

Biology 125:

Molecular Biology and Genomics

This course focuses on Genomics, Molecular Evolution, Phylogenetics, Gene Expression, Molecular Dynamics and Bioinformatics (integrated creation, storage and utilization of molecular biology information).

It is a “project-based” course which means students engage in active learning through:

- *Review of theoretical backgrounds to biological processes
- *Hands-on participation in group research projects.
- *Working individually and in groups to design and conduct experiments on the molecular basis of human colon cancer.

- *Work effectively with peers
- *Demonstrate skills in analysis and critical thinking to solve problems and apply scientific knowledge.
- *Demonstrate skills in designing and implementing controlled scientific experiments.
- *Use and interpret relevant quantitative data
- *Access, evaluate, use primary scientific literature as learning resource.
- *Effectively communicate understanding of scientific/experimental concepts in the form of a comprehensive scientific report.
- *Demonstrate understanding of molecular dynamics regulating gene expression, genome organization/maintenance, and cell cycle control.
- *Demonstrate understanding of molecular evolution as it reflects adaptation in the relationship of structure and function at the molecular level.
- *Apply informatics tools and approaches in analyzing genes & genomes.

At the end of the Course

Students should have:

Developed scientific skills of inquiry, experimental design, data collection and analysis and hypothesis testing.

Acquired the skills to practice being a scientist rather than just learning about what scientists discover.

Become prepared for advanced study in the biology major, and set on a lifelong path of scientific discovery whatever their professional goal!

Gene Expression and Molecular Genetics

Molecular basis of inheritance, Gene Sequences, Genetic Code, Prokaryote/Eukaryote DNA, Plasmids and vectors, Wild type versus mutant genes, Gene Expression in Human Colorectal Cancer. (Plasmid Isolation)

Molecular Cloning and Biotechnology

DNA Structure, Replication, Central Dogma, Transcription and Translation, Plasmids and vectors, DNA Isolation and Analysis using Restriction/gel electrophoresis, Wild type versus mutants, PCR amplification, Site-directed Mutagenesis, Recombinant DNA Technology and gene cloning, DNA and Protein Sequencing

(Plasmid Isolation/Restriction/Gel Electrophoresis)

Molecular Basis of Cancer (Cancer Genetics)

Molecular basis of cancer, Mutations, Cancer and DNA Repair, Cancer, Oncogenes and Cellular Signaling, Cell Cycle Regulation

(PCR, Site-directed Mutagenesis, Transforming Yeast Cells, Selecting Transformants, Complementation Analysis, Phenotypic Analysis*****Poster Presentation)

Genomics and Phylogenetics

Organization of Eukaryote Genomes, Genomics, Phylogenetics Approaches.

(Extracting Total Protein, Immunoblot Analysis, ***Progress Report/Poster Presentation)

Key Terms

Genomics	study of genomes (sum total of all genes in an organism), DNA sequence, fine scale genetic mapping, gene expression (transcriptome/proteome), gene interactions
Molecular Biology	focuses on single genes, their functions and roles
Bioinformatics	is the application of information technology to the field of molecular biology. It involves algorithms, computation, statistics, theory, database management and analysis, sequence mapping, homology alignment, structure prediction and analysis, interaction networks, evolution modeling etc.
Cancer	Phylogeny

THE BIG PICTURE

Biology Study of Life

Molecular Biology Study of Molecular Components

Disease is Alteration from the Normal

Colon Cancer

MSH2 Gene

Bioinformatics and Getting Info for MSH2 Project

Webct has all the information you need for working on your problem sets and doing bioinformatics.

All the information you will need can be obtained from literature search (Pubmed, NCBI), there are web links in Webct and the MMR site will give you more info on the MSH2 gene. You have Biology Workbench to do your analysis. I have also included some links below that you might find interesting.

Literature:

*****This should be your first read. It is the most relevant literature to your project.

Gammie et al, Functional Characterization of Pathogenic Human MSH2 Missense Mutations in *Saccharomyces cerevisiae* Genetics, 2007; 177(2): 707 - 721.

-The site below gives you a list of the MSH2 mutations in yeast relevant to HNPCC. What are their human homologs?. The site gives you a bunch of additional info on each mutant.

<http://www.genetics.org/cgi/content/full/177/2/707/TBL2>

Look for it in Pubmed and you may want to review some of the other articles related to this citation. It has very important info relevant to the successful execution of your project. Note: it has supplements that have more specific info!!

I suggest browsing these other links, you might find them interesting and useful.

[http://www.ncbi.nlm.nih.gov/sites/entrez?Db=gene&Cmd=retrieve&dopt=full_report&list_uids=4436&log\\$=genesensorsearch&logdbfrom=pubmed](http://www.ncbi.nlm.nih.gov/sites/entrez?Db=gene&Cmd=retrieve&dopt=full_report&list_uids=4436&log$=genesensorsearch&logdbfrom=pubmed)

<http://biowww.net/sms2/>

http://www.ornl.gov/sci/techresources/Human_Genome/publicat/urllist.shtml

Biology Workbench

molbiol-tools.ca/

molecularstation.com

Human Genome

ornl.gov/sci

mfold.bioinfo.rpi.edu

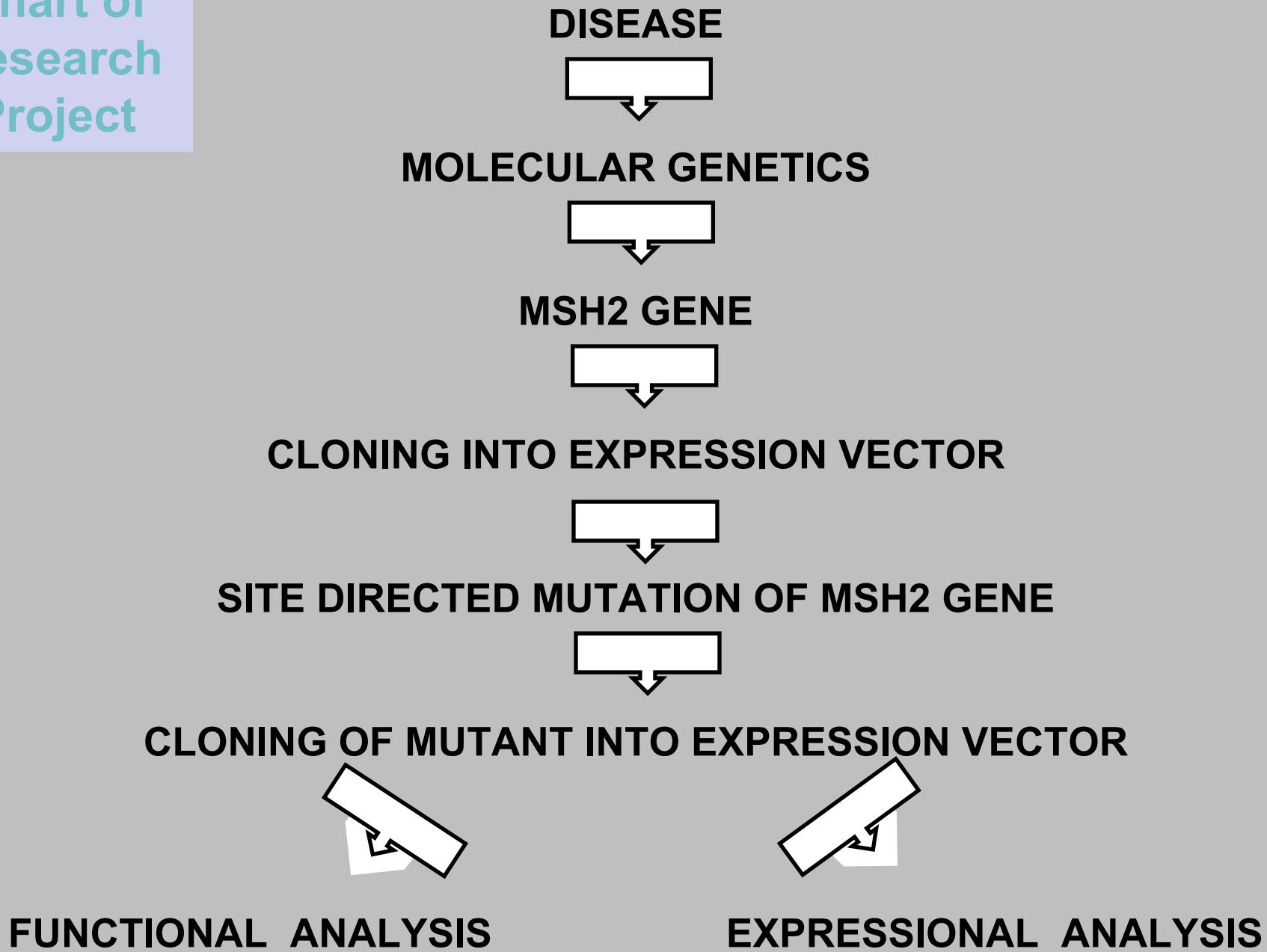
NCBI

Pubmed

MMR database

Integrating Theoretical Knowledge into Practical Experimental Exercises

Chart of Research Project



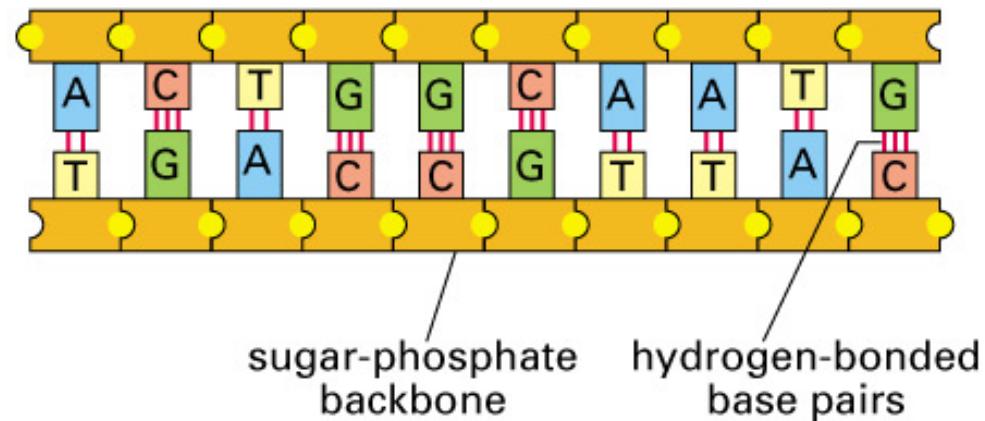
THE BIG PICTURE

THEORY CONCEPTS BIOINFORMATICS

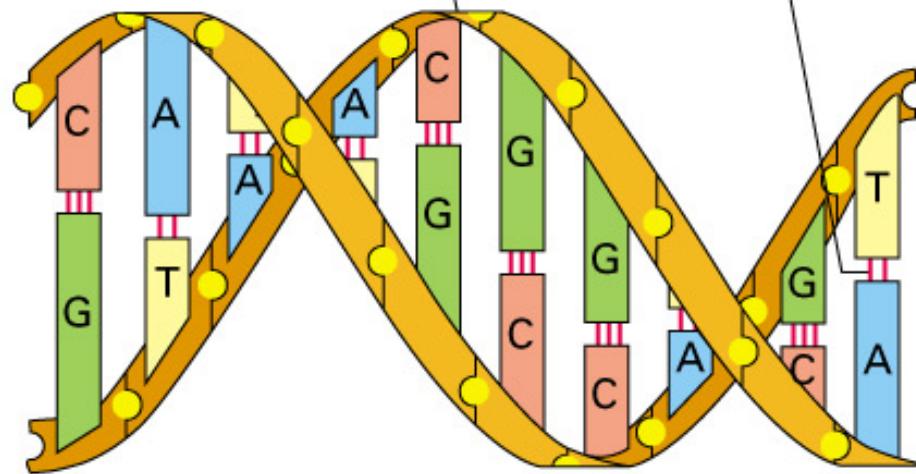
MSH2 IN MISMATCH REPAIR →
MUTATION → DISRUPTION OF CELL CYCLE →
COLON CANCER

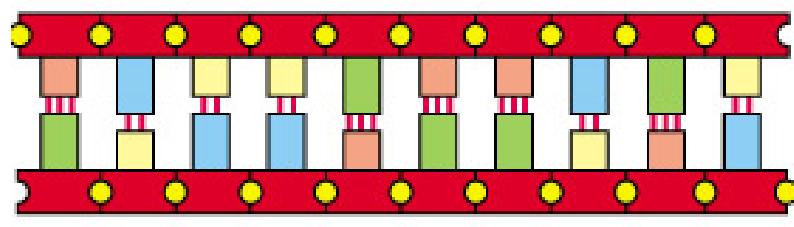
PLASMID PREPARATION
PRIMER DESIGN
PCR AMPLIFICATION
RESTRICTION ANALYSIS
DNA GEL ELECTROPHORESIS
YEAST TRANSFORMATION
TRANSFORMANTS IN COMPLEMENTATION ASSAY
TRANSFORMANT PROTEIN EXTRACTION
PROTEIN GEL ELECTROPHORESIS

(D) double-stranded DNA



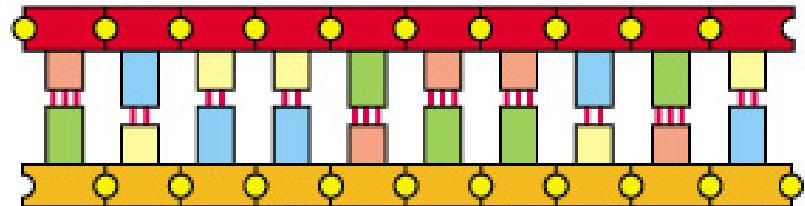
(E) DNA double helix





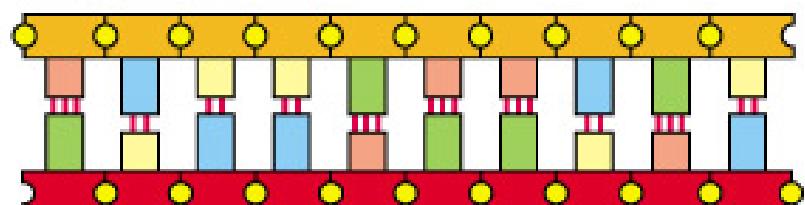
parent DNA double helix

template strand

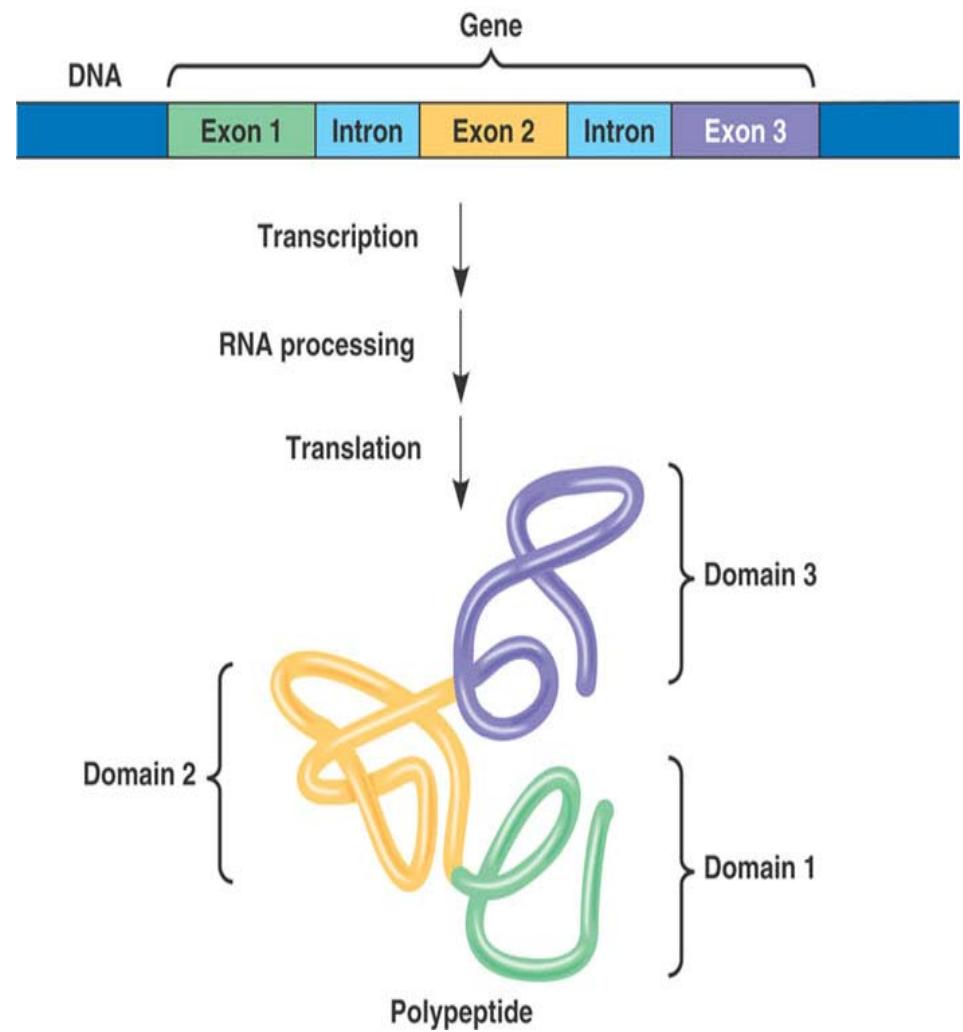
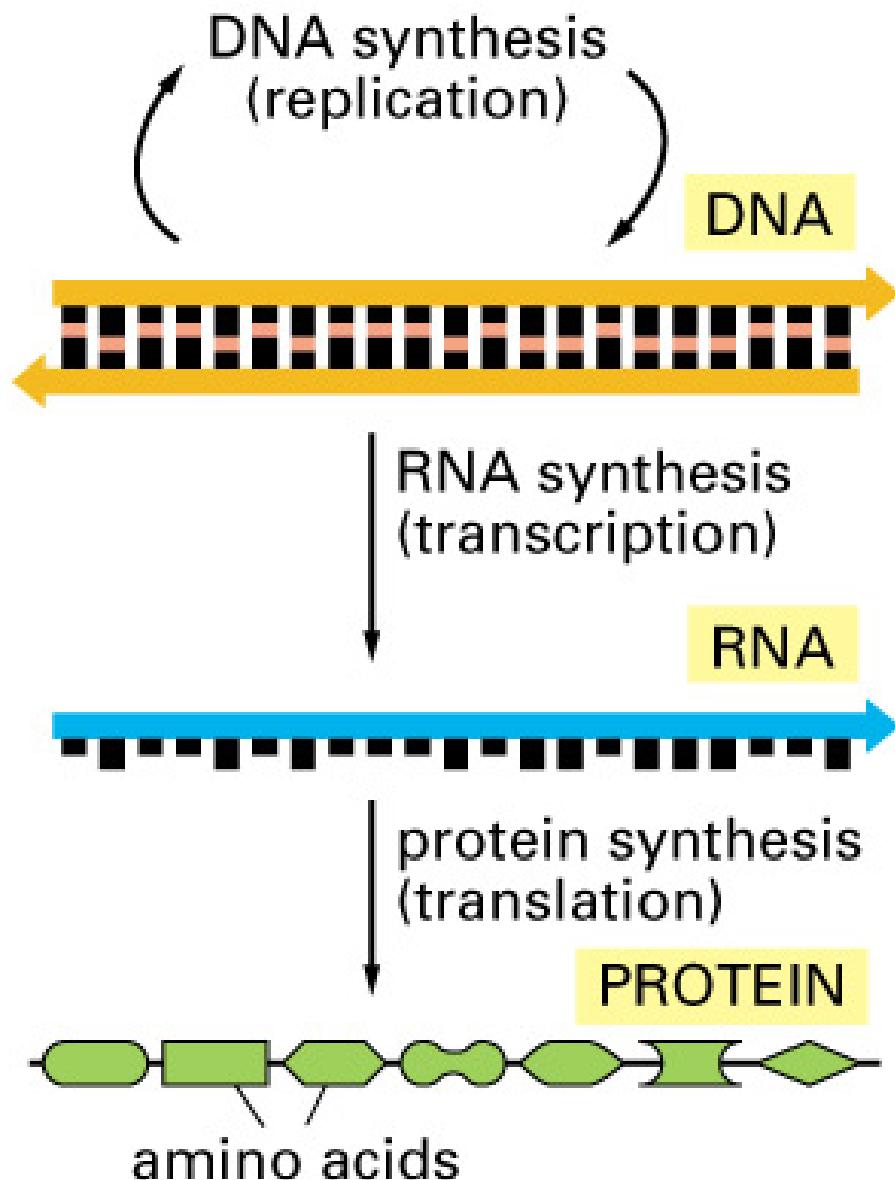


new strand

new strand



template strand



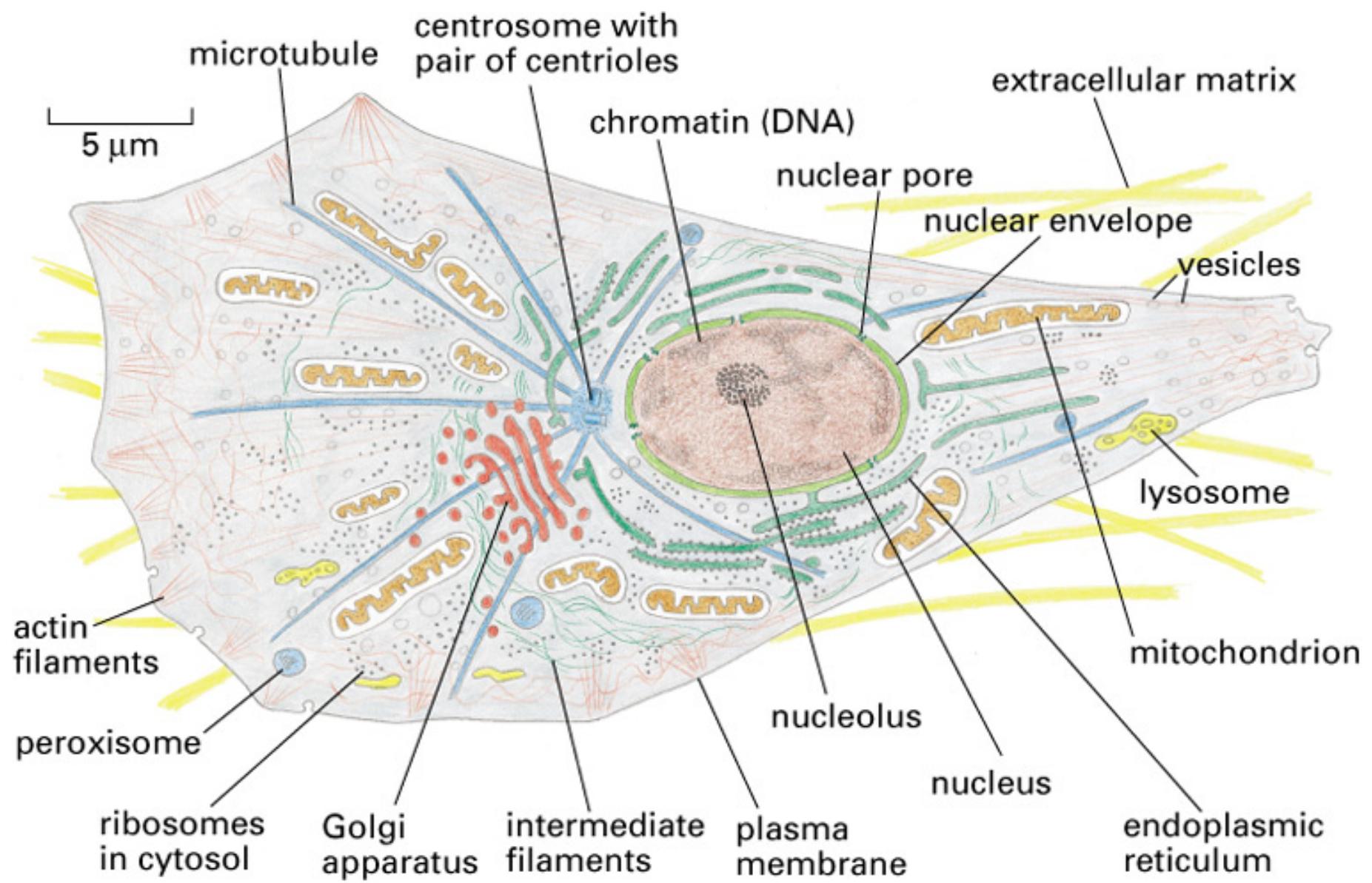
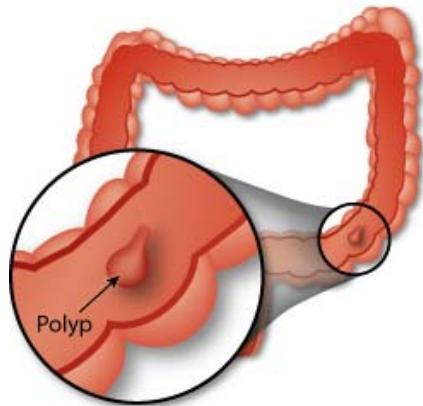


Figure 1–31. Molecular Biology of the Cell, 4th Edition.

Colorectal Cancer (CRC)

Two Genetically Different Forms

▪Familial Adenomatous Polyposis (FAP)

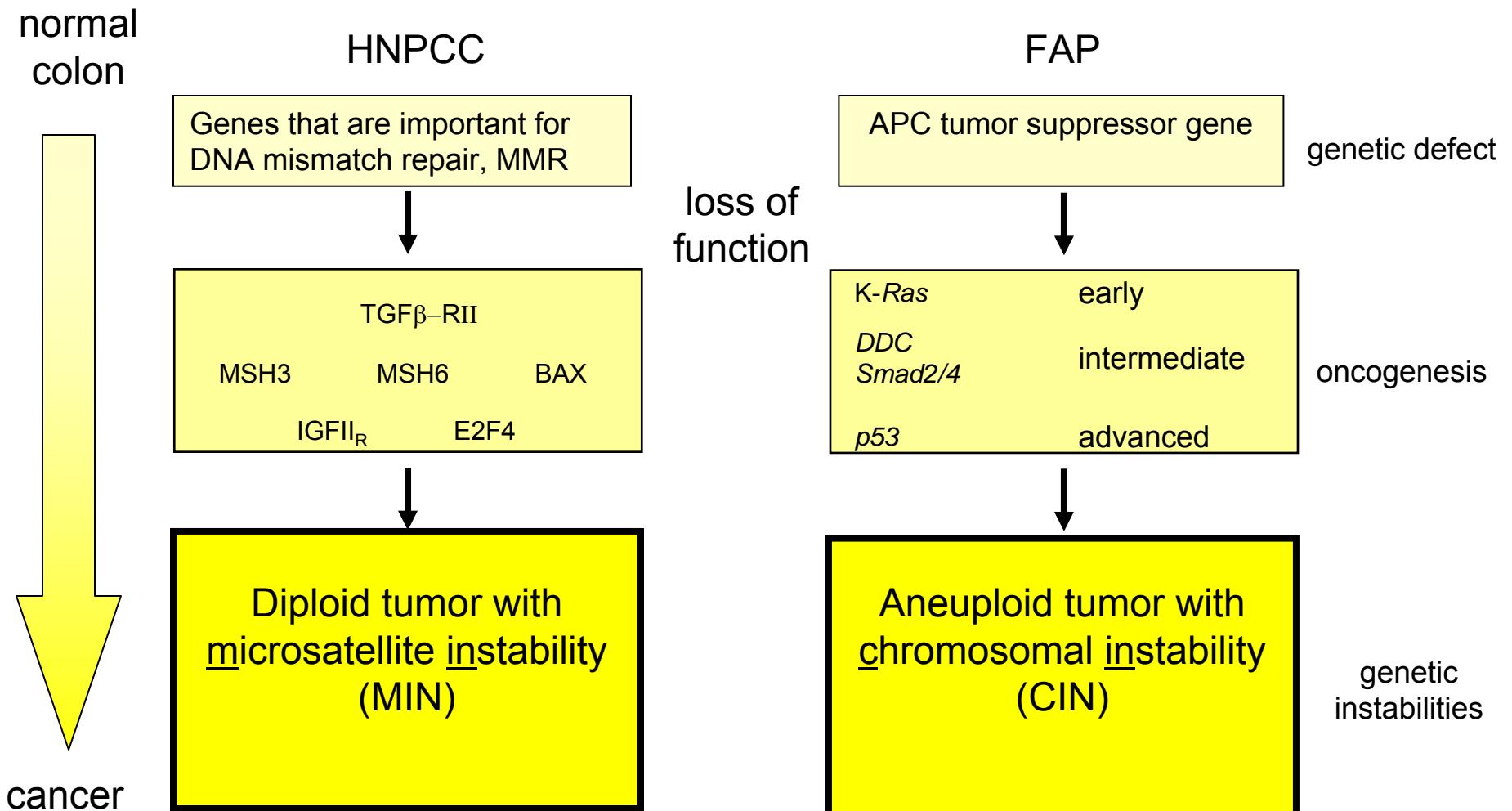


APC tumor suppressor gene
Adenomatous Polyposis Coli

Hereditary Non-Polyposis
Colorectal Cancer (HNPCC)

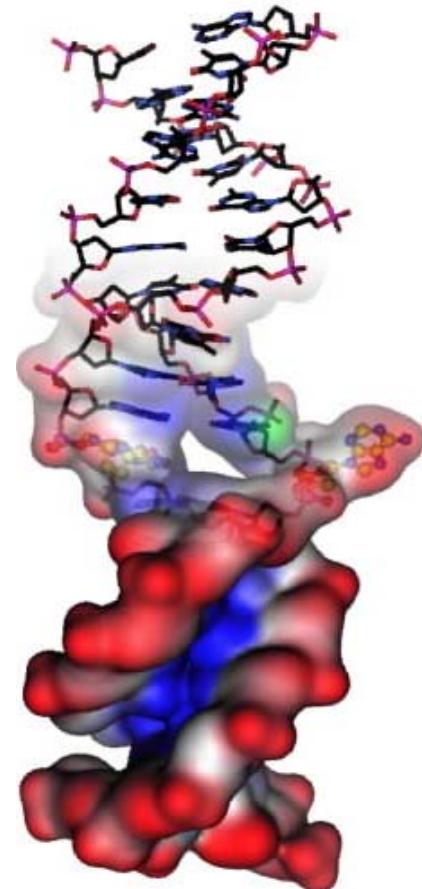
MMR DNA mismatch repair genes
important for DNA damage repair

Differences in FAP and HNPCC: genetic defect, oncogenesis and genetic instabilities (MIN vs. CIN)

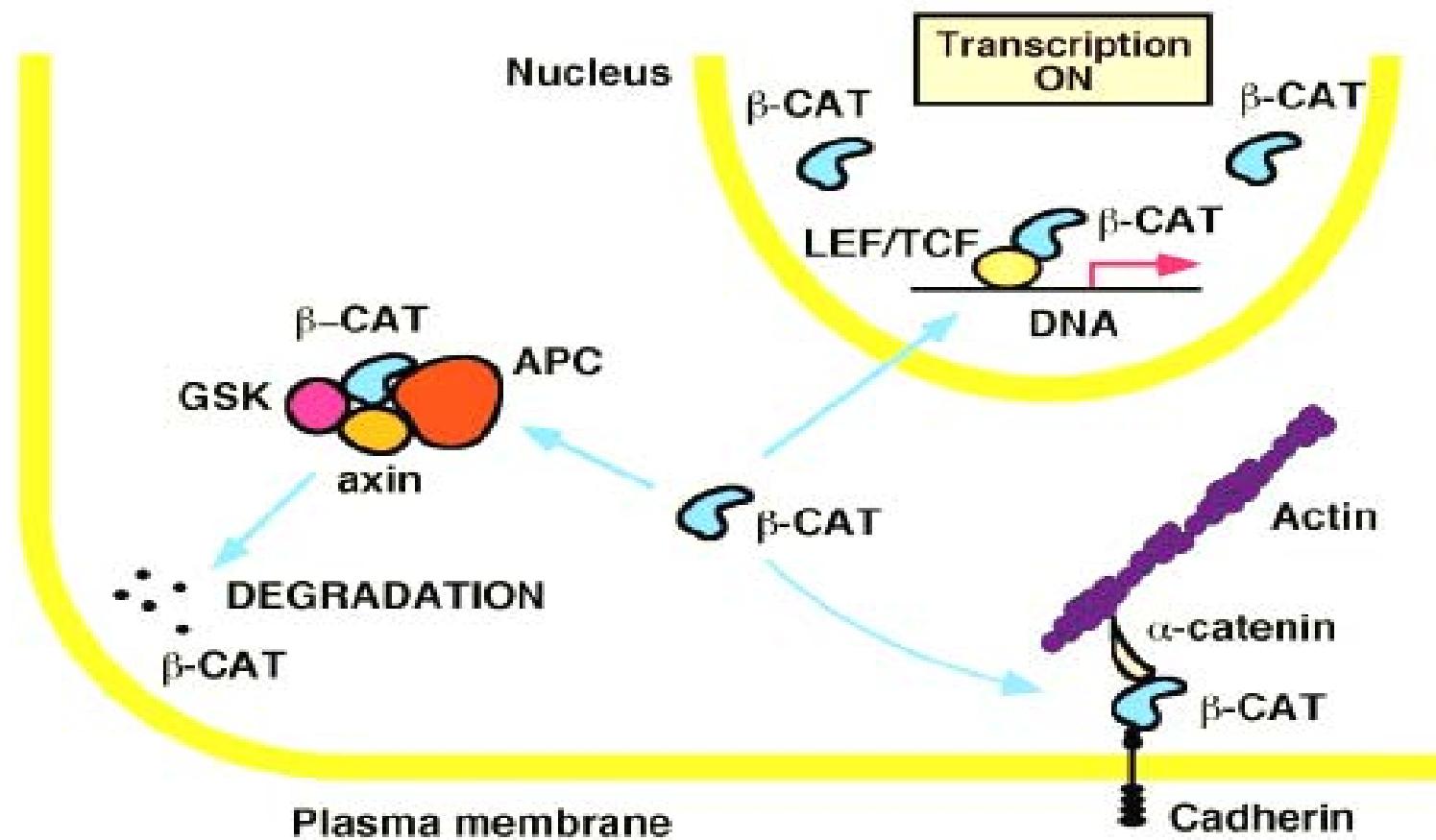


Agents and Factors that cause DNA Damage

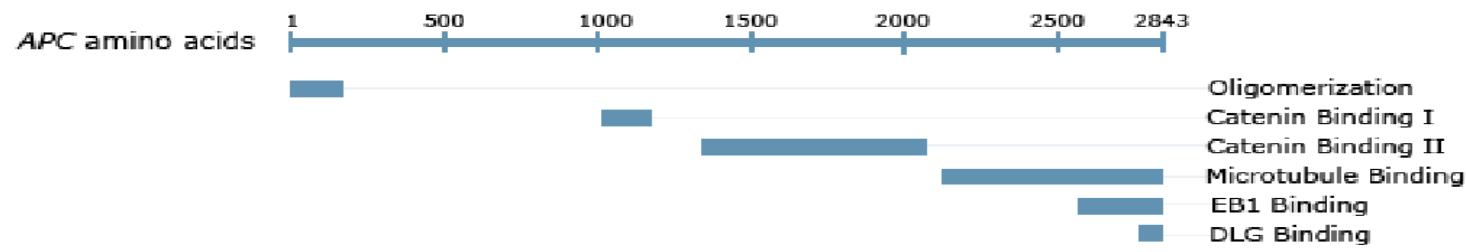
- | | |
|-----------------------|---|
| • Nucleotide analogs | BrdUTP |
| • Oxidizing agents | nitrous acid |
| • Alkylating agents | nitrosoguanidine |
| • Frameshift mutagens | Benz(a)pyrene
(polycyclic aromatic hydrocarbons) |
| • Ionizing radiation | X-rays, g-rays |
| • UV | UV, 260 nm |
| • Altered DNA Pol III | DNA PolIII (α) |
| • Error-prone repair | MutS, DNA PolIII |
| • Other mutator genes | <i>mutM, mutT, mutY</i> |



Signaling by β -catenin



Regions of APC that bind to other proteins.



