BAYLOR
COLLEGE OF
MEDICINE

One Baylor Plaza
Houston, Texas 77030-3498
Department of Pathology
Section of Molecular
Pathobiology
TEL: (713) 798-4661
FAX: (713) 798-5838

June 6, 1995

Philip Migliore, M.D.
Research Director
Moran Foundation

Dear Dr. Migliore:

Enclosed please find my progress report for Moran Foundation Project number 1-94-0074. We have made significant progress on this work in the past funding period. I would like to express my appreciation for the support provided by the Moran Foundation.

Enclosed are 4 copies of a publication acknowledging support from the Moran Foundation (#1 listed below). The preliminary results from this work were recently presented at the RNA Processing Meeting in Cold Spring Harbor, New York on May 17-21, 1995. Below is a list of recent publications. Publication #4 describes work supported by the Moran Foundation in 1993-1994.

1. Lee, A.B. and Cooper. T.A. (1995) An improved direct PCR screen of bacterial colonies: wooden toothpicks inhibit PCR amplification. BioTechniques 18, 225-226.
2. Ramchatesingh, J., Zahler, A.M., Neugebauer, K.M., Roth, M.B. and Cooper, T.A. (1995) A subset of SR proteins activates splicing of the cardiac troponin T alternative exon by direct interactions with an exonic enhancer (submitted)
3. Humphrey, M.B., Bryan, J., Cooper. T.A., Berget, S.M. (1995) A 32 nucleotide exon splicing enhancer regulates usage of competing 5' splice sites in a differential internal exon. Mol. Cell. Biol. (in press).
4. Coulter, L., Landree, M., and Cooper, T.A. In vivo selection of exonic splicing enhancers. (in preparation)

If you have any questions, please feel free to contact me.

Sincerely,

Thomas A. Cooper, M.D.
798-3141 office
798-5838 fax
tcooper@bcm.tmc.edu

MORAN FOUNDATION PROJECT (1-94-0074)

A cell-free assay for regulated pre-mRNA alternative splicing

The aims of this proposal are to establish a cell free complementation assay for positive-acting factors that regulate alternative splicing during striated muscle development. We have made significant progress toward the goals of this project.

To establish a cell free complementation assay for factors in embryonic muscle that activate exon inclusion, we first reproduced default alternative splicing of exon 5 in nuclear extracts from a human nonmuscle cell line (HeLa). A truncated RNA substrate containing exons 4, 5, and 6 was spliced in vitro. All in vitro splicing products and intermediates were identified. As expected of non-muscle cell extracts, exon 5 is predominantly skipped (10-20% of total spliced RNA included exon 5, depending on the nuclear extract preparation). During the funding period we have significantly improved our in vitro splicing assay. The spliced products are subject to degradation due to 3' exonuclease activity causing size heterogeneity causing smearing on electrophoresis gels. To more accurately measure the level of exon inclusion, we perform primer extension on whole in vitro splicing reactions using a 32P-labeled oligonucleotide that anneals to exon 6. Relative mRNA levels are quantitated directly from the gel using a Betagen Betascope 610.

To identify a source of muscle nuclear extract containing factors that activate exon inclusion, we screened several skeletal muscle cell lines for appropriate regulation of the endogenous cTNT gene. The quail muscle cell line, QM7 was found to express high levels of endogenous cTNT mRNA in which >90% of the mRNAs included exon 5. Therefore, alternative splicing was appropriately regulated in these cells. Nuclear extracts were prepared from differentiated QM7 cultures using our current small-scale procedure. When 2 or 5 p.l of this extract (approximately 5 lig protein/p1) was added to a 25 p1 splicing reaction containing 10 of HeLa nuclear extract, the level of exon inclusion is significantly increased. Even 1 gl (5 jig) of QM7 nuclear extract had detectable modulatory activity. In control reactions, addition of 2 or 5 p1 of HeLa extract did not activate exon inclusion. Both HeLa and QM7 nuclear extracts were in the same buffer so that alterations in the level of exon inclusion is not due to differences in ionic conditions but rather a trans-acting factor present in muscle nuclear extracts that are absent in nonmuscle nuclear extracts. These results have been reproduced using three different QM7 nuclear extract preparations.

Therefore. we have established a complementation assay for factors that activate muscle-specific exon inclusion. It is important to note that QM7 extracts alone do not contain splicing activity yet complement HeLa extracts. Given that a relatively small amount of muscle nuclear protein activated inclusion, we believe that QM7 muscle extracts contain a relatively abundant activity that modulates the default splicing pattern of the HeLa constitutive splicing machinery. These results provide strong evidence for a positive-acting regulator in differentiated muscle extracts. This is an important step towards characterizing and isolating factors that regulate alternative splicing.

