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**STRATEGIES
FOR TEACHING DNA EFFECTIVELY
IN A SECONDARY HONORS BIOLOGY CLASS**

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STRATEGIES
FOR TEACHING DNA EFFECTIVELY
IN A SECONDARY HONORS BIOLOGY CLASS

By

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ABSTRACT

STRATEGIES
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by

Charles K. Sharp

Although our understanding of the nature and importance of DNA began in 1953 (37 years ago), it is only recently that students in secondary schools have been able to work with the new biotechnology that has developed around this understanding.

This thesis proposes an approach to teaching an Honors Biology class that centers as its final goal on a more thorough comprehension by the student of the nature and role of DNA in the cell.

Significant laboratory exercises illustrating the new biotechnologies are included as a central focus to aid in discussion and conceptualization.

This work is dedicated to my wife,

Marilyn Sharp

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INTRODUCTION.

There is probably no more important biological knowledge that a high school student of today must have than a basic understanding of the DNA molecule, its role in the cell, and its role in making an organism uniquely what it is.

Recently scientists have begun to sequence the entire human genome in an effort to understand the complexity of the human organism. This is a project that, it is estimated, will take fifteen years by a large number of dedicated scientists working in laboratories throughout the United States and the rest of the world. (1) The implications for the human race are so immense as to be unimaginable.

If the human genome project is accomplished, it will mean the probability of not only a complete understanding of human inheritance, but also a deeper understanding of many of the hereditary diseases of mankind. Scientists will be able to understand defective enzymes and polypeptides well enough to be able to treat their adverse effects and perhaps someday to insert corrective genes into affected tissues and bring about their expression.

New techniques for the analysis of DNA will be needed; an army of scientists and peripheral workers will be necessary to accomplish this task. Our schools will have to provide those young people who have a basic training in the biotechnology of DNA analysis. They will be needed to contribute not only to this immense task, but also to the many applications of genetic manipulation in knowing and understanding the nature of every kind of living thing.

We will need young men and women, too, who will enter the field of plant and livestock genetics to improve food production. In addition, scientists in pharmacology and health medicine will require a disciplined knowledge of DNA technology.

It is important for the teacher to understand that even though these young men and women may not now choose the field of biotechnology many of them will be involved directly or indirectly in what is becoming a major commercial, industrial, agricultural and social influence in our society.

It will be important, as well, to educate young people to the nature of the genetic options from which they might have to choose as they raise families and provide for their care (2,3)

The new technology requires that an educated person not only have a familiarity with the nature of DNA, but also a familiarity with the techniques used to manipulate and analyze it.

Laboratory exercises involving biotechnology which high school students can perform have been slow in coming. Few veteran teachers have a familiarity with the methods and procedures. Beginning teachers have difficulty introducing what seem to be new and radical concepts into the high school. Even fewer teachers understand that by using simple and inexpensive materials, many of which are already in place in the biology laboratory, students can perform and experience some of the more sophisticated tasks of biotechnology.

The school in this study, Holy Name High School, is a Catholic parochial school of about 1300 students in the diocese of Cleveland, Ohio. Students are drawn from a number of Catholic elementary schools in Cleveland and surrounding suburbs. Student backgrounds are

heterogeneous and repesent all social and intellectual facets of the community. Students are not selected on the basis of intellectual superiority. There are special classes for students determined to be learning disabled and honors classes for those of high ability who choose to take them. Most of the students would be classified as "average".

The classes in which the labs presented in this thesis were used included forty-three tenth grade secondary students and seven ninth grade students in two Honors Biology groups. Students were selected on the basis of their academic grade point average: 3.2 or better. They were then given an "invitation" to take the Honors Biology class which required parental permission and a request submitted to the counselor before acceptance into the course.

There is no freshman general science course in this school. It was known and understood from previous experience as well as a questionaire given this year that at least 90 per cent of the students had little or no background in science. Those with some background had, in elementary school, dissected invertebrates or primitive vertebrates or had made leaf collections. Some few had studied cells and two or three had a rudimentary knowledge of DNA.

Because of such varied and, in most cases, limited backgrounds, the entire first quarter is spent in teaching scientific notation, dimensional analysis, the metric system, the concept of the atom, and basic chemistry. So the entire first quarter is spent in preparing the students for the regular course of studies.

The text book used in the course is Biology by Wessells and Hopson and is accompanied by a study guide. (See No. 9 in the bibliography.)

The classes were taught using what might be called a modified conceptual method (4, 5), i. e. the students were led to concepts through questions put forth by the teacher. This was most frequently the manner in which new subject matter was introduced, without prior reference to the text. Text assignments would be given, following the introduction of the basic concepts, and the learning experience was subsequently filled in with necessary terminology and more precise details. Sometimes, but only occasionally, a lecture method was used.

Periods are 40 minutes long five days a week and there are double periods on Monday and Wednesday that may be used for laboratory work. Laboratory exercises, however, sometimes ran for three or four sequential days through both single and double periods.

These would be the kinds of laboratory exercises, for example, involving measurements in learning the metric system where time was not a critical factor. Or in some laboratory exercises where enzymes were studied or bacteria were transformed, the materials could be placed in the refrigerator overnight at specific stages and work completed on the following day. In labs involving the growth of already transformed bacteria, the bacteria were placed in incubators overnight and observed the next day. In the case of gel electrophoresis, materials had to be kept overnight in the refrigerator for completion of the staining process.

This thesis is an attempt to present understandable and "do-able" laboratory exercises. It is the hope that through the use of already adapted and developed laboratory experiences and through the modification and development of others that the technology can be experienced and the biochemistry understood.

CHAPTER ONE

GENERAL STATEMENT OF A PROBLEM.

The subject to be taught, DNA, is really a year long project. No single unit can cover the complexities or the implications of DNA. It is knowledge that must be introduced with a basic simplicity, reinforced and reinforced again, and more knowledge added. There are at least four areas, widely separated, to which the study of and the applications to DNA apply:

1. In the basic introductory lesson on proteins, carbohydrates, lipids and nucleic acids.
2. In the study of Prokaryotes including viruses and plasmids.
3. In the study of Gametogenesis and the Cell Cycle.
4. Finally and most intensively in the study of Molecular Genetics.

It is the purpose of this thesis not to present a single module or isolated block of teaching, but rather to show an application to the four areas of study.

The course that is taught is by design a course in cellular and subcellular biology and therefore its main concern is for an understanding of molecular events within the cell. And even though all aspects will be included in this thesis, it should be pointed out that the basics of nucleic acids, carbohydrates, proteins and lipids must be constantly reinforced. Through this continual reinforcement, effective understanding can result: the students will develop a sense of the cell

as a highly regulated entity in which are found the biochemical interactions that cause sickness and health, life and death.

Most recently M. Mitchell Waldrop wrote about "spontaneous self-organization"; cellular life (Which, after all, is all life.) defies the Second Law of Thermodynamics. Dynamical systems such as the cell do evolve from disorder into order, from chaos into organization at an extremely complex level. Cellular systems do "unscramble the egg". (6)

Those of us who still cling to the idea of a "vitalism" hidden somewhere in the forces of nature, perceive in this mystery a beauty which no simple "mechanistics" can resolve. The paradox is that life, itself, opposes nature, while obeying all her edicts: energy levels, universal forces, and all other physical rules which must be obeyed.

And finally before I turn to the business at hand, it must be said that students need to be reminded that there is a beauty in all of these events that can be perceived. There is in me a reverential awe that seems to grow exponentially with time for these highly integrated cellular events. This thesis describes the manner in which I feel DNA in its major roles is to be taught.

THE APPLICATION

1. The basic studies should begin with an understanding of biochemical molecules: carbohydrates, lipids, proteins and nucleic acids. In this area we study the structures of the pentose sugars through carbohydrates. The primary structure of proteins leads to the simple statement that genes or DNA determine the primary structure. The structure of the nucleic acid is determined early on by the sequence of nucleotides. In this area students first learn of the nitrogenous bases, the phosphates and the pentose sugars. Here, also, they learn some basic differences between DNA and RNA: Thymine in DNA and Uracil in RNA and the differences in the pentose sugars. (7,8)

2. In the study of Prokaryotes, including the study of viruses, the students will study plasmids and will be introduced to the concept: DNA (transcription) ---->RNA(translation)---->Protein. There will be a major reinforcement of the previous work on DNA. Plasmids contain the message for the proteins and enzymes, i. e. their amino acid sequence such as for beta lactamase that destroys the antibiotic, ampicillin. At this point a lab exercise is introduced that demonstrates the transfer of plasmids and the transformation of a species of E. coli. Specific plasmids are mentioned: pBR 322, pUC 18, pAMP, and others. Genetic engineering, restriction enzymes, cloning, DNA replication are major areas of discussion and consideration. (9 - 15)

3. Gametogenesis and the cell cycle require an understanding of the formation of chromosomes from chromatin, replication, nucleosomes, crossing over, prophase I -which includes the understanding that during

this phase transcription stops and translation is reduced to 25 % of its original activity. Transcription and translation have not as yet been introduced. Much has been inferred and alluded to, but nothing has actually been defined in exact terms. We are preparing students through all of this for the "final act" which is the full explanation of transcription and translation. Perhaps the best understanding and reinforcement can occur when students build models of double stranded DNA. These models, as part of a lab and take-home exercise demonstrate 3′ and 5′ orientation of the sugars, double and triple hydrogen bonds, a close approximation of the molecular structures of the bases, the sugars and the phosphates. (16 - 18)

4. Genetics and Molecular Genetics. After the basics of inheritance which includes major Mendelian principles and principles of sex influence, sex linked genes, sex limited genes, incomplete and non-dominance, we can then turn to molecular genetics and all of the beautiful and highly organized simplicities of transcription and translation. Students will construct with construction paper and tag board models of transcription and translation. (9)

PROGRAM GOALS

Understanding biology in this decade also requires that students have an awareness and working knowledge of biotechnology. It is requisite that the teacher also have a background in molecular biology to teach it. Likewise, it is important for his students to be able to experience the biotechnology of molecular biology through laboratory experience. Much of his future is going to be influenced by this new branch of biology which began about 1939. (19)

The content of this new science has been taught in secondary schools for the last 20 or so years, but laboratory exercises relating to it have lagged far behind. Introducing the laboratory experiences will familiarize students with several aspects of working with DNA in the laboratory.

This thesis introduces new, simple laboratory exercises with DNA which will enable both the teacher and the student to develop self-confidence and understanding.

SUBJECT OBJECTIVES

1. Background preparation for the teaching.

a. It is important for teachers to develop a freedom and an intimacy in dealing with DNA, restriction enzymes, plasmids, lambda DNA, and gel electrophoresis.

b. It is important to be able to work with DNA in its many forms through simple, easily understood lab experiences.

2. Subject objectives for the students.

a. Students will appreciate the historical development of thoughts that lead to scientific truth and discovery.

b. Students will understand that the scientific world is a "real" world of people and personalities that are stubborn, selfish, generous, egotistic, ambitious, thoughtful, religious, atheistic; containing all the qualities of human beings.

c. Students will describe a DNA molecule, accurately.

d. Students will have an understanding of plasmids, bacteriophages, restriction enzymes, genetic engineering, transcription and translation.

e. Students will make, load and run agarose gel to do electrophoretic studies of lambda DNA, restricted lambda DNA, plasmids, and restriction digests of plasmids.

f. Students will make methylene blue staining solution and employ the correct technique for staining gels.

g. Students will understand how to isolate and culture bacteria for study.

h. Students will perform the laboratory procedures for the isolation of plasmids from bacterial cultures that are in mid-log growth.

i. Students will use, with ease, the spectrophotometer, Bausch and Lomb - Spec 20, and to use it for determining mid-log bacterial growth.

3. Concepts to be Presented.

a. Perhaps the most important concept is that the student be able to visualize the DNA molecule through the construction of a model of the molecule.

b. The cell is the unit of life for most living things. (Viruses, viroids, prions, are these exceptions? The existence of these complex forms of life should be emphasized without providing answers.)

c. DNA is the molecule that provides the information to the molecules that cause life.

d. Life, sickness, health, death are subcellular events involving change to the stability of regular molecular structures within the cell.

e. Subcellular events involve interactions between molecules within cells. (Examples are the Embden-Meyerhoff pathway, Krebs Cycle and Oxidative Phosphorylation.)

f. The shape of an organism is dependent upon the molecules produced by the genes (DNA) within the cell.(At this point I initiate such questions as: Why are dogs, for example, shaped differently from cows or humans or trees or marigolds? Their cells are much the same and do most of the same things, i. e. "e", above, so their enzymes are

generally the same (at least have the same active sites, although they may have evolved slightly different "non-critical parts".) The only thing that is essentially different is their DNA. So the question must follow: What is different about their DNA? And the answer must be the sequence of bases in the DNA molecule.)

g. How do we know that there are differences and similarities in the DNA? We have to be able to look at the DNA of different organisms and compare the differences in the sequences of the bases. How are we able to see the differences in the sequencing of bases? The most commonly used tool is gel electrophoresis.

h. The concept of agarose gel electrophoresis as a tool to separate different amounts of DNA and to see the presence of DNA by simple staining of the gel is important. Here a lab involving lambda DNA can be introduced so students can see the DNA for themselves. Here is the DNA, now, how do we find its sequence?

i. The sequencing of DNA is too complicated a procedure for high school or even undergraduate courses in college, but the concept of restriction enzymes can be introduced and the manner in which they work, like molecular scissors, to hydrolyze DNA at predictable places into pieces. Here, too, the nomenclature for restriction enzymes can be introduced with several examples such as EcoR1, Hind III, and BamH1 along with their cleavage sites.

j. Now the study of viruses, most notably lambda phage must be introduced. Viruses may be grown as a lab exercise to demonstrate their existence. Also, the structure of lambda DNA which is understood can now be stated. And the concept of how viruses attack and invade

bacterial cells and are reproduced can be described. This can be related to the common cold, the flu and other viral diseases.

k. The understanding and study of lambda DNA continues with the study of the effect of restriction enzymes, EcoR1 and BamH1, on lambda DNA. A simple laboratory exercise demonstrates this plainly by having the students prepare restriction digests and electrophoresing them on agarose gels. The student should learn how to use restriction digests and to make restriction maps after they have measured the distances travelled by each of the bands. Here comparisons of bands in lanes from single and double digests can be shown as well as the ligated, circular form of the phage lambda DNA.

l. The concept of cloning would come next. Teaching this concept involves an explanation of the use of DNA in cloning experiments in plants. Here, as well, one might describe the use of the plasmid from the bacterium Agrobacter tumifaciens (This bacterium causes crown gall in plants.) as a cloning vector to introduce cloned genes of choice into plants whose characteristics one wishes to alter through those genes.

m. To continue the concept of cloning and genetic engineering I use a laboratory exercise involving "natural" genetic engineering. Two strains of E. coli are grown each of which has a resistance to a different antibiotic. One of the bacteria possesses the "F" or fertility plasmid, the other does not. In this very workable lab exercise bacteria are produced which have immunities to both antibiotics. We call these "mutant" strains.

n. In discussing the events of the aforementioned lab exercise, students become more familiar with plasmids by reading the

the Scientific American article, Plasmids, (15) which clearly states how plasmids behave in bacterial cells. Using this exercise along with the previous work gives students a good understanding of these "endosymbionts".

o. Now we introduce a simple laboratory exercise in which we isolate plasmids from bacterial cultures which are in the mid-log stage of growth. Existence of plasmids can be shown through gel electrophoresis. Those students who wish to pursue the subject further, can restrict the plasmids and subject them to electrophoresis.

p. There is at least one workable exercise that will enable us to clone into an ampicillin resistant plasmid a gene for kanamycin resistance. The opportunity to do this work should be available to any student who desires to do further laboratory research.

q. To further continue our study of plasmids, students may introduce ampicillin resistant plasmids containing the Lac Z gene into E. coli strains made competent through treatment with cold calcium chloride and heat shock. Bacteria would be grown on medium containing IPTG (isopropylthiogalactoside) and X-Gal (5-bromo-4-chloro-3-indolyl-betaD galactoside). The colonies can be easily identified because those containing the plasmid will appear as blue dots on the agar plates. (20, 21)

4. Applications of Biotechnology of DNA in the World Today

a. Present day genetic engineering of plants and animals:

(1) The development of transgenic mice to study cancer causing genes (oncogenes) and the aids virus.

(2) The development of transgenic strains of cattle, pigs, sheep, rabbits and chickens in order to produce more desirable food products. (22)

(3) The development of genetically engineered strains of various agricultural plants to modify and improve crops. (23)

b. The understanding of, control of and possible alteration of human gene expression.

(1) This means, someday, being able to introduce healthy genes into the cells of individuals who have defective genes and causing those healthy genes to be expressed.

(2) It also means the future possibility of being able to prevent the expression of genes for degenerative diseases such as Alzheimer's and Huntington's Chorea.

(3) It means being able to control and eventually cure diseases which are now incurable: the common cold and influenza will eventually be curable.

d. Through our understanding of viruses and DNA we can now immunize the population against various diseases:

(1) Immunizations can be obtained against such diseases as smallpox, measles, three forms of polio, some influenza viruses, and other diseases. Immunity can be obtained against some new and mutant forms of influenza viruses almost as soon as they develop.

e. It also means jobs in the future:

(1) The biotechnology industry has grown extremely rapidly and is taking all of the trained technicians and scientists which are available.

CHAPTER TWO

A SYNOPSIS OF THE MAJOR HISTORICAL SCIENTIFIC DEVELOPMENTS THAT LED TO OUR PRESENT UNDERSTANDING OF DNA.

No work written today better or more clearly documents the history of the development of our understanding of DNA than Horace Judson's work The Eighth Day of Creation. Therefore, apart from past information obtained from general references, and in the interest of accuracy, I have chosen Mr. Judson's book as a primary source for the chronology, the events and the quotations.

1868. Johann Friedrich Miescher, with whom the history seriously begins, went to Tuebingen, to study with Ernst Felix Hoppe-Seyler (The man who named hemoglobin.) . Miescher was interested in studying the nucleus of cells. He began by studying white blood cells which he collected from pus from discarded surgical bandages. He discovered in the isolated nuclei an acidic substance rich in phosphorus, and made up of very large molecules. He named this substance in 1869 and called it NUCLEIN.

The following year he began to study salmon sperm for nuclein and a student of his, Richard Altmann, introduced the term NUCLEIC ACID.

Remarkably in 1892, Miescher pointed out in a letter to an uncle that "some of the large molecules encountered in biology, composed of a repetition of a few similar but not identical small chemical pieces, could express all the rich variety of the hereditary message, just as the words and concepts of all languages can find expression in twenty-four to thirty letters of the alphabet."*

*Judson, Horace Freeland, The Eighth Day of Creation, (New York: Simon and Schuster, 1979), p. 28.

Much of the elementary chemistry of nucleic acids was done by Miescher and his students and by other laboratories at that time. From the discovery of Guanine in 1844, eventually all of the bases were isolated by 1900 and by the 1920's, it was known that there were two kinds of nucleic acids, deoxyribose nucleic acid and ribonucleic acid.

In 1928, Fred Griffith discovered two strains of a bacterium, Streptococcus pneumoniae, one of which was lethal to mice and another which was non-virulent. He labelled them type II or R (R stands for rough surface.) which was non-virulent and type III or S (S stands for smooth surface...this form was enclosed in a capsule.). Type III was virulent and caused pneumonia in mice and their subsequent death.

When he injected mice with live Type II (R form) and dead Type III (S form), virulent strains of Streptococcus pneumoniae were produced which killed the mice from pneumonia.

Griffith's work was really the first time that transformation of bacterial cells was described. DNA from one form, which in this case was the dead "S" form, is able to enter a living form which was non-virulent and transform it into a virulent strain.

The transformation of bacteria was confirmed in 1929.

1944. Oswald Avery, Colin MacLeod, and Maclyn McCarty at the Rockefeller Institute in New York published on of the first papers describing the transformation of bacteria. "R" forms of Streptococcus pneumoniae which are harmless mutants that do not cause pneumonia, were transformed into "S" forms, which are virulent strains and cause pneumonia, by mixing them with the purified DNA extracted from killed "S" forms. Basing their work on the originial work of Fred Griffith they

determined that the "transforming material"..contained no demonstrable protein, lipid or polysaccharide, but was a highly polymerized, viscous form of DNA. (24)

The work of Avery, MacLeod and McCarty took many years. Avery's work was subtle and exhaustive and ingenious. He noted that DNA was both Autocatalytic and Heterocatalytic. In a letter to a brother he noted: " If we are right, and of course that's not yet proven, then it means that nucleic acids are not merely structurally important but functionally active substances in determining the biochemical activities and specific characteristics of cells -& that by means of a known chemical substance it is possible to induce predictable and hereditary changes in cells.....Sounds like a virus - may be a gene."*

1951- 52. Alfred Hershey and Martha Chase determined that it was the DNA of the bacteriophage that contained the genetic component for which everyone was then searching. They grew viruses on bacterial lawns containing radioactive phosphorus, 32P. Since phosphorus is present in DNA and not in proteins, the radioactive phosphorus would indicate the presence of DNA. They also used radioactive sulfur, 35S as a marker to determine the presence of protein. They infected E. coli with phage particles grown in bacterial cells containing 35S and 32P.

Since protein contains sulfur and no phosphorus, it then became a relatively simple matter to determine where the protein was and where the DNA was. The DNA would be present where the radioactive phosphorus appeared and the protein would be present where the radioactive sulfur appeared.

The experiment which they performed is referred to as the "Waring Blender" experiment. They observed that the electron micrograph of the

*Ibid, P. 39.

phages on the outside of the bacterial walls appeared to be empty. Their problem was to separate the bacteria from the viral fragments on the outside of the bacterial wall and determine which of the radioactive isotopes were present and where they were: in the bacteria or in the viral wall.

They took a chilled suspension of bacterial cells recently infected with a phage virus and placed it in a Waring blender to separate the viral shells from the bacterial walls by blending. They then centrifuged the mixture to pull down the bacterial cells which would be heavier and separate them from the bacteriophages that were present on the surface of the bacterial cell. The bacteriophages remained in suspension.

The question had to be, were the DNA and the protein together? Salvadore Luria had just proposed that the phage protein had the genetic role and that the DNA was only added in the last step before the cell burst.

Hershey and Chase discovered that the radioactive sulfur remained in the supernatant liquid with the viral shells (protein) and that the DNA was present in the bacterial cells, which were in the centrifugate. The virus then attached itself to the outside of the bacterium and injected its DNA into the cell. Their modest conclusion inferred that sulfur containing protein had no function in phage replication and that "DNA had some function." (25)

This proved conclusively the work begun by Avery that the transforming principle was DNA, that DNA was the actual genetic material and protein was not.

Now the scientific community began to realize that the important discovery of the future was to be the discovery of the structure of DNA and eventually the manner in which DNA carried out its genetic activity.

1953. James D. Watson, F. H. C. Crick and Maurice Wilkins determine the structure of the beta form of the DNA double helix. The paper, a single page, was published in May of 1953 in Nature magazine.(18) This is the most revolutionary work of modern science and yet without a lot of luck and all of the work that preceded it, it would have been impossible.

1940. Now the work of Beadle and Tatum from 10 years previous to the discovery of Watson and Crick took shape in the minds of the scientific community. Using a common bread mold, Neurospora crassa, they determined that it was the undertaking of a gene to make an enzyme. Recalling this classical series of experiments made it apparent that the DNA in some way made enzymes.

