The Moran Foundation
DEPARTMENT OF PATHOLOGY
BAYLOR COLLEGE OF MEDICINE
TEXAS MEDICAL CENTER
HOUSTON, TEXAS 77030

June 23, 1992

Russell M. Lebovitz, M.D., Ph.D.
Department of Pathology
Baylor College of Medicine

Dear Dr. Lebovitz:

Please update me on the status of your Moran Foundation project (1-90-0046) entitled "Identification of Specific DNA-Binding Proteins Using Photoactive Cross-linking Agents".

Since approval and funding is generally for a one-year period, all projects approved in or prior to June 1991 should now be "complete", or nearly so.

I need a progress and/or final report regarding your project, including dates and times of any presentations, and information regarding any publications.

Please submit this to me within the next 30 days.

Sincerely yours,

Philip J. Migliore, M.D.
Research Director

PJM/ms

c: Dr. Michael Lieberman
Mr. John Moran

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June 30, 1992

Philip J. Migliore, M.D., Research Director
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Dr. Migliore:

I am pleased and grateful to report to The Moran Foundation that our project (1-90-0046) entitled "Identification of Specific DNA-Binding Proteins Using Photoactive Cross-Linking Agents" has resulted in a well-received formal presentation at the Texas Society of Pahologists December 1990 meeting in San Antonio (presented by Dr. Samreung Rangdqeng, a senior resident in our program), as well as a recently-submitted manuscript.

Our initial goal was to evaluate several different methods for chemically-linking DNA-binding proteins to synthetic oligonucleotides containing specific protein binding sites. We compared the relative efficacy of direct cross-linking via UV-activated thymidine residues with that mediated by azidophenyl glyoxal. UV-activated thymidine exhibits a very low efficiency, but a high degree of specificity due primarily to the zero-length nature of the direct crosslink. UV activated thymidines are only able to crosslink to amino acid residues lying immediately adjacent (distance less than approximately 4 angstroms) to the UV-activated DNA; this accounts for both the low efficiency and high specificity of the crosslink. Azidophenyl glyoxal (APG), in contrast, has an effective linear span of 8-14 angstroms, and the activated intermediate has a half-life considerably greater than that of UV-activated thymidine. As a result, APG binds DNA-binding proteins with great efficiency, but is able to bind proteins which are only loosely associated with a given DNA sequence.

Our strategy was as follows. APG was pre-bound to double-stranded oligonucleotides synthesized in the Department of Pathology and carrying specific protein-binding sites. These APG/oligonucleotide complexes were incubated with nuclear extracts from normal and ras-transformed liver epithelial cells, and specific DNA-protein complexes were allowed to form quantitatively. The reaction mix was treated with UV light at a wavelength of 310 nm which activates the azido moiety of APG, but not the endogenous thymidines. Parallel reactions in the absence of APG were illuminated with 254nm UV light which can activate thymidine. Since the oligonucleotides were end-labeled at their 5' ends with ^{32}P-phosphate, the unbound and the cross-linked oligonucleotides were easily distinguished by electrophoresis under denaturing conditions. Our results indicate that APG can efficiently crosslink DNA to sequence-specific binding proteins, and that the background of non-specific cross-linking can be reduced substantially by decreasing the total amount of nuclear protein in the assay. Unfortunately, the glyoxal moiety of APG binds preferentially to single-stranded DNA, and the binding of more than one APG per oligonucleotide is sufficient to cause complete denaturation and separation of the strands; this may be overcome by using longer stretches of DNA, although we have not yet confirm ed this directly. Our results also indicate that the methods we have developed would be particularly useful for probing the specific interactions between single-stranded RNA and sequence-specific proteins.