The in vitro assay has been established by Kathy Ryan, a third year graduate student in the lab. Ms. Ryan has made significant progress in the past year having established a primer extension assay for alternative splicing in vitro, screened a

number of stable muscle cell lines, identified and prepared nuclear extracts from an appropriate cell line, and established conditions to detect a muscle-specific response in vitro. She is now beginning the biochemical characterization/fractionation of this splicing activity.

These preliminary results were recently presented at the RNA Processing Meeting in Cold Spring Harbor, New York on May 17-21, 1995. These preliminary studies have been included in a proposal to obtain funding from an outside agency.

Improved Direct PCR Screen for Bacterial Colonies: Wooden Toothpicks Inhibit PCR Amplification

The polymerase chain reaction (PCR) can be used for rapid analysis of recombinant DNA directly from bacterial or yeast colonies, or viral plaques (1-6). In a particularly convenient modification of this procedure, colonies are transferred directly into a complete PCR and cells are lysed during an extended denaturation step prior to cycling (1,3,5). While optimizing conditions for a direct PCR screen of bacterial colonies, we were surprised to discover that the wooden toothpicks used to transfer colonies inhibited PCR reactions containing low amounts of *Taq* DNA Polymerase. A comparison of the effects of wooden and plastic toothpicks on PCR reactions containing 0.1, 0.3, 1.0 and 3.0 units of *Taq* DNA Polymerase is shown in Figure 1. The results can be summarized as follows: *(i)* The expected amplicon was obtained from colonies picked using plastic toothpicks in all amounts of *Taq* DNA Polymerase tested (lane 2). In contrast, PCR products were not detected in reactions containing less than 3 units of polymerase when colonies were picked using wooden toothpicks (lane 3). *(ii)* This difference is due to inhibition of the PCR by the wooden toothpick, since amplification of the same amplicon from 50 ng of plasmid is inhibited at all levels of enzyme tested by briefly swirling a wooden toothpick in the reaction (lane 4). *(iii)* The nature of the inhibitor is unknown; however, it is water soluble since presoaking wooden toothpicks in water for 10 min reduces the effect (lane 5). In addition, water that is pre-treated with wooden toothpicks by brief swirling also inhibits the PCR reaction (data not shown). This inhibition was observed with *Taq* DNA Polymerase (Perkin-Elmer, Norwalk, CT, USA) and Vent ® DNA polymerase (New England Biolabs, Beverly, MA, USA) and two major brands of wooden toothpicks (Diamond, Minneapolis, MN, USA, and Forster, Wilton, ME, USA) taken directly from the box or following autoclaving (data not shown). The results shown in Figure 1 have been reproduced with two different amplicons.

According to a representative of Forster, no chemicals are added during toothpick production, suggesting the interesting possibility that the inhibitor is intrinsic to the wood. All toothpicks made in the U.S. are from white birch.

The salient point from these investigations is that the amount of enzyme can be significantly reduced when plastic toothpicks or pipet tips, rather than wooden toothpicks, are used to transfer colonies. Based on this, we have established a reliable PCR colony screening that significantly reduces enzyme costs in comparison with previously published procedures. In our laboratory, this procedure has been successful for every oligo pair tested (>15) on several different amplicons. The oligonucleotides used for these screens were on hand from previous work. We screen for recombinant clones using DNA minipreps only when an appropriate oligo pair is not available.