Many afflicted families have mutations in MSH2
345 mapped (as of Jan 4, 2007)

~75% cause an obvious change

NO PROTEIN PRODUCED

~25% cause a very small change

MISSENSE MUTATIONS

Can we be sure that the “little” changes cause the disease?
we must be sure for genetic counseling

Types of DNA Damage Repair

Direct Repair of Damage

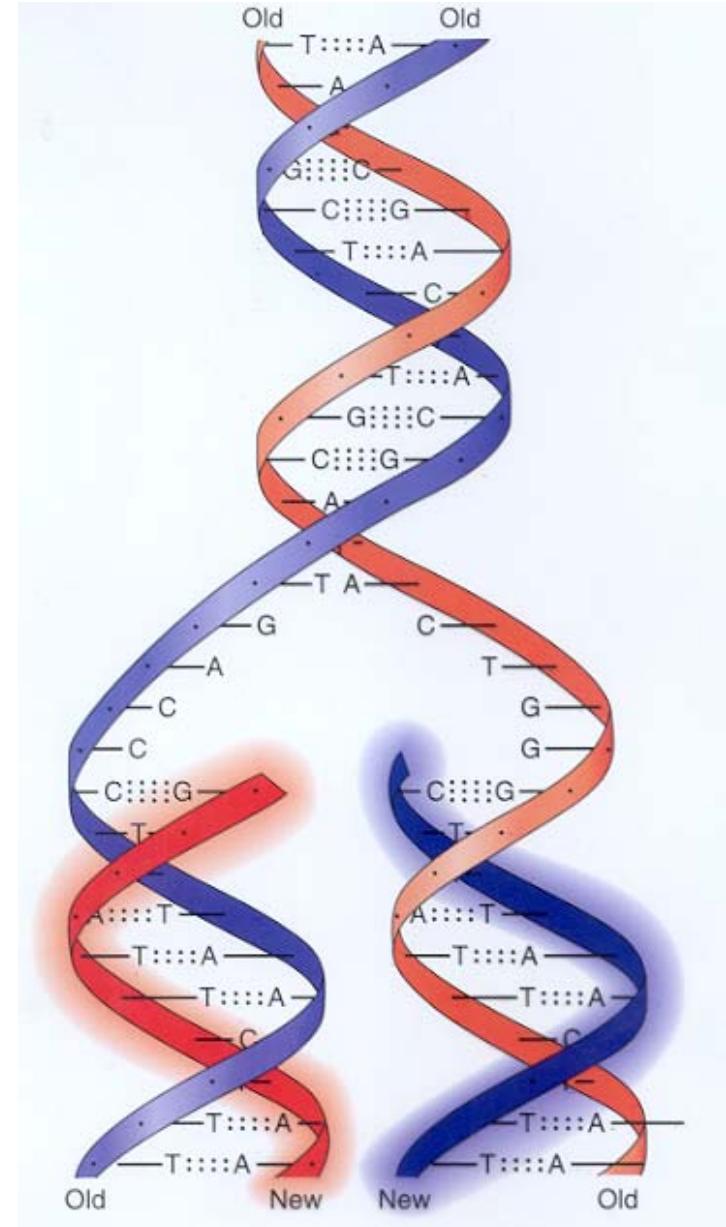
Mismatch Repair

Base Excision Repair

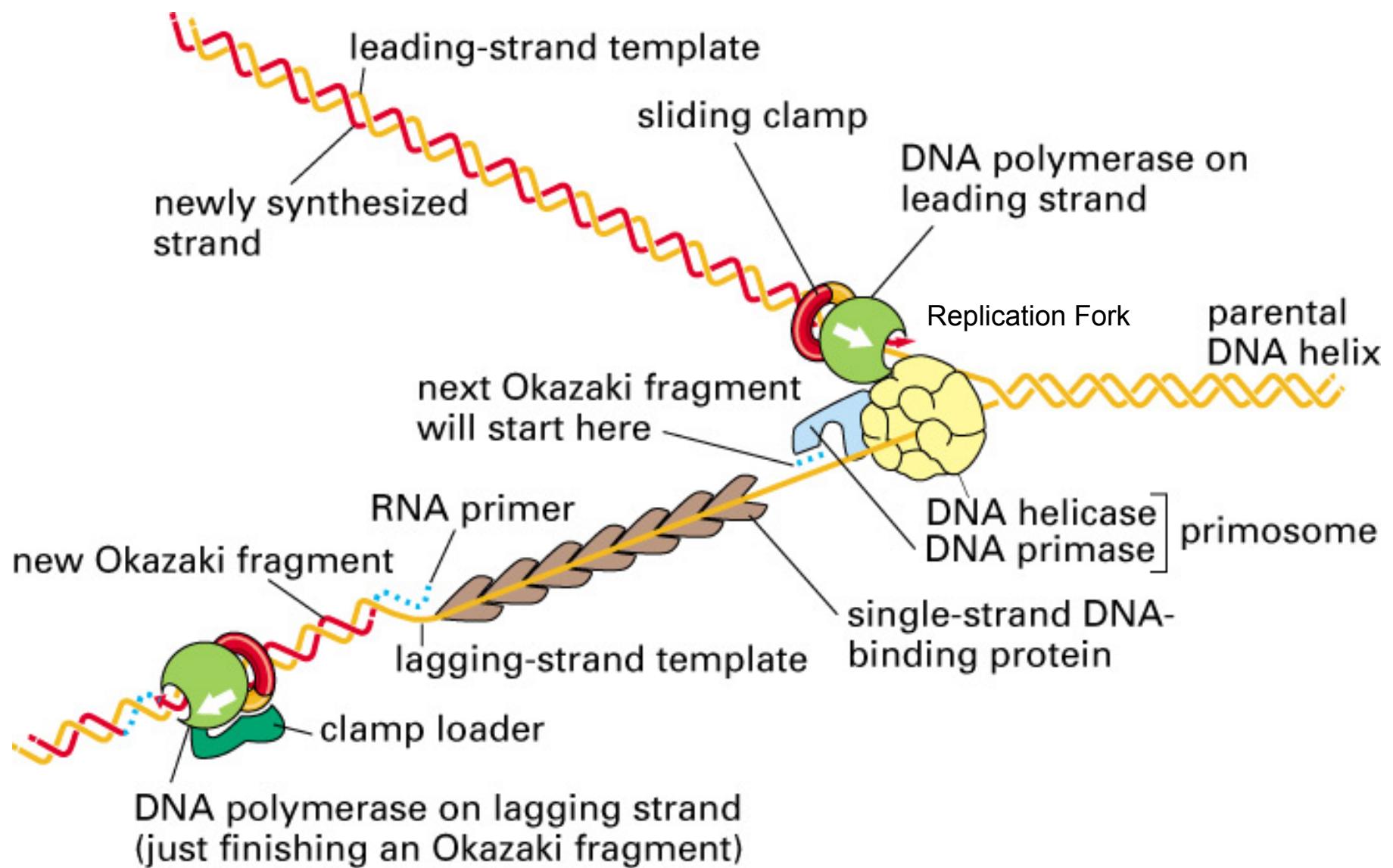
Nucleotide Excision Repair

Double-strand Break Repair

Damage Bypass



Review of Replication



From, Molecular Biology of the Cell

Mechanism of Mismatch repair

Recognition

Protein Binding

Incision

Degradation

Synthesis of new strand

Complement

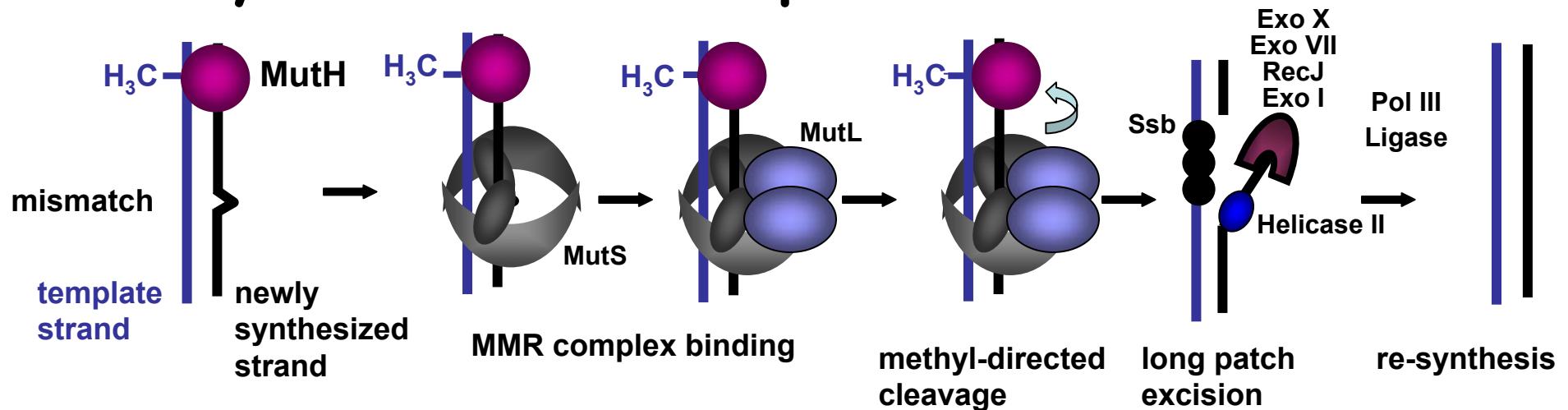
Closing gaps

Genes Encoding Enzymes of Mismatch Repair

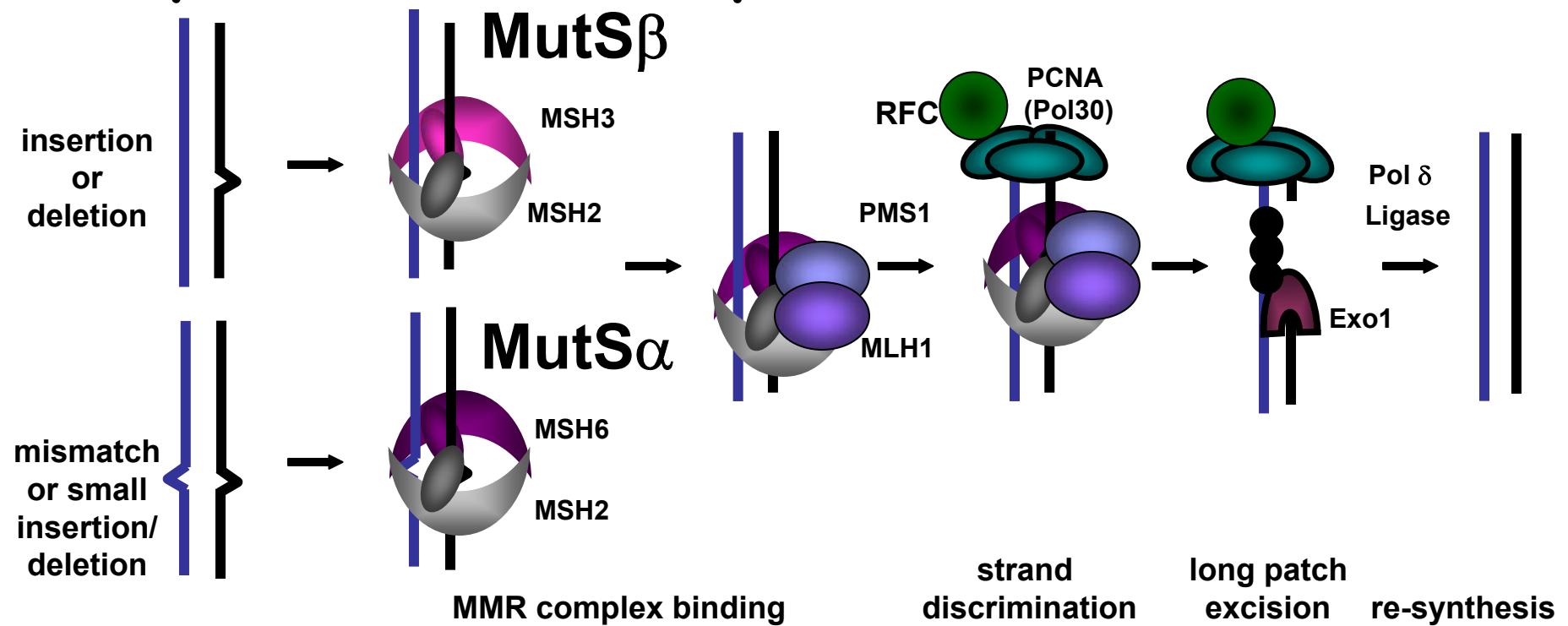
<i>E. coli</i>	<i>S. cerevisiae</i>	Human	Functions of Eukaryotic Proteins
MutS	MSH2	MSH2	MutS α (with MSH6; 80-90%); MutS β (with MSH3)
"	MSH3	MSH3	MutS β (with MSH2); repair of larger loops
"	MSH6	MSH6	MutS α (with MSH2); repair of mismatches and small loops
MutL	MLH1	MLH1	Forms heterodimers with the other three MutL homologs
"	PMS1	PMS2	MutL α (90%); Mismatch repair; endonuclease motif
"	MLH2	PMS1	MutL β ; Role unknown
"	MLH3	MLH3	MutL γ ; Mismatch repair; endonuclease motif
MutH	?	?	?
uvrD	?	?	?
?	Exonuclease I	Exonuclease I	Excision (5' to 3' polarity)
?	RFC, PCNA, Poly γ	RFC, PCNA, Poly γ	Nick identification; gap filling

The eukaryotic genes are homologs of the corresponding *E. coli* genes both in terms of amino acid sequence and in terms of functional similarities. Whereas **MutS** and **MutL** function as homodimers, the eukaryotic proteins function as heterodimers. Heterodimers of MutS homologs are responsible for initial recognition of mismatches and small insertions/deletions, and heterodimers of MutL homologs interact with the resulting complex, as in *E. coli*.

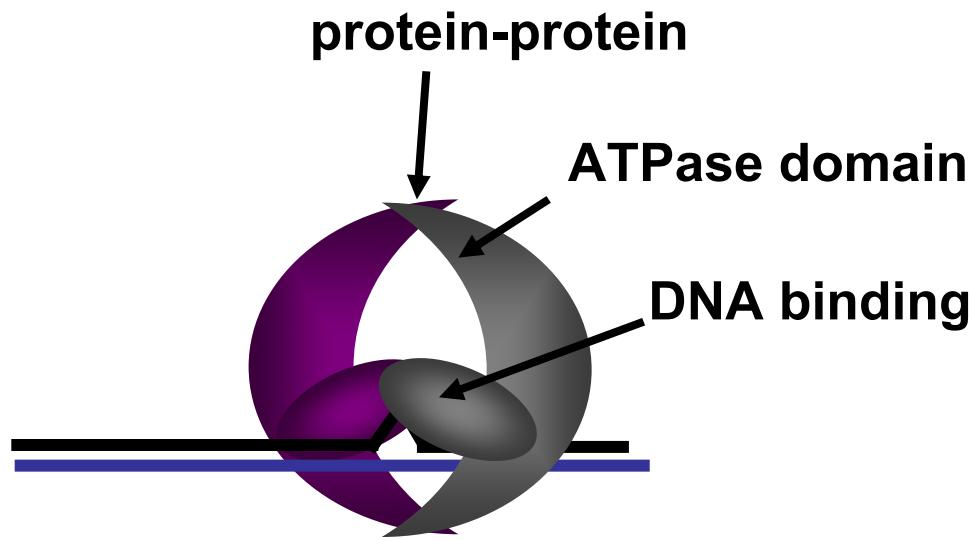
Prokaryotic Mismatch Repair



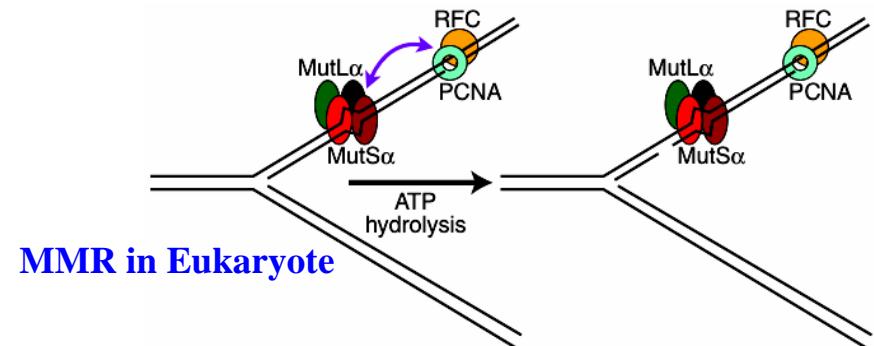
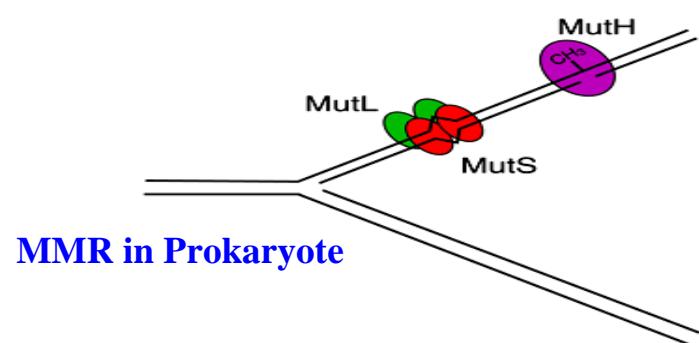
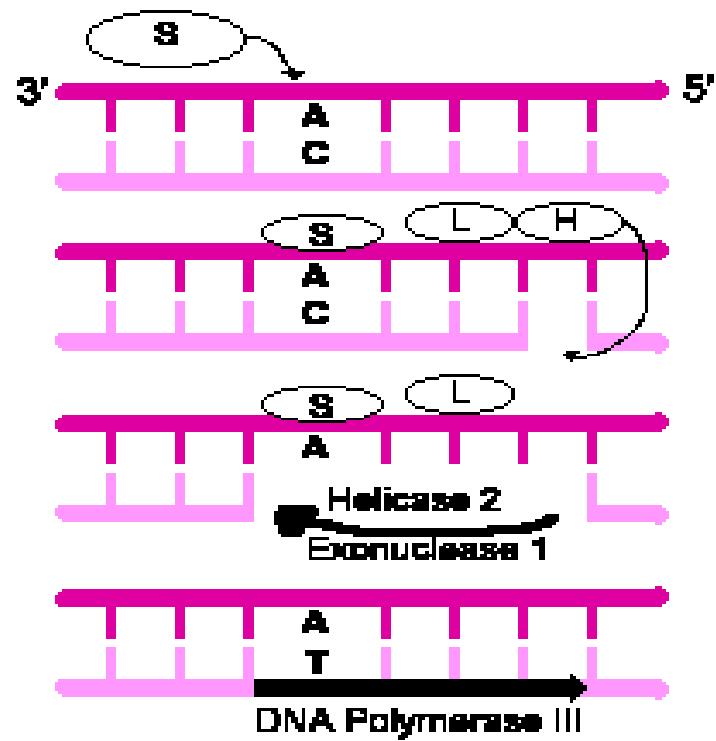
Eukaryotic Mismatch Repair



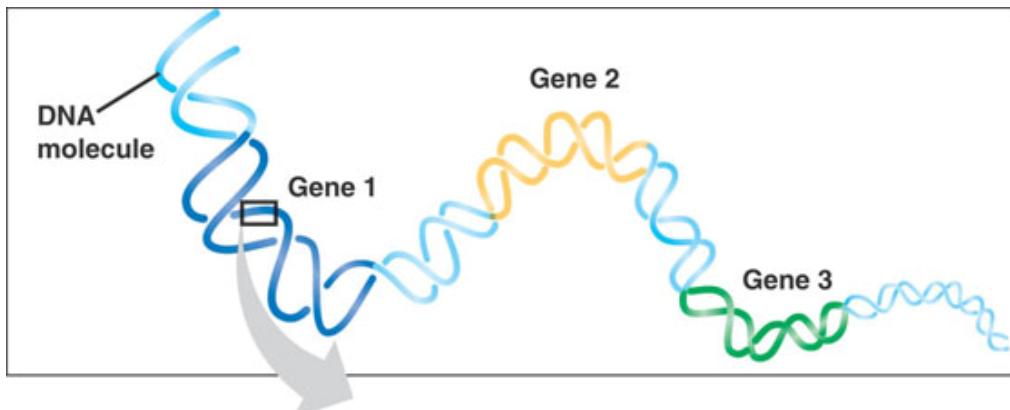
Msh2p Important Domains



- Structural integrity residues (throughout)
- DNA binding region
- Protein-protein interacting regions
- ATPase domain



Review of Gene Expression



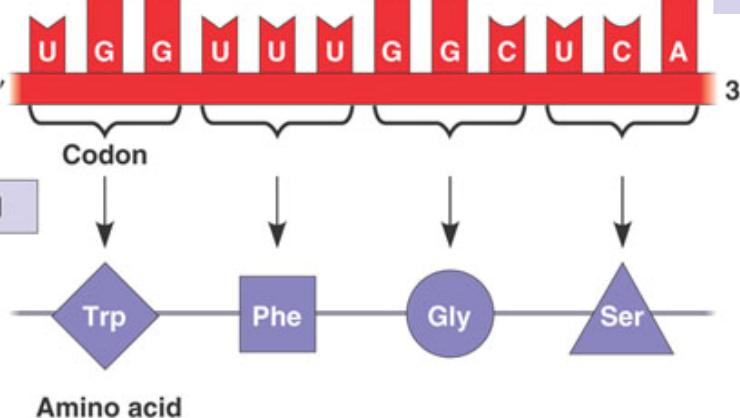
DNA strand (template) 3' A C C A A A C C G A G T 5' ORF Open Reading Frame

TRANSCRIPTION

mRNA

TRANSLATION

Protein



Flow of Genetic Information: Gene to Protein



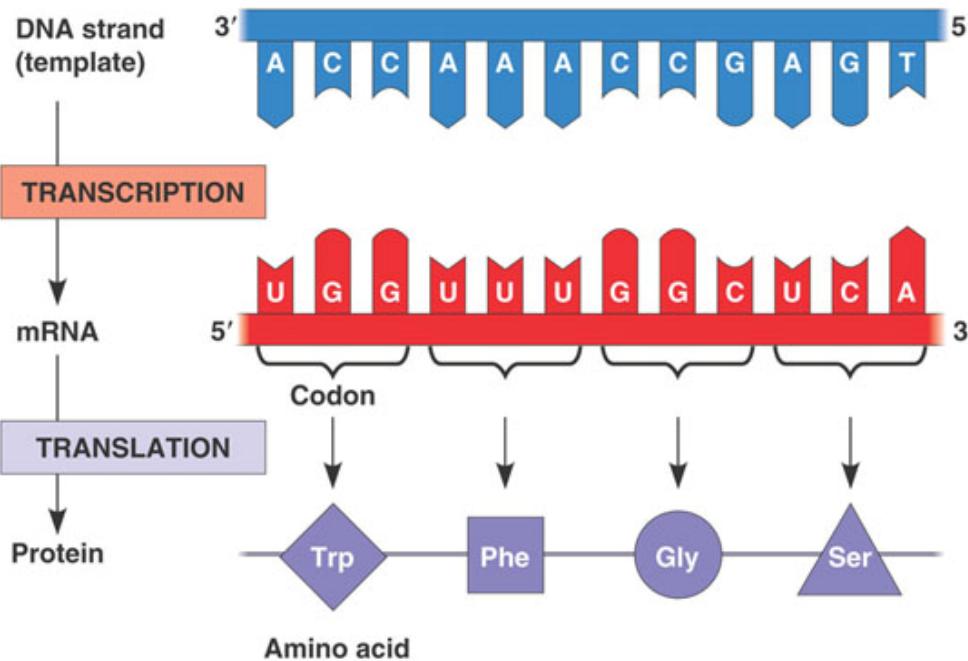
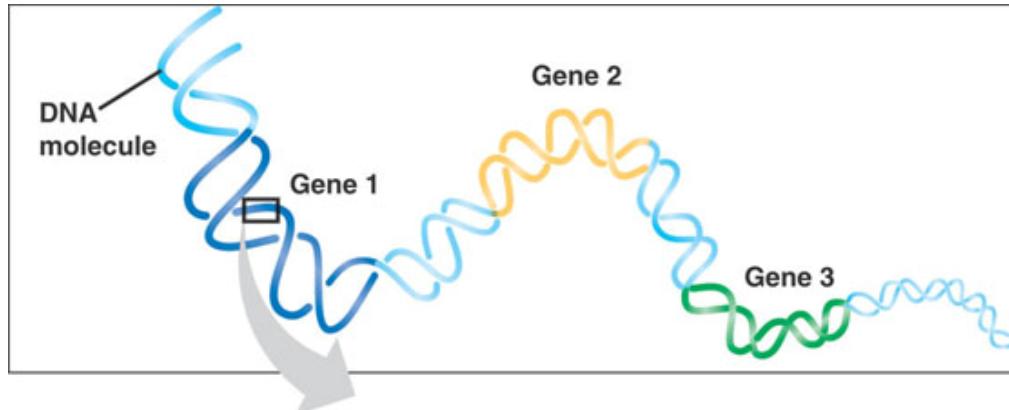
AMINO ACID

Aspartic acid	Asp	D
Glutamic acid	Glu	E
Arginine	Arg	R
Lysine	Lys	K
Histidine	His	H
Asparagine	Asn	N
Glutamine	Gln	Q
Serine	Ser	S
Threonine	Thr	T
Tyrosine	Tyr	Y

AMINO ACID

Alanine	Ala	A
Glycine	Gly	G
Valine	Val	V
Leucine	Leu	L
Isoleucine	Ile	I
Proline	Pro	P
Phenylalanine	Phe	F
Methionine	Met	M
Tryptophan	Trp	W
Cysteine	Cys	C

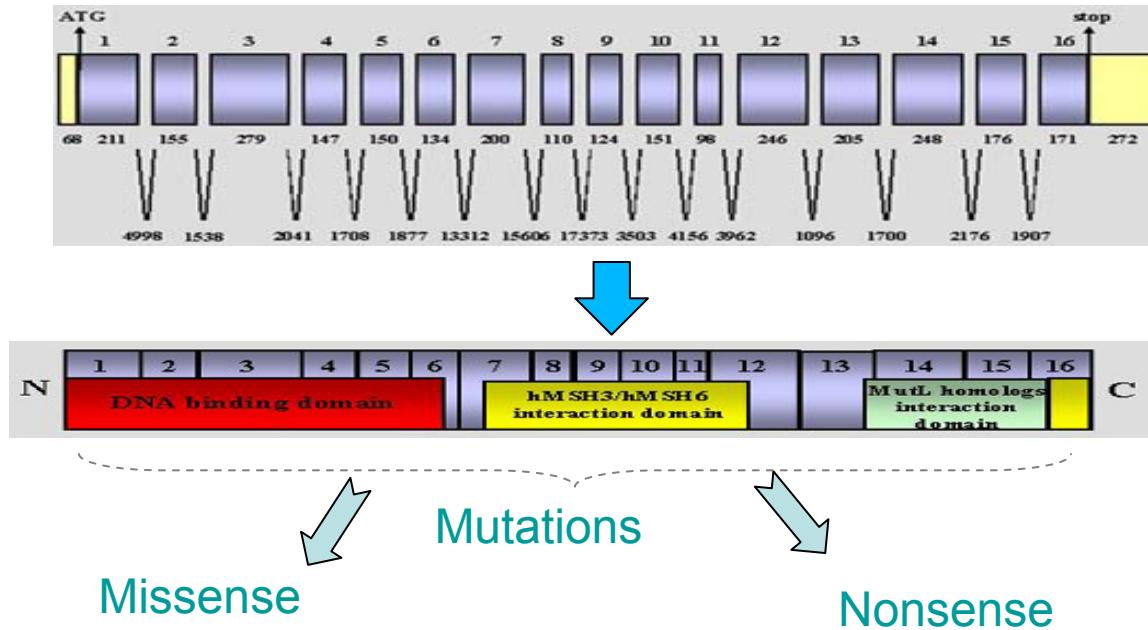
Review of Gene Expression



Flow of Genetic Information: Gene to Protein

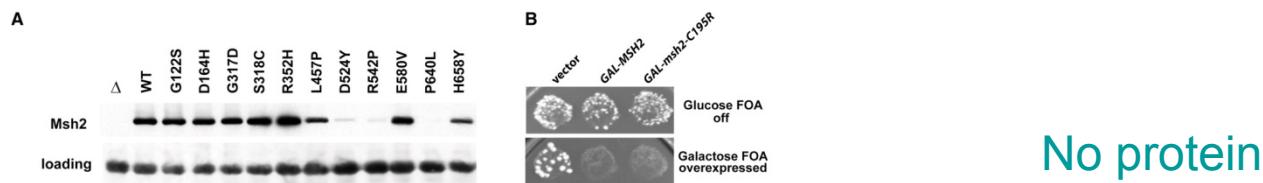
ORF Open Reading Frame				
	2nd base			
	U	C	A	
U	UUU (Phe/F) Phenylalanine	UCU (Ser/S) Serine	UAU (Tyr/Y) Tyrosine	UGU (Cys/C) Cysteine
	UUC (Phe/F) Phenylalanine	UCC (Ser/S) Serine	UAC (Tyr/Y) Tyrosine	UGC (Cys/C) Cysteine
	UUA (Leu/L) Leucine	UCA (Ser/S) Serine	UAA Ochre (Stop)	UGA Opal (Stop)
	UUG (Leu/L) Leucine	UCG (Ser/S) Serine	UAG Amber (Stop)	UGG (Trp/W) Tryptophan
	CUU (Leu/L) Leucine	CCU (Pro/P) Proline	CAU (His/H) Histidine	CGU (Arg/R) Arginine
	CUC (Leu/L) Leucine	CCC (Pro/P) Proline	CAC (His/H) Histidine	CGC (Arg/R) Arginine
	CUA (Leu/L) Leucine	CCA (Pro/P) Proline	CAA (Gln/Q) Glutamine	CGA (Arg/R) Arginine
	CUG (Leu/L) Leucine	CCG (Pro/P) Proline	CAG (Gln/Q) Glutamine	CGG (Arg/R) Arginine
	AUU (Ile/I) Isoleucine	ACU (Thr/T) Threonine	AAU (Asn/N) Asparagine	AGU (Ser/S) Serine
	AUC (Ile/I) Isoleucine	ACC (Thr/T) Threonine	AAC (Asn/N) Asparagine	AGC (Ser/S) Serine
	AUA (Ile/I) Isoleucine	ACA (Thr/T) Threonine	AAA (Lys/K) Lysine	AGA (Arg/R) Arginine
	AUG (Met/M) Methionine, Start [A]	ACG (Thr/T) Threonine	AAG (Lys/K) Lysine	AGG (Arg/R) Arginine
C	GUU (Val/V) Valine	GCU (Ala/A) Alanine	GAU (Asp/D) Aspartic acid	GGU (Gly/G) Glycine
	GUC (Val/V) Valine	GCC (Ala/A) Alanine	GAC (Asp/D) Aspartic acid	GGC (Gly/G) Glycine
	GUA (Val/V) Valine	GCA (Ala/A) Alanine	GAA (Glu/E) Glutamic acid	GGAG (Gly/G) Glycine
	GUG (Val/V) Valine	GCG (Ala/A) Alanine	GAG (Glu/E) Glutamic acid	GGG (Gly/G) Glycine

Human MSH2 gene has 16 exons, coding for one protein with three functional domains:

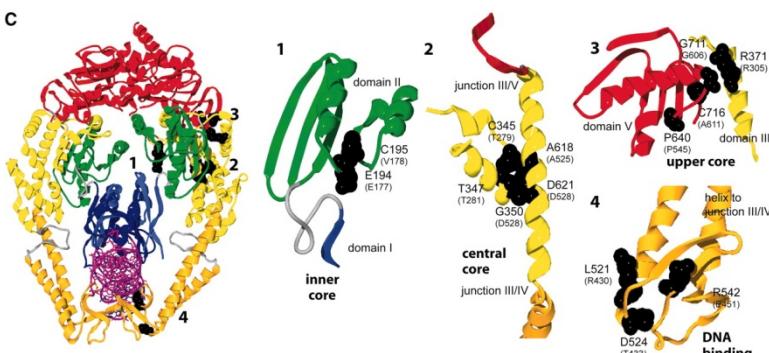


hMSH2 gene

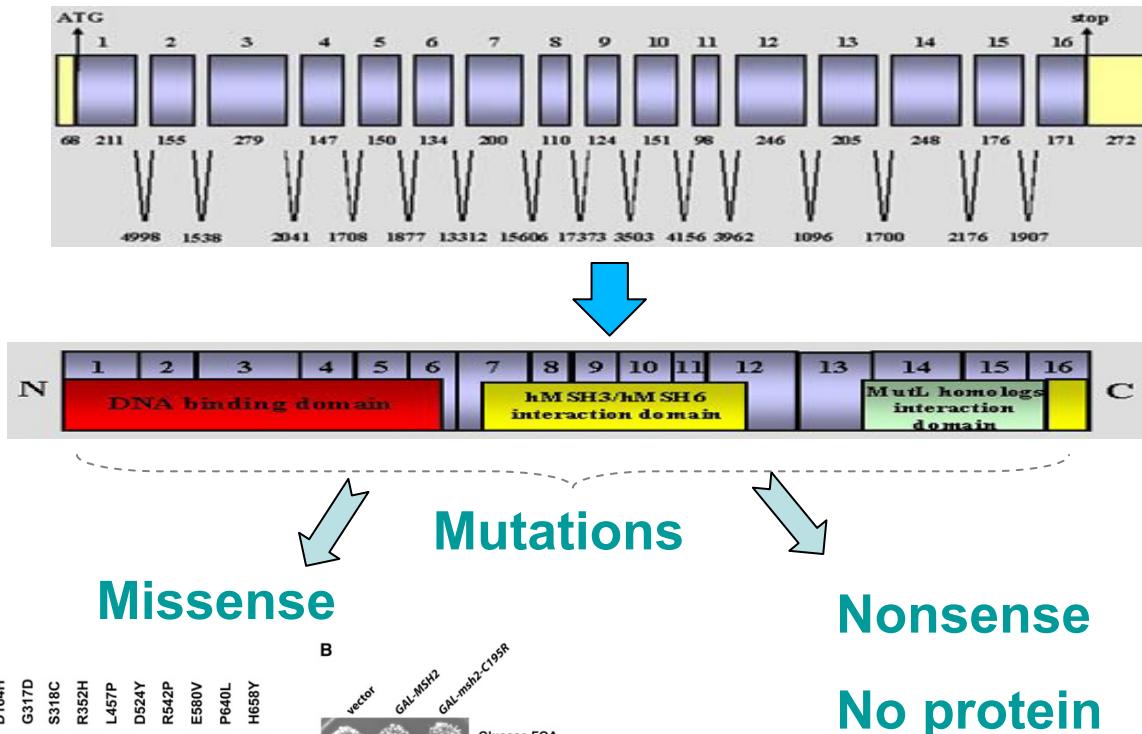
hMSH2 gene
product (protein)



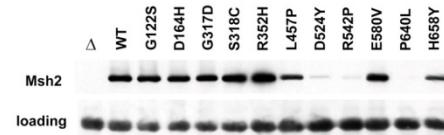
No protein



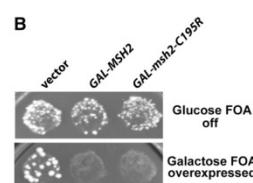
Human MSH2 gene has 16 exons, coding for one protein with three functional domains:



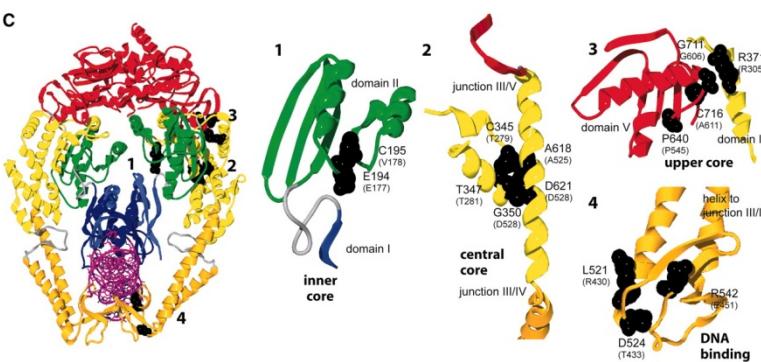
A



B



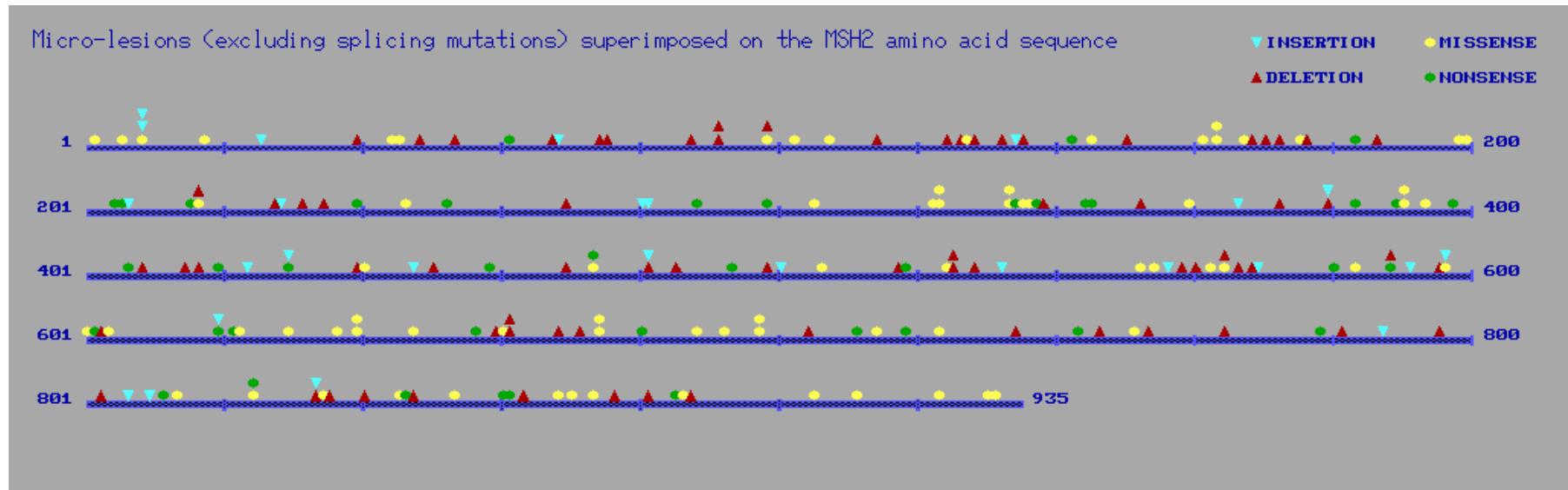
C



AMINO ACID

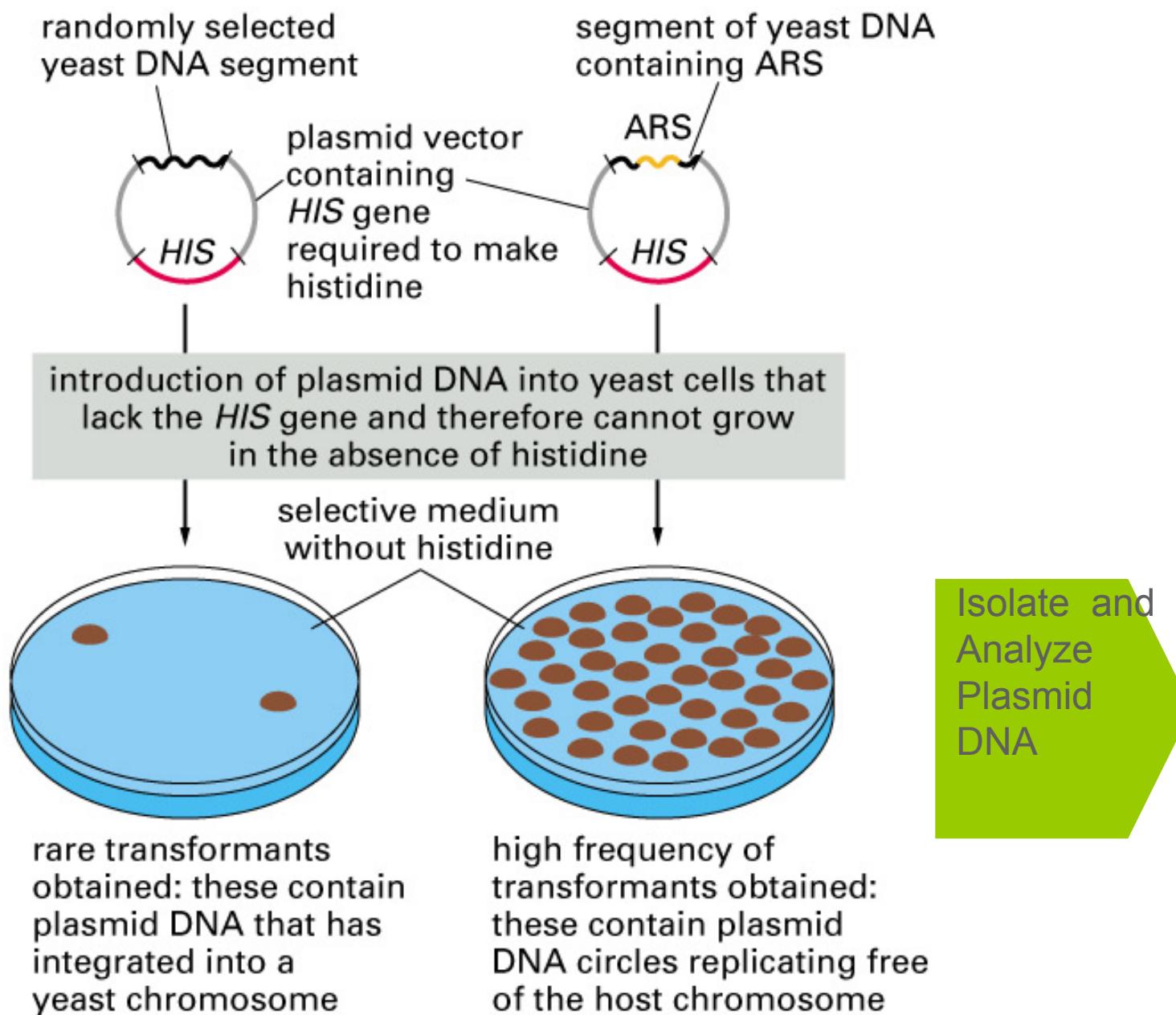
Aspartic acid	Asp	D	Alanine	Ala	A
Glutamic acid	Glu	E	Glycine	Gly	G
Arginine	Arg	R	Valine	Val	V
Lysine	Lys	K	Leucine	Leu	L
Histidine	His	H	Isoleucine	Ile	I
Asparagine	Asn	N	Proline	Pro	P
Glutamine	Gln	Q	Phenylalanine	Phe	F
Serine	Ser	S	Methionine	Met	M
Threonine	Thr	T	Tryptophan	Trp	W
Tyrosine	Tyr	Y	Cysteine	Cys	C

Project Overview

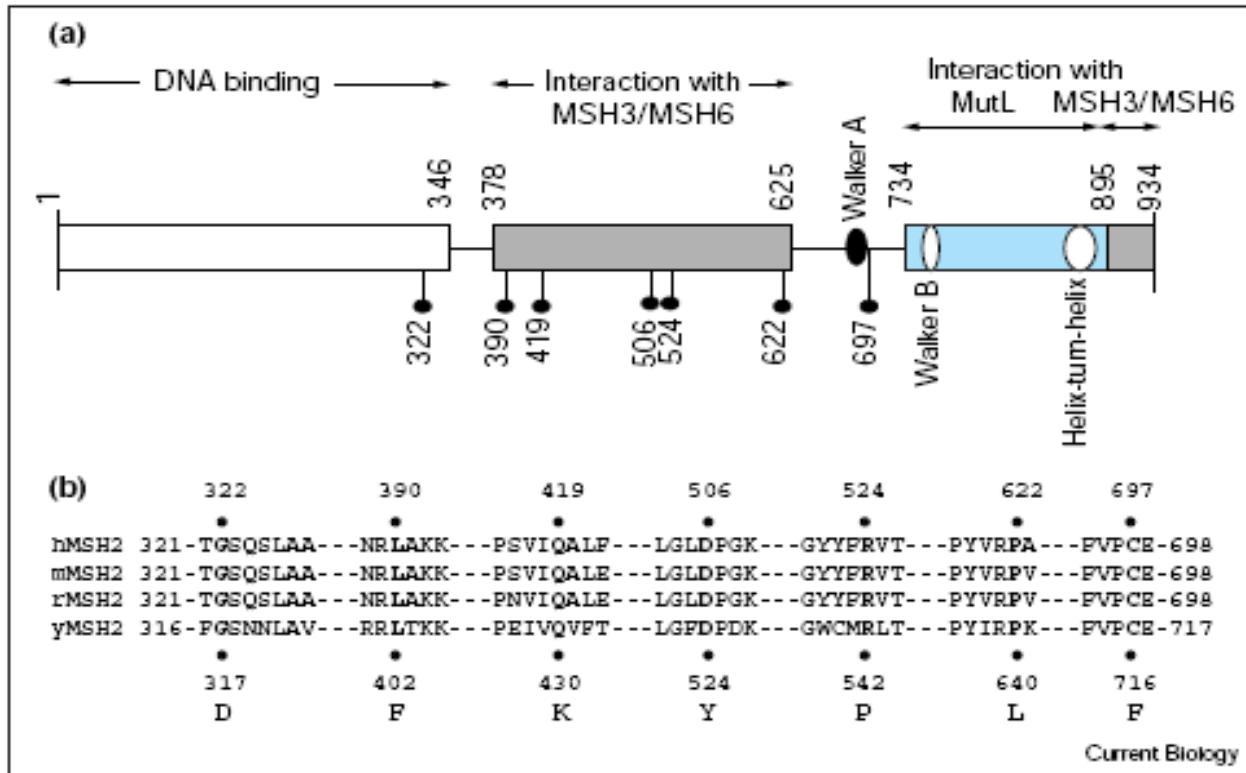


Manipulate the yeast *MSH2* gene to determine which human missense mutations are likely to be benign or pathogenic in nature.

Examine the defect at a molecular level to determine why the Msh2 variants are dysfunctional.



From, MolecularBiology of the Cell



Representations of MSH2. (a) Putative functional regions of hMSH2. An amino-terminal DNA-binding region, a region that interacts with the mismatch-repair protein MutL (blue), and a carboxy-terminal MutS dimerization region are suggested by studies of deletion mutants of *E. coli* MutS [20]. Amino acids 827–846 comprise a helix-turn-helix motif suggested to interact with yMSH6 [21]. Studies of hMSH2 have implicated two regions in heterodimerization of hMSH2 with MSH3 and MSH6 (grey) [22]. Also indicated are the Walker A and B motifs required for ATP binding and hydrolysis [23]. The numbers above the boxes indicate amino acids delineating the functional regions; those below correspond to the missense mutations. (b) Alignment of human, mouse, rat and yeast MSH2. The numbers above the alignment correspond to hMSH2 amino-acid positions; those below correspond to yMSH2 residues. The missense mutations are indicated below the alignment.

MSH2 missense mutations examined in this study.

Mutation in hMSH2	Homologous mutation in yMSH2	Allele frequency	Role in HNPCC*	Role in sporadic or early onset colon cancer*
R524P	R542P	ND	+†	-
P622L	P640L	ND	+ [7]	-
C697F	C716F	ND	+ [8]	-
D506Y	D524Y	ND	-	+ [9]
G322D	G317D	1–6% [8,10–14]	+ [17]	+ [15,16]
Q419K	Q430K	1% [18]	-	-
L390F	L402F	2% [18]	+ [19]	-

The mutations are indicated in the single-letter amino-acid code.

*These columns indicate whether a particular polymorphism has (+) or has not (–) been implicated in the development of HNPCC, or of

sporadic or early onset colon cancer. †Although described as HNPCC [4], this patient did not fulfill the Amsterdam criteria and had ovarian cancer. ND, not determined.

Mid-Term Exam is March 5

Questions on Everything we've done so far.
Multiple Choice, Written, Concept, Workbench

Plasmid Preparation

Design Primer for your mutant.

PCR all plasmid Preps

Restriction Analysis of PCR products

Prepare Poster Abstract (due next week)

Focusing on Mismatch Repair

Cloning Strategy for MSH2 Gene

The cloning of a gene uses recombinant DNA technology. It is the process by which a group of identical DNA (gene) molecules can be created from a single ancestor.

When a target DNA fragment (gene) is inserted in a cloning vector (plasmid, viral etc), the recombinant cloning vector can be amplified, in which case the inserted DNA fragment is also amplified.

Cloning Strategy for MSH2 Gene

The recombinant cloning vector can be transferred into living cells. All progenies of the transformed cell will contain identical copies of the vehicle and the DNA fragment inserted into it.

The DNA insert can also be amplified using Polymerase Chain Reaction (PCR).

Mechanism of Mismatch repair

Recognition

Protein Binding

Incision

Degradation

Synthesis of new strand

Complement

Closing gaps

In prokaryotes, the enzyme complex MutH-MutL-MutS catalyzes mismatch repair. The MutS is a homodimer that binds mismatch DNA

After mismatch recognition in prokaryotes, MutL homodimer complexes with MutS and initiates subsequent repair events

In eukaryotes, MutS homolog (MSH) is a heterodimer

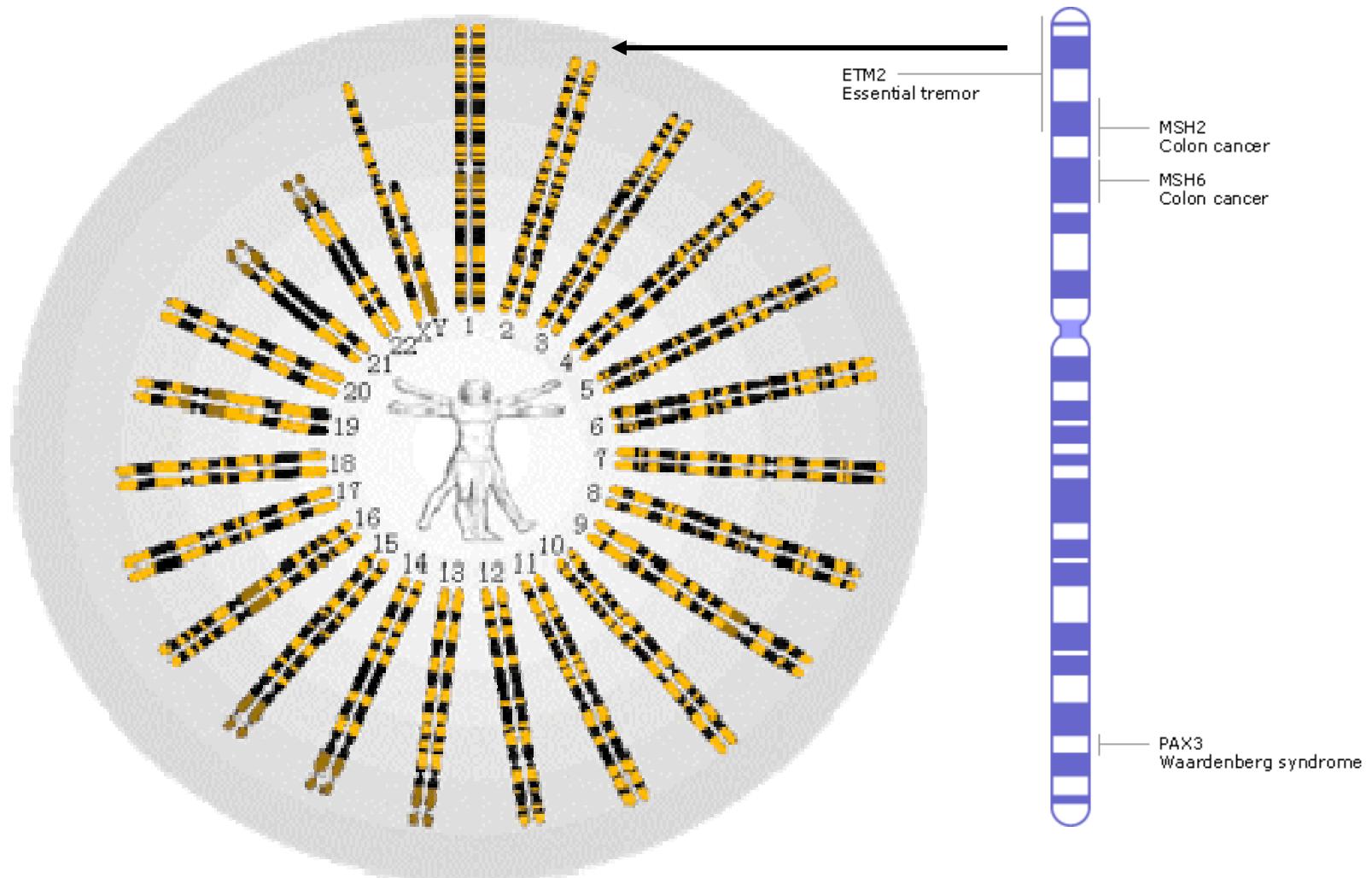
- 1) MutS α (Msh2 + Msh6)
- 2) MutS β (Msh2 + Msh3)

MutS α binds single base pair mismatches, small insertions and deletions

MutS β binds larger insertions/deletion loops up to 16 nucleotides

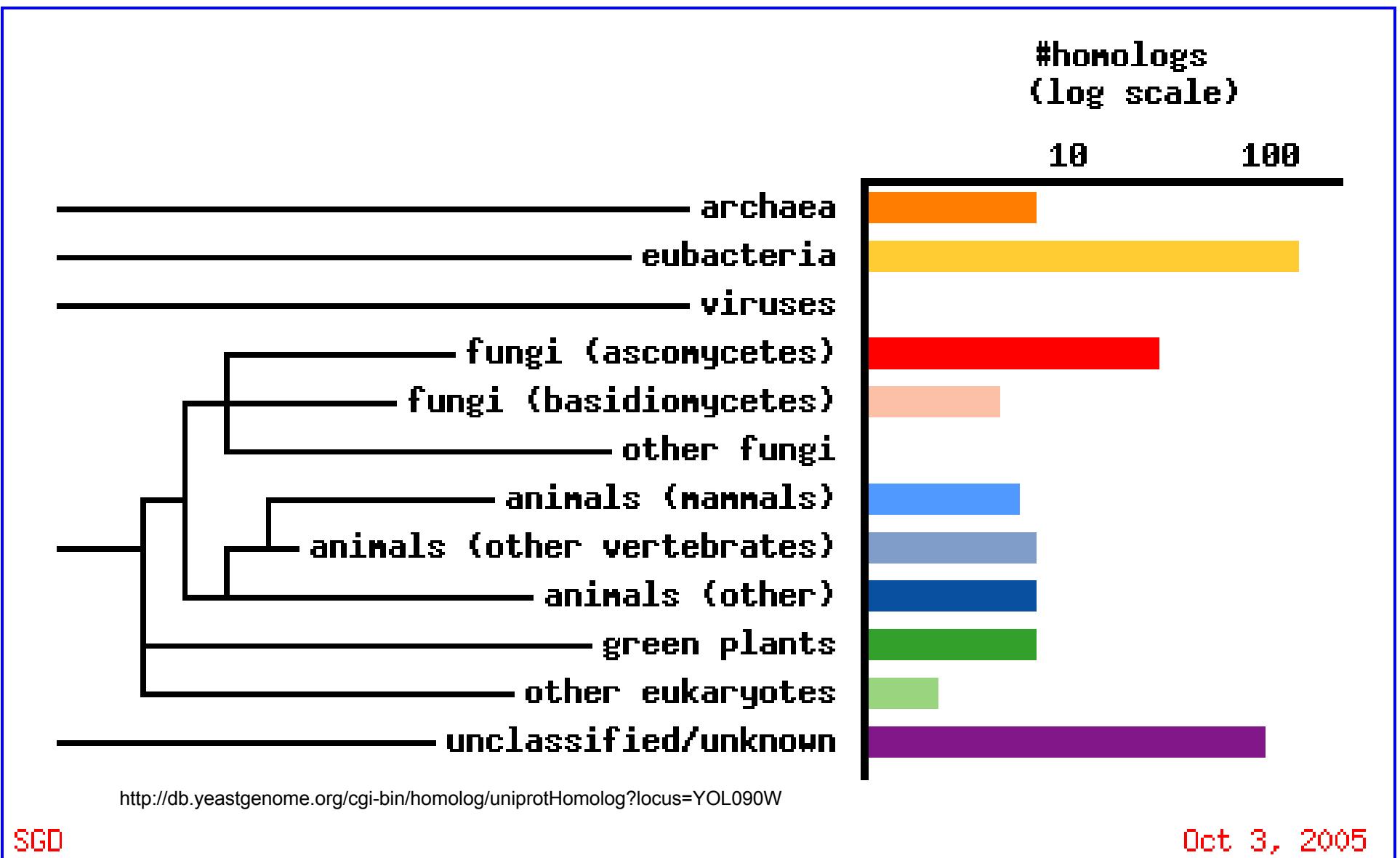
In eukaryotes, MutLa (MLH1 + PMS2), bind Msh2 to initiate repair process.

<i>E. coli</i>	<i>S. cerevisiae</i>	Human	Functions of Eukaryotic Proteins
MutS	MSH2	MSH2	MutS α (with MSH6; 80-90%); MutS β (with MSH3)
"	MSH3	MSH3	MutS β (with MSH2); repair of larger loops
"	MSH6	MSH6	MutS α (with MSH2); repair of mismatches and small loops
MutL	MLH1	MLH1	Forms heterodimers with the other three MutL homologs
"	PMS1	PMS2	MutL α (90%); Mismatch repair; endonuclease motif
"	MLH2	PMS1	MutL β ; Role unknown
"	MLH3	MLH3	MutL γ ; Mismatch repair; endonuclease motif

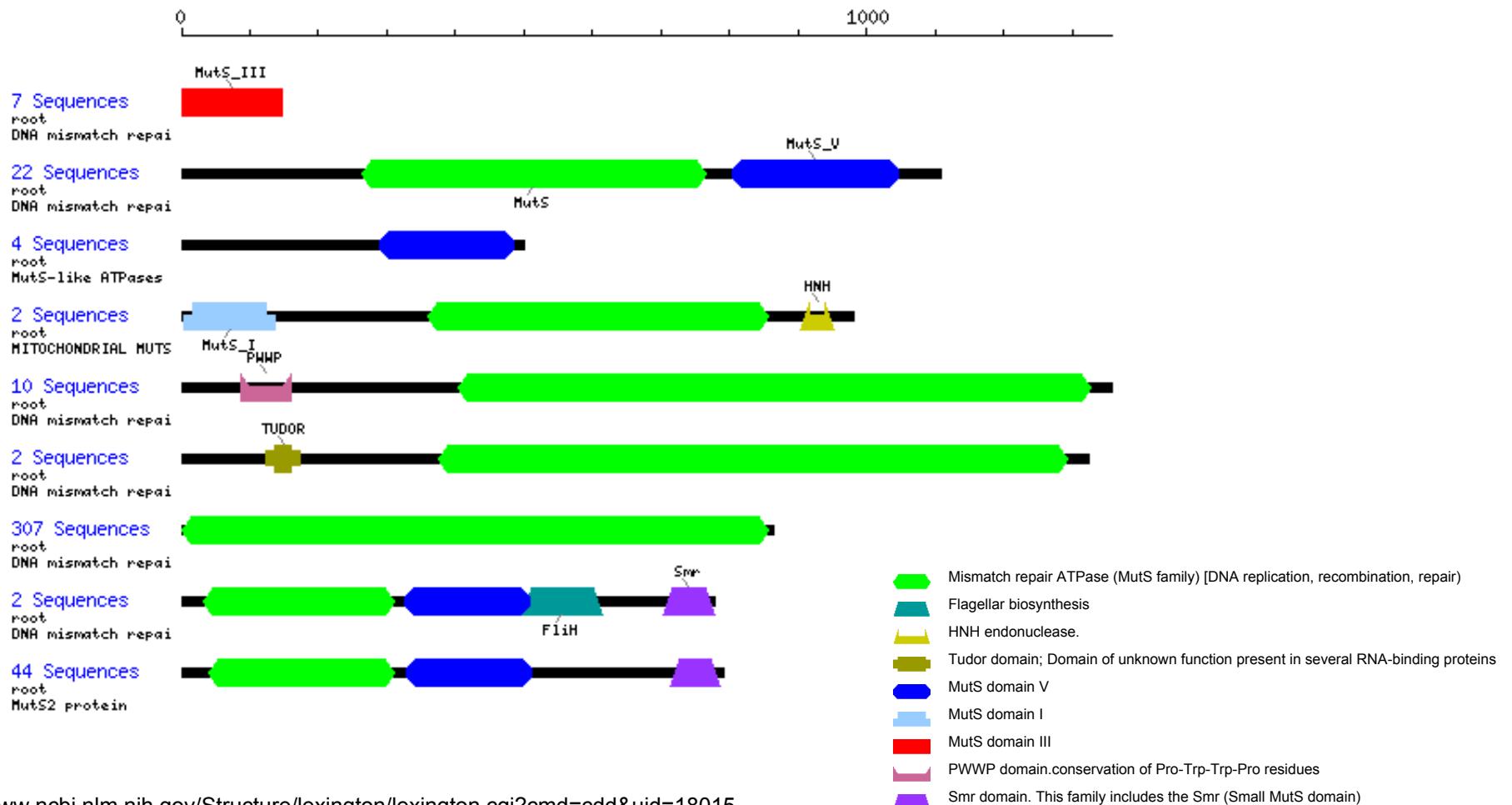


hMSH2 mapped to Chromosome 2
Homolog of *yMSH2* and *mutS*.

