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Biotinylated oligonucleotides for DNA-protein methylation protection

Lorri Kay Lockyer
San Jose State University

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BIOTINYLATED OLIGONUCLEOTIDES FOR DNA-PROTEIN
METHYLATION PROTECTION

A Thesis

Presented to

The Faculty of the Department of Chemistry

San Jose State University

In Partial Fulfillment

of the Requirements for the Degree

Master of Science

by

Lorri Kay Lockyer

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APPROVED FOR THE DEPARTMENT OF CHEMISTRY

Dr. Pamela C. Stacks

Dr. Daryl K. Eggers

Dr. Stephen E. Branz

APPROVED FOR THE UNIVERSITY

ABSTRACT

BIOTINYLATED OLIGONUCLEOTIDES FOR DNA-PROTEIN METHYLATION PROTECTION

by Lorri Kay Lockyer

Some proteins preferentially bind stronger to either the major groove or minor groove of DNA. The major and minor grooves have different geometries and functional groups. Dimethyl sulfate methylates DNA at N-7 of guanine in the major groove, and N-3 of adenine in the minor groove. When a protein binds to DNA, that area will be protected from methylation. Exposed DNA will be methylated, and with further treatment, cleavage will result in various sizes of DNA fragments. The results will help determine DNA-protein binding locations.

A protocol was developed using a 15 base pair oligonucleotide that was 5' end-labeled with biotin to test methylation and cleavage sites of adenine and guanine. Transferability of small DNA fragments to a nylon membrane and detection of the biotinylated DNA by chemiluminescence were determined. Technique optimization parameters included amounts of DNA needed to transfer and detect, required exposure times, and stop solution effectiveness.

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LIST OF ABBREVIATIONS

A	adenine
b	biotin
bp	base pair
C	cytosine
DMS	dimethyl sulfate
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetraacetic acid
fmol	femto mole (10^{-15} mole)
G	guanine
M	molar (moles/liter)
nmol	nano mole (10^{-9} mole)
pmol	pico mole (10^{-12} mole)
SS	single stranded
T	thymine
TEMED	tetramethylethylenediamine
tRNA	transfer ribonucleic acid
TB	tris-borate
μ L	micro liter (10^{-6} liter)

Introduction

The answer to many diseases and problems involves an understanding of the reactions of proteins with DNA and the types of proteins that are made or not made from the DNA template. DNA is packaged according to the space available in the nucleus of each cell. A comparison between somatic cell nuclei and sperm cell nuclei demonstrates the structural differences. In a somatic cell nucleus, DNA is coiled twice around an octamer of histones (a group of basic small proteins) through hydrophobic and electrostatic interactions, thus forming nucleosomes. The nucleosomes are coiled further into solenoids to form chromatin (1). It would not be possible for DNA in the sperm nucleus to form nucleosomes. This type of structure would require 213% of the total sperm nuclear volume. Since the nucleus of a sperm cell is much smaller than a somatic cell nucleus, a completely different type of DNA packaging is needed. Table 1 shows a comparison between the volume occupied by DNA in a liver nucleus, mitotic chromosome and sperm nucleus (1).

Table 1. DNA Packaging Volumes in Somatic and Sperm Nuclei.

	% Liver Nucleus	% Mitotic Chromosome	% Sperm Nucleus
DNA	5	12	91
Nucleosomes	9	35	0
Solenoid	20	85	0

The percent volume of DNA that is occupied with somatic nuclei, such as a mouse liver nucleus, and mitotic chromosome is compared to a mouse sperm nucleus. The somatic nuclei contain nucleosomes and solenoids, whereas the sperm nucleus does not.

Protamine, the category of protein that interacts with DNA in the nucleus of a sperm cell, condenses the DNA forty-fold, compared to a somatic cell (2), and six-fold more than DNA in the mitotic chromosome (1, 3).

During mammalian spermatogenesis, spermatogonia proliferate mitotically (a process that maintains the amount of DNA while replicating cells) to form primary spermatocytes. Spermatocytes undergo meiosis to yield haploid spermatids, decreasing the total amount of DNA by half in each spermatid. Spermatids eventually transform during the last phase, spermiogenesis. Spermiogenesis is a differential phase to form mature spermatozoa through morphological, biochemical, and physiological changes. About half way through spermiogenesis, the nucleus begins to elongate, and the chromatin condenses (4). These nuclear changes coincide with the replacement of histones by transition proteins. Transition proteins are then removed, and the DNA is bound by protamine. This is the most important event in determining the nuclear status of the sperm cell (3). Protamines are low-molecular weight, very basic proteins. Human sperm contain equal amounts of P1 protamine and P2 protamine. The protamine neutralizes the DNA charge while coiling it into toroidal structures, which could hold up to 60 kb of DNA (26). Sperm cells can package 50,000 toroids in the nucleus due to protamine's high binding affinity for DNA (5). In this compact structure, where about

85% of the DNA is packaged, the packaged DNA is protected from enzymatic hydrolysis, and transcription is totally inhibited (3). The stabilization of this sperm chromatin is essential for proper sperm morphology and mobility. P1 and P2 protamines with reduced affinity for DNA cause poor sperm morphology, which has been associated with infertility in humans (6, 7). Subfertility in man can be screened to look for sperm that are protamine-deficient or have nicked and partially denatured DNA (8).

