# CHAPTER 13

# DNA REPLICATION AND REPAIR

## OBJECTIVES

- Outline the possible models for replication.
- Describe the experiments that proved that replication is semiconservative.
- Describe the process of bacterial replication, the enzymes involved and its major features.
- Emphasize the bidirectional and semidiscontinuous nature of replication and the reasons for it.
- Outline the properties of DNA polymerases and how they dictate certain aspects of replication.
- Describe the use of RNA primers in replication and how they are removed from DNA before replication is completed.
- Describe the exonuclease activities of DNA polymerases and their role in "proofreading".
- Describe eukaryotic replication, pointing out its similarities to and differences from the prokaryotic process.
- Emphasize the importance of multiple initiation sites to replication in eukaryotes.
- Outline the different types of DNA damage caused by various environmental conditions.
- Outline what is known about the different types of DNA repair mechanisms.

## LECTURE OUTLINE

### DNA Replication: Background Information and Overview

I. Reproduction is a fundamental property of all living systems & can be observed at several levels
   A. Organisms duplicate by asexual or sexual reproduction
   B. Cells duplicate by cellular division
   C. Genetic material duplicates by DNA replication – replication machinery is also used to repair the genetic material after it has sustained damage

II. Capacity for self-duplication is presumed to have been one of first critical properties to have appeared in the evolution of the first primitive life forms
   A. Without propagation, any primitive assemblage of biological molecules would be destined for oblivion
   B. First carriers of genetic information were probably RNA molecules that could self-replicate
   C. Later RNA was replaced by DNA as the primary storehouse of genetic information
      1. Replication then became more complex & required a large number of auxiliary components
      2. DNA contains information for its own duplication, but cannot perform the activity itself like RNA

III. Watson & Crick (1953) - proposed DNA structure & suggested how it might "self-duplicate"
   A. Suggested that replication occurred by gradual double helix strand separation via successive breakage of H bonds, much like the separation of the two halves of a zipper

B. Since each strand is complementary to the other, each has the information needed to construct the other; once separated, each strand can serve as template to direct the formation of the other strand

IV. Semiconservative nature of replication - Watson & Crick predicted that new DNA should consist of one old strand (from parental duplex) & one newly synthesized chain
   A. Possible types of replication
      1. Semiconservative - daughter duplex made of one parental & one newly synthesized strand
      2. Conservative - 2 original strands stay together after serving as templates for 2 new strands that also stay together; one contains only "old" DNA, the other only "new" DNA
      3. Dispersive – integrity of both parental strands disrupted; new duplex strands made of old & new DNA; neither the parental strands nor the parental duplex is preserved
   B. Of the suggested mechanisms, the third is the most unlikely, but it is the only one that avoided the seemingly impossible task of unwinding the 2 intertwined DNA duplex strands during replication
   C. Matthew Meselson & Franklin Stahl (1957, Caltech) - grew bacteria in media with $^{15}NH_4Cl$ as sole nitrogen source for many generations; DNA bases contain "heavy" nitrogen
      1. Wash out $^{15}NH_4Cl$; put bacteria in $^{14}NH_4Cl$ ("light"); remove samples at intervals over several generations; use $^{14}N$ & $^{15}N$ to distinguish between newly synthesized & parental strands, respectively
      2. Extract DNA & subject it to CsCl equilibrium density-gradient centrifugation to find buoyant density
      3. Mix DNA with concentrated CsCl solution, centrifuge until double-stranded DNAs reach equilibrium according to their density; density of DNA is directly related to percentage of $^{14}N$ or $^{15}N$ it contains
      4. Density of DNA decreases until one generation time when it is halfway between the density of totally heavy & totally light DNA; it is a hybrid — half new & half old
      5. After 2 generation times, half of the DNA is totally light & half is hybrid (half light, half heavy)
      6. While semiconservative replication continues, original heavy parental strands remain intact & present in hybrid DNA molecules, but they occupy a smaller & smaller percentage of total DNA
      7. With time, the vast majority of DNA present is fully light with 2 light strands
   D. Eukaryotes also semiconservative - cultured mammalian cells were allowed to undergo replication in presence of bromodeoxyuridine (BrdU), which replaces thymidine (a T analog) in DNA
      1. After replication, a chromosome is composed of 2 chromatids; after one round of replication in BrdU, one chromatid of each chromosome contained BrdU; the other contained only thymidine
      2. After 2 replication rounds in BrdU, 50% of the chromosomes are hybrids with one BrdU chromatid and one T-containing chromatid; the other 50% have both chromatids containing BrdU

## Replication in Bacterial Cells: The Overall Process

I. Genetic & biochemical approaches have revealed at least 30 proteins needed for *E. coli* replication; the following approaches have driven progress in understanding prokaryotic replication (eukaryotes trickier):

A. Availability of mutants unable to synthesize one or another protein required for the replication process – how can such cells be cultured?; shouldn't they die if they cannot replicate?

1. Temperature-sensitive (ts) mutants - deficiency seen only at higher (nonpermissive or restrictive temperatures); at low (permissive) temperature, mutant protein holds together well enough to work
2. With ts mutant, the cells can continue to grow & divide at permissive temperature
3. ts mutants have been isolated that affect virtually every type of physiological activity & have been very important in studies of DNA synthesis in replication, DNA repair & genetic recombination

B. Development of *in vitro* systems in which replication can be studied using purified cellular components

1. Replicate DNA with extracts from which specific proteins thought to be essential were removed **or**
2. Incubate DNA with a variety of purified proteins whose activity is to be tested

C. Replication in prokaryotes & eukaryotes occurs by very similar mechanisms so most information presented on bacterial replication applies to eukaryotes

II. Replication forks & bidirectional replication

A. Replication begins at a specific site on bacterial chromosome (**origin**)

1. It starts at specific sequence (called *oriC* in *E. coli*; ~245 bp sequence) on bacterial chromosome
2. Many proteins bind at *oriC* to initiate replication; bacterial replication origin analogous to promoter for transcription; both bind sequence-specific, DNA-binding proteins to start process at specific site
3. Replication moves out from origin in both (opposite) directions (**bidirectional**)

B. **Replication forks** are sites where the pair of replicated segments come together & join the nonreplicated segments; each replication fork corresponds to a site where the:

1. Parental double helix is undergoing strand separation, and
2. Nucleotides are being incorporated into the newly synthesized complementary strands

C. The 2 replication forks move in opposite directions & meet at point across the circle from origin, where replication terminates —> newly replicated duplexes detach from one another & go to different cells

III. Unwinding the duplex & separating the strands – separation of the strands of a circular, helical DNA duplex or a giant, linear eukaryotic chromosome poses major topological problems

A. Analogy: take two-stranded helical rope & place linear piece of it on the ground

1. Grab both strands at one end & begin pulling them apart (just like replicating DNA does)
2. Strand separation of a double helix also involves the process of unwinding the structure
3. A rope is free to rotate around its axis so separation of strands at one end is accompanied by rotation of the entire fiber as it resists the development of tension

B. What happens if rope is attached to hook on wall? – strand separation at free end generates increasing torsional stress in rope; unseparated portion is wound more tightly

1. Separation of 2 strands of circular DNA or linear DNA that is not free to rotate (like eukaryotic chromosome) is analogous to attaching one end of linear molecule to a wall
2. In all of the above cases, tension cannot be relieved by rotation of the entire molecule

