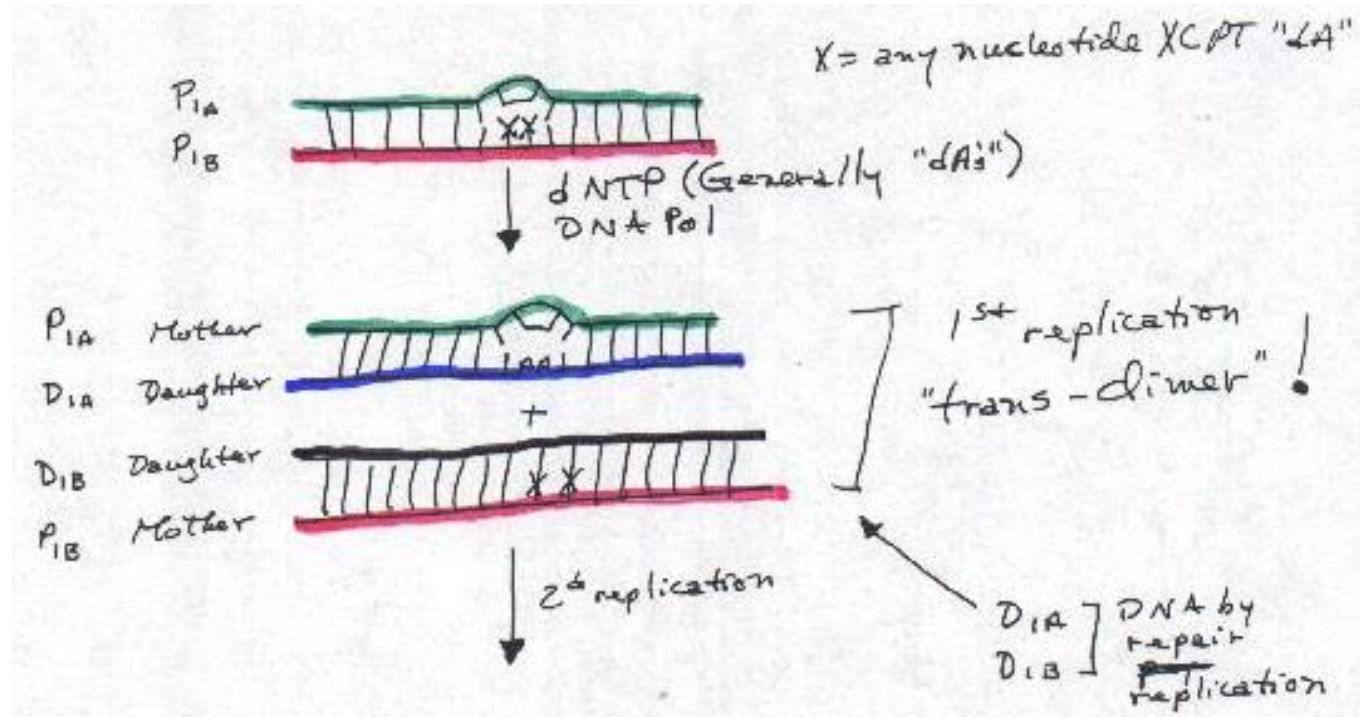


The Second Mechanism is Excision Repair.

- The second mechanism is excision repair.
- This is similar to the endonuclease activity repair we examined earlier, but is of sufficient enough difference that it has another name.
- Two endonuclease activities are required: 1 to clip on the 5' end of the dimer about 12 nucleotides from the dimer ("nick") and the 2d to clip on the 3' end of the dimer 4-5 nucleotides from the dimer ("slice"), at right.
- This "nick" and "slice" leaves 3'-OH's and 5' phosphates on both the excised dimer and the intact strand.
- Once the dimer is removed, DNA Pol and ligase with dNTP's will "fill in" the excision and leave repaired DNA.