It took the work of the 50's following the discovery of the structure of DNA by Watson and Crick to discover how the gene produced enzymes. Even though there were many previous hints even as early as the early 40's: the research of Torbjorn Caspersson and the Karolinska Institute in Stockholm and Jean Brachet in Paris. Their work indicated that" vigorous protein synthesis was always associated with plentiful RNA. And further: this RNA was located in the cytoplasm, while DNA was confined to the cell nucleus."*

In 1950 it was proposed by two bacteriologists in Strasbourg, Andre Boivin and Roger Venrely that DNA makes RNA makes protein.

It must be noted here, too, that a major problem was the development of the energetics to drive the expression of the gene. For

*Ibid, p. 236.

this Fritz Lipmann must be credited with his development of the understanding of the role of ATP in taking the energy out of the metabolic flow and conducting it to where it was needed to produce reactions.

Judson's account of the stimulating and restless period of the 50's reveals the names of many scientists who contributed to this exciting era. Alexander Dounce at the University of Rochester suggested that RNA ought to be the template for proteins and that the RNA originated on the template of the DNA gene. George Gamow, the Cosmologist responsible for the "Big Bang" theory, proposed a model for DNA synthesis that dominated the thoughts of many most especially Francis Crick's for several years.

But, according to Judson, it was Francis Crick, who through correspondence and conferences and personal diplomacy, directed and drove the research to discover the mechanisms of genetic action. It was Crick who deduced the role of transfer RNA; (He spoke at a symposium on Feb. 18, 1956, and said, "One possibility is that each amino acid is combined with its special 'adaptor'"...);* it was he who introduced the term degenerate, a term from quantum physics.

But many, many men published many, many research papers. Fred Sanger published the amino acid sequence for insulin in 1955. Vernon Ingram's chemical analysis of the peptides and eventually of the single amino acid differences between normal and sickle cell hemoglobin, developed the understanding of what was the fundamental role of the gene.

Ribosomes began to appear in electron micrographs. They were called microsomes and by the end of 1956 it was apparent that soluble RNA molecules were an intermediate in protein synthesis, picking up

*Ibid, P. 316.

activated amino acids, bringing them to the microsomes and depositing them there. Robert Holley identified the transfer RNA's, all of them, for which he won the Nobel prize in 1968. The structure of ribosomes were to be determined and proven as the site of protein synthesis. Richard Roberts named these microsomes, ribosomes, and demonstrated their activity at the Carnegie Institute of Washington.

But it was at the Fifth International Congress of Biochemistry that began on the 10th of August 1961 in Moscow that an American scientist from the National Institute of Health, Marshall Nirenberg, presented the final answer to the whole equation. His paper, "The Dependence of Cell-free Protein Synthesis in E. coli Upon Naturally Occurring or Synthetic Template RNA." was produced with the collaboration of his colleague, Heinrich Matthaei. He finally solved the coding problem and the exact role of messenger RNA. His lecture was poorly attended and lasted only about 15 minutes; the only scientists in attendance of note were: Matthew Meselson, Walter Gilbert and Alfred Tissieres. Nirenberg and Matthaei had synthesized a long chain of phenylalanine from polyuridylic acid and demonstrated that one or more uridylic acid residues coded for phenylalanine.

Judson states, "Their [Nirenberg's and Matthaei's] aim was Avery's too: to understand the specificity of the nucleic acids, by putting in some known thing to see what came out. Avery's proof that the transforming principle was DNA set the agenda for biology. Watson's and Crick's elucidation of the structure of DNA confirmed rather than concluded that agenda. But the questions that Avery had asked, Nirenberg and Mathaei began at last to answer." * Nirenberg was asked to

*Ibid, p. 481.

give a second reading of his paper in Moscow, this time to hundreds of scientists.

Following the revelation in Moscow, it was Har Gobind Khorana at the University of Wisconsin who finally elucidated the entire code.

Nirenberg, Holley and Khorana received the Nobel prize in physiology and medicine in 1968.

REFLECTION

There is one man who stands out for his imposing mentality as well as for his contribution to molecular biology. His name is Erwin Chargaff. He was an outstanding and highly disciplined scientist, a polemicist, a moralist, a prophet who forsaw dangers in genetic engineering, in tampering with nature. His words printed in a letter to Science magazine, "..the future will curse us for it" [He refers to the manipulation of heredity through transduction of foreign genes into organisms.]. But his important contribution to science was pivitol: he noted in 1950 that the ratio of Adenine to Thymine and Cytosine to Guanine, and purines to pyrimidines was about 1. His work was to shape the internal structure of the DNA molecule for Watson and Crick.

Francis Crick emerges in Judson's work as the imposing force, the scientist who coordinated and directed the efforts of other scientists.

Horace Judson writes of many of the major scientists of this period in molecular biology who, after significant discoveries go other ways, abandoning research for various things: writing, admininstration, supervision of graduate students and post docs. But Francis Crick continues to do science, albeit a new science.

Judson suggests that after the discovery of Watson and Crick, they wanted to abandon the project. They were lionized, in demand; yet somehow they were compelled to continue, to complete the task: to determine transcription and translation. They became bored with "DNA". Crick seems to have carried on with vigor to the end. Watson's efforts and contributions diminish considerably, although Watson now is in

charge of the Human Genome Project. Still, the work is administrative rather than bench science.

CHAPTER THREE

The laboratory exercises that accompany this work are presented in this chapter. These activities form the basis for classroom discussions from which proper conceptualization and understanding should occur.

Laboratory Exercise No. 1.

TRANSFORMATION OF E. COLI

Instructor's Guide.

Target Group.

Entry level, advanced and Honors biology students.

Time Frame.

The time commitment for the instructor is much longer than the time it takes for the class to do the lab. Preparing the materials is most time consuming. Student assistants can be used to assist in the mixing and sterilizing medium.

The time in preparation of the lab is about 3 - 5 hours. The time spent in actual lab work by the students depends upon how much time is allowed to prepare the students to do "sterile technique", and actual student time in doing the lab. Actual student time should be about 20 minutes.

Background Information for the Teacher.

The teacher needs to understand something of plasmids and plasmid transfer between bacteria. This exercise involves two strains of E. coli, called J53-R and Ht-99.

There is a plasmid in some bacteria called the "F" factor which enables the bacterium to form a conjugation tube (sex pilus) with

other bacteria in order to transfer plasmids from the one strain to another. Those bacteria possessing the "F" factor are termed "F+"; those not having the fertility factor are termed "F-".

In this instance, the teacher will work with a "male" or donor or "F+" strain of bacteria which contains a plasmid for the fertility factor. This bacterium also has, in addition, a plasmid (designated pBR322) with the gene that enables the bacterium to produce the enzyme that will chemically decompose the antibiotic, chloramphenicol. The bacterium is a strain called Ht 99, and has this specially "engineered" plasmid. It is engineered to be "male" and thus is able to donate its plasmid containing the chloramphenicol decomposing gene to another or recipient bacterium.

The students will also be working with the "female" or recipient bacterium, an "F-" strain, which possesses a plasmid (also designated pBR322) which enables it to be immune to an antibiotic, rifampicin. Its plasmid possesses the gene that enables it to produce the enzyme that will decompose the antibiotic rifampicin. Since it is an "F-" strain it does not possess a plasmid for the formation of a conjugation tube or sex pilus and so is not able to donate its plasmid to another bacterium.

Both strains of bacteria will be grown separately in a Luria broth nutrient medium, and when they have reached mid-log growth, a portion of each will be mixed together in the same kind of sterile nutrient broth in a separate flask. Some of the "F+" strains may then form conjugation tubes with the "F-" strains when placed together in the mixed culture and susequently produce a "mutant" strain with the characteristics of both parents. The formation of the sex pilus, which

will result in the transfer of plasmids, can be stimulated by frequent agitation or aeration with a simple aquarium pump.

Plasmid, pBR322, is a synthetic plasmid that has been constructed from several naturally occuring plasmids. It consists of, depending upon the genes which have been inserted, of about 4400 base pairs. In this laboratory exercise the two bacterial strains contain the plasmid, pBR322, but the plasmids in each are engineered slightly differently. One has the plasmid with the the gene for chloramphenicol resistance and the other has the gene for rifampicin resistance. (13, 14)

Preparation of Materials.

The teacher should prepare the agar plates beforehand and also mix the antibiotic solutions of chloramphenicol and rifampicin.

The preparation of the Antibiotics: (14)

1. Chloramphenicol: Dissolve 250 mg Chloramphenicol in a mixture of 5 mL of methanol and 5 mL of distilled water.

2. Take 1 mL of the solution in #1, and dissolve in 9 mL of methanol. THIS IS CALLED SOLUTION A.

3. Add 1 mL of solution A to 9 mL of sterile, distilled water. This solution, which is solution B, is mixed with cooling agar before the agar is poured into the pour plates.

The Preparation of Rifampicin.

1. Dissolve 250 mg of Rifampicin in 25 mL of methanol. THIS IS CALLED SOLUTION B.

2. Dissolve 1 mL of solution B in 9 mL of sterile distilled water. This solution, which is solution C, is mixed with the cooling agar to be poured into the petri dishes. N.B. (Nota bene, note well) the agar should be cooled to less than 60 deg. C. before the antibiotics are mixed with the agar.

Making the Agar Plates:

1. Dissolve three (3) grams of Nutrient Agar in 90 mL of distilled water and autoclave at 250 F for 15 min.

2. When the agar has cooled to just below 60 deg. C, add 10 mL of the chloramphenicol, solution B, AND UNDER STERILE CONDITIONS POUR CAREFULLY INTO PREVIOUSLY STERILIZED PETRI DISHES. (One can use sterile plastic disposable petri dishes to save time.)

3. The Rifampicin plates are made in the same way as the chloramphenicol plates, except that rifampicin solution C is poured into the cooling agar medium.

4. You will need to make plates containing both antibiotics to determine whether conjugation and transfer of plasmids will have taken place. Make them in the following manner:

a. One (1) mL of SOLUTION A (containing concentrated chloramphenicol) and one (1) mL of SOLUTION B (containing rifampicin in concentrated state.) are mixed with 8 mL of sterile, Dist. water.

b. The 10 mL aliquot described in 4a, is mixed with the cooling agar as previously described and subsequently poured into the pour plates.

5. Preparation of Bacterial Cultures. Obtain freeze-dried cultures of E. coli J53-R and Ht-99 from Kemtec.

a. Aseptically add approximately 0.5 ml of nutrient broth to each of the glass vials containing the lyophilized cultures. Shake the vials gently until the bacteria are suspended and the solution is cloudy.

b. Label one 250 ml Erlenmeyer flask containing sterile Luria broth J53-R and another Ht-99.

c. Transfer the contents of each vial, aseptically to the properly labelled 250 ml Erlenmeyer flask.

d. Incubate overnight in a 37 deg. C. water bath.

Preparation of the Mating Mixture.

1. Transfer, aseptically, 0.1 ml of the overnight culture of E. coli Ht-99 and 0.9 ml of the overnight culture of J53-R into a 250 ml Erlenmeyer flask containing sterile Luria broth.

2. Incubate with aeration or in a shaking 37 deg. C water bath for three to four hours.

3. Maintaining the Cultures. The bacterial cultures can be maintained in liquid suspension in the refrigerator for several days prior to usage.

Using one or two student "volunteer" assistants makes it possible to complete the preparations described above in a short time. Most of the time is spent waiting for the autoclave to come to temperature to complete the sterilization cycle and then cool down.

Problems Encountered.

1. It is necessary to demonstrate sterile technique to students beforehand.

2. Students may not complete sterile technique well and so some of the plates may be improperly inoculated.

3. You have to decide whether you want each student to inoculate the three plates or whether you want them to work in teams. This will determine how much medium you prepare and how many plates to pour.

Sources of Materials:

Kemtec has the "engineered" bacterial strains and "kits" that are already prepared. However, one can purchase the materials separately from them: Kemtec, 9889 Crescent Park Dr., West Chester, Ohio 45069, (1-513 777 3535) just outside of Cincinnati.

Background Information for the Student. The students will need to have the background information on plasmids and the two strains of E. coli mentioned in the Background Information for the Teacher. It will be usefull to the student to have available to him the Scientific American article from December 1980 entitled "Plasmids". It will be usefull to read at least the first four pages in class before doing the lab.