3. Unlike a rope, which can become tightly overwound, an overwound DNA molecule becomes positively supercoiled
4. Thus, replication fork movement generates positive supercoils in unreplicated portion of DNA ahead of fork
5. This is a big problem, since the complete circular *E. coli* chromosome has ~400,000 turns & is replicated by 2 forks within ~40 min

C. Cells contain enzymes (**topoisomerases**) that can change state of DNA supercoiling - one is DNA gyrase (a type II topoisomerase); it relieves mechanical strain that builds up during replication in *E. coli*

1. DNA gyrase travels along DNA ahead of fork removing positive supercoils & changing positively supercoiled DNA into negatively supercoiled DNA
2. It cleaves both DNA duplex strands, passing a segment of DNA through the double-stranded break to the other side & then seals the cuts; process is driven by energy released during ATP hydrolysis
3. Eukaryotic cells possess similar enzymes that carry out this required function

IV. DNA polymerase properties - synthesizes new DNA strands; first studied by Arthur Kornberg et al. (1950s; Washington U.); purified from bacterial extracts that could incorporate DNA precursors into polymer

A. DNA polymerase I (first found; received the number in its name when other DNA polymerases were discovered) - incorporated hot (radioactive) DNA precursors into acid-insoluble DNA polymer

1. Required presence of DNA & all 4 deoxyribonucleoside triphosphates (dTTP, dATP, dCTP, dGTP)
2. New hot DNA had same base composition as original unlabeled DNA (it was template for new DNA)

B. DNA polymerase catalyzes a reaction similar to that of RNA polymerase

1. Both polymerases move along template DNA strand & incorporate a complementary nucleotide onto the end of strand being assembled (reaction is essentially the same as RNA synthesis)
2. Differences in the 2 reactions – precursors are deoxyribonucleotide triphosphates in DNA synthesis instead of ribonucleotide triphosphates; also DNA polymerase requires primer

C. Template DNA had to meet certain structural requirements to promote labeled precursor incorporation

1. Intact, linear, double-stranded DNA did not stimulate incorporation – not surprising since strands of helix had to be separated for replication to occur
2. Single-stranded, circular DNAs also cannot serve as template less obvious why it did not work; in fact, one might expect it to be an ideal template
3. In contrast, partially double-stranded DNA works well & yields immediate nucleotide incorporation

D. It was soon discovered that a single-stranded DNA circle does not serve as DNA polymerase template because enzyme cannot initiate DNA strand formation

1. Polymerase can only add nucleotides to 3'-OH end of an existing strand; this strand called **primer**
2. All prokaryotic & eukaryotic DNA polymerases have these same 2 basic requirements: a primer strand to which nucleotides can be added & a template DNA strand to copy

E. Requirements explain why certain structures mentioned above failed to promote DNA synthesis

1. Intact, linear double helix has 3'-OH terminus, but lacks a template
2. Single-stranded, circular DNA has template strand, but lacks a primer

3. Partially double-stranded DNA satisfies both requirements & thus promotes incorporation

F. DNA polymerase findings raised questions due to their shared properties (eukaryotic & prokaryotic), including the finding that the DNA polymerase only polymerizes DNA in 5' to 3' (5'—>3') direction
   1. Watson & Crick originally predicted that one strand at replication fork would be made in 5'—>3' direction, while the other was made in 3'—>5' direction — how does 5'—>3' polymerase do this?
   2. If it cannot initiate strands on its own, how does DNA polymerase initiate synthesis of a new strand in the cell?

G. Soon new DNA polymerases (II & III) were discovered in mutants – a mutant *E. coli* had <1% of normal activity of DNA polymerase I (Kornberg enzyme), but multiplied at normal rate (1969)
   1. Typical bacterium has 300 - 400 copies of polymerase I, ~40 copies of II & 10 copies of III
   2. Much greater amounts of DNA polymerase I had masked presence of polymerases II & III
   3. Still did not answer the 2 basic questions – II & III also need primer & only work in 5'—>3' direction

V. Semidiscontinuous replication - all DNA polymerases lay down nucleotides in 5'—>3' direction & move along template in 3'—>5' direction; both strands at fork synthesized in that direction

A. During polymerization, the 3'-OH at end of primer carries out nucleophilic attack on 5'-α-phosphate of incoming nucleotide triphosphate
   1. Polymerases building both strands move in 3' to 5' direction along the template; both make a chain that grows from its 5'-phosphate terminus
   2. One DNA strand grows toward replication fork; the other away from the fork; both grow 5'—>3'

B. The 2 newly synthesized strands are synthesized by very different processes
   1. Strand growing toward fork grows continuously in 5'—>3' direction (**leading strand**) as the replication fork advances
   2. Strand growing away from fork grows discontinuously as fragments (**lagging strand**); initiation of each fragment must wait for parental strands to separate & expose more template; fork must move
   3. The lagging strand fragment grows away from the replication fork toward the 5' end of the previously synthesized fragment to which it is subsequently linked
   4. Both strands are probably made at same time so the leading & lagging terms may not be as appropriate as was thought when they were first coined
   5. Since one strand is synthesized continuously & the other discontinuously, replication is said to be **semidiscontinuous**

C. Reiji Okazaki (Nagoya Univ.; Japan) - discovered that one strand is synthesized as small fragments
   1. Incubated bacteria for a few seconds in [$^3$H]-thymidine & immediately killed them
   2. Radiolabel found as part of small DNA fragments (1000 - 2000 nucleotides long)
   3. If cells were incubated in labeled DNA precursor for 1 – 2 minutes, most of the incorporated radioactivity became part of much larger DNA molecules
   4. Some DNA was constructed in small segments (later called **Okazaki fragments**) that were rapidly linked to longer pieces of DNA synthesized earlier

5. The enzyme that joins Okazaki fragments into a continuous strand is called **DNA ligase**

D. Problem - fragments could not be initiated by DNA polymerase because there was no free 3'-OH

1. It was found that strand initiation is done by an enzyme that makes a short RNA primer, a distinct type of RNA polymerase, called **primase,** that constructs a short primer made of RNA, not DNA
2. Leading strand synthesis is initiated at the replication origin by a primase molecule
3. Short RNAs made by primase at 5' end of leading strand & the 5' end of each Okazaki fragment serve as the required primer for synthesis of DNA by a DNA polymerase
4. The RNA primers are subsequently removed & the resulting gap in the strand is filled with DNA & then sealed by DNA ligase
5. The process may decrease mistakes, since mistakes are more likely during initiation than during elongation; the use of a short, removable RNA segment avoids the inclusion of mismatched bases

## ~~Replication in Bacterial Cells: The Machinery Operating at the Replication Fork~~

~~I. DNA gyrase converts positively supercoiled DNA developing ahead of the replication fork into negatively supercoiled DNA that is under mechanical stress & already primed to unwind & separate~~

~~A. Even with the motive force behind it, duplex unwinding & strand separation requires the aid of 2 types of proteins that bind to DNA, a helicase & single-stranded DNA binding (SSB) proteins~~

~~II. DNA helicase (DNA unwinding enzyme) - unwinds DNA in reaction using energy from ATP hydrolysis to break H bonds holding 2 strands together, thus exposing the single-stranded DNA template~~

~~A. *E. coli* has at least 12 different helicases used in various aspects of DNA (& RNA) metabolism~~

~~B. DnaB helicase, the product of the *dnaB* gene, is the major unwinding machine during replication; it consists of 6 subunits arranged to form a ring-shaped protein that encircles a single DNA strand~~

~~1. DNA helicase operation - first loaded onto DNA at replication origin with the help of the protein DnaC & moves in 5' -> 3' direction along lagging-strand template, unwinding helix as it proceeds~~

~~2. DNA unwinding by helicase is aided by attachment of SSB proteins to separated DNA strands~~

~~3. SSBs bind selectively to single-stranded DNA - keep it extended & prevent it from being rewound~~

~~III. Primase enzyme initiates synthesis of each Okazaki fragment - helicase closely associates transiently with primase in bacteria forming **primosome**~~

~~A. Helicase moves along the lagging strand template processively (without being released from the template strand during the lifetime of the replication fork)~~

~~B. As helicase moves ("motors") along lagging-strand template opening duplex strands, primase periodically binds to helicase & synthesizes short RNA primers that begin formation of Okazaki fragment~~

~~1. Primers are subsequently extended as DNA by a DNA polymerase~~

C. Does the same DNA polymerase III synthesize successive Okazaki fragments of lagging strand or are new polymerases recruited to do it? – given that Okazaki fragments **are** made very rapidly

1. Approached by replicating DNA *in vitro* and suddenly diluting the reaction mixture
2. Reaction does not slow so DNA polymerase must stay attached through direct or indirect interactions with other proteins as it synthesizes successive Okazaki fragments
3. If recruitment of new polymerases were needed, reaction should have slowed greatly & it did not
4. Results support hypothesis that a single DNA polymerase III molecule is recycled, finishing Okazaki fragment & then moving to next site along lagging strand template (closer to replication fork)
5. At new site, the polymerase attaches to 3' OH of RNA primer just laid down by primosome & adds new DNA nucleotides onto the end of the short RNA

D. How does DNA polymerase III move from one site on the template to a site closer to replication fork?

1. It hitches a ride with the DNA polymerase that is moving that way along the leading strand template
2. The 2 polymerases are part of a single protein complex even though they move in opposite directions with respect to each of their templates
3. Replication of both strands by the 2 tethered polymerases can be done by having the DNA of the lagging strand looped back on itself so it has same orientation as the leading strand template
4. Both polymerases can then move together as part of a single replicative complex (**replisome**) without violating the 5' –>3' directionality rule for synthesis of a DNA strand
5. Once the polymerase assembling the lagging strand reaches the 5' end of the Okazaki fragment made during the previous round, the lagging-strand template is released
6. The polymerase then begins work at the 3' end of the next RNA primer toward the fork
7. This model is often referred to as the "trombone model", since the looping DNA repeatedly grows & shortens during lagging strand replication; reminiscent of brass loop of trombone being played

## The Structure and Functions of DNA Polymerases

I. All DNA polymerases in both prokaryotes & eukaryotes have basically same activity, so why do they need >1 polymerase? - their roles in cell differ; functions determined based mostly on studies of mutant strains

A. DNA polymerase I - mostly involved in DNA repair to correct damaged DNA sections; consists of a single subunit; it removes RNA primers at 5' Okazaki fragment end & replaces the RNA with DNA

B. DNA polymerase II – as yet uncertain; bacterial mutants have been isolated & have no evident deficiency

C. DNA polymerase III (replicase) - acts in DNA strand formation during replication in *E. coli*; part of a large complex called DNA polymerase III holoenzyme or **replisome**, a large replication machine

II. DNA polymerase III structure – the holoenzyme is much larger than the other 2 polymerases; 10 different subunits with various coordinated functions in replication process

A. β clamp – a noncatalytic component of replisome; keeps polymerase associated with the DNA template

B. DNA polymerases, like RNA polymerases, have two somewhat contrasting properties:

1. Must stay associated with template over long stretches to make a continuous complementary strand
2. Must be attached loosely enough to template to move from one nucleotide to the next

C. These contrasting properties are provided by the doughnut-shaped β clamp that encircles the DNA (DNA extends through doughnut central hole) & slides freely along it
1. As long as it is attached to a β "sliding clamp", DNA polymerase can move processively from one nucleotide to the next without diffusing away from the template
2. The polymerase on the leading strand template stays attached to a single β clamp during replication
3. In contrast, when the polymerase on the lagging strand template completes the synthesis of an Okazaki fragment, it disengages from β clamp
4. It is then cycled to a new β clamp that has been assembled at an RNA primer-DNA template junction located closer to the replication fork

D. The assembly of the β clamp around the DNA requires a multisubunit clamp loader that is also part of replisome; one clamp-loader subunit, the "wrench", opens the β clamp so that it can fit around the DNA

III. Exonuclease activities of DNA polymerases – DNA polymerases also degrade nucleic acid polymers; all bacterial polymerases possess exonuclease activity; a seeming contradiction; certainly curious

A. Kornberg was first to find that DNA polymerase I preps always contained exonuclease activity; exonucleases are enzymes that degrade DNA polymers by removing ≥1 terminal nucleotides at a time
1. Kornberg initially thought that this activity was due to a contaminating enzyme, since the action of exonuclease is so dramatically opposed to that of DNA synthesis
2. But exonuclease activity could not be removed from polymerase prep & was, in fact, a true activity of polymerase; it was subsequently shown that all bacterial polymerases had exonuclease activity
3. Exonucleases are divided into 5'—>3' & 3'—>5' exonucleases, depending on the direction in which the strand is degraded

B. DNA polymerase I has both 5'—>3' & 3'—>5' exonuclease activities, along with its polymerase activity
1. These 3 activities reside in 3 different domains of the single polypeptide - DNA polymerase I is 3 enzymes in one; the 2 exonuclease activities have entirely different roles in replication

C. DNA polymerase I 5'—>3' exonuclease activity degrades both RNA & DNA (unusual, since usually exonucleases are specific for & degrade one or the other)
1. Exonuclease removes the RNA primer laid down by the primase at Okazaki fragment 5' end; polymerase simultaneously fills in the resulting gap with deoxyribonucleotides
2. DNA ligase covalently joins the last deoxyribonucleotide added during RNA primer digestion & the 5' end of the previously synthesized & adjacent DNA fragment

~~IV. Ensuring high fidelity of DNA replication – organism survival depends on accurate genome duplication~~

~~A. A mistake made during mRNA synthesis by RNA polymerase results in synthesis of defective proteins~~
~~1. But an mRNA is only one short-lived template among a large population of such molecules~~
~~2. Thus, little lasting damage is done as a result of the mistake~~

B. In contrast, a mistake in DNA replication results in a permanent mutation & the possible elimination of that cell's progeny
   1. In *E. coli*, the chance that an incorrect nucleotide will be incorporated into DNA during replication & remain there is $<10^{-9}$ or fewer than 1 out of 1 billion nucleotides
   2. Since the *E. coli* genome contains ~4 x $10^6$ nucleotide pairs, this error rate corresponds <1 nucleotide alteration for every 100 replication cycles, the spontaneous mutation rate in this bacterium