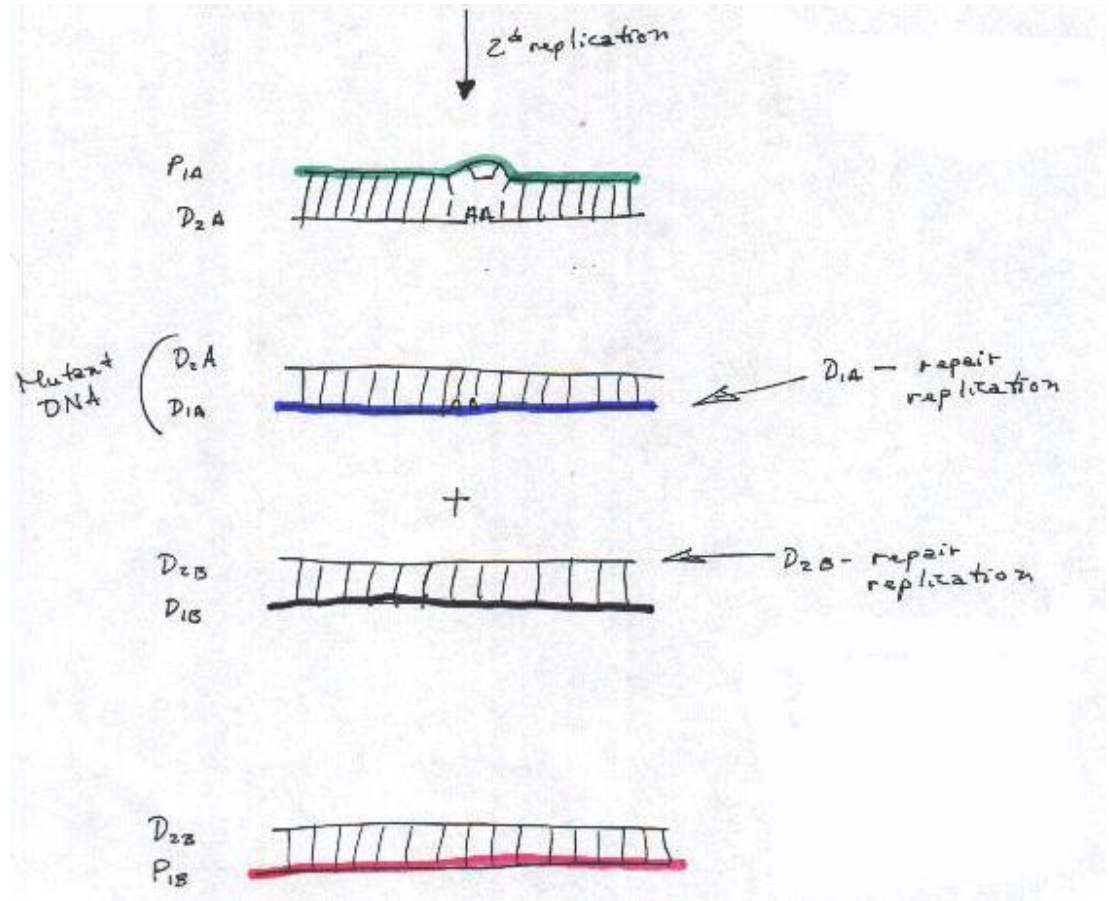


The Third Mechanism is Called SOS Repair



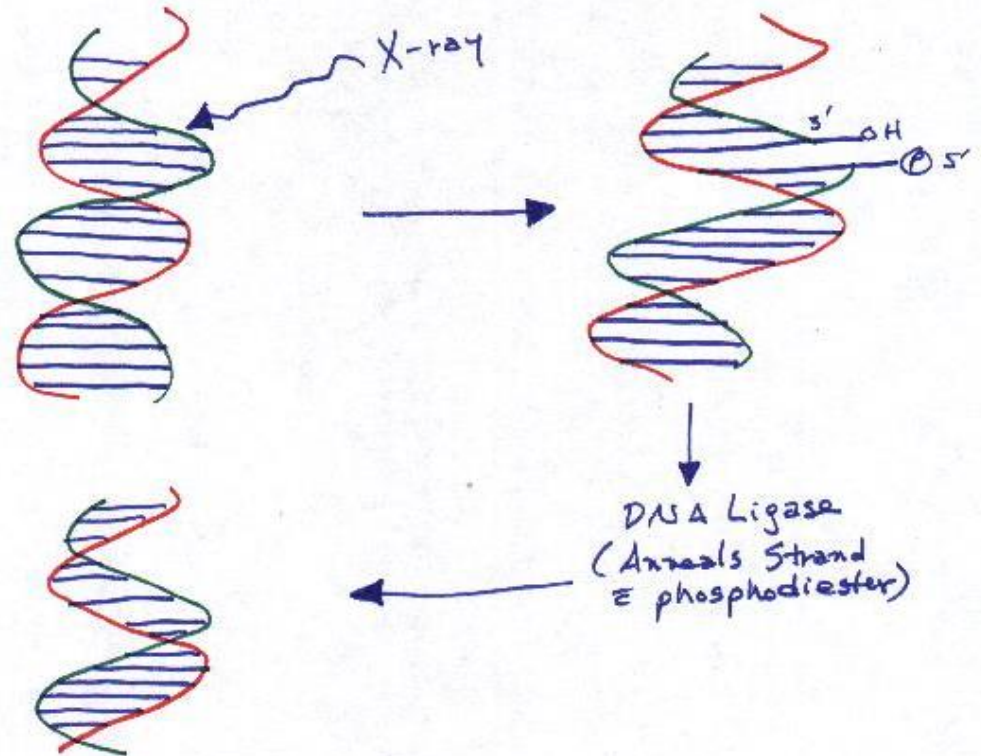
- This is the system that, when induced, wreaks havoc with DNA.
- The way in which this mechanism works is as follows: a dimer is induced in one strand of the DNA.
- DNA Pol and dNTP's (generally dATP's) induce DNA replication.
- The product of this first replication is two double strands of DNA: 1 with the dimer still there and 1 that is, more or less, repaired.
- This replication is said to be "trans-dimer" as the process read right over (across) the dimer and permitted its semiconservative replication and repair replication.
- Note also that the first generation of daughter cells is half parent (P) and half daughter (D) DNA.

- The second replication/generation is when and where the problems really fire up: now these four strands of DNA from the first replication will ALSO be semi-conservatively replicated.
- Of the four new sets of DS DNA, 1 has the dimer still intact and one has no error -- both of these contain half-original parent DNA and half brand-spanking, new DNA.
- The cell will eventually discard these.
- The other two contain half of their DNA from the first replication and the other half from the second replication.
- These two DS DNA are the product of repair replication.
- One has no errors; the other (designated D2A, D1A in the figure) is the mutant DNA.
- The SOS system has to crank up its protein synthetic machinery to effect its "repair".
- It is also the MAJOR cause of UV-induced mutagenesis.



The Last Repair Mechanism to be Examined is The Effect of Ionizing Radiation (e.g., x-ray, gamma ray) on DNA

