LA-SUB--96-6

**PROJECT REPORT TO STB/UO
NORTHERN NEW MEXICO COMMUNITY COLLEGE
TWO-YEAR COLLEGE INITIATIVE - BIOTECHNOLOGY**

FEB 11 1996

OSTI

Abstract

This report summarizes a project funded by the Los Alamos National Laboratory's Science and Technology Base Programs/University Outreach Division during calendar year 1995. The project, a faculty directed undergraduate research experience, focused on biotechnology research and its applications. Researchers and collaborators at Northern New Mexico Community College and at the Life Sciences Division at LANL will be pleased to provide additional details or explanation of this project.

Overview

The biology program at Northern New Mexico Community College has been involved in screening for mutations in human DNA for over three years. This research effort has been in collaboration with the Cell Growth Damage and Repair Group (*currently the DNA Damage and Repair Group*) of the Life Sciences Division at Los Alamos National Laboratory (LANL). With support from the NSF and other federal sources, this program has developed the ability to perform many standard and advanced molecular biology techniques. Most of these are based around the polymerase chain reaction (PCR) and include the use of single strand conformation polymorphism analysis (SSCP) and denaturing gradient gel electrophoresis (DGGE) in the screening for mutant DNA molecules. Recently, Northern has acquired the capability to sequence PCR generated fragments of DNA using non-isotopic imaging. At Northern, these activities have a two-fold objective: 1) to bring current molecular biology techniques to the teaching laboratory, and 2) to support the training of minority undergraduates in research areas that stimulate them to pursue advanced degrees in the sciences. The college has supported the development of its biology faculty in this area through NSF, NIH, and Department of Energy (DOE) training programs. The faculty, in turn, serve as mentors to undergraduate science majors through undergraduate research experiences (URE's). Students in the URE's receive support from the New Mexico Alliance for Minority Participation (AMP), Arizona State University's Coalition to Increase Minority Degrees (CIMD), the University of New Mexico's Minority Engineering Mathematics and Science project (MEMS), and LANL's Science and Technology Based/University Outreach (STB/UO) program.

The LANL-STB/UO Biotechnology Project

On September 8, 1994 a forum held at LANL's University House asked about the feasibility of using molecular genetic approaches to address ethnohistorical and medically related research questions. Anthony Sena, chairman of Northern's biology program, and a visiting scientist at LANL's LS division, was among the participants in these discussions. Several of these questions directly relate to disease associations and genetic markers in the Pueblo and Hispanic populations of northern New Mexico. From these discussions a project was developed and submitted to LANL proposing to perform preliminary molecular genetic analysis at the Espanola campus. In December of 1994, Los Alamos National Laboratory through its STB/UO program awarded the Mathematics, Science and Engineering department at Northern New Mexico Community College a small grant to pursue this research. The primary focus of the project was to utilize the URE format to train undergraduates in the use of molecular genetic techniques in uncovering mutations and/or polymorphism's in alleles known to be associated with certain diseases. An interesting question raised by these associations is that certain haplotypes (specific genetic polymorphisms) tend to be associated with particular ethnic groups. In addition to Northern's MSE department, this project also involved LANL's LS Division.

In addition to technical assistance, LS Division provided supplies and materials, reagents, and construction of oligonucleotide primers to target a genetic region of interest. Through the assistance of the STB/UO office, LS provided Anthony Sena with a transport pass in order to get these materials to the Espanola Campus.

MASTER

In February 1995, two undergraduate interns were selected for this project. The URE format includes a total of six hours a week in the laboratory and three hours each week in seminar sessions to discuss the nature and progress of the effort. The interns were required to maintain laboratory notes and present these during the seminar session.

Conceptual Background and Experimental Design

Sensitive molecular genetics techniques are made possible by having sufficient quantities of DNA for analysis. The PCR is an ideal means of generating large amounts of DNA through amplification of specifically defined fragments of genetic regions. The PCR uses specific primer pairs to amplify a DNA sequence in a particular region of a chromosome (Figure 1). Each cycle of amplification will double the amount of this specific molecule. With a typical PCR consisting of 30 -32 rounds, the theoretical yield of PCR product is over 1 billion. The PCR generates such large quantities of molecules that it is possible to differentiate and record polymorphic molecules using conventional ethidium bromide staining. It is through this amplification that DNA is prepared for DGGE analysis.