C. Incorporation of particular nucleotide onto end of growing strand depends on incoming nucleoside triphosphate being able to form an acceptable base pair with the nucleotide in template strand
   1. Analysis of distances between atoms & bond angles indicates that A-T & G-C base pairs have nearly identical geometry; any deviation from those pairings results in a different geometry
   2. DNA polymerase discriminates among 4 different precursors as they move in & out of active site
   3. Only one of them forms a proper geometric fit with the template, producing an A-T or G-C base pair that fits in the enzyme active site
   4. If incoming nucleotide is perceived by the enzyme as correct, a conformational change occurs in which the "fingers" of the polymerase rotate toward the "palm", gripping the incoming nucleotide
   5. If the newly formed base pair exhibits improper geometry, the active site cannot achieve the conformation required for catalysis & the incorrect nucleotide is not incorporated
   6. In contrast, if the base pair exhibits proper geometry, the incoming nucleotide is covalently linked to the end of the growing strand

D. Sometimes the polymerase incorporates an incorrect nucleotide leading to a mismatched base pair (other than A-T of G-C) ~1 time for every $10^5$ – $10^6$ nucleotides incorporated
   1. This frequency is ~$10^3$ – $10^4$ times greater than the spontaneous mutation rate of ~$10^{-9}$

E. How is the mutation rate kept so low? - the polymerase's 3' ––>5' exonuclease activity takes care of this; the 3' ––>5' exonuclease activity is found in DNA polymerases I, II & III
   1. When an incorrect nucleotide is incorporated by polymerase, the new end of the strand has an increased tendency to separate from the template & form a single-stranded 3'-end
   2. When this occurs, the enzyme undergoes a conformational change that directs the end of the newly synthesized strand into the 3' ––>5' exonuclease active site, which removes the mismatched nucleotide
   3. This proofreading is one of the most remarkable of all enzymatic activities; it illustrates the sophistication to which biological molecular machinery has evolved
   4. The 3' ––>5' exonuclease removes ~99 out of every 100 mismatched bases; raises fidelity to $10^{-7}$ - $10^{-8}$
   5. Bacteria also possess a mechanism called mismatch repair that operates after replication & corrects nearly all of mismatches that escape proofreading step, reducing overall observed error rate to ~$10^{-9}$

F. Thus, the fidelity of DNA replication can be traced to 3 distinct activities:
   1. Accurate selection of nucleotides
   2. Immediate proofreading
   3. Postreplicative mismatch repair

V. Speed of replication fork is remarkable - bacterial chromosome replicates in ~40 minutes at 37°C (1,000 nucleotides/sec) - equivalent to length of entire Okazaki fragment

A. Whole process of Okazaki fragment synthesis (RNA primer formation, DNA elongation & simultaneous proofreading by polymerase, RNA excision/replacement with DNA, strand ligation) occurs within ~1 sec

B. In *E. coli*, a new round of replication can begin before the previous round has been completed - thus when growing at maximal rate, they can double their numbers in ~20 minutes

## Replication in Eukaryotic Cells: Background Information

I. Relatively new eukaryotic experimental systems (see below) are used to study eukaryotic replication; the gap in our understanding of eukaryotic replication relative to that of prokaryotes is closing

A. Isolation of mutant yeast cells unable to make specific gene products needed for various aspects of replication

1. Replication proteins used by yeast are similar in structure to those in higher eukaryotic cells so the information obtained in yeast studies is applicable to mammals

B. Development of *in vitro* systems where replication can occur in cellular extracts or mixtures of purified proteins

1. *Xenopus*, an aquatic frog, starts life as a huge egg stocked with all of the proteins needed to carry it through a dozen or so rounds of cell division
2. Extracts can be prepared from these eggs that will replicate added DNA, regardless of sequence; such extracts also support the division of mammalian nuclei, so it is a very useful system
3. Antibodies can be used to delete the extracts of particular proteins & the replication ability of the extract can then be tested in the absence of the affected protein

II. Initiation of replication in eukaryotic cells - subject to much regulation

A. Higher organisms' cells have much more DNA than bacteria & incorporate DNA at much slower rates; thus, they initiate replication at many sites rather than just one as with the circular chromosome of *E. coli*

1. Cells of higher organisms may have 1000 times as much DNA as *E. coli*
2. To accommodate the differences, eukaryotes replicate their genomes in small portions (**replicons**)
3. Eukaryotic replicons are generally 15 - 100 μm in length (50 - 300 kb)
4. Each has its own origin from which replication forks proceed outward in both directions; in human cell, replication starts at about 10,000 to 100,000 different replication origins
5. The existence of replicons was first demonstrated in autoradiographic experiments in which single DNA molecules were shown to be replicated simultaneously at several sites along their length

B. Initiation of DNA synthesis in a given replicon is subject to regulation

1. Replicons close to each other on a given chromosome tend to replicate simultaneously
2. Those replicons active at a particular time during one round of DNA synthesis tend to be active at a comparable stage in succeeding rounds
3. In mammals, timing of replication in a chromosomal region may be determined primarily by the activity of genes in the region and/or its state of compaction

C. The less active, more tightly compacted the DNA, the later is the stage at which it is replicated

1. The *DHFR* gene is actively transcribed in a particular cell type, while the β-globin gene is not transcribed in that same cell type; the *DHFR* gene replicates in these cells before the β-globin gene
2. The presence of acetylated histones, which is closely correlated with gene transcription, is a likely factor in determining the early replication of active gene loci

D. The most highly compacted, least acetylated chromosome regions are packaged into heterochromatin; they are the last regions to be replicated

1. Sequence unimportant in timing – the inactive, heterochromatized X chromosome in female mammals replicates late in S phase; the active, euchromatic X chromosome replicates at earlier stage
2. There must be a control that allows each section of chromosome to replicate only once each cycle

III. Yeast origins of replication can be removed & placed into bacterial DNAs - useful in studies of replication initiation & they have led to much progress in this area

A. Yeast origins can then replicate in yeast cells or in cellular extracts with the required replication proteins

B. They promote the replication of the DNA in which they reside; thus they are called **a**utonomous **r**eplicating **s**equences (ARSs); there are ~400 ARSs scattered throughout yeast chromosomes

IV. Those ARSs that have been isolated & analyzed share several distinct elements and properties:

A. ARS core element has conserved 11-bp-sequence (functions as specific binding site for essential multiprotein complex [**o**rigin **r**ecognition **c**omplex; *ORC*])

B. If the ARS is mutated so that ORC cannot bind—> replication cannot occur

C. *ORC* remains bound to each origin throughout cell cycle; initiation of replication is triggered by binding of other proteins to *ORC*-origin complexes

V. Replication origins have proven more difficult to study in vertebrate cells than in yeast

A. Problem - virtually any type of purified, naked, vertebrate DNA when tested as template *in vitro* is suitable for replication using cellular extracts from frog eggs - makes study of replication origins difficult

1. Suggests that, unlike yeast, vertebrate DNA may not have specific ARSs at which replication is initiated
2. But studies of intact mammalian chromosomes *in vivo* suggest that replication does begin at specific sites along DNA, rather than by random selection as happens with frog egg extract *in vitro*
3. It appears that a DNA molecule contains many sites where replication can be initiated, but positioned nucleosomes & higher-order chromatin structure suppress initiation at most of these sites
4. This same structure promotes replication initiation at specific sites that serve as replication origins

B. A bidirectional replication initiation site has been discovered within the human β-globin gene cluster

1. If this specific DNA segment is transplanted experimentally to a new chromosomal location in a monkey cell, it functions as an origin of replication in its new DNA environment
2. Thus, a mammalian origin of replication can depend on the presence of a specific DNA segment