Protamine

Protamine P1 is a small protein [~50 amino acid residues] that is about half the size of core histones. It is extremely basic, consisting of 50-70% arginine residues depending upon the particular species. Serine and threonine residues are phosphorylated and dephosphorylated, an event proposed to assist the protamine positioning onto DNA for maximum compaction (9). In mammals, there are cysteine residues generating intra- and inter-molecular disulfide linkages, that ensure stability of the chromatin structure. The amino acid sequence of protamine in mammals, such as the bull (2), rat (10), boar, ram, stallion and humans (11), reveals a homology in arginine domains (3). However, there are considerable differences observed in fish protamines in both size and composition. Whereas human P1 protamine has a molecular weight of 6700 Daltons and human P2 protamine is 7500 Daltons, fish (salmon) protamine is only 4000 Daltons and does not contain cysteine or histidine residues (7, 27). The human P1 family is composed of protamine HP1b and the phosphorylated form, HP1a. The human P2 family includes protamines HP2, HP3 and HP4, which vary only in their terminal amino acid sequence

(12). In the studies thus far, P1 has been detected in all mammals and is processed into a mature protein. P2 is present in spermatozoa of the mouse, hamster, cotton rat (13), stallion and man (14). The protamine P2 gene is transcribed in the cat, bull, boar, ram and monkey, but the P2 protein has not been detected (15).

Determining where protamine binds to DNA has been explored for many years. Protamine binding has been studied by X-ray diffraction methods, optical detection of magnetic resonance (2), spectroscopic techniques, atomic force microscopy (16), Raman spectroscopy (27), chemical modifications, and electron microscopy (7). Bianchi and co-workers proposed three different structural models (7):

- Protamine binds to DNA by winding around the double helix in the minor groove.
- Protamine adopts an alpha helical confirmation and binds in the major groove.
- DNA wraps around protamine in a supercoil conformation.

However, the work presented by Bianchi and coworkers did not allow differentiation between these possibilities. Bull P1 protamine has been used for previous *in vitro* studies at San Jose State University to determine if protamine has a higher binding affinity for the major or minor groove of DNA (17). The results were inconclusive and required the development of a more effective method of blocking methylation before the protein was removed and prior to cleavage reactions.

The 50 amino acid sequence for bull protamine P1 (using the one letter abbreviation found below Figure 1) is

ARYRCCLTH¹⁰SGSRCRRRRR²⁰RRRRRRRRRF³⁰GRRRRRRVCC⁴⁰RRYTVIRCTR⁵⁰

(2). The three underlined central arginine groups have been suggested to be the DNA-

binding domains. In the model proposed by Balhorn, one protamine molecule is bound to each helical turn (~ 11 bp) of DNA (2). The arginine-rich domains are also found in proteins that compact DNA in several viruses, but these proteins have fewer binding domains per molecule (5). There are also seven cysteines that form intra- and inter-molecular disulfide linkages and result in a staple-like shape of the protamine (Fig. 1).

Figure 1. A Bull Protamine Molecule. The lines making the staple shape indicate the intra-molecular disulfide linkages between cysteines. Three arginine domains are underlined. Modified drawing from *Molecular Biology of Chromosome Function* (28).

Amino Acid abbreviations: Alanine, A; Arginine, R; Cysteine, C; Glycine, G; Histidine, H; Isoleucine, I; Leucine, L; Phenylalanine, F; Serine, S; Threonine, T; Tyrosine, Y; Valine, V.

In order to study the length of the binding domain, DNA oligomers consisting of 10, 12, and 14 base pairs were bound to P1 protamine and their dissociation rate was measured. The 10 base pair oligomer dissociated 4 to 8 times faster than the 12 or 14 base pair oligomer. The difference in binding affinity between the 10 base pair oligomer and the 12 and 14 base pair oligomer indicates that the binding site required by P1 is

longer than provided by the 10 base pair oligomer. It was concluded that the length of DNA bound to protamine appeared to be between 10 and 12 base pairs (18).

Guanine / Adenine Cleavage

In order to determine whether protamine binds in the major groove or the minor groove, the chemical sequencing protocol by Maxam and Gilbert (25) was utilized in this study. This protocol allows the determination of where the DNA has been methylated. Dimethyl sulfate methylates the guanines in the major groove of DNA at the N7 position and the adenines in the minor groove at the N3. The glycosidic bond of a methylated guanine is less stable than the unmethylated base and breaks easily on heating at a neutral pH, resulting in a free sugar. Treatment with a heated alkali will then cleave the sugar from neighboring phosphate groups resulting in a fragmented piece of DNA. The glycosidic bond of a methylated adenine is less stable than that of a methylated guanine. A gentle treatment with dilute acid releases methylated adenines preferentially (19).

Methylation Protection Experiments

Methylation protection experiments have been used widely to determine where protein is bound to DNA. When a methylating agent, such as dimethyl sulfate is added to a DNA-protein complex, the individual nucleic acid bases that are bound to the protein are protected from the methylating agents. A stop solution is added to bind to all the remaining methylating agents in the solution so that, when the protein is removed, no further methylation of the DNA bases occurs. Samples of the DNA are treated with

different solutions to cleave the DNA at the different methylated sites (19). The resulting DNA fragments are then separated utilizing polyacrylamide gel electrophoresis.

Yafe and co-workers (30) used methylation protection experiments to show how multiple guanine residues form into hairpin and tetraplex structures. A muscle-specific transcription factor bound to the guanine-rich domain. The DNA that did not have protein bound to it was methylated using dimethyl sulfate. The guanines that were protected by the protein were not methylated, indicating that the protein was bound to the major groove of the DNA.

Biotin

Biotin-labeled probes have been used extensively in DNA studies using Southern blots for *in situ* detection. Southern blotting (a technique developed by E. M. Southern) is a method in which DNA probes are used to detect a specific complementary DNA sequence that has been separated by gel electrophoresis and transferred (blotted) to a nylon membrane or nitrocellulose filter (29). Biotinylated dATP and dCTP nucleotide analogs are synthesized with biotin linked to the N-6 and N-4 positions of the bases, respectively, through linkers which can vary in length between 3 and 17 atoms (20). One advantage of biotin compared to radioactive labels is the stability of biotin. Biotin probes have a longer shelf life than radioactive isotopes such as ^{32}P and ^{33}P . They are also safer to use and create no radioactive waste. Detection is also faster with biotin. The signal can be detected in as little as 30 seconds. Radioactive isotopes can take from hours to a few days to detect by radiography depending upon the specific activity used. There are

some drawbacks to non-radioactive labeling. Reagents are sensitive to quality of preparation and cleanliness of glassware. Target materials must be handled with care. Fingerprints and scratches on the surface can show up as dark artifacts, that cannot be removed (21).