Goals.

The major thrust will be to introduce students to the concept of plasmids; this becomes a good introduction to recombinant DNA technology.

Performance Objectives.

1. The student will learn sterile technique in the inoculation of bacterial plates.

2. The student will observe, indirectly through cause and effect, the behavior of plasmids in bacteria and will experience the results of bacterial conjugation.

3. The student will observe the growth of normal and mutant bacterial strains. He will observe differences in their growth patterns and in their resistances to antibiotics.

4. The student will experience classic scientific methodology: simple and direct experimentation with controls and experimental parameters.

Materials.

Sterile petri dishes (100mm X 10mm or almost any size that is available.), three per team.

Chloramphenicol

Rifampicin

Nutrient Agar, Luria Broth Agar.

Luria Broth

Inoculating loops

Alcohol lamps

Sterile test tubes containing Luria Broth

Methanol

Sterile distilled water in test tubes.

Incubator, microbiological

Ht-99 bacteria, freeze dried.

J53-R bacteria, freeze dried.

Procedures.

1. Prepare petri dishes as previously indicated so that there are enough for three for each person or three per team. (It is better if each person does his own; this procedure provides more data, and gives better results.

2. Prepare bacterial cultures in Luria Broth at least two days before doing the lab. Using sterile technique, inoculate sterile Luria broth in a 25 ml Erlenmeyer flask with the J53-R strain of E. coli. Place in an incubator for 24 hrs. Culture the Ht-99 strain in the same way.

3. Prepare a mixed culture of J53-R and Ht-99 by mixing, using a sterile pipet and sterile technique, 0.9 mL of the J53-R with 0.1 mL of the Ht-99 culture. Incubate these for 24 hours.

4. It is suggested that you give each student or each team test tubes containing each of the three cultures, again using previously prepared sterile test tubes with aluminum foil caps and containing Luria Broth. These can be inoculated with the original cultures of Ht-99, J53-R and the mixed culture, respectively, with sterile, disposable pipets.

5. Students should obtain the three cultures and inoculate them in the following manner:

a. The rifampicin containing pour plates can be inoculated on the left side of the plate with the J53-R strain, making a large "R" with the inoculating loop, being careful not to scratch the agar in the dish.

b. The same plates can be inoculated on the right side with the Ht-99 strain, making a large "H" with the inoculating loop, again being careful not to scratch the agar in the dish.

c. The same should be done with the chloramphenicol plates and in the same manner by dividing the plate in half and inoculating each side with a different strain using the same letters, "R" and "H".

d. The plates containing the mixed antibiotics, i. e. both the chloramphenicol and rifampicin, should be divided into thirds. One of the three sections should be inoculated with the J53-R strain making a large "R", another of the three sections with the Ht-99, making a large "H", the third section of the dish should be inoculated with the mixed strain using a large "M".

6. The petri dishes are now placed upside-down in an incubator overnight.

Results.

1. Each student or team reads his own plates and records his data on a chart on the board. He should indicate with a (+) where there

is growth of bacteria and what kind of bacteria, and with a (-) where there is no growth. Each student copies down the class results into his lab book.

2. The class should then discuss and evaluate their results.

The Preparation of the Laboratory Report.

The student will prepare a laboratory report which will contain the following:

1. Purpose. The student will prepare a single sentence statement describing the purpose of the laboratory exercise.

2. Introduction. He will draw his introductory material from the December 1980 Scientific American article by Robert Novick on Plasmids and from other background material supplied by the teacher.. He will write a thorough explanation of plasmids concentrating on conjugation and the transfer of plasmids from one bacterium to another.

3. Procedures. The students will indicate the followed steps in carrying out the experiment.

4. Data. The student will prepare a data table for his results and for the class results.

5. Graph. In this lab the student will prepare a bar graph of the per cent of positive growth obtained in the cultures. The graph is to be done as both hand drawn and computer drawn.

6. Results and Conclusion. The student will be asked to draw conclusions from the experiment. He will answer such questions as: Was

the experiment successful? What did it prove? Did the bacterial growth demonstrate the presence of plasmids? How? Were there errors or mistakes in the experiment? What were they? Why do you think they occurred? Were the errors sufficient to say the experiment did not succeed in proving what it set out to do?

LABORATORY EXERCISE NO. 2.

COLONY TRANSFORMATION

Target Group.

This laboratory exercise is designed for advanced placement or honors biology students.

Instructors Guide.

1. Background Information.

The purpose of this laboratory exercise will be to transform bacteria, which would be killed by the antibiotic ampicillin, to ampicillin resistant strains. Students will attempt to do this by making bacteria "competent" to receive plasmid and subsequently introducing those plasmids into bacterial suspensions allowing the bacteria present to "take them in". These suspensions will be inoculated onto agar plates containing ampicillin and should produce visible colony growth.

The control will be bacterial suspensions which have not been inoculated with plasmids. These are "untransformed" strains that also will be inoculated on agar plates containing ampicillin; these should not grow.

The instructor will work with strains of E. coli that have no plasmids in them. In this instance this instructor used strains of E. coli designated MM294 which were obtained from Carolina Biologicals and are plasmid free.

Plasmids must also be obtained that will be introduced into the bacterium. This instructor used two different plasmids, both having an ampicillin resistant gene. One plasmid, pUC18, was obtained from Modern Biology, Inc. (Dayton, Indiana) and the other plasmid used was pAMP which was obtained from Carolina Biologicals.

Preparation of Materials.

Agar plates have to be prepared or obtained already prepared for use in the laboratory exercise.

The Preparation of the Agar Plates.

1. It will be necessary to have four plates per team of students with 2 to 4 students per team.

2. The petri dishes (plates) will have to be sterilized before hand.

3. Two of the plates will contain Luria Broth Agar: 25 grams per liter of distilled water.

a. The Luria Broth Agar is placed in the distilled water in a beaker and heated until it is dissolved. It is then poured, 100 ml, into 150 ml Erlenmeyer flasks which are covered with aluminum foil that is held in place with rubber bands and the flasks are autoclaved (sterilized) at 250 deg. F (121 deg. C) for 15 minutes.

b. After the flasks are removed from the autoclave or sterilizer, they are allowed to cool to about 100 deg. F (about 40 deg. C). To half of the flasks a preparation of 1 ml of ampicillin is added. The ampicillin is dispensed with sterile, 1 ml pipets.

The Preparation of Ampicillin.

1. Add 1.06 g Ampicillin (sodium salt) to 100 ml of sterile distilled water, mix thoroughly with a clean stirring rod or a magnetic spin bar or by swirling the solution. Pour into sterile, 10 ml test tubes previously covered with aluminum foil and freeze or keep cold, if the ampicillin is to be used immediately, until usage.

Pouring the Plates.

1. Add the agar preparation (6 - 10 ml) minus the ampicillin to each of two plates. One plate is marked Agar "+" and the other is marked Agar "-".

2. To each of the remaining two plates the agar preparation containing the ampicillin. One plate is to be marked Agar/Amp + and the other is to be marked Agar/Amp -.

There are now four petri dishes containing Luria Broth Agar or Luria Broth Agar/Ampicillin. Each plate is marked as indicated.

Additional Materials Needed.

1. It will also be necessary to prepare a solution of ice-cold 0.05 M Calcium chloride. This solution is prepared by mixing 5.55 g Calcium chloride in 1 liter of distilled water. It is to be kept cold.

2. A water bath is needed that will enable the students to heat-shock their sample at 42 deg. C. for 90 seconds.

3. A small, 10 ml test tube is prepared for each team containing about 5 ml of Luria broth. The test tube is covered with foil and placed in the autoclave and sterilized as before.

4. Students will also need two foil covered, 10 ml test tubes that are sterilized.

Time Frame.

The lab itself takes about 40 minutes to complete. Considerably more time is required beforehand for preparation. Plates pre-prepared and marked as indicated can be obtained from Carolina Biologicals so the time spent in preparing plates can be reduced to nothing. About three hours of time is needed to prepare the plates for two classes. Preparing Ampicillin requires about 15 minutes. Most of the time is spent autoclaving petri dishes and Luria Broth agar and in pouring the plates. The preparation and sterilization of test tubes with and without Luria broth takes another 45 minutes.

Content Skills Required of the Teacher.

1. The use of a micropipet.

2. To prepare and pour the plates using sterile technique.

Problems Students Might Encounter.

1. The cultures could become contaminated if students do not inoculate the cultures carefully and handle all materials using sterile technique.

Student Goals.

1. The student will develop greater skill in using sterile technique.

2. The student will observe the function of plasmids.

3. The student will become familiar with techniques used in making competent cells.

4. The student will observe the transformation of bacteria.

Background Information for the Student.

1. The student will need to know how to use a micropipet or its equivalent.

2. Everything is to be kept on ice when not being manipulated.

3. The student will have to understand and use sterile technique.

Procedures for the Student. (26)

1. Using transfer pipet add 25 microliters of cold calcium chloride solution to each of two 10 ml test tubes. Label one tube "+" and the other tube "-". Place the test tubes in a beaker of ice.

2. Using sterile technique, transfer a cell mass from the cell culture into the "+" tube. (Be careful not to transfer agar with the cells.) Vigorously tap the loop to dislodge the cells from the loop

and get them into the solution. Flick the tube until the cell mass has disappeared and the solution is cloudy. Place the tube on ice.

3. Using sterile technique transfer a cell mass from the cell culture into the "-" tube, and proceed as in No. 2, above, to suspend the cells in the solution, placing them on ice.

4. A sterile inoculating loop is now used to add about 10 microliters of either pAMP or pUC18 to the "+" tube. If the loop has a film of liquid covering the inside of the loop, that will be about 10 microliters. If you can't get a film filling the loop, the smallest visible drop will probably be sufficient. Tap the tube to release the plasmids from the loop and swirl to mix the plasmids with the bacterial suspension. Place the tube back on ice.

5. Incubate the "+" and "-" tubes for 15 minutes on ice.

6. Obtain four petri dishes containing medium: two should be labelled LB and two should be labelled LB/AMP. Label one LB plate "+" and the other "-". Do the same with the LB/AMP plates.

7. After the test tubes have incubated for 15 minutes on ice remove the tubes and "heat shock" them in a 42 deg. C. water bath for exactly 90 seconds. The 90 second period of time is most critical. After the tubes have been "heat shocked" return the tubes to the ice for one or more minutes.

8. With a transfer pipet, add 250 microliters of Luria broth to each tube. Mix well by tapping with your finger and set the tubes at room temperature.

9. Take the "-" tube and add 100 microliters of cell suspension to the center of the LB "-" plate and another 100 microliters to the center of the LB/AMP "-" plate. Using a sterile inoculating loop spread the cells over the surface of each plate being careful not to scratch the agar surface. Be careful to sterilize the inoculating loop after each use.

10. Do the same with the "+" tube and spread the cell suspensions over the surface of the plates.

11. Allow the plates to sit for a minute, then label them with your team No. or name and place them together upside down in an incubator for from 12 to 24 hours.

12. Examine your plates the following day and draw and record the sizes and shapes of the bacterial colonies that grow.

The Preparation of the Laboratory Report.

I. Purpose. A brief single sentence statement of the reason for doing the laboratory exercise.

II. Introduction. This introduction should contain information about specific plasmids, notably pUC18. The teacher may supply the students with information concerning the size of the plasmid and the genes it contains. The student should do additional research. Students need to note the purpose of the calcium chloride in making cells competent. And they should note the purpose of the "heat shock". The teacher may supplement their knowledge where they are unable to obtain it on their own.

III. Procedures. The student will note what he has done to perform the experiment.

IV. Data. The student will prepare a data table of the results of his own experiment and a data table of class results.

V. Graph. The student will prepare a bar graph indicating the class results: per cent of Luria broth agar and Luria broth agar/ampicillin plates showing positive growth of plasmid "+" strains and plasmid "_" strains of bacteria growing on each of the plates prepared and used by the class.