Two or more otherwise identical DNA molecules that differ in base pair sequence have certain properties under differing conditions. At increased temperatures ($>80^{\circ}\text{C}$) a two-stranded DNA molecule will begin to dissociate due to disruption of the hydrogen bonding holding the duplex base pairs to each other. This dissociation (called "melting") does not result in complete strand separation but rather will melt differentially depending on the type and number of base pairs in particular regions of the molecule. There are two hydrogen bonds holding each adenine (A) and thymine (T) pair together while three hydrogen bonds hold paired guanine (G) and cytosine (C) together. Because of this, regions rich in G-C pairs will require higher temperatures or temperature equivalents before strand separation occurs compared to regions rich in A-T pairings (Figure 2).

Figure 2. Hydrogen bonding between base pairs in a DNA duplex molecule. Each G-C pair has three hydrogen bonds, Each A-T pair has two hydrogen bonds.

DGGE exploits this differential strand separation of DNA by subjecting the molecules to a chemical matrix representing varying temperature equivalents (i.e., % chemical denaturants). More specifically, DGGE involves the differentiation of homologous segments of DNA through dissociation of DNA molecules as they migrate through a denaturing electrophoretic gel. Unlike standard, or non-denaturing, gel electrophoresis (which sorts DNA molecules by size; small molecules moving at a faster rate than large ones) denaturing gradient gels are constructed with a chemical denaturant gradient that induces differential melting by different DNA molecules. As DNA molecules move through the denaturing gradient, differential melting of the respective duplex will result in a decreased rate of migration by these molecules in the gel relative to the other non-denatured (non-melted) molecules (Figure 3). This technique can be sensitive enough to detect a single base-pair difference between two homologous molecules.

In order to be assured that differential melting is due to base differences in the sequences being studied, PCR generated fragments must first be artificially constructed to contain a "high-melt domain". In our analyses, the DNA molecules from the first PCR provide the target for a second round PCR which incorporates a 40 base pair CG (cytosine-guanine) rich "high-melt clamp" to one end of the amplified DNA. The CG clamp assures that all the DNA molecules will have a standard melting domain so that any differences observed through DGGE will be

reflective of specific polymorphisms. Attachment assures that the homologous DNA molecules will remain paired at this region as they move through the denaturing gel so that differences in migration rate will be due to any base pair differences in the molecules being studied.

Two kinds of DGGE analyses are performed on the clamped PCR samples. The first, called perpendicular DGGE, allows for the determination of optimal denaturant conditions for the DNA fragments under study. The second, called parallel DGGE, allows for the resolution of differences in two or more homologous fragments by providing a unique "fingerprint" for a mixture of two kinds of DNA samples (Figure 4).

Experiments

Our first set of experiments were designed to test the efficiency of the screening methods on PCR generated fragments by comparative analysis of untreated and treated DNA samples. These genomic DNA samples were provided by the LS division. The untreated sample, derived from a TK-6 cell line, was designated as the "wild-type". The other samples (α W5 and WTK1) are derived from TK-6 but have been subjected to varying dosages of ionizing radiation. These were the treated samples and were designated as "mutant". The wild-type sample provided a standard with which to compare the effectiveness of the screening techniques. Because ionizing radiation has been shown to cause genetic changes in DNA, the mutant samples are likely to contain molecular differences in their genetic composition.

Our initial experiments used the PCR to amplify DNA from a region within our genomic samples (see flowchart in Figure 5). The *p53* gene was chosen because exon 7 of this loci is known to be associated with radiation damage, and would thus serve as a genetic region likely to contain genetic changes. Once a polymorphism is indicated by the DGGE these DNA samples are sequenced to identify the specific biochemical difference in the molecules. DNA sequence information provides the basis for asking questions about linkage of specific sequences to other alleles.

Undergraduate interns were successful in generating enough product from exon 7 for us to attempt DGGE analysis. The clamped product was subjected to perpendicular DGGE to test for molecular differences in the DNA and also to give us an indication of the optimal denaturing conditions. The data (Figure 6) indicated that a mixture of wild-type and mutant samples contained different molecular sequences.