Chemiluminescence

In order to detect the biotin labels, the DNA is transferred from the polyacrylamide gel to a positively charged nylon membrane. Nylon membranes come in pore sizes from 0.45 μm to 0.2 μm , with a moderately or very high positive charge. After the DNA is transferred, it is cross-linked to the membrane by heating in order to immobilize the DNA on the nylon membrane. Chemiluminescence is the emission of light (luminescence) as the result of a chemical reaction. In order to achieve this, the membrane is treated with a series of washing and blocking buffers, and then streptavidin-alkaline phosphatase is added. The streptavidin has a high affinity (10^{-15}M^{-1}) for biotin (20), and the alkaline phosphatase reacts with a chemiluminescent substrate that decomposes into two compounds. One of the compounds is in an excited state that gives off light. The light will only be detected by an X-ray film if it is in the ultraviolet to blue spectral regions. An X-ray film is exposed to the light emission to generate a permanent record. Chemiluminescence is the most sensitive of any non-radioactive method, allowing pico-mole amounts of target molecules to be detected (22).

Several enzymes are in use for non-radioactive detection, including alkaline phosphatase (AP) and horseradish peroxidase (HRP). HRP has the benefit of a higher

turnover rate, meaning one photon of light per molecule, leading to faster signal production. Alkaline phosphatase is stable at elevated hybridization temperatures (e.g., 50 °C) and under high stringency conditions (high salt concentration), including the presence of sodium dodecyl sulfate (SDS). In addition, alkaline phosphatase is a versatile enzyme that can be used with either colorimetric (measuring the change in color) or chemiluminescent (giving off light) substrates (23, 24).

Research Objective

The objective of this research project is to design a protocol for a methylation protection experiment using a biotin 5'-labeled 15 base pair oligonucleotide. In order for a methylation protection experiment to be successful, a stop solution to remove all active methylating groups (a “methyl sink”) must be proven effective. Thus, all active methylating groups still in solution must be neutralized by the stop solution. The naked DNA in a DNA-protein complex becomes methylated in locations where the protein does not protect the purine (adenine or guanine) bases. The importance of this step can be seen when the protein is removed from the DNA after the methylation has occurred. If there are any active methylating groups remaining, all of the purine bases will be methylated and it will not be possible to determine where the protein was bound to the DNA. This will be tested by adding the stop solution to dimethyl sulfate thereby providing a “methyl sink.” Then the dimethyl sulfate, which has reacted with the stop solution, will be added to the DNA. If no methylation occurs, then the stop solution has been effective.

Another part of the study involves the testing of procedures to cleave methylated-DNA at guanine and adenine, using two separate methods that were described previously. The final objective is to separate and transfer oligomers ranging in size from 5-15 base pairs in length. After size separation by polyacrylamide gel electrophoresis under denaturing conditions, the oligomers will be transferred from the gel to a moderately positively-charged, small pore size (0.2 μm) nylon membrane using an electrophoretic transfer cell. The goal is to cross-link the oligomers to the nylon membrane before they move or diffuse through the pores. There was no evidence in the literature that this technique had been successful on oligomers of this size. Even the company that provided the membrane had no data available for small oligomers. If the oligomers do stick to the membrane, strepavidin-conjugated alkaline phosphatase will be applied to bind with the biotin on the DNA. A chemiluminescent substrate will be added to react with the strepavidin-conjugated alkaline phosphatase. The DNA bands will then emit light and expose an X-ray film in order to determine the different bands of DNA fragments. By analyzing the size of the DNA fragments, it will be possible to determine where the DNA was methylated and if the stop solution was effective. Optimization of DNA concentration and amount of dimethyl sulfate will be tested. This protocol was developed from an analysis of the literature. A successful protocol could be tested with protamine to determine if protamine binds in the major or minor groove of DNA.

Materials and Methods

Buffers

A 0.5 M EDTA solution was prepared from solid, dissolved in deionized water, and adjusted to pH 8.0 using HCl or NaOH. A 0.89 M Tris-borate solution (10X TB) was made from a stock solution of Tris base and boric acid, prepared volumetrically in deionized water, and adjusted to pH 8.3 with the addition of Tris base or boric acid. The transfer buffer (5X TBE) was a stock solution consisting of 0.5 M Tris, 0.5 M boric acid, and 10 mM EDTA prepared volumetrically in millipore water. This was further diluted ten-fold for a working solution. DNA loading buffer was prepared volumetrically using 80% formamide, 10 mM NaOH, 1 mM EDTA and 0.1% (w/v) xylene cyanol as a tracking dye. The electrophoresis running buffer consisted of 12.1 g Tris base, 57.6 g glycine, 4.0 g SDS, and deionized water added to make a volume of 4.0 liters.

DNA Oligomers

The customized, single stranded DNA oligomers were chemically synthesized (Fisher Scientific Genosys) to 15 bases in length. The annealing buffer was comprised of 10 mM Tris, (pH 7.5-8.0), 50 mM NaCl, 1 mM EDTA (all made from solid), and 2.5 ml of sterile 10X TB buffer. Sterile water was added to bring the final volume to 25 ml. The single strands were annealed by adding 200- μ l of annealing buffer to each tube of dried DNA, mixed by tickling (flicking gently with finger), and placed in a 37 °C water bath for 15 minutes.

After incubation, aliquots of 6 µl DNA were removed and added to 994 µl of annealing buffer in a 1 ml cuvette. The initial concentration of DNA was determined by UV absorbance at 260 nm. The 5'OH end of only one strand in the double stranded DNA was labeled with biotin denoted by "b". There was a 12-carbon linker between the 5' OH and biotin. The nucleotide sequence of the oligomer is shown below.