VI. Results and Conclusion. The student will determine the success of the experiment, individually and for the class. He will present his results and draw conclusions concerning the growth or lack of growth of plasmid and non plasmid containing bacteria on Luria broth agar and Luria broth agar/ampicillin plates.

LABORATORY EXERCISE NO. 3

DNA RESTRICTION ANALYSIS

Background Information for the Teacher.

We will restrict (cut) the DNA from bacteriophage lambda with the restriction enzymes EcoRI and/or BamHI. There will be three preparations by the student: one test tube containing the lambda DNA and the restriction endonuclease EcoRI, a second test tube with lambda DNA and two restriction enzymes, EcoRI and BamHI, and a third test tube, the control, with just lambda DNA and no restriction enzyme.

Phage lambda DNA is viral DNA and consists of 48,502 base pairs. It is made up of about 50 genes: genes for head and tail proteins of the virus, genes for attachment, integration, and excision and a gene for lysing the cell after sufficient viruses have been produced by the cell.

There are five specific sites where it can be cut with the restriction enzyme EcoRI. Each site contains the DNA base sequence: G A A T T C and attached to this sequence is the complementary strand C T T A A G. The restriction enzyme cuts between the two complementary strands cutting each end first between the G and the A.
The restriction cuts occur at 21,226 base pairs (bp), at 26,104 bp, at 31,747 bp, at 39,168 bp, and at 44,972 bp. If it cuts in five places there are six pieces of lambda DNA that result from the cuts. (Figure 1.) Students will have to know the exact number of base pairs for each fragment of DNA, so it will be necessary to subtract two adjacent numbers to determine the number of base pairs between them.

The DNA fragments will migrate during electrophoresis at a speed which is inversely proportional to size. The smallest will move fastest and farthest. Students can then determine which segment is which by its relationship to other fragments of larger and smaller sizes.

From this information students should, using two cycle semilog graph paper, produce a standard curve for the EcoRI digest.

BamHI is a restriction enzyme that also cuts lambda DNA in five places. It cuts, a staggered cleavage as in EcoRI, between the sequences G G A T C C and its complement C C T A G G. The cut is between the first and second G and the G A T C. BamHI restricts at 5,505 bp, at 22,346 bp, at 27,972 bp, at 34,499 bp, and at 41,732 bp. (Figure 1) (21, 27 - 32)

When the mixed digest is produced there should be 11 pieces of lambda DNA. Although because of the smallness of the size of some of the bands, they may not be totally visible. And because of the closeness of the sizes of some of the bands there may be double bands which appear as one.

Preparation of Materials.

1. The teacher will need to have available a buffer for the enzymes which will be added to the reaction tube before the enzyme. These buffers are usually included separately by the supplier when the restriction enzymes are sent. Boehringer Mannheim gives the following recipe for the storage and dilution buffer which they send with the restriction enzyme:

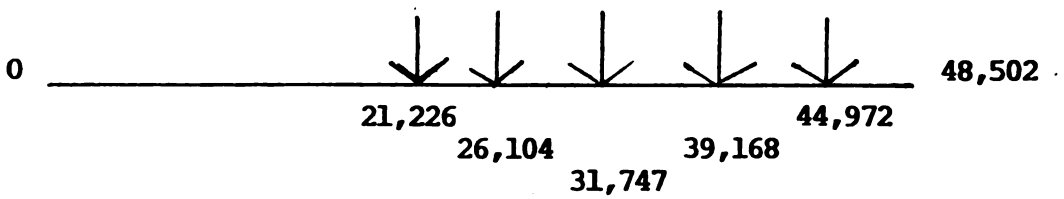
a. For EcoR1: TrisHCl, 10 mmole/liter (1.58 g) ; NaCl, 200 mmole/l (11.7 g); EDTA, 1 mmole/l (0.37 g); dithioerythritol, 0.5

PROPERTIES OF TWO RESTRICTION ENZYMES ALONG WITH THEIR RESTRICTION MAPS.

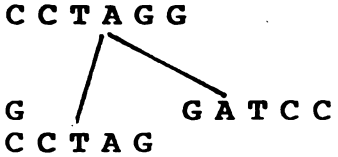
Restriction Enzyme	Bacterial Source	Recognition Sequence
EcoRl	Escherichia coli	G A A T T C C T T A A G

Restriction Map of Lambda DNA - EcoRl

Arrows indicate the position of the recognition sites where EcoRl restricts. Sites are in number of base pairs from the left end of Lambda DNA which is 48,502 base pairs long.



Restriction Enzyme	Bacterial Source	Recognition Sequence
Bam Hl	Bacillus amyloliquefaciens	G G A T C C C C T A G G



Restriction Map of Lambda DNA - Bam Hl

Arrows indicate the position of the recognition sites where Bam Hl restricts. Sites are in number of base pairs fromthe left end of Lambda DNA.

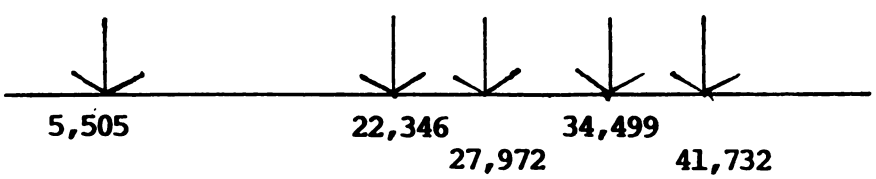


Figure No. 1. RESTRICTION MAPS OF LAMBDA DNA.

mmole/l (0.08 g); Triton X-100 (a polyoxyethylene ether, a surfactant), 0.2% (v/v) (2ml); glycerol, 50% (v/v) (500 ml); pH ca 7.

b. Hacket, Fuchs and Messing (20) offer the following recipe in their lab manual, An Introduction to Recombinant DNA Techniques,: for one liter of a 10X EcoR1 buffer (pH7.2): 0.5 M Tris (pH 7.2) (70.2 g Tris HCl + 5.45 g Tris base); 0.1 M Magnesium chloride (9.5 g); 0.5 M NaCl (29.25 g); 0.02 M dithiothreitol (3.09 g); and store at -20 deg. C.

Electrophoresis gels will need to be prepared either in class or prior to class before the lab can be completed.

1. Preparation of concentrated TBE buffer (Tris - borate - EDTA): Trizma base - 54 grams, Boric Acid - 27.5 grams, EDTA (Ethylenediaminetetracetic acid) - .05 M at pH 8.0 - 20 ml of this to the above.

2. Dissolve Tris (Trizma) base and boric acid in a 500 or 800 ml beaker with stirring, pour this into a 1 liter volumetric flask or 1 liter graduated cylinder, add 20 ml of EDTA at pH 8.0 and add water to make 1 liter. This solution is a concentrated solution; it will need to be diluted: 1 part concentrated solution to 4 parts distilled water to make the running buffer and the buffer in which you will mix the agarose to make the gel.

a. To make EDTA: dissolve 0.93 grams EDTA in 50 ml of distilled water. Use 1 N NaOH or 1 N HCl to adjust the EDTA solution to pH 8.0. Then add 20 ml of this to the TBE buffer, as above.

3. To prepare the gels:

a. Make 500 ml of diluted buffer by taking 100 ml of concentrated buffer and adding 400 ml of distilled water.

b. To make enough medium for one gel: take 0.6 grams of agarose and add to 50 ml of dilute buffer. Heat until boiling and the solution clears. Pour the dissolved agarose into the mold for the gel and place the comb which will form the wells about 65 mm from one end of the mold.

4. Methylene Blue Stain. The gel will be stained in a solution of methylene blue: 0.25 grams/liter of distilled water. (33)

5. A two liter beaker of ice should be obtained before hand since students and the teacher will need to have beakers of ice available in which to keep their reaction tubes.

6. The restriction enzymes and lambda DNA should be thawed and kept cold on ice throughout their usage. They may be refrozen afterwards with little loss of activity.

Time Frame.

For this laboratory exercise, about two hours of preparation time before hand if one makes one's own buffer and makes and pours the gels before class. If the buffer is already on hand and is ready for use, there is essentially no preparation time for the buffer.

About 30 minutes to one hour is needed to make the TBE buffer.

With a microwave oven each student can prepare his own gel in about 3-4 minutes. If one prepares seven gels for a normal class, it would take about 40 minutes to heat and pour the TBE - agarose mixture

and into the already prepared form. About 20 minutes or more is needed for cooling each of the gels and for removing the tape and the combs from the gels.

About 15 minutes is needed to make the methylene blue stain.

One needs to take the materials out of the freezing compartment of the refrigerator and thaw them on ice before starting the lab.

Problems Students Might Encounter.

If the forms for the gel are not properly taped the hot, liquified gel will flow out of the form. The form needs to be double taped with ordinary masking tape, if one is using the homemade variety of electrophoresis chamber. The combs need to be straight up and down and straight across in the gel, and should not be removed until completely cool. (When the gel is cool, it will be translucent.)

Student Goals.

1. The student will make his own gel.

2. He will prepare the restriction digest and load the restriction digest into the wells.

3. The student will connect his gel to a power source and electrophorese his samples.

4. The student will know and understand the action of restriction enzymes on Lambda DNA.

5. The student will relate this laboratory exercise to an understanding of how the sequence DNA is determined and of the methods used.

Background Information for the Student.

In this laboratory exercise, you will digest the DNA from the Bacteriophage Lambda with one and then with two restriction endonucleases. You will separate the fragments that you digest by gel electrophoresis.

Both restriction enzymes cut the lambda DNA in five places. You will need to determine the number of bands of DNA which will appear on the gel for the single digest and then for the double digest. You will need to measure the distance each band travels from the center of the well.

Procedures for Preparing the Restriction Digest.

1. Obtain 3 - 1.5 ml sterile micro-test tubes and label them as follows: "A" (EcoR1); "B" (EcoR1/Bam H1); and "C" (Lambda DNA). Test tube "C" will be the control.

ALL REAGENTS MUST BE STORED ON ICE THROUGHOUT THE FOLLOWING STEPS.

2. Set up the following matrix to use as a checklist as you add reagents with micropipetors to each reaction tube. (To reaction tube C, 1.5 microliters of water are also added.)

BUFFER MUST BE ADDED BEFORE THE ENZYME/ENZYMES.

USE A FRESH, STERILE MICROPIPET TIP FOR EACH REAGENT.

THE FOLLOWING AMOUNTS ARE IN MICROLITER MEASUREMENTS:

	DNA	2X Buffer	EcoR1	EcoR1/Bam H1
A	6	6	1.5	0
B	6	6	0	1.5
C	6	6	0	0

3. Pulse the micro-test tubes for 1 - 2 seconds in the centrifuge.

4. Tap the tubes gently to mix the reactants.

5. Place the tubes in a 37 deg. C. water bath and incubate them for 20 minutes.

AT THIS POINT THE MICRO-TEST TUBES CAN BE FROZEN AND HELD UNTIL ELECTROPHORESIS.

6. Remove the tubes from the 37 deg. C. water bath.

7. Using fresh, sterile micropipet tips load the entire contents of each reaction tube into separate wells in the gel. Since we are only loading three wells with reaction mixture, load only every other well.

a. In loading the well, steady the pipet with both hands resting your elbows on the table for additional support.

b. Be careful to get all of the contents into the well and not to push the pipet tip through the bottom of the well.

c. Depress the pipet plunger slowly when the pipet tip is in the well and continue to depress the pipet plunger until after the tip has been removed from the well.

d. Use a fresh, sterile tip for each reaction mixture.

8. Connect the gels to the power source with the wells farthest away from the positive electrode. (Migration of the DNA will be from the negative side toward the positive side.) If you use 100 volts at about 1.5 amps, the migration will take about 1 hr. Weaker power sources will require a longer period of time.

9. Load the marker dye mixture in the center well. This will indicate when the electrophoresing is done. (The marker dyes are: Xylene Cyanol which moves with the equivalency of 2800 base pairs; this dye is an aqua- marine color. Bromophenol blue moves with the weight equivalency of 250 base pairs; this dye is a purple color. Orange G moves with the equivalency of 70 base pairs and is a yellowish - orange color. (21,28)

10. After the gels have been electrophoresed, they will need to be stained and destained. (33)

a. Making sure the power source is disconnected, carefully remove the gel from the form in the electrophoresis chamber.

b. Place the gel in a container suitable for staining; the gel should remain flat.

c. Pour the methylene blue stain over the gel and stain for 15 minutes.

d. After 15 minutes carefully pour the stain into a beaker (It can be reused.), and cover the gel with distilled or tap water and allow the gel to destain for two minutes.

e. Discard the destain and destain again for another two minutes. And then destain a third time for two minutes.

f. Carefully wrap the gel in plastic wrap or seal the staining chamber with plastic wrap and place in the refrigerator for 24 hours.

11. Remove the gel from its container and examine over a light box. If no light box is available, holding the gel carefully to the window will enable students to see and measure the distance the bands have moved.

a. Measure the distance each DNA band has moved from the center of the well and record the distances. Record the distances in millimeters.

b. Sketch the gel on plain, white paper with a pencil and try to indicate the relative heaviness of the bands by making them darker or lighter with the pencil.

12. Wrap the gel and place it back in the refrigerator for future reference after you have made the drawings and measured the distances the bands have moved.

13. Obtain a sheet of two cycle semi-log graph paper (Figure No. 2) and plot the size of the bands in base pairs (Y axis) against the distance in mm moved (X axis)..

a. Plot the distance moved by the bands in the EcoR1 lane of the gel against the number of base pairs. (Remember that the smallest number of base pairs will be the band farthest away from the center of the well.) This line you draw between the points will constitute your standard curve. You can use this curve to determine base pair sizes of other bands that have moved known distances, but whose sizes you may not know.

Questions for the Student.

1. What is the size of each of the fragments of the EcoR1 restriction?

2. What is the size of each of the fragments from the EcoR1/Bam H1 restriction?

3. Plot the points on the standard curve which represent the number of base pairs of each of the bands of the mixed digest. Read down to the "X" axis and determine the distance each of them should have moved. Do the distances on the graph coincide with the distances actually moved? If there is an error calculate the per cent error for each band.

4. Draw a straight line to a scale of 1 cm = 4 kilobases (4000 base pairs). Indicate with arrows, as precisely as possible, the location of the recognition sites (restriction digest sites) for EcoR1. Indicate the size in base pairs of each of the fragments.

5. Do the same thing for the mixed digest.

6. Explain how you might make a restriction map of Lambda DNA from these two pieces of information.

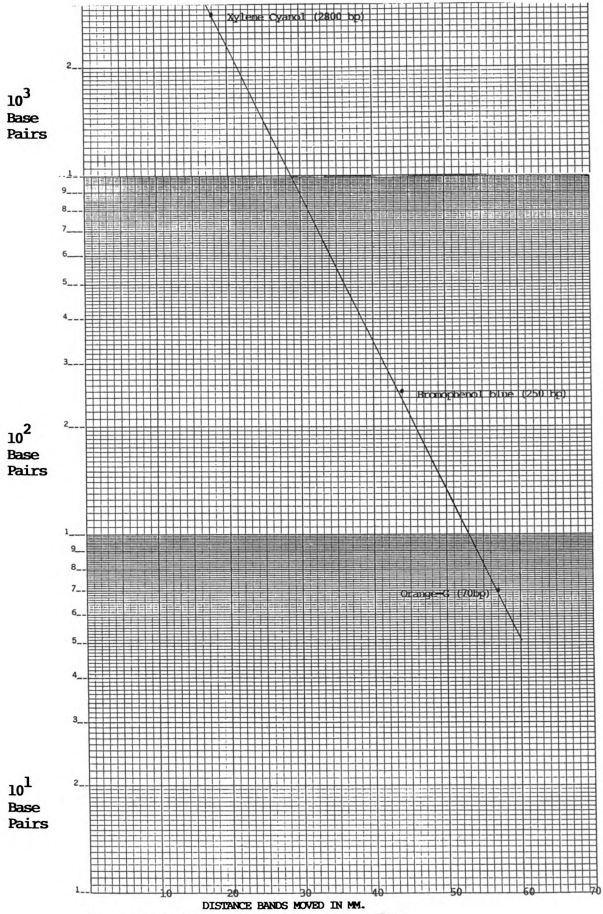


Figure No. 2. A plot of the marker dyes.

SUMMARY AND DISCUSSION

The laboratory exercises that were used in the classroom were: Laboratory Exercise No. 1, Transformation of E. coli; Laboratory Exercise No. 2, Colony Transformation; and Laboratory Exercise No. 3, DNA Restriction Analysis.

During the laboratory exercises, students worked in pairs. Each student was required to write a laboratory report. Students wrote laboratory reports on Laboratory Exercises 1 and 2, and were asked to answer scaffolded type questions following Laboratory Exercise No. 3. See Appendix B for the questions.

The answers to the questions were such that 80 % of the students showed a clear understanding of what had been done in the lab i.e. received a grade of 21 or better out of 25 possible points.

For all other laboratory exercises a laboratory report was required in which the student wrote an Introduction, with a Purpose, a set of Procedures, a Data Table or Tables, and a Graph or Graphs and included some sort of Conclusion.

Procedures from these laboratory exercises and Laboratory Exercise No. 4 as well as additional methods were used by students who completed science fair projects for the Northeast Ohio Regional Science Fair held at Case Western Reserve University in March of this year.

Laboratory Exercise No. 1 was done as it was written with small revisions by a senior student, Kimberley Anne S.; she received a first place for her work and her explanation which had to include a complete understanding.

Another science fair project, done by two students, Thomas P. and Anne Marie P., a senior and a junior, respectively, involved procedures from laboratory exercises 2, 3, and 4 and the use of agar plates with I.P.T.G. and X-Gal. The title of this more elaborate project was: Gene Fragment Insertion in Plasmid Puc 18.

For this project they received first place also. They were invited to make a presentation at the Cleveland convention of the American Society of Medical Technologists. They received cash prizes from the ASMT and a number of items of equipment were donated to our school by the members of the convention who rewarded them with a standing ovation.

In Thomas P.'s case, his work with the science fair project has influenced his choice of career. Ann Marie P. is doing research now, under my supervision, on the Human Genome Project and hopes to be a Westinghouse Scholar applicant for next year.

As a final note to the year, thirteen students received questionnaires to be filled out after they had completed their second semester final exam. (34) They were told to complete the questionnaire to the best of their ability.

All students, without exception, were able to demonstrate a satisfactory understanding of the chemical composition of DNA. That is, they knew the names of the bases, the base pairs, the location of the linkage bonds to the sugar and the location of the phosphate to deoxyribose sugar bonds.

To the question, "Did the construction of the cardboard model of DNA help in your understanding? (Students were asked to construct a DNA model showing adenine, thymine, cytosine and guanine on one side with their complements on the other side with 3′ to 5′ bonding on one side

and 5′ to 3′ bonding on the other.) eleven students answered that it helped them to understand the 3′ and 5′ bonding of the phosphates to the sugars, and the bonding of the bases to each other. Two students felt that the construction of the model was confusing to them and not helpful.

To the question, "We did two labs involving plasmids. Do you recall what happened in each of them? Were they successful?

The general response was: "We inserted plasmids into bacteria to make them immune to antibiobics." Students used terms such as "injected plasmids into bacteria", "plasmids can transform bacteria" and "if successful plasmids that are placed in a cell will express genes contained on the DNA"

All students agreed that the labs were successful, and they were. Two of the students showed confusion between names of plasmids and strains of bacteria; one student recalled that "we inserted a plasmid into agar that was inoculated with ampicillin to see if it was immune to ampicillin."

To the question, "We did a lab involving the cutting of a piece of DNA with restriction enzymes. Do you recall what was cut? where was it cut? with what was it cut? where did the restriction enzyme come from? why restrict a piece of DNA?

The responses were not as complete as I would have liked. Nevertheless they showed an acceptable level of comprehension. "Lambda DNA was cut with a restriction enzyme called EcoR1. We cut up the DNA to help determine the lengths of the segments.." All thirteen students stated that we cut the DNA with EcoR1. Nine students noted with clear understanding that Lambda DNA was "cut". Eight of the students noted

that the restriction enzyme came from Escherichia coli. Nine students noted that the reason for cutting the DNA was to "help map the chromosome" or "to determine where nucleotide sequences were located" or "to study the fragments easier" or "to discover the lengths of the fragments" or "to gain a further understanding of each particular band" or similar answers. Five students remembered that we also used the restriction enzyme, BamHi, although none could state that it came from Bacillus amyloliquifaceans - one student did mention "Bacilles" Three students were able to remember that EcoRi cut at the sequence G A A T T C and its compliment and indicated the correct "cut" with a sketch. One student noted, "Bacteriophage lambda DNA which is 48,502 base pairs. The cutter was pUC18 (restriction enzyme) (to) study and differentiate base pair length."

It has been the purpose of this thesis to present laboratory exercises that would be invocative and provocative and "do-able". The degree of understanding that might grow from these labs is indicated by the answers to the questionaire presented above.

At no time did I feel that I had achieved 100% success in my teaching. But I am heartened by what the students did learn, as of June 8, 1990.

Two of these labs were done for the first time this year as classroom activities: the ampicillin transformation lab with bacterial strain mm294, and the lab involving restriction digests. Laboratory exercise No. 1, has been done for at least four years and with increasing success each time. The success is related to, in part, the deeper understanding of the lab experience that I, myself, develop from study and working with the students. The two, new introduced labs will,

in time, increase in adaptability to student ability to comprehend and to use the knowledge in the real world.

These laboratory exercises did stimulate discussion and motivate classroom work. They did familiarize the student with the basics of biotechnology. They did begin to answer the questions, "How are these things done?";"How do these things work?" and "How do we obtain meaning from this technology?"

These exercises, I believe, bridge the gap between knowing and doing, between knowledge and understanding, between imagination and reality.

There is a serious need for students to understand the knowledge which they obtain. Most of them do not. Even in an Honors Biology class it is impossible to have complete understanding of any concept.

The most important tasks in Education are for the teacher to motivate, to stimulate and to create an attitude for a desire to learn. The appreciation for the seriousness of the learning concept is sometimes, but certainly not always felt by the student. There is always the one or two and sometimes three or four students who respond to the teaching concept with enthusiasm and desire. These are the students for whom the teaching may provide the inspiration for a future career.

For the other students, it can only be hoped that enough of an intelligent understanding may result so that future decisions may be directed by reasonable choices.

APPENDIX A

APPENDIX A

LABORATORY EXERCISE NO. 4.

THE ISOLATION OF PLASMID DNA FROM BACTERIAL CELLS

(E.COLI , STRAIN MC 1061, CONTAINING THE SYNTHETIC PLASMID, pBR 322.)

BACKGROUND INFORMATION.

A plasmid is a piece of double stranded DNA that occurs naturally in the form of a ring or circle. It exists, mostly, in bacterial cells, and is considered an endosymbiont. It possesses the genes that control its own replication number. In other words it replicates using the bacterial enzymes and metabolic systems. It can also be cut into pieces with special enzymes called restriction enzymes.

If the plasmid ring is cut in one place the ends can be "engineered" to be "sticky" and a gene with compatible "sticky" ends can be mixed with the plasmid culture and will attach itself chemically to the cut ends. The circle will close and the plasmid, now a vector carrying a gene, can be put back into the same bacterial cell or into another one.

There are a number of reasons for isolating plasmids from bacterial cells. Perhaps the most common use of plasmids is as cloning vectors. ThE technology for isolating plasmids and inserting genes into

the plasmids has been available for some time. Once the plasmids have been reintroduced into the bacterium, the genes inserted into them can then be cloned by the bacterium as it makes multiple copies of the plasmid.

