Ch 8A Revamp

INTRO:

In this exercise, you will be investigating the role of ribonucleic acid (RNA) in the cell's central pathway for information transfer. This pathway is sometimes referred to as the Central Dogma of Molecular Biology. The dogma is shown in Figure 8-1 and has three steps: first is the replication of DNA, second is the transfer of information to an RNA transcript, and third is the use of this transcript to encode a specific polymer of amino acids. You will be performing two of these steps with your own hands in a single test tube! These two steps are called transcription and translation; transcription is the copying of a gene into an RNA molecule and translation is the conversion of this sequence of nucleotides to a sequence of amino acids for an encoded protein. Both of these processes involve the activity of RNA molecules.

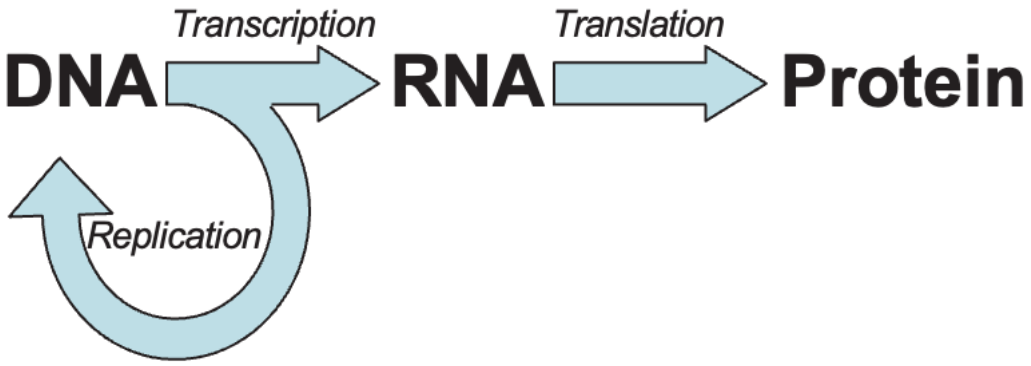


FIGURE 8-1

The Central Dogma of Molecular Biology. The arrows indicate the transfer of information among these three macromolecules, DNA, RNA, and protein. The names for each transfer process are in italics.

RNA is a very important macromolecule required for many of the steps involved in the gene expression process described above. One, messenger RNA (mRNA) carries the information for the synthesis of the enzymes and proteins that perform the work of the cell. Two, small nuclear RNA (snRNA) and/or micro RNA (miRNA) are used as components, along with many proteins, for the processing of these information molecules. Three, ribosomal RNA (rRNA) is used as a central part of the ribosome to translate mRNA into protein and catalyzes the peptide-bond formation. Four, transfer RNA (tRNA) is the key molecule for interpretation of the genetic code. A single mammalian cell contains about 10–50 pg (picograms; 10^{-12} g) of total RNA, 80–85% of which is rRNA. Most of the remaining 15–20% consists of a variety of low molecular weight species (tRNAs, snRNAs, miRNAs, etc.). These RNAs are abundant and of a defined size and sequence. They are easily isolated in virtually pure form by gel electrophoresis, density gradient centrifugation, ion exchange chromatography, or high-pressure liquid chromatography (HPLC). In contrast, mRNA, which comprises 1–2% of the total cellular RNA, is heterogeneous in both size (ranging from hundreds to thousands of bases in length) and sequence. However, most eukaryotic mRNAs carry a 3′-terminal tract of adenine residues, or polyadenylic acid. This is called the "3′-poly(A) tail." After isolation of total RNA, it is easy to purify the mRNA away from the other major RNA species by affinity chromatography (see Chapter 3) using oligo(dT)- cellulose. The resulting heterogeneous population of mRNA molecules (poly(A)$^+$ fraction) collectively encodes virtually all the polypeptides needed for that particular cell.

The amounts of mRNA for most genes in any given cell are often too small to isolate in quantities sufficient for study. Recombinant DNA methods have been devised to circumvent the isolation of specific mRNAs prior to their translation. Such proteins are instead made directly after the mRNA is made from the gene (*i.e.*, all in the same tube), which has been cloned into a plasmid vector (see Chapter 6). Such genes have been cloned into vectors that have the proper promoter sequences that will inform the transcription and translation of the cloned gene. These plasmids can be introduced into a reaction that contains all the necessary factors for transcription and protein synthesis, including an RNA polymerase to synthesize the specific mRNA corresponding to the cloned gene. This mRNA is immediately available for translation into a protein in the same reaction.