Since our results with APG clearly indicated that oligonucleotides smaller than 100 base pairs are too small to retain the double stranded character after extensive coupling to APG, we have attempted to produce longer templates by *in vitro* mutagenesis of circular plasmids. During these efforts we have devised a method which greatly improves the efficiency of mutagenesis using standard methods. Briefly, the method of choice at present is to prepare circular single-stranded DNA template in bacterial strains which allow substitution of Uracil for Thymidine residues. Mutagenesis is performed by hybridizing an oligonucleotide which is mostly complementary to the template except for a small region in which the desired mutation lies. The mutagenic oligonucleotide is used to prime synthesis of a second strand with DNA polymerase followed by ligation to produce a double-stranded covalently closed circular DNA (cccDNA). The cccDNA is transfected into wild type bacterial strains that degrade the Uracil containing strand and allow preferential replication of the oligonucleotide-primed mutated strand.

We have consistently encountered a problem with this method involving unavoidable small amounts of degraded DNA contaminating our single-stranded circular DNA template. This degraded DNA serves as an effective primer for the second strand, but does not carry the desired mutation. The net effect of this contaminant is to greatly reduce the frequency and thus the efficiency of the desired mutation. We have modified the mutagenesis protocol to include a single incubation with E coli Exonuclease III, which removes the endogenous primers, but leaves the single-stranded circular DNA template intact. This single step increases the frequency of desired mutations as much as 10-20 fold. We have recently submitted a brief manuscript on this procedure, and have explicitly credited The Moran Foundation for support.

I am grateful to The Moran Foundation, and to Mr. Moran for supporting this project.

Sincerely,

Russell M. Lebovitz, M.D., Ph.D.
Assistant Professor of Pathology and Cell Biology

RML/nb

A simple method for improving the efficiency of mutagenesis during oligonucleotide-directed *in vitro* mutagenesis

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Site-directed *in vitro* mutagenesis is a valuable and widely used procedure for the study of structure-function relationships involving regulatory and coding regions of DNA (1). Most commonly accepted methods introduce directed mutations on single-stranded circular templates using mutagenic oligonucleotides complementary to the region to be mutated except for a small region of mismatch. Using the mutagenic oligonucleotide as a primer, the single-stranded circular template is copied with bacterial or phage DNA polymerase and sealed with DNA ligase to produce a closed-circular double-stranded DNA with targeted mutations in one strand. The yield of mutant progeny using this procedure theoretically should be 50%, but in practice the actual yield is much lower due to factors such as i) host-directed mismatch repair mechanisms (2); ii) incomplete *in vitro* polymerization ; and iii) primer displacement by DNA polymerase *in vitro*.

Kunkel (3) has described a method which provides a strong selection against the original non-mutagenized template. Single-stranded (ss) DNA templates are synthesized in *dut ung* double mutant bacterial strains resulting in occasional substitution of uridine for thymidine because of the *dut* mutation. The *ung* mutation inactivates Uracil N-glycosylase, which allows the incorporated uridine to remain in the DNA. After annealing with mutagenic oligonucleotides and conversion to covalently closed circular (ccc) DNA with DNA polymerase and ligase, the DNA is transfected into bacterial strains (*ung*$^+$) that preferentially inactivate the non-mutant uracil-containing template strands through the action of Uracil N-glycosylase. One potential problem with this procedure is non-specific priming caused by contaminating nucleic acid in the ssDNA template. These contaminants can increase the background significantly, since the DNA polymerase elongates endogenous and exogenous mutant primers with approximately equal efficiencies. In the event of endogenous priming activities, Bio Rad recommends the preparation of a new template. However, despite costly and time-consuming re-preparation of single-stranded templates, we frequently observe mutation efficiencies below 50%.

The modified procedure we report here requires only one additional enzymatic step and increases the efficiency of mutagenesis to 90-100% using our "contaminated" template preparations. This increased efficiency of mutagenesis dramatically reduces the time and material spent sequencing potential mutants. Briefly, bluescript clones of the 5′ cfos regulatory region were transfected into *dut ung* bacteria by electroporation. Infections of transformed bacteria with helper phage, preparation of phagemids and isolation of uracil containing ssDNA were performed as suggested by the supplier. Two different

strategies to eliminate endogenous priming of single-stranded templates were attempted : 1) heat denaturation at 90°C for 5 minutes followed by rapid chilling on ice and 2) incubation with E. coli Pol I (in the absence of dNTPs) or Exo III at 37°C for 30 minutes. After heat inactivation, the reaction was phenol extracted (once), phenol-chloroform extracted (once) and EtOH precipitated. The pellet was dried and resuspended in 1X TE(8) buffer (10mM Tris HCl pH8, 1mM EDTA).