The plasmids from the bacterial cell are first restricted with a restriction enzyme. Next a gene or piece of DNA of choice is allowed to interact with the restricted filament and ligated with DNA ligase. The plasmid can then be put back into the bacterial cell. The bacterial cells will be cultured and grown to produce the product of the gene that was inserted into the plasmid which is replicated many times. This is cloning.

The plasmids will be isolated in one laboratory period and subjected to electrophoresis analysis in the second period.

In this laboratory exercise we are shall isolate the synthetic plasmid, pBR 322, from a strain of bacterial cells called MC1061. We shall use gel electrophoresis to demonstrate its isolation.

The plasmid will then be subjected to digestion with the restriction enzyme, EcoRI; the cut and uncut plasmids will be differentiated by gel electrophoresis.

Plasmid pBR322 is a circular, double stranded DNA. It was constructed from parts of several other naturally occurring plasmids. It consists of 4363 base pairs with one EcoRI restriction site. It has an ampicillin resistant gene that produces the enzyme beta lactamase that destroys ampicillin and also a tetracycline resistant gene. (13,20)

One of the first products to be produced commercially in this way was the human growth hormone. It was produced from a gene cloned into a strain of E.coli through the use of a plasmid vector.

Perhaps the first major product having a truly momentous impact was the cloning of the human insulin gene. This was available for the first time commercially in December of 1983. The product is called Humulin and it's availability abrogates the necessity of using bovine (from cows) and/or porcine (from pigs) insulin.

Before plasmid vectors can be used, their structures have to be known. One has to know where to cut the circular plasmid so as not to interfere with its ability to replicate itself, mobilizing the enzymes of the bacteria.

An additional note: the first living organism was patented by a research scientist by the name of Ananda Chakrabarty. On June 16, 1980 the Supreme Court of the United States granted Dr. Chakrabarty and his colleagues the right to patent a form a soil bacterium called Pseudomonas. This bacterium was especially selected for the plasmid which enabled the bacterium to use a major environmental poison, 2,4,5-T (2,4,5-trichlorophenoxyacetic acid) the principle ingredient in Agent Orange, as a source of food.

In this case, the plasmid occurred naturally. It was not engineered. Dr. Chakrabarty and others simply had to grow enough cultures of these bacteria until they found the one that had the "right" plasmid. Then it was just a matter of growing the bacteria in large quantities.

In this way the Love Canal in Niagara Falls, New York, a neighborhood that had to be evacuated because of the environmental toxins that were buried near the homes of the residents, has been restored to normal use.

BEHAVIORAL OBJECTIVES:

1. The student will learn the use of the clinical centrifuge in biotechnology.

2. The student will reinforce his previous learning about plasmids.

3. The student will learn how plasmids are extracted for use in genetic engineering.

4. The student will reinforce his learning and practice of sterile technique.

5. The student will be able to determine the major events in each step of the lab procedure and their purposes.

6. The student will become familiar with and practice genetic engineering techniques.

7. The student will reinforce and develop a deeper understanding of the relationship between bacteria and plasmids.

8. The student will have a deeper understanding of genetic engineering.

TIME REQUIRED:

Teacher Preparation: If the teacher uses freeze dried or lyophilized cultures of bacteria, it will involve activating the bacterial culture and allowing it to incubate for about 12 hours. Fifteen minutes are required for inoculation.

Preparation of the STET (10% sucrose, 5% Triton X-100, 50 mM Tris-HCl pH 7.5, 50 mM EDTA) solution and Storage Buffer solutions takes about 30 minutes.

Student Lab Time: The student can stop at the end of each centrifugation, store the contents in the refrigerator and proceed the following day to the next stage. About 40 minutes each day for three days.

Material Preparation:

1. STET: To prepare 50 ml: 0.33 grams TrisHCl; .05 grams Tris (base); 0.93 grams EDTA; 5 grams sucrose; and 2.5 ml of Triton X-100. Adjust pH 7.5 with 0.1 M NaOH.

2. Storage Buffer: TE buffer for storage: 0.137 grams of Tris HCl; 0.016 grams of Tris (base); 0.037 grams of EDTA. Dissolve in sterile distilled water and dilute to 100 ml. Adjust to pH 7.4 with 0.1 M NaOH. (21)

Activation of the Bacterial Cultures.

1. Prepare one liter of Luria broth and put 200 ml of broth in five 250 ml Erlenmeyer flasks.

2. Cover each flask with a sponge stopper such as those used in culturing fruit flies.

3. Sterilize at 121 deg. C for 15 minutes.

4. Cool the flasks and using sterile technique inoculate bacterial cultures into one or more of the flasks.

5. Insert a sterile 1 ml pipet through the sponge stopper and into the inoculated Luria broth solution.

6. Connect the pipet to an air pump, using sterile neoprene tubing. Place cotton in the tubing to trap any particles that might be pumped into the culture from the air pump. (This is a substitute for a shaking water bath usually required when growing bacterial cells to their most positively growth state.)

7. Place the flask in a water bath at 37 deg. C. and allow to incubate with the bubbling of the air through the culture until the bacterial culture reaches mid-log growth. Mid-log growth occurs when the optical density in a spectrophotometer at 550 nanometers reaches between 0.35 and 0.55.

Procedures: (35)

1. Carefully withdraw 100 microliters of plasmid-harboring bacterial cells from the bacterial cultures.

2. Suspend the cells in 50 microliters of cold STET in a 1.5 millliliter microfuge tube. Tap the tube with your finger until all of the cells are suspended and the solution becomes cloudy.

3. Add 4 microliters of a fresh lysozyme solution. (Ten mg/ml in water.)

4. Suspend the microfuge tube in a boiling water bath for 40 - 60 seconds.

5. Centrifuge the microfuge tube for 10 minutes at room temperature.

6. Remove the supernatant with a micropipet and place in a clean microfuge tube.

7. Add 40 microliters of isopropanol to the supernatant.

8. Chill for 5 minutes in a dry ice-ethanol bath.

9. Centrifuge in the refrigerator for 5 minutes to collect the precipitate (This should be the plasmids).

10. Remove the supernatant and dry the pellet under vacuum.

11. Resuspend the pellet in 20 microliters of sterile distilled water.

12. The pellet may then be restricted with the restriction enzyme EcoR1 and electrophoresed in an agarose gel.

Questions and Answers.

Question 1. How can we determine whether or not we have plasmids? Name two ways.

Answer 1. We can determine whether or not we have plasmids by electrophoresis or by taking bacterial cells that lack the plasmid, making them competent and putting the plasmids into them. Then grow these bacteria on plates containing ampicillin and tetracycline.

Question 2. What formed when the isopropyl alcohol was added to the solution? What was the precipitate?

Answer 2. A cloudy, gelatinous, white precipitate formed. The precipitate was DNA.

Queston 3. What was the first centrifugate?

Answer 3. The first centrifugate was bacterial cells.

Question 4. What should the final centrifugate have been?

Answer 4. The final centrifugate should have been plasmids and perhaps some RNA.

Question 5. Why do we use such rigid sterile technique?

Answer 5. The use of sterile technique is to prevent the destruction of the DNA by nucleases.

SOURCES OF MATERIALS.

Sigma Chemical Co.
P.O. Box 14508
St. Louis, Mo. 63178
1-800-325-3010

Boehringer Mannheim Biochemicals
P.O. Box 50414
Indianapolis, Ind. 46250
1-800-262-1640

LABORATORY EXERCISE NO. 5.

THE ISOLATION OF PLASMID DNA FROM BACTERIAL CELLS

(E. COLI, STRAIN MC1061, CONTAINING THE PLASMID PBR322)

An Alternate, Shorter Method

INTRODUCTION TO THE LAB.

See introduction to Laboratory Exercise No. 4.

PITFALLS AND CAUTIONS.

Precise timing of centrifugation will be important. Therefore it is important to know the speed of the centrifuge. For example if the lab procedure calls for centrifuging the sample at 15,000 rpm's for 10 minutes, that would mean centrifuging for 15 minutes at 10,000 rpm's or 50 minutes at 3000 rpm's.

Dry ice should be obtained ahead of time; it is important that the plasmids and other DNA be quickly frozen before the final centrifugation.

Always, when dealing with DNA, absolute sterile technique must be used. The work area should be thoroughly washed with soap and water and alcohol, gloves should be worn and frequently replaced or frequently washed. Anything that touches the bacterial culture or DNA should not touch anything else beforehand.

MATERIALS.

1. A bacterial culture of cells at mid-log growth that has been stimulated to produce multiple copies of plasmids.

2. Centrifuge tubes, sterilized and with tops covered with aluminum foil, or otherwise sealed. Depending upon the size you will need about 12 of these. Microfuge tubes are best, but only if you have a microfuge.

3. Clinical centrifuge or faster centrifuge, if possible.

4. STET. A solution of 10% sucrose, 5 % Triton X-100 (a detergent), 50 mM Tris HCl pH 7.5 and 50 mM EDTA. (Adjust the pH with a 0.1 M NaOH solution.) Fifty millliliters of this should be enough for several teams.

5. Dry ice.

6. Two ten millliliter sterile test tubes in which to pour and mix the products.

7. A vortexing machine, if one is available. Otherwise, mixing can be done by flicking with the fingers.

8. Ordinary ice in a beaker.

9. A boiling water bath in a small beaker; this comes later on in the procedure so it can be prepared as the materials are being centrifuged.

10. Lyophilized lysozyme which is suspended in the STET solution at a concentration of 10 milligrams/millliliter. One millliliter

of this should be enough because you will use only 200 microliters or 0.2 ml of this for each team.

11. Micropipets and their plunger; these will need to have been sterilized beforehand. The sizes to be used varies from 20 microliters to 200 microliters. If a pipetman is available, use it with sterilized tips.

12. Isopropyl alcohol. You will use 3 mls of this per team.

13. A dessicating chamber or vacuum chamber.

14. 12 Sterile 15 ml centrifuge tubes.

PROCEDURES: (35)

1. Obtain a bacterial culture that has been grown to mid-log stage in a medium treated to stimulate rapid plasmid replication. You will need about 60 ml.

2. Place the culture medium into 4 - 15 ml sterile centrifuge tubes and centrifuge the cells for 5 minutes at 10,000 rpm. This should be done at 4 deg. C. The centrifuge can be placed in a refrigerator and centrifuged inside the refrigerator.

3. Discard the supernatant liquid.

4. Add 1 ml of STET to each of the centrifuge tubes and mix the centrifugate with the STET by flicking with a finger or vortexing until the cells are resuspended.

5. Pour the contents of all four centrifuge tubes into a small, 10 ml sterile test tube and add 200 microliters of lysozyme suspended in STET. Mix by flicking with your finger.

6. Incubate on ice for 5 minutes.

7. Transfer the tube to a boiling water bath and incubate for exactly 1 minute.

8. Put the tube back in the ice and cool the sample.

9. Place the sample in a sterile centrifuge tube, balance the tube in the centrifuge with a centrifuge tube on the opposite side of the centrifuge that has a volume of water equal to that of the material in the sample tube, centrifuge the sample at 10,000 rpm for 15 min, or preferably at 15,000 rpm for 10 minute.

10. Carefully pour the supernatant liquid into a clean, sterile test tube. DO NOT TRANSFER ANY OF THE GELATINOUS MATERIAL AT THE BOTTOM TO THE NEXT TUBE.

11. To the supernatant in the clean, sterile test tube add 3 ml of isopropyl alcohol. Mix by flicking.

12. Incubate the sample on Dry Ice for 15 minutes or until the sample is completely frozen.

13. Centrifuge the sample after pouring back into a clean, sterile centrifuge tube at 15,000 rpm for 15 minutes or at 10,000 rpm for 22.5 minutes, or at 3000 rpm for 60 minutes.(21)

14. Carefully remove the supernatant with a micropipet. Try not to disturb the centrifugate at the bottom...it contains the plasmids.

15. Dry the pellet in a vacuum chamber or in a dessicating chamber or air dry the sample.

16. Resuspend the pellet in 300 microliters of TE buffer or some other storage buffer as indicated for the particular type of plasmid you are isolating.