PROPERTIES OF RNA

Regardless of whether RNA is isolated or made in situ (a Latin phrase meaning "as it sits" or "in position"), it is necessary to minimize the activity of enzymes called ribonucleases, or RNases, that are liberated during cell lysis and are present on your hands. These enzymes can be inhibited or inactivated in three ways: one, by using compounds that react with the active site histidine present in most RNases; or two, by using methods that denature and wash away these enzymes; or three, by heating to very high temperatures (>200 °C), which destroys the polypeptide polymer. Due to the difficulty of inactivating RNases present in a biological sample, it is important to avoid the accidental introduction of trace amounts of RNases from other laboratory sources in the laboratory. These enzymes are ubiquitous and are found on your skin and in your breath.

Therefore, you should use gloves to protect your experiment from YOU, in addition to protecting you from your experiment! A number of precautions can be used

to avoid problems with RNases. The Appendix lists precautions to take when contamination with RNases is a problem.

IN VITRO TRANSLATION

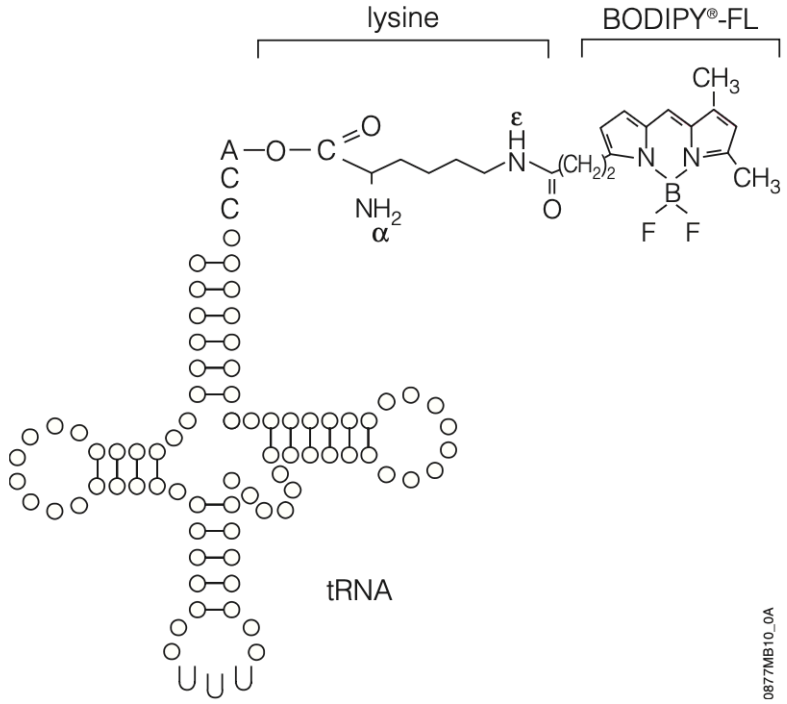
The synthesis of protein in vitro is a useful tool in biochemical and molecular biology research. Translation systems are used to rapidly characterize recombinant DNA clones, study mutations, examine translation signals, make radiolabeled protein for further study, and characterize mRNA populations. Two basic approaches are available: 1) in vitro systems programmed with RNA (translation only systems), or 2) those programmed with DNA (coupled transcription/translation systems). Many other factors go into the choice of an in vitro system. We will be using a coupled transcription/translation system to characterize the protein encoded in the plasmids you isolated in Chapter 6A.

In vitro transcription/translation systems are modified versions of in vitro translation systems, which are described in detail in entry #13 in the References section. The modification is the addition of all four nucleotide triphosphates (ATP, GTP, CTP, and UTP = NTPs) needed for RNA synthesis, and an appropriate RNA polymerase. The most common translation system for these coupled systems is wheat-germ extract. This extract is prepared by grinding wheat germ in an extraction buffer followed by centrifugation to remove cellular debris, which is sometimes called the "S30" fraction. This S30-supernatant fraction is then subjected to gel filtration chromatography to separate small molecules, such as endogenous amino acids and plant pigments, from the extract. What remains are the components needed for protein synthesis: ribosomes, tRNAs, aminoacyl-tRNA synthetases, and initiation/elongation/termination factors.