3. If this site is deleted, as it is in patients with a specific hemoglobin deficiency (hemoglobin Lepore syndrome), a new origin of replication does not appear at the site
4. Instead, DNA in this part of the chromosome is replicated by a replication fork that moves into the region from an unidentified origin outside the β-globin cluster
5. Thus, the removal of an origin does not stimulate initiation at a new origin sequence; instead, the cell takes advantage of the presence of a distant, existing origin

VI. Restricting replication to once per cell cycle – it is essential that each portion of genome replicates once, and only once, only once during each cell cycle
   A. Thus, a mechanism must exist to prevent reinitiation of replication at an already-duplicated site
   B. It is believed that for replication initiation at a particular origin, it must pass through several distinct states; this may be hard to reproduce during S phase after replication fork has passed an initiation site
      1. Similar steps requiring homologous proteins take place in plants & animals, suggesting that the basic mechanism of replication initiation is conserved among eukaryotes

VII. Steps that lead to replication in yeast cell
   A. Replication origin is bound by ORC protein complex, which remains associated with origin throughout the cell cycle; the ORC is called a molecular landing pad since it binds subsequently needed proteins

   B. Licensing factors (proteins) bind to the ORC to assemble a protein-DNA complex (prereplication complex [pre-RC]) that is licensed (competent) to initiate replication
      1. Studies of the molecular nature of licensing factors have focused on a set of 6 related Mcm proteins (Mcm2 – Mcm7)
      2. Mcm proteins are loaded onto the replication origin at a late stage of mitosis or soon after mitosis has completed
   C. Assembly of pre-RC can be followed by treating isolated chromatin with DNA-digesting enzymes
      1. Prior to assembly of pre-RCs, only a small region of DNA at each replication origin is protected from nuclease digestion by the bound ORC proteins
      2. The protected region of DNA (a **DNA footprint**) becomes greatly expanded after association of Mcm proteins with the previously bound ORC
   D. Just before the cell cycle S phase starts, activation of key protein kinases leads to initiation of replication
      1. One of the protein kinases is a cyclin-dependent kinase (Cdk); its activity remains high from S phase through mitosis, which suppresses the formation of new pre-RCs
      2. Thus, each origin can only be activated once per cell cycle
   E. Once replication is initiated at the start of S phase, the Mcm proteins move with the replication fork & are essential for completion of replicon replication
      1. Studies show that Mcm2-Mcm7 proteins associate into ring-shaped complex with helicase activity
      2. Researchers have had great difficulty identifying the major replicative helicase (the one that unwinds DNA at the replication fork)
      3. The Mcm protein complex is a strong candidate for this helicase (analogous to DnaB in *E. coli*)
   F. The fate of the Mcm proteins after replication depends on the species studied

1. In yeast, the Mcm proteins are displaced from the chromatin & exported from the nucleus
2. In contrast, mammalian cell Mcm proteins are displaced from the DNA, but remain in the nucleus
3. Regardless, Mcm proteins cannot reassociate with a replication origin that has already fired; thus, each origin can only be activated once per cell cycle

VIII. Eukaryotic replication fork – events occurring at replication forks are very similar whether genome being replicated is viral, prokaryotic or eukaryotic; requires same collection of enzymes as prokaryotic fork

A. All replication systems require helicases, single-stranded DNA-binding proteins, topoisomerases, primase, DNA polymerase & DNA ligase
   1. The helicase that does DNA unwinding during replication has not been identified with certainty
   2. Thus, *in vitro* eukaryotic replication studies often use viral SV40 large T antigen (initiates replication & has helicase activity) to replace unknown helicase along with mammalian replication proteins
   3. Large T antigen, encoded by SV40 viral genome, induces strand separation at the SV40 origin of replication & unwinds the DNA as the replication fork progresses

B. As in prokaryotes, DNA is made semidiscontinuously, but Okazaki fragments in lagging strand are considerably smaller (~150 nucleotides in length) than in bacteria

C. Like *E. coli* DNA polymerase III, the eukaryotic replicative DNA polymerase δ is present as a dimer; suggests leading & lagging strands are made in coordinate manner by 1 replicative complex (**replisome**)

## Replication in Eukaryotic Cells: DNA Polymerases

I. Eukaryotes have 5 different DNA polymerases instead of 3 - α, β, γ, δ & ε; 3 involved in replication; 2 are not

A. All eukaryotic polymerases elongate DNA strands in a 5' —> 3' direction by addition of nucleotides to a 3'-hydroxyl group; none of them is able to the initiate synthesis of a DNA chain without a primer

B. Polymerases γ, δ & ε possess a 3' —> 5' exonuclease. whose proofreading activity ensures that replication occurs with very high accuracy

II. Polymerase γ - replicates mitochondrial DNA; not involved in nuclear DNA replication

III. Polymerase β - functions in DNA repair; not involved in nuclear DNA replication

IV. Polymerase α - tightly bound to primase; together they initiate the synthesis of each Okazaki fragment; primase lays down a short primer; then polymerase α extends it with ~20 deoxyribonucleotides

V. Polymerase δ - assembles leading strand & most of lagging strand fragments; thought to be the primary replicative enzyme; requires "sliding clamp" structure (PCNA), like polymerase III in *E. coli*

A. Sliding clamp tethers the enzyme to DNA, allowing it to move processively along template; the eukaryotic sliding clamp is very similar in structure & function to the *E. coli* polymerase III β subunit
   1. In eukaryotes, the sliding clamp is called PCNA - **p**roliferating **c**ell **n**uclear **a**ntigen; first found as antigen that reacted to autoantibodies in serum of lupus erythematosus patients
   2. Later, it was localized in the nuclei of rapidly proliferating cells (role in replication discovered later)
   3. The clamp loader that loads PCNA onto DNA is called RFC & is analogous to the *E. coli* polymerase III clamp loader complex

B. After making an RNA primer & short DNA segment, primase-polymerase α complex is replaced at template-primer junction by PCNA-polymerase δ complex (completes Okazaki fragment synthesis)

C. When newly made strand reaches 5' end of previously synthesized Okazaki fragment, the RNA primer of that next fragment is removed, along with short stretch of DNA made by polymerase α
   1. The gap is filled by the continued movement of polymerase δ

D. Eukaryotic DNA polymerases do not have 5' —> 3' exonuclease activity, unlike DNA polymerase I; the primers are removed by at least 2 separate nucleases, RNase H1 & FEN-1

E. Adjoining fragments are ultimately sealed by DNA ligase

VI. Polymerase ε - determining its role has been difficult; it appears to play a role in nuclear DNA replication (replication cannot be finished in cells lacking this polymerase)
   A. But it is not required for *in vitro* replication of SV40 DNA; its exact role unknown

VII. Several other DNA polymerases (η, σ & ι) have a specialized function that allows cells to replicate damaged DNA

## Replication in Eukaryotic Cells: Replication and Nuclear Structure

I. Replication apparatus consists of huge complex of proteins operating within confines of a structured nucleus; increasing evidence suggests that replication machinery is present in tight association with nuclear matrix
   A. Give cells very short, hot nucleotide DNA precursor pulse —> incorporated label (>80%) is associated with nuclear matrix; suggests that replication machinery is tightly associated with matrix
      1. Give cells a hot pulse, then incorporate unlabeled precursors for hour or so before fixation —> most label is chased from the matrix into the surrounding DNA loops
      2. Conclude that replicating DNA moves like conveyer belt through immobilized replication apparatus, rather than remaining stationary
   B. Forks that are active at a given time are not randomly distributed throughout cell nucleus, but instead are localized within 50 - 250 sites (**replication foci**)
      1. Each of these replication foci may have up to ~40 replication forks/site incorporating nucleotides into DNA strands simultaneously
      2. Clustering of replication forks may provide a mechanism for coordinating the replication of adjacent replicons on individual chromosomes
      3. If cells are treated to remove most of chromatin, replication foci remain attached to nuclear matrix