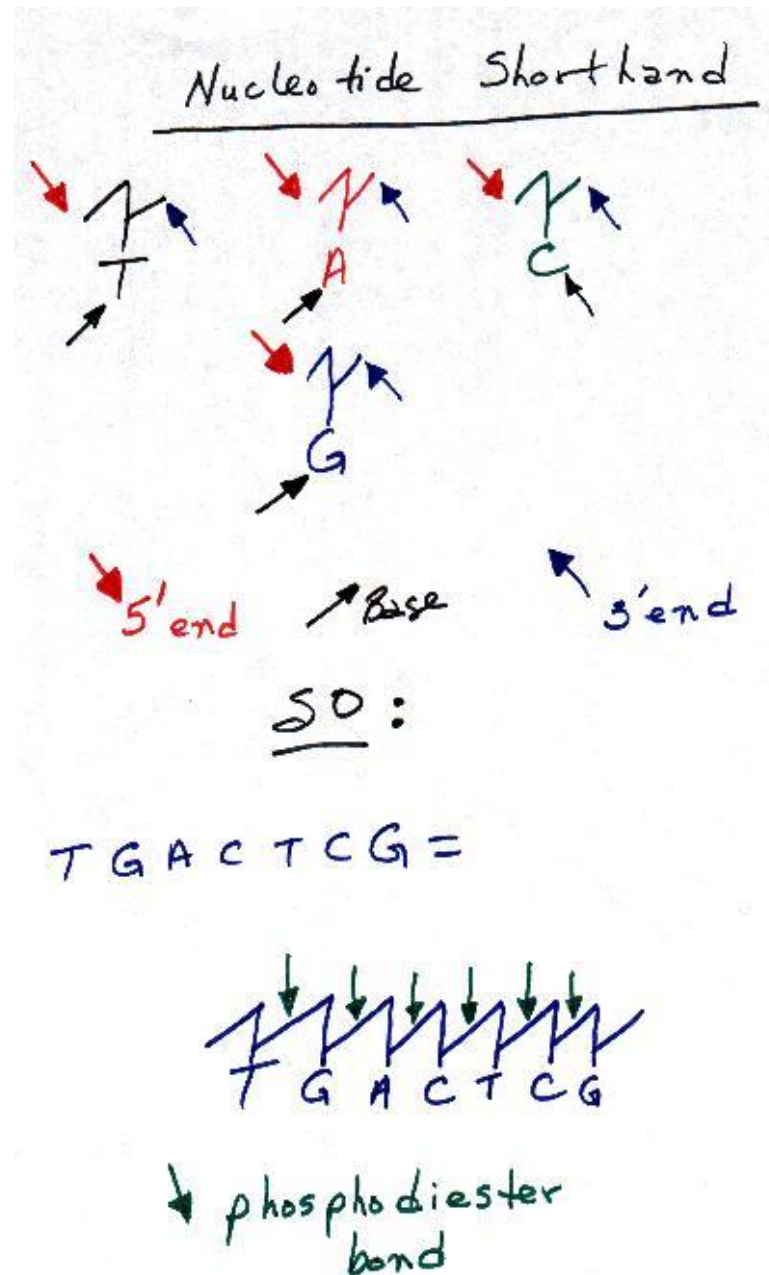
- When ionizing radiation interacts with DNA, it causes a "nick" in the DNA.
- DNA ligase anneals the "nick" with a phosphodiester bond at the expense of ATP to repair the DNA.



Techniques for Isolating, Identifying and Sequencing Nucleic Acids

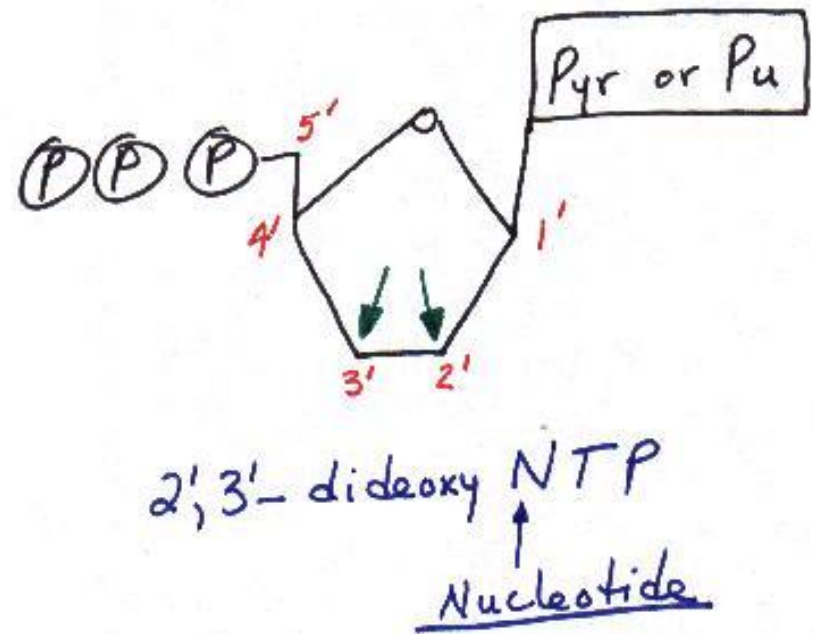
Emphasis on DNA

- Several techniques have been developed to determine the sequence of DNA: the Maxam-Gilbert and Sanger methods. In order to follow how the techniques work, it is necessary to understand a little nucleotide shorthand, image at right:
- Note: Maxam-Gilbert will not be discussed as the Sanger method has been digitized due to its more simple and rapid approach in determining DNA sequences.



Sanger Method

- It is also known as the chain termination, controlled interruption of enzymatic replication and dideoxy methods of DNA sequencing.
- The key to this method is the utilization of a 2',3'-dideoxy compound, Figure, right, green arrows.



- In this compound, besides having no 2' -OH, there is also no 3' -OH.
- Without this -OH, it is impossible to make 3',5'-phosphodiester bonds and the DNA chain will not continue to elongate.

- In order to follow this method, let's consider the sequence:

3'-CTAACCCGGTTACAA-5'

- In addition, this method requires a primer that typically comes from a restriction digest. The primer for our sequence is GATTG, 5' to 3'.
- Note that the primer is complimentary to the first 5 nucleotides in the sequence we want to study

- The DNA fragment for sequencing is reacted with the complementary primer, DNA Pol, radioactively labeled dNTP's (dNTP*) and ddGTP (dideoxy-GTP*;
represented by the backwards G in the figure)

3'-CTAACCCGGTTACCAA-5'
5'-GATTG -- PRIMER!!!!

↓ DNA Pol
dNTP's
ddGTP (⌂)

5'-GATTG⌂

+

5'-GATTGG⌂

+

5'-GATTGGGCCAAT⌂

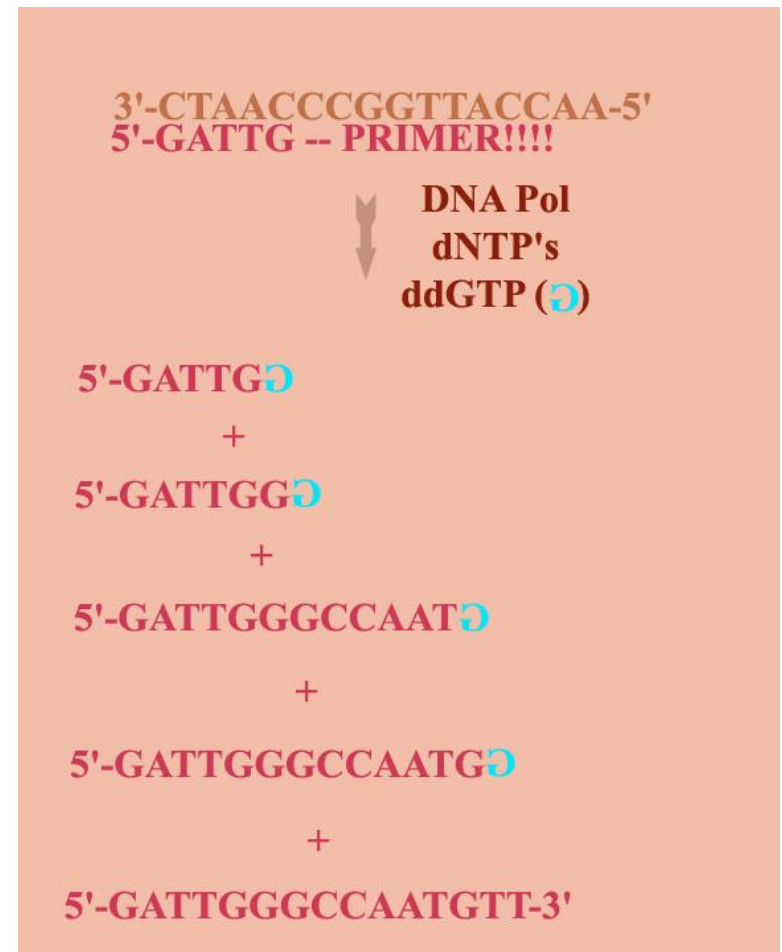
+

5'-GATTGGGCCAATG⌂

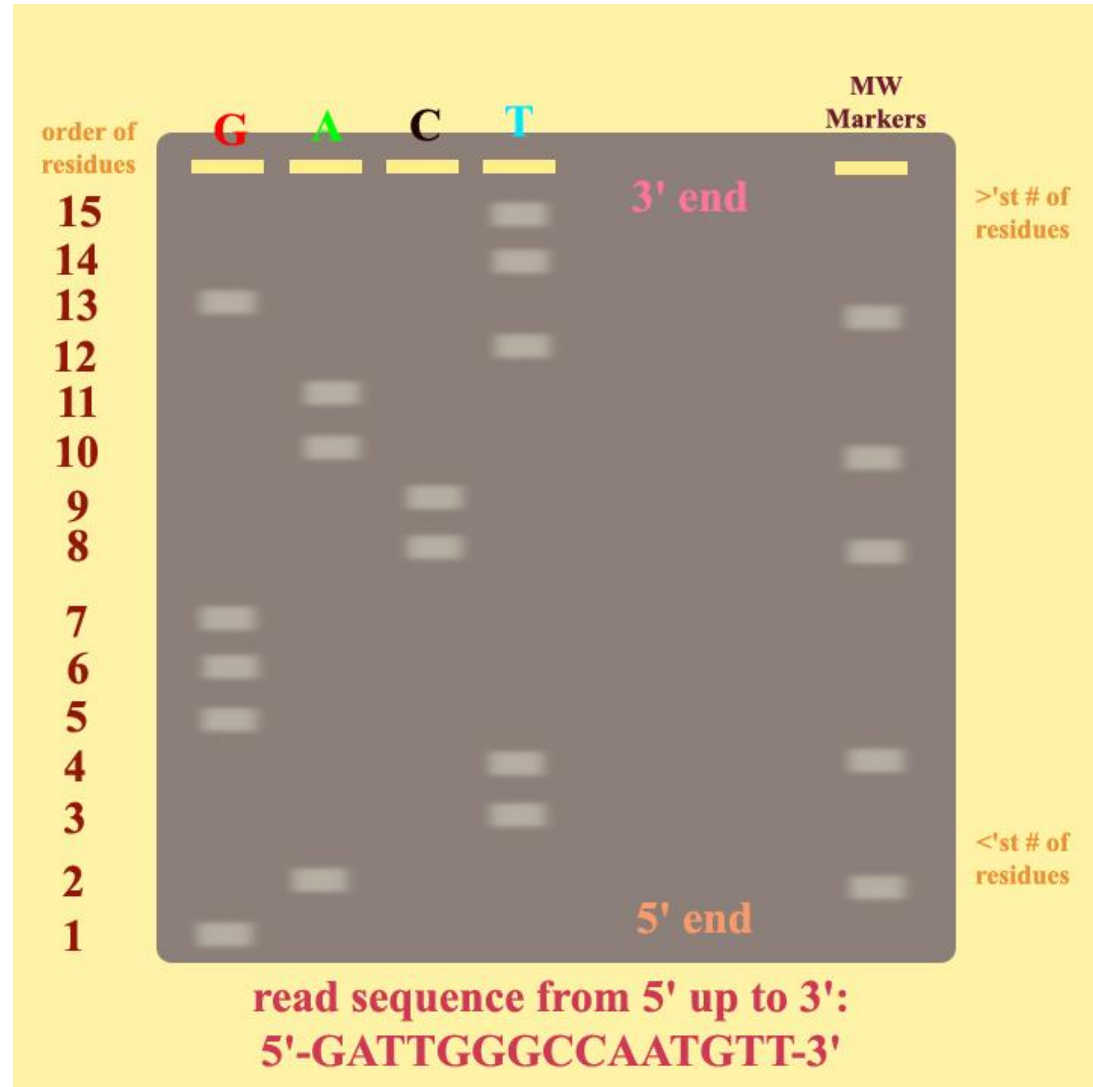
+

5'-GATTGGGCCAATGTT-3'

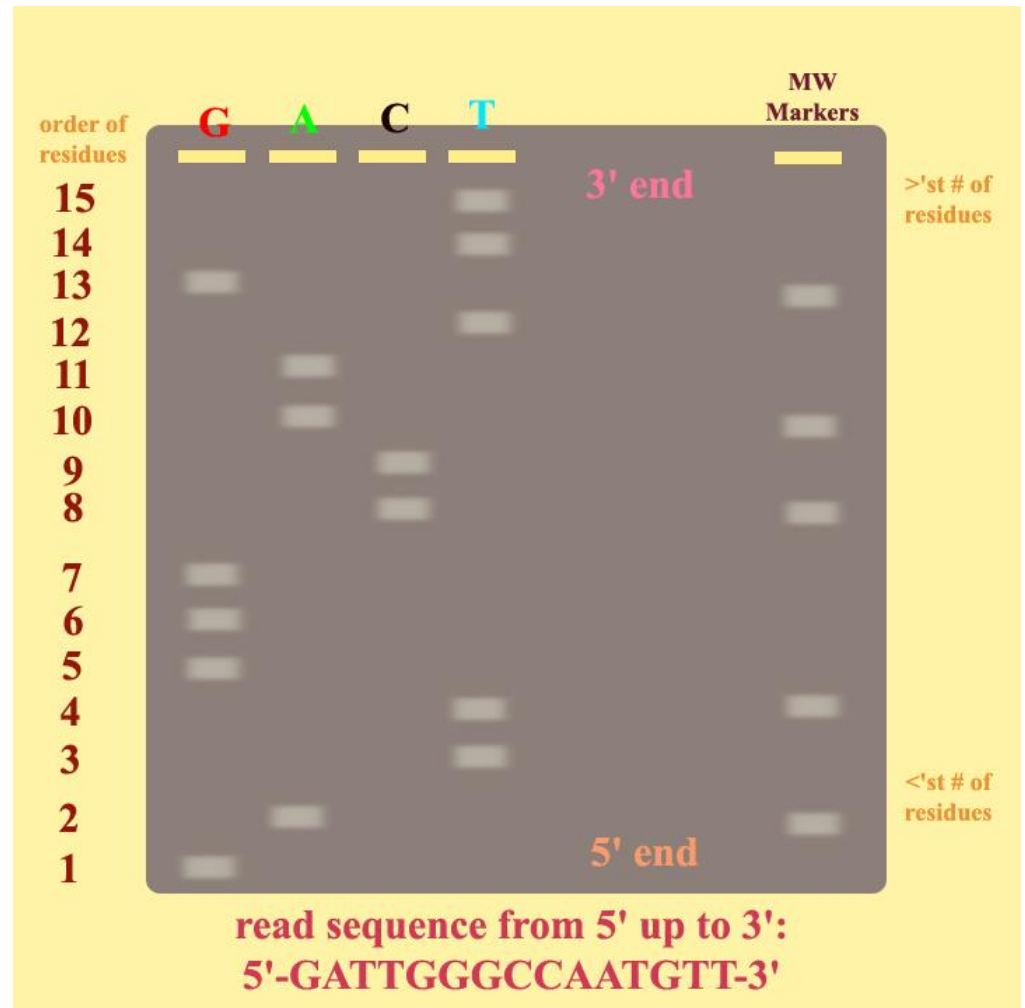
- Note that the sequence being synthesized is complimentary to the original sample.
- Whenever the newly synthesizing sequence is determined to need a "G" added to its sequence, either dGTP* or ddGTP will add on.
- If ddGTP adds on, that fragment will no longer elongate, i.e., the chain terminates -- the last residue of that fragment is a ddGTP.
- This cycle continues as desired, *ad nauseum*.



- Each ddNTP will be utilized in the Sanger method so that, essentially, 4 samples will be obtained, all of which are dideoxy-derivatives of G, A, C, T.
- These samples will be electrophoresed in the much the same manner as with the Maxam-Gilbert method



- In the Sanger method, the first residue is the first nucleotide from the original nucleotide's newly synthesized complimentary DNA after the primer.



- The sequence is read directly from the autoradiograph and the original sequence deduced from its complimentary strand sequence.
- Although both methods are used, the Sanger method has been automated and is much faster and useful in DNA sequencing in the lab.

Sanger Sequence

5'-GATTGGGCCAATGTT-3'

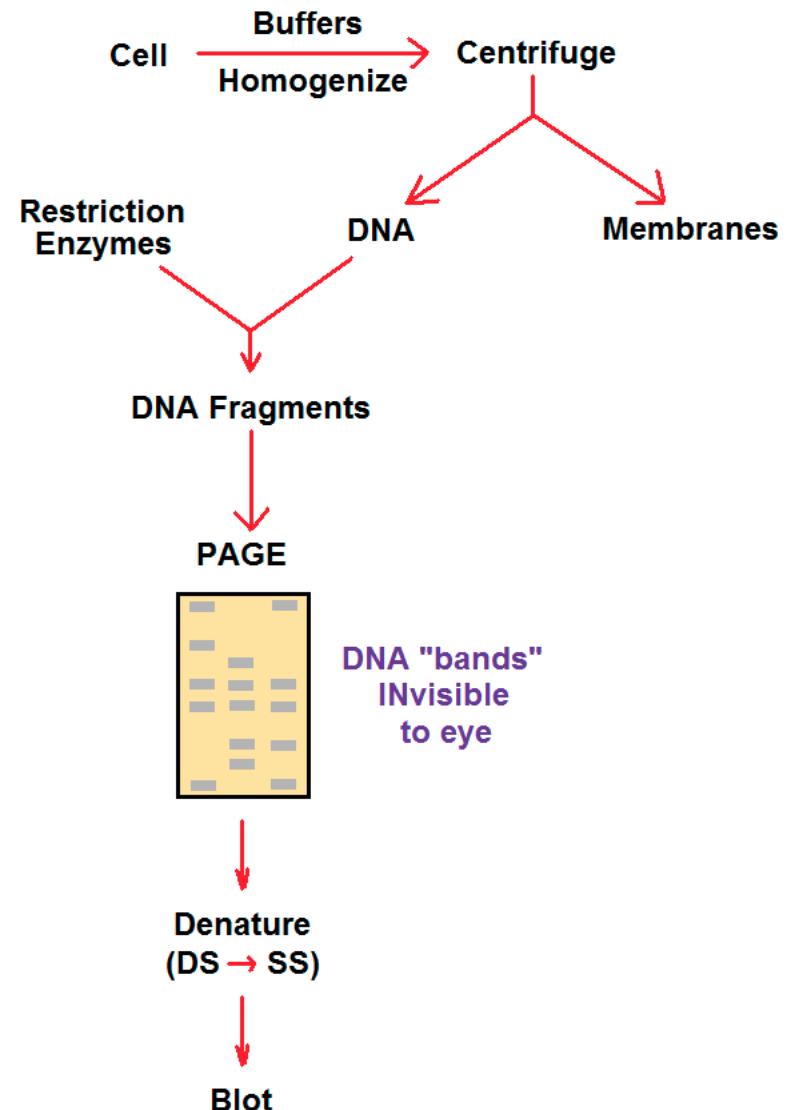
3'-CTAACCCGGTTACAA-5'