Like a similar translation system, the reticulocyte lysate, the extract is treated with micrococcal nuclease to destroy endogenous mRNAs and thus minimize background translation. In addition, the wheat germ extract is further optimized for mRNA translation by adding an energy generating system consisting of phosphocreatine kinase, phosphocreatine, a mixture of tRNAs to expand the range of mRNAs that can be translated, and potassium and magnesium acetates that are optimized for most exogenous mRNAs.

The RNA polymerases used in the coupled transcription/translation are usually from bacteriophage sources, such as T3, T7, or SP6 phage. These polymerases are used because their promoter sequences are unique and different from those used by Escherichia coli RNA polymerases or any eukaryotic RNA polymerases. Therefore, transcription will only start at sites that have these specific promoter sequences, which are easily incorporated into plasmid DNAs just upstream from the cloning sites for any open reading frame desired. The plasmids used in this experiment have this type of promoter sequence (see Chapter 6).

We will be measuring the incorporation of fluorescently labeled lysine, shown below coupled to tRNA:



tRNA graphic from https://www.promega.com/products/protein-detection/protein-labeling/fluorotect-greenlys-in-vitro-translation-labeling-system/?catNum=L5001#protocols

LEARNING GOALS:

- To use an in vitro transcription and translation system to synthesize proteins from genes cloned into a plasmid.
- Introduction to working with fluorescent compounds

IN YOUR PRELAB NOTEBOOK PAGES, INCLUDE:

- Everything normally required for the class, as well as
- A table to keep track of the color coding of the different reagent tubes
- A flowchart of the protocol

PROCEDURES

First Week (Parts A and B)

1. Use a combined RNA polymerase transcription system and a wheat germ lysate-translation system for the synthesis of proteins from genes cloned into a plasmid. [NOTE: the plasmids used are the same as those used in Chapter 6. If possible, students should use their own preparations]

Obtain aliquots of water, buffer, amino acids, RNasin, RNA polymerase, and Fluortect GreenLys tRNA. If frozen, these components can be thawed at room temperature and then stored on ice. Make sure they are thawed and in solution, then

immediately place on ice. Note: The TNT® Reaction Buffer may contain a precipitate after thawing on ice. Re-dissolve the precipitate by vortexing at room temperature for 30 sec. The RNA polymerase is in 50% glycerol and should not be frozen solid. Be sure to place all reagents on ice immediately.

TABLE IV

TNT® Wheat Germ Extract Reactions: using Fluortect Green$_{Lys}$ tRNA

Component	Vol. per reaction (μL)	Cocktail for 4 reactions (μL)
Nuclease-Free Water	6.3	28.4
TNT® Reaction Buffer	0.8	3.6
Amino Acid Mixture, 1 mM	0.4	1.8
RNasin® Ribonuclease Inhibitor (40 U/μL)	0.4	1.8
TNT® RNA Polymerase (SP6)	0.4	1.8
Fluortect Green$_{Lys}$ tRNA	0.8	3.6
TNT® Wheat Germ Extract	10	45
TOTAL Volume		86
Remove 5 µL (2 times) before aliquoting into reactions		-10
Aliquot 19 µL into each of 4 tubes	19	76

In four *amber or black* 0.5-mL microcentrifuge tubes (reaction tubes), add the appropriate negative control (water), positive control DNA, or experimental DNA samples. In a fifth reaction tube, assemble the reaction components in the order listed for 4 reactions (Table IV), such that you add the fluorescent tRNA last into the cocktail. Obtain the TNT® Wheat Germ Extract LAST. Thaw your aliquot by hand warming and then place on ice if not already done for you by your *Instructor*. Quantitatively transfer the correct amount of Wheat Germ Extract to the combined other components of the reaction 1–6. Gently mix. Each pair/subgroup of students will remove 5 μL of this reaction cocktail to a clean microcentrifuge tube and keep on ice until the rest of the samples are ready. A total of 10 μL will have been removed. Aliquot 19 μL of this reaction cocktail (now containing components 1-7) into each of four amber reaction tubes. You can add the last aliquot to the water (negative) control in case there isn't enough cocktail remaining. Cap each tube and gently mix the reaction by flicking with your finger. Incubate all four reactions at 30 °C for 90 min. Keep covered from light.

After the 90 min incubation, stop the reactions by putting the tubes on ice. While the reactions are still on ice, split them in half (remove 10 μL of each to a new tubes), one for each group. Now each group should have their own reactions, including the 5-μL aliquot removed before mixing the cocktail with the DNA. Each group works separately henceforth.

Add 10 μL of 2x SDS-PAGE loading gel to each reaction, then store at -20 °C until the next week's lab.

SET UP AND MATERIALS:

WEEK ONE

Equipment:

3+	Pipetman P2 or P10 with tips
3+	Microcentrifuges, at 25 °C
1	**Incubator, 30° C (preferably)**
	OR
1	Water bath, 30° C, with shallow dish of water for tube rack lots
lots	Gloves
1/pr	Ice bucket
1/pr	◊Pipetman P20, P200 with tips for each

Glassware:

5/pr	amber or black microcentrifuge tubes, 0.5 mL with caps

Reagents:

50 μL/2 pairs	DEPC water: Treat with diethylpyrocarbonate by adding to 0.1% (1 mL) to 1 liter deionized water and incubate at 37 °C for 12 hr, then autoclave. Alternatively, this may be purchased (Promega; Nuclease-Free Water (Cat. #P1193)). Make ALIQUOTS and freeze.
4 μL/2 pairs	TNT® Reaction Buffer. Use from Promega Kit (L4130), but available separately (Cat. #L486A). ALIQUOT 4 μL for 2 pairs of students. Store frozen at <70 °C until use.
2 μL/2 pairs	TNT® Amino acid mixture, 50X. Use from Promega Kit (L4130), but available separately (Cat. #L9961). ALIQUOT 3 μL for 2 pairs of students. Store frozen at <70 °C until use.
3 μL/2 pairs	RNasin® Ribonuclease Inhibitor (Cat. #N2111 or N2511). ALIQUOT 3 μL for 2 pairs of students. Store frozen at <70 °C until use.
3 μL/2 pairs	TNT® SP6 RNA Polymerase. Use from Promega Kit (L4130), but available separately (Cat. #L515A). ALIQUOT 3 μL for 2 pairs of students. Store frozen at <70 °C until use.
4 μL/2 pairs	FluoroTect™ GreenLys in vitro Translation Labeling System (Cat. #L5001). ALIQUOT 3 μL for 2 pairs of students. Store frozen at <70 °C until use.
46 μL/2 pairs	TNT® Wheat Germ Extract: Use from Promega Kit (L4130), but available separately (Cat. #L4380). Each kit has enough reagents for 40 student pairs. The lysate contains buffer, tRNAs, ribosomes, and the energy system. Storage and Stability: Store all components at <70

	°C. The product is sensitive to CO_2 (avoid prolonged exposure) and multiple freeze-thaw cycles, which may have an adverse effect on activity/performance. Extracts are stable for at least 12 months if stored and handled properly. Except for the actual translation incubation, all handling of the extract components should be done at 4 °C or on ice. Any unused extract should be refrozen in an ethanol/dry ice bath as soon as possible after thawing to minimize loss of translational activity. Do not expose the extracts to more than two freeze-thaw cycles. ALIQUOT 50 μL for 2 pairs of students. Store frozen at <70 °C (on dry ice) until use.
1 μL/2 pairs	DNA control: Use from Promega Kit (L4130), but available separately (Cat. #L4741A). Kit only provides enough for 10 reactions (20 student pairs), the separate product (L4741) provides enough for 20 reactions. Both are provided at 0.5 μg/μL. ALIQUOT 1 μL for 2 pairs of students. Store frozen at <70 °C until use.
2 μL ea/2 pairs	DNA (unknowns): Dilute preparations of pGEM3-REL and pGEM4-REL to 0.5 μg/μL with 50 μL DEPC water. The DNA preparations from Chapter 6 are sufficiently purified to work in this assay, although the concentration might not be sufficient (>200 ng/μL). Other purification methods can be used, such as DNA that is prepared using the Wizard® Plus SV DNA Isolation System (Cat. #A1330) or by the standard alkaline-lysis method described by Sambrook et al. (2001). ALIQUOT ~2 μL of each plasmid for 2 pairs of students. Store frozen at -20 °C until use.
2-3 sq. ft/pair	Aluminum foil