Figure 2: Sequence of Customized, Synthetic DNA Oligomer.

Methylation

The 15 base pair, biotinylated DNA oligonucleotide was designed and synthesized to give different banding patterns using polyacrylamide gel electrophoresis, depending on whether the major (N-7 of guanine) or minor groove (N-3 of adenine) was methylated. If the protein was present in the minor groove, the DNA in the minor groove would be protected from methylation. If the protein was present in the major groove, the DNA in the major groove would be protected from methylation. Figure 3 indicates the different sizes of biotinylated DNA bands that would be produced under the two circumstances.

G Cleavage

This will yield bands at 5, 8, and 12 base pairs in length.

A Cleavage

This will yield bands at 6, 10, and 14 base pairs in length.

Figure 3. DNA Sequence and Cleavage of Adenines and Guanines

Dimethyl sulfate will methylate adenines (A) and guanines(G). Cleavage occurs at these locations under the conditions used.

It was important to ensure that the methylation reaction could be stopped. The purpose of this protocol was to verify the methylation and cleavage products of adenine and guanine, as well as to verify that the stop solution was an effective methyl sink, to verify that all excess methylating agent had reacted with the methyl sink. The methylation process began by placing a 1.5 ml microfuge tube in an ice bath and by mixing 5 μ L of 2 μ M biotin-labeled double stranded DNA ($\sim$ 10 pmol) with 200 μ L of 1X sterile TE buffer and 1 μ L of dimethyl sulfate (Sigma). After incubating for twenty minutes at 20 $^{\circ}$ C, 100 μ L of the stop solution was added. The stop solution (Sigma) consisted of 1.5 M sodium acetate, 0.5 M dithiothreitol, and 100 μ g/ml yeast tRNA. The reaction was placed in a 0 $^{\circ}$ C ice water bath, and 750 μ L 95% ethanol (0 $^{\circ}$ C) was added. The tube was inverted four times and chilled at -70 $^{\circ}$ C for five minutes.

The procedure was a method to concentrate DNA by centrifugation followed by lyophilization. The steps were performed in the cold room (4 $^{\circ}$ C). The DNA mixture was centrifuged for fifteen minutes at 12,000 x g in a microfuge resulting in a DNA pellet. The orientation of the tubes was marked prior to placing them in the centrifuge in order to determine the DNA pellet location. The supernatant was removed and discarded into 5 M NaOH. The DNA pellet was resuspended by adding 250 μ L 0.3 M sodium acetate (0 $^{\circ}$ C) and vortexed. A 750 μ L solution of 95% ethanol (0 $^{\circ}$ C) was added to the DNA and mixed by inverting the tube. The solution was chilled at -70 $^{\circ}$ C for ten minutes and then centrifuged as above. The supernatant was removed and discarded. The DNA pellet was gently rinsed with 500 μ L of 95% ethanol (0 $^{\circ}$ C) and centrifuged at 12,000 x g

for fifteen seconds. The supernatant was removed and discarded and the DNA pellet was dried by lyophilization.

Adenine Cleavage

The chemical sequencing protocol by Maxam and Gilbert (25) was utilized to cleave methylated adenines and guanines. The methylated DNA was dissolved in 20 μL sterile water and chilled to 0 $^{\circ}\text{C}$ in an ice water bath. A solution of 5 μL 0.5 M HCl was added and kept on ice for two hours while mixing continuously. The DNA was then mixed with 200 μL sodium acetate (cold) and 750 μL 95 % ethanol (cold) and placed in a -70 $^{\circ}\text{C}$ freezer. The steps were performed in the cold room (0 $^{\circ}\text{C}$). The DNA mixture was pelleted by centrifugation for fifteen minutes at 12,000 x g in a microfuge. The supernatant was removed and discarded. The DNA pellet was gently rinsed with 500 μL of 95 % ethanol (0 $^{\circ}\text{C}$) and centrifuged at 12,000 x g for fifteen seconds. The supernatant was removed and discarded, ending the procedures in the cold room. The DNA pellet was dried by lyophilization. The DNA was dissolved in 10 μL 0.1 M NaOH / 1 mM EDTA and heated at 90 $^{\circ}\text{C}$ for thirty minutes. A loading buffer of 5 μL formamide was added, mixed, and centrifuged for a few seconds. The mixture was heated to 90 $^{\circ}\text{C}$ for three minutes to denature the DNA and quickly cooled in an ice water bath to prevent reannealing. The treated sample could be stored until loaded onto the polyacrylamide gel.

Guanine Cleavage

The methylated DNA was dissolved in 100 μ L of freshly dilute 0.1 M piperidine. The solution was vortexed, inverted a few times and centrifuged for a few seconds. Incubation took place in a 90 °C water bath for thirty minutes. The solution was allowed to sit at room temperature for five minutes, then centrifuged for a few seconds and placed in a -20 °C freezer. The solution was lyophilized to dryness, which took approximately one hour. The DNA pellet was resuspended by adding 10 μ L cold sterile water and placed in a -20 °C freezer. The solution was lyophilized to dryness, which took approximately twenty-five minutes. The DNA pellet was resuspended a second time by adding 10 μ L cold sterile water and placed in a -20 °C freezer. The solution was lyophilized to dryness, which took approximately twenty-five minutes. If the experiment needed to be stopped, the DNA pellet could be frozen at -20 °C. The DNA pellet was dissolved in 4 μ L sterile water and 2 μ L formamide loading buffer was added. The solution was heated to 90 °C for three minutes and quickly cooled in an ice bath. The sample could be stored until loaded onto the polyacrylamide gel.