II. Chromatin structure & replication – eukaryotic chromosomes consist of DNA tightly complexed to regular arrays of histone proteins

A. EM micrographs of replicating DNA – show nucleosomes forming on both daughter duplexes very near replication fork; indicates that assembly of DNA into nucleosomes is a very rapid event

B. The core histone octamer of a nucleosome consists of an $(H3H4)_2$ tetramer together with a pair of H2A/H2B dimers

1. *In vitro* studies indicate that the $(H3H4)_2$ tetramers present prior to replication remain intact & are distributed randomly between 2 daughter duplexes
2. As a result, old & new $(H3H4)_2$ tetramers are intermixed on each daughter DNA molecule
3. In contrast, the 2 H2A/H2B dimers of each parental nucleosome do not remain together as the replication fork moves through the chromatin
4. Instead, the dimers of a nucleosome separate from one another & appear to bind randomly to the new & old $(H3H4)_2$ tetramers already present on daughter duplexes
5. The stepwise assembly of nucleosomes & their orderly spacing along the DNA is facilitated by a network of accessory proteins

## ~~DNA Repair: Introduction~~

~~I. DNA is one of the most susceptible cell molecules to environmental damage even though it must remain essentially unchanged – many destructive forces exist in internal & external environments of an organism~~

~~A. Types of damage experienced by DNA~~

~~1. Ionizing radiation can break DNA backbone~~

~~2. Exposure to a variety of reactive chemicals, some made by cell metabolism, can alter DNA bases structurally~~

~~3. Ultraviolet radiation causes adjacent pyrimidines to interact covalently, forming a dimer~~

~~4. Absorption of thermal energy generated by metabolism in a warm-blooded bird or mammal can split adenine & guanine from their attachment to DNA backbone sugars~~

~~B. These spontaneous alterations or lesions are quite plentiful; it is estimated that each cell of a warm-blooded mammal loses ~10,000 bases/day~~

~~II. Failure to repair such lesions produces permanent alterations or mutations in DNA (so it must be repaired)~~

~~A. If mutation occurs in a cell destined to become a gamete, genetic alteration may be passed on to next generation~~

~~B. Mutation can affect nonreproductive (somatic; cells not in germline) cells, too; can cause lesions~~

~~1. Can interfere with transcription and replication~~

~~2. Can lead to the malignant transformation of a cell~~

~~3. Can speed the process by which an organism ages~~

~~C. Considering the potentially drastic consequences of DNA alterations & the high frequency at which they occur, it is essential that cells possess mechanisms for repairing this damage~~

~~1. In fact, cells have a bewildering array of repair systems that correct virtually any kind of damage to which DNA is subjected; it is estimated that <1 base change in a thousand escapes cell repair systems~~

~~2. These systems are an excellent example of molecular mechanisms that maintain cell homeostasis~~

~~3. DNA repair importance is shown by examining what happens to humans with DNA repair deficiencies~~

D. Many proteins patrol DNA searching for alterations & distortions that they can recognize & repair (eukaryotes & prokaryotes); sometimes damage can be fixed directly; often it is excised & replaced
   1. Humans possess enzymes that can directly repair damage from cancer-producing alkylating agents
   2. One enzyme uses sunlight energy to break bonds holding pyrimidine dimers together (direct repair) - returns molecule to its original condition
   3. Most repair systems excise damaged section selectively; excised strand is replaced via the undamaged complementary strand, which serves as a template to replace excised, damaged nucleotides

E. Repair can be observed by following $^{3}$H-thymidine incorporation when cell is not doing replication; DNA replication & repair have many features in common & share the same parts & services

## DNA Repair: *N*ucleotide *E*xcision *R*epair (NER)

I. Removes part of strand having certain types of bulky lesions: pyrimidine dimers & nucleotides to which various chemical groups are attached; "cut-and-patch" mechanism; 2 distinct NER pathways distinguished
   A. Transcription-coupled pathway - template strands of genes that are being actively transcribed are preferentially repaired; repair of template strand is thought to occur as DNA is being transcribed
      1. The presence of the lesion may be signaled by a stalled RNA polymerase
      2. This preferential repair pathway ensures that those genes of greatest importance to the cell, the genes being actively transcribed, receive the highest priority on the repair list
   B. Global pathway - slower, less efficient pathway that corrects DNA strands in remainder of genome

II. Steps in repair process in eukaryotic cells - first step is lesion recognition by proteins scanning DNA & the recruitment of repair enzymes to distorted sites in helix
   A. Lesion recognition is probably done by different proteins in the 2 NER pathways, but the subsequent repair steps are thought to be very similar

   B. Two of TFIIH's various subunits (XPB & XPD) are proteins with helicase activity; they separate the 2 strands of the duplex in preparation for removal of the lesion
      1. TFIIH (huge protein that also participates in transcription initiation) is a key repair machinery component; its involvement established a crucial link between transcription & DNA repair
      2. These 2 processes were previously assumed to be independent of one another
   C. The damaged strand is then cut on both sides of the lesion by a pair of endonucleases; the segment of damaged DNA is now held in position only by H bonds
   E. Segment of DNA between the incisions is then released
   F. Once excised, the gap is filled by DNA polymerase & the strand is sealed by DNA ligase

## DNA Repair: *B*ase *E*xcision *R*epair (BER)

I. Prokaryotes & eukaryotes remove altered nucleotides generated by reactive chemicals present in diet or produced by metabolism; these alterations distort the helix less

II. Steps in repair process in eukaryotes
   A. BER is initiated by a DNA glycosylase that recognizes alteration & removes base by cleavage of glycosidic bond holding the base to the deoxyribose sugar moiety
   B. Once the altered purine or pyrimidine is removed, the "beheaded" deoxyribose phosphate remaining in the site is removed by the combined action of a specialized (AP) endonuclease & a DNA polymerase
      1. The AP endonuclease cleaves the DNA backbone
      2. Phosphodiesterase activity of polymerase β removes the sugar-phosphate remnant that had been attached to the excised base
   C. Gap is then filled by DNA polymerase β, which inserts a nucleotide complementary to the undamaged strand, & the strand is sealed by DNA ligase III

III. A number of different DNA glycosylases have been identified; each is more-or-less specific for a particular type of altered base, like those below:
   A. Uracil - forms by hydrolytic removal of cytosine's amino group
   B. 8-oxo-guanine - caused by damage from oxygen free radicals
   C. 3-methyladenine - caused by alkylating agents; transfer of methyl group from a methyl donor

IV. Uracil formation from cytosine may explain why natural selection favored thymine's use as a base in DNA instead of uracil even though uracil was presumably in RNA, when it was life's original genetic material
   A. If uracil had been retained as a DNA base, repair systems could not distinguish between a uracil that resulted from damage to cytosine & a normal uracil that belonged at a particular site
   B. An enzyme that removes uracil from DNA (uracil-DNA glycosylase) is one of the most conserved proteins between *E. coli* & humans (sharing 56% identity in amino acid sequence)
      1. Structural studies show that uracil-DNA glycosylase binds DNA & causes uracil to flip out of helix & into the active site of the enzyme where it is removed