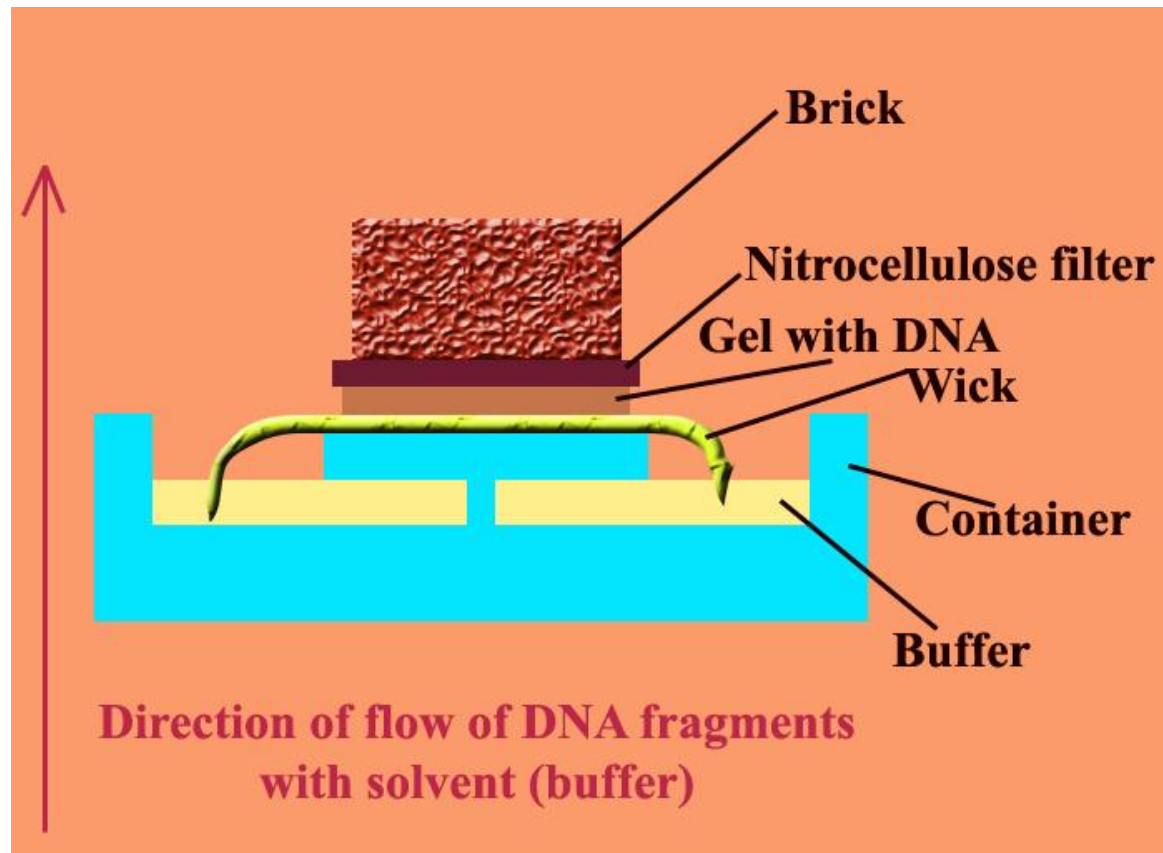
**Original Strand
complimentary to Sanger's strand**

- With several questions now answered about DNA and its structure, there is still a burning question regarding techniques of identifying either specific DNA regions or individuals by their DNA sequences.
- About 25% of the human genome is in many different alleles (identical genes with different sequences). These are called polymorphisms.
- Each is an inherited pattern and segregate according to Mendel's rules (coming up in the future). These **restriction fragment length polymorphisms (RFLP's)** may be used to identify diseases, criminal acts or the release of suspects for lack of correct evidence, *ad nauseum*. Only identical twins have identical RFLP's, but NOT identical fingerprints or retinal scans.
- **VNTR's (variable numbers of tandemly repeated units)** comprise very unique RFLP's. They serve as "molecular fingerprints" of an individual. Both of these fragments may be studied by Southern blot and PCR.

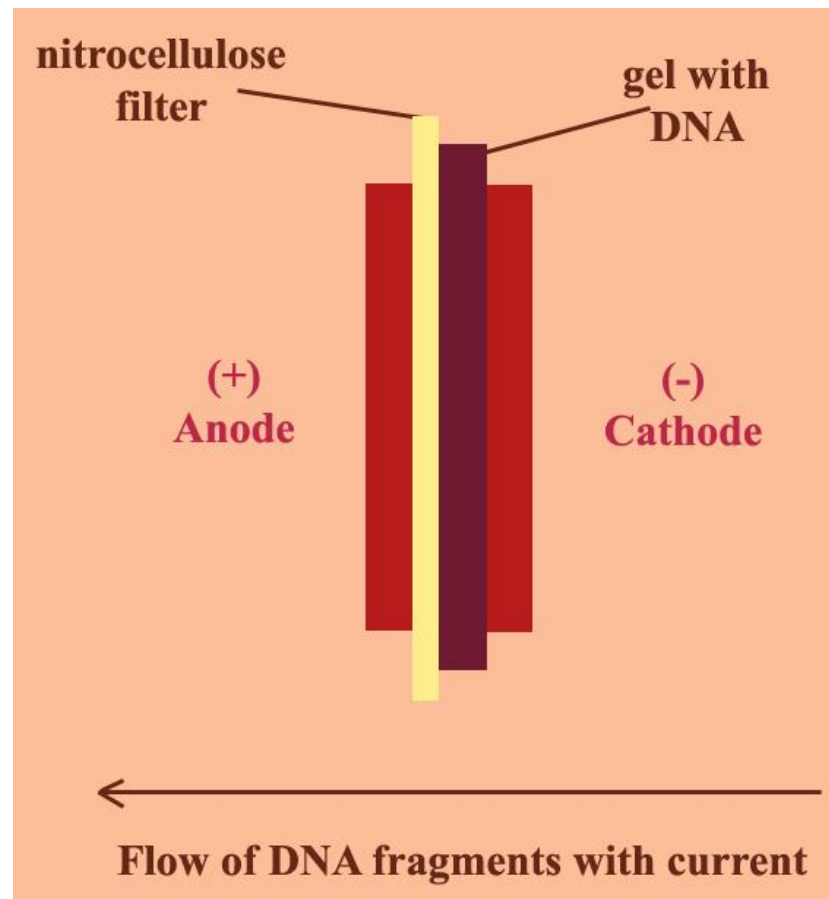
Southern Blot

- Cells are mixed with appropriate buffers and homogenized. The mixture is centrifuged to give various cell fractions, which include DNA.
- The DNA is digested by restriction enzymes to fragments and submitted to PAGE.
- There are NO fragments visible to the human eye in this gel -- yet. The gel is treated in such a manner that the DS DNA is denatured to SS DNA and then the gel is blotted onto a nitrocellulose filter.





- Buffer is poured into a "pan" with a stand in its center. A wick (some just use paper towels) is laid over the stand, upon which the gel is placed. A nitrocellulose filter is placed on top of the gel and a brick (or some heavy weight) is placed on top of the filter. The solvent (buffer) flows up the wick and under the pressure of the brick, causes the fragments to be transferred to the nitrocellulose filter.



- The second way in which material is transferred from the PAGE gel to a nitrocellulose filter: by electrophoresis. The gel with the SS DNA is placed between two electrodes with a nitrocellulose filter placed on the positively charged electrode (anode) side. Electrophoresis is run as described earlier. The DNA (negatively charged) is attracted to the anode and the SS DNA is transferred to the nitrocellulose filter.

- There are **STILL** no bands visible on the nitrocellulose filter, yet, Figure, right.
- A radioactively (^{32}P) labeled "probe", a specific sequence of DNA complimentary to what you are looking for (cDNA -- complimentary DNA) is added to the nitrocellulose filter and incubated. Once incubation is complete, the irradiated filter is incubated with a piece of film and, then, developed. The only bands, which show up on the autoradiograph, are those bands that are complimentary to the probe and light up on the film. Those fragments may be identified on the nitrocellulose filters by matching it up with the film; the fragments may then be studied as desired.