Polyacrylamide Urea Stock Solution

Stock solution was made in quantities of 500 ml and stored in the refrigerator at 4 °C. The solution consisted of 95 g acrylamide, 5 g bisacrylamide, 250 g urea, 50 ml 1 M tris-borate and 50 ml 20 mM EDTA, which was heated and stirred until the urea had completely dissolved. Solution was transferred to a volumetric flask. The container was

rinsed with sterile water. Sterile water was added to bring the volume to 500 ml. The solution was then transferred to a brown container to protect it from light.

Sequencing Gel

Two different electrophoresis apparatuses were utilized to separate the different oligomer sizes. One was comprised of two glass plates (20 cm x 15 cm) with side and bottom spacers. The 0.5 mm spacers determined the thickness of the gel. The other was a Hoefer Mighty Small II unit consisting of two plastic molds (8 cm x 7-1/2 cm x 0.75 cm). The 19% polyacrylamide-urea sequencing gel consisted of 25 ml polyacrylamide-urea stock solution and 170 μ L 10% w/v ammonium persulfate that were degassed for ten minutes. TEMED (15 μ L) was added; the solution swirled and immediately poured. A comb was placed at the top to create the wells within which the DNA would be loaded. The polymerization was complete in 15-20 minutes. The polymerization was monitored by observing the remaining solution in the flask. The bottom spacer and comb were carefully removed. The wells were rinsed with 1X tris –borate buffer to remove any loose gel pieces, and then the gel was placed in the electrophoresis apparatus. The upper chamber was filled first with electrophoresis running buffer, then the lower chamber was filled. Air bubbles were removed from the wells and between the plates at the bottom of the gel. Pre-electrophoresis of the gel, to eliminate salt and ion contaminants, was performed for one hour at 300 V. Before loading the DNA samples, the wells were rinsed with TBE buffer to remove urea that had surfaced during the pre-electrophoresis. The urea increases the density of the running buffer, causing the DNA samples to not

settle to the bottom of the wells. This happened several times, until I realized that I had skipped the step of rinsing the well before loading the DNA. After loading the 3 μ L DNA samples in the gel, electrophoresis was conducted at 500 V (constant voltage) for one hour.

Semi Dry Electrophoretic Transfer of DNA

In order to transfer the DNA oligomers from the gel to a positively-charged nylon membrane, the gel was equilibrated in 0.5X transfer buffer, while the Nytran® N nylon membrane (Schleicher & Schuell) was cut to fit the gel. The Nytran® N membrane and 6 filter papers were soaked in 0.5X transfer buffer for fifteen minutes. A Trans-Blot® SD (Bio Rad) was used to transfer the oligomers from the gel to the Nytran® N membrane. Current started at the cathode and traveled to the anode, taking the DNA from the gel onto the positively charged nylon membrane. There was no evidence in the literature indicating whether or not oligomers would adhere to the membrane. See Figure 4 for Trans-Blot® SD assembly.

Figure 4. Trans-Blot® SD Semi Dry Electrophoretic Transfer Cell Assembly

The Trans Blot SD started at 25 V and the amperage was increased up to 300 mA in ten minutes for a total of forty minutes. After blotting, the membrane was equilibrated in transfer buffer for five minutes to remove gel particles. The membrane was baked at 80 °C for twenty minutes to ensure cross-linking of the DNA onto the membrane. The membrane was washed twice in 1X wash buffer (used for detection of the chemiluminescence by X-ray film exposure) for five minutes. The experiment could be halted at this point by wrapping the membrane in plastic and placing in the refrigerator. The membrane was allowed to come to room temperature for the chemiluminescence detection process.

Chemiluminescence

Chemiluminescence was chosen as an alternative to using radioactive isotopes. The BrightStar™ BioDetect™ Kit (Ambion) was used because it does not suffer from the usual problems associated with detection of biotinylated DNA, which includes high background and low sensitivity. This process had extremely high signal to noise ratios with a limit of detection of about 100 fg. The kit contained a wash buffer, blocking buffer and assay buffer that are proprietary, so their ingredients are not available. Streptavidin was covalently bound to alkaline phosphatase, which was then bound to the biotin on the DNA. The substrate consisted of an alkaline phosphatase-reactive; 1, 2-dioxane chemiluminescent reagent, CDP-Star®.

The membrane was washed twice in blocking buffer (0.5 ml/cm²) at five minute intervals. The blocking buffer was increased in volume to 1 ml/cm² to wash the

membrane for thirty minutes. A conjugate solution made of 10 ml blocking buffer and 1 μ l streptavidin-alkaline phosphatase per 100 cm^2 was made to wash the membrane for thirty minutes. (More washing and blocking steps may be added to decrease non-specific background luminescence.) The membrane was then washed in blocking buffer (0.5 ml/ cm^2) for ten minutes. Wash buffer was used three times at five minute intervals at a volume of 1 ml/ cm^2 . Then there were two final washes in assay buffer (0.5 ml/ cm^2) at two minute intervals. The membrane was now ready for the reaction with the substrate CDP-Star®. The substrate was added at 5 ml/100 cm^2 . The excess substrate was shaken off and blotted quickly with some filter paper. It was important to keep the membrane moist to prevent drying. The membrane was wrapped in a single layer of plastic wrap while exposed to a film at room temperature. In the dark room, the light from the CDP-Star® was visible. The membrane was exposed to the film for 10-40 minutes and then processed through the San Jose State University Health Department X-ray processing machine.

Figure 5. Flow Chart of Experiment

Results

Detection of DNA and Transferability

The goal of this thesis project was to determine if the small DNA oligomers would transfer from a sizing polyacrylamide electrophoresis gel and become immobilized on a nylon membrane. It was also a test to determine the amount of DNA required for detection using chemiluminescence and the optimal exposure time of the chemiluminescent nylon membrane to the X-ray film. This experiment utilized a single electrophoretic separation of single stranded (SS) 15-base oligomer in amounts ranging from 0.01-70 pmol (10^{-12} mol). To determine the optimal exposure times of the chemiluminescent nylon membrane to the X-ray film, several time exposures were completed and compared.