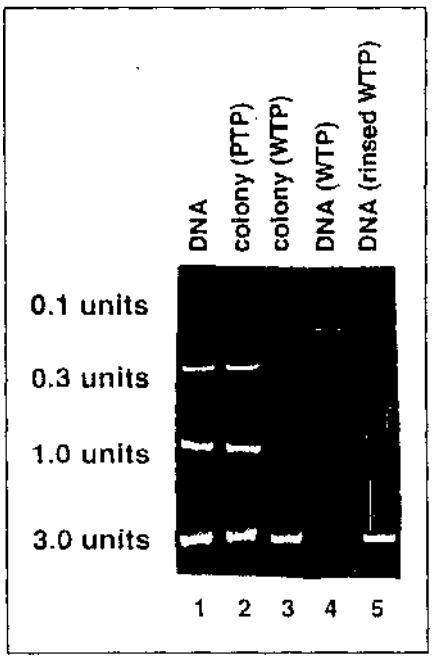

Figure 1. Wooden toothpicks inhibit PCRs
PCRs containing 0.1, 0.3, 1.0 or 3.0 units of *Taq* DNA Polymerase were used to compare the effects of wooden and plastic toothpicks. Reactions contained 50 ng plasmid DNA (lane I). Single bacterial colonies containing the same plasmid were introduced into the PCR using plastic (PTP, lane 2) or wooden (WTP, lane 3) toothpicks. A wooden toothpick was briefly swirled in a reaction containing 50 ng plasmid DNA (lane 4). A wooden toothpick soaked in distilled water for 10 min and then dried was briefly swirled in a reaction containing 50 ng plasmid DNA (lane 5). After 20 cycles, 10 p1. (of 40 pL) were loaded on a 4% nondenaturing acrylamide gel and stained with ethidium bromide. The amplicon is 244 bp.

Procedure

1. Make up and aliquot reaction cocktail for 40-pt reactions: lx Perkin-Elmer buffer [10 mM Tris-HCI (pH 9.0 at 25°C), 50 mM KCI, 0.1% Triton® X-100); 1.75 mM $MgC1_2$; 0.2 mM dGATC. For each reaction, include 70 ng each oligonucleotide and 0.3 units of *Taq* DNA Polymerase.
2. Use plastic toothpicks (or yellow tips) to transfer colonies (.^ 1 mm in diameter) into aliquots with brief swirling, and then stab an agar plate containing the appropriate antibiotic. Up to 60 stabs can fit on one 100-mm plate.
3. Add one drop light mineral oil and run the following program: 95°C for 10 min; then 20 cycles of 92°C x 1 min, 42°C x 1 min, 72°C x 1 min and a final 5-min step at 72°C. We use a PTC-100, Model 60 thermal cycler (MJ Research, Watertown, MA, USA).
4. For analysis, load 10-20 pi- directly on an appropriate sizing gel or add directly into a 40-FL restriction digest.

REFERENCES

1. Gussow, D. and T. Clackson. 1989. Direct clone characterization from plaques and colonies by the polymerase chain reaction. Nucleic Acids Res. 17:4000.
2. **Hawker, K.J. and J•J•** Billadello. 1993. A multiplex polymerase chain reaction colony miniprep. BioTechniques /4:764-765.
3. Rapley, R. and M. Walker. 1992. PCR screening of DNA cloned into polylinker-containing vectors with MI3 sequencing primers. BioTechniques 12:516.
4. Sandhu, GS., J•W. Precup and B.C. Kline. 1989. Rapid one-step characterization of recombinant vectors by direct analysis of transformed *Escherichia coli* colonies. BioTechniques 7:689-690.
5. Sathe, G.M., S. O'Brien, M.M. McLaughlin, F. Watson and G.P. Livi. 1991. Use of polymerase chain reaction for rapid detection of gene insertions in whole yeast cells. Nucleic Acids Res. /9:4775.
6. Zon L.I., **D.M.** Dorfman and S.H. Orkin. 1989. The polymerase chain reaction colony miniprep. BioTechniques 7:696-698.

This procedure was developed as part of a project supported by the Moran Foundation. Thanks to Russ Lebovitz and

Benchmarks

Vaughan Lee for helpful suggestions. Address correspondence to T.A. Cooper; Molecular Pathobiologv Section, Department of Pathology, Baylor College of Medicine, One Baylor Plaza, Houston, TX 77030, USA. Internet: tcooperghcm.unc.edu

Received 21 December 1993; accepted 18 October 1994.

Angie B. Lee and Thomas A. Cooper
Baylor College of Medicine
Houston, DC, USA

Cycle DNA Sequencing from a Gel Band

Since repetitive polymerase chain reaction (PCR) can introduce multiple mutations at a certain rate, it is risky to use only one subclone of the PCR products for DNA sequencing. Several clones should be isolated and sequenced to confirm uniformity of the target sequence. This is time-consuming and questions about the sequence may still remain.

Direct sequencing is possible by isolating the target fragments from an electrophoresis separation. Since the mutated fragments are not dominant among the PCR products, they will not produce a sufficient signal for autoradiography. Many different techniques are now available to isolate the fragments from both agarose and polyacrylamide gels (2). However, those procedures can take a long time and recoveries can be low. Frequently, DNA is lost at the precipitation step due to its low quantity.

We have previously reported that reamplification of the PCR products was possible from the gel bands using toothpicks (1). We have now applied this technique with slight modifications to recover template DNA for cycle DNA sequencing and obtained excellent results. The following procedure will lead to reproducible DNA sequencing results.