To introduce mutations within the 5'-flanking regions of the cfos gene, the primers FP1 (5' CGTGGAAACCTG**C**T**A**AGGCAGATGTCCTAAT 3') and DS1(5'TCCTAATATGGACATC**ACT**TGTAAAGGAGG 3'; mutated bases **underlined**) were used to prime untreated and exonuclease treated ssDNA. Annealing, elongation and ligation were performed in strict accordance with the protocol provided by Bio Rad. After polymerization and ligation, the reactions were monitored on TAE-1% agarose gels. Aliquots of the reactions products were transfected into *dut*$^+$ *ung*$^+$ bacteria by electroporation. Ampicillin-resistant colonies were picked randomly; plasmid DNA was prepared and sequenced (4).

Figure 1 shows results obtained when the *in vitro* mutagenesis reactions were analyzed on ethidium bromide-agarose gels. The second strand synthesis reaction results in the formation of cccDNA, which migrates more slowly than ssDNA in the TEA/agarose/EtBr gel system. It is clear that ssDNA templates prepared in the absence of Exo III or Pol I (lanes 2 and 3) contain considerable endogenous priming activities, since there is almost as much cccDNA in the control lane (lane 2) as in the lane with added mutagenic primer (lane 3). For templates heated alone or treated with Pol I, the amount of cccDNA in the absence of exogenous primers is visibly reduced compared with untreated template(compare lane 2 with lane 4), but enough nonspecific priming still occurs to give a relatively high percentage (>30%) of non-mutagenized plasmids upon screening. In contrast, after treatment of the template with Exo III (Pharmacia; reaction performed in 1X Exo III buffer), the amount of cccDNA in the control lane (lane 5) is reduced to levels undetectable by EtBr staining Furthermore, subsequent transfection and screening of colonies produced from Exo III-treated templates results in greater than 90% mutants as confirmed by sequencing.

Figure 2 shows autoradiographs of sequencing reactions of wild type and mutant clones in two different regions of the cfos promoter when synthetic oligonucleotides FP1 and DS1 were used as mutagenic oligonucleotide primers with our modified

procedure. We have also successfully used additional oligonucleotides (not shown) to introduce a variety of different mutations into this region. Because of the high efficiency of this modified system, we have been able to reduce both the time and cost of producing many different types of mutations including substitutions, deletions and insertions.

1. Kramer B, Kramer W, Fritz HJ. *Cell* 1984; 38:879-887.
2. Kunkel TA. *Proceedings of the National Academy of Sciences USA* 1985; 82:488-492.
3. Promega Corp. *Sequencing protocol using end-labeled primer. fmole DNA sequencing system technical manual.* 1992.
4. *Recombinant DNA Methodology, Chapters 17-21*, Wu R, Grossman L, ed. New York, NY: Academic Press, 1987.

FIGURE LEGENDS

Figure 1. Effect of ExoIII, pol I, or heat treatment on endogenous priming. Single-stranded circular templates (lanes 3-8) were incubated at 95°C for 5 min., rapidly cooled on ice, and treated with Exo III (lanes 7,8) or pol I (lanes 5, 6) for 30 min. at 37°C. Mutagenic oligonucleotide primers were added to half of the samples (lanes 4,6,8), followed by elongation (T7 DNA polymerase), ligation and electrophoretic analysis according to the manufacturers instructions (Bio Rad). Hind III/Eco RI-lambda DNA fragments (lane 1) and single-stranded circular phagemid DNA (lane 2) were used as size standards.

Figure 2. Double-stranded sequencing of wild type and mutant clones. Mutated bases are indicated by asterisks. Panel 1 shows wild type (A) and mutated (B) sequences corresponding to the FP1 oligonucleotide (B). Panel 2 shows WT (A) and mutated (B) sequences corresponding to the DS1 oligonucleotide.