The data shown in Figures 6A and 6B demonstrated that small oligomers would transfer from a gel and stick to a nylon membrane. Also, the clear separation of bands indicated that the proper gel composition was used. Figure 6A displays the 2-minute exposure and shows the entire film with the majority of SS DNA traveling 4.5 cm. The middle set of bands traveled with the tracking dye. Samples of 0.1-70 pmol DNA were initial amounts applied to the gel, but the smallest application (0.01 pmol) is only faintly visible in the 2-minute exposure. (The 1.0 pmol-application was visible clearly in the 3-, 4-, and 5-minute exposures, not shown.) The top doublets at 1.6 cm may be due to double stranded DNA which renatured from the SS DNA, even though this was a denaturing gel. Another possibility was that the doublets were due to the DNA not settling to the bottoms of the wells when injected into the gel.

Figure 6A. Two-Minute Exposure of DNA Transferability and Detection Test.

Starting on the left, the amounts of DNA were 70, 60, 50, 40, 30, 20, 10, 1 pmol. The lines at the top indicate the bottom of the loading wells (origin). The tracking dye traveled approximately 3 centimeters (cm). The DNA was electrophoresed for 2 hours and 10 minutes on a 19% gel. The dot on the left of the bands is the CDP-Star® control that was added to the membrane.

Figure 6B. Thirty-Second Exposure of DNA Transferability and Detection Test.

The chemiluminescent nylon membrane was exposed to the X-ray film for 30 seconds, and then developed. Bands traveled 4.5 cm. The 0.10 pmol application did not show up in this exposure.

The wells were not rinsed after pre-electrophoresis or loading of DNA. Urea diffused from the gel and increased the density of the solution in the wells, thus the DNA did not settle completely to the bottom of the wells.

Figure 6B displays the thirty-second exposure, with only the bands for 15 base oligomers that traveled 4.5 cm are shown. The 0.1 pmol amount was not visible at this exposure. The lesser exposure time was successful in producing bands that were very clear and distinct for such small sized oligomers.

The experiment did verify that 15-base oligomers can be transferred successfully to a nylon membrane. It also showed that 0.1 pmol DNA application was too small for adequate transfer and viewing. The detection of larger amounts of DNA (10-70 pmol) was optimal with 30 seconds to 2-minute exposures. A smaller quantity, 1 pmol, required a minimum 3-minute exposure in order to be detected. Exposure times of 1, 3, 4, and 5 minutes are not shown in the Figures.

The results of the experiment raised the question: does the tracking dye affect the travel of the DNA through the gel? An electrophoretic analysis was performed with three different amounts of DNA, with and without tracking dye to test the effect of the presence of tracking dye. Figure 7 shows the comparison of DNA consisting of 0.1, 1.0, and 10 pmol amounts. The tracking dye was only applied to lanes 1, 3, and 5. In each of these lanes, the tracking dye traveled 3.5 cm. All three application amounts traveled a maximum of 4.8 cm in all six lanes.

Figure 7. DNA Electrophoresis and Transfer With and Without Tracking Dye.

From left to right, lanes 1 and 2 contained 10 pmol, lanes 3 and 4 contained 1 pmol, and lanes 5 and 6 contained .01 pmol. Lanes 1, 3, and 5 contained the tracking dye. This was a 10-minute exposure. The electrophoresis was run for 145 minutes on a 19% gel.

Exposure of the chemiluminescent nylon membrane to the X-ray film was done at 1-, 2-, 3-, 4-, and 10-minute intervals. All sample amounts that traveled 4.8 cm were visible after a 10-minute exposure (Figure 7). The 1-minute exposure only showed the bands at 4.8 cm for lanes 1 and 2 (data not shown). The 2-minute exposure included bands at 4.8 cm and 3.5 cm for lanes 1 and 2 (data not shown). The 3- and 4-minute exposures showed bands at 1.3 cm and 1.7 cm for lanes 1 and 2, as well as 4.8 cm and 3.5 cm (data not shown). When the DNA samples were injected into the gel, they did not lie flat on the bottom of the wells in the gel. This was very evident when looking at lane 3 with 1 pmol DNA in the absence of tracking dye. The uneven distribution of DNA likely was due to the wells not being rinsed after the gel was pre-electrophoresed.

This experiment indicated that the presence of the tracking dye did not make a difference in rate of migration through the gel. It also shows a consistent band separation of the small oligomers.

Optimization of DNA: DMS and Stop Solution Effectiveness

To determine whether protamine binding occurred in the major or minor groove of DNA, a procedure of methylating the exposed bases was used. The N-7 of guanine would be exposed to methylation in the major groove if the protamine was bound in the minor groove. The N-3 of adenine would be exposed to methylation in the minor groove if the protamine was bound in the major groove. The stop solution was applied to act as a methyl sink. All methylation must discontinue prior to the separation of the DNA and protamine. Conditions were optimized to ensure that no additional methylation would occur after the separation of the complex. After the methylation has occurred and the stop solution applied, the DNA and protamine would be separated. The methylated DNA would be cleaved after separation at the methylated bases using a chemical sequencing protocol by Maxam and Gilbert (19, 25). Since protamine was not being utilized in these experiments, the optimization experiment was a test of the procedure to methylate the bases (adenine or guanine) and subsequent cleavage of the DNA, and to determine the optimum conditions to ensure that no additional methylation would occur after complex separation.

One experiment compared the use of 10 pmol DNA with 12 nmol DMS or 1.2 nmol of DMS, a 1200-fold excess versus a 120-fold excess of DMS, respectively. The optimization of DNA and DMS in Fig. 8 lanes 7 -10, showed that lane 9, which had a 1200 fold higher amount of DMS created smaller bands than lane 7. This was exciting to see that oligomers with less than 15 bases successfully transferred from the gel to the nylon membrane and were detectable on the film.