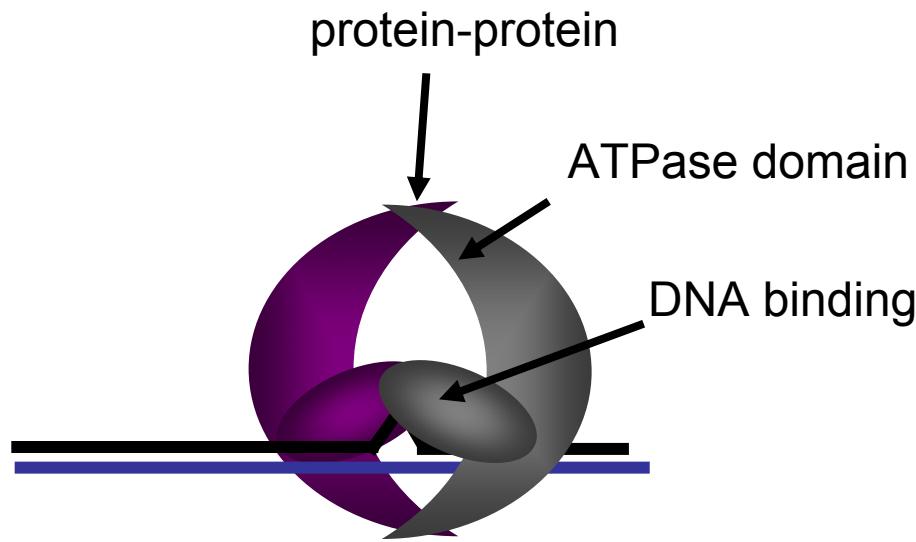
MSH, or MutS homologs found in all branches of the phylogenetic tree



The homology is based on amino acid sequence and function

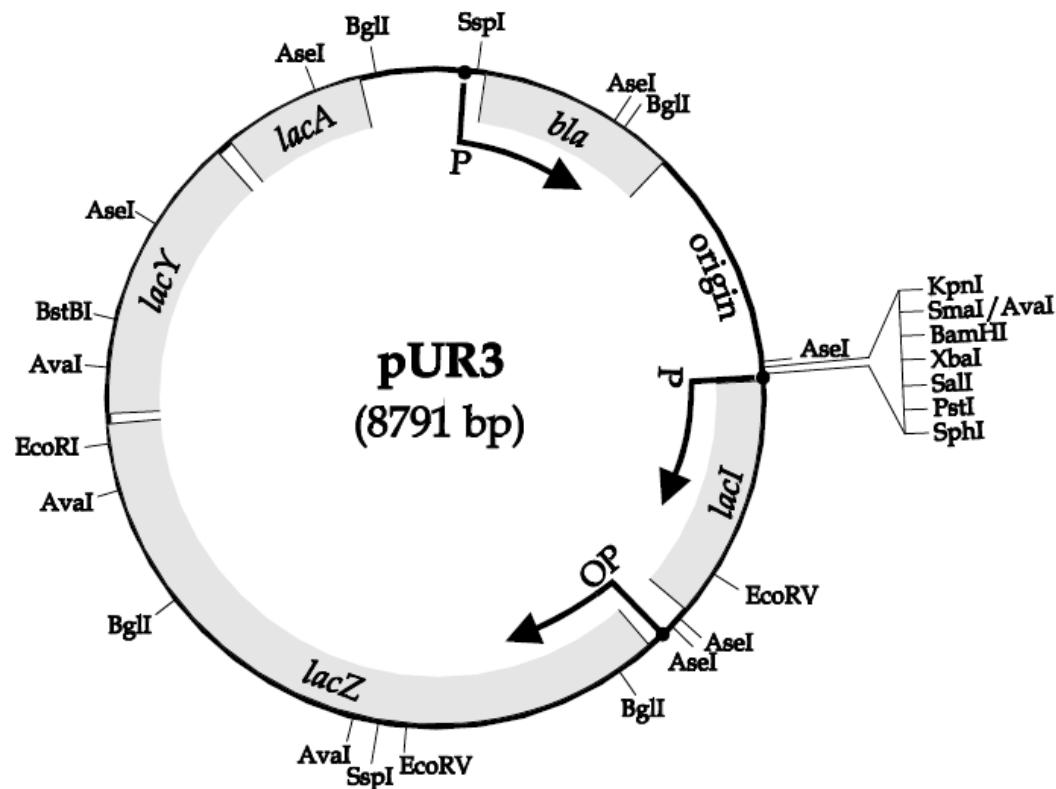


Msh2p Important Domains



- Structural integrity residues (throughout)
- DNA binding region
- Protein-protein interacting regions
- ATPase domain

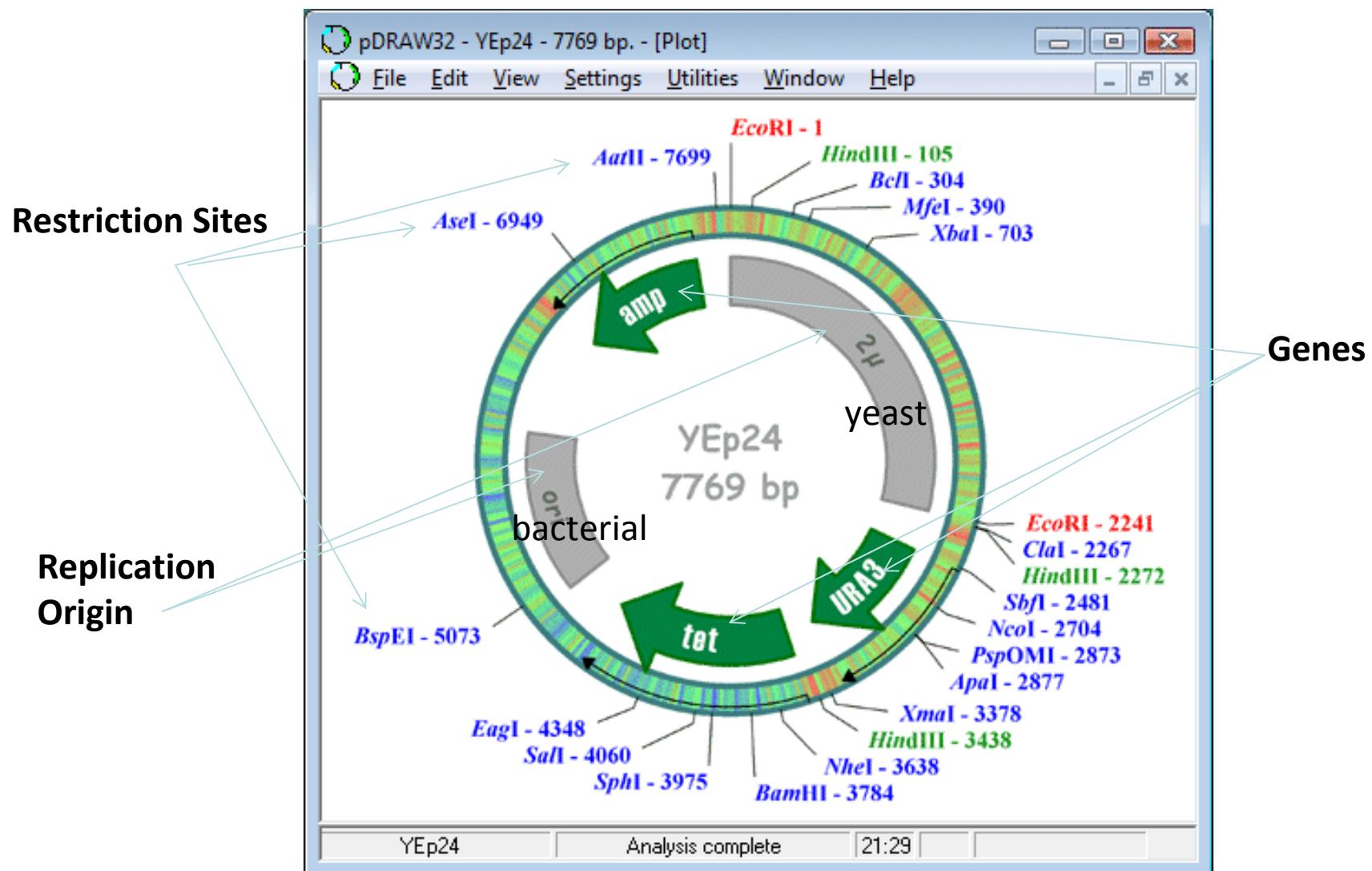
Plasmid Isolation: Understand Plasmids (circular versus linear DNA). Understand Restriction Enzymes and Restriction Sites



DNA fragments can be analyzed by agarose gel electrophoresis. Electrophoresis of restriction fragments provide indication of restriction sites in DNA sample.

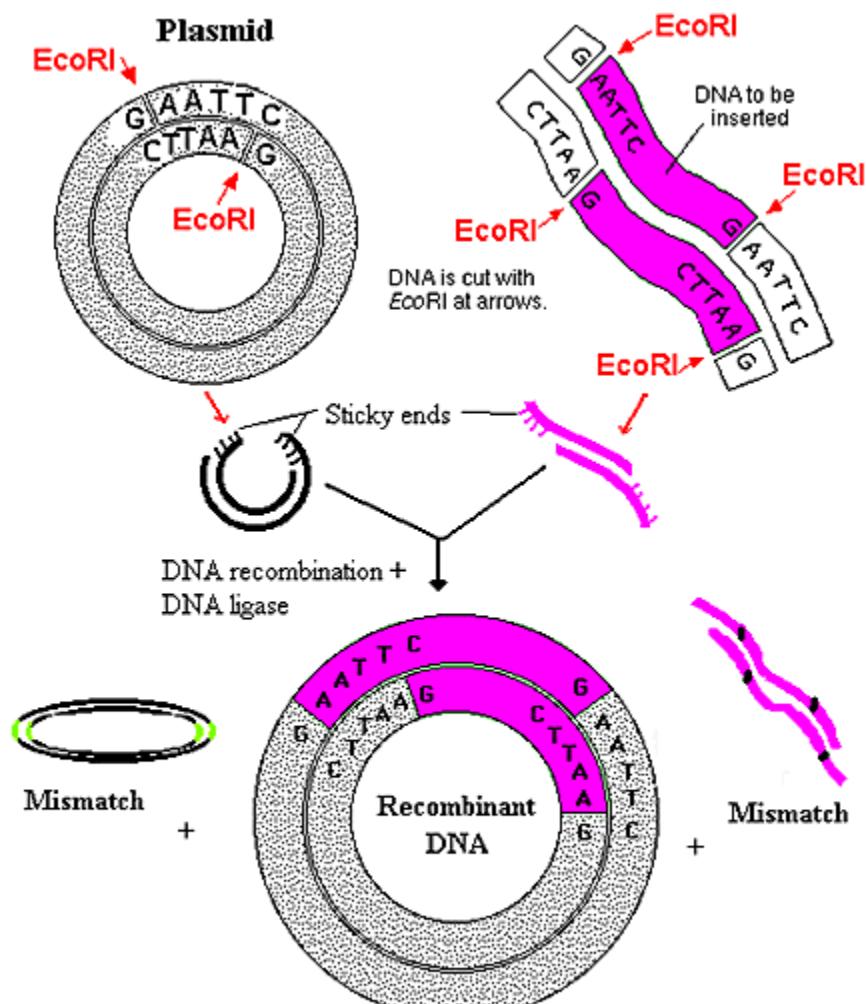
Structure of plasmid pUR3. Diagram shows genes (shaded), promoters (P) that initiate transcription (arrows), and selected restriction sites. The plasmid is manmade and is derived from multiple sources: *bla* (encoding β -lactamase, which confers resistance to penicillins) from transposon Tn1, the origin of replication from the natural plasmid pMB1, *lacI* and the *lac* operon from *E. coli*, and a short, synthetic stretch of DNA (often called a polylinker or multiple cloning site) containing several restriction sites useful for cloning foreign DNA fragments.

Another View: Plasmid Maps



This plasmid is a bacterial:yeast shuttle vector!

Strategy for subcloning DNA fragments into a plasmid vector



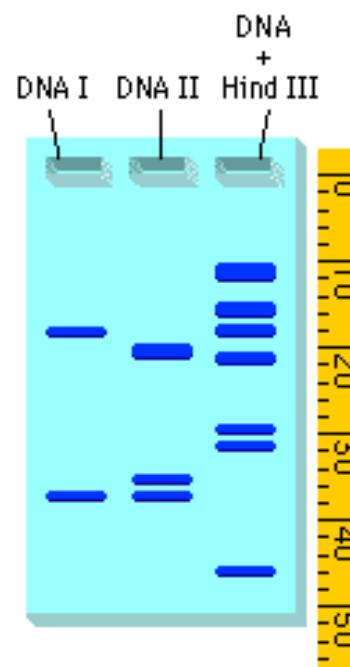
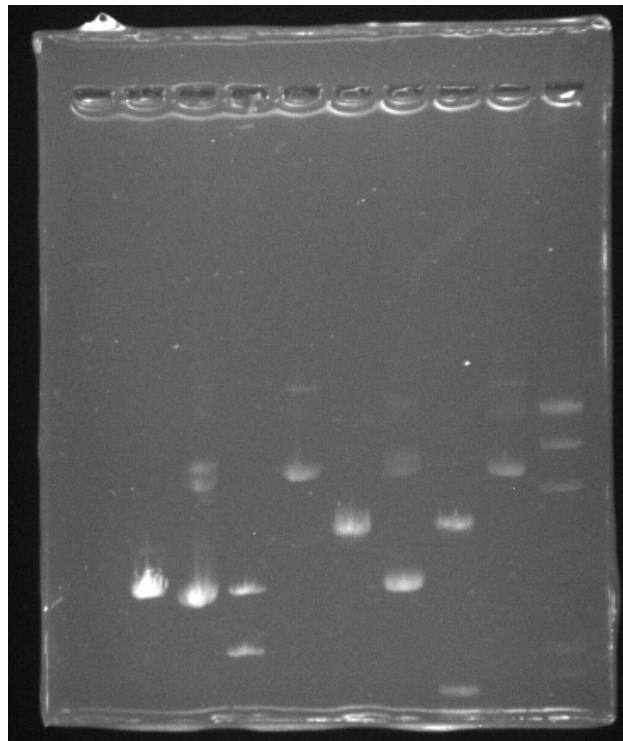
Restriction Analysis with Agarose Gel Electrophoresis

Analyzing your Gel

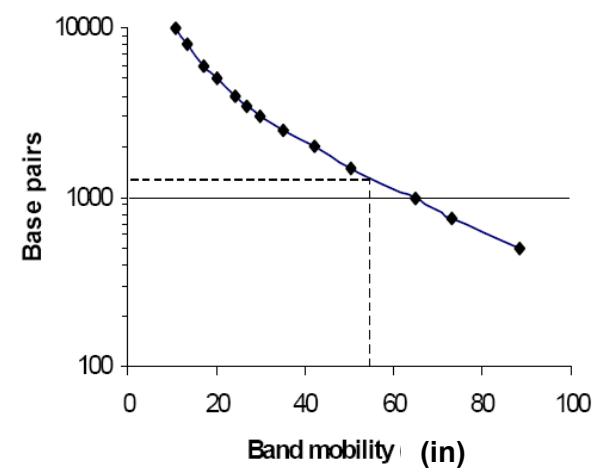
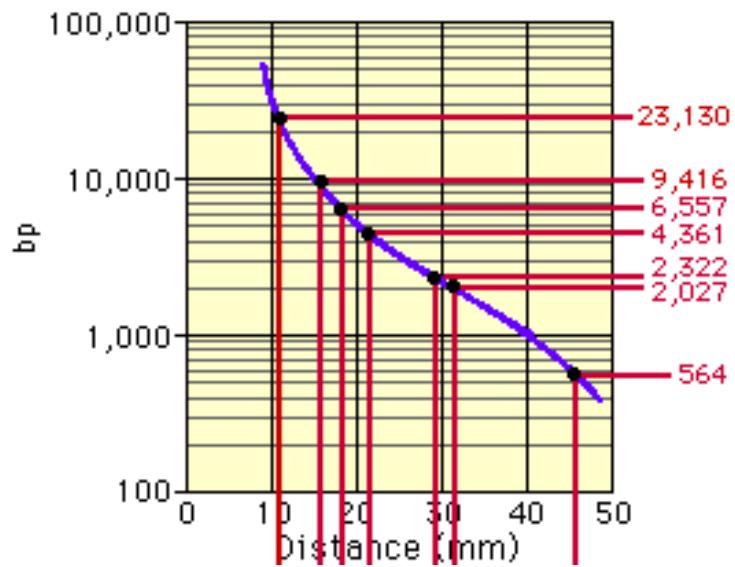
First draw a standard curve:

Copy and paste a gel that you want to work with. Print the image and then you can begin to do the measurement. You will generate 2 data sets, 1 = Base pair standards, 2 = distance of base pair from gel (in inches or mm). Draw standard graph of distance against base pair.

Then determine for each lane of your sample wells, the base pair size for each of the fragments identified.



Actual Base Pairs (bp)	Measured Distance (mm)
23,130	
9416	
6557	
4361	
2322	
2207	
564	



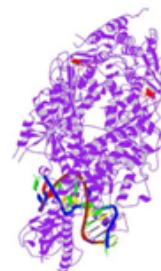
<http://arbl.cvmbs.colostate.edu/hbooks/genetics/biotech/gels/>

Shaila Strayhorn

Dr. Tokunbo Yerokun

Molecular Biology 125, 8gm WF

Comparing the DNA of MSH2 Mutant and the Wild Type



Introduction

Colorectal Cancer is responsible ---Incidence, genetic disposition

genetic basis, MSH2 gene linked, gene function

purpose of experiment (project), experiment

Materials/Methods

E coli, microfuge tubes, centrifuge

Isolating Plasmid DNA from Host Bacterial Strains Using Promega Wizard Mini-Prep Method

1. Label 2mL mirofuge tubes with a Sharpie and transfer 2mL of the incubated culture by use of sterile 2mL pipettes
2. Then Centrifuge your tubes for two minutes with the micorcentrifuge or until cell pellet is visible. Decant the excess liquid.
3. Transfer 200 μ L of Cell Resuspension Solution to allow cell pellet to resuspend.

Restriction Enzyme Digestion of Plasmid DNA

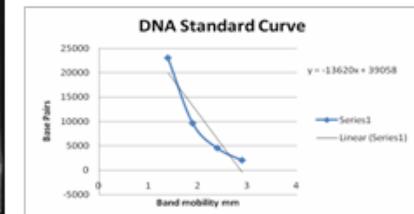
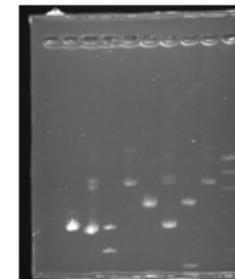
1. Label four 1.5 microfuge tubes-pMSH2 cut, pMSH2 uncut, vector cut, vector uncut.
2. Then discover the concentration of DNA within your solutions by using a Nano drop machine.
3. Obtain a sample from a vector tube and a pMSH2 tube as well (be use to black the Nano drop machine with TE before beginning). Record results in mg/mL

Agarose Gel Electrophoresis of Nucleic Acids

1. Carefully Pour readymade Agarose Gel into tray of electrophoresis (try to prevent bubbles).
2. Place comb were black line of the tray is so wells can form.
3. While gel is hardening, add a blue dye to cell solution so that DNA bands may appear more visible while migrating within the gel.

Results

DNA Concentration		
	Wild Type	Vector
260/280	1.83	1.82
ng/ μ L	39.3	58.8
mg/mL	3.93x10 $^{-2}$	5.88x10 $^{-2}$

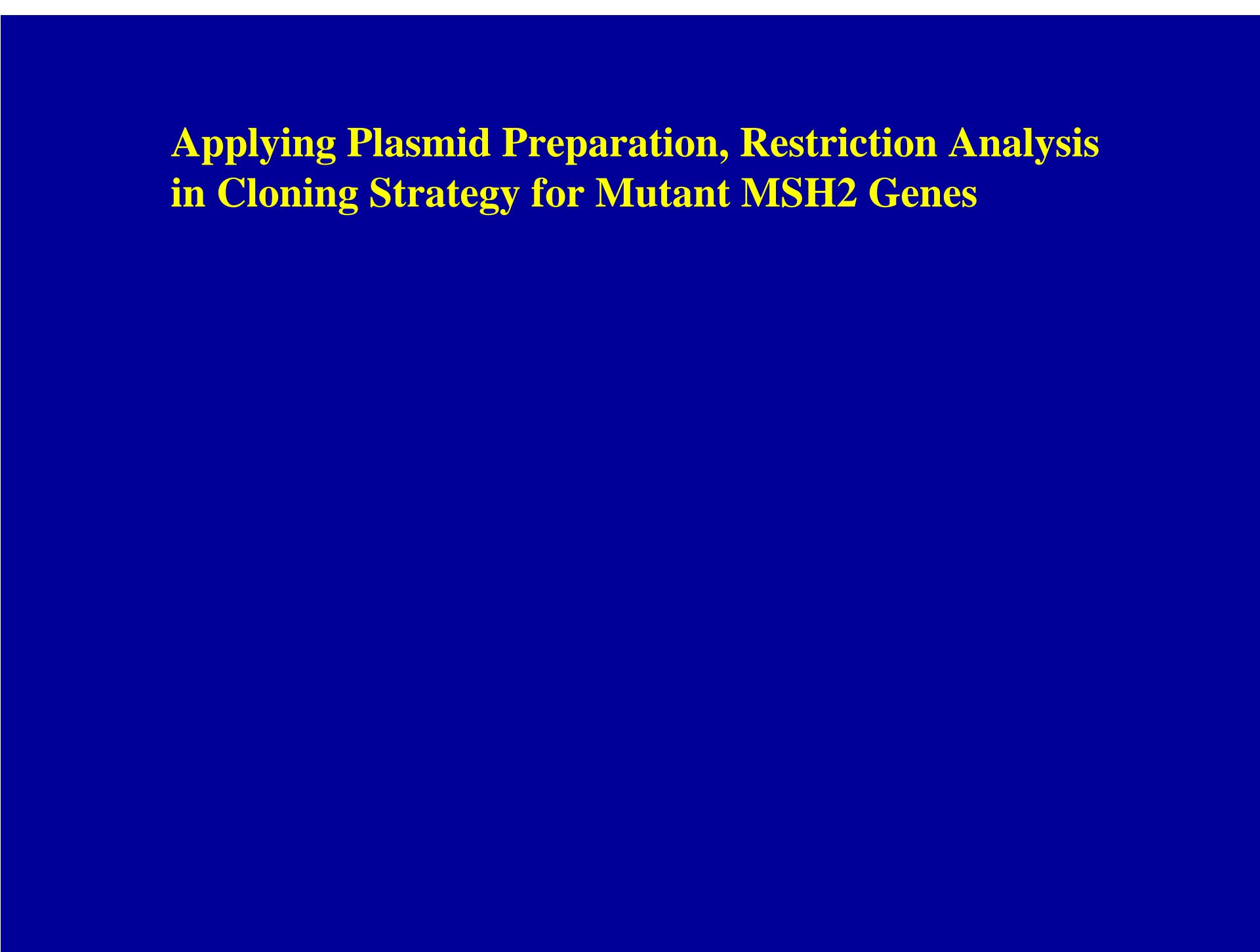


Lanes	Distance Traveled	Base Pairs (mm)
2	1.9	36470.2
2	2.2	36061.6
3	1.4	37151.2
3	1.8	36606.4
3	2.1	36197.8
3	2.4	35789.2
4	1.5	37015
4	1.6	36878.8
4	1.9	36470.2
4	2.3	35925.4

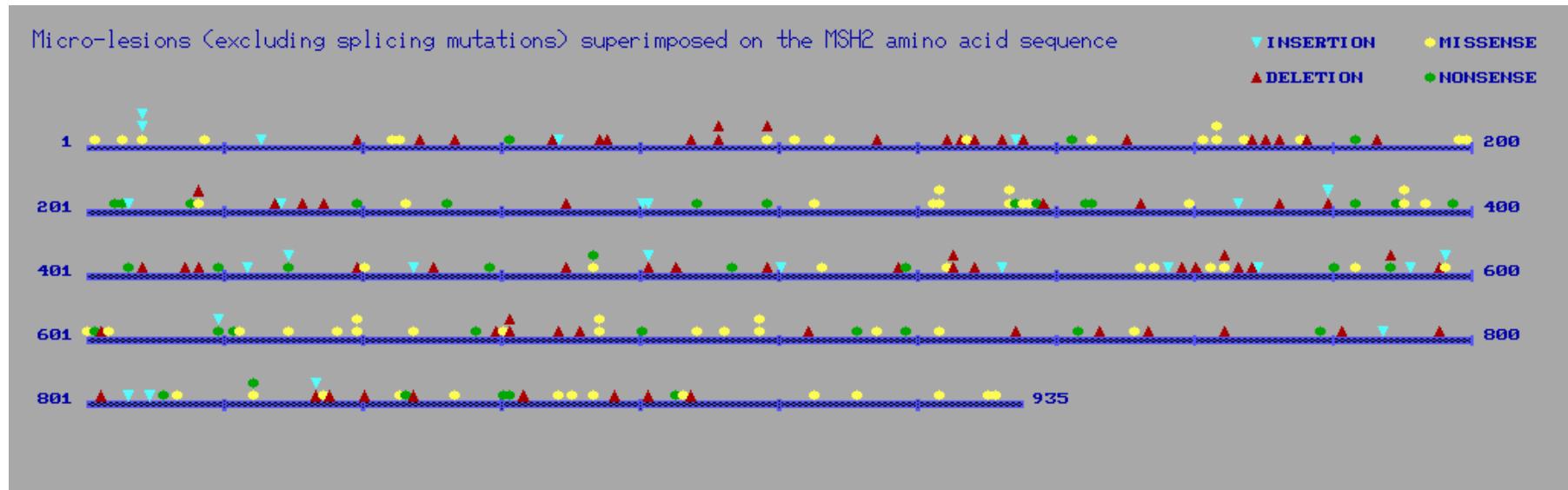
Conclusion

As seen within the table the bands which had the greatest Base Pair value, migrated within the gel at the shortest distance. The bands which exhibited the smallest

Applying Plasmid Preparation, Restriction Analysis in Cloning Strategy for Mutant MSH2 Genes

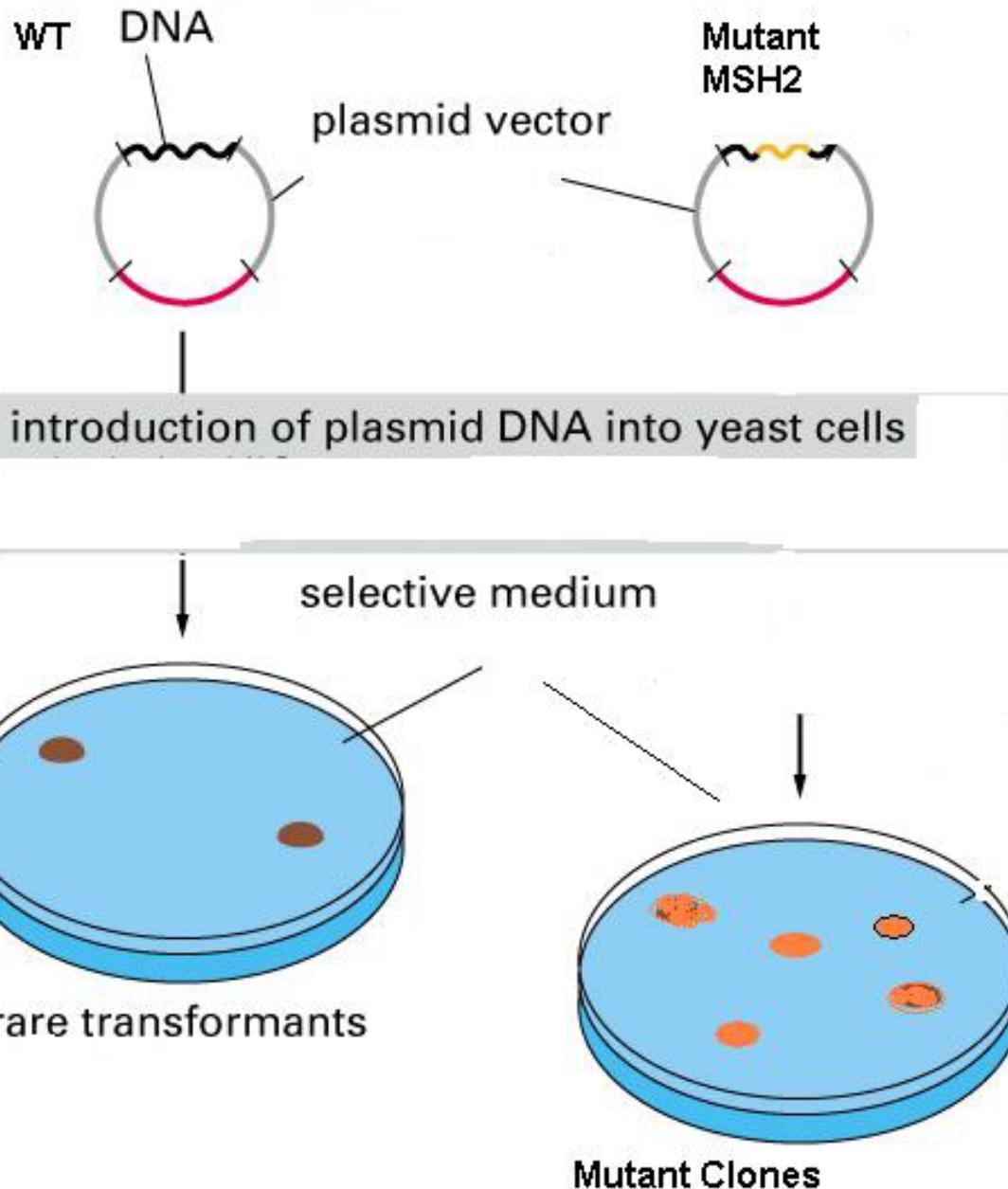


Project Overview



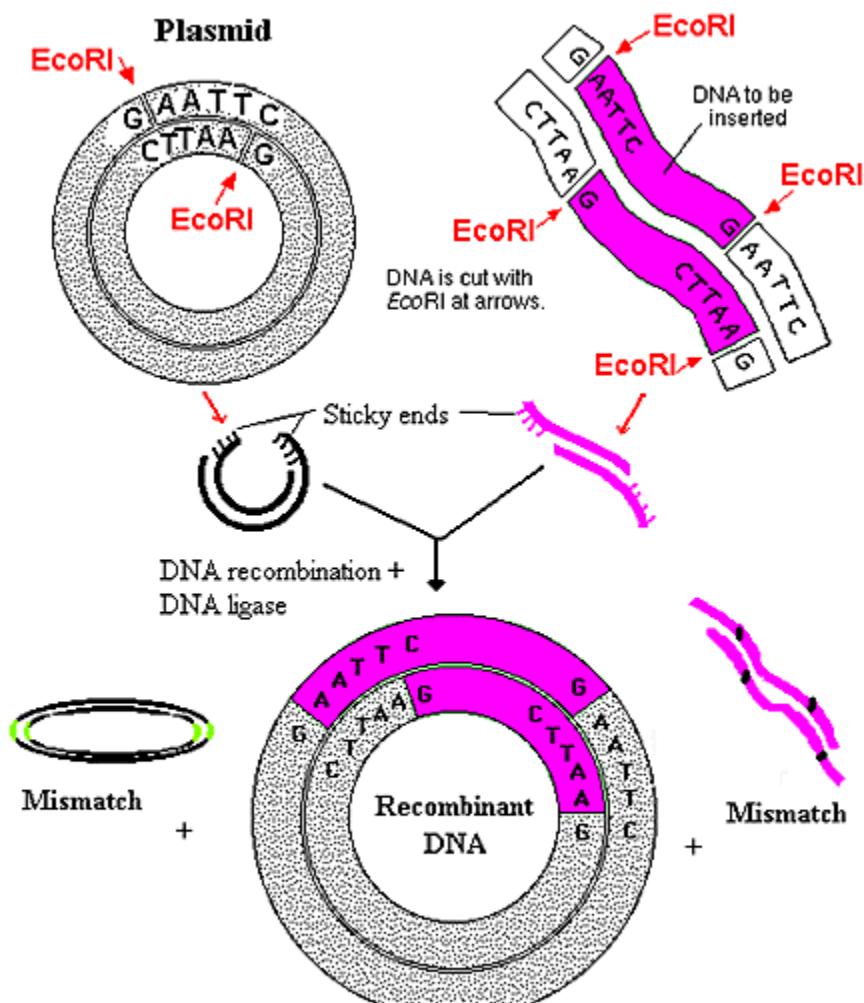
Manipulate the yeast *MSH2* gene to determine which human missense mutations are likely to be benign or pathogenic in nature.

Examine the defect at a molecular level to determine why the Msh2 variants are dysfunctional.



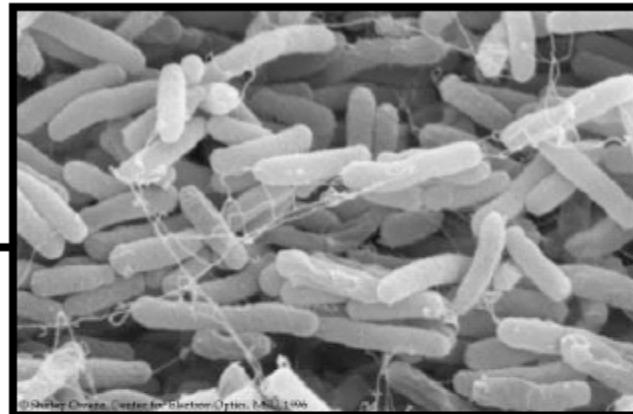
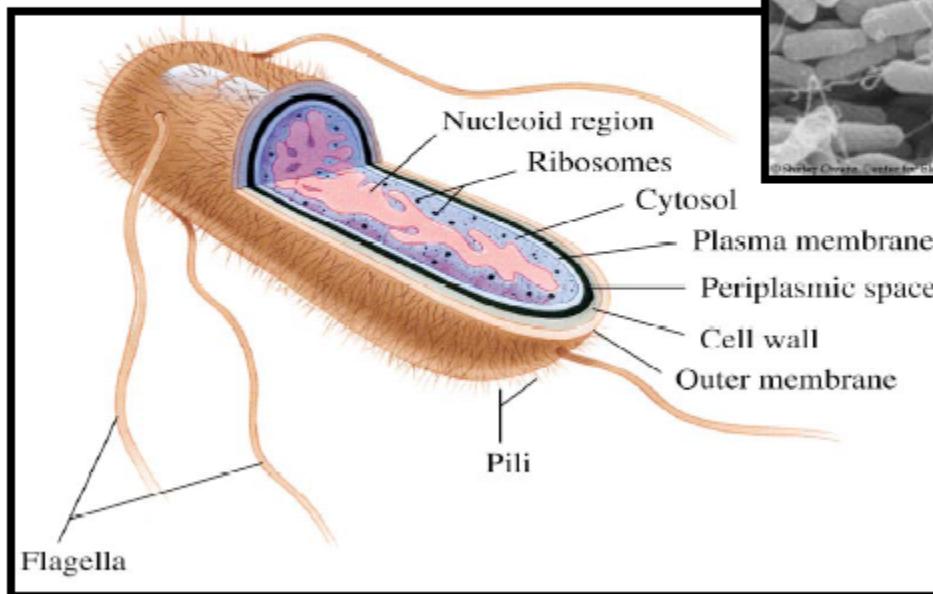
From, MolecularBiology of the Cell

Strategy for subcloning DNA fragments into a plasmid vector



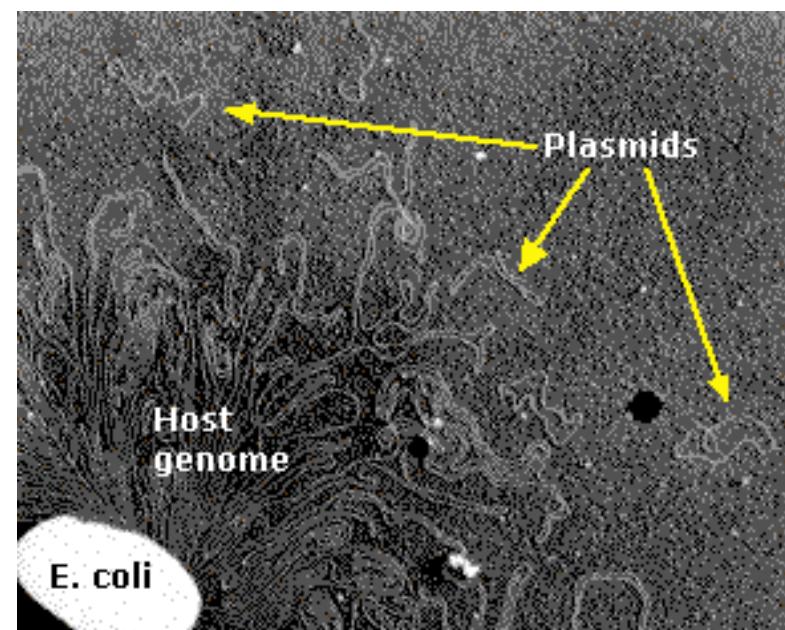
Inserting a DNA Sample into a Plasmid

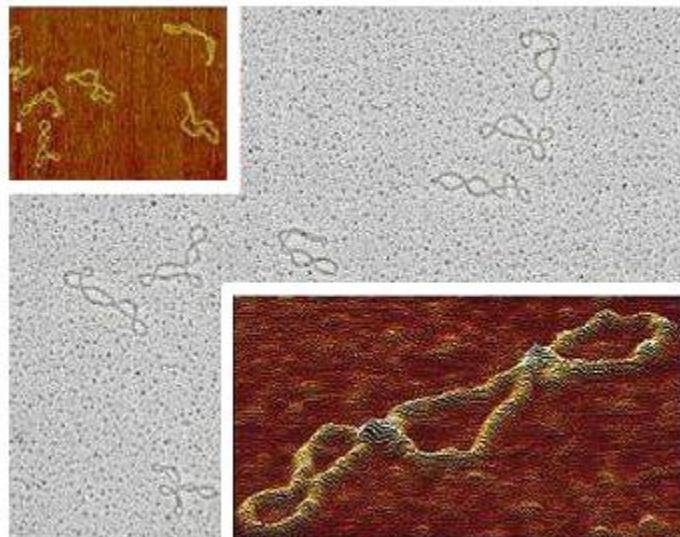
E. Coli as a host organism in molecular biology



Electron micrograph of an *E. coli* cell ruptured to release its DNA. The tangle is a portion of a single DNA molecule containing over 4.6 million base pairs encoding approximately 4,300 genes. The small circlets are plasmids.

(Courtesy of Huntington Potter and David Dressler, Harvard Medical School, accessed at <http://users.rcn.com/jkimball.ma.ultranet/BiologyPages/R/RecombinantDNA.html>)



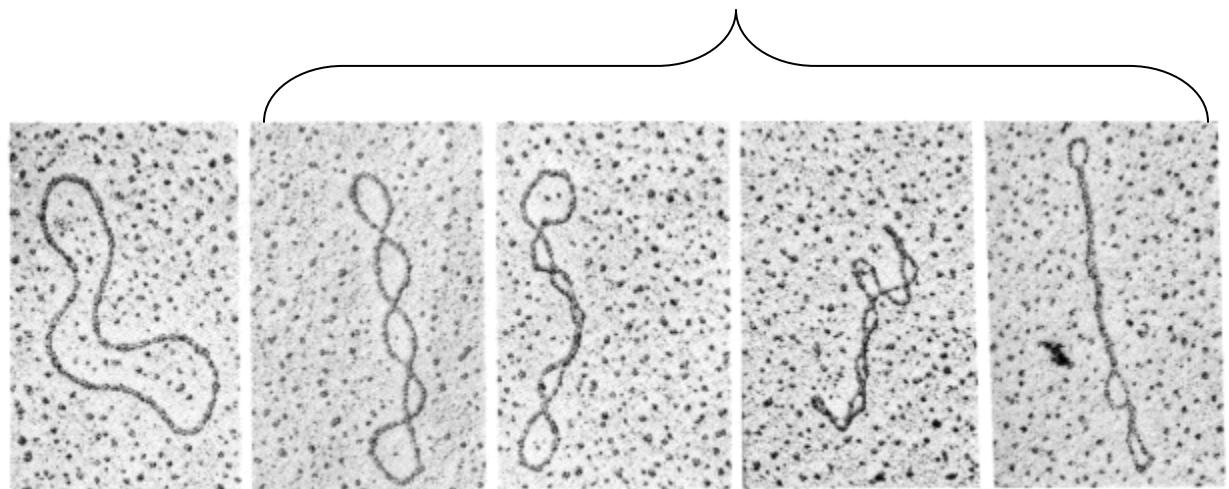


[http://w3.uniroma1.it/centric
nr-can/edocs/gme3.htm](http://w3.uniroma1.it/centric_nr-can/edocs/gme3.htm)

relaxed

Plasmid DNA: extrachromosomal circular molecules in the bacterial cell

supercoiled



<http://intranet.siu.edu/~mbmb/451resources/supercoiling.html>

0.2 μ m

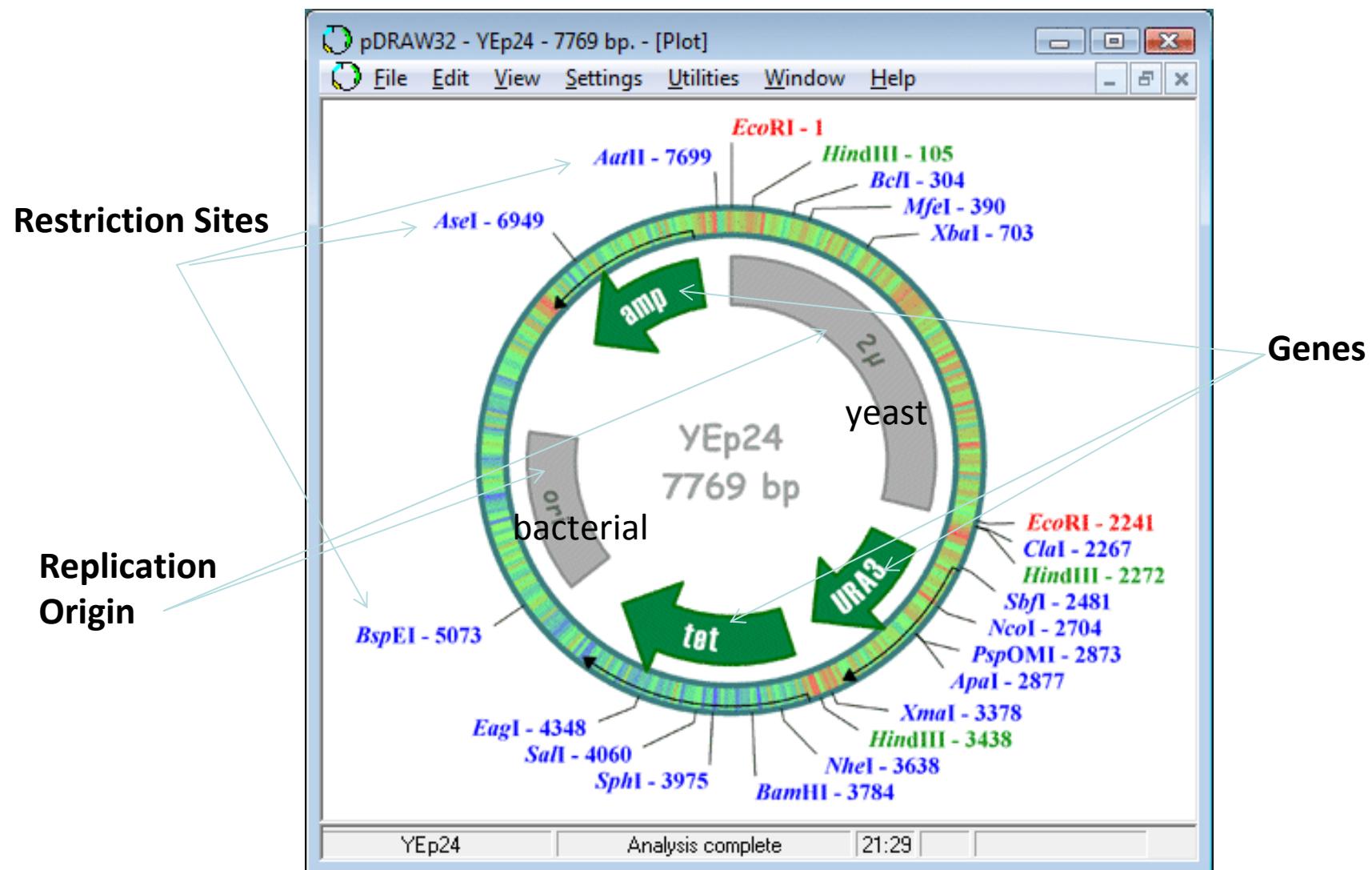
Shigella Outbreaks in Japan, 1953-1960: occurrence of antibiotic-resistant strains

Year	# Strains tested	Sm	Tc	Cm	Sm + Cm	Sm + Tc	Cm + Tc	Sm, Cm & Tc
1953	4900	5	2	0	0	0	0	0
1954	4876	11	0	0	0	0	0	0
1955	5327	4	0	0	0	0	0	1
1956	4399	8	4	0	0	0	1	0
1957	4873	13	46	0	2	2	0	37
1958	6563	18	20	0	7	2	0	193
1959	4071	16	32	0	71	0	0	74
1960	3396	29	36	0	61	9	7	308

(From <http://www.mun.ca/biochem/courses/4103/topics/plasmids.html>)

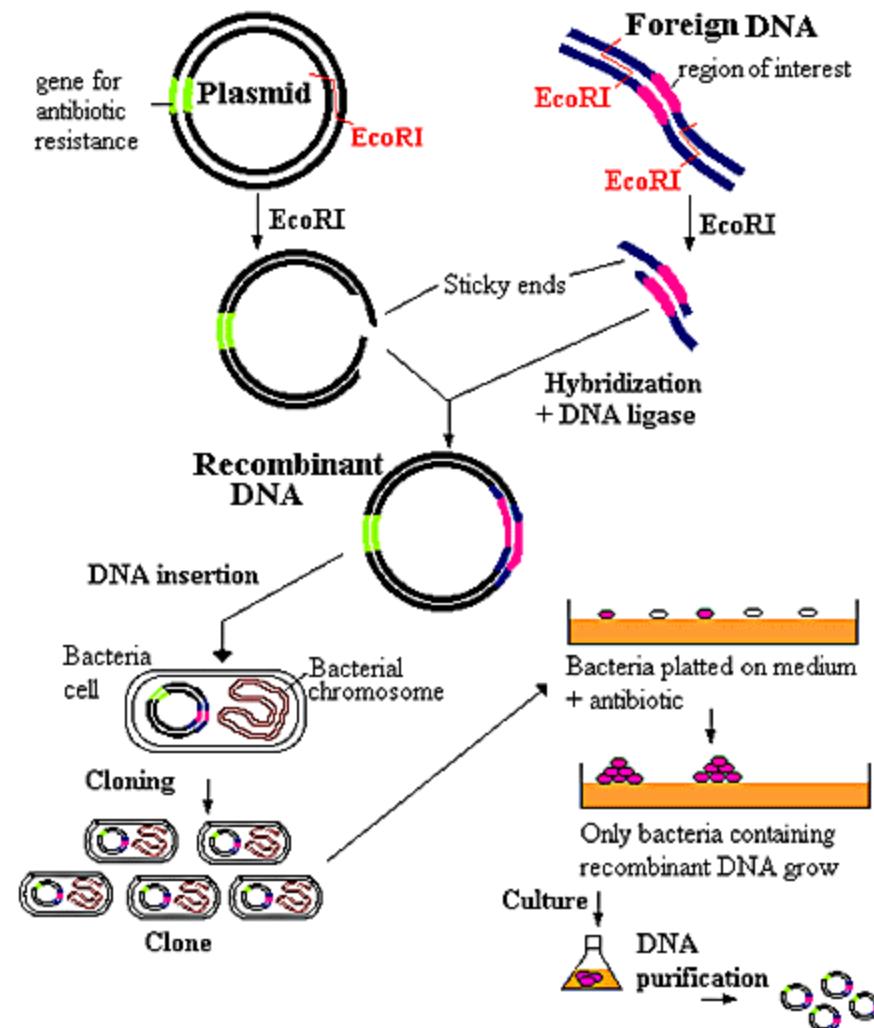
- Plasmids confer antibiotic resistance to strains harboring them!
- Plasmids can be “shared” between strains by lateral transfer!

Another View: Plasmid Maps



This plasmid is a bacterial:yeast shuttle vector!

Plasmids can be used as vectors to carry a gene of interest

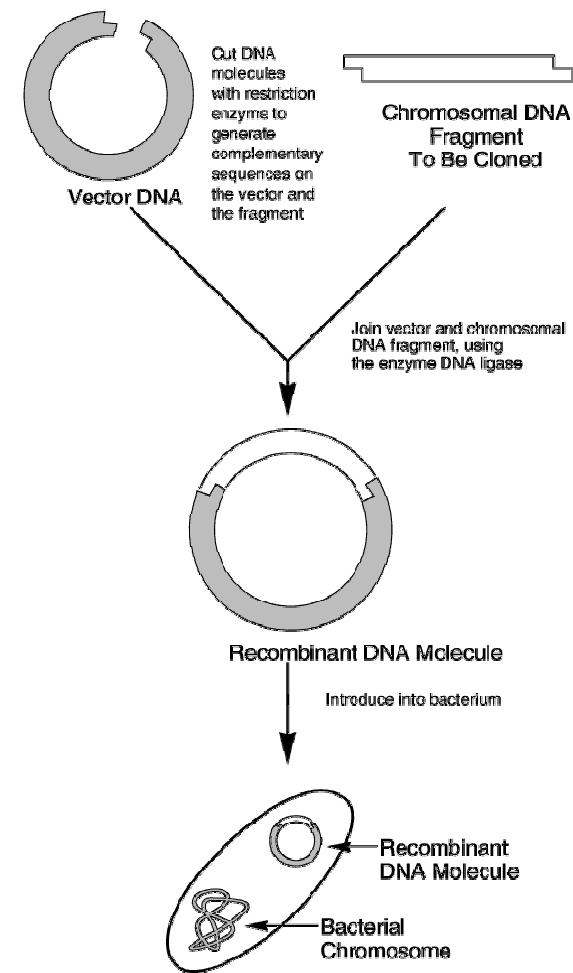


Cloning into a plasmid

(from <http://www.accessexcellence.org/RC/VL/GG/plasmid.html>)

Gene Cloning I

- Provides an easily replicated source of a gene.
- Enables detailed analysis of gene structure.
- Permits production of large quantities of the gene product for functional and structural analysis.

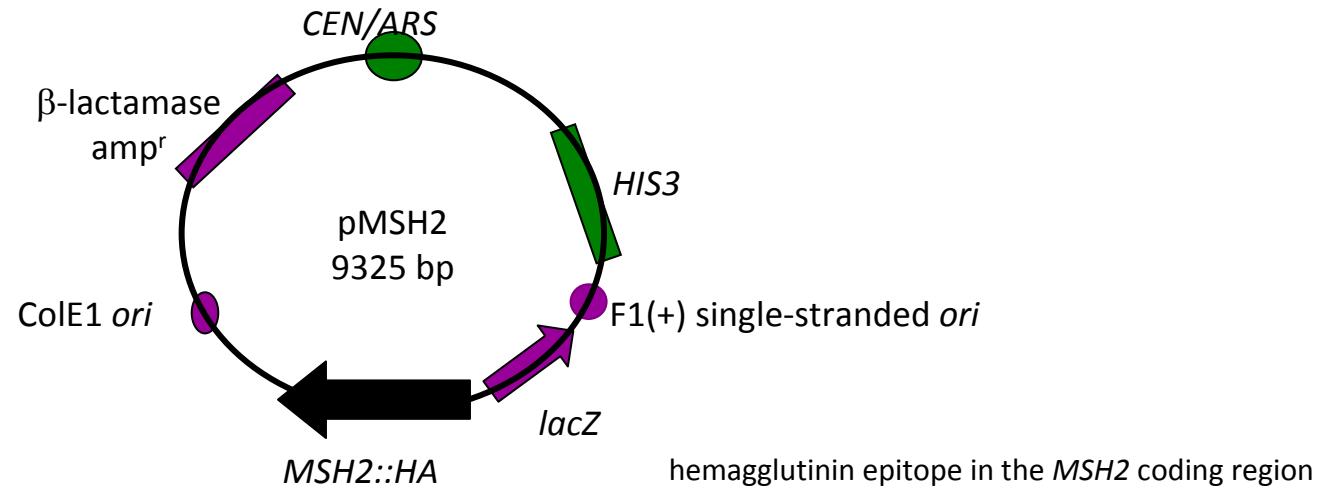


Courtesy of U.S. Department of Energy Genome Program

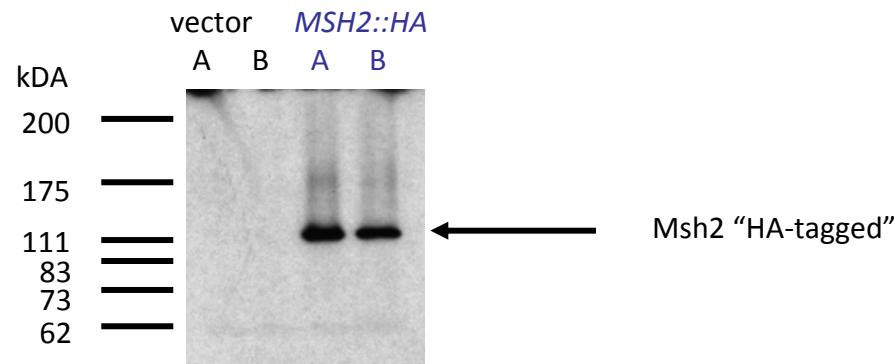


Taking a closer look at our plasmid, pMSH2

MSH2 construct for mutagenesis and functional assays

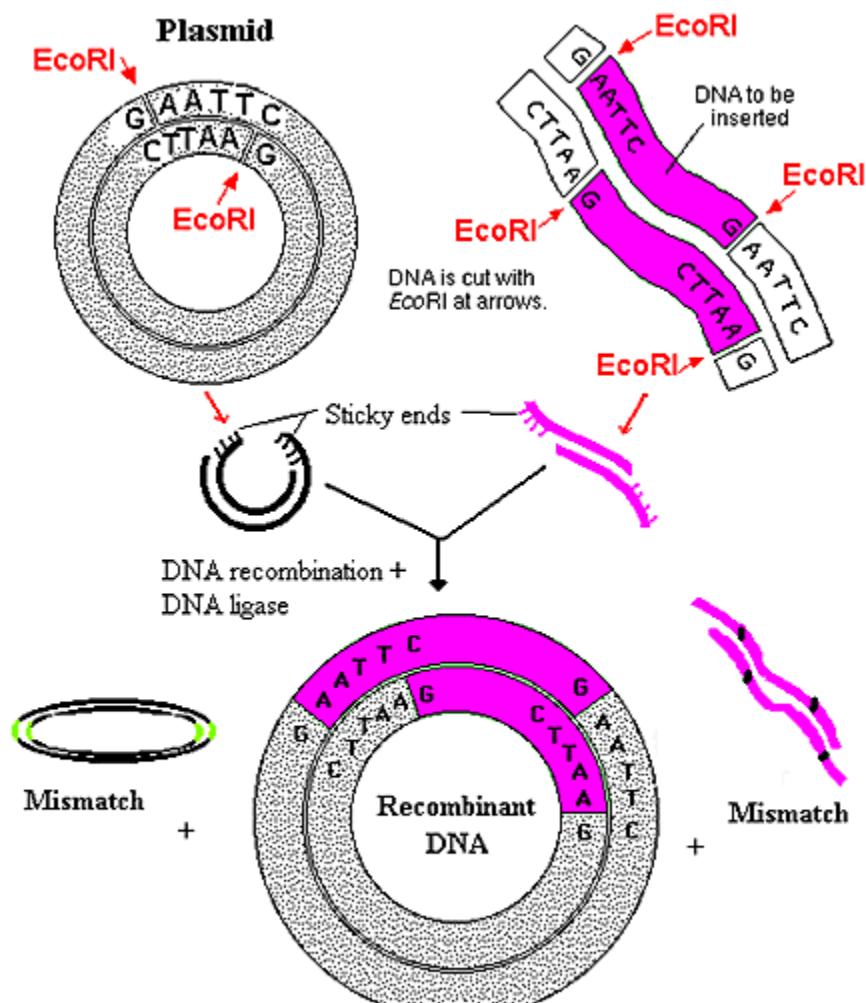


-produces a protein product of the expected molecular weight



-complements *msh2Δ* defects, therefore the construct is functional

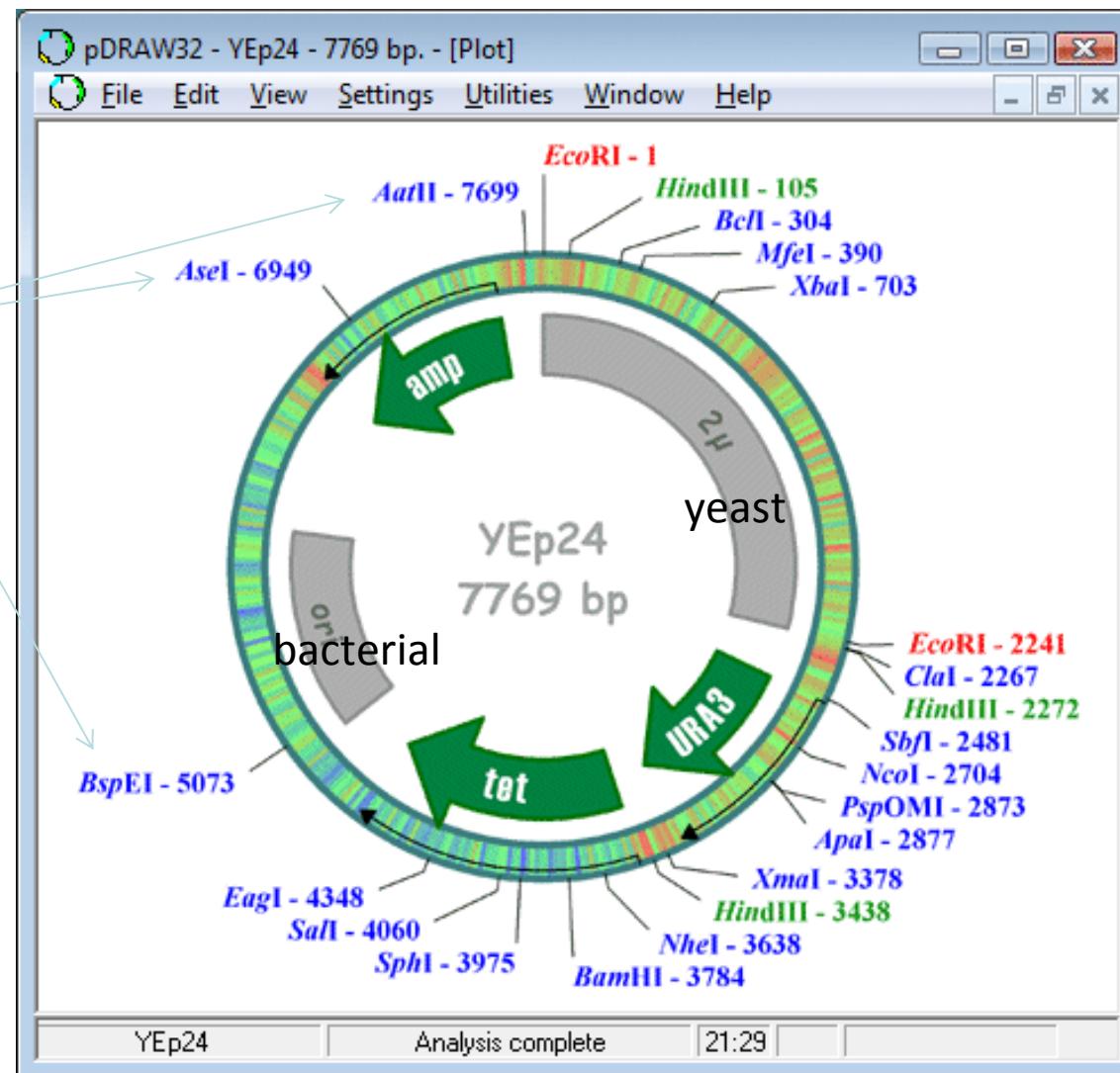
Strategy for subcloning DNA fragments into a plasmid vector



Inserting a DNA Sample into a Plasmid

Another View: Plasmid Maps

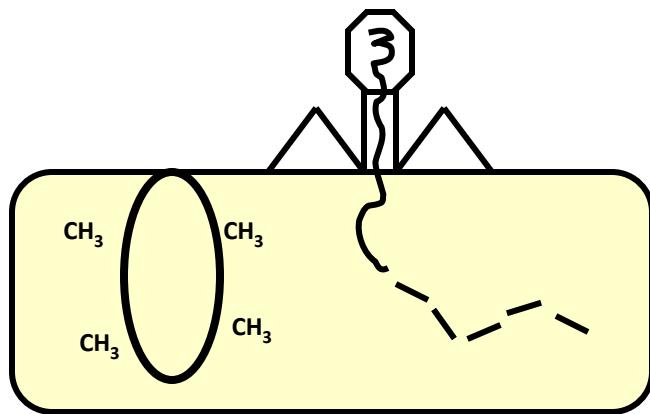
Restriction Sites



What are restriction sites for??

Biological function of restriction endonucleases is to protect cells from foreign DNA

Infecting DNA is cleaved (restricted) by the restriction enzymes, preventing it from successfully replicating and parasitizing the cell



most lab strains are completely “domesticated” (R-M systems have been inactivated)

some examples in the genotypes:

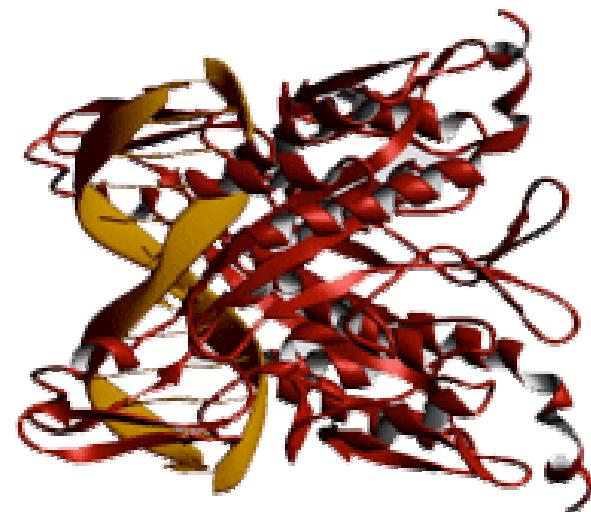
hsd

mcrA

mcrBC

Restriction Endonucleases

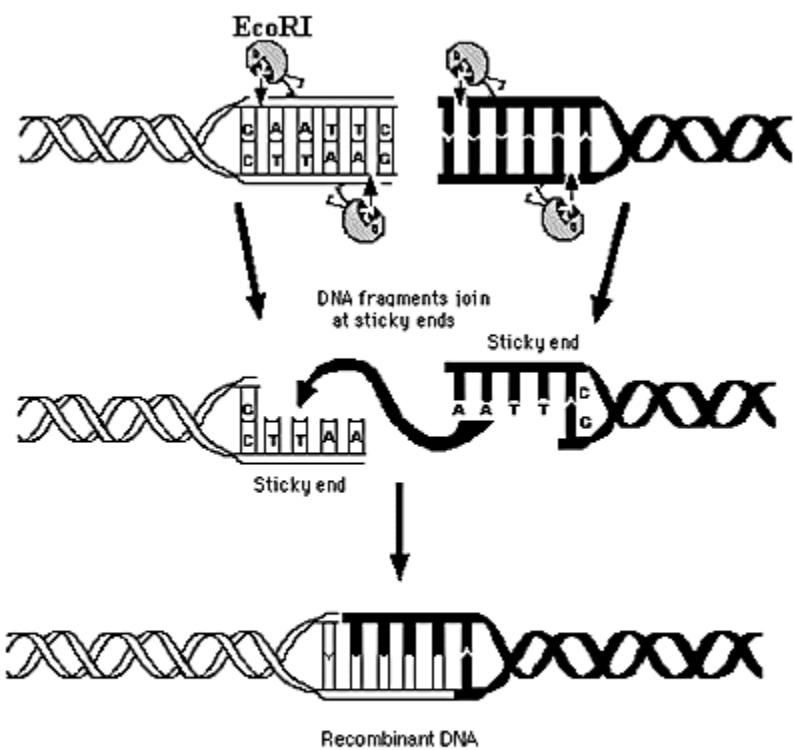
- Over 10,000 bacteria and archaea have been screened for restriction enzymes
- Restriction enzymes are not confined exclusively to bacteria
- Nearly 3,000 enzymes have been found, exhibiting over 200 different specificities (many of the 3000 are isoschizomers; different enzyme, same recognition site)



EcoRI binding DNA

http://www.biophysics.pitt.edu/john_m_rosenberg.htm

Activity of a typical Restriction Enzyme Enzyme



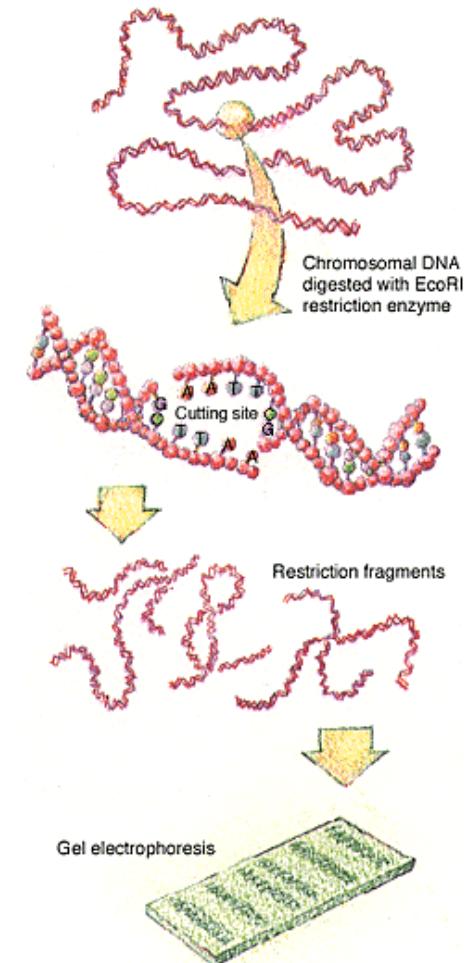
**Restriction Enzyme
Action of EcoRI**

ends generated from a Type II restriction endonucleases

	<u>enzyme</u>	<u>recognition site</u>	<u>end generated</u>		
defined ends	<i>PvuII</i>	5' CAGCTG 3' GTCGAC	5' CAG	CTG	blunt
	<i>BamHI</i>	5' GGATCC 3' CCTAGG	3' GTC	GAC	
	<i>PstI</i>	5' CTGCAG 3' GACGTC	5' G	GATCC	
variable ends	<i>XmnI</i>	5' GAANNNNNTTC 3' CTTNNNNNAAG	3' CCTNN	G	5' overhang
	<i>BanI</i>	5' GGP _y PuCC 3' CCP _p PyGG	5' G	GPyPuCC	
	<i>BstXI</i>	5' CCANNNNNNTGG 3' GGTNNNNNNNACC	3' CCP _p PyG	G	
			5' CCANNNNN	NTGG	3' overhang
			3' GGTN	NNNNNACC	

Restriction Enzymes I

- Restriction enzymes can be used:
 - to cut DNA at specific nucleotide sequences.
 - Example – to cut chromosomes into smaller pieces for analysis by gel electrophoresis.
 - for cloning or the generation of genetic libraries.

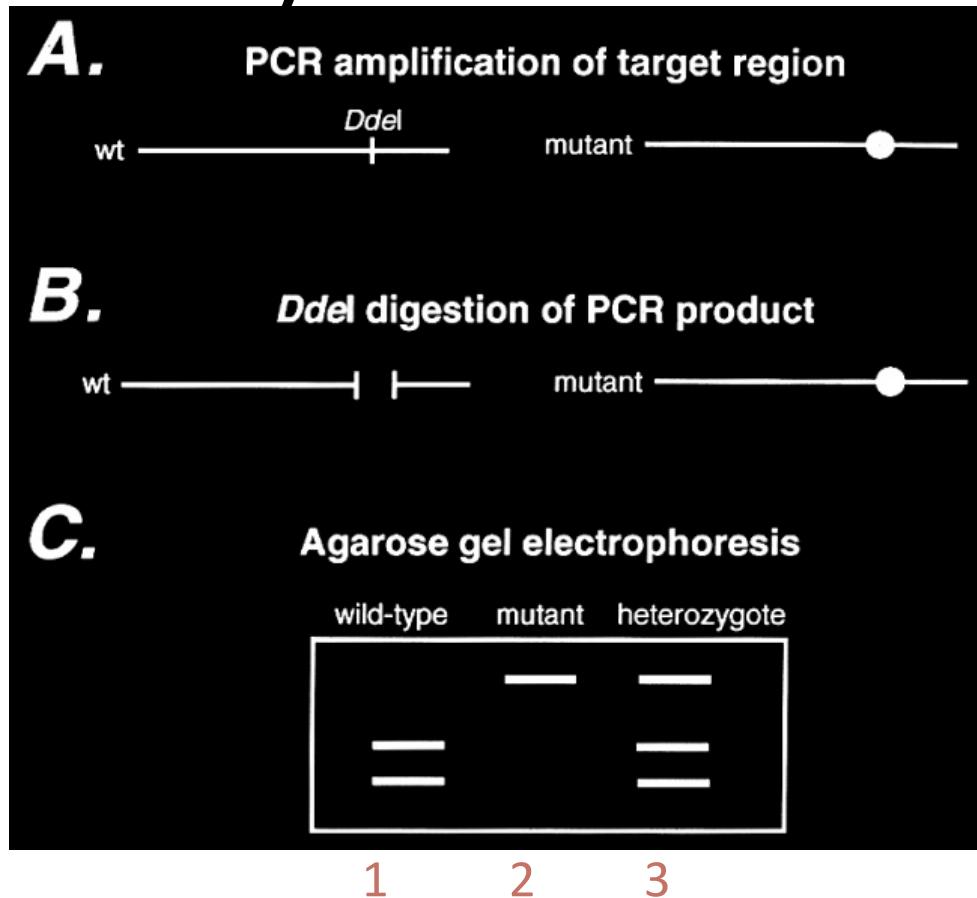


Courtesy of U. S. Department of Energy



Restriction Enzymes II

- Can be used to detect mutations in DNA.
 - Example – The enzyme called Ddel can identify the mutation that causes sickle cell anemia.
 - The mutation changes the DNA sequence so that Ddel cannot cut the DNA if the mutation is present.



Courtesy of Alford, Rossiter, and Caskey.



To simplify the analysis, a shorter gene fragment can be generated using the nucleic editing tool

	L L P N I G K L Y P L A T F H L A Q A Q	
1	ttgttacctaacaataggtaagttgtaccctcttgcaacgttcacttggcgcaagccaa	60
	D F E G V R A A L A N V Y E Y R G F L E	
61	gatttcgaaggaggtagagctgcttagctaacgtctatgagtatagggattttggag	120
	T G N L E D H F Y Q L E M E L C R D A F	
121	actggtaacttagaagatcactttaccaattggaaatggaactatgttagagatgcttc	180
	T Q Q F A I S T V W A W M K S K E Q E V	
181	acgcaacaatttgcacatcagcactgttggcctggatgaaatccaaggaacaa gaa gtt	240
	R N I T W I A E C I A Q N Q R E R I N N	
241	aggaatattacctggattgcagaatgtatcgacaaaaccaaagagaaagaatcaacaat	300
	Y I S V Y *	
301	tatattccgttattga	318

sequence can be analyzed with TACG tool to identify restriction sites in the region of the codon to be targeted:

it is helpful to know what each restriction site is, and this information is conveniently provided for you in the TACG tool:

<u>Enzyme</u>	<u>Restriction Site</u>
StyI	C' C _{ww} G_G
BsaJI	C' C _{nn} G_G
BtsCI	GGATG_nn '
FokI	GGATG _{nnnnnnnnnn} ' nnnn_
TspDTI	ATGA _{nnnnnnnnnn} _nn '
SspI	AAT'ATT

This is the coding region we are interested in:

SspI

5' -atgaaatccaaggaacaagagtttaggaatattacctggattgcagaatgtatc-3'

M K S K E Q **E** V R N I T W I A E C

To design a primer that 1) alters the glutamate codon (E) to code for lysine (K), and 2) destroys the SspI restriction site:

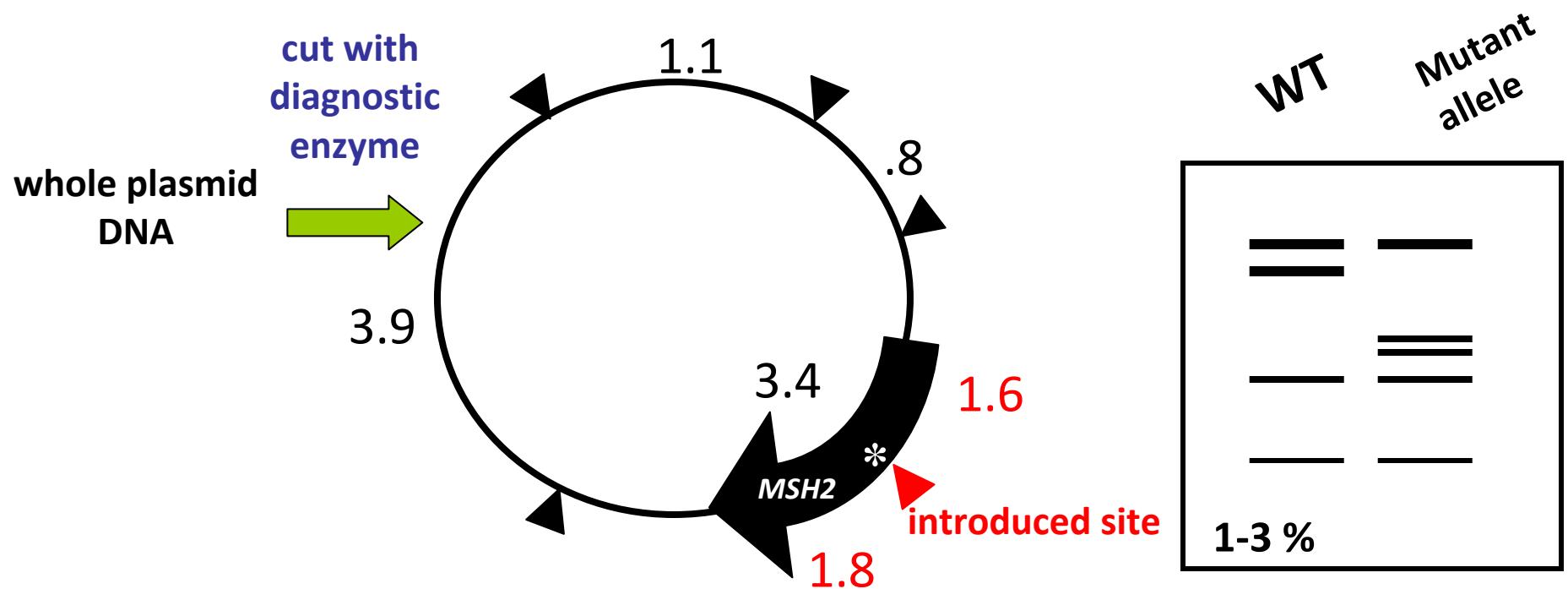
Gene Sequence: 5' -gqaacaa**qaa**gttaqq**aatattac**cctqq-3' E SspI

For the final step, it is important to design a second primer that will anneal to the complementary strand:

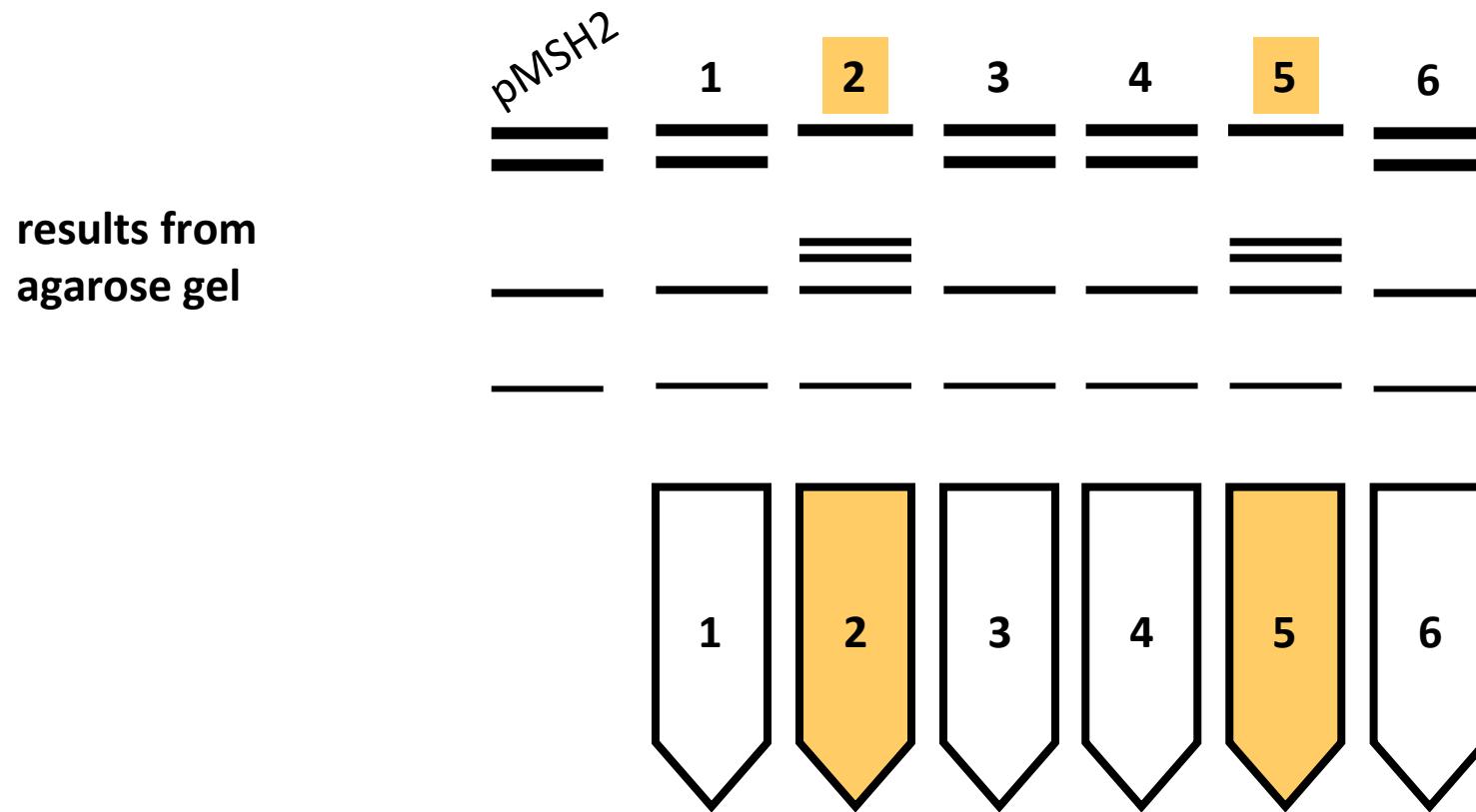
5' -gaacaaaaagtttaggaatgttacc-3' (referred to as the "forward" primer)

3' -cttgttttcaatccttacaatgg-5' (referred to as the "reverse" primer)

We will use restriction digestion to distinguish between WT and mutagenized alleles of our pMSH2 vector

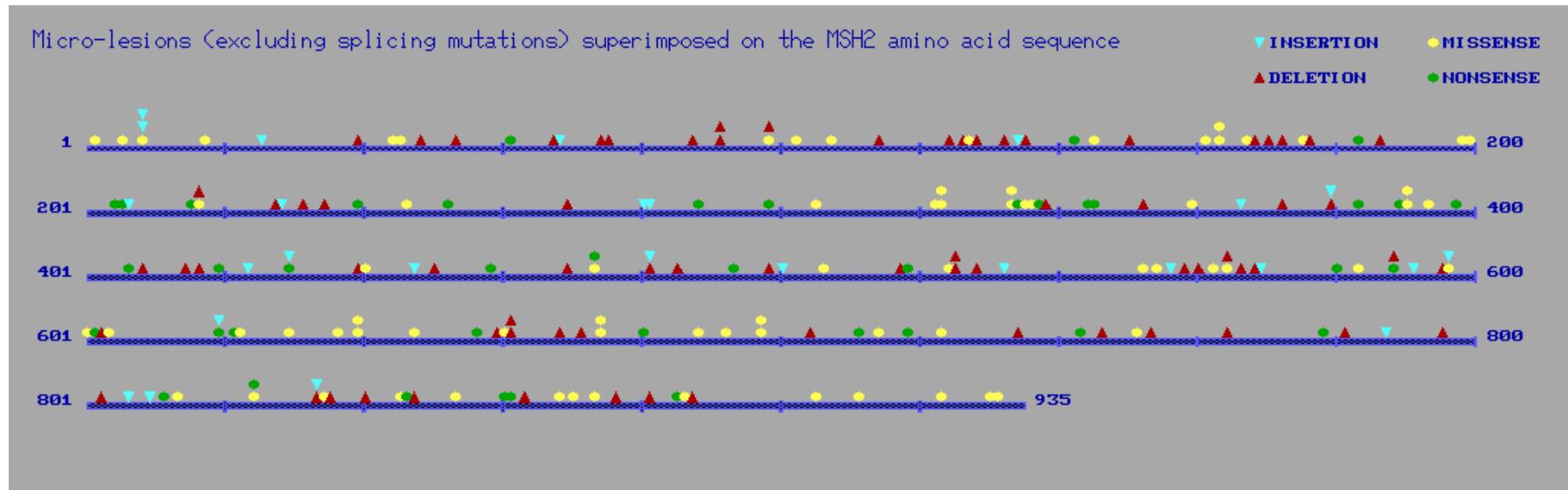


After the diagnostic gel, only proceed with a plasmid showing the altered restriction endonuclease digestion pattern (e.g. 2 or 5)



- ✓ Inoculate 25 ml of media with the correct bacterial colony for a midi-scale plasmid preparation (anion exchange chromatography)
- ✓ Use miniprep DNA to transform yeast to begin the functional analyses

Project Overview



Manipulate the yeast *MSH2* gene to determine which human missense mutations are likely to be benign or pathogenic in nature.

Examine the defect at a molecular level to determine why the Msh2 variants are dysfunctional.

Locating the codon to be mutagenized

Note that the two sequences don't have corresponding codon numbers

for example Ala 719 in yeast is Ala 700 in humans

Yeast 600	INITLTYTPVFEKLSLVLAHLDVIASFAHTSSYAPIPYIRPKLHPMDERRTHLISSRHP	659
	+NI+ Y + L+ VLA LD + SFAH S+ AP+PY+RP + + R L +SRH	
Human 582	VNISSGYVEPMQTLNDVLAQLDAVVSFAHVSNGAPVPYVRPAILE-KGQGRRIILKASRHA	640
Yeast 660	VLEMQDDISFISNDVTLESKGDFLIITGPNMGKSTYIRQGVVISLMAQIGCFVPCEEA	719
	+E+QD+I+FI NDV E K F IITGPNMGKSTYIRQ GVI LMAQIGCFVPCE A	
Human 641	CVEVQDEIAFIPNDVYFEKDKQMFHIITGPNMGKSTYIRQTGVIVLMAQIGCFVPCESA	700
Yeast 720	EIAIVDAILCRVGAGDSQLKGVSTFMVEILETASILKNASKNSLIIIVDELGRGTSTYDGF	779
	E++IVD IL RVGAGDSQLKGVSTFM E+LETASIL++A+K+SLII+DELGRGTSTYDGF	
Human 701	EVSIVDCILARVGAGDSQLKGVSTFMAEMLETASILRSATKDSLIIIDELGRGTSTYDGF	760
Yeast 780	GLAWAIAEHIAASKIGCFALFATHFHELTTELSEKLPNVKNMHVVAHIEKNLKEQKHEDDEDI	839
	GLAWAI+E+IA+KIG F +FATHFHELT L+ ++P V N+HV A +E +	
Human 761	GLAWAISEYIATKIGAFCMFATHFHELTALANQIPTVNNLHVTA-----LTTEETL	811
Yeast 840	TLLYKVEPGISDQSFGIHVAEVVQFPEKIVKMAKRKANELEDDLKTNNE----DLKK--AK	893
	T+LY+V+ G+ DQSFGIHVAE+ FP+ +++ AK+KA EL++ + E D+ + AK	
Human 812	TMLYQVKKGVCDQSFGIHVAELANFPKHVIECAKQKALELEEFQYIGESQGYDIMEPAAK	871

Designing a mutagenic oligonucleotide - choosing the codon guided by a yeast codon usage table

yeast codon 122 changed from glycine (G) to serine (S)
termed G122S mutation

wild-type *MSH2* coding sequence

A S P G N I E
5' CGCATCTCCA GGGAACATTGAGC

missense mutation in *MSH2* coding sequence

A S P S N I E
5' CGCATCTCCA TCT AACATTGAGC

A S P S N I E
5' CGCATCTCCA TCA AACATTGAGC

A S P S N I E
5' CGCATCTCCA TCC AACATTGAGC

A S P S N I E
5' CGCATCTCCA AGT AACATTGAGC

top four candidates

Codon usage in yeast (Appendix of manual)

AmAcid	Codon	Number	/1000	Fraction
Ser	AGT	2411.00	11.14	0.14
Ser	AGC	1599.00	7.39	0.09
Ser	TCG	1411.00	6.52	0.08
Ser	TCA	3316.00	15.33	0.19
Ser	TCT	5495.00	25.40	0.32
Ser	TCC	3198.00	14.78	0.18

Designing a mutagenic oligonucleotide:

choosing the codon to create or destroy a restriction endonuclease site

wild-type *MSH2* coding sequence

A S P G N I E
5' CGCATCTCCA**GGG**AACATTGAGC
GCGTAGAGGT**CCC**TTGTAACTCG 5'

lose a *Bst*NI site
↓

missense mutation in *MSH2* coding sequence

A S P **S** N I E
5' CGCATCTCCA**TCA**AACATTGAGC
GCGTAGAGGT**AGA**TTGTAACTCG 5'

✓best codon choice

✓lose a *Bst*NI site

Designing a mutagenic oligonucleotide - choosing the codon guided by a yeast codon usage table

yeast codon 122 changed from glycine (G) to serine (S)
termed G122S mutation

wild-type *MSH2* coding sequence

A S P G N I E
5' CGCATCTCCA **G**GGGAACATTGAGC

missense mutation in *MSH2* coding sequence

A S P **S** N I E
5' CGCATCTCCA **TCT**AACATTGAGC

A S P **S** N I E
5' CGCATCTCCA **TCA**AACATTGAGC

A S P **S** N I E
5' CGCATCTCCA **TCC**AACATTGAGC

A S P **S** N I E
5' CGCATCTCCA **AGT**AACATTGAGC

top four candidates

Codon usage in yeast (Appendix of manual)

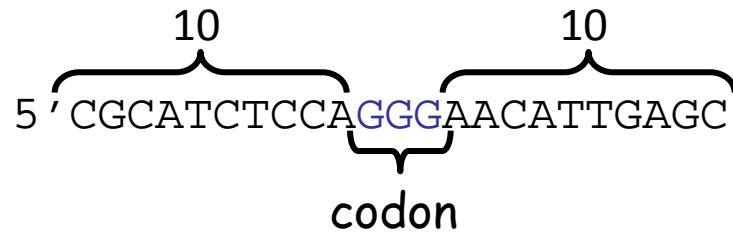
AmAcid	Codon	Number	/1000	Fraction
Ser	AGT	2411.00	11.14	0.14
Ser	AGC	1599.00	7.39	0.09
Ser	TCG	1411.00	6.52	0.08
Ser	TCA	3316.00	15.33	0.19
Ser	TCT	5495.00	25.40	0.32
Ser	TCC	3198.00	14.78	0.18

Laboratory Session 3 (Mon/Tue)

designing the mutagenic oligonucleotide

important considerations:

- 23 nucleotide long (for efficient annealing to the template)

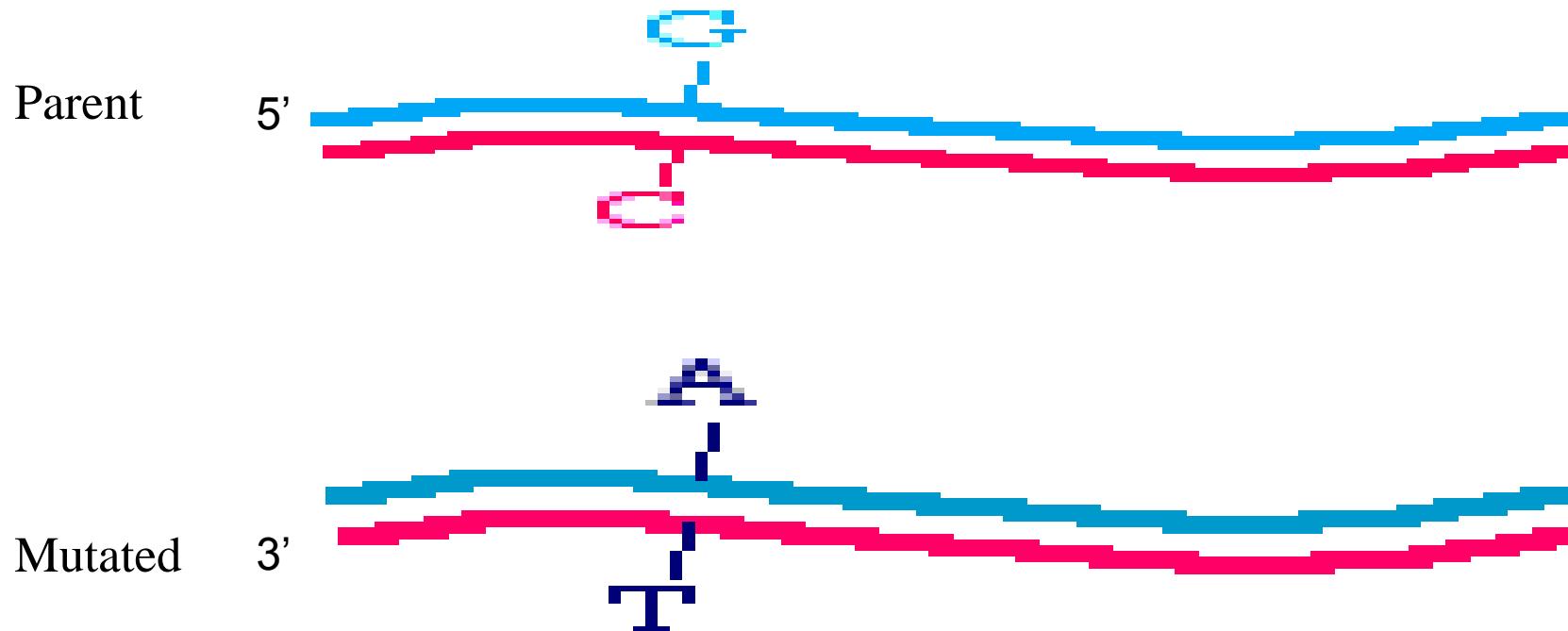


- codon preference for expression in yeast
- creating/destroying a restriction site for screening
- strand must be complementary to template

Cloning Strategy for MSH2 Mutant Gene

The principle of site-directed mutagenesis is that a mismatched oligonucleotide is extended, incorporating the "mutation" into a strand of DNA that can be cloned.

When making a specific mutation, the starting molecule, the one without the mutation, is called the "parent" molecule (wild type).

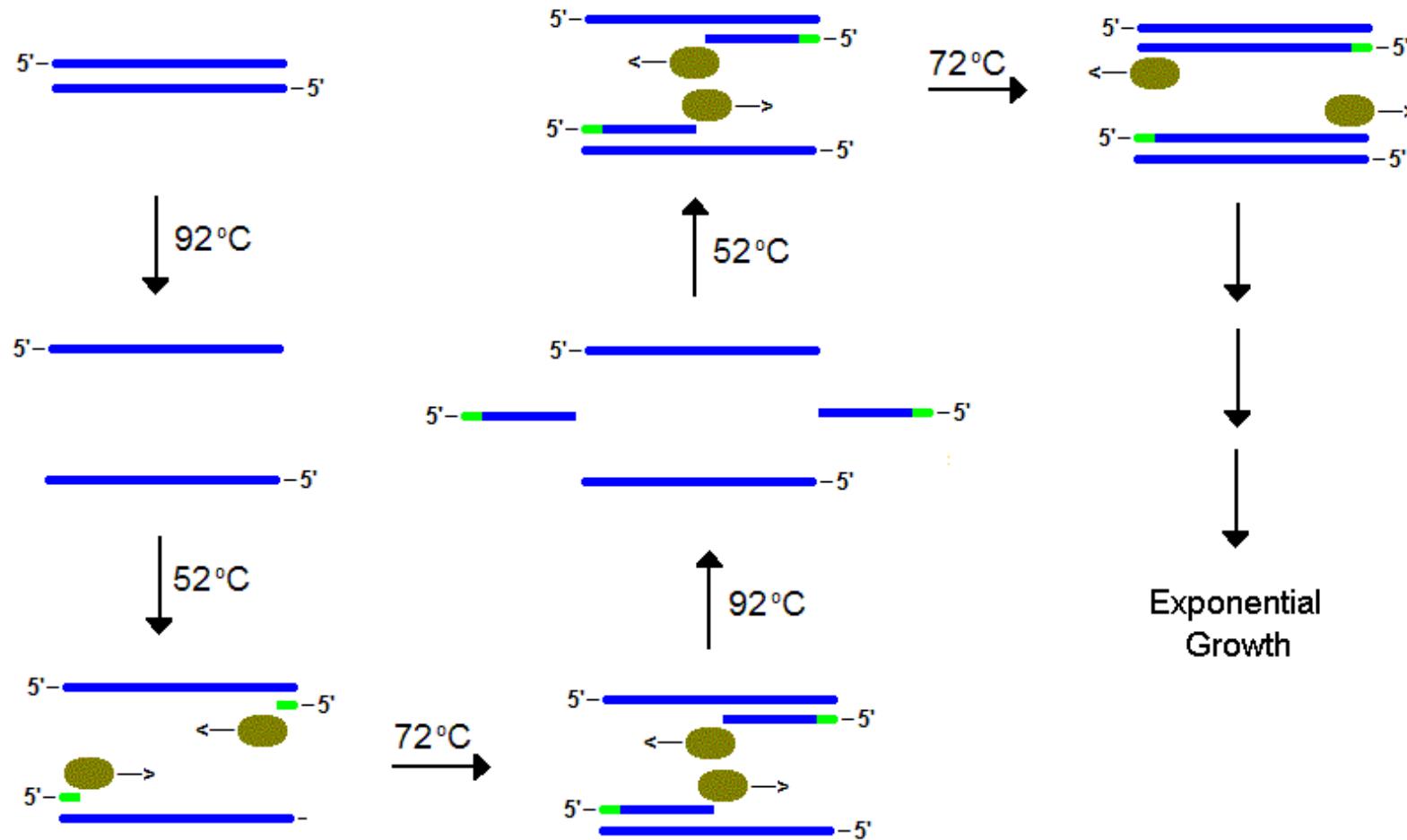


Cloning Strategy for MSH2 Mutant Gene

A common approach is to take the parent molecule and convert it to the mutated version by PCR. The mutation is made by having a mismatch between the parental template and one (or more) oligonucleotide primers.

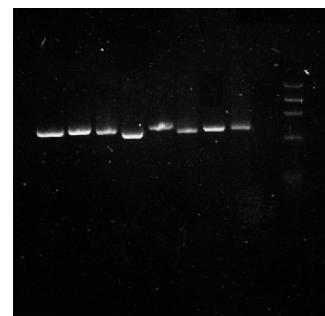
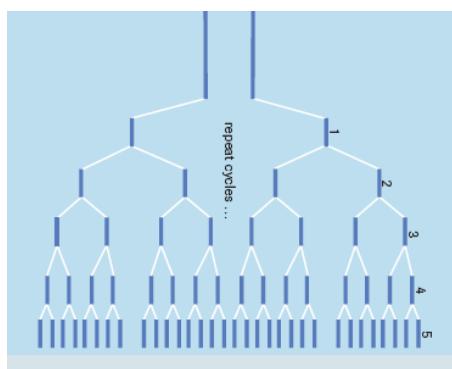
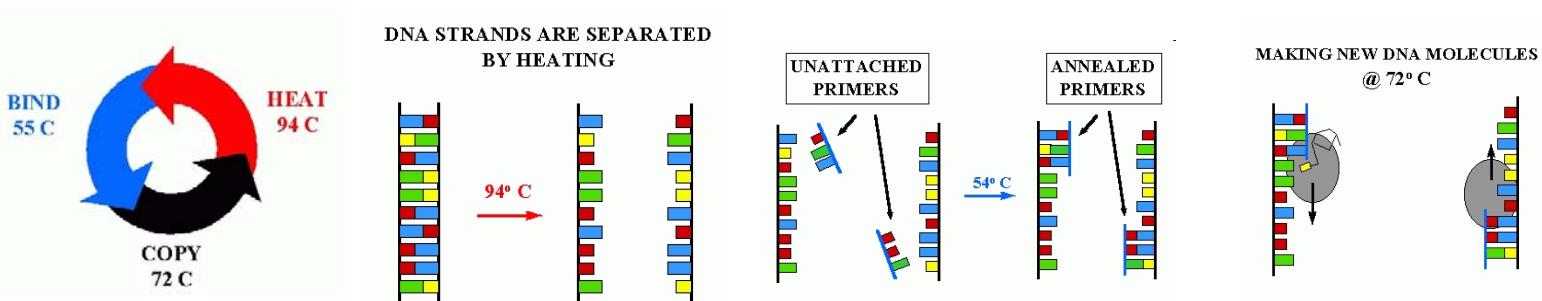
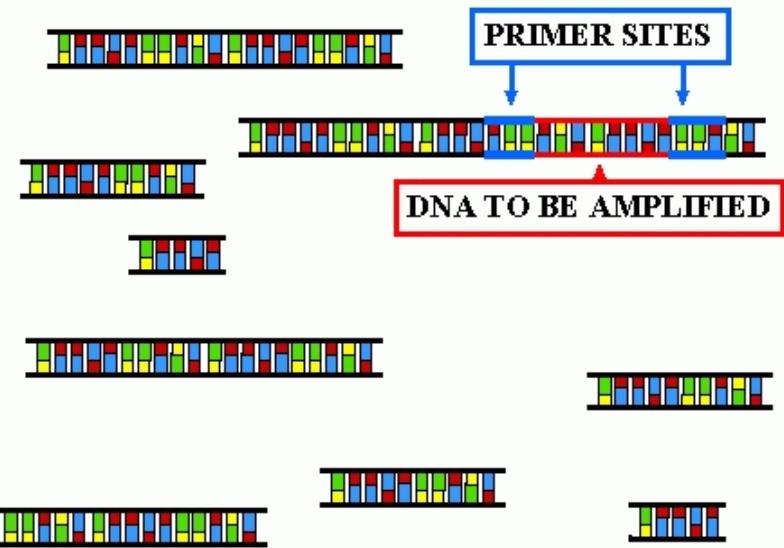
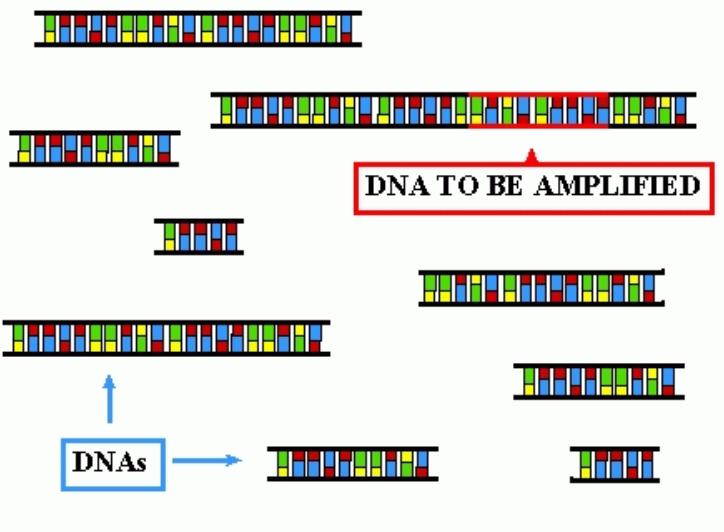
To do this requires learning a bit more about the design of primers (PCR) and making primers that are slightly mismatched with the template.

Normal PCR Schematic Diagram

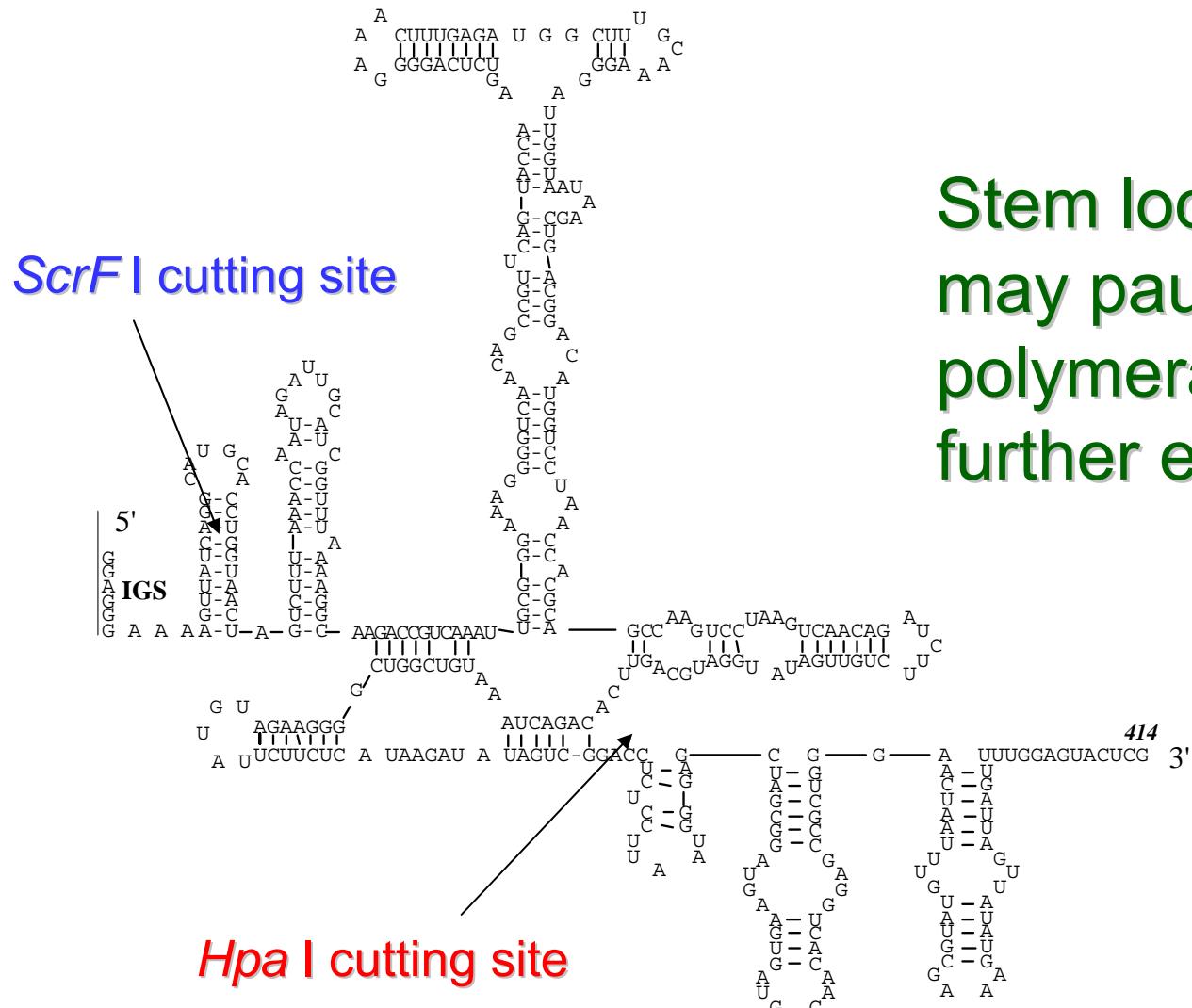


<http://www.promega.com/paguide/animation/selector.htm?coreName=pcr01>

Mullis KB, Faloona FA: Specific synthesis of DNA in vitro via a polymerase-catalyzed chain reaction. *Methods Enzymol* 155: 335–350, 1987



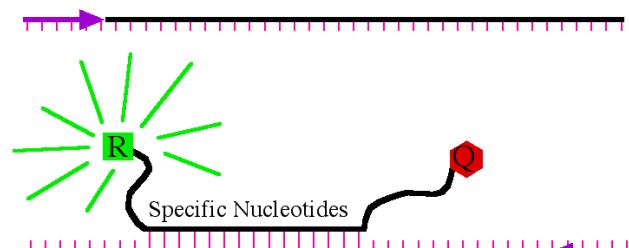
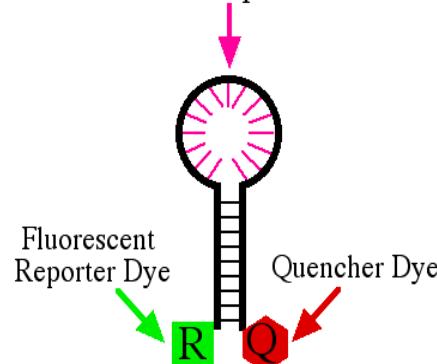
Why Secondary Structure Matters?



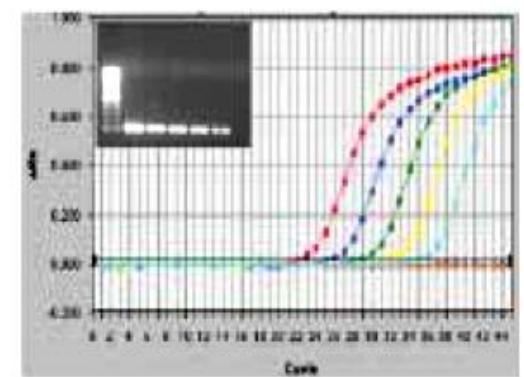
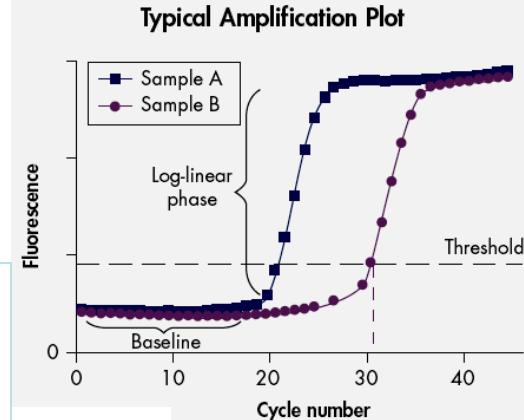
Stem loop structures
may pause *Taq*
polymerase from
further extension?

Real Time Versus Reverse Transcription PCR

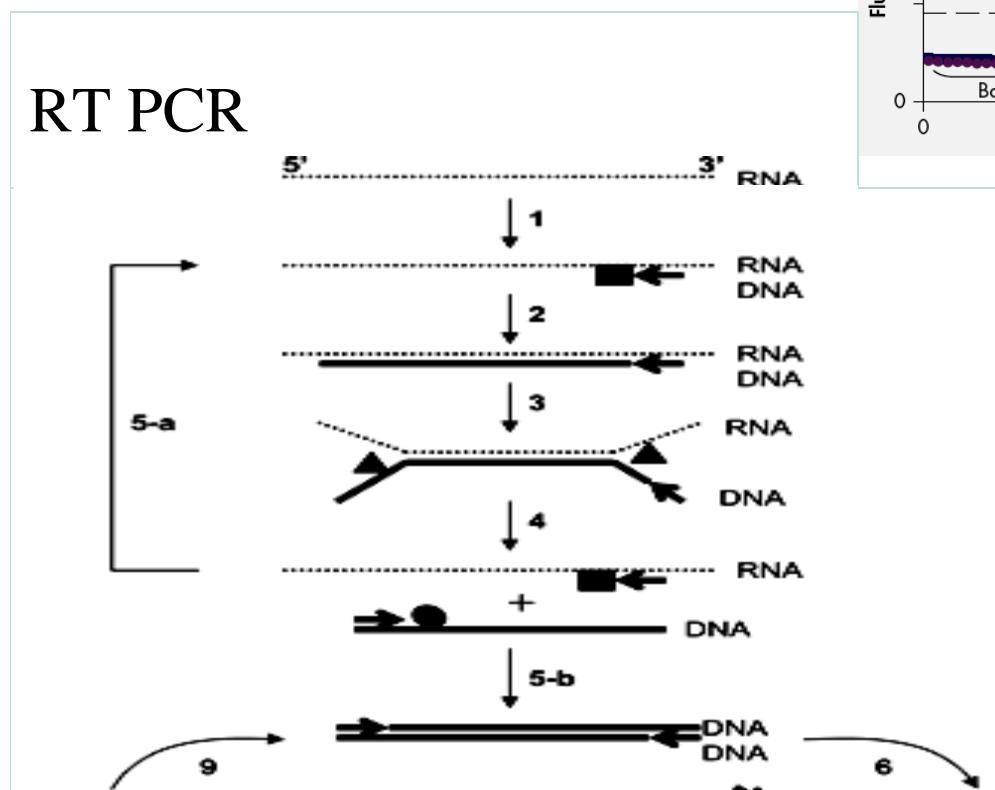
PCR Product-Specific Nucleotides

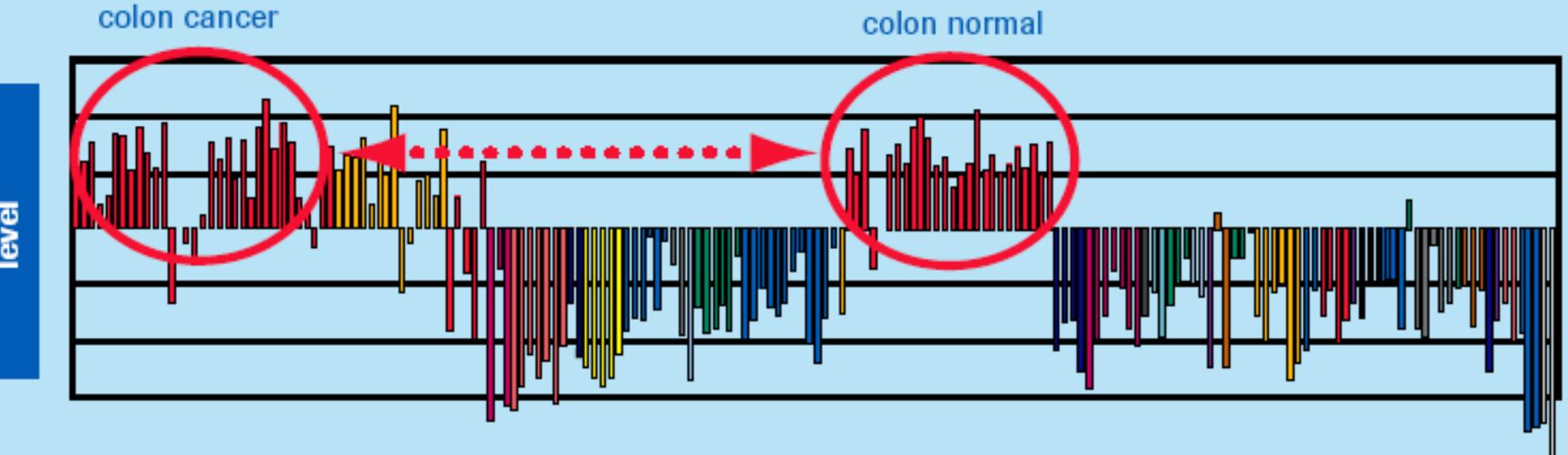


Typical Amplification Plot



RT PCR





Cloning Strategy for MSH2 Mutant Gene

A common approach is to take the parent molecule and convert it to the mutated version by PCR. The mutation is made by having a mismatch between the parental template and one (or more) oligonucleotide primers.

To do this requires learning a bit more about the design of primers (PCR) and making primers that are slightly mismatched with the template.

TCGATGGACCAGTACGATA_CCCAGTA.....CGACCTACGTAGACTAGACGGATAGAG
AGCTACCTGGTCATGCTATGGTCAT.....GCTGGATGCATCT GATCTGCCTATCTC

ends generated from Type II restriction endonucleases

	<u>enzyme</u>	<u>recognition site</u>	<u>end generated</u>		
defined ends	<i>Pvu</i> I	5' CAGCTG	5' CAG	CTG	blunt
		3' GTCGAC	3' GTC	GAC	
	<i>Bam</i> H I	5' GGATCC	5' G	GATCC	5' overhang
variable ends	<i>Pst</i> I	3' CCTAGG	3' CCTAG	G	
		5' CTGCAG	5' CTGCA	G	3' overhang
	<i>Xba</i> I	3' GACGTC	3' G	ACGTC	
	<i>Xba</i> I	5' GAANNNNTTC	5' GAANN	NNNTTC	blunt
		3' CTTNNNNNAAG	3' CTTNN	NNAAG	
	<i>Ban</i> I	5' GGP _y PuCC	5' G	GPyPuCC	5' overhang
	<i>Bst</i> XI	3' CCPuPyGG	3' CCPuPyG	G	
		5' CCANNNNNNTGG	5' CCANNNNN	NTGG	3' overhang
	<i>Bst</i> XI	3' GGTNNNNNNNACC	3' GGTN	NNNNNACC	

Cloning Strategy for MSH2 Mutant Gene

Adding restriction sites is important for cloning. To add an EcoRI site (GAATTC) to the end on the left, and a BamHI site (GGATCC) to the end on the right.

GCGAATTCTCTATGGACCAGTACGAT

GCGGATCCCTCTATCCGTCTAGTCTA

T

GCGAATT~~C~~GCTATGGACCAGTACGAT

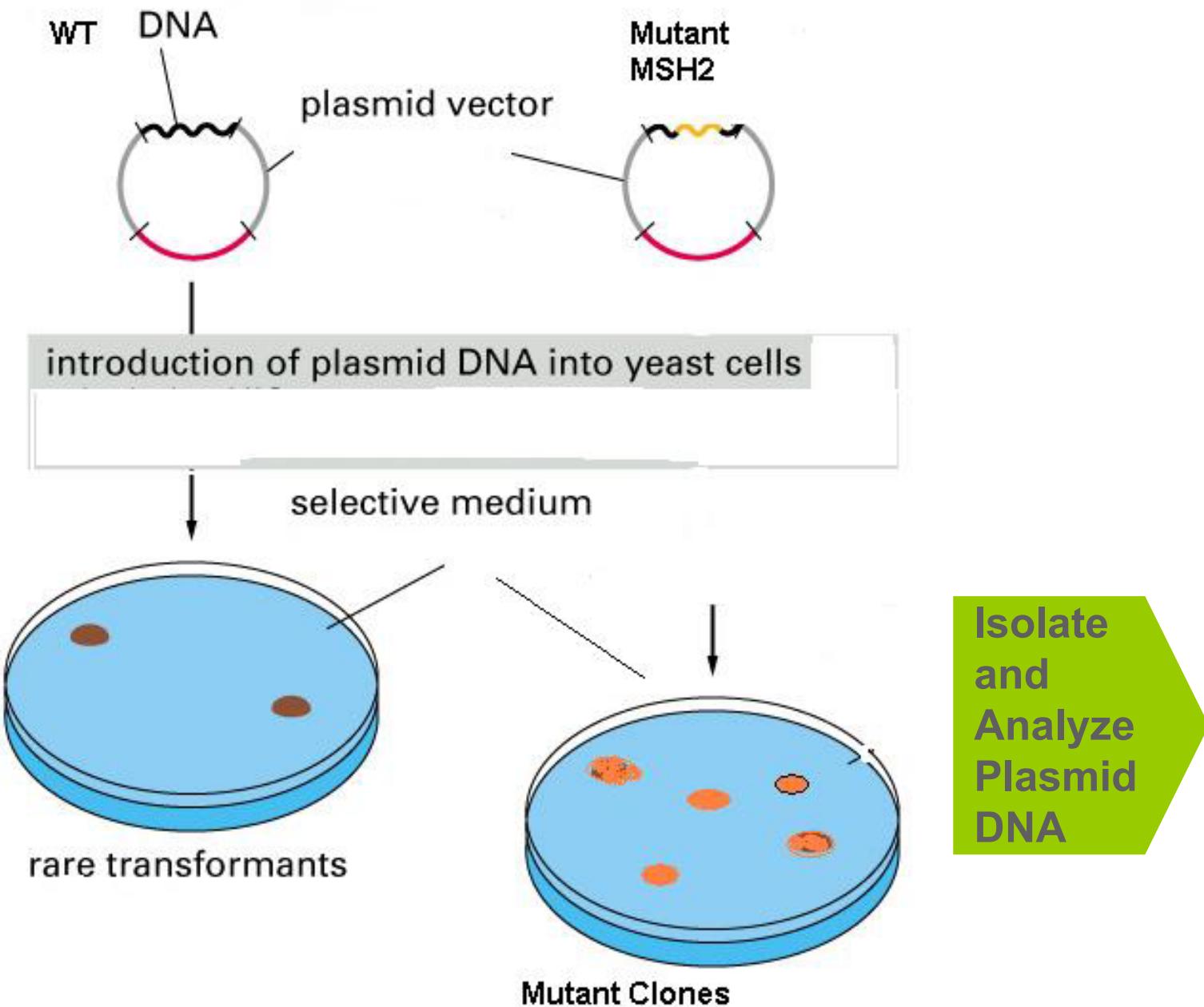
GCGGATCCCTCTATCCGTCTAGTCTA

GCGAATT~~T~~CTATGGACCAGTACGAT

GCGGATCCCTCTATCCGTCTAGTCTA

TCTATGGACCAGTACGATA~~CCAGTA.....CGACCTACGTAGACTAGACGGATAGAG~~
~~AGATA~~~~ACCTGGTCATGCTATGGTCAT.....GCTGGATGCATCT GATCTGCCTATCTC~~

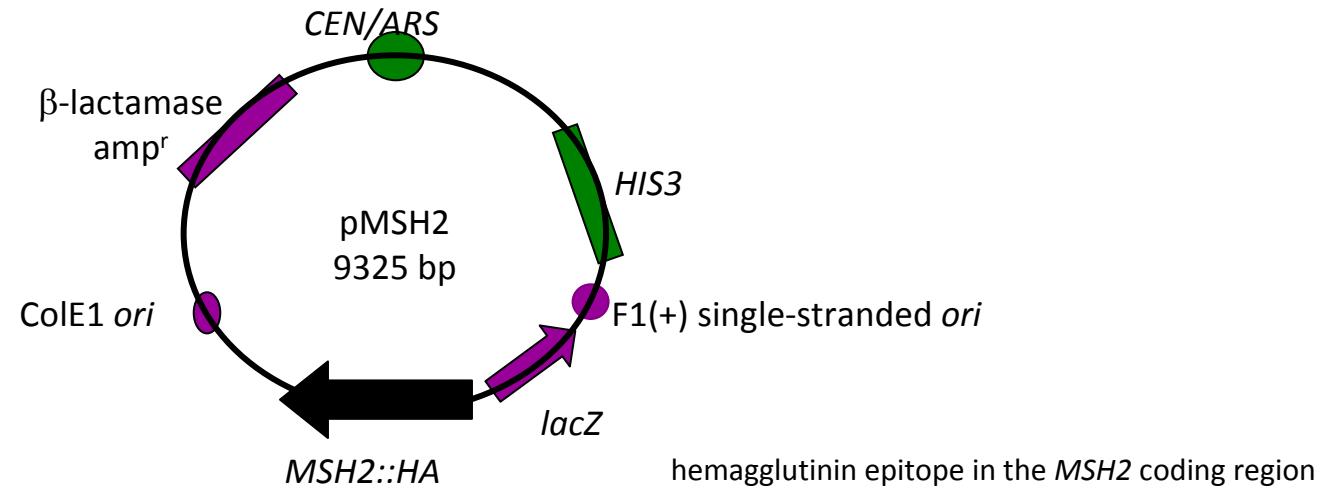
The project would also require using knowledge gained from plasmid extraction, Gel electrophoresis, PCR-----



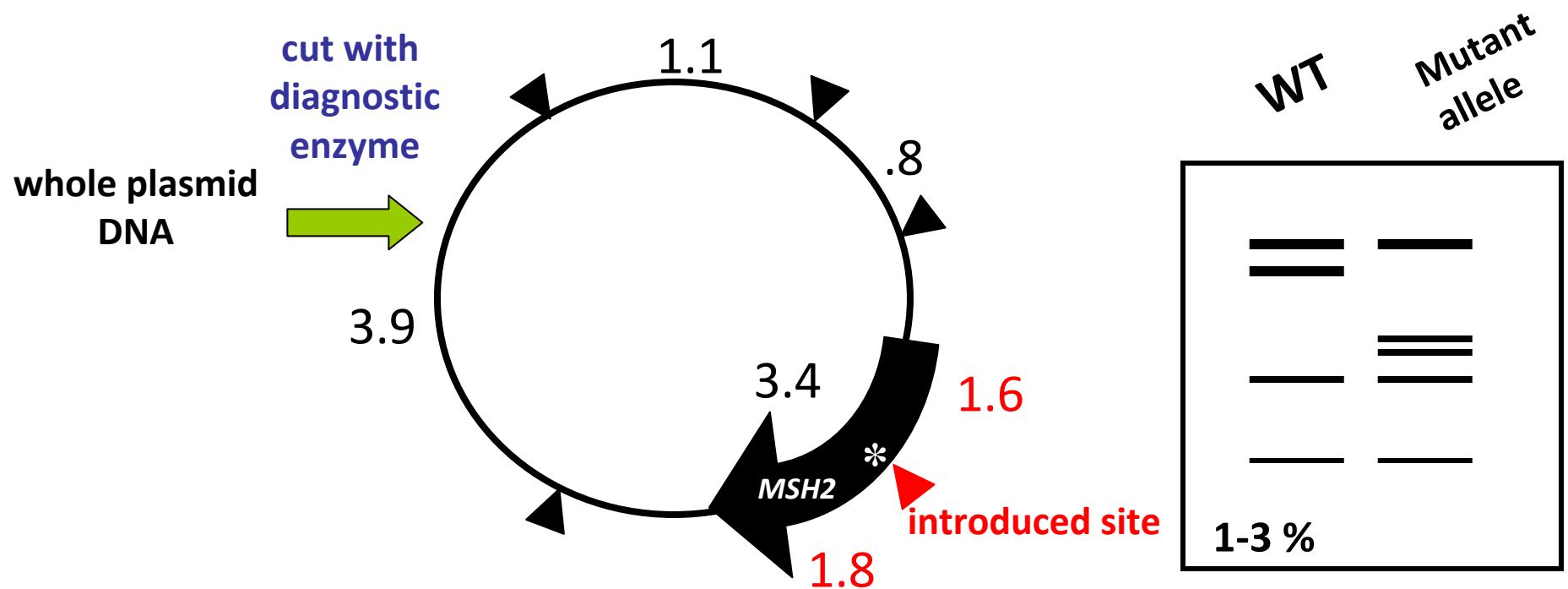
From, MolecularBiology of the Cell

Taking a closer look at our plasmid, pMSH2

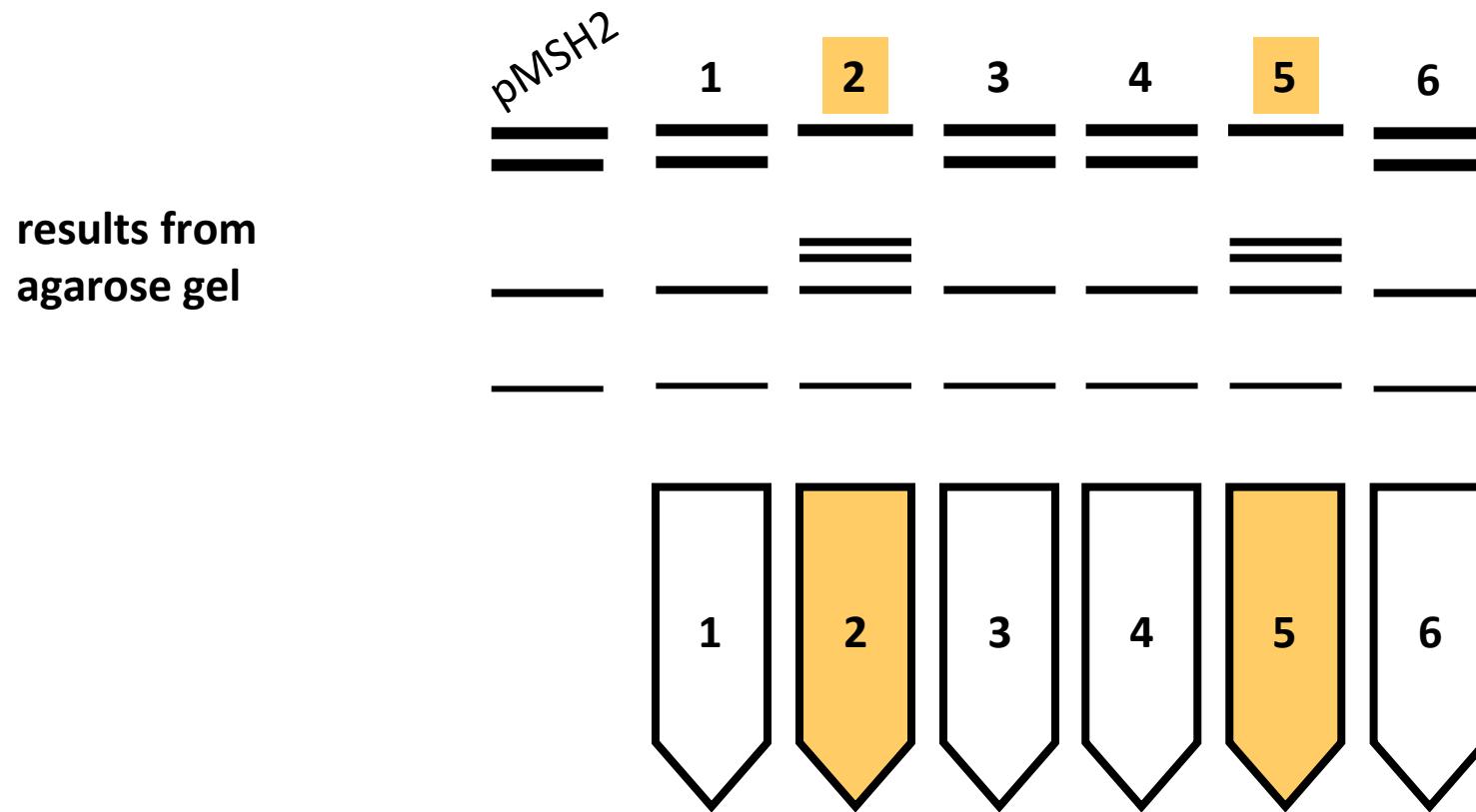
MSH2 construct for mutagenesis and functional assays



We will use restriction digestion to distinguish between WT and mutagenized alleles of our pMSH2 vector



After the diagnostic gel, only proceed with a plasmid showing the altered restriction endonuclease digestion pattern (e.g. 2 or 5)



- ✓ Inoculate 25 ml of media with the correct bacterial colony for a midi-scale plasmid preparation (anion exchange chromatography)
- ✓ Use miniprep DNA to transform yeast to begin the functional analyses

Locating the codon to be mutagenized

Note that the two sequences don't have corresponding codon numbers

for example Ala 719 in yeast is Ala 700 in humans

Yeast 600	INITLTYTPVFEKLSLVLAHLDVIASFAHTSSYAPIPYIRPKLHPMDERRTHLISSRHP	659
	+NI+ Y + L+ VLA LD + SFAH S+ AP+PY+RP + + R L +SRH	
Human 582	VNISSGYVEPMQTLNDVLAQLDAVVSFAHVSNGAPVPYVRPAILE-KGQGRRIILKASRHA	640
Yeast 660	VLEMQDDISFISNDVTLESKGDFLIITGPNMGKSTYIRQGVVISLMAQIGCFVPCEEA	719
	+E+QD+I+FI NDV E K F IITGPNMGKSTYIRQ GVI LMAQIGCFVPCE A	
Human 641	CVEVQDEIAFIPNDVYFEKDKQMFHIITGPNMGKSTYIRQTGVIVLMAQIGCFVPCESA	700
Yeast 720	EIAIVDAILCRVGAGDSQLKGVSTFMVEILETASILKNASKNSLIIIVDELGRGTSTYDGF	779
	E++IVD IL RVGAGDSQLKGVSTFM E+LETASIL++A+K+SLII+DELGRGTSTYDGF	
Human 701	EVSIVDCILARVGAGDSQLKGVSTFMAEMLETASILRSATKDSLIIIDELGRGTSTYDGF	760
Yeast 780	GLAWAIAEHIAASKIGCFALFATHFHELTTELSEKLPNVKNMHVVAHIEKNLKEQKHEDDEDI	839
	GLAWAI+E+IA+KIG F +FATHFHELT L+ ++P V N+HV A +E +	
Human 761	GLAWAISEYIATKIGAFCMFATHFHELTALANQIPTVNNLHVTA-----LTTEETL	811
Yeast 840	TLLYKVEPGISDQSFGIHVAEVVQFPEKIVKMAKRKANELEDDLKTNNE----DLKK--AK	893
	T+LY+V+ G+ DQSFGIHVAE+ FP+ +++ AK+KA EL++ + E D+ + AK	
Human 812	TMLYQVKKGVCDQSFGIHVAELANFPKHVIECAKQKALELEEFQYIGESQGYDIMEPAAK	871

Designing a mutagenic oligonucleotide - choosing the codon guided by a yeast codon usage table

yeast codon 122 changed from glycine (G) to serine (S)
termed G122S mutation

wild-type *MSH2* coding sequence

A S P G N I E
5' CGCATCTCCA **G**GGGAACATTGAGC

missense mutation in *MSH2* coding sequence

A S P **S** N I E
5' CGCATCTCCA **TCT**AACATTGAGC

A S P **S** N I E
5' CGCATCTCCA **TCA**AACATTGAGC

A S P **S** N I E
5' CGCATCTCCA **TCC**AACATTGAGC

A S P **S** N I E
5' CGCATCTCCA **AGT**AACATTGAGC

top four candidates

Codon usage in yeast (Appendix of manual)

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Designing a mutagenic oligonucleotide:

choosing the codon to create or destroy a restriction endonuclease site

wild-type *MSH2* coding sequence



lose a *Bst*NI site

missense mutation in *MSH2* coding sequence



✓best codon choice

✓lose a *Bst*NI site

Designing a mutagenic oligonucleotide - choosing the codon guided by a yeast codon usage table

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A S P **S** N I E
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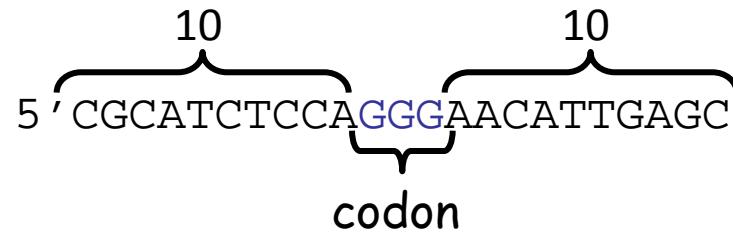
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designing the mutagenic oligonucleotide

important considerations:

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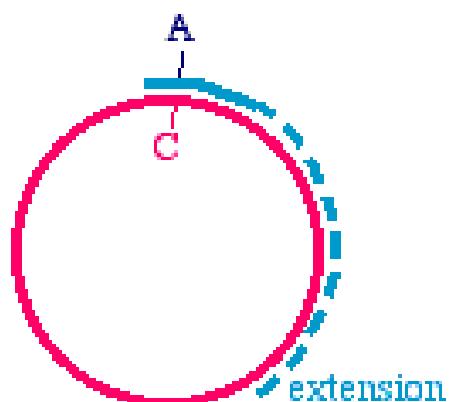


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- strand must be complementary to template

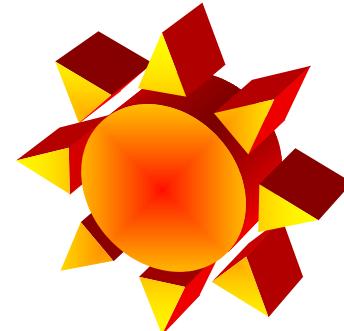
Cloning Strategy for Mutant Genes

It should be noted that there are several approaches to site-directed mutagenesis. The **QuikChange site-directed mutagenesis** kit (Stratagene) is a commercial kit that uses *PfuTurbo*® DNA polymerase** and temperature cycling as in PCR. The DNA polymerase replicates both plasmid strands with high fidelity, without displacing the mutant oligonucleotide primers.

Extension of the insert containing the desired mutation during temperature cycling by *PfuTurbo* generates a mutated plasmid containing staggered nicks. Following temperature cycling, the product is treated with *Dpn* I endonuclease (target sequence: 5'-Gm6ATC-3') which is specific for methylated and hemimethylated DNA and is used to digest the parental DNA template. Mutation-containing synthesized DNA is left intact and can be isolated, then used in transforming supercompetent cells.



Mutagenic PCR



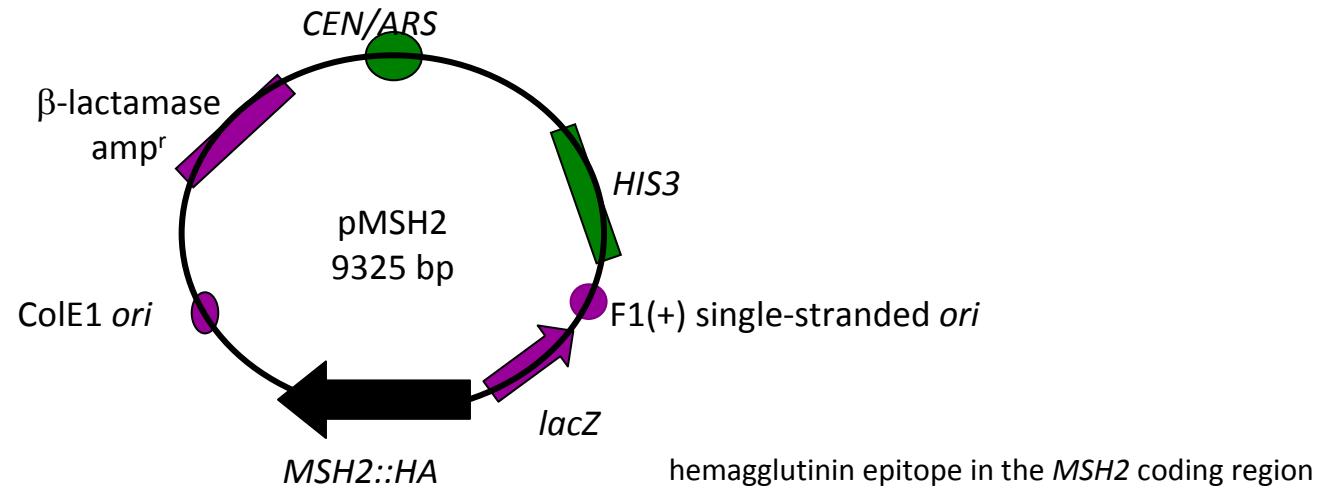
- Making *Taq* more Error Prone:
 - Site-directed
 - compared to old technique of randomized oligos

1: Cadwell, C. R.; Joyce, G. F. *PCR Meth. Appl.*, **2**, 28-33 (1992)

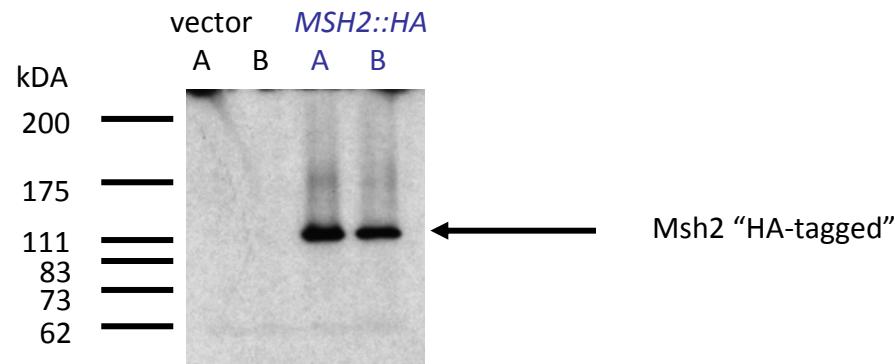
2: Vartanian, J-P.; Henry, M.; Wain-Hobson, S. *Nucleic Acids Research*, **24**, 2627-31 (1996)

Taking a closer look at our plasmid, pMSH2

MSH2 construct for mutagenesis and functional assays



-produces a protein product of the expected molecular weight



-complements *msh2Δ* defects, therefore the construct is functional

Problem Set

Creating mutagenic primers for site-directed mutagenesis in yeast VMA6

Here's the nucleotide sequence of the yeast VMA6 gene:

```
1 ATGGAAGGCG TGTATTCAA TATTGACAAT GGGTTTATTG AAGGTGTA GT GAGAGGCTAC
 61 AGAAATGGGT TGTTATCTAA TAACCAATAC ATCAACTTAA CACAATGTGA CACGTTGGAA
121 GATCTAAAAT TACAATTATC ATCAACTGAT TATGGTAATT TTCTTCCCTC TGTTTCCTCA
181 GAGTCTTGA CCACGTCA TT GATTCAAGAA TATGCTTCTA GCAAGTTGTA CCACGAATTG
241 AACTACATAA GAGACCAATC CAGTGGATCC ACGAGAAAGT TCATGGACTA TATCACTTAT
301 GGTTACATGA TCGACAATGT AGCATTGATG ATTACAGGTA CTATTCATGA TCGTGATAAG
361 GGTGAAATTT TACAACGTTG TCATCCGCTA GGTTGGTTTG ATACTTTGCC TACGTTGAGT
421 GTTGCTACTG ATCTTGAATC CCTATACGAA ACCGTATTGG TGGATACCCC ACTGGCACCT
481 TACTTCAAAA ACTGTTTGA CACGGCAGAG GAGCTAGACG ATATGAACAT TGAAATTATT
541 AGAAATAAGC TGTACAAGGC TTATTTAGAA GACTTTACA ATTTTGTAC TGAAGAAATT
601 CCGAACCTG CTAAAGAATG TATGCAAACA TTACTAGGGT TTGAAGCTGA CAGAAGAAGT
661 ATCAATATTG CACTCAACTC TTTGCAAAGT TCAGATATTG ACCCAGATTG GAAAAGTGAC
721 TTGTTACCTA ACATAGGTAA GTTGTACCCCT CTTGCAACGT TTCACTTGGC GCAAGCCCAA
781 GATTTCGAAG GAGTTAGAGC TGCTTTAGCT AACGTCTATG AGTATAGGGG ATTTTTGGAG
841 ACTGGTAACT TAGAAGATCA CTTTTACCAA TTGGAAATGG AACTATGTAG AGATGCTTTC
901 ACGCAACAAT TTGCCATCAG CACTGTTGG GCCTGGATGA AATCCAAGGA ACAAGAAGTT
961 AGGAATATTA CCTGGATTGC AGAATGTATC GCACAAAACC AAAGAGAAAG AATCAACAAT
1021 TATATTTCCG TTTATTGA
```