In order to discern a specific "fingerprint" for each sample, the clamped product was then analyzed on parallel DGGE. In this analysis DNA samples move parallel to the denaturing gradient. Melting is dependent on the sequence of the samples and, if different, when melted the sample will slow down in the gel. Because of this it was possible to differentiate between the TK-6 and the α W5 samples (Figure 7).

Before each sample is analyzed by DGGE it is placed at 100° C for 5 minutes and then allowed to cool to room temperature for 10 minutes. The 100° temperature causes complete strand dissociation of the PCR product, while slow cooling to room temperature allows each single strand to reassociate with a complementary strand. If the genomic DNA from which the PCR product was amplified is derived from an individual that has two different alleles (and therefore two differing DNA sequences), each of the separated strands can come together with any complementary strand. For this reason, it is possible to end up with two strands that have a "mismatch" at the site where a difference exists. Such a molecule is called a "heteroduplex". In Figure 6, the TK-6 molecules migrate to a single position on the gel indicating that these molecules were all of the same sequence (called "homoduplexes"). This is also true for the WTK1 and the α W5 DNA samples, but note that the WTK1 melts at a lower denaturant condition than either the TK-6 or the α W5. Differential melting tells us that the WTK1 molecule is different from either TK-6 and α W5. The TK-6 and the α W5 samples, on the other hand, migrate to the same position on the gel. This makes it impossible to tell if the TK-6 and the α W5 samples are different or identical. The heating and reassociation of a mixture of these samples does allow us to make this determination. If the two samples are identical they will reassociate into identical molecules and should therefore migrate to the same position on the gel. If, on the other hand, the samples are different heteroduplexes can be expected to form and each of these would then migrate in a different manner on the gel. This is shown very clearly in lanes 4

and 6 of the parallel gel which contain a mixture of TK-6 + WTK1 and WTK1 + α W5. Lane 5 on the other hand, which contains a mixture of TK-6 + α W5 shows the same migration pattern as both TK-6 and α W5 alone, indicating that these are identical. The subsequent phase of this analysis is to determine the actual biochemical basis for the difference in the WTK1 molecule through DNA sequencing.

The ability to discern different molecules by the creation of heteroduplexes, each of which has different melting characteristics, makes DGGE and DNA sequencing powerful diagnostic and research tools. The undergraduates in this project were able to show that they could perform this relatively sophisticated procedure in a college laboratory setting. With this training the project team turned its focus on the use of this technology to questions of molecular genetics and disease.

Our general focus is on the relationship between genetic polymorphism and disease. We are particularly interested in the autoimmune disease known as *pemphigus vulgaris*, a rare disease which occurs with relatively high incidence in Hispanic populations. This disease is also associated with alleles located on the P (short arm) arm of human chromosome 6. This region called the major histocompatibility complex (MHC), is a region of extreme genetic diversity. The MHC contains several genes associated with the immune response. The MHC-type II subregion encompasses several loci (*principally loci classified as DR, DQ and DP*) each of which contains many alleles. Recent genetic evidence associates specific alleles in the MHC-type II DR loci with susceptibility to several autoimmune diseases (*among these are rheumatoid arthritis, insulin dependent diabetes and pemphigus vulgaris*).

Figure 8. Experimental design for DGGE analysis of pemphigus DNA.

Since we now know that we can use DGGE to screen for polymorphisms in homologous DNA sequences, we are currently asking about the practicality of using polymorphisms as markers for genetic disease. At this point it is not clear whether these mutations are directly involved in causation of certain diseases, or if a particular polymorphism is associated to a specific disease through linkage disequilibrium with other loci. Genomic DNA from several human cell lines,

along with primer sets for the MHC DR, DQ, and DP region of chromosome 6 were provided by LS Division. We tested the suitability of the DNA and the effectiveness of the primers in preliminary PCR's of 10 independent genomic samples.