In the same experiment, the ratio of DMS to DNA was varied with the stop solution to determine an optimal ratio. The stop solution effectiveness was tested by combining the stop solution (which contains 1.5 M sodium acetate, 0.5 M dithioreitol and 100 µg/ml yeast tRNA) with varying concentrations of DMS. The stop solution acts as a methylamine sink and inactivates the DMS. The stop solution and DMS were allowed to incubate for twenty minutes, and then DNA was added. The goal was not to see any methylation on the DNA due to residual DMS. The samples were divided to perform adenine the cleavage protocol and guanine cleavage protocol. The results were compared to the DNA that had no contact with DMS but treated with the same cleavage protocols to act as a negative control.

Figure 8 shows that the stop solution was effective in producing the same results when compared to the samples that had not undergone methylation, but did go through the cleavage procedures. This can be seen by comparing lanes 3 and 4, which had no methylation, but went through the adenine and guanine cleavage procedures, respectfully, alongside the stop solution test lanes 5 and 6. Lanes 3, 5, and 7 were lighter due to loss of sample during procedures. A 6 % polyacrylamide gel was utilized to test band

separation. This proved not to be sufficient, resulting in long, blurry bands, and the gel was difficult to handle. This experiment was also affected by the wells not being rinsed after pre-electrophoresis causing the samples to load unevenly.

The experiment was repeated using a 19% polyacrylamide gel to test if conditions would improve (Figure 9). There was a slight improvement in clarity of the bands with a higher percentage gel. This was the first experiment in which the wells of the polyacrylamide gel were flushed to remove the urea, prior to loading the samples. All samples went directly to the bottom of the wells.

The DNA was doubled for the adenine samples, which contained 20 pmol, and the guanine samples contained 10 pmol DNA. This was done to see if the bands would be darker than in Figure 8. A larger amount of DMS was utilized to test if more methylation would result in more cleavage and even smaller oligomers. The amount of DMS was increased to compare 12 nmol to 21 nmol. Figure 9 showed there was not a detectable difference between lanes 2, 3, 4, and 5, which compared 21 nmol of DMS to 12 nmol DMS. There was separation in both samples. The sample in lane 4 was lost during the guanine cleavage process. The adenine cleavage (lanes 3 and 5) produced bands at 14, 10, and 6 bases. It appears some of the 15 base DNA did not cleave. Cleavage for guanine, in lane 2, produces bands at 12 and 8 bases.

The size of the DNA in the various bands is approximated based on the distance traveled. There should have been a band at 5 bases, but it did not show on this film. There is also the possibility that so short an oligomer did not stick to the nylon membrane and thus was not detected. There is no conclusion between using 12 nmol versus 24 nmol

DMS in this experiment, both amounts of which are in excess of the DNA, since the sample in lane 4 was lost.

Figure 8. Optimization of DNA:DMS Ratio and Stop Solution Test 1.

Starting from the left, lanes 1 and 2 are double stranded and single-stranded DNA, respectively (controls). Lanes 3 and 4 are the negative controls (DNA that was not exposed to DMS, but went through cleavage protocols). The stop solution test was lanes 5 and 6 for which DNA was added after quenching the DMS with stop solution. Methylation experiments were conducted on lanes 7 and 8 (1.2 nmol DMS) and lanes 9 and 10 (12 nmol DMS). Lanes 3, 5, 7, and 9 followed the adenine cleavage procedures. Lanes 4, 6, 8, and 10 followed the guanine cleavage procedure. The dot in the upper left hand corner is the CDP-Star® control that was added to the membrane. The resulting 10 pmol DNA samples were analyzed using a 6% polyacrylamide sequencing gel that ran for 1 hour and thirty minutes.

Figure 9. Optimization of DNA: DMS Ratio and Stop Solution Test 2.

Lane 1 was single-stranded DNA (control). Lanes 3, 5, 7, 9 had adenine cleavage using 20 pmol DNA. Lanes 2, 4, 6, 8 had guanine cleavage using 10 pmol DNA. Lanes 2 and 3 were methylated using 21 nmol DMS. Lanes 4 and 5 were methylated using 12 nmol DMS. Lanes 6 and 7 were the stop solution tests, and lanes 8 and 9 were controls (no methylation, but subject to cleavage). The DNA samples were analyzed using a 19% polyacrylamide sequencing gel that ran for fifty minutes.

The stop solution test results (lanes 6 and 7) are the same as the negative controls in lanes 8 and 9, which had no methylation. One may conclude that 50 μ L stop solution with 12 nmol DMS was effective in preventing methylation of DNA. These results also suggested that there was a difference in intensity between the 10 pmol and 20 pmol DNA sample amounts throughout the experiment.

Methylation/Cleavage/Stop Solution Test – Adenine Only

Using adenine samples from the last experiment, the gel loading methodology was examined by using different gel loading tips and syringes. The smaller oligomers do transfer and stick to the nylon membrane, however, with more dispersed bands. The goal of this experiment was to use longer exposure times to examine if clearer, more distinct bands would be present. Figure 10 displays the gel loading methodology using gel loading tips (lanes 1-5) which may have contributed to the slightly better separation in samples that had less than 15 bases. The results of the stop solution test (lanes 3 and 8) were identical to samples with no methylation but which were subjected to the cleavage protocol, shown in lanes 2 and 7. Lanes 4, 5, 9, and 10 were methylation experiments and there was more cleavage of bases compared to the non-methylated samples. It was evident that such short oligomers do not yield clear, distinct bands. Figure 10 was a 10-minute exposure. The 20-minute exposure did not have any considerable difference and the 40-minute exposure was much darker (not shown). Shorter exposure times may have produced sharper bands.

Final DNA: DMS and Test Solution Optimization

The final optimization test utilized a smaller quantity of DNA with the goal of getting clearer, more distinct bands with small size oligomers. Smaller amounts of DNA did make a slight difference, though the bands were still dispersed. Figure 11 shows that the stop solution was effective in lanes 4 and 5. Lanes 10 through 13 show that the optimal amount of DNA, based on visualization, was 5 pmol. There was no observable

difference based on whether 12 nmol or 21 nmol DMS was used. The SS DNA used as a control was very dispersed in lane 1. It appears to yield multiple bands and may have been a sample that had been cleaved. The variance in the final picture is also due to technique. There are several steps in which sample can be lost or damaged. This can be seen by comparing lanes 10 and 12, which were identical in procedure (only change in the amount of DMS), and lane 10 had better separation with less DMS.

Figure 10. Ten-Minute Exposure of Adenine Methylation and Stop Solution Test.

Starting from the left, lane 1 was the SS DNA control. Lane 2 had no methylation, just the cleavage procedure. Lane 3 was a stop solution test combining DMS and stop solution, then DNA. Lane 4 was a methylation using 12 nmol of DMS. Lane 5 was a methylation using 24 nmol of DMS. Lanes 6 through 10 repeated in the same order using a different loading syringe and different loading tips. The dot in the upper right hand corner is the CDP-Star® control that was added to the membrane. The 20 pmol DNA samples were analyzed using a 19% polyacrylamide sequencing gel that was electrophoresed for 1 hour.

Figure 11. Final Optimization Test.

In lanes 1-9, 1 pmol DNA was used. Starting from the left, lane 1 was a control sample with 1 pmol SS DNA that had no methylation or cleavage. Lanes 2 and 3 had no methylation reactions, but had adenine or guanine cleavage procedures, respectively. Lane 4 and 5 samples had a stop solution test with 12 nmol DMS, then 1 pmol DNA added and either the adenine or guanine cleavage reaction, respectively. Lane 6 and 7 had 1 pmol DNA methylated with 12 nmol DMS and adenine or guanine cleavage, respectively. Lane 8 and 9 had 1 pmol DNA methylated with 21 nmol DMS and either adenine or guanine cleavage, respectively. Lanes 10-13 had 5 pmol DNA samples. Lane 10 and 11 had 5 pmol DNA methylated with 12 nmol DMS and either adenine or guanine cleavage, respectively. Lane 12 and 13 had 5 pmol DNA methylated with 21 nmol DMS and adenine or guanine cleavage, respectively. A portion of the exposed film is displayed. The lines and arrow on the film point out band separation. The resulting samples were analyzed using a 19% polyacrylamide sequencing gel that electrophoresed for 1 hour.

Discussion

There is no literature evidence or commercial sources that state that oligomers in the range of 5-15 bases can be detected with biotin-labeled DNA. Radioactive markers have been used to detect such small oligomers. This experiment demonstrated that using biotin for detection can be achieved without the bio-hazard and issues associated with the need to continually purchase radioactive materials with short half-lives. The shelf life of biotin is longer than radioactive material. The biotin was attached to the 5'-OH of DNA via a 12-carbon linker.

The experimental procedure was successful for transferring small oligomers onto a nylon membrane. This was another procedure for which results were not available in the literature or in the industry proprietary materials. Concerns about the possibility that the small oligomers would go right through the nylon membrane with a pore size of 0.2 μm or that some of the smaller sample amounts would not stick proved unfounded. All DNA samples were transferred to the nylon membrane and went through multiple washings for the chemiluminescent procedure. To prevent loss of the DNA sample during the washings, the nylon membrane was baked prior to the chemiluminescent procedure in order to fix the DNA to the membrane.

The chemical sequencing protocol by Maxam and Gilbert (25) was successful using small oligomers. The oligomer was designed to yield bands of 5, 8, and 12 bases in length when methylated and utilizing the guanine cleavage protocol. Figure 10, lanes 11 and 13, show the guanine cleavage separation experiment and a band that could be 12 bases in length. The 8-base oligomer may be faintly apparent, but was not conclusively

detected. The 5-base oligomers were not detected. The oligomer was also designed to cut lengths of 14, 10, and 6 bases when methylated and utilizing the adenine cleavage protocol. Bands of 14 and 10 bases in length were easily detected. The 6-base oligomer was not detectable.

There was always a major band at 15 bases, the original length of the oligomer. This could have been due to the bases not being methylated. The cleavage procedure may not have been efficient in cleaving every base that was methylated. This needs further study.

The stop solution was successful as a methyl sink and preventing further methylation of DNA by DMS. When the DMS and stop solutions were allowed to react and then DNA was added, no methylation of the DNA was apparent. The next step would be to test the stop solution with a protamine-DNA structure in future studies.

A 19% polyacrylamide gel was easier to handle and revealed better separation than a 6 % polyacrylamide gel. Increasing the sieving of the matrix slowed down the migration for DNA bases, but also may have made it more difficult for the entire sample to move down the gel consistently. The smaller bases did go further, but there is not an explanation why some samples were dispersed all the way from top to bottom. The first few experiments were also affected by the wells not being rinsed prior to loading.

Experimental technique was also a factor. Optimizing each step was a constant goal by using different instruments, using gel loading tips, following directions, and carefully handling of such small amounts of substances.

Conclusion and Future Studies

The experimental protocol was a success, but there are still many questions that need to be answered through future studies. The protocol is ready to be tested using protamine, but a larger; 20-30 base oligomer should be utilized. The larger size would give larger oligomers that may give clearer results. This is still assuming that the protamine would bind in the major or minor groove of DNA, but not both.

Future studies could also investigate the difference between using soluble protamine verses protamine that was bound to a dyna bead (insoluble matrix). This would be helpful to increase our knowledge about DNA-protamine complexes. By increasing our knowledge of DNA-protamine complexes, procedures to maximize the interactions and bonding could be discovered. This could lead to an increase in fertility or possibly a non-surgical sterilization procedure in men.

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