**DNA "bands"
INvisible
to eye
on Nitrocellulose
Filter**



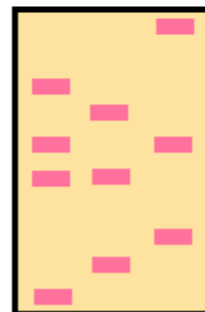
***probe
(radioactive)
(cDNA)**



**STILL
Invisible**



**Incubate
with film
and develop**

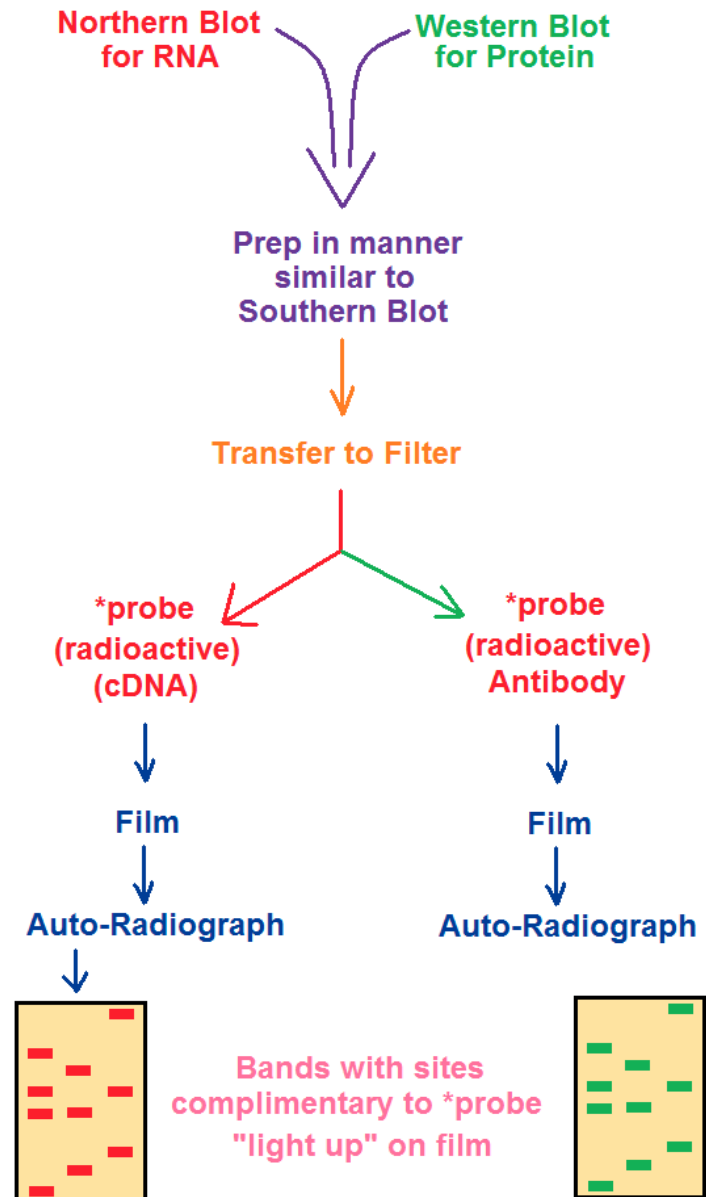


**Bands with sites
complimentary to *probe
"light up" on film**

Northern and Western Blots

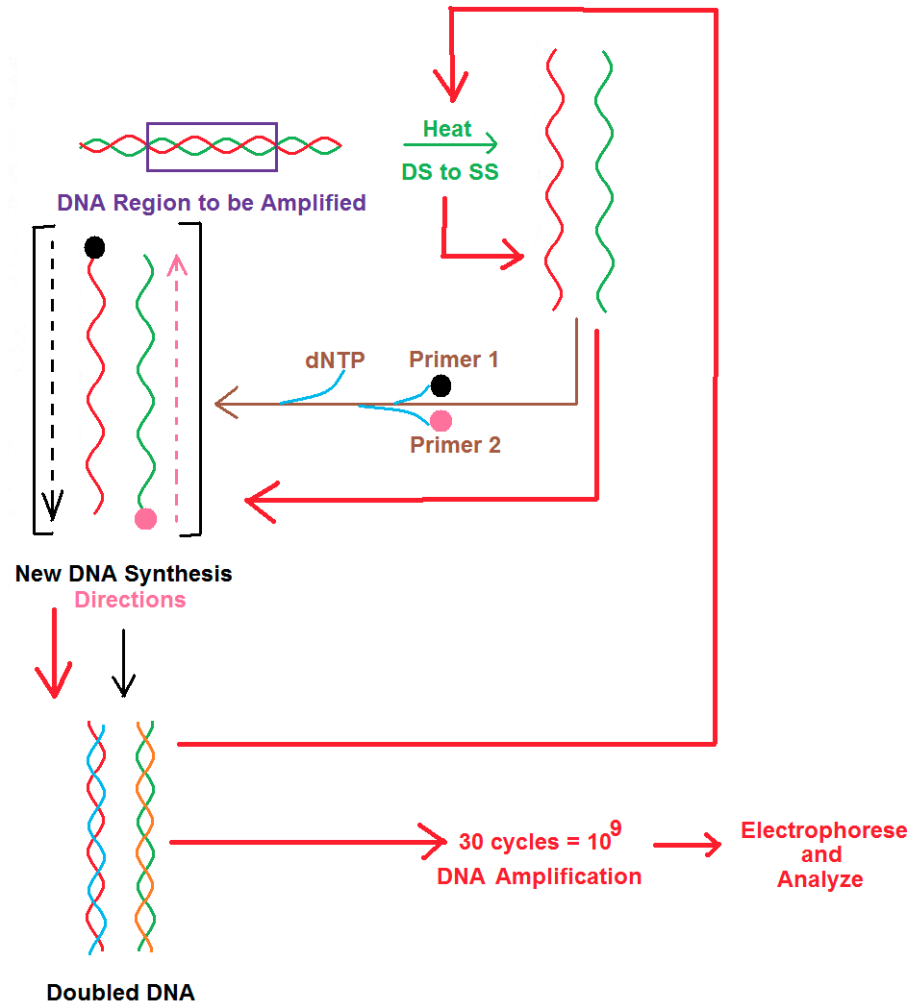
- Two other blots are of significance, as well:
 - the Northern and
 - Western blots.
- While the techniques to do these blots are similar, they examine DIFFERENT macromolecules.

- The Northern blot (named for geography rather than someone's name) examines RNA fragments.
- The probe for the Northern blot is radioactively labeled cDNA.
- The Western blot (more warped geneticist's humor) **looks for proteins**, Figure, right.
- The probe for the Western blot is radioactively labeled antibodies (more on this in the future).
- The Western blot has gained notoriety as it is the confirming test to positively diagnose people who are infected with HIV.



Polymerase Chain Reaction

- What happens, though, if there isn't enough DNA in a sample to be studied adequately? A technique called the PCR (polymerase chain reaction) was developed just for this purpose.
- In brief, a fragment of DS DNA is heated to denature it.
- To the two strands of SS DNA, two primers are added, as well as DNA Pol and dNTP's.
- As this chain reaction gets going, after 20 cycles, this small amount of, previously unstudyable, DNA is amplified one million fold! After 30 cycles, this DNA is amplified a billion-fold!



- This technique has proven useful in numerous trials.
- The PCR is also used
 - 1) to detect viruses of a "sneaky" nature (e.g., HIV),
 - 2) prenatally, e.g., to identify genetic defects,
 - 3) to detect polymorphisms,
 - 4) in tissue typing to reduce tissue rejections,
 - 5) in old DNA samples, e.g., bacteria from pyramids in amber to track evolutionary changes and
 - 6) in forensics, e.g., to identify perpetrators and separate them from innocent suspects.

CRISPR

- CRISPR is an abbreviation of **C**lustered **R**egularly **I**nterspaced **S**hort **P**alindromic **R**epeats.
 - Small clusters of *cas* (CRISPR-associated) genes are located next to CRISPR sequences in *S. pyogenes*.
- A simple version of the CRISPR/Cas system, CRISPR/Cas9, has been modified to edit genomes. By delivering the Cas9 nuclease complexed with a synthetic guide RNA (gRNA) into a cell, the cell's genome can be cut at a desired location, allowing existing genes to be removed and/or new ones added.
- CRISPR/Cas genome editing techniques have many potential applications, including medicine and crop seed enhancement.
- Recently, issues with CRISPR's accuracy have arisen.
- CRISPR technology is a simple yet powerful tool for editing genomes.
 - It allows researchers to easily alter DNA sequences and modify gene function.
 - Its many potential applications include correcting genetic defects, treating and preventing the spread of diseases and improving crops.
 - However, its promise also raises ethical concerns.