Review from last time

- While the lecture primarily dealt with the human genome, other genomes have different conformations – i.e. the Fugu genome is very compact.
- The human genome is large and complex – details were discussed
  - Be aware of relative proportions of different genomic components
- The basic strategies for sequencing a genome are hierarchical and whole genome shotgun sequencing – be familiar with them
- BLAT and BLAST are tools to explore our genome and others leading to a greater understanding of genome structure and function
- Variation exist among human genomes and that variation can be exploited to investigate aspects of human population biology and origins
  - The example discussed illustrates how the existing variation suggests that the human population arose in Africa and later spread across the planet
Manipulating Genes and Cells
Manipulating Genes and Cells

- Humans have been manipulating cells and the genomes of many organisms for thousands of years.
- Techniques have just gotten more precise and effective over time.
- We have recently gained the ability to directly manipulate DNA and even transfer genes between organisms.
Artificial selection is the process of intentional or unintentional modification of a species through human actions which encourage the breeding of certain traits over others.
Isolating and Growing Cells

• To study and characterize individual cell types, they must be dissected from the surrounding tissue and grown as a uniform population
  – Often done by using enzymes that break down the bonds between cells in a tissue
  – The cells can then be isolated as individuals
  – The cells are sorted in several ways
Isolating and Growing Cells

• Fluorescent cell sorting
  – Use antibodies to bind to different cell types
  – The antibodies bind to specific fluorescent dyes
  – The dyes can be distinguished in a fluorescence activated cell sorter which diverts cells according to the dye
ultrasonic nozzle vibrator

cell suspension

sheath fluid

detectors

laser

Analyzer

drop-charging signal

small groups of drops positively charged due to detection of single fluorescent cell

small groups of drops negatively charged due to detection of single nonfluorescent cell

-2000 V

+2000 V

cell collector
cell collector

flask for undeflected droplets
Isolating and Growing Cells

• Cells are then grown as a monoculture
  – Given the right conditions, most cells can be grown in culture
  – Most cell types continue to express their particular products and behave as they do in tissues while being cultured
    • Fibroblasts secrete collagen
    • Muscle cells fuse and contract spontaneously
Isolating and Growing Cells

• Maintaining cell cultures is not easy
  – Most cell types have a finite number of divisions and thus eventually die in culture
    • Telomerase not expressed in all cell types
  – Cell lines that express telomerase naturally or by being engineered to do so are called *immortalized*
  – Embryonic stem cells are undifferentiated cells
    • They have the ability to become any cell type
DNA Analysis

• The analysis of DNA was not an easy proposition until very recently (~1970’s)
  – Isolating a single gene even from a simple genome like *E. coli* was essentially impossible without molecular tools
  – DNA shearing was as close as we could get but it was inefficient and not reproducible
  – Restriction endonucleases (not ‘nucleases’) are enzymes that cut at specific nucleotide sequences in a DNA molecule
DNA Analysis: Restriction Endonucleases

• Restriction endonucleases (RE) were discovered in bacteria when it was noticed that any foreign DNA was quickly degraded
• REs recognize specific DNA sequences (usually palindromes) and cleave the DNA in specific ways
• The bacteria’s own DNA is protected by chemical modification (methylation usually)
Figure 10-4 Essential Cell Biology, 2/e. (© 2004 Garland Science)
DNA Analysis

- Different REs cut at different degrees in relation to their site specificity
  - 4-base cutter (e.g. HaeIII) generates fragments averaging 256 bp \( (4^4) \)
  - 6-base cutter (e.g. HindIII) generates fragments averaging 4096 bp \( (4^6) \)
  - 8-base cutter (e.g. NotI) generates fragments averaging 65,536 bp \( (4^8) \)
DNA Analysis: Gel Electrophoresis

- Separating DNA fragments is usually based on their size
  - DNA has a negative charge
  - By placing the DNA in a restrictive medium and applying a current, the DNA will move toward the positive end at a rate dependent on its length
  - DNA ranging in size from Mb differences to differences of 1 nucleotide can be distinguished
Gel Electrophoresis

(A) double-stranded lambda DNA
- cut with Eco RI
- cut with Hind III

(B) gel electrophoresis image

(C) slab of agarose gel
- nucleotide pairs (x1000)
- direction of migration
- top
- bottom

DEVELOP FILM

developed autoradiograph

sheet of photographic paper
DNA Analysis: Gel Electrophoresis

• Visualizing the fragments is accomplished in several ways
  – Fluorescence – ethidium bromide is an intercalating agent that fluoresces when exposed to UV radiation
  – Radiolabeling – Different isotopes can be chemically attached to DNA and the radiation can be detected using photographic film
Review from last time

- Humans have been manipulating DNA and cells for thousands of years, primarily through artificial selection
- Manipulation of cells involves separating them from their natural environment and sorting them
  - Fluorescent cell sorting
- Most cell types can be cultured but only cells that express telomerase can be immortalized
- DNA can be cut reliably and in a repeatable manner using restriction enzymes
  - Be aware of the details of restriction endonucleases
- DNA of various sizes can be separated using gele electrophoresis
DNA Analysis: DNA Cloning

- Individual fragments of DNA that have been digested by REs can be inserted combined with other fragments and then copied inside bacteria

- Terminology
  - DNA ligase – an enzyme that creates covalent bonds between DNA fragments
  - Plasmid – a circular, extragenomic piece of DNA found in bacteria; a type of vector
  - Transformation – the introduction of foreign DNA into a bacterium.
DNA Analysis: DNA Cloning

- The DNA to be cloned must first be digested using a RE.
- The vector (plasmid) must also be digested using either the same RE or one that cuts in the same way.
- The ends of the target DNA and the plasmid DNA can be joined by DNA ligase.
DNA Analysis: DNA Cloning

Plasmid DNA
DNA Analysis: DNA Cloning

Cohesive ends and DNA ligase

(A) JOINING TWO COMPLEMENTARY STAGGERED ENDS

$5'\text{C T T A A} 3' + 5'\text{A A T T C} 3' \xrightarrow{\text{ligase} + \text{ATP}} 3'\text{C T T A A} 5' \text{recombinant DNA}$

(B) JOINING TWO BLUNT ENDS

$\text{CGG} + \text{CTG} \xrightarrow{\text{ligase} + \text{ATP}} \text{CGG}$

recombinant DNA

(C) JOINING A BLUNT END WITH A STAGGERED END

$\text{CGC} G A \xrightarrow{\text{deoxyribonucleotides} + \text{polymerase}} 3'\text{C T T A A} 5' \xrightarrow{\text{ligase} + \text{ATP}} 3'\text{C T T A A} 5' \text{recombinant DNA}$
DNA Analysis: DNA Cloning

DNA cloning

circular double-stranded plasmid DNA (cloning vector)

CLEAVAGE WITH RESTRICTION NUCLEASE

DNA fragment to be cloned

COVALENT LINKAGE BY DNA LIGASE

recombinant DNA

200 nm
DNA Analysis: DNA Cloning

• Transformation, uptake of the cloned fragment by a bacterium, is accomplished in two ways
  – Heat shock – cells are exposed to heat for a short time
  – Electroporation – cells are exposed to an electrical current
  – Both methods induce the opening of pores in the cell membrane and uptake of DNA from the surroundings
DNA Analysis: DNA Cloning

- The cells are cultured and grown
- The vector+fragment DNA in the cells is copied along with them
- After growth, the bacterial culture containing millions of copies of the original plasmid will be lysed and the DNA purified
DNA Analysis: DNA Cloning

- A DNA library can be created through cloning
- DNA from a genome is fragmented and all of the fragments are inserted into vectors
- Thus, various cells contain representative fragments of the genome being studied
- The library can be screened to determine which cells contain fragments of particular interest
human DNA

CLEAVE WITH RESTRICTION NUCLEASE

millions of genomic DNA fragments

DNA FRAGMENTS INSERTED INTO PLASMIDS USING LIGASE

recombinant DNA molecules

INTRODUCTION OF PLASMIDS INTO BACTERIA

genomic library
DNA Analysis: Nucleic Acid Hybridization

- How is the library screened to locate fragments of interest?
- Hybridization (renaturation) is the process of denaturing DNA from different sources and allowing those single strands to renature.
DNA Analysis: Nucleic Acid Hybridization

- By labeling ‘probe’ DNA with radioactive isotopes and allowing it to hybridize with clones from a genomic DNA library, we can isolate clones that contain the DNA of interest.
Figure 10-24  Essential Cell Biology, 2/e. (© 2004 Garland Science)
DNA Analysis: Nucleic Acid Hybridization

- A similar process can be used to screen isolated fragments of DNA for the sequences of interest
  - Southern blotting – detecting DNA fragments
  - Northern blotting – detecting RNA fragments
DNA FRAGMENTS SEPARATED BY AGAROSE GEL ELECTROPHORESIS

(A) unlabeled DNA cut with a restriction nuclease

(B) stack of paper towels

(C) remove nitrocellulose paper with tightly bound DNA

SEPARATED DNA FRAGMENTS BLOTTED ONTO NITROCELLULOSE PAPER

Labeled DNA of known sizes as size markers

Labeled DNA probe hybridized to separated DNA
Labeled DNA probe hybridized to complementary DNA bands visualized by autoradiography.
Review from last time

- DNA cloning is the process of taking fragments of DNA and inserting them into appropriate DNA vectors, usually plasmids
  - The DNA is usually fragmented with REs. The vector must have a complementary end in order to incorporate the fragment
  - The vector usually consists of a circular DNA molecule with its own origin of replication and an antibiotic resistance gene
- Bacteria can be transformed, induced to take up the recombinant vector by heat, electricity, or chemical methods
- Creating DNA library is to develop a pool of bacteria containing representative fragments from an entire genome.
- DNA libraries as well as electrophoretically separated DNA can be screened for fragments of interest using hybridization techniques
- Northern and Southern blotting are two of those hybridization techniques
DNA Analysis: DNA Microarrays

- A DNA microarray is a glass microscope slide studded with a large number of DNA fragments.
- Each fragment can serve as a probe for a specific gene.
- The expression products from a cell (mRNA) can be hybridized to this microarray to determine what genes were active in the cell.
DNA Analysis: DNA Microarrays

• The following 5 slides dealing with DNA microarrays have been added or altered. You can download them at the class website.
DNA Analysis: DNA Microarrays

• DNA microarray example:
  – As a scientist, you are interested in the differences in expression patterns between normal human cells and cancer cells
  – A DNA microarray can be constructed that has enough space for 40,000 – 60,000 known genes
    • Remember that it is estimated that the human genome only has ~30,000+ genes
  – There are several ways to construct your microarray
    • Deposit known exons onto the chip chemically
    • Photolithography
Photolithography

An empty glass slide

Attach nucleotides and an associated protector group

The protector group is photoliable. It is removed when exposed to UV light
DNA Analysis: DNA Microarrays

• DNA microarray example:
  – As a scientist, you are interested in the differences in expression patterns between normal human cells and cancer cells

• Once the microarray is ready, you can collect your normal and cancer cells and extract the gene expression products – mRNA

• Animation
DNA Analysis: *In situ* hybridization

- DNA can be hybridized to individual chromosomes to determine the location of a gene - FISH

- DNA can be hybridized to cell preparations to determine which cells are expressing a particular mRNA
DNA Analysis: Polymerase Chain Reaction

- Polymerase chain reaction (PCR) is used to amplify selected DNA sequences
  - Limitation: You must know the sequence of the DNA surrounding the target to be amplified
  - Advantage: Can be performed entirely in vitro, no living cells are needed; fast (hours vs. days for cloning)
Denaturation (94°C) → Annealing (30-70°C) → Extension (72°C)

- **Denaturation**: Heat to separate strands.
- **Annealing**: Hybridization of primers.
- **Extension**: DNA synthesis from primers.

**First Cycle**
Figure 10-27 part 2 of 2  Essential Cell Biology, 2/e. (© 2004 Garland Science)
isolate DNA

SEPARATE STRANDS AND ADD PRIMERS

PCR AMPLIFICATION

genomic clones

isolate mRNA

mRNA sequence to be cloned

ADD FIRST PRIMER, REVERSE TRANSCRIPTASE, AND DEOXYRIBONUCLEOSIDE TRIPHOSPHATES

DNA

RNA

SEPARATE STRANDS AND ADD SECOND PRIMER

PCR AMPLIFICATION

cDNA clones

Figure 10-28  Essential Cell Biology, 2/e. (© 2004 Garland Science)
DNA Analysis: Polymerase Chain Reaction

• Applications of PCR include:
  – DNA sequencing
  – Disease diagnosis
  – Paternity testing
  – Many, many others
blood sample from infected person

rare HIV particle in serum of infected person

EXTRACT VIRAL RNA GENOME

RNA

REVERSE TRANSCRIPTASE/PCR AMPLIFICATION

control, using blood from noninfected person

GEL ELECTROPHORESIS

REMOVE CELLS BY CENTRIFUGATION
Figure 10-30 part 1 of 2  Essential Cell Biology, 2/e. (© 2004 Garland Science)
(B) 

- Individual A 
- Individual B 
- Individual C 
- Forensic sample F

3 pairs of homologous chromosomes

VNTR 1 
VNTR 2 
VNTR 3

PCR

A B C F

Number of repeats:
- 0
- 5
- 10
- 15
- 20
- 25
- 30
- 35

Electrophoresis
DNA Analysis: Polymerase Chain Reaction

• Applications of PCR include:
  – Forensic identification
    • FBI CODIS (Combined DNA Identification System)
    • 13 polymorphic microsatellite loci
  • In the hypothetical case here, the chance for an exact match with another human is ~1 in 7.7 quadrillion

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<td>3.52%</td>
<td>7.2%</td>
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DNA Analysis: PCR

- The following 5 slides dealing with DNA microarrays have been added or altered. You can download them at the class website.
DNA Analysis: Polymerase Chain Reaction

• Remember the limitations?
  – You must know the sequence of the primer sites to use PCR
  – How do you go about sequencing regions of a genome about which you know little?
  – Several techniques exist and you already know the tools to use
  – Example: you know the sequence of an expressed gene (mRNA sequence) but want to amplify the entire gene sequence from end to end including exons
  – Using the tools you have already learned about, there are several ways to accomplish your task
DNA Analysis: Polymerase Chain Reaction

• Solution 1:
  – If you are dealing with a human gene or a gene from an already sequenced genome, no problem.
  – Search BLAT or BLAST for the sequence and determine the flanking sequences for the gene
  – Design your primers and amplify from the genome
DNA Analysis: Polymerase Chain Reaction

• Solution 2:
  – Create a genomic library using a vector
  – Hybridize your cDNA probe to the library to locate the clone containing the gene you are interested in
  – Sequence the clone and determine the flanking sequences of the gene
  – Design your primers and amplify from the genome
DNA Analysis: Polymerase Chain Reaction

• Solution 2: Linker PCR
  – Fragment your genome and anneal double-stranded DNA ‘linkers’ using DNA ligase
  – The linkers serve as known ‘handles’ that can be used as the sequence for one primer and then the other
Using linkers to amplify unknown portions of a genome

Known sequence from cDNA

Sticky ends from restriction digest

YYYYYYYYYYYYTTAATT????????????XXXXXXXXX??????????AATTAAYYYYYYYYYYY
YYYYYYYYYYYYAATTAAYYYYYYYYYX??????????TTAATTYYYYYYYYYY

Unknown sequences flanking the gene

Design new primers to amplify the gene from the genome

YYYYYYYYYYYYTTAATTTNNNNNNNNNXXXXXXX&AATTAAYYYYYYYYY
YYYYYYYYYYYYAATTAANNNNNNNNNXXXXXXXAAATTAAYYYYYYYYYY
YYYYYYYYYYYYTTAATT????????????XXX
YYYYYYYYYYYYTTAATT????????????XXXXX

Sequence both fragments to determine flanking sequences
Review from last time

- DNA microarrays are valuable tools for investigating gene expression and other aspects of a genome
  - Be aware of how a microarray is constructed and used
- Polymerase chain reaction (PCR) is a method of creating millions of copies of a single section of a genome
  - By creating these millions of copies, you can now ignore all of the complexity of the genome and focus on the region of interest
  - There are advantages and limitations to PCR. Be aware of them and how PCR works.
  - Applications of PCR include paternity testing, forensic identification, etc.
  - There are ways to overcome the limitations inherent in PCR methodology
DNA Analysis: DNA Sequencing

- Currently, the most commonly used method to determine the sequence of a DNA molecule is the one devised by Fred Sanger in 1975 – chain termination sequencing
- Involves the use of dideoxyribonucleotides (ddNTPs) to terminate a growing chain of DNA during a PCR-like reaction
- The ddNTPs are missing the hydroxyl group that is normally the target for covalent bonding when a DNA chain is being formed, thus the phosphate backbone (chain) is terminated
- The ddNTPs are labeled either radioactively or fluorescently to allow visualization of the chain-terminated fragments
DNA Analysis: DNA Sequencing

- ddNTPs are analogous to faulty LEGOOs

Normal LEGOOs have little pegs that allow them to stack

Faulty LEGOOs lack the little pegs and nothing can stack on them – thus, they ‘terminate’ the stack
POLY-THYMINE DNA TEMPLATE
Unique, let's use poly-thymine DNA as an example.
POLY-THYMINE DNA TEMPLATE
POLY-THYMINE DNA TEMPLATE
(A) deoxyribonucleoside triphosphate

allows strand extension at 3’ end

(dideoxyribonucleoside triphosphate)

prevents strand extension at 3’ end

(B) normal deoxyribonucleoside triphosphate precursors (dATP, dCTP, dGTP, and dTTP)

oligonucleotide primer for DNA polymerase

single-stranded DNA molecule to be sequenced

small amount of one dideoxyribonucleoside triphosphate (ddATP)

rare incorporation of dideoxyribonucleoside by DNA polymerase blocks further growth of the DNA molecule