Using SIXFRAME, the correct open reading frame (ORF 1) can be identified:

```
M E G V Y F N I D N G F I E G V V R G Y
1 atggaaaggcgtgtatattcaatattgacaatgggtttattgaaggtagtgagaggctac 60
R N G L L S N N Q Y I N L T Q C D T L E
61 agaaaatgggttgttataactaaccatacatcaacttaacacaatgtgacacgttggaa 120
D L K L Q L S S T D Y G N F L S S V S S
121 gatctaaaattacaattatcatcaactgattatggtaatttcttcctgtttccctca 180
E S L T T S L I Q E Y A S S S K L Y H E F
181 gagtccttgcaccacgtcattgtatccaagaatatgctctagcaagtgtaccacgaaatc 240
N Y I R D Q S S G S T R K F M D Y I T Y
241 aactacataagagaccaaattccaggatccacgagaaaatgttcatggactatatacttat 300
G Y M I D N V A L M I T G T I H D R D K
301 ggttacatgatcgacaatgttagcattgtgattacaggtactattcatgatcgtgataag 360
G E I L Q R C H P L G W F D T L P T L S
361 ggtgaaattttacaacgttgtcatccgcgttaggtgggttgcatacttgcctacgttgagt 420
V A T D L E S L Y E T V L V D T P L A P
421 gttgctactgatctgaatccctatacgaaaccgtattggtgatcacccactggcacct 480
Y F K N C F D T A E E L D D M N I E I I
481 tacttcaaaaactgtttgacacggcagaggagctagacgatatacgatggaaattatt 540
R N K L Y K A Y L E D F Y N F V T E E I
541 agaaataagctgtacaaggctttttagaagacttttacaattttgtcactgaagaaattt 600
P E P A K E C M Q T L L G F E A D R R S
601 ccggAACCTGCTAAAGAATGTATGCAAACATTACTAGGGTTGAAGCTGACAGAGAAAGT 660
I N I A L N S L Q S S D I D P D L K S D
661 atcaatattgcactcaactcttgcattttcaatggatattgacccagatttggaaatgt 720
L L P N I G K L Y P L A T F H L A Q A Q
721 ttgttacctaatacgatggtaagttgttacccctctgtcaacgtttcacttggcgcaagccccaa 780
D F E G V R A A L A N V Y E Y R G F L E
781 gatttcgaaggagtttagagctgttttagctaacgtctatgagttataggggatttttggag 840
T G N L E D H F Y Q L E M E L C R D A F
841 actggtaactttagaagatcactttaccatggaaatggaaactatgttagagatgtttc 900
T Q Q F A I S T V W A W M K S K E Q E V
901 acgcaacaatttgcatttcacgactgtttggcctggatgaaatccaaggaaacaagaatgtt 960
R N I T W I A E C I A Q N Q R E R I N N
961 aggaatattacctggattgcagaatgtatgcacaaaaaccaaagagaaagaatcaacaat 1020
Y I S V Y *
1021 tatattccgttattga 1038
```

To simplify the analysis, a shorter gene fragment can be generated using the nucleic editing tool

	L L P N I G K L Y P L A T F H L A Q A Q	
1	ttgttacctaacaataggtaagttgtaccctcttgcaacgttcacttggcgcaagccaa	60
	D F E G V R A A L A N V Y E Y R G F L E	
61	gatttcgaaggaggtagagctgcttagctaacgtctatgagtatagggattttggag	120
	T G N L E D H F Y Q L E M E L C R D A F	
121	actggtaacttagaagatcactttaccaattggaaatggaactatgttagagatgcttc	180
	T Q Q F A I S T V W A W M K S K E Q E V	
181	acgcaacaatttgcacatcagcactgttggcctggatgaaatccaaggaacaa gaa gtt	240
	R N I T W I A E C I A Q N Q R E R I N N	
241	aggaatattacctggattgcagaatgtatcgacaaaaccaaagagaaagaatcaacaat	300
	Y I S V Y *	
301	tatattccgttattga	318

sequence can be analyzed with TACG tool to identify restriction sites in the region of the codon to be targeted:

it is helpful to know what each restriction site is, and this information is conveniently provided for you in the TACG tool:

<u>Enzyme</u>	<u>Restriction Site</u>
StyI	C' C _{ww} G_G
BsaJI	C' C _{nn} G_G
BtsCI	GGATG_nn '
FokI	GGATG _{nnnnnnnnnn} ' nnnn_
TspDTI	ATGA _{nnnnnnnnnn} _nn '
SspI	AAT'ATT

This is the coding region we are interested in:

SspI

5' -atgaaatccaaggaacaagagtttaggaatattacctggattgcagaatgtatc-3'

M K S K E Q **E** V R N I T W I A E C

To design a primer that 1) alters the glutamate codon (E) to code for lysine (K), and 2) destroys the Sspl restriction site:

Gene Sequence: 5'-gqaacaa**qaa**gttaqq**aatattac**cgtt-3' E SspI

For the final step, it is important to design a second primer that will anneal to the complementary strand:

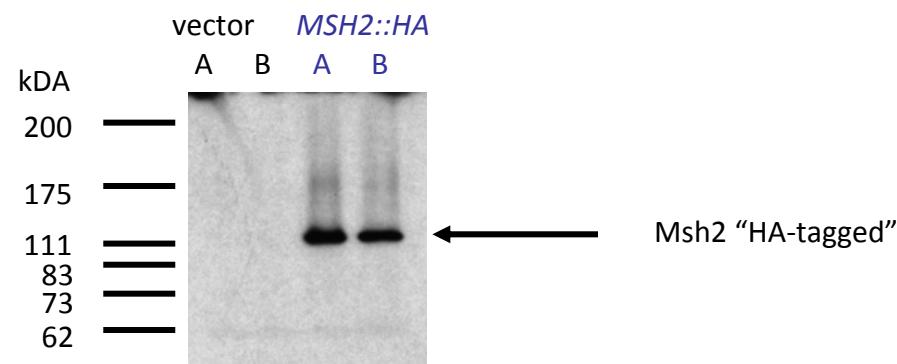
5' -gaacaaaaagtttaggaatgttacc-3' (referred to as the "forward" primer)

3' -cttgttttcaatccttacaatgg-5' (referred to as the "reverse" primer)

Functional Analysis

Functional Analysis

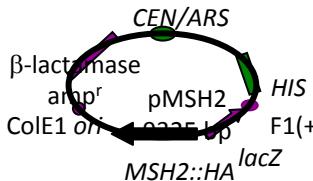
-produces a protein product of the expected molecular weight



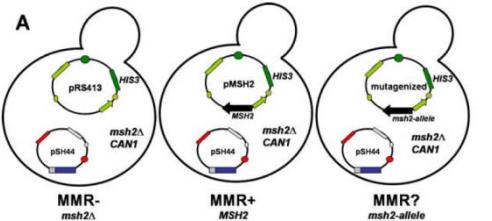
-complements *msh2Δ* defects, therefore the construct is functional

Molecular Biology Genomics

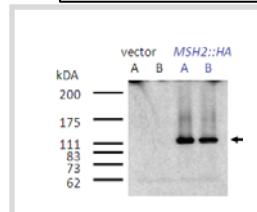
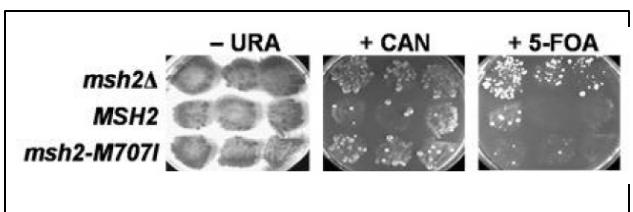
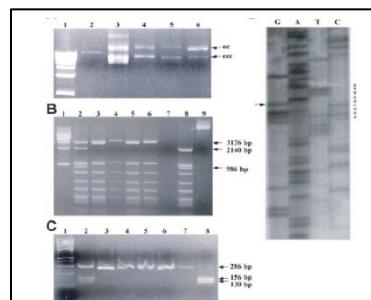
PCR and Plasmid Constructs

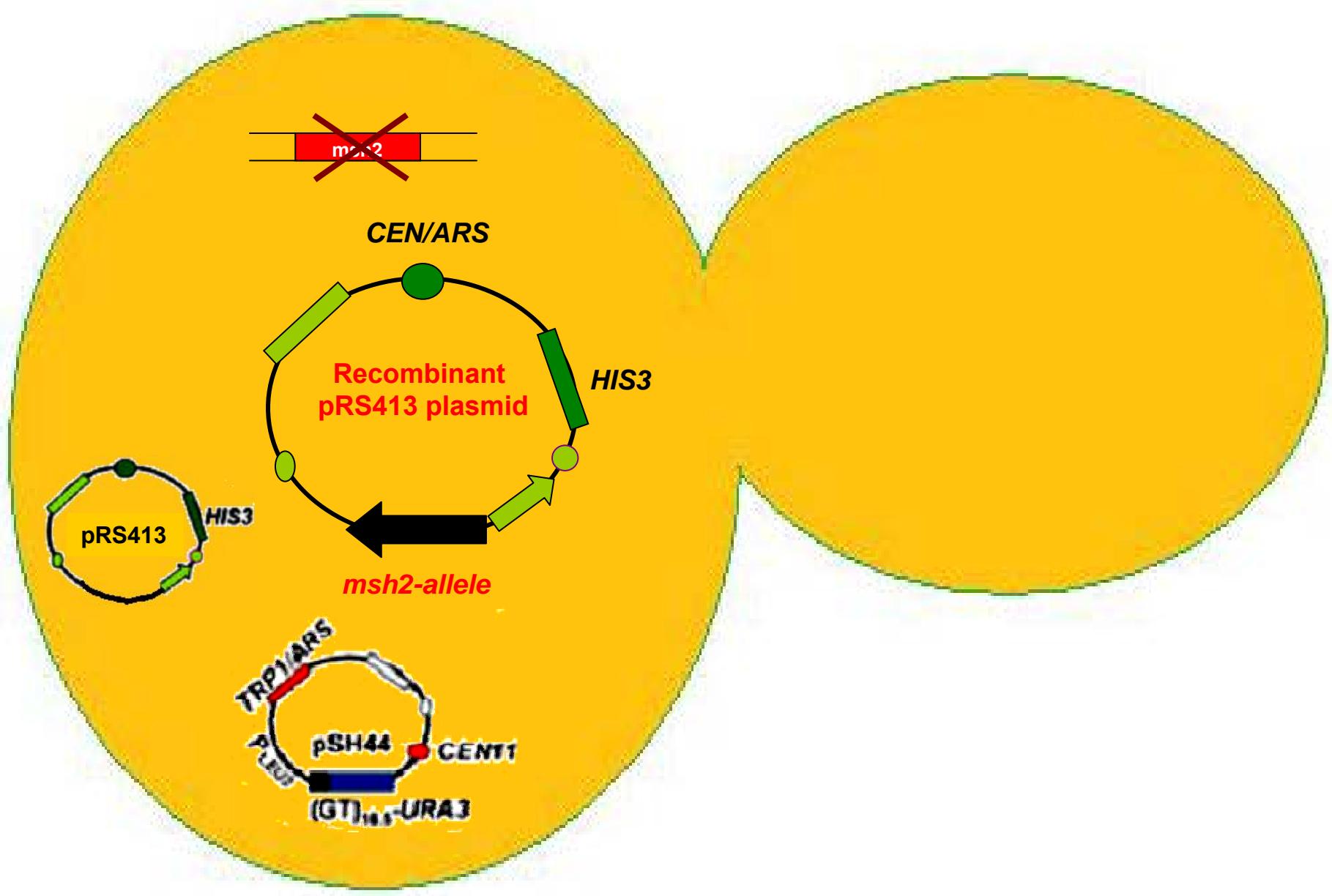


MSH2
Gene

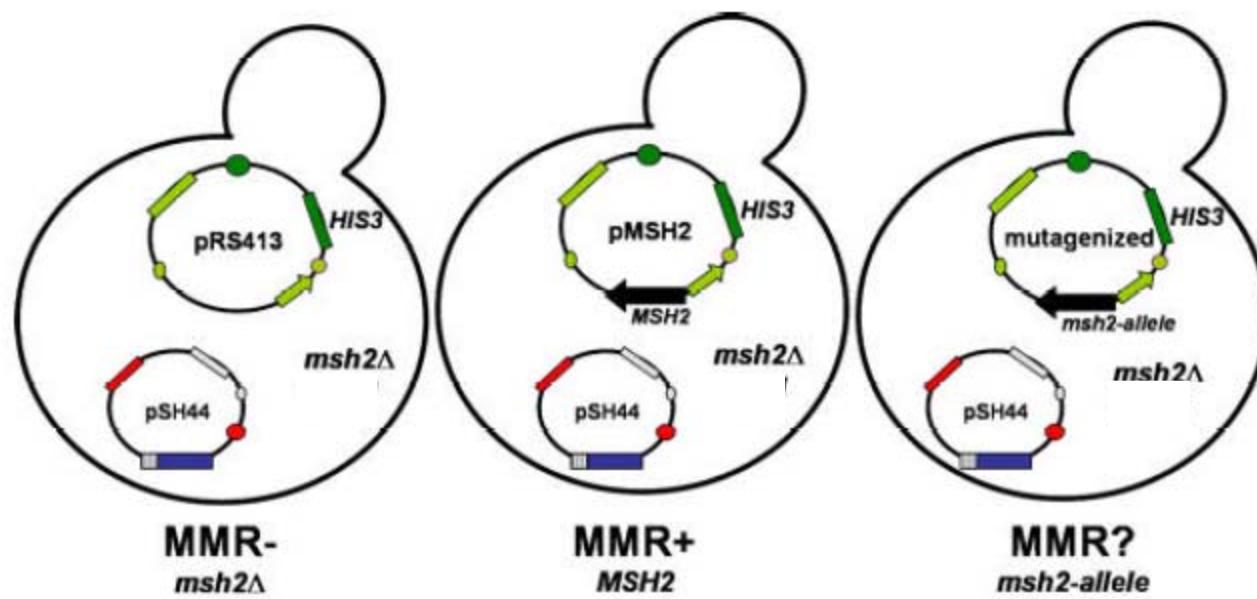


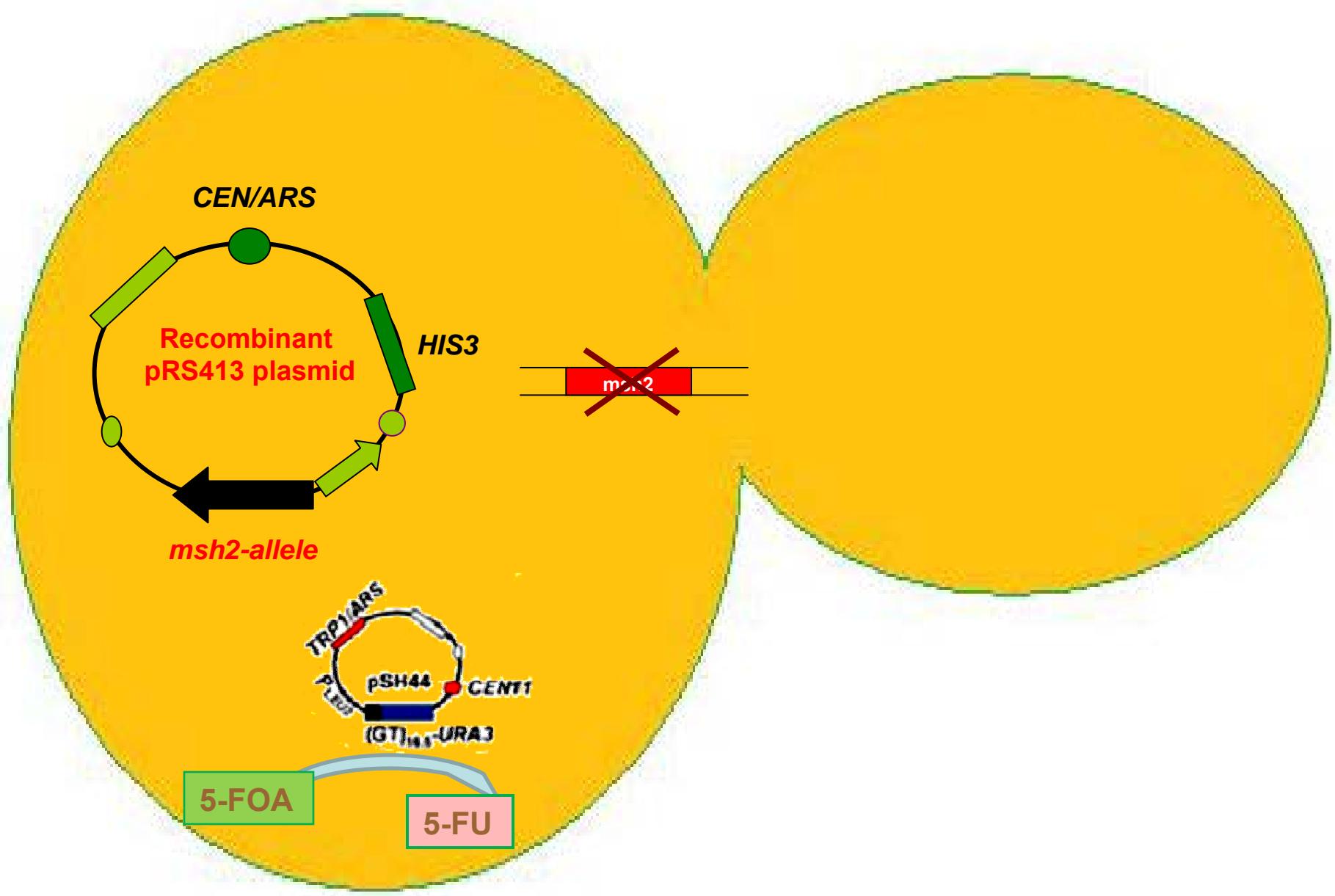
Plasmid
Constructs





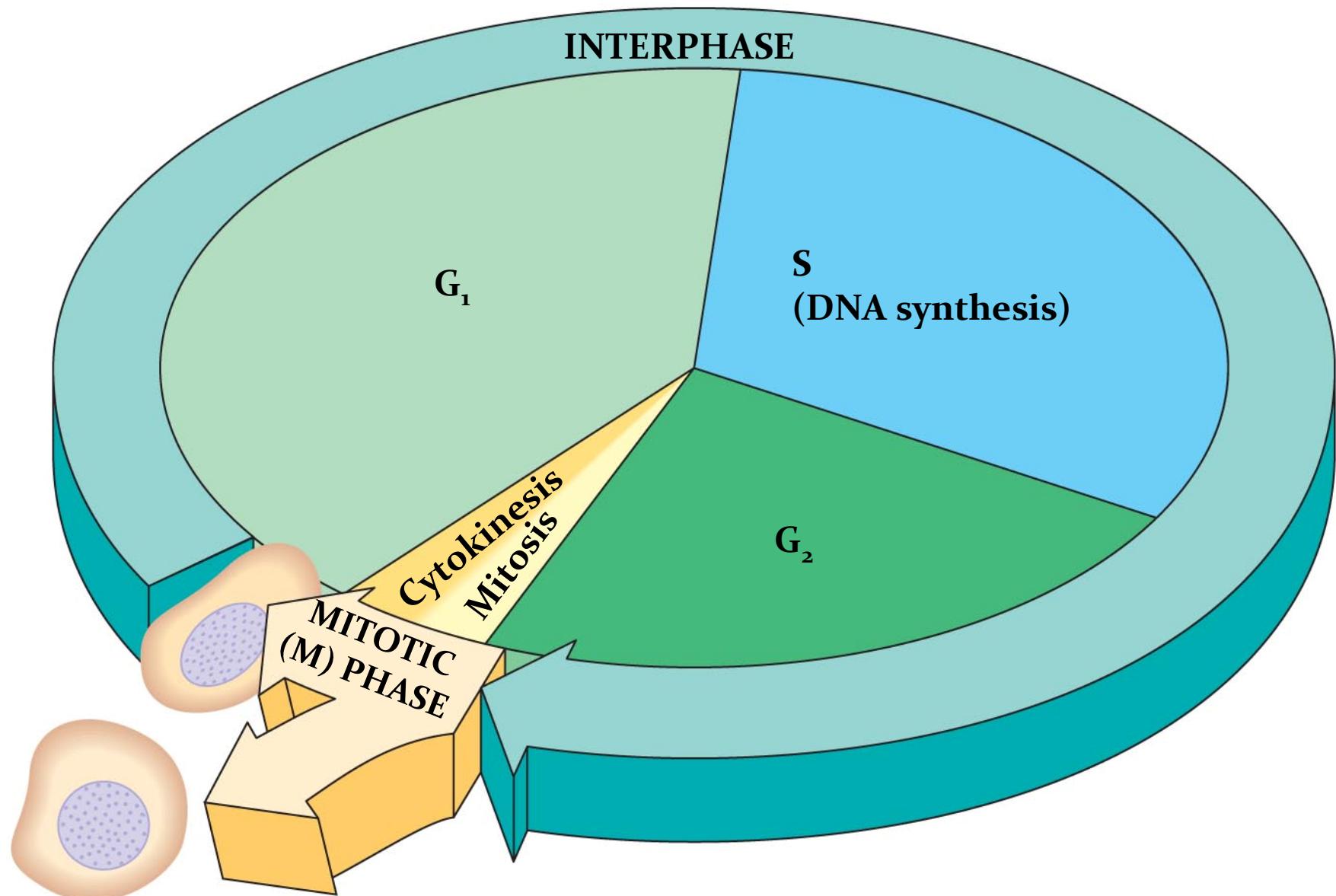
Outline of Yeast Transformation Experiment



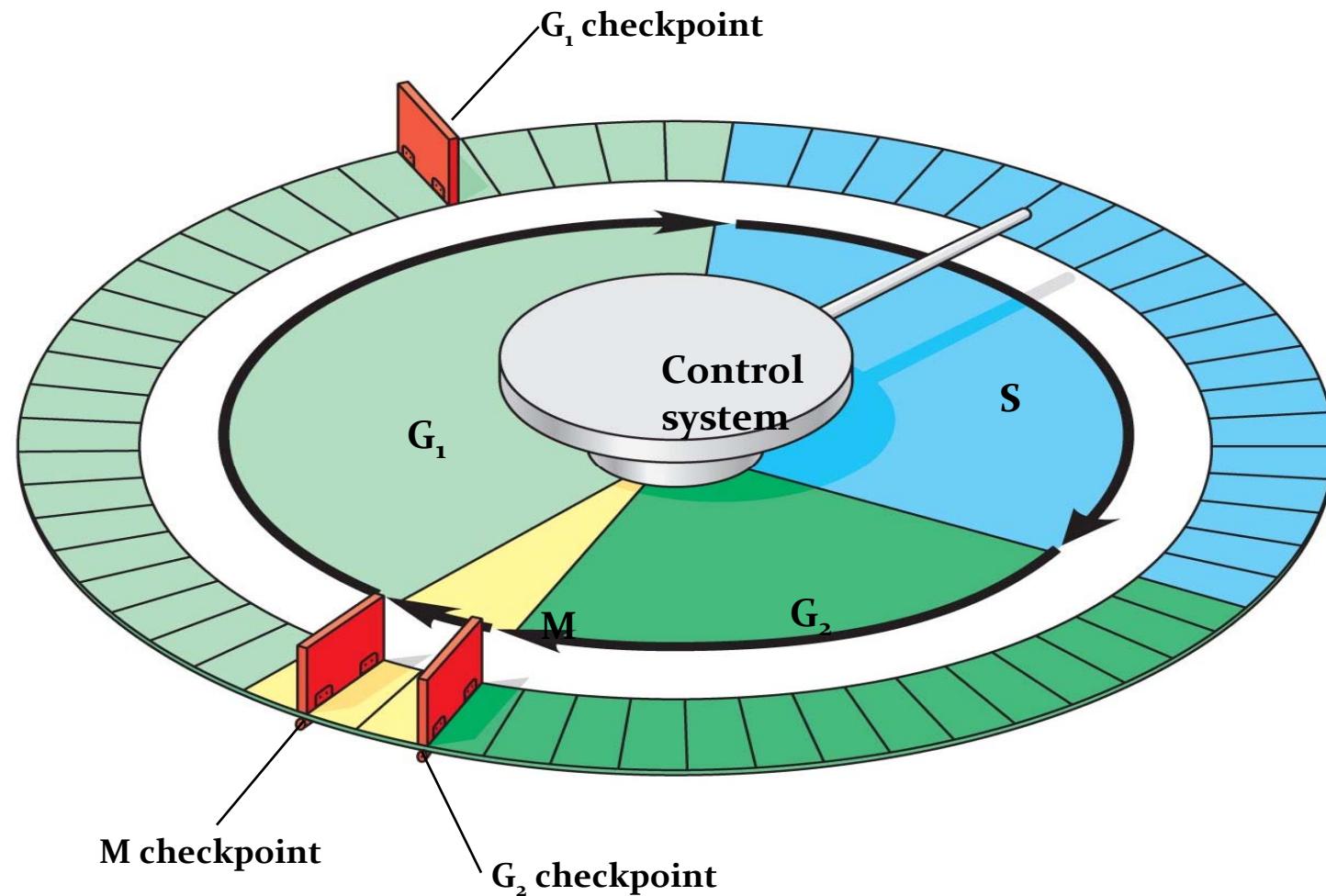


Phases of the Cell Cycle

- The cell cycle consists of
 - Mitotic (M) phase (mitosis and cytokinesis)
 - Interphase (cell growth and copying of chromosomes in preparation for cell division)
- Interphase (about 90% of the cell cycle) can be divided into subphases:
 - G₁ phase (“first gap”)
 - S phase (“synthesis”)
 - G₂ phase (“second gap”)



Cell Cycle Checkpoints



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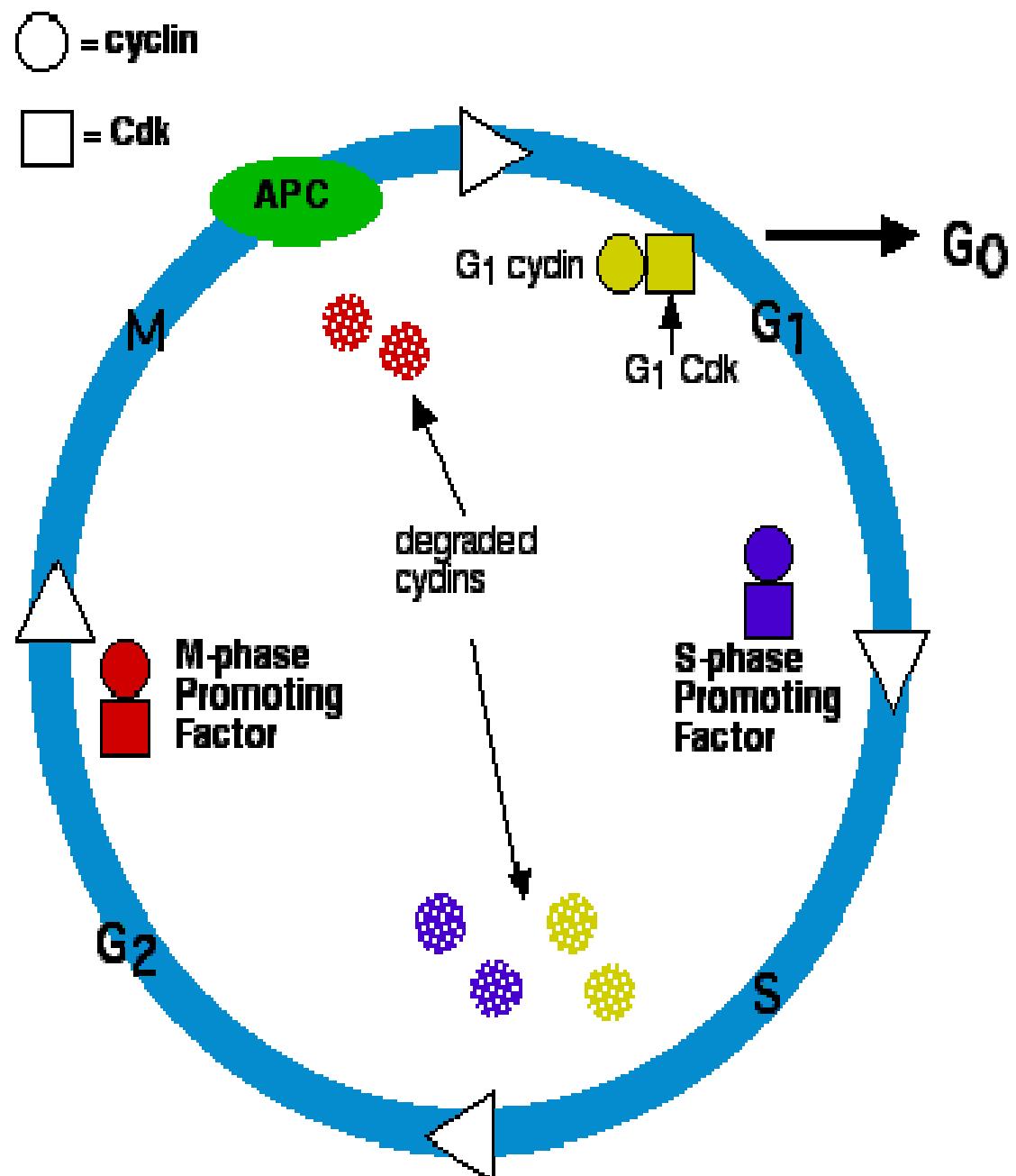
For cell cycle, Cyclin D binds CDK4/6. The active complex phosphorylates the tumor suppressor retinoblastoma (Rb), this relieves the inhibition of the transcription factor E2F, which causes the expression of cyclin E. Cyclin E interacts with CDK2 to allow for G1-S phase transition.

The first checkpoint (G1 checkpoint) is located at the end of the cell cycle's G₁ phase, just before entry into S-phase, making the key decision of whether the cell should divide, delay division, or enter a resting stage.

This first checkpoint involves p16, which inhibits Cyclin D-CDK4 binding.

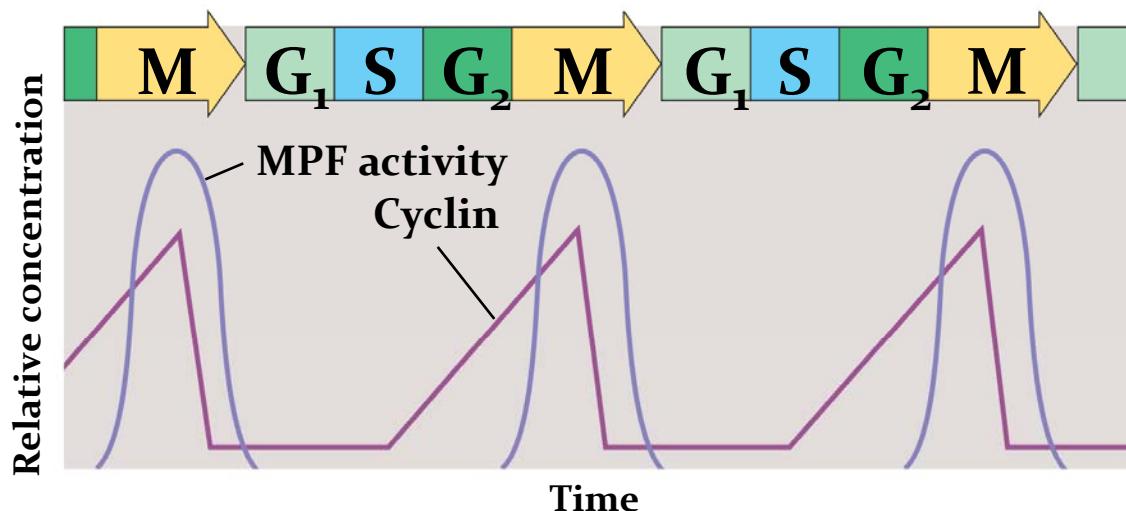
For the second checkpoint (G2 checkpoint), MPF activates the CDKs involved.

Then there is Replication checkpoint, and Mitosis checkpoint.



The Cell Cycle Clock: Cyclins and Cyclin-Dependent Kinases

- Two types of regulatory proteins are involved in cell cycle control: cyclins and cyclin-dependent kinases (Cdks)
- The activity of cyclins and Cdks fluctuates during the cell cycle



(a) Fluctuation of MPF activity and cyclin concentration during the cell cycle

Loss of Cell Cycle Controls in Cancer Cells

- Cancer cells do not respond normally to the body's control mechanisms
- Cancer cells form tumors, masses of abnormal cells within otherwise normal tissue
- If abnormal cells remain at the original site, the lump is called a primary tumor
- Malignant tumors invade surrounding tissues and can metastasize, exporting cancer cells

Gene Categories Important in Cancer

- Tumor suppressor genes-normally act to inhibit cell growth.
 - When damaged cell growth is no longer regulated
 - Mutation results in nonfunctioning gene (loss-of-function)
- Proto-oncogenes- normally act to regulate cell growth and division.
 - Oncogene is the defective version
 - Mutations results in constitutively active gene (gain-of-function)
- Chromo stability gene normally,