1. Electrophorese the PCR sample on a proper agarose gel. We used the small Mupid 2 unit (Cosmo Bio. Tokyo, Japan, or WyoBiGen, Laramie, WY, USA). For successful reactions, each hand to be sequenced should have at least 0.1 pg of DNA.

2. Prepare the reaction mixture for cycle sequencing according to the supplier's protocol (e.g., CircumVentTM DNA Sequencing Kit; New England Biolabs, Beverly, MA, USA). However, the radioisotope (e.g., la-^{32}PIdCTP 13000 Ci/mmol; 10 1.1Ci/mL, Du Pont NEN, Boston, MA, USA! or IdATP [300 Ci/mmol; 3.15 Ki411.., Du Pont NEN[) or the end-labeled primer should not be added at this stage to avoid contaminating the toothpicks. Store the reaction tube on ice until the gel is ready.

3. Poke into the target band with a sterile toothpick at least three times from the back side of the gel.

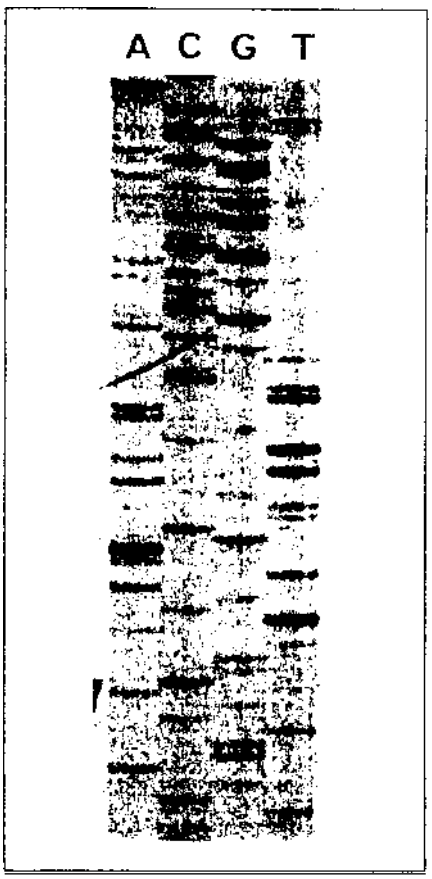

Figure 1. Typical sequencing pattern with a toothpick method. The PCR fragments produced from spider genomic DNA with two minor ampul late-specific primers (M11'2+ and NI1P1-, Mark A. Colgin, University of Wyoming) were electrophoresed on a 0.8% agarose gel (Agarose I; AMRESCO, Solon, OH, USA), picked with a toothpick and sequenced with an MI P2 primer. as described in the text.

4. Rinse the toothpick in the reaction mixture to release the DNA. Since the wooden toothpick can work as a wick and absorb the small amount of reaction mixture in a short time, rinsing should be completed in 5 s.

5. Add radioisotope and enzyme.

6. Boil the mixture for 2 min, cool on ice, then briefly centrifuge.

7. Aliquot 3.2 pL of the mixture into each of four small tubes to which 3 pL of each dideoxy/deoxy mixture have been al iquoted.

8. Mix by pipetting and overlay a drop of mineral oil.

9. Continue the reaction according to the supplier's protocol.

Figure I demonstrates a typical DNA sequencing pattern. We could read consistently approximately 250 bases by a single loading, and the length of sequence read was nearly as good as the standard method for cycle DNA sequencing using the recommended amount of purified template DNA. However, since the toothpicks can carry only a limited amount of DNA, only the cycle number should be extended to 40. Also, autoradiography should be carried out for at least two days, even if the ^{32}P incorporation method is used, in order to obtain an equivalent resolution to the standard method. We recommend the use of ^{32}P rather than ^{35}S to reduce the total sequencing time.

REFERENCES

1. Kadokami, Y. and R.V. Lewis. 1994. Repeated PCR of a gel band can be used to obtain a single PCR hand. BioTechniques 17:438.
2. Samhrook, J., E.F. Fritsch and T. Maniatis. 1989. Molecular Cloning: A Laboratory Mannual. 2nd Edition. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Address correspondence to R. V Lewis, Department of Molecular Biology, University of Wyoming, P.O. Box 3944, Laramie, WY 82071-3944, USA.

Received 14 September 1994; accepted 25 October 1994.

Yoichi Kadokami, Stephanie L. Ruess, Brady W. Mills and Randolph V. Lewis
University of Wyoming
Laramie, WY, USA