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Quantitative Analysis of Diepoxybutane Damage within the Chicken β-globin Domain

André M. Pilon
Colby College

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Quantitative Analysis of Diepoxybutane Damage Within the Chicken β-Globin Domain

André M. Pilon

A thesis submitted to the Department of Chemistry in partial fulfillment of the requirements for graduation with HONORS IN RESEARCH in Chemistry

Colby College
Department of Chemistry
Waterville, ME 04901
May 2003
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Approved by:

Dr. Julie T. Millard
Mentor

Dr. Stephen U. Dunham
Reader

Colby College
Waterville, ME 04901
May 2003
Epigraph

I am neither especially clever
nor especially gifted.
I am only very, very curious.

--Albert Einstein

Experience is not what happens to a man;
it is what a man does with what happens to him.

--Aldous Huxley

There is no such thing as failure,
only results,
with some more successful than others.

--Jeff Keller

Consider the work of God:
for who can make straight,
which He has made crooked?

-- Ecclesiastes 7:13

Although I can accept talking scarecrows, lions,
and great wizards of emerald cities, I find it
hard to believe there is no paperwork
involved when your house lands on a witch.

--Dave James
Vitae

André Michel Pilon was born in Springfield, Massachusetts, the eldest son of Wayne P. and Eveline J. Pilon. He has one brother, Dylan Nicholas. André attended East Longmeadow High School and was valedictorian of the graduating class of 1998.

André began his research career in January, 2000, with Dr. Shari U. Dunham, in the Colby College Department of Chemistry, followed by a summer stint at the University of Florida's Whitney Marine Biological Laboratory. He resumed work with Dr. Dunham during the 2000-2001 academic year, subsequently taking a summer position with Dr. Julie T. Millard, also of the Department of Chemistry. This was followed by a year-long sabbatical from Colby, during which André joined the lab of Colby alumnus, Dr. David M. Bodine '76, in the Hematopoiesis Section of the Genetics and Molecular Biology Branch of the National Human Genome Research Institute at the National Institutes of Health in Bethesda, