

Chapter 12
**Experiments with the Structure and
Function of DNA**

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181

101

101

Introduction

It is difficult to design laboratory experiments which allow students to study both the structure and function of DNA during a two or three hour laboratory period. Studies of molecular models and isolation of DNA tend to leave the student with a feeling for the structure of DNA but without an understanding for how this structure is packaged into chromosomes and how it then serves to control the synthesis of proteins within the cell. The following exercises were designed to introduce students to the study of chromatin structure, organization of eukaryotic chromosomes, and the mechanisms of transcription and translation. The exercises may be used individually or in sequence during several laboratory periods and for several different levels of students as follows:

<i>Exercise</i>	<i>Time Developed</i>	<i>Recommended level</i>
I. Isolation of Nucleic Acids and Chromatin Proteins		
A. Isolation of DNA from <i>E.coli</i> .	1½ hrs. (other activities may be carried out during incubations)	freshman–senior
B. Isolation of Nucleic Acids from Beef Liver.	1 hour; activity is fairly constant	freshman–senior
C. Isolation and Identification of Histones and Acidic Proteins from Eukaryotic Chromatin.	instructor preparation time—3 days (may be done in advance and material can be frozen); student time—3 hours plus several 1 hour periods of returning to the laboratory	senior (small groups of students in advanced courses only)
II. The Structure of Polytene Chromosomes from <i>Drosophila</i> .	½ hour	sophomore–senior

III. Understanding Transcription and Translation—A Model	2 hours	freshman
IV. Effects of Base Substitution on the Final Gene Product.	1 hour	freshman–senior

Student Materials

Background Information

DNA is the major genetic material in both prokaryotic and eukaryotic cells. Within prokaryotic cells the DNA remains relatively free of structural proteins except for a few types of basic proteins, but in eukaryotic cells the DNA is complexed with both basic histone and acidic non-histone proteins to form *chromatin*. Non-histone proteins are very heterogeneous and occur in different amounts in different tissues. They include such molecules as DNA and RNA polymerases, unwinding proteins and countless regulatory proteins which can associate and disassociate with the DNA at different times in the cell cycle. The general role of non-histone proteins is one of regulation associated with gene function. Histones on the other hand are found in the same amounts in all tissues of most organisms and in a 1:1 ratio with DNA. There are only 5 types of histones, H₁, (very lysine rich), H₂A, H₂B (moderately lysine rich), H₃ and H₄ (arginine rich). The amino acid sequences for all but histone H are evolutionarily conserved, demonstrating little diversity among different organisms. Histone proteins appear to be important to maintenance of chromosome structure, and by controlling this structure they are also involved less directly in regulation of gene function.

In human diploid cells, approximately 174 cm of chromatin are partitioned among the 46 chromosomes, and the DNA in each chromosome is compacted more than 7000 fold. Every 140 base pairs of DNA are associated with an octamer of histones; two molecules each of histones H₂A, H₂B, H₃ and H₄ to form a core particle. An additional stretch of 60 nucleotides is associated with histone H₁ and forms a linker between core particles. When eukaryotic chromosomes are spread for electron microscopy histone H₁ is usually lost during preparation, and the chromatin appears to be composed of beads (core particles) on a string (linkers). If prepared by gentle means the linker piece

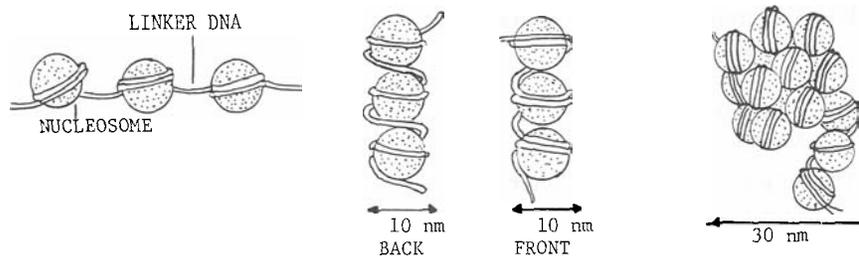


Figure 12.1. Chromatin structure.

remains closely associated with the core particle to form a bead-like nucleosome or η body 10 nm in diameter. Each nucleosome is thus composed of histones plus 200 base pairs of DNA. Contiguous nucleosomes in close apposition for a 10 nm fiber, but this represents only a 5 to 6 fold packing of chromatin. To achieve the compactness of metaphase chromosomes this fiber must be folded 1000X more. The 10 nm fibers are coiled into 30 nm fibers which are then looped and folded to form the chromomeres so obvious in the giant polytene chromosomes of insects, where they are associated side by side to form bands along the chromosomes.

Chromatin packing and chromosome structure have been studied extensively in relation to the giant polytene chromosomes characteristic of dipteran insects. Banded chromosome regions contain genes which, when transcriptionally active, form chromosomal puffs. The nature of interband regions is less well understood. Recently Z DNA has been shown to be present in the interband regions, but to date no function for this DNA has been assigned. The presence of band and interband regions in human chromosomes is also apparent when special staining methods are used. Heterochromatic bands on human chromosomes include Q,G,C,R and T bands. Q and G and R bands represent regions of facultative heterochromatin while C and T banding may be used to identify regions of constitutive heterochromatin. Constitutive heterochromatin is believed to have no genetic function, while facultative heterochromatin may be transcriptionally active, indicating that chromatin packaging at this level is also important to the regulation of gene function.

As further intricacies of chromosome structure are resolved it will be possible to understand the structure of genes and regulation of gene function more precisely. Information encoded in the nucleotide sequence of DNA is known to serve as the key for directing semiconservative replication of DNA, transcription of messenger RNA and subsequent translation into polypeptide chains. These processes will be examined further in the following exercises.

Instructors' Materials

I. Isolation of Nucleic Acids and Chromatin Proteins

A. Isolation of DNA from *E. coli*

Since *E. coli* are prokaryotic cells and do not contain a nucleus, it is fairly simple to isolate the DNA from ruptured cells. The following exercise has been adapted from Clark, J. M. and R. L. Switzer, *Experimental Biochemistry*, W. H. Freeman and Company, San Francisco.

1000ml

1. Suspend 2 g of bacterial paste in 25 ml of 0.15 M NaCl and 0.1 M Na₂ EDTA. (Use a 20 × 150 mm screw-top glass tube if you do not have access to a centrifuge for later steps.)
2. Add 1 ml lysozyme solution (10 mg/ml)
3. Incubate at 37°C for 30 min
4. Add 2 ml 25% SDS (Sodium lauryl sulfate)
5. Incubate at 50° for 10 min or until supernatant is clear
6. Add 7.5 ml of 5 M NaClO₄ and stir
7. Add an equal volume (35 ml) of chloroform/isoamyl alcohol (50:1)
8. Shake well
9. If you have access to a centrifuge, then centrifuge the mixture at 10,000 rpm for 10 min. A protein pellet will form at the interface between the two solutions. Remove the clear aqueous upper phase (this contains the DNA) and save in a beaker.
If you do not have access to a centrifuge, allow the solution to settle out and pipette out the clear aqueous phase (upper layer) and place in a large, glass test tube or beaker. This will take approximately 45 min.
10. Add two volumes of cold 85% EtOH slowly down the side of the beaker or tube containing the aqueous phase.
11. Stir with a glass rod (heated or acid-washed to remove any nucleases). Stir gently and spool out the DNA. Scoring the end of the glass rod with a diamond pencil will make it easier to spool out the DNA.

B. Isolation of Nucleic Acids from Beef Liver

Quick isolation procedures for DNA from eukaryotes will yield DNA which is more contaminated with RNA and protein. Many of the chromatin proteins will still be associated with the DNA. Chromosomal or nascent RNA will also be present.

Caution: Steps 1–9 must be completed with all solutions and materials kept cold in an ice chest. The following exercise has been adapted from Zimmerman, J. K. and C. S. Brown, *A Biochemistry Laboratory Manual*, Burgess Publishing Company, Minneapolis.

1. Homogenize 10 g fresh beef liver in 5 volumes ice-cold distilled water in a chilled Waring blender.
2. Filter homogenate through cheesecloth into a 500 ml chilled erlenmeyer.
3. Add an equal volume of cold 30% TCA to homogenate and swirl or shake for 20 min.
4. Transfer homogenate to precooled centrifuge tubes and centrifuge at 5000 rpm for 10 min.
5. Discard supernatant
6. Place 1 g precipitate into a chilled centrifuge tube.
7. Add 3 ml chilled acetone, resuspend pellet and centrifuge at 5000 rpm for 10 min.
8. Discard supernatant and resuspend precipitate in acetone, repeating Step 7.
9. Wash precipitate in 3 ml acetone:ether (50/50) and centrifuge at 5000 rpm for 10 min.
10. Wash precipitate in 3 ml ether and centrifuge at 5000 rpm for 10 min.
11. Air dry chromatin.

Note: Diphenylamine and orcinol tests can be done to test for DNA and RNA with this material. Weigh out 0.1 g of chromatin powder and suspend in 2 ml distilled water. Run standard orcinol test or diphenylamine test. (See Appendix A).

Materials

- A. *E. coli* paste—prepare a bacterial cell paste from *E. coli* by centrifuging cells (grown in tryptic soy broth [30g/1]) in late log phase at 6,000 rpm for 10 min. You may freeze the paste for use as needed.

You may purchase *E. coli* from Carolina Biological Supply Company #15-5065; tube/\$4.40—suspend with enough tryptic soy broth to make a turbid mixture. Use 1 ml to inoculate 100 ml medium. Grow overnight. Use this to inoculate 1000 ml until slightly cloudy. In approximately 4 hrs the cells should be in late

log phase (just when you cannot see through the medium/ $A_{660} = 0.5$) generation time is approximately 1/2 hr at 37°C. Bacterial paste may also be purchased from Grain Processing Co., P.O. Box 341, Muscatine, Iowa, 52761 (*E. coli* k₁₂:1/2 log is 500 g).

Lysozyme—10 mg/ml

25 ml (0.15 M NaCl, 0.1 M Na₂EDTA) per 2 g of bacterial paste

NaClO₄ solution—5M

SDS solution—25%

chloroform-isoamyl alcohol (50:1)

95% Ethanol—cold! (Store in freezer)

100 ml erlenmeyer flask

Large polyethylene centrifuge tubes (for SS34 Sorvall rotor or similar type)

Glass rods

B. liver (beef preferred)

anhydrous ether

acetone

cheesecloth

500 ml erlenmeyer

refrigerated centrifuge (Sorvall SS34 rotor)

centrifuge tubes

C. *Isolation and Identification of Histones and Acidic Proteins from Eukaryotic Chromatin*

In order to isolate chromatin proteins from eukaryotes it is first necessary to isolate nuclei. Chromatin may then be isolated relatively free from contamination by cytoplasmic proteins. (This procedure is not complicated, but takes time. It would be best to isolate chromosomal proteins during the summer months and then freeze them for later use in the laboratory. The procedure is given in Appendix B.) This procedure was developed by Gordhan H. Patel, Department of Zoology, University of Georgia, and the author would like to take this opportunity to thank Dr. Patel for his guidance.

1. Resuspend lyophilized histones or acidic proteins by dialyzing overnight against dialysis buffer (see Materials for preparation of dialysis tubing and dialysis buffer). Determine histone and acidic protein concentrations before dialysis. ($1.55 \times A_{280} - 0.76 \times A_{260} = \mu\text{g protein/ml}$)
2. Prepare electrophoresis disc gels as described in Materials section.

3. When ready to run gels remove parafilm from bottom of gels and place in electrophoresis chamber. Bottom chamber should already be filled with electrophoresis running buffer. Make sure there are no air bubbles present in the bottoms of the gel tubes.
4. Prepare dialyzed samples by boiling for 1 min. Add 5 μ l of β mercaptoethanol plus 5 ml of Solution G (see Materials section). Use samples of approximately 100 μ l for acidic protein gels and 80 μ g for histone gels. Sample volumes should not exceed 100–250 μ l.
5. Remove water from the top of gels using a laboratory tissue.
6. Layer appropriate amount of sample onto gel using a microliter pipette, and layer reservoir buffer on top of sample using pasteur pipette, filling the gel tube to the top.
7. Pour reservoir buffer into upper chamber (gently!!)
8. Run gels at 2 mA (milliamps) per gel for 2 hrs; normal polarity = top (-) bottom (+).
9. Rim gels with 21 gauge long needle using distilled water and place each gel into a test tube.
10. Stain gels in either 1% Buffalo Black (1% amido black, 40% ethanol, 7% acetic acid) or in 1% Coomassie Blue (1% Coomassie Brilliant Blue in 7% acetic acid) for at least two hours, preferably overnight. Stain gels in test tubes.
11. Destain Buffalo Black gels in 7% acetic acid or destain Coomassie gels in methanol, water, glacial acetic acid, 5:5:1.
12. Examine gels. Store in 7% acetic acid.

Five bands will appear on the histone gels. These are (from top to bottom) histones H1, H3, H2B, H2A, and H4. Many bands will appear on the acidic protein gels—these represent the many structural and regulatory proteins associated with eukaryotic chromatin.

Materials

Histones—Histone proteins may be prepared from rat liver as described in Appendix B.

Acidic Proteins—Acidic proteins may be prepared from rat liver as described in Appendix B.

Electrophoresis Solutions

I. Dialysis Buffer

8 M urea	800 ml 10 M urea
0.1% SDS (sodium lauryl sulfate)	10 ml of 10% SDS
1% β -mercaptoethanol	10 ml β mercaptoethanol
0.01 M PO_4 buffer pH 7.0	100 ml 0.1 M NaPO_4 pH 7
(Make up to 1 liter with distilled water—Adjust to pH 7)	

II. Preparation of Dialysis Tubing

- A. Boil tubing in 0.001 M EDTA for 1 hour.
- B. Rinse in distilled water.
- C. Boil tubing in water containing a small amount of sodium bicarbonate.
- D. Rinse thoroughly.
- E. Store tubing in 50% EtOH and wash with distilled water before use.

III. Solutions for Gel Preparation. (A–D are per 100 ml distilled H_2O)

- A. 48 ml 1 N HCl
36.6 g Tris
0.23 ml TEMED pH 8.9
- B. 48 ml 1 N HCl
5.98 g Tris
0.46 ml TEMED pH 7.6
- C. 40 g acrylamide
0.6 g bis-acrylamide
filter through #1 Whatman
- D. 10 g acrylamide
2.5 g bis-acrylamide
filter through #1 Whatman
- E. 4 mg Riboflavin/up to 100 ml H_2O (distilled)
- F. 70 mg ammonium persulfate to 50 ml in (8 M urea, 0.2% SDS) (80 ml 10 M urea + 18 ml H_2O + 2 ml 10% SDS)
Prepare weekly

G. 0.05% Bromphenol Blue in H₂O (distilled)

For all solutions—prepare every 1–2 months. Keep refrigerated if solutions do not contain SDS (sodium lauryl sulfate). Let come up to room temperature before use (for accurate vol).

IV. *Reservoir Buffer*

6 g Trizma Base
28.8 g glycine
2 g SDS
Prepare 2 liters (pH 8.4)

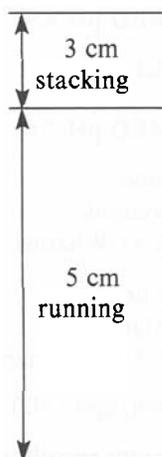
V. *Stain*

1% amido black
40% ethanol
7% glacial acetic acid

or

1% Coomassie Brilliant Blue
7% acetic acid

VI. Preparation of Gels—Mark tubes as shown and cover bottom of tube with parafilm.



A. Running gels

<i>For 6 gels:</i>	<i>For 12 gels:</i>
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1 ml A	2 ml A
3 ml C	6 ml C
4 ml F	8 ml F

Mix acrylamide in 25 ml side arm erlenmeyer. Degas for 1 min using vacuum. Load tubes to 5 cm mark using pasteur pipette. Layer with small amount of distilled H₂O using a clean pasteur pipette.

Polymerize 2 hr at rm. temp. Remove H₂O layer (absorb with laboratory tissue) and add:

B. Stacking gels

<i>For 6 gels:</i>	<i>For 12 gels:</i>
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1 ml B	2 ml B
2 ml D	4 ml D
1 ml E	2 ml E
4 ml [8 M urea, 0.02% SDS]	8 ml [8 M urea, 0.2% SDS]

Mix in 25 ml side arm erlenmeyer. Degas for 1 min using vacuum. Load tubes to 8 cm mark by adding 3 cm of stacking gel to each tube. Layer with distilled H₂O using clean pasteur pipette.

Polymerize 1/2 hr under fluorescent light. Place gels approximately 1 ft from light source.

Gels may be made in the morning for afternoon class. We usually make students prepare gels during the laboratory period just for experience, but we use gels prepared earlier so that the electrophoresis can be completed within a 3 hr laboratory period.

II. The Structure of Polytene Chromosomes from *Drosophila*

1. Select 3rd instar larvae: large, active, well-fed individuals grown at 25°C or lower temperatures. (See Appendix C.) Place on dissecting stage (see Fig. 12.2).
2. Dissect out glands in 45% acetic acid and quickly transfer on the tip of a dissecting needle to a small drop of aceto-orcein stain in a siliconized glass coverslip.

II. Mounting Medium

Euparal and Euparal thinner (1:1) from Flatters and Garnett Ltd., 309 Oxford Road, Manchester 13, England.

III. Siliconized Coverslips

Concentrated silicone solution is obtained from General Electric Co. as #Sc-87 "Dri-film".

- A. Dip coverslip in concentrated silicone solution.
- B. Scrub thoroughly in detergent or soap solution to remove as much excess silicone as possible.
- C. Wipe with laboratory tissue.
- D. Store in 95% EtOH.
- E. Wipe with laboratory tissue before using.

IV. Dissecting Stage

Two regular microscope slides with a triangular piece of carbon paper between are glued together with Permount.

When this mount is dry, dip it quickly in concentrated silicone solution; then rinse briefly in warm soap or detergent solution and dry. (Re-dip when silicone wears off).

Dissect salivary glands by placing drop of 45% acetic acid on slide and place 3rd instar larva in drop. This slide provides alternatively a black background for dissecting translucent salivary glands or other larval organs; or a white background when over a white plate for examining such larval markers as yellow, ebony, etc..

III: Understanding Transcription and Translation

Ultimately the base sequence of DNA is responsible for the linear sequencing of amino acids into a polypeptide chain, i.e. DNA functions as the genetic material because it can determine the types of proteins to be manufactured by the cell. Since these proteins are both structural and metabolic, they determine the characteristics of individual cells and, in a broader perspective, the entire organism. Let us now turn our attention to understanding the processes of transcription (mRNA production) and translation (protein formation).

Kits for this exercise can be inexpensively prepared using green, blue, black and orange posterboard. (Appendix D.) Pieces should be cut according to sizes given in the Materials section. DNA and RNA nucleotide and amino acid structures are mimeographed onto blue, green and yellow paper respectively.

The role of protein factors is expressed in a non-committal way throughout this exercise for two reasons. First, in general biology courses learning the specific functions of each factor only confuses the students who are trying to learn about the process of transcription and translation. Second, the perceived role of protein factors continually changes as more is learned about their structure and function. For our majors' course in biology, we supplement this exercise with a handout sheet on the role of protein factors. The following directions are given to each student as part of the kit or laboratory manual.

Each kit includes the following materials:

- One DNA molecule—white
- One sheet of nucleotides—blue
- One sheet of nucleotides—green
- One sheet of amino acids—gold
- One ribosome (black)
- Four aa-tRNA-synthetase enzymes (green)
- Four tRNA molecules (blue)
- One ATP molecule (orange)
- GTP-GDP, EFT, EFG, R₁ R₂ R₃, IF₁ IF₂IF₃ (white envelope)
- Genetic code chart

Replication

1. Using the white DNA molecule, separate the two nucleotide chains which make up DNA by “cutting the hydrogen bonds” which hold the two chains together. Use a pair of scissors to cut the molecule.
2. Replication of DNA is semiconservative, each half of the original DNA serving as a template or pattern for the making of its other half. New nucleotides (A, T, G, and C) are sequestered from the nuclear protoplasm. The bases pair up in a specific manner, A with T and C with G according to their steric configuration and the number of hydrogen bonding sites on each nucleotide. The two new DNA molecules which result

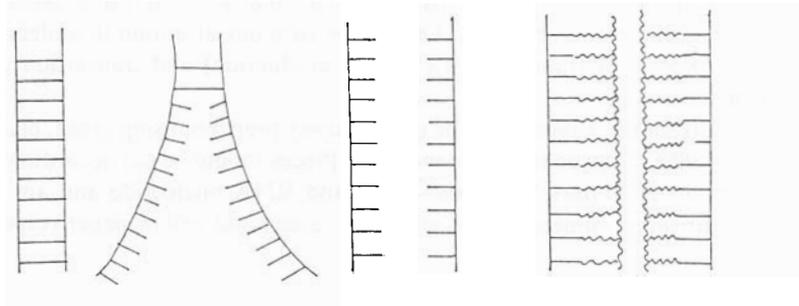


Figure 12.3. Semiconservative replication of DNA.

from this process of semi-conservative replication are each made up of one old half of the original DNA duplex and one new half made from nucleotides sequestered from the nucleotide "pool" or reserve present in the nucleoplasm.

- Using the blue nucleotides, semiconservatively replicate DNA. (a) Cut out the proper blue nucleotides; (b) line them up in the proper order according to the base composition of the original DNA; (c) tape the nucleotides together and indicate which is made using strand I DNA as a template and which is made using strand II DNA as a template by writing I and II on their ends. Label the 3' end and 5' end of each polynucleotide strand. Save these molecules to show to your laboratory assistant who will be coming around to check your work.

Transcription

- Using your blue DNA made from strand I of the white DNA, synthesize a molecule of messenger RNA (mRNA) using the *green* nucleotides. This process is called *transcription*. Cut out the nucleotides and tape them together in the proper sequence.
- Label the 3' and 5' ends of the message you have made.
- Usually in a cell only one side of the DNA molecule serves as a template for transcription into mRNA. This is known as "copy choice."

In the space provided below record the nucleotide sequence of your messenger RNA.

3' end ————— 5' end

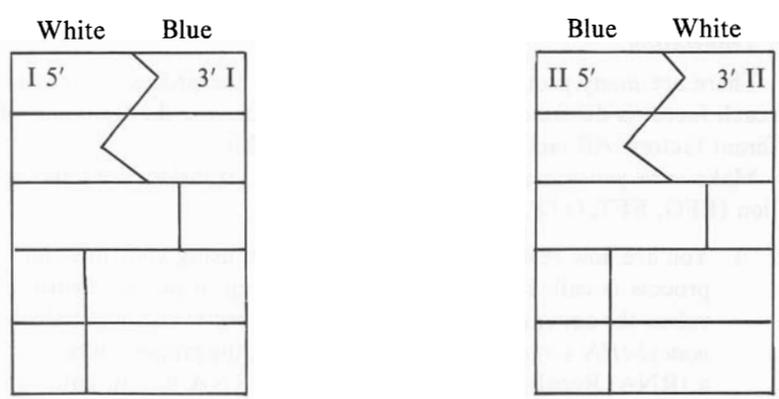


Figure 12.4. Using the DNA kit to simulate semi-conservative replication.

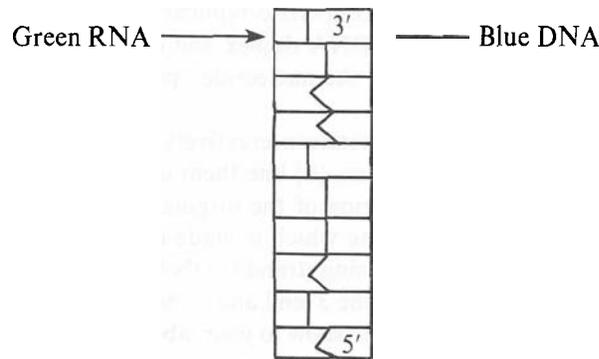


Figure 12.5. Using the DNA kit to simulate transcription.

Record the messenger RNA sequence which could have been synthesized from the other half of the DNA molecule (the white half which represents the “old” part of the DNA which was semiconservatively replicated)

3' end ————— 5' end

Did you record the messages in the proper direction? In which direction is the mRNA read while being used for the synthesis of proteins? Record your message in the proper direction.

- Each group of 3 nucleotides in a messenger RNA molecule is called a *codon*. What is the importance of these nucleotide triplets? Using brackets, indicate the nucleotide triplets of your messenger RNA (green copy) on the sequence written above.

Translation

There are many protein “factors” involved in the process of translation. As each factor is discussed, make a chart to summarize the functions of the different factors. All factors are included in your kit.

Make sure you use all factors for elongation, translocation, and termination (EFG, EFT, GTP, IF₁, IF₂, IF₃, R₁, R₂, R₃)

- You are now ready to synthesize a protein using your message. This process is called *translation*. The first step in protein synthesis involves the activation of the amino acids. Special enzymes called *aminoacyl-tRNA-synthetases* are used to hook the proper amino acid onto a tRNA. Recall from lecture that each tRNA has an anticodon on one end which must pair up with a codon on messenger RNA to insure that the amino acids are put together in the proper sequence. The correct amino acid must become attached to the tRNA carrying its specific anticodon before that amino acid can be slipped into place

by a specific pairing between tRNA anticodon and mRNA codon. *Amino-acyl-tRNA synthetase* is responsible for seeing that a particular tRNA molecule carrying a specific anticodon nucleotide triplet is “charged” with the corresponding amino acid.

- a. Place one of the green enzymes on your desk.
- b. Find the amino acid which fits into the enzyme. The enzyme is shaped so that the tRNA anticodon bases fit into one end and the amino acid fits into the other. Do *NOT* insert the amino acid yet.

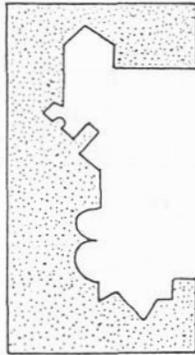


Figure 12.6. The amino-acyl tRNA synthetase enzyme.

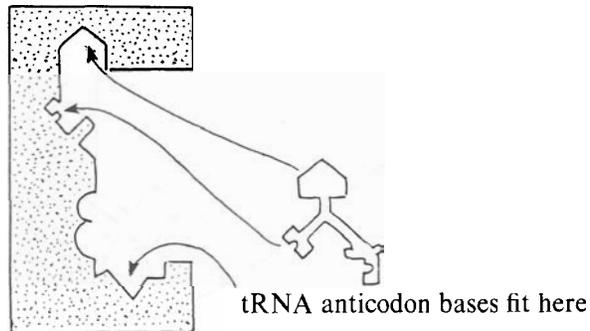


Figure 12.7. Active sites of the amino-acyl tRNA synthetase enzyme.

- c. Find the ATP molecule (orange). ATP is used to activate the amino acid so that it can be inserted into the enzyme. When this happens $ATP \rightarrow AMP + PP_i$ releasing energy for the insertion. The AMP fits into a groove on the side of the enzyme and is also attached to the amino acid. The amino acid loses an OH group which is incorporated into the PP_i . Cut out the OH.

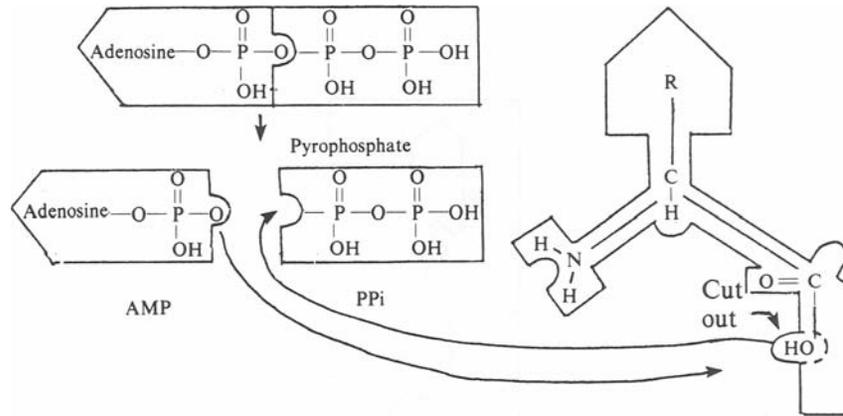
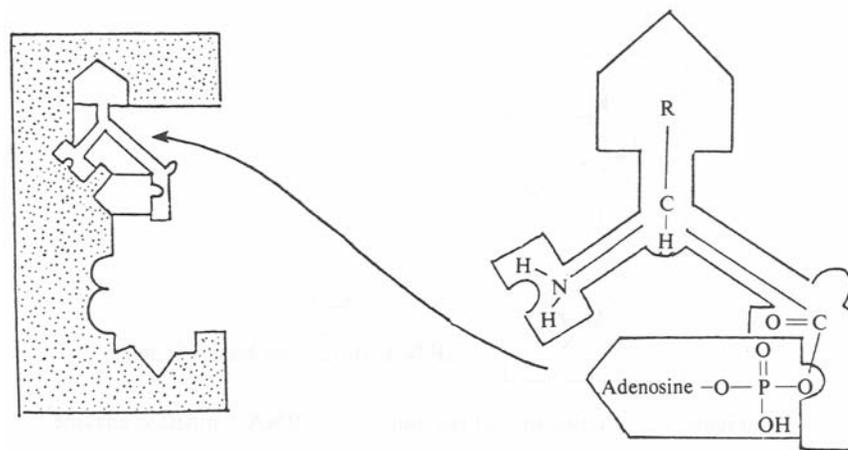


Figure 12.8. Activation of amino acids.



Aminoacyl-tRNA-synthetase

Figure 12.9. Insertion of activated amino acid into one of the active sites of the amino-acyl tRNA synthetase enzyme.

- d. Now tRNA comes in. The tRNA has a triplet of nucleotides CCA on one end and the anticodon nucleotides on the other end. The adenosine nucleotide or "A" fits into the same groove that the adenosine of AMP fits into. When AMP comes out, the energy given off in breaking the bond between AMP and the amino acid can be used to attach the amino acid to the tRNA. Attach the amino acid to the tRNA using a paper clip. The tRNA now has the correct amino acid hooked to it and is said to be a *charged* tRNA. The tRNA carrying the appropriate amino acid will break loose from the tRNA synthetase molecule and is then ready to participate in the process of protein synthesis.
- e. Now "charge" your 3 other tRNA's with amino acids as you did above.

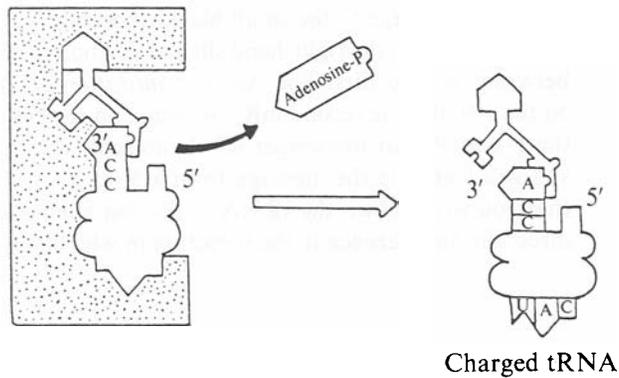


Figure 12.10. Charging the tRNA molecule.

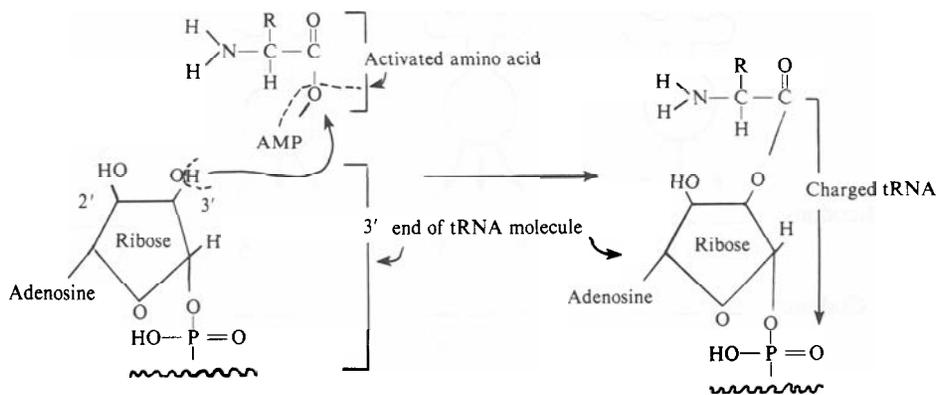


Figure 12.11. Linking the 3' end of the CCA terminus of a tRNA molecule to the amino acid which it will carry.

Make sure that you understand the nature of the biochemical bond between the adenosine residue of tRNA and the amino acid.

In the space provided below, indicate the anticodon nucleotides for each of your four tRNA molecules. Notice that you are writing the anticodons in the 3' to 5' directions left to right. Indicate the complementary codon for each anticodon. Notice that you are writing the codons in the 5' to 3' direction, left to right. Recall that complementary polynucleotide chains must always be antiparallel. Indicate the amino acids being carried by each of your four tRNA molecules by using the copy of the genetic code contained in your kit. Do you look for the anticodon or codon in this chart?

2. During protein synthesis messenger RNA moves from the nucleus to the cytoplasm in eukaryotes (remember that prokaryotes do not have a nuclear membrane and message is already in their cytoplasm).
 - a. Messenger RNA attaches to the small ribosomal subunit. Attach your message to the small black ribosomal subunit by sliding it up through the right-hand slit and position the first 2 codons between the two slits. The AUG or *initiation codon* should be on the left and the second mRNA codon on the right. Note that the 5' end of your messenger RNA molecule is on the left, thus you will translate the message from a 5' to 3' direction. Recall that you wrote down the tRNA anticodon nucleotides in a 3'5' direction. In reference to the direction in which you read a mes-

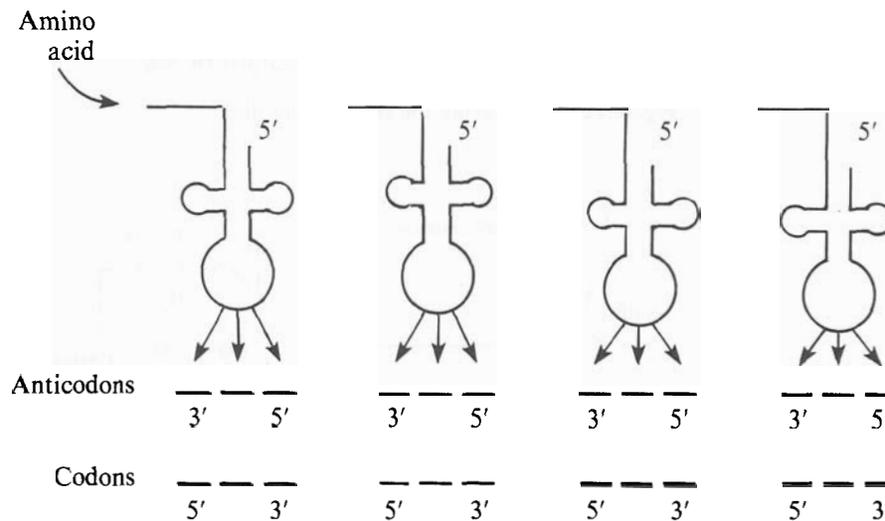


Figure 12.12. Codons and anticodons.

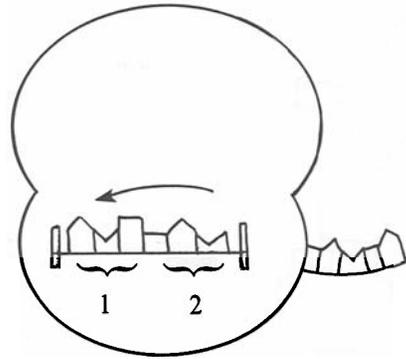


Figure 12.13. Attachment of mRNA to the small ribosomal subunit.

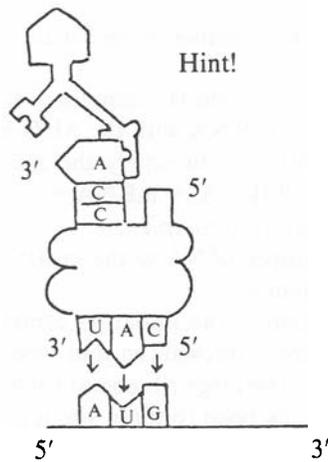


Figure 12.14. Alignment of the tRNA anticodon with the mRNA codon.

sage, each anticodon will pair with a codon on mRNA in a *antiparallel* fashion. What does this mean?

- b. A protein factor IF_3 is involved in the attachment of mRNA to the small ribosomal subunit. Attach IF_3 to the small ribosomal subunit along with the attachment of mRNA.
- c. A tRNA carrying the amino acid formyl-methionine attaches to the first initiation AUG codon by pairing the tRNA anticodon with the mRNA codon in an antiparallel fashion. (This is true for prokaryotes. Evidence indicates that methyl methionine is the initiation amino acid in eukaryotes.)

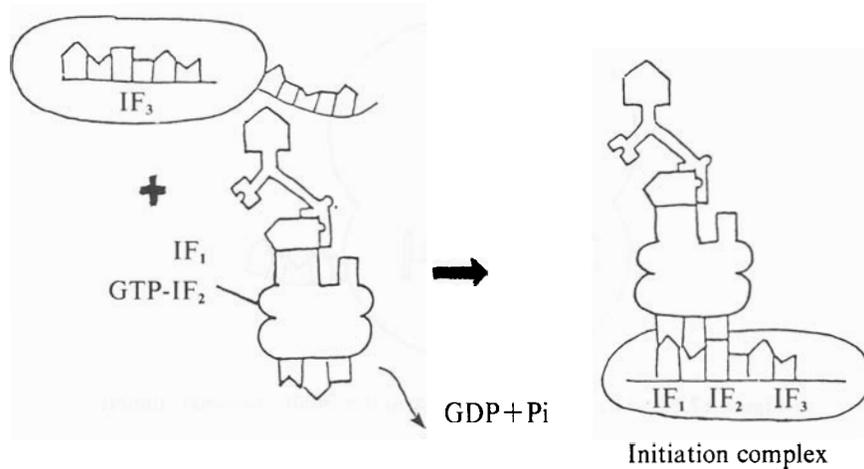


Figure 12.15. Formation of the initiation complex.

Protein factors IF₁ and IF₂ complexed with GTP are involved in pairing this first tRNA with the AUG initiation codon. GTP is split into GDP + Pi to supply the energy for the attachment. The complex of tRNA + mRNA + small ribosomal subunit is called the *initiation complex*.

Attach the proper tRNA to the initiation codon using the correct protein factors.

- d. After formation of the initiation complex the large ribosomal subunit becomes attached (on your model this subunit is already represented). The large ribosomal subunit contains 2 sites. The P site or peptide bond forming site is on the left and the A site or arrival site is on the right.
- e. A second tRNA now attaches to ribosome-tRNA-mRNA complex. This tRNA fits into the A site of the large ribosomal subunit and its anticodon complements with the second mRNA codon. A protein factor (EFT or elongation factor) which is complexed with GTP aids in insertion of the second tRNA and all subsequent tRNA molecules carrying amino acids. Again $\text{GTP} \rightarrow \text{GDP} + \text{Pi}$ to supply energy for the insertion of the tRNA.

Attach the second tRNA carrying amino acid #2 to the A site on the mRNA—ribosome complex. Use the EFT-GTP factor.

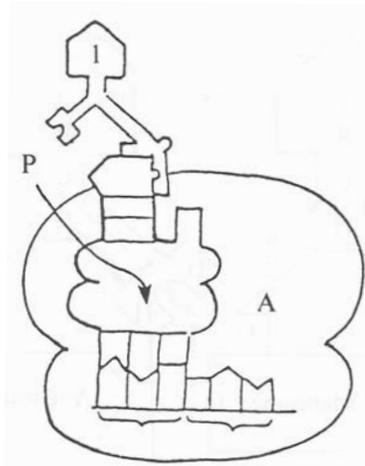


Figure 12.16. Attachment of the large ribosomal subunit.

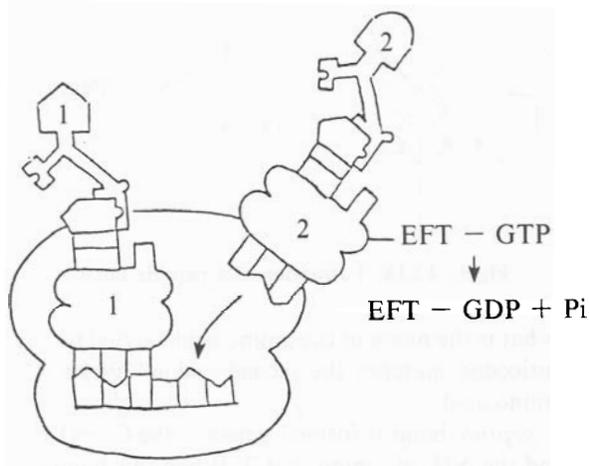


Figure 12.17. Elongation of the peptide chain.

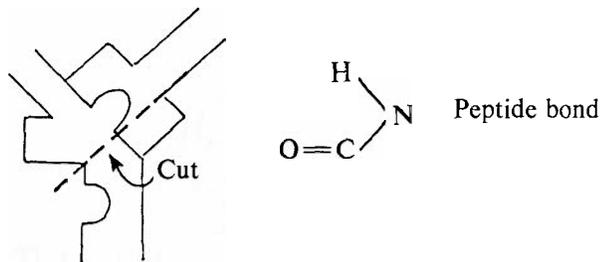
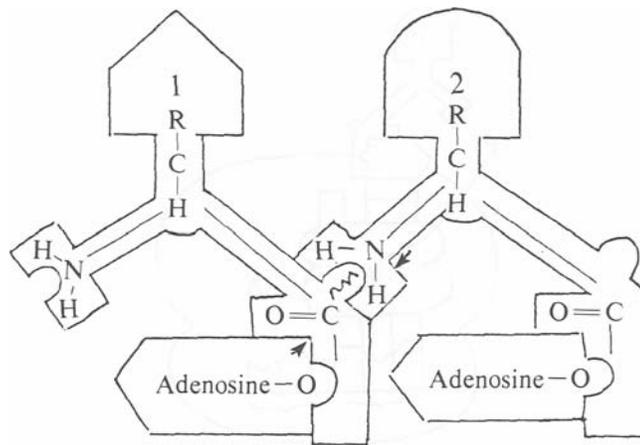


Figure 12.18. Formation of a peptide bond.

- f. What is the name of the amino acid carried by the tRNA whose anticodon matches the second codon? Write the name on the amino acid.
- g. A *peptide* bond is formed between the $\text{C}=\text{O}$ of amino acid 1 and the NH_2 of amino acid 2. When this happens, the bond between the tRNA and amino acid one breaks. The first amino acid is now held by the peptide bond to the second amino acid on the second tRNA. The H from NH_2 of amino acid 2 is added to the 3' position on the A residue of tRNA, to restore it to the 3' OH group.
- h. Attach the two amino acids together with a piece of tape. Remove the extra H on NH_2 using your scissors at arrows on Fig. 12.18. A peptide bond has been formed and your protein chain

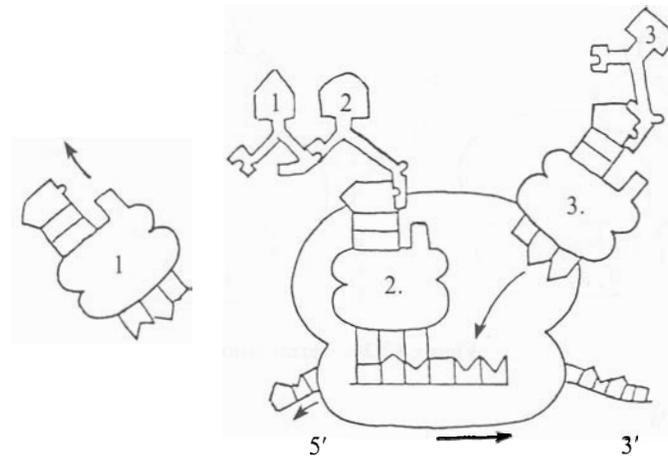


Figure 12.19. Translocation.

is now two amino acids long. Each time a tRNA carrying an amino acid is added and a peptide bond is formed the chain gets longer. Thus, this process is known as *elongation*. NOTE: Peptidyl transferase is the enzyme involved in peptide bond formation.

- i. The ribosome now moves along the message. Place your fingers on the message and move your ribosome to the right. The tRNA associated with the first codon leaves, since it is no longer bound to the amino acid. This process of ribosome movement from one codon to the next is known as *translocation*.
A protein factor (EFG, translocation factor) complexed with GTP is involved in the movement of a ribosome along the message in a 5' 3' direction. Again $\text{GTP} \rightarrow \text{GDP} + \text{P}_i$ supplies the energy for translocation.
- j. Now tRNA number two is on your left and the first two amino acids are attached to it. Match the anticodon of the tRNA number three carrying amino acid number three to the next codon, and repeat steps (g) and (h). You should now have 3 amino acids attached to tRNA number three.
- k. Repeat steps (i) and (j), until the last mRNA codon is in the A site.

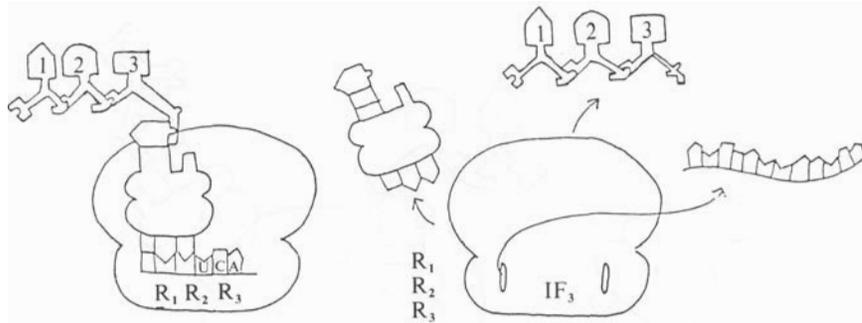


Figure 12.20. Termination.

Materials

DNA kit (designed by the author. Appendix D.)
 tape
 scissors
 paper clips

IV. The Effects of Base Substitution on Production of Messenger RNA and the Final Gene Product

If hemoglobin structure has not been previously discussed in lecture, students should be provided with the following information by way of introduction. This exercise is usually done as a demonstration in large introductory laboratories, but for smaller groups of students it may be done individually or in pairs.

In humans, Hemoglobin A (normal adult hemoglobin), Hemoglobin S (sickle-cell hemoglobin) and Hemoglobin C all contain 2 α and 2 β chains. In the latter two, however, a single base change in the DNA coding for these protein products has resulted in a single amino acid change in the sixth amino acid residue of the β chain. Sickle-cell hemoglobin contains 2 normal α chains and 2 β chains with valine substituted for glutamic acid at the sixth amino acid position. Hemoglobin-C contains 2 normal α chains and 2 β chains with lysine substituted for glutamic acid at the sixth amino acid position. The different amino acid composition of the various hemoglobins allows for their easy separation by electrophoresis. Hemoglobin F (fetal hemoglobin) which contains 2 α and 2 γ chains can also be separated from hemoglobins A, C and F in this manner.

Procedure

1. Collect blood on Whatman #1 filter paper.
2. Using a hole punch, punch out a 1/4" circle of dried blood.
3. Elute blood in 1 drop of Hemolysate reagent for 1 hour.
4. Prior to use, soak zip zone cellulose acetate plates in buffer for 15 min. Be careful to lower plates into buffer slowly so cellulose acetate will not peel off.
5. Fill spotting well in the sample well plate with 5 μ l of eluted blood and place A, FSC control blood in one of the wells also.
6. Blot cellulose acetate plate between several layers of bibulous paper.
7. Spot using Helena applicator.
8. Lay plates with the cellulose acetate downward onto the two electrophoresis wicks in the electrophoresis apparatus. Place sample end at the cathode. (Electrophoresis wicks can be made from Whatman # 1 filter paper)
9. Electrophorese towards anode at 350 volts for 25 min in Supre-heme buffer for electrophoresis.
10. Stain in Ponceau S for 10 min.
11. Wash in several changes of 5% glacial acetic acid until red color disappears.

Note: From origin hemoglobins will be HbA, HbF, HbS, HbC.

Table 12.1. Showing how the different amino acids in position six of HbS and HbC result from changing the second triplet base from T to A, or the first triplet base from C to T.

Type Hemoglobin	A.A. in position #6	DNA Triplet	mRNA Codon
HbA	glutamic acid	CTT or CTC	GAA or GAG
HbS	valine	CAT or CAC	GUA or GUG
HbC	lysine	TTT or TTC	AAA or AAG

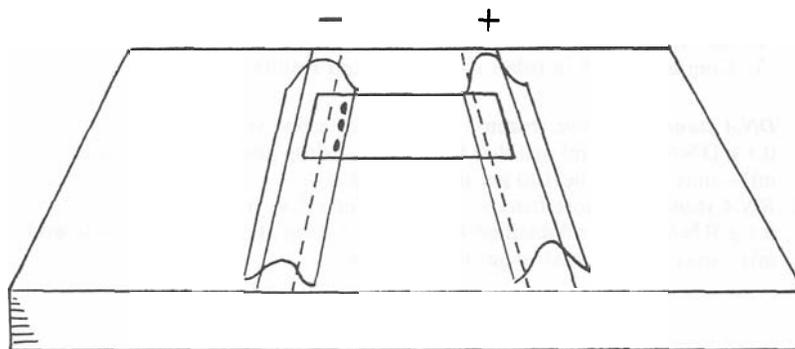


Figure 12.21. Electrophoresis apparatus for hemoglobin preparation.

Materials:

All materials can be obtained from Helena Laboratories, P.O. Box 752, Beaumont, Texas, 77704 (800-231-5663)

Zip Zone Cellulose Acetate plates (Cat# 3023) \$25.75 for 25

Supre-heme buffer for electrophoresis—(Cat# 5802) \$31.00 for 12 packages—buffer can be used many times—good for 1 month

A₁FSC Control—(Cat# 5331) \$55.00

Hemolysate Reagent—(Cat# 5125) \$33.00

Zip Zone Applicator System—(Zip Zone Applicator, Cat# 4080, \$61.25; Zip Zone Sample Well Plate, Cat# 4081, \$20.50; Zip Zone Aligning Base, Cat# 4082, \$32.50)

Ponceau S stain—(Cat # 5525, \$30.00) or (0.5% Ponceau S, 3.5% sulfosalicylic acid, 3.5% TCA)

APPENDIX A

*Colorimetric Tests for DNA and RNA*A. *Diphenylamine Test for DNA*

1. Pour 3 ml of 4% NaCl or saline citrate into a clean test tube.
2. Use an applicator stick to remove some of your isolated DNA from the glass stirring rod and redissolve this material in the NaCl.
3. Mark the tube with an "I" to indicate that it contains your *isolated* material.
4. Place 3 ml of a DNA standard solution into a second test tube and 3 ml of water into a third test tube. Mark these with an appropriate indication of their contents. These tubes will provide you with a standard and a control with which to compare your results.
5. Add 3 ml diphenylamine reagent to each tube.
6. Boil for 15 min.
7. Compare colors in tubes and record your results.

B. *Orcinol Test for RNA*

1. Pour 2 ml of 4% NaCl or saline citrate into a clean test tube.
2. Dissolve part of your isolated nucleic acid in the NaCl. Label.
3. Place 2 ml of the RNA standard solution into a second test tube. Label.
4. Place 2 ml distilled water into a third tube. Label.
5. Add 2 ml orcinol reagent to each tube.
6. Boil for 20 min.
7. Compare colors in tubes and record your results.

1. *DNA standard*—store frozen—will keep for a few yrs.

0.1 g DNA to 200 ml distilled H₂O (place 1 drop glacial acetic acid to every 200 ml)—may need to heat to get into solution

2. *RNA standard*—store frozen—will keep for a few yrs.

0.1 g RNA to 200 ml distilled H₂O (place 1 drop glacial acetic acid to every 200 ml)—may need to heat to get into solution

Sigma Chemical Co.				
DNA	D1626	250 mg		\$4.00
		1 g		11.00
RNA	R6626	25 g		2.00
Fisher				
DNA (sodium salt-less pure)		11453	25 g	\$8.65
RNA		10010-P	500 g	\$7.65

3. *Diphenylamine*—Fisher 0-2611 100 g \$14.50
 1. Dissolve 15 g diphenylamine in 1 liter of glacial acetic acid (use new bottle)
 2. Add 15 ml concentrated H₂SO₄ (new bottle)
 3. Store in dark bottle.
 4. On day of use add 1 ml acetaldehyde (Fisher 0 1004 250 ml \$10.10—keep in fire cabinet) solution (1 ml acetaldehyde to 500 ml distilled H₂O) per every 100 ml diphenylamine
4. *Orcinol*
 1. 5g of FeCl₃ to 1 liter of concentrated HCl
 2. Prepare fresh before use:
0.1g orcinol + 10 ml FeCl₃ solvent

APPENDIX B

Isolation of Chromosomal Proteins

- Day 1*
- Rat liver (50 g)
 - Pass through tissue press
 - Add 500 ml buffer (0.34 M sucrose, .003 M Ca Cl₂)
 - Homogenize (Omni Mixer)
 - Filter thru 4 thicknesses cheesecloth
 - Centrifuge 2500, 15' in GSA bottles
 - Pool and wash pellets (0.25 M sucrose, .003 M Ca Cl₂)
 - Centrifuge 3000 rpm, 15'
 - Pool pellets; discard supernatant
 - Resuspend (2.4 M sucrose, .003 M Ca Cl₂)
 - Homogenize (motor-driven homogenizer)
 - Filter (miracloth)
 - Centrifuge 19 K, 1 hr
 - Pool pellets in 0.25 M sucrose, .003 M Ca Cl₂; discard supernatant
 - Homogenize
 - Centrifuge 15 K, 15'
 - Freeze pelleted nuclei; decant

Day 2 Resuspend pelleted nuclei (100 ml, 0.14 M NaCl, 0.05 M Tris pH 7.5)
Homogenize (motor-driven homogenizer)
Stir 30 min.
Centrifuge 15 K, 15'
Resuspend in 100 ml (0.14 M NaCl, 0.05 M Tris pH 7.5); NSP I
(nuclear sap proteins-discard)
Homogenize
Stir 30'
Centrifuge 15 K, 15'
Resuspend 150 ml (0.14 M NaCl, 0.05 M Tris pH 7.5); NSP II
Stir 15'
Centrifuge 15 K, 15'
Nuclear pellet
Resuspend in 2 M NaCl 5 M urea 0.01 M Tris pH 7.5; NSP III
Homogenize
Stir 2 hr
Rehomogenize
Adjust OD to 10 OD₂₆₀ units/ml
Added PMSF to total conc. of 0.0001 M *
Stir overnight
Centrifuge 19 K, 1 hr.
Dialyze vs 13 vol H₂O made 0.1% with PMSF; discard pellet residue
Centrifuge 15 K, 30'
Resuspend pellets; Supernatant contains Chromatin acidic proteins
Homogenize
Stir 2 hrs
Rehomogenize
Stir overnight
Centrifuge 19K, 1 hr
Dialyze vs. 13 vol distilled water; pellet residue discard
Centrifuge 15K, 30'
2x Reconstituted Chromatin; Supernatant contains chromatin acidic
proteins—pool with above
Resuspend in 50 ml (buffer =
200 ml 5M NaCl
250 ml 10M urea
1 ml 0.5 M sodium phosphate pH 8

* PMSF Phenylmethyl Sulfonylfluoride Sigma P-7626

Adjust buffer to pH 8
 Homogenize
 Stir 2 hrs
 Sonicate 2 min
 Stir 10 min
 Histones

APPENDIX C *Drosophila Food*

1. BOIL: 3000 ml H₂O
2. WEIGH:
 300 g cornmeal
 76 g brewers yeast
 60 g agar
3. STIR IN: 800 ml H₂O and 400 ml molasses

Add the above mixture (2) & (3) to the boiling H₂O in (1). Let simmer for 10 min. Let cool for 10 min. Add 26 ml propionic acid & mix. Pour into vials and put into a refrigerator or 4°C chamber for cooling and storage. Sufficient for about 2 trays of vials with 20 ml per vial.

Rearing Drosophila for Large Chromosomes from Salivary Glands

1. Rear flies at 18°C
2. Daily add THICK suspension of baker's yeast (from packet or whatever) 1–2 drops to each vial.
 Keep yeast covered and refrigerated and as fresh as possible.

APPENDIX D *Patterns for the DNA Kit*

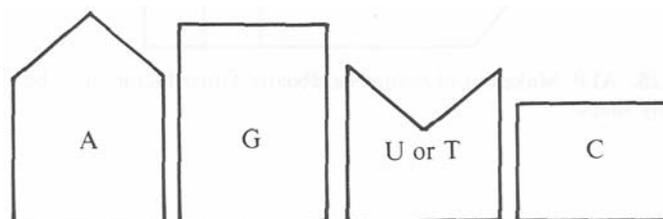


Figure 12.22. Patterns for nucleotides.

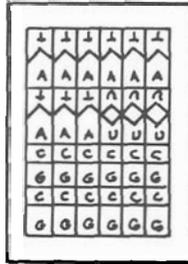


Figure 12.23. Arrangement of nucleotides on handout sheet for the students to cut out. Mimeograph these on blue paper (DNA) and green paper (RNA). Use pattern for nucleotides above, or enlarge all patterns by same proportion.

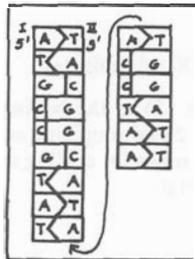


Figure 12.24. DNA mimeographed on white paper. Use pattern for nucleotides from above to draw proper size. Attach shorter piece to end of longer piece with tape.

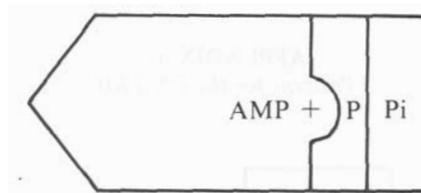


Figure 12.25. ATP. Make out of orange cardboard. Other factors may be of similar size and any shape.

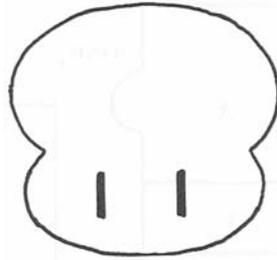


Figure 12.26. Ribosome. Make out of black cardboard. Widest dimension must be approximately 10X width of nucleotide in Figure 12.22.

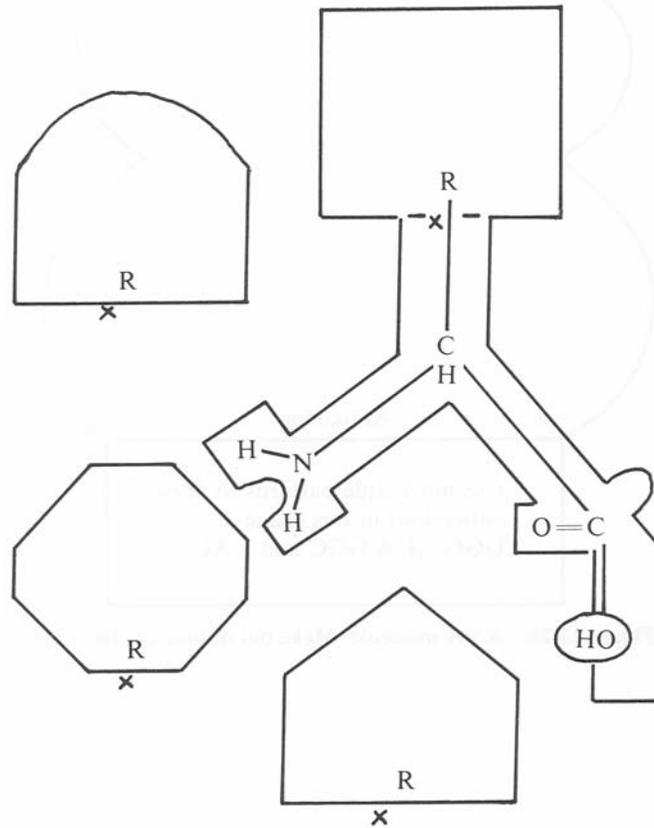


Figure 12.27. Amino acids. Mimeograph on yellow paper. Four different amino acids should be made and drawn separately on the yellow paper by substituting the three extra R groups for the square R group at point "X".

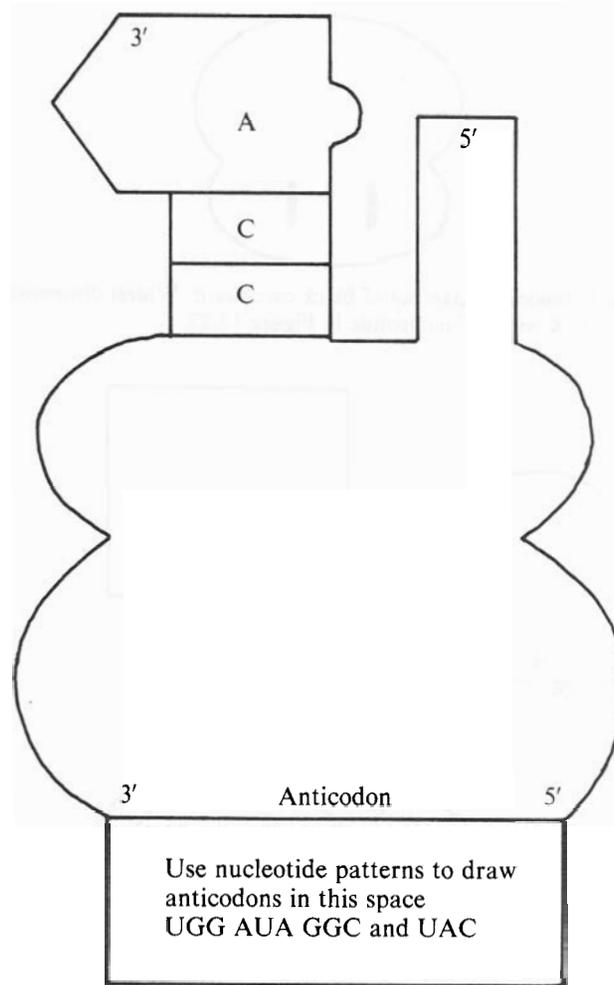
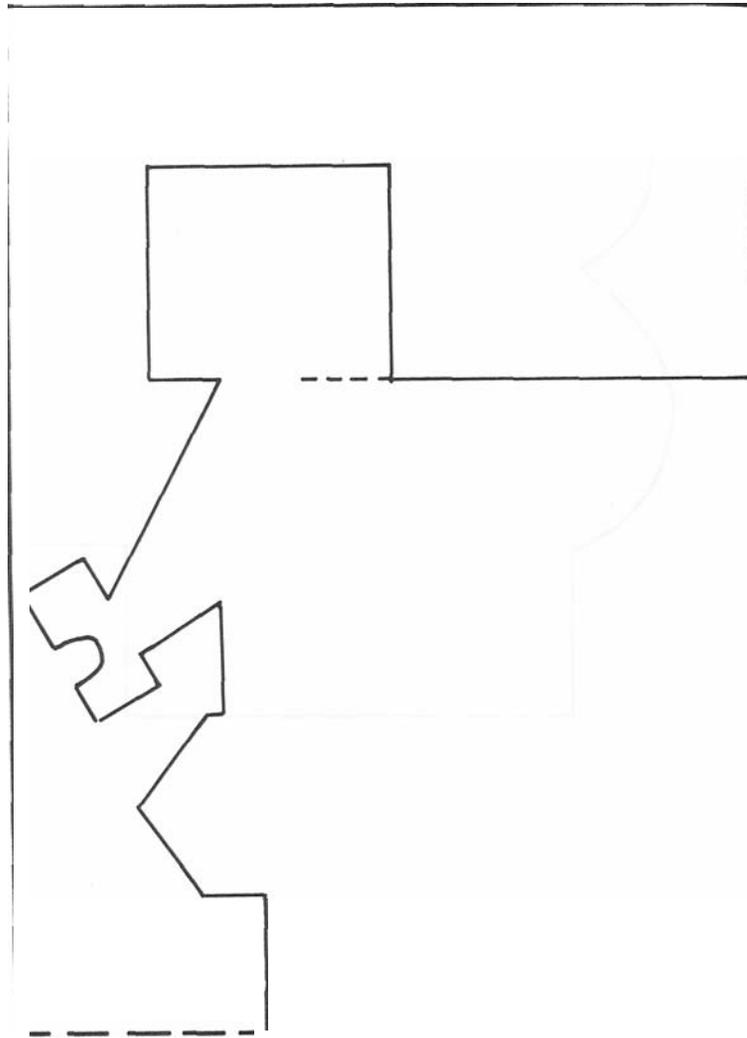


Figure 12.28. tRNA molecule. Make out of blue cardboard.



Attach to bottom half here

Figure 12.29a. Aminoacyl -tRNA synthetase enzyme (top half). Make out of green cardboard. Left side can be extended for more stability and easier handling.

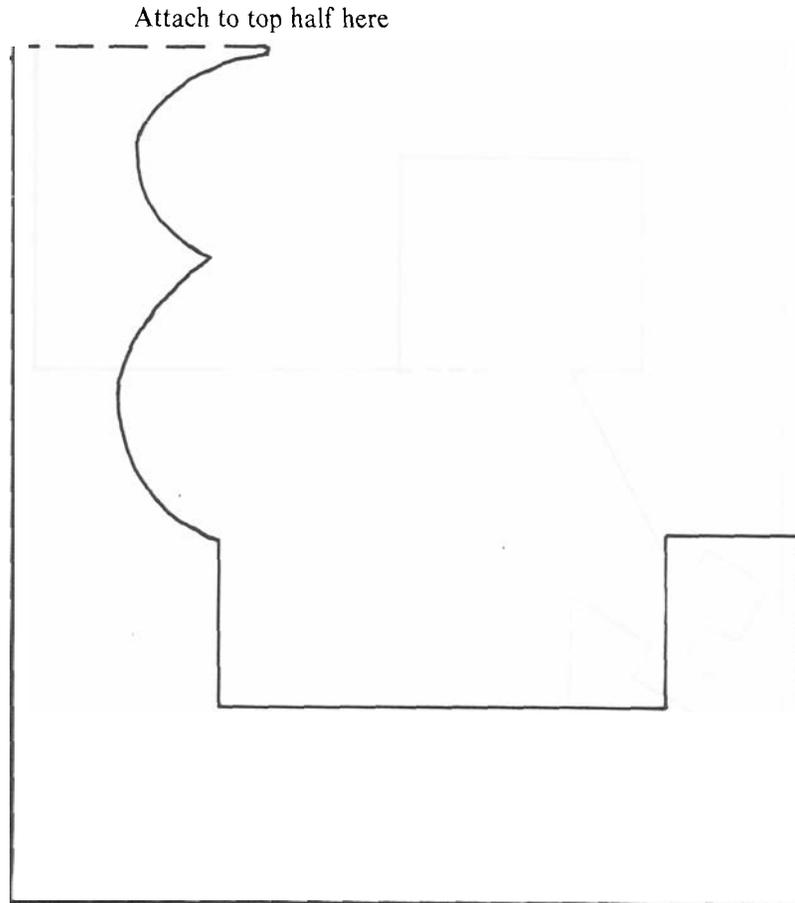


Figure 12.29b. (cont'd.) Aminoacyl-tRNA synthetase enzyme (bottom half). Make the entire enzyme as one piece even though the pattern must be put together first from parts a. and b.