Figure 10-7 part 1 of 2 Essential Cell Biology, 2/e. (© 2004 Garland Science)
Figure 10-7 part 2 of 2 Essential Cell Biology, 2/e. (© 2004 Garland Science)
DNA Analysis: DNA Sequencing

DNA Engineering

• By manipulating DNA using combinations of the processes described above:
  – Novel DNA molecules can be created
  – Large amounts of rare proteins can be made using expression vectors
  – We can learn when and where genes are expressed
  – We can alter the genomes of organisms to suit our purposes
Figure 10-31  *Essential Cell Biology, 2/e.* (© 2004 Garland Science)
Examples:
Insulin
Growth Hormone
Factor VIII
DNA Engineering

• Learning when and where genes are expressed involves using reporter genes
  – Reporter gene – a gene whose activity can be easily monitored, usually through fluorescence
  – By replacing the sequence of the gene under study by a reporter gene we can determine how regulatory sequences effect expression of the gene, when the gene is expressed and what cells express the protein
(A) STARTING DNA MOLECULES

normal

1 2 3

regulatory DNA sequences that determine the expression of gene X

coding sequence for protein X

start site for RNA synthesis

pattern of normal gene X expression

cells

A B C D E F

(B) TEST DNA MOLECULES

RECOMBINANT

1 2 3

coding sequence for reporter protein Y

EXPRESSION PATTERN OF REPORTER GENE Y

EXPRESSION PATTERN OF GENE X

(C) CONCLUSIONS

—regulatory sequence 3 turns on gene X in cell B

—regulatory sequence 2 turns on gene X in cells D, E, and F

—regulatory sequence 1 turns off gene X in cell D

Figure 10-34 Essential Cell Biology, 2/e. (© 2004 Garland Science)
DNA Engineering

• Learning what a gene does is often best accomplished by creating organisms that carry mutant versions of a gene
  – Isolate a gene, change it, and clone it into a vector
  – Insert the vector into an organism and see what differences in phenotype the altered gene produces
  – Site directed mutagenesis is a method for changing the amino-acid sequence of an expressed gene by altering the DNA sequence of the gene
(A) inserted normal gene  
plasmid cloning vector  

STRAND SEPARATION  
synthetic oligonucleotide primer containing desired mutated sequence  

(B)  

(C) STRAND COMPLETION BY DNA POLYMERASE AND DNA LIGASE  

INTRODUCTION INTO CELLS. REPPLICATION AND SEGREGATION INTO DAUGHTER CELLS  

(D)  

TRANSCRIPTION  
5' GAC 3' mRNA  

TRANSLATION  
Asp normal protein made by half the progeny cells  

proteins  

5' GOC 3'  

TRANSLATION  
Ala protein with the single desired amino acid change made by half the progeny cells  

Figure 10-36 Essential Cell Biology, 2/e. (© 2004 Garland Science)
DNA Engineering

- *Transgenic* organisms have a new gene introduced into their genome
- There are ways to create transgenic organisms
  - Gene replacement
  - Gene knockout
  - Gene addition
- In simple, haploid organisms this is easily accomplished through introduction of a vector and recombination with the organism’s genomic DNA
NORMAL GENE X

**GENE REPLACEMENT**
- only mutant gene is active
- (A)

**GENE KNOCKOUT**
- no active gene present
- (B)

**GENE ADDITION**
- both genes are active
- (C)
DNA Engineering

- In more complex, diploid organisms creating transgenic organisms is more difficult but still possible
- The genes must be inserted into the germ line
ES cells growing in tissue culture

- Introduced a DNA fragment containing altered gene into many cells
- Let each cell grow to form a colony
- Test for the rare colony in which the DNA fragment has replaced one copy of the normal gene

Female mouse

- Mated and wait 3 days
- Injected ES cells into early embryo
- Early embryo partly formed from ES cells

- Introduced early embryo into pseudopregnant mouse
- Birth
- Somatic cells of offspring tested for presence of altered gene, and selected mice bred to test for gene in germ-line cells

ES cells with one copy of target gene replaced by mutant gene

Transgenic mouse with one copy of target gene replaced by altered gene in germ line

Figure 10-38  Essential Cell Biology, 2/e. (© 2004 Garland Science)