## DNA Repair: Mismatch Repair

I. Mismatch repair – cells can remove mismatched bases that are incorporated by DNA polymerase & escape the enzyme's proofreading exonuclease

II. A mismatched base pair causes distortion in double helix geometry; recognized by a repair enzyme - how does it know which member of a mismatched pair is the incorrect nucleotide?
   A. If it removed one of the nucleotides at random, 50% of the time it would remove the wrong one & cause a permanent mutation at the site
   B. To repair mismatch after DNA polymerase has moved past site, it is important for repair system to distinguish newly made strand (contains incorrect nucleotide); parental strand contains correct one
      1. *E. coli* – the 2 strands are distinguished by the presence or absence of methyl groups; parental strand has methyl groups linked to certain adenosine residues; not added to new strand right away
      2. Repair system patrols DNA looking for mismatch before methylation of new strand
      3. If mismatch is found, the enzyme always removes & replaces the nucleotide from nonmethylated strand, guaranteeing that it restores the original base pairs
   C. DNA methylation does not appear to be used by mismatch repair system in eukaryotes; the mechanism of identification of newly synthesized strand remains unclear

## DNA Repair: Double-Strand Breakage Repair

I. Ionizing radiation (X-rays, gamma rays, particles emitted by radioactive atoms) generate ions as they pass through matter; if this radiation collides with fragile DNA molecule, both double helix strands often break
   A. Double-stranded breaks (DSBs) can also be caused by certain chemicals, including several used in cancer chemotherapy (bleomycin) & free radicals produced by normal cellular metabolism
      1. DSBs are also introduced during replication of damaged DNA
   B. A single DSB can cause serious chromosome abnormalities & ultimately prove lethal for cell

II. Double-strand breaks (DSBs) can be repaired by several alternate pathways – two are described below; defects in both repair pathways have been linked to increased cancer susceptibility
   A. In mammalian cells, the simplest pathway is called **nonhomologous end joining (NHEJ)**,
      1. A complex of proteins binds to the broken ends of the DNA duplex & catalyzes a series of reactions that rejoin the broken strands
      2. Cells that lack one of the proteins required for NHEJ are very sensitive to ionizing radiation
   B. Another DSB repair pathway includes genetic recombination & is considerably more complex

III. DNA repair is important because unrepaired damage caused by deficiencies in DNA repair can lead to human diseases; studying these diseases has helped to understand DNA repair

## Between Replication and Repair

I. Xeroderma pigmentosum (XP) – inherited disease; leaves patients with an inability to repair certain lesions caused by exposure to ultraviolet radiation
   A. Patients with the classical form of XP have a defect in 1 of 7 different genes involved in nucleotide excision repair (NER); these genes are designated XPA, XPB, XPC, XPD, XPE, XPF & XPG
   B. Highly susceptible to developing skin cancer as a result of sun exposure

II. Another group of patients was identified that was highly susceptible to developing skin cancer as the result of sun exposure, like people with XP
   A. Cells from these patients were capable of nucleotide excision repair & were only slightly more sensitive to UV light than normal cells
   B. The heightened UV sensitivity revealed itself during replication - these cells often produced fragmented daughter strands after UV irradiation
   C. Patients in this group were classified as having a variant form of XP, designated XP-V

III. While cells are able to repair a variety of DNA lesions, sometimes a lesion is not repaired by the time that segment of DNA is scheduled to undergo replication
   A. On these occasions, the replication machinery arrives at the site of damage on the template strand & gets stalled there
   B. When this happens, some type of signal is emitted that leads to recruitment of a specialized polymerase that is able to bypass the lesion
      1. Suppose the lesion is a thymidine dimer in a skin cell that was caused by exposure to UV radiation
      2. When the replicative polymerase (pol δ or ε) reaches the obstacle, the enzyme is temporarily replaced by a DNA polymerase designated pol η
      3. This enzyme is able to insert 2 A residues into the newly synthesized strand across from the 2 T residues that are covalently linked as part of the dimer

4. Once this damage bypass is accomplished, the cell switches back to the normal replicative polymerase & DNA synthesis continues without leaving any trace of the problem

C. Patients with XP-V have a mutation in the gene encoding pol η & thus have difficulty replicating past thymidine dimers

IV. Polymerase η is member of a relatively large family of DNA polymerases that are specialized for incorporating nucleotides opposite various types of DNA lesions in the template strand

A. The polymerases of this family engage in translesion synthesis (TLS) & they have an uncanny ability to incorporate the nucleotide that would have paired with the undamaged version of the template base

1. Also, they are only capable of incorporating one to a few nucleotides into a DNA strand (they lack processivity)
2. They have no proofreading capability
3. They are much more likely to incorporate an incorrect (noncomplementary) nucleotide than the classic polymerases

B. According to one controversial proposal, these error-prone polymerases may be induced in some organisms during periods of adverse conditions

1. This could prove adaptive by elevating the mutation rate
2. This could increase the likelihood of producing individuals that can survive the environmental stress

## The Human Perspective: The Consequences of DNA Repair Deficiencies

I. The sun constantly emits a stream of ultraviolet (UV) rays that age & mutate the cells of our skin

A. Rare, recessive genetic disorder xeroderma pigmentosum (XP) illustrates these hazardous effects of sun

B. Patients with XP possess a deficient repair system that cannot remove segments of DNA damaged by UV radiation; they are thus extremely sensitive to sunlight

1. Even very limited exposure to direct rays of the sun can produce many dark-pigmented spots on exposed areas of the body & a greatly elevated risk of developing disfiguring & fatal skin cancers

C. Some help may coming for XP patients in the form of skin creams containing DNA repair enzymes

II. Cockayne syndrome (CS) - an inherited disorder characterized by acute sensitivity to light, neurological dysfunction due to neuron demyelination & dwarfism without an evident rise in skin cancer frequency

A. Cells from CS patients are deficient in preferential pathway by which transcriptionally active DNA is repaired; the rest of genome is repaired at normal rate, perhaps explaining normal skin cancer rates

B. Where does dwarfism come from? – most CS cases are traced to a mutation in one of 2 genes, either CSA or CSB, which are thought to be involved in coupling transcription to DNA repair

1. They may also disturb transcription of certain genes, leading to growth retardation & abnormal nervous system development

C. In rare cases, CS symptoms can also occur in people with XP who carry specific mutations in the *XPD* gene (encodes a subunit of the transcription factor TFIIH required for transcription initiation)

D. Mutations in *XPD* could lead to defects in both DNA repair & transcription

III. Certain other mutations in the *XPD* gene are responsible for another disease trichothiodystrophy (TTD), which also combines symptoms suggestive of both DNA repair & transcription defects
A. Like CS patients, individuals with TTD exhibit increased sun sensitivity without elevated risk of cancer development; they have additional symptoms, including brittle hair & scaly skin
B. Thus 3 distinct disorders (XP, CS & TTD) are caused by defects in a single gene, with the particular disease outcome determined by the specific mutation present in the gene