17. Store the plasmid in the storage buffer at 4 deg. C.

FOR FURTHER INVESTIGATION.

1. Continue this lab by electrophoresing the sample and see if you really successfully isolated plasmids.

2. How would you determine the concentration of the plasmids in your sample?

3. What would be your next steps in preparing a sample for cloning a gene into the plasmid?

LABORATORY EXERCISE NO. 6

METHOD FOR DETERMINING

THE SENSITIVITY OF METHYLENE BLUE

FOR STAINING DNA

Background Information for the Teacher:

Lambda DNA is the "guts" of Bacteriophage lambda. It is one of the best understood of the viruses. There are many electron photomicrographs of this phage so its appearance is clearly understood.

The entire sequence of nucleotides of the DNA is known and it contains 48,502 base pairs having a molecular weight of 3 X 10E7 Daltons. (36) The 4.8 kilobase structure contains about 50 genes. These genes direct the bacterial cell to make: head proteins, tail proteins, attachment, integration, and excision proteins as well as lysis proteins.

The lambda DNA integrates itself into the bacterial genome (chromosome) and then directs the rest of the formation of complete viruses finally producing the enzymes that lyse the cell releasing about a hundred completed viruses into the environment to attack other bacterial cells.

When you observe the results of the experiment, you should detect a single band. If you do observe two bands, then there should be a smaller, fainter band farthest from the well and a larger, heavier band closer to the wells. The smaller band will be a supercoiled form of the viral DNA. It takes the shape of a "T" and flows through the gel

pores faster than the circular form. Most of the lambda DNA will be of the circular form which will appear as a denser band.

Before the gels are fixed and stained, the marker dyes should be measured in terms of how far they have moved from the wells. Plot the distance the bromophenol blue and xylene cyanol have moved on two cycle semilog graph paper: the first section for hundreds and the second for thousands. You will be graphing distance on the "X" or horizontal axis and base pairs on the "Y" or vertical axis. (Figure No. 2) If you extrapolate the line, i. e. extend it beyond the points you have graphed you will be able to determine the equivalent base pair values for lambda DNA. All you will need to do is measure the distance the lambda band has moved from the well. Find that place on the line you have drawn on the graph and then read straight over to the "Y" axis. This should tell you within reasonable accuracy the number of base pairs in the viral DNA.

Target Group:

This lab is designed for an Honors Biology or an Advanced Placement class in biology. It can be used in either a first or a second year class.

Course and Topic the Lab Would Reinforce:

1. This lab would be suitable for a module on molecular genetics because it deals with DNA.

2. It would be suitable for a module on viruses or microbiology because it deals with the DNA of the lambda phage.

3. It would be a suitable module as an introduction to the biochemistry of DNA.

4. It would be suitable to reinforce learning in the metric system because it deals with nanograms, micrograms, millliliters, and microliters.

Purposes of the Lab:

1. The purpose of the lab is to familiarize students with electrophoresis and to determine the sensitivity of DNA to methylene blue stain.

Special Equipment and Supplies Needed:

1. One needs electrophoresis equipment, whether the home made variety or the more sophisticated equipment such as Gelteach. The source of these could be the kinds of kits made up at the Michigan State University Molecular Biology Institute. Modern Biology in Dayton, Indiana produces good, simple and practical electrophoresis equipment, as does Geltech of Salem, Ohio.

2. One needs lambda DNA and the chemicals for buffers: Agarose, Boric Acid, Tris and Tris-HCl, EDTA, Methylene Blue. Sources of these could be Sigma Chemical Co., or Boehringer Mannheim or other chemical companies.

3. One needs pipetting devices and small sterile test tubes or centrifuge tubes.

Time Frame:

1. The gels and buffer need to be prepared beforehand; this would take about 40 minutes. (This could be done before or after school.)

2. At least one period of preparation and explanation and demonstration of technique before the lab period.

3. Then about 40 minutes to prepare the dilutions and load the gels for electrophoresing. (The teacher would have to take responsibility for turning off the apparatus when the electrophoresis is finished.)

4. The gel may then be put into a fixative overnight and stained and destained on the following day.

5. Staining and destaining take about 30 minutes. The gels are then wrapped in Saran and placed in a refrigerator until the following day.

6. They are then measured with a metric ruler and the distances from the starting point noted. This three day long project includes instruction and preparation. (If the teacher decides to do his own dilutions, etc., a two successive lab period interval is sufficient.)

Content Skills and Knowledge Required of the Teacher.

1. The teacher needs to understand the use of the equipment.

2. There would need to be some knowledge of lambda DNA.

3. Their would need to be some understanding of marking dyes and how they work.

4. The teacher would need to know how to make and electrophorese gels.

5. It would be necessary to have the ability to graph with semilog graph paper.

6. The teacher would also have to have a good understanding of "sterile technique".

Problems Students Might Have and Suggestions for Overcoming Them.

1. Mixing solutions correctly, if they are to mix them. The teacher might have to watch over their procedures and correct mistakes as they are made.

2. Loading the wells carefully so as not to overload or dispense the materials into the buffer instead of the well. The teacher might demonstrate good technique and again watch them as they inoculate the wells.

3. Using sterile technique and avoiding contamination. Encourage students to wash frequently, to wear rubber gloves and to avoid touching contaminating surfaces with the equipment. DNA nucleases are everywhere, on skin, table tops on any surface that comes human in contact with our bodies. They will catalyze the hydrolysis of DNA so that bands will not appear on the gels; you will get nothing that is useful.

4. Following the proper lengths of time in staining and destaining and handling the gels properly. Oversee this part of the lab diligently.

Guide for Preparation of Materials:

1. Glycerine-sterile distilled water mix (20% v/v): Place 0.4 ml of glycerine in 1.6 ml of sterile distilled water. Mix this into sterile test tube. Prepare one 2 ml test tube per team.

2. Electrophoresis gels will need to be prepared either in class or prior to class before the lab can be completed.

3. Preparation of 1 liter of concentrated TBE buffer (Tris - borate - EDTA): Trizma base - 54 grams, Boric Acid - 27.5 grams, EDTA (Ethylenediaminetetracetic acid) - .05 M at pH 8.0 - 20 ml of this to the above.

Dissolve Tris (Trizma) base and boric acid in a 500 or 800 ml beaker with stirring, pour this into a 1 liter volumetric flask or 1 liter graduated cylinder, add 20 ml of EDTA at pH 8.0 and add water to make 1 liter. This solution is a concentrated solution; it will need to be diluted: 1 part concentrated solution to 4 parts distilled water to make the running buffer and the buffer in which you will mix the agarose to make the gel.

4. To make EDTA: dissolve 0.93 grams EDTA in 50 ml of distilled water. Use 1 N NaOH or 1 N HCl to adjust the EDTA solution to pH 8.0. Then add 20 ml of this to the TBE buffer, as above.

5. To prepare the gels:

a. Make 500 ml of diluted buffer by taking 100 ml of concentrated buffer and adding 400 ml of distilled water.

b. To make enough medium for one gel: take 0.6 grams of agarose and add to 50 ml of dilute buffer. Heat until boiling and the

solution clears. Pour the dissolved agarose into the mold for the gel and place the comb which will form the wells about 65 mm from one end of the mold.

Alternatives for Organizing, Dispensing and Removal of Materials.

The students can do as little or as much of the work as the teacher feels necessary. The teacher can do all of the preparations, if he or she desires it.

Disposal of Used Material:

The electrophoresis buffer can be saved to be reused. (Buffers have been used three times with no loss of effectiveness.) The gels can eventually be discarded in the waste basket after they have been used.

Procedure:

1. Obtain a fresh sample of lambda DNA of known quantity. (An example might be a sample that is obtained from Boehringer Mannheim that contains 0.25 micrograms/microliter in a quantity of 100 microliters of storage buffer. The amounts will be clearly marked on the container which contains the lambda DNA.)

Since we are going to be measuring amounts of DNA in nanograms, it is worthwhile to note that that there are 1000 nanograms per microgram. Thus 0.25 micrograms is 250 nanograms.

2. Dilute the sample so that small enough quantities of lambda DNA can be carried in larger quantities of solution.

a. The manner of diluting a sample containing 0.25 micrograms/ microliter would be: Take 5 microliters (This will contain 1.25 micrograms or 1250 nanograms of lambda DNA.) of the sample indicated above and dilute to 1 millliliter (1000 microliters) in a mixture of sterile distilled water and glycerine. (36) Now each microliter of sample will contain 1.25 nanograms of lambda DNA. (The glycerine will add to the specific gravity of the water and enable the sample to sink to the bottom of the well and stay there. For the preparation of the glycerine, see the Preparation of Materials, No. 1, as indicated previously in this exercise.)

3. Set up the following matrix to follow in placing the samples in the wells of the gel:

Well No.	Microliters of lambda DNA dilution	Glycerine-water Diluent	Quantity of Lamda DNA in ng.
1	24	6	30
2	5	25	6.25
3	10	20	12.5
4	2 microliters of dye mixture.		
5	12	18	15
6	14	16	17.5
7	20	10	25
8	22	8	27.5

4. The gel should be electrophoresed at 100 volts for approximately 1 hr. [The marker, Orange G, will indicate when the electrophoresis is complete (equivalent to 70 bp) when the dye has reached the end of the gel.]

5. The gel will be removed from the electrophoresis chamber (Disconnect the power source first!) and placed in a container for staining.

6. Pour methylene blue stain over the gel and allow to stain with gentle agitation for 15 minutes.

7. Pour off the stain (It may be reused.) and destain with tap or distilled water at 2 minute intervals each three times.

8. Wrap the gel carefully in plastic wrap and place in the refrigerator overnight to allow the stain to concentrate in the DNA bands.

Results and Conclusion.

A class discussion can provide an effective conclusion to this laboratory exercise. The background information can be discussed and reviewed. The student can be quizzed on the background information and the purposes and results of the lab.

APPENDIX B

APPENDIX B

HONORS BIOLOGY

RESTRICTION DIGEST LABORATORY

QUESTIONS

Write the answers in complete sentences skipping a line after each answer. Incorporate the meaning of the question into the answer and whereever appropriate underline the words that constitute the answer.

1. What is EcoRI?

2. What is phage lambda DNA?

2a. What is the size of lambda DNA?

2b. Draw a picture of a bacteriophage virus, labelling all the parts. (Text reference is a good picture of bacteriophage lambda.)

3. What does a restriction enzyme do?

4. How many EcoRI restriction sites are there on Lambda DNA?

4a. Describe the nucleotide sequence where EcoRI cuts. (Text Reference.)

4b. Where does EcoRI cut Lambda DNA? (Draw a straight line to a scale of 1 Cm = 4 kilobases and indicate with arrows as precisely as possible the places where the restriction enzyme cuts the line.)

5. What is the size of each of the fragments of an EcoRI cut of lambda DNA? (Notes)

6. Where does EcoRI get its name?

7. Where does BamHI get its name?

7a. How many BamHI sites are there on lambda DNA?

8. Where does BamHI cut Lambda DNA? (Answer this in the same way as question 4b.) (Notes.)

9. Describe the nucelotide sequence where BamHI cuts. (Text Reference)

10. How many pieces would there be in a mixed digest (EcoRI and BamHI)?

11. Draw a straight line map of lambda DNA, to the same scale as indicated above, and indicate with an arrow where each cut would be in a mixed digest. On the bottom of the line map indicate the total length from the start of the lambda DNA of each cut. Indicate above the line the actual size in base pairs of each of the fragments. (3 points)

12. From the semilog graph that you have already made of your own individual EcoRI digest and the distance moved by each of the bands (your standard curve) determine the distances that the bands of of digest of BamHI should move. (Use red dots and red circles around the dots and indicate the size in kilobases, example: 3,600 bp = 3.6 Kb. Round the numbers off to the nearest 0.1) (3 points)

13. Determine the distances that bands would move in a mixed digest of EcoRI and BamHI. (Use green dots and green circles around the dots, and indicate the size in kilobases.) (5 points)

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