Overview of Project:

In this project, group members will investigate the association in different people between the type of skin and hair pigmentation and single nucleotide polymorphisms (SNPs) in the melanocyte-stimulating hormone receptor gene, \textit{MSHR} (also known as \textit{MC1R}, for melanocortin 1 receptor). The melanocytes in skin and hair (or fur of non-human mammals) produce two major types of pigment: eumelanin, which is a brown/black pigment, and pheomelanin, which is a red/yellow pigment. Multiple intracellular pathways control the relative production of the two different types of melanin. The pigmentation process is regulated by the release of melanocyte-stimulating hormone (MSH) from the pituitary gland, which circulates through the blood to the skin, where it binds to the melanocyte-stimulating hormone receptor (MSHR) in the plasma membrane of the melanocytes. Binding of MSH to its receptor activates the eumelanin intracellular signaling cascade, which leads to a darkening of the skin (Lin and Fisher, 2007).

In humans, the number of melanocytes is the same for all ethnic groups, and the difference in skin and hair color is due primarily to the relative amounts of the different melanins produced. High levels of MSHR signaling lead to a high amount of eumelanin production and darker skin and hair, whereas lower levels lead to a higher production of pheomelanin and lighter skin and hair color. Sequencing of the \textit{MC1R} gene in different people revealed that the gene is highly polymorphic, with over 30 variant alleles identified to date (NCBI Gene, 2011). Moreover, certain single nucleotide polymorphisms (SNPs) result in amino acid substitutions in the polypeptide chain that are often associated with different human skin/hair types (Valverde et al., 1995). For example, the valine at position 92 is replaced by a methionine in many Caucasians of European descent, while the arginine at position 163 is replaced by a glutamine in many people of European and Asian descent (Harding et al., 2000). Hair/fur color in non-human mammals (e.g., the chestnut coat of horses and the light hair of yellow Labradors) is also associated with SNP’s in their \textit{MC1R} genes (Marklund et al., 1996; Everts et al., 2000).

The single exon of the \textit{MC1R} gene encodes a G-protein linked receptor, 317 amino acids in length, with seven-transmembrane domains (NCBI Gene, 2011). One question to explore in this project is how the particular SNPs ultimately alter the structure and/or function of the MSH receptor, leading to decreased signaling by the receptor (and lower eumelanin production). Reduced MSHR signaling may be because (a) the MSH receptor is not present in the plasma membranes of melanocytes; (b) the variant MSH receptor may not bind MSH with the same affinity as the non-variant (consensus) receptor; or (c) the variant receptor may bind MSH but is defective in a downstream activation step. Research group members should consider these additional questions as they explore this topic: What is the normal structure and function of the MSH receptor? Where within the structure do the variant amino acids reside? What is the side chain structure of the variant and non-variant amino acids at the polymorphic sites? How might the variant amino acids alter the structure and/or function of the protein? For answers to some of these questions, group members should read the paper by Más and co-workers (2002), in which they describe three-dimensional modeling of the protein. You may also want to do an on-line search for more recent references.
A final, yet highly intriguing, question is what selective pressures have been in force to bring about a lightening of the skin in certain populations during human evolution. For answers to this question, you will certainly want to read papers by Nina Jablonski and co-workers.

References:


A missense mutation in the gene for melanocyte-stimulating hormone receptor (MC1R) is associated with the chestnut coat color in horses. Marklund L, Moller MJ, Sandberg K, Andersson L. Mamm Genome 7:895-899, 1996


Skin deep. Jablonski, NG and Chaplin, G. Scientific American 287:74-81, 2002


MC1R melanocortin 1 receptor (alpha melanocyte stimulating hormone receptor) [Homo sapiens], NCBI Gene, Gene ID: 4157, 26-Jun-2011

Summary of Project Protocol:

On Day 1 of this project, each group member will amplify the entire protein-coding region of the MC1R gene (contained within a PCR fragment of 1013 bp). Each researcher should prepare three cheek cell DNA samples for PCR: his/her own DNA and that of two other individuals (e.g., other teachers in the workshop and/or faculty/staff or students in the Biology Department at Amherst). Each human volunteer should fill out a consent form and questionnaire, which asks them to self-categorize themselves into different skin types.

The success of the PCR reactions will be determined by gel electrophoresis on Day 2 of the project, and DNA from successful PCR reactions will be purified and prepared for shipping to the Biotechnology Resource Center at Cornell University for sequencing on Day 3. Group members will then use NCBI’s BLAST search engine to compare each of their sequences with
those in NCBI’s gene database to make sure they have in fact amplified the correct region of the human genome. More detailed sequence analyses to identify the SNPs and the corresponding MSHR amino acid variants (if any) for each individual will then be done using the Lasergene software suite. All members of the group will compile their individual results in a chart, to assess the correlation between MSHR amino acid variations and skin type. Finally, group members will explore the effects of amino acid variants on the structure and function of the MSH receptor. A flow chart for the experimental steps to complete the project is below.

| Day 1: Volunteers fill out Hair & Skin Type Questionnaire | DNA isolation from human cheek cells
| Day 2: Analytical agarose gel of PCR products | Day 3: Spin purification of PCR products
| | Analytical agarose gel of purified PCR products and quantification of DNA amounts
| | Sequencing reactions* set up and shipped to sequencing center via overnight delivery
| Day 4: DNA sequencing by BRC at Cornell University | Days 5 & 6: DNA sequence analysis and presentation preparation
| Day 7: Project Presentation |

*Molecular Identification of Single Nucleotide Polymorphisms in the Human Melanocyte-Stimulating Hormone Receptor Gene (MC1R)

Materials Needed:
See Excel spreadsheet and recipes in Appendix
Protocol:

NOTE: PCR is extremely sensitive to contamination. A few foreign cells in your preparation can ruin your results. For this reason, it is extremely important to maintain sterility at all times while conducting the following procedures.

Day 1:

A. Consent and Questionnaire
   1. Have each subject fill out a Human Subject Consent Form and Questionnaire.

B. Cell Extraction and DNA Isolation (adapted from Princeton University)
   http://www.hhmi.princeton.edu/Manual/04%20HHMI%20Lab_04%20D1S80.pdf
   1. You will obtain cheek cell DNA from two (or more) people. Obtain one 1.5 ml microcentrifuge tube containing 10% Chelex for each volunteer and use a permanent marker to label the top of each with an assigned human DNA sample number. Note that Chelex is not actually a solution, but a slurry of resin coated beads in water. You will see the beads settled at the bottom of the tube. Also, obtain one 15-ml centrifuge tube containing 10 ml of sterile saline (0.9% NaCl) for each volunteer and label the side of the centrifuge tube with the assigned DNA sample number.
   2. Have the volunteer put on gloves and pour the contents (saline) of the conical centrifuge into his/her mouth and agitate vigorously for 2 min, using his/her teeth to gently rub the inner surface of his/her mouth to dislodge as many cells as possible. The more cells in the sample, the higher the yield of DNA will be. [Note: this works best if people have not eaten/chewed gum for an hour. If they just ate something crumbly like Doritos, they should try to rinse their mouth a bit first with water, because otherwise you end up with lots of food particles in the cell suspension.]
   3. The volunteer should then expel (i.e., spit) the solution carefully into a clean 50 ml centrifuge tube, then carefully pour the saline/cheek cell suspension back into the original 15 ml centrifuge tube.
   4. If so desired, the volunteers can rinse their mouths out with water, at the water fountain in the hall. Thank the subject(s) and invite them to have some cookies in the lobby to get the salt taste out of their mouths.
   5. Place the 15-ml tubes in the centrifuge, putting each tube across the rotor from another for balance. Spin the tubes in the centrifuge for 5 min at 3,000 rpm.
   6. When the spin ends, gently pour off the supernatant into the sink, leaving behind the pellet of cheek cells (and whatever else). You should see a white pellet of cells in each tube. Quickly compare the relative size of the pellet to others in your group.
   7. Use a sterile, graduated transfer pipette to draw the water away from the settled Chelex beads from one microcentrifuge tube. Use this water and pipette to gently resuspend the cells in one 15-ml tube. Then, transfer the cell suspension back to the microcentrifuge tube with the Chelex. Mix the cell/Chelex suspension well. Repeat this step for each of the other cheek cell samples you have (using a new transfer pipette for each sample).
   8. Firmly shut the microcentrifuge tubes and place them in a floating rack in the boiling water bath for 10 min. DO NOT DELAY BETWEEN STEP 7 AND 8.
   9. When the 10 min boiling step has ended, use forceps to remove the tubes from the water bath, and place your boiled samples on ice for 1-2 minutes.
   10. Spin your tubes with others (making sure the rotor is balanced) for 5 min in the high-speed microcentrifuge. This will pellet the Chelex beads and cell debris, leaving the DNA
suspended in the supernatant above the beads. IF YOU HAVE TO WAIT FOR OTHERS TO SPIN, KEEP YOUR TUBE ON ICE WHILE WAITING.

11. Wearing gloves, use a P200 micropipettor to transfer ONLY 150 µl of the clear supernatant from the top of the solution to a new, sterile microcentrifuge tube. Be careful not to transfer any of the material in the pellet because this will inhibit the PCR reaction.

12. Label each new tube with its respective DNA sample number. You have now isolated a small quantity of human genomic DNA. Keep the tubes on ice until you are ready to set up your PCR reactions. The samples can also be frozen for later use.

C. PCR Amplification

1. Put on gloves. Use a permanent marker to label the top of a sterile 0.2 ml PCR tube. You will run one PCR reaction for each DNA sample that you have (each person should do a minimum of two different DNA samples). Label the PCR tubes with the respective human DNA sample number and make a key.

2. One person in the project group should also prepare one PCR reaction tube for the negative control (water) and the positive control (DNA known to work in assay).

3. I will provide you with MC1R-Amp Mix*. Use a P-200 micropipettor to pipet 46 µl of this amplification mixture into each of your PCR reaction tubes. This mixture contains MC1R forward (MC1R-F) and reverse primers (MC1R-R) to sequences in the 5' and 3' flanking regions, respectively, of the human MC1R gene, the four deoxynucleotides (A, T, C, G), GoTaq® Flexi DNA polymerase, MgCl₂, buffer and other chemicals.

4. Using a P-20 micropipettor, pipet 4 µl of each extracted DNA sample into its respective reaction tube.

5. The person who set up the controls in Step 2 should pipet 4 µl of Chelex water (negative control) and 4 µl of JAE DNA (positive control) into their respective tubes.

6. Mix and briefly spin the samples in a microfuge.

7. The DNA is now ready for amplification in the thermocycler. The lab instructor will collect your reaction tubes, place them in the thermocycler, and start the program. The reaction will proceed as follows:

<table>
<thead>
<tr>
<th>Cycle</th>
<th>Temperature/Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 cycle</td>
<td>95°C for 3 minutes</td>
</tr>
<tr>
<td>40 cycles</td>
<td>95°C for 1 min</td>
</tr>
<tr>
<td></td>
<td>62°C for 1 min</td>
</tr>
<tr>
<td></td>
<td>72°C for 3 min</td>
</tr>
<tr>
<td>1 cycle</td>
<td>72°C for 10 minutes</td>
</tr>
<tr>
<td>Hold</td>
<td>4°C overnight</td>
</tr>
</tbody>
</table>

*MC1R-Amp Mix consists of:
1X PCR buffer w/ 2.5 mM MgCl₂
0.25 µM PCR Nucleotide Mix
0.5 µM MC1R-F primer
0.5 µM MC1R-R primer
2 units Taq polymerase/sample
dH₂O (to 46 µl/sample)
Day 2:
A. **Analytical Mini-Gel of Primary PCR Products**

1. **Put on gloves.** Remove your sample tubes from the thermocycler (or refrigerator). Your reaction tubes now contain your primary PCR products.
2. Centrifuge the tubes for 2-3 seconds before opening the lid.
3. Remove 2 µl of each sample and put it into a clean 1.5 ml microcentrifuge tube. Put your PCR tubes back on ice or in the refrigerator.
4. Add 1 µl of 10X sample buffer and 7 µl of dH₂O to the DNA in each new 1.5 ml microcentrifuge tube. Mix and briefly spin in microfuge.
5. For each sample, load the entire 10 µl into one well of a 1% agarose gel that contains ethidium bromide.
6. Record your human DNA numbers on the gel diagram sheet next to the appropriate well numbers for your PCR products. This is so you will know which lanes of the gel contain your PCR products, since we will be running large gels with multiple samples.
7. Load 10 µl of the 1-kb DNA ladder (see figure to right) into one outside well per row of samples.
8. Run the gel for ~1 hour at 100 V. Bring gel to the darkroom to examine/photograph it with the UV transilluminator.
9. If you have a discrete band of DNA that is 1013 bp in length for your experimental DNA samples, proceed to Day 3. If you do not have bands in the experimental AND the positive control sample, repeat only the PCR reaction. If you have a band in the positive control lane but no bands in your experimental lanes, repeat BOTH Day 1 procedures.

Day 3:
A. **Spin purification of the PCR Products**

1. Use an Amicon Ultra-0.5 Spin Filter Unit and follow the next two pages of directions to purify the PCR products away from unincorporated nucleotides, etc. **Wear gloves.**
2. For step #2, add 400 µl of sterile dH₂O to the filter unit. Carefully remove the rest of the PCR sample (~48 µl) and add it to the water in the filter unit.
3. Follow the rest of the Amicon directions (steps 3-6).
4. **Make sure that the microcentrifuge is set to rcf (NOT rpm).**
PCR Product Clean-Up with Amicon Ultra-0.5 Spin Filter Units

Materials Supplied

The Amicon Ultra-0.5 device is supplied with two microcentrifuge tubes. During operation, one tube is used to collect filtrate; the other to recover the concentrated sample.

How to Use Amicon Ultra-0.5 Centrifugal Filter Devices

1. Insert the Amicon Ultra-0.5 device into one of the provided microcentrifuge tubes.
2. Add up to 500 µL of sample to the Amicon Ultra filter device and cap it.
3. Place capped filter device into the centrifuge rotor, aligning the cap strap toward the center of the rotor; counterbalance with a similar device.
4. Spin the device at 14,000 × g for approximately 10–30 minutes depending on the NMWL of the device used. Refer to Figure 1 and table 3 for typical spin times.

Step 4 above: spin for 11 minutes to obtain a final, concentrated volume of ~22 µl
How to Use Amicon Ultra-0.5 Filter Devices, continued

5. Remove the assembled device from the centrifuge and separate the Amicon Ultra filter device from the microcentrifuge tube.

6. To recover the concentrated solute, place the Amicon Ultra filter device upside down in a clean microcentrifuge tube. Place in centrifuge, aligning open cap towards the center of the rotor; counterbalance with a similar device. Spin for 2 minutes at 1,000 × g to transfer the concentrated sample from the device to the tube. The ultrafiltrate can be stored in the centrifuge tube.

NOTE: For optimal recovery, perform the reverse spin immediately.

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Performance - DNA Concentration

The Amicon Ultra-0.5 30K device provides the best balance between recovery and spin time for double-stranded DNA for base pairs ranging from 137 to 1159. To achieve maximum PCR product recovery and primer removal with primers greater than 20 bases, one or two additional spins with Tris-EDTA (TE) buffer are recommended.

Table 2. Typical recovery of nucleotides from Amicon Ultra-0.5 30K device

<table>
<thead>
<tr>
<th>PCR Product (base pairs)</th>
<th>PCR Primer (bases)</th>
<th>PCR Recovery (%)</th>
<th>PCR Primer Removal (%)</th>
<th>TE Washes (number)</th>
</tr>
</thead>
<tbody>
<tr>
<td>137</td>
<td>10</td>
<td>≥ 95</td>
<td>≥ 90</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>≥ 90</td>
<td>≥ 85</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>≥ 90</td>
<td>≥ 75</td>
<td>2</td>
</tr>
<tr>
<td>301</td>
<td>10</td>
<td>≥ 90</td>
<td>≥ 90</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>≥ 85</td>
<td>≥ 90</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>≥ 90</td>
<td>≥ 80</td>
<td>2</td>
</tr>
<tr>
<td>648</td>
<td>10</td>
<td>≥ 95</td>
<td>≥ 90</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>≥ 90</td>
<td>≥ 90</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>≥ 95</td>
<td>≥ 90</td>
<td>2</td>
</tr>
<tr>
<td>1159</td>
<td>10</td>
<td>≥ 90</td>
<td>≥ 90</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>≥ 90</td>
<td>≥ 95</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>≥ 95</td>
<td>≥ 95</td>
<td>2</td>
</tr>
</tbody>
</table>

Spin conditions: 40° fixed angle rotor, 14,000 × g, room temperature, 500 μL starting volume, 20–30 μL final volume, 10 minute spin, n=12.
B. Analytical Mini-Gel of Spin-Purified PCR Products
   1. Put on gloves. Pipet 8 µl of dH2O and 1 µl of 10X sample buffer into a new, labeled 1.5 ml microcentrifuge tube for each DNA sample.
   2. Remove 1 µl of each DNA sample and put it into the corresponding microcentrifuge tube from step 1. Mix and briefly spin in microfuge. Put your DNA samples on ice.
   3. For each sample, load the entire 10 µl into one well of a 1% agarose gel that contains ethidium bromide.
   4. Record your name on the gel diagram sheet next to the appropriate well numbers for your PCR products. This is so you will know which lanes of the gel contain your PCR products, since we will be running large gels with multiple samples.
   5. Quick spin the tube, then load 10 µl of the Bio-Rad Precision Molecular Mass marker ladder (see figure to right) into one outside well per row of samples.
   6. Run the gel for ~1 hour at 100 V. Bring gel to the darkroom to examine/photograph it with the UV transilluminator.
   7. Estimate how much DNA is in the MC1R band by comparing it to the staining intensities of bands in the marker lane.
   8. Calculate the volume of DNA solution that you need to use to have 200 ng of PCR product. If this volume is less than 1 µl, dilute the DNA in sterile dH2O.

C. Set Up Sequencing Reactions
   1. Put on gloves.
   2. You will have two sets of two tubes each: Human DNA 1 and Human DNA 2, with each DNA sample to be sequenced using both the S1 and S2 primers. Obtain four 0.5 ml, screw-cap skirted microcentrifuge tubes.
   3. Check with your lab instructor for the correct numbering sequence to use for labeling the tubes (on the cap and the side with a Sharpie), and use the following table to fill in and keep track of how much of each item to add to each tube:

<table>
<thead>
<tr>
<th>Sequencing #</th>
<th>DNA Sample #:</th>
<th>Vol. for 200 ng DNA</th>
<th>Primer (8 pmol)</th>
<th>dH2O (to 18 µl final vol.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>#_____-F</td>
<td>2 µl MC1R S1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>#_____-R</td>
<td>2 µl MC1R S2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>#_____-F</td>
<td>2 µl MC1R S1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>#_____-R</td>
<td>2 µl MC1R S2</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

   4. Use a P20 pipettor and sterile yellow tips to pipet each of the above solutions into the respective tubes. Cap and Parafilm the tubes and give to the lab instructor for shipment to the DNA Sequencing Facility at Cornell University.

Days 4-5:
   A. DNA sequencing at the Biotechnology Resource Center at Cornell University
Days 5-6:
A. Analyzing the sequence data - see instructions starting below  
B. Preparation of project presentation - see guidelines on p. 16

Day 7:
A. Project presentation and questions

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Days 5-6: DNA Sequence Analysis

*Note: Lasergene can be used for free for a limited time each year by educators. Visit [https://www.dnastar.com/forms.aspx?forms=edu](https://www.dnastar.com/forms.aspx?forms=edu) for more information.*

A. Examining the DNA Sequence Files:

Proceed as follows to examine the DNA sequence and chromatogram for each sequencing reaction:

1. Download Chromas Lite onto your computer  
   *(Note: this already should have been down for the Amherst computers)*
   a. Google Chromas Lite.
   b. Follow directions from the Technelysium website—free and easy.
   c. Chose to save the compressed file to your disk; Desktop is convenient.
   d. Right Click on the new Chromas.exe icon on your desktop. **OPEN** it and **Extract** it using the default Program directory. Also elect to save shortcuts on the desktop and elsewhere if you like.
   e. Click on the newly created Chromas Lite icon to check that you’ve got it. It should be able to open .abi files from Cornell or elsewhere to display the raw sequence data.
   f. You may now discard the original Chromas icon (looks like a computer), which is needed only to install the software.

2. Download and unzip the BRC sequence files and save the folder of unzipped files onto the desktop. Copy your individual DNA sequence files into a new subfolder. Open the .abi files with **Chromas Lite** and examine the raw chromatograms to see where and why ABI is unable to call bases unambiguously (ambiguous bases are represented by an “N”) in regions of your sequence. **After** you have aligned two (or more) sequence reads using Lasergene’s SeqMan (see below), you will want to return, more than once, to the chromatograms to see if you believe one “read” or the other. The choice depends upon your confidence or lack thereof in nearby bases, and other aspects: Is the region too close (often in the first 10-20 bases) to your primer? Or too far? Some sequences turn unreadable after 300 bases; 500 bases or even 700 is nice; and few are readable after 800 bases.

B. Did You Amplify and Sequence the Correct Gene?

Before going any further, it is important to verify that you have in fact amplified and sequenced the gene region that you thought you did. So, you will now compare your sequence with those in the National Center for Biotechnology Information (NCBI) database using BLAST.
1. There are two ways to do this:

   a. Go to the NCBI Web site (http://www.ncbi.nlm.nih.gov/), click on BLAST under ‘Popular Resources’ on the right and select the “nucleotide blast” option under the Basic BLAST header. Paste the DNA sequence copied from the Chromas Lite window into the box that appears in the next window below Enter Query Sequence, select ‘Others (nr etc.):’ next to ‘Database’ under ‘Choose Search Set’, click on ‘Show results in a new window’ next to the BLAST button, then click on the BLAST button in the lower left of the window and see what you get. Experiment! From NCBI, you can link directly to publications via the PubMed search engine, which is very convenient.

   b. Or, even simpler, click on “Net Search” in the top menu bar of SeqMan (see below), select “Blast selection” and proceed as directed; the default selections of the blastn program and the nr (non-redundant nucleotide sequences) database should work fine. Note that you cannot link to the returned “hits” if you choose “b.”

2. Do this for both sequence (.seq) files for each PCR sample. Examine the hits returned, and think! Have you sequenced the correct gene?

C. Creation of a Sequence Contig

If all goes well, you will have sequence data for two sequencing reactions per PCR product. For this project, the two sequencing primers should generate a few hundred base pairs of overlapping sequence from the MC1R gene. Since the coding region is 951 bp in length, however, this means that you will only have single coverage (e.g., sequence from only the one sense strand) for much of the length of the PCR product. You will first identify a region of consensus between your pair of sequence reads by aligning the two sequence files to come up with a “contig” (“contig” refers to a stretch of contiguous sequence). You should then use the best sequence (from the S1 or S2 primer) for the regions that are outside of this contig. Proceed as follows to create your consensus sequence:

1. Open Lasergene’s SeqMan program: go to Start → All Programs → Course Related → Biology → DNASTAR Lasergene 8 Core Suite → SeqMan.

2. In the (upper) menu bar of the SeqMan window, click on the Sequence tab then select “Add…”. A new window will appear entitled “Enter Sequences.” Use the scroll buttons next to the “Look in:” box to find and select (highlight) the folder of your sequences on the desktop. Then click the “Open” tab under the blank window on the left. All the project sequences should then appear in the above window. In this window, for each sample that you sequenced, highlight the first sequence (J##.seq) file for your PCR product and click “Add ->” in the lower-right side of the window, which adds the sequence file to the box in the upper-right side of the window. Repeat for the second sequence file for the PCR product. Don’t (at this point) intermix your sequences with those of other clones/amplifications. We usually work from Cornell’s .seq files but the .abi files work, too. Now click the “Done” button.

3. Your two sequences should now appear in the “Unassembled Sequences” window. Next, click on the “Assemble” button in the upper-left part of the menu bar. To see the result, double click on the new “Contig1” which hopefully has just been created in the “Untitled Window.” Yet another new window entitled “Alignment of Contig 1” should then appear. If SeqMan insists that your two (or more) reads fail to join into a single contig, something has gone awry.
4. Note that the consensus need not be displaying the sense sequence. Determine the orientation of the two sequences by looking at the small red and green arrows next to the sequence file names. You may want to choose the “Complement Contig” selection in the dropdown “Contig” menu list, if the sense sequence is not displayed in the 5’ to 3’ (left to right) direction (which for some reason it usually is not!).

5. Examine the contig and note all points of discrepancy within the region of overlap (look for red letters or dashes or purple N’s). You should now decide which sequence reaction you believe and whether or not you agree with the computer’s “read.”
   a. To do so, you will have to go back to the raw chromatograms in Chromas Lite. Use the scale bar in the upper menu bar of Chromas to spread the chromatogram peaks apart for easier reading. You can print the chromatogram if that helps (especially if you can get to a colored printer). You can also RevComp a sequence directly in Chromas Lite, which often makes that read easier to align with data as presented in SeqMan.
   b. Are there places where you must accept an ambiguity? (R=purine; Y=pyrimidine; other uncertainties also possible)
   c. Are any of the ambiguous bases possibly due to heterozygosity? This would be discernible as two peaks at a single position on the sequencing chromatogram. If the person is heterozygous, make sure you note where, in case the nucleotide is in a codon for a variant amino acid (see D.4 below).
   d. Can you rule one read “in” and the other “out” at a given position? If so, manually edit the sequence by highlighting the nucleotide letter and typing in your letter of choice. **Write down on a piece of paper what you have done to create the consensus. Save the new consensus file you have just created, named in some inimitable fashion.**

D. Additional Analysis

1. For this project, your PCR product encodes a protein, and your task is to identify whether or not there are amino acid variants along the polypeptide chain for each human volunteer. Use Lasergene to translate each of your consensus DNA sequences into a polypeptide chain. Although you can translate reading frames one at a time with EditSeq, multiple translations are most easily done in SeqBuilder, as follows:
   a. Make a note of the position of the start codon (ATG) in the saved sequence from part C. Copy and paste the sense strand sequence for the MSHR protein into SeqBuilder (go to Start → All Programs → Course Related → Biology → Lasergene 9 → SeqBuilder). Note how the software automatically enters the complementary strand (though you can make it disappear under “Residues” if you wish: we suggest you leave both strands showing for now).
   b. SeqBuilder also shows you, by default, 3 translation frames, but for now, click these “off” using the left menu panel. Click on ORFs and also on Full Translation. Examine the 6-fold translation products (or 3-fold, if you already have put the sequence in the correct 5’ to 3’ orientation). Decide which Open Reading Frame (ORF) you wish to pursue, and observe its translation product.

2. Presumably, at least one reading frame is open and full-length, and with luck not many others. There is a print out of the amino acid sequence encoded by the \textit{MC1R} gene in Appendix II on page 15. Compare this baseline (ancestral) amino acid sequence with each of yours. **Note: due to skips or staggers by the DNA polymerase during the sequencing reaction, you may have to**
move back and forth from one reading frame to another to create one complete, contiguous reading frame. You can then edit the sequence file (to remove or add a nucleotide, as needed) to obtain the contiguous reading frame (you may have to do the editing using Lasergene’s EditSeq program). Save the protein sequence as a .pro file.

3. Examine the amino acid sequence of each of your PCR samples for the presence of amino acid variants. You can do this by doing a straight, visual comparison of the published amino acid sequence with each of your consensus sequences, although this may be somewhat tedious, as there are 317 amino acids to investigate. Alternatively, you can use NCBI’s ‘protein BLAST (blastp)’ search engine to align your sequences with the NCBI sequence (Protein database, accession #Q01726, scroll down to the amino acid sequence at the bottom of the page), which will then quickly identify the amino acids that are different. After selecting ‘protein blast’, check the box ‘Align two or more sequences’ in the ‘Enter Query Sequence’ box, paste in your consensus sequence in the Query Sequence box and the NCBI sequence from above (which should be the same as on p. 17) in the Subject Sequence box.

Work with other members of your group to align your full-length protein sequence(s) with all of theirs and with the baseline MSHR sequence from NCBI’s Protein database (accession # Q01726). Note: clicking on the link for this number brings you to a very informative UniProt page for the protein. If you prefer Lasergene to BLAST, you may also align multiple sequences (from your group or from the databases) using MegAlign, as follows:

a. Open Lasergene’s MegAlign (All Programs…-> Lasergene -> MegAlign). To grab sequences from NCBI using EditSeq or SeqBuilder go to “File,” “Open Entrez Sequence” and type in the accession number (Q01726) of the file you want. Be sure to specify the proper database (e.g., protein). Once you have the files of interest, add the sequences one at a time or in groups to MegAlign, pick an alignment method, and observe the results.

b. Create a chart of the position and identity of any amino acid variants for each PCR product that was analyzed by your group, paying special attention to the polymorphisms listed in Table 1 of the Valverde et al. (1995) and Rana et al. (1999) papers, respectively. Note: many more variants are listed on the UniProt Web page for the MSHR protein.

c. View also the various reports: sequence distances, residue substitutions and phylogenetic trees.

4. Do any of the amino acid variants correspond with those in the literature or on the UniProt Web page for the MSHR?

5. If you do have one or more amino acid variants in any of your samples, use Figure 2 in the Rana et al. (1999) paper and any other images of the receptor you may find on-line to determine where (within the 3-D protein structure) each of the polymorphisms is. How might amino acid substitutions in these regions affect the function of the MSHR protein? (A chart of the amino acid codes is on the next page.) Opening your brand new “.pro” files from Step 2 above using Lasergene’s Protean and Protein 3D software may also help in this analysis. Behold the structural overview!

6. Finally, is there any correlation between the level of skin pigmentation, UV skin response and hair color of each human subject and the amino acid variants that your group found?
The Single-Letter and Triplet Amino Acid Codes:

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<thead>
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<tbody>
<tr>
<td>G</td>
<td>Gly</td>
<td>P</td>
<td>Pro</td>
</tr>
<tr>
<td>A</td>
<td>Ala</td>
<td>V</td>
<td>Val</td>
</tr>
<tr>
<td>L</td>
<td>Leu</td>
<td>I</td>
<td>Ile</td>
</tr>
<tr>
<td>M</td>
<td>Met</td>
<td>C</td>
<td>Cys</td>
</tr>
<tr>
<td>F</td>
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<td>Y</td>
<td>Tyr</td>
</tr>
<tr>
<td>W</td>
<td>Trp</td>
<td>H</td>
<td>His</td>
</tr>
<tr>
<td>K</td>
<td>Lys</td>
<td>R</td>
<td>Arg</td>
</tr>
<tr>
<td>Q</td>
<td>Glu</td>
<td>N</td>
<td>Asn</td>
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<tr>
<td>E</td>
<td>Glu</td>
<td>D</td>
<td>Asp</td>
</tr>
<tr>
<td>S</td>
<td>Ser</td>
<td>T</td>
<td>Thr</td>
</tr>
</tbody>
</table>

E. Preparation of Project Presentation

1. Your group should prepare a Power Point-based presentation, in which each member of the group spends ~2-3 minutes talking about the information on particular slides. The overall presentation should not exceed 25 minutes, which will allow up to 5 minutes of questions/discussion afterwards.

2. The presentation should be organized in the format of a scientific paper, with Introduction, Methods, Results and Conclusions sections. Please also include a Bibliography slide, with a list of any cited references.

3. The most effective Power Point slides are those that are visually pleasing to the eye and not too dense with text. Please talk with the instructional staff if you have any questions about using Power Point.
Appendix I: Human MC1R gene sequence, coding plus 5'- and 3'- flanking sequences.  
NCBI Accession # X65634 (Mountjoy et al., 1992, Science 257:1248-1251)

Coding sequences are highlighted in green, and the start site of translation is underlined.  PCR amplification of the coding region was done using the MC1R (forward) and MC1R (reverse, complement) primers. The sequences of these primers are below, and their locations are indicated above in orange/boxed (MC1R-F) and purple (MC1R-R), respectively. All but the very 3' end of the translated region of the gene is sequenced using primers S1 (complement, 3' end, location underlined above) and S2 (complement, 5' end, location highlighted in blue above). The sequences of these primers are also below.

**MC1R-F primer** = 5' T TCC TGC TTC CTG GAC AGG ACT AT 3'

**MC1R-R primer** = 5' CCC TCT GCC CAG CAC ACT TAA A 3'

**S1 primer** = 5' TGC CCA GCA CAC TTA AAG CGC GTG CA 3'

**S2 primer** = 5' TGA TCA CGT CAA TGA CAT TGT 3'

Since the S1 primer is quite close to the 3' end of the gene, the codons for the last 4-7 amino acids will likely be missing from the sequenced PCR product.

* Amino acid sequence is on the next page.*
Appendix II: Single-letter amino acid sequence of the human melanocyte-stimulating hormone receptor (the protein product of the MC1R gene).

#Q01726 - MSHR Amino Acid Sequence (ancestral/baseline sequence) - 317 a.a.
Note: amino acids are in groups of 10, with 60 to a line

ORIGIN

1 MAVQGSQRRL LGSLNSTPTA IPQGLAANQ TGARCLEVSI SDGLFLSLGL VSLVENALVV
61 ATIAKNRNLH SPMYCFICCL ALSDLLVSGS NVLETA底盘 LEAGALVARA AVLQQLDNVI
121 DVITCSSMLS SLCFLGAIAV DRYISIFYAL RYHSIVTLPR ARRAGAIWV ASVVFSTLFI
181 AYYDHAVVLL CLCVFPLAML VLMVLYHVHM LARACQHAQG IARLHKQRPV VHQGFGKGA
241 VTLTILLGIF FLGWGPFPLL LTLLVLCEPH PTGCCIFKNF NLFALIIICN AIDDIYAF
301 HSQELRRTLK EVLTCSW

Appendix III: Reagent Recipes

10% Chelex Suspension

- Initial preparation:
  - Sterilize two 25 mL graduated cylinders with foil covering.
  - Sterilize six 50 mL foil-covered beakers with small stir bars.
  - Sterilize six weighing spatulas, foil wrapped separately.
  - Sterilize 4 x 50 mL of dH₂O (in 125-ml glass bottles). Label ‘dH₂O – PCR ONLY’.
  - Obtain 1 packet clipped P1000 pipet tips, sterile (for pipetting Chelex suspension).

- Chelex Suspension preparation (makes ~50 tubes, store at 4°C):
  - Using a sterile spatula, add 2.5 g Chelex beads (Sigma cat. no. C-7901) to a 50 mL sterile beaker with sterile stir bar.
  - Use sterile graduated cylinder to add 25 mL sterile dH₂O to the beaker. Place the beaker on a stir plate (set at 5-7).
  - While the solution is stirring, keeping the beads in suspension, pipet 0.5 mL aliquots of the slurry into 1.5 mL micro centrifuge tubes. Pipet the suspension using clipped and sterilized blue tips.

Oligonucleotide Primers for PCR Reactions

MC1R-F primer = 5’ T TCC TGC TTC CTG GAC AGG ACT AT 3’
MC1R-R primer = 5’ CCC TCT GCC CAG CAC ACT TAA A 3’

  - Purification = standard desalting.
  - Unit size = 25 nmol.
  - Bases = 25.

- Use IDT nm amounts as guide and add ~appropriate amount of PCR-grade dH₂O to each tube to make a 100 µM stock solution.
- Make a 1:4 dilution of the 100 µM solutions (20 µl of primer and 60 µl of PCR dH₂O) to have working stocks of 25 µM. Store at -20 °C until needed.
- Thaw on ice when using to make MC1R-Amp Mix.
Amplification Mix
1 tube = enough for 9 reactions (make as many tubes as needed)

<table>
<thead>
<tr>
<th></th>
<th>Amount</th>
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<tbody>
<tr>
<td>dH$_2$O</td>
<td>246.2 µl</td>
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<tr>
<td>5X Colorless GoTaq® Flexi PCR Buffer</td>
<td>90 µl</td>
</tr>
<tr>
<td>PCR Nucleotide Mix (Promega)</td>
<td>11.25 µl</td>
</tr>
<tr>
<td>MC1R-F primer working stock (25 µM)</td>
<td>9 µl</td>
</tr>
<tr>
<td>MC1R-R primer working stock (25 µM)</td>
<td>9 µl</td>
</tr>
<tr>
<td>25 mM MgCl$_2$ (for 2.5 mM final conc.)</td>
<td>45 µl</td>
</tr>
<tr>
<td>GoTaq® Flexi DNA Polymerase (5 units/µl) (2 u/reaction final)</td>
<td>3.6 µl</td>
</tr>
</tbody>
</table>

- Store all components at -20 °C until needed.
- Mix in a sterile 1.5 ml microcentrifuge tube. Keep mix on ice during lab.
- Students should add 4 µl of a DNA sample to 46 µl of the mix.

10X Tris/Borate/EDTA (TBE) Electrophoresis Buffer (from p. 538 in DNA Science)
Makes 1 liter. Store at room temperature (indefinitely).

1. Add the following dry ingredients to 700 ml of distilled or deionized water.
   - 1 g of NaOH (m.w. = 40.0)
   - 108 g of Tris (m.w. = 121.10)
   - 55 g of boric acid (m.w. = 61.83)
   - 7.4 g of EDTA (disodium salt, m.w. = 372.24)
2. Stir to dissolve, preferably using a magnetic stir bar.
3. Add distilled or deionized water to bring solution total to 1 liter.
4. Mix well and filter through #1 Whatman paper into a glass 1-L bottle.

Working concentration is 1X. Add 100 ml of 10X TBE to 900 ml of distilled or deionized water and mix well.

10X Sample Buffer (from p. 539 in DNA Science)

1. Dissolve the following in 40 ml of deionized water:

<table>
<thead>
<tr>
<th></th>
<th>Amount</th>
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<tbody>
<tr>
<td>Bromophenol blue (MW=669.96)</td>
<td>0.25 g</td>
</tr>
<tr>
<td>Xylene cyanol (MW=538.60)</td>
<td>0.25 g</td>
</tr>
<tr>
<td>1 M Tris, pH 8.0</td>
<td>1 ml</td>
</tr>
</tbody>
</table>

2. Stir in 50 ml of glycerol and bring final volume up to 100 ml with deionized water.
3. Aliquot and store indefinitely at room temperature.

1 kb DNA Ladder
- Purchase from Promega (50% discount for teachers; # G5711).
- Add 100 µl of stock DNA Ladder to 80 µl dH$_2$O and 20 µl of 10X Sample Buffer.
- Make twenty 10 µl aliquots. Store at -20 °C until needed.
- Use 10 µl when loading marker lane in gel.
Precision Molecular Mass Standards
- Purchase from Bio-Rad (#170-8207)
- Add 50 µl of stock standards to 110 µl of sterile TE and 40 µl of 5X Orange G Sample Buffer. Make twenty 10 µl aliquots. Store at -20 °C until needed.
- Load 10 µl in the marker lane of gel.

Tris/EDTA (TE) Buffer
Store at room temperature (indefinitely)
Mix:

1 ml of 1 M Tris (pH 7.9) (10 mM)
200 µl of 0.5 M EDTA (1 mM)
99 ml of distilled water

Sequencing Primers:
- MC1R S1 primer = 5' TGC CCA GCA CAC TTA AAG CGC GTG CA 3'
- MC1R S2 primer = 5' TGA TCA CGT CAA TGA CAT TGT 3'
  - Purification = standard desalting.
  - Unit size = 25 nmol.
  - Bases = 25.
- Use IDT nm amounts as guide and add ~appropriate amount of PCR-grade dH₂O to each tube to make a 100 µM solution.
- Make a 1:25 dilution of the 100 µM solutions (4 µl of primer and 96 µl of PCR dH₂O) to have a working stock of 4 pmol/µl. Store at -20 °C until needed.
- Thaw on ice when setting up sequencing reactions.

0.9% NaCl (sterile)
- Dissolve 9 gm of NaCl in 1 L of water.
- Add 100 ml to each of ten 125-ml bottles. Loosely cap bottles.
- Autoclave for 15 minutes on liquid cycle.
- Using sterile technique, pipette 10 ml each into 20 sterile 15-ml conical centrifuge tubes