IV. Persons with DNA-repair disorders are not the only individuals who should worry about sun exposure
A. Even in a skin cell whose repair enzymes function at optimal levels, a small fraction of lesions fail to be excised & replaced - alterations in DNA lead to mutations that can cause a cell to become malignant
B. One consequence of the failure to correct UV-induced damage is the risk of skin cancer
1. >1 million persons develop one of three forms of skin cancer every year in the U. S.
2. Most of these cases are attributed to overexposure to the sun's UV rays
C. The 2 most common forms of skin cancer, basal cell carcinoma & squamous cell carcinoma, rarely spread to other parts of the body & can usually be excised in a doctor's office
1. Both of these types of cancer originate from the skin's epithelial cells
D. Malignant melanoma, 3rd type of skin cancer, is potential killer – it develops from pigment cells in skin; cases diagnosed in U. S. are rising at alarming rate of 4%/year due to rise in time people spend in sun
1. One of greatest risks for developing melanoma as adult is severe, blistering sunburn as child or adolescent; it is obviously important to prevent such burns in children

V. XP is very rare, but colon cancer is relatively common – it is estimated that up to 15% of colon cancer cases can be attributed to mutations in genes that encode the proteins required for mismatch repair
A. Mutations that cripple the mismatch repair system inevitably lead to a higher mutation rate in other genes because mistakes made during replication are not corrected

B. Highest frequency of replication errors occurs when DNA polymerases copy short, repeated sequences (microsatellites), which are sites where the enzyme tends to slip as it moves along template
1. A gene that codes for the receptor of a growth factor (TGF-β) is very susceptible to mutation since its sequence contains an A residue string where replication enzymes tend to make mistakes
2. If both copies of the gene become mutated, the cell can no longer respond to the growth factor & has a greatly increased likelihood of developing into a tumor

VI. Cancer is also a consequence of double-strand DNA breaks that have either gone unrepaired or been repaired incorrectly
A. Breaks in DNA can be caused by a variety of environmental agents to which we are commonly exposed (X-rays, gamma rays, radioactive emissions)
1. The most serious environmental hazard of this type is probably radon (specifically $^{222}Rn$), a radioactive isotope formed during the disintegration of uranium
2. Some areas of planet contain relatively high levels of uranium in the soil; houses built in these regions can contain dangerous radon gas levels
3. Estimates are that ~1% of U. S. houses have radon levels that yield >10 picocuries/liter of radiation

4. When the gas is breathed into lungs, it can lead to double-strand DNA breaks that raise risk of lung cancer; a significant fraction of lung-cancer deaths in nonsmokers is likely due to radon exposure

# LECTURE HINTS

## DNA Replication

Describe the three different types of replication that are possible: conservative, semiconservative and dispersive. Then lay out the Meselson - Stahl experiment. This experiment has been a favorite of mine since it was assigned to me as a report topic in my freshman biology course. It was the first time I had to read an actual journal article. Much to my surprise, I could read and, after a couple of times through the paper, understand the experiment fully. It is elegant. Once the class understands how equilibrium density centrifugation works and what the data "look like", you can get the class to predict the results that would be expected for each of the three hypotheses. You can then show the class the results that were actually obtained and ask them to draw their own conclusions. I have also found that variations on the Meselson - Stahl experiment make great test questions (see above), for example, asking the students what results they would expect if replication were conservative or what results they would expect in the third or fourth generation. The latter, of course, requires some math skill, often something students seem to resent having tested nowadays.

## Replication in Bacterial Cells

The use of mutants in studies such as these has been emphasized before. You may wish to reiterate their importance and mention how temperature-sensitive mutants work. You may also wish to mention the use of *in vitro* systems in studying replication.

The Cairns experiment, which was used to visualize replication in bacteria, while not included in this edition of the text, is an excellent experiment to mention to the class. It is another classic experiment that is relatively simple to understand and explain, elegant in its conception and one that makes use of oft-used molecular biological techniques. The Cairns experiment and others revealed that replication is bidirectional and appears to occur as the DNA strands separate in a region called the replication fork.

Mention the discovery of the DNA polymerases and the revelation that they only lay down nucleotides in a 5' ->3' direction. Mention the contradiction inherent in this finding due to the antiparallel structure of the DNA duplex. At each replication fork, one of the strands grows in the 3' ->5' direction, the other in the 5' ->3' direction. Ask the class how this can be and then talk about the idea of semidiscontinuous replication and the experimental results that led to the discovery of Okazaki fragments. Another difficulty arose in thinking about replication when it was realized that DNA polymerase required a primer. The problem was solved when it was realized that short RNA molecules are used as primers and that these short RNA segments are made by a special RNA polymerase called primase (that synthesizes them on both the leading and lagging strands). Mention the roles of ligase, SSBs and helicases as well and emphasize their overall importance to the process.

## Structure and Function of DNA Polymerases

Some of this material has been covered above. Outline the properties of all of the DNA polymerases emphasizing how each one fits into the scheme of the process of replication. Perhaps the least expected property in a DNA polymerase is exonuclease activity (both 5' ->3' and 3' ->5'). Ask your class why that statement makes sense. If they have difficulty answering

the question, ask them what an exonuclease is. The necessity for removing the RNA primers and the repair functions explain the 5'—>3' exonuclease activity. The enzyme's proofreading ability, of course, explains the 3'—>5' exonuclease activity.

*Analogy*

**The IBM Selectric (Proofreading) Analogy**

Back when I was a graduate student, a marvelous new invention showed up on typewriters (I guess I'm dating myself). For people like me, who were and are rotten typists, this invention was a definite winner. It was that little correction tape that had been added to the typewriter. If the typist made a mistake, typing the wrong letter, for instance an "E" instead of an "R", (s)he would hit a special key that would back the carriage up one letter. Next, the typist would hit the key representing, in this example, the "E". The machine would then type an "E", but it would use the correction tape so that the white material on the tape would cover the letter typed by mistake. Under these circumstances, however, the carriage does not advance as it usually does. Now the typist can type in the correct letter (an "R", in this case). The letter will be typed with the black ribbon this time covering over the mistake further. DNA polymerase acts in much the same way. If it detects a mistake, it basically backs up and excises the incorrect base. Subsequently, the enzyme inserts the correct base and moves forward until another error is made. This analogy can now be updated using a word-processing program. We didn't have them back there in the seventies when dinosaurs still walked the Earth.

## Replication in Eukaryotic Cells

Point out the similarities and differences between replication in eukaryotes and prokaryotes and the molecules involved in the processes in both types of organisms. Perhaps one of the biggest differences between them is the initiation of replication at numerous sites in the eukaryotic genome, instead of just one site as is the case in prokaryotes. Ask the students the reason for this difference (the much greater size of the eukaryotic genome, for example). Stress that the timing of the replication of particular chromosomes is regulated. The pattern of replication is the same from generation to generation. Discuss the role of ARSs in yeast cells and describe their role in research that is teaching us about eukaryotic replication.

## DNA Repair

First, describe the types of damage that can be done to DNA and the sources of that damage. Students have already been informed as to the consequences of having no repair mechanisms. You may, however, wish to refresh their memories, if you feel it necessary. Once you have described the sources of damage, summarize the different types of repair and the mechanisms by which this repair occurs: nucleotide excision repair, base excision repair, mismatch repair and double-strand breakage. If you have time, explain the postulated evolutionary reason for the use of thymidine in DNA instead of uracil. It is a fascinating story and gets to the core of the development of life on our planet. Also mention the relatively newly discovered polymerases that are involved in translesion synthesis.