To date we have performed DGGE on two of these samples (which will serve as our controls) and have had limited success in resolving differences in migration rate. The problem appears in non-specific amplification of the control samples. We are currently collaborating with LS division and the University of New Mexico Medical School to obtain genomic samples from pemphigus patients. Also LS has recently obtained sequence information for the DRB1 allele and a genetic haplotype called DRB1 0402 which occurs most frequently in patients than in New Mexico Hispanic controls. This information will allow us to construct additional primer sets and further refine our PCR amplifications.

Project Personnel

This project involved personnel from LS Division along with faculty members and student interns. Rachel Kear and Victoria Baros participated in this project from February to April 1995. In May both of these students accepted internships at other sites and were unable to continue in the project. Trinidad Lopez and Yolanda Martinez were selected to continue during the summer months (June, July, and August). Richard Okinaka, LS staff member, served as the consultant and mentor for the project. Anthony Sena, served as on-site mentor and laboratory facilitator.

The *p53* data obtained in this project has been of use for one of the post-doctoral fellows at the LS-Division. This data shows that one of the samples tested has been changed at this locus. While this is not a new finding, it does represent the first time that DGGE analysis has been

used to identify this mutant. The testing of non-isotopic technology for these analyses also provides information to LS Division on the efficacy of using such methods in lieu of analysis using radioactivity. DGGE analysis of the MHC loci of the pemphigus DNA will complement different approaches currently under study at LS Division.

The value of this project to Northern has been in the experience, skills, and knowledge that all four interns have acquired. This process has also made our students aware of similar programs throughout the nation. The project has introduced the students to biotechnology and biomedical research and, in the process, has brought the concepts of molecular biology out of the realm of textbook abstraction. This project continues with support from CIMD and UNM-MEMS. The undergraduate interns will be asked to prepare their research data and encouraged to present their results at either a regional AMP or SACNAS meeting.

Acknowledgments

Northern New Mexico Community College extends its gratitude to LANL's STB/UO and LS Divisions for their encouragement and support of this project. Special thanks are extended to Abad Sandoval (STB) and Rich Okinaka (LS), for their kindness and help in developing this project. The continuing progress of this project is made possible by the understanding and support of Connie Valdez, President, and Jose Griego, Dean of Instruction at Northern New Mexico Community College.

References

1. Erlich, H. A. and T. L. Bugawan. HLA Typing, In: *PCR Protocols, A Guide to Methods and Applications*. 1995. Academic Press.
2. Klein, J., N. Takahata, , and F. J. Ayala. 1993. MHC polymorphisms and Human Origins. *Scientific American*, December, 1993.
3. Okinaka, R. T., S. L. Anzick A. Oller, and W. G. Thilly. 1993. Analysis of large x-ray induced mutant populations by denaturing gradient gel electrophoresis, *Radiation Research*, 135, 212-221.
4. Scharf, S. J., A. Friedman, et al. 1988. HLA class II allelic variation and susceptibility to pemphigus vulgaris. *Proceedings of the National Academy of Science*, 87: 3504-3508.
5. Scharf, S. J., G. Horn, H. Erlich. 1986. Direct cloning and sequence analysis of enzymatically amplified genomic sequences. *Science*, 223: 1076-1078.
6. Steinman, Lawrence. 1993. Autoimmune disease. *Scientific American*, September, 107-114.
7. Trowsdale, J., Young, J. A. T, Kelly, A. P. (et al.). 1986. Structure, Sequence and Polymorphism in the HLA-D region. *Immunological Reviews*, 85: 1- 43.
8. Wordsworth, P. 1991. PCR-SSO Typing in HLA disease association studies. *European Journal of Immunogenetics* 18: 139-146.

DISCLAIMER

This report was prepared as an account of work sponsored by an agency of the United States Government. Neither the United States Government nor any agency thereof, nor any of their employees, makes any warranty, express or implied, or assumes any legal liability or responsibility for the accuracy, completeness, or usefulness of any information, apparatus, product, or process disclosed, or represents that its use would not infringe privately owned rights. Reference herein to any specific commercial product, process, or service by trade name, trademark, manufacturer, or otherwise does not necessarily constitute or imply its endorsement, recommendation, or favoring by the United States Government or any agency thereof. The views and opinions of authors expressed herein do not necessarily state or reflect those of the United States Government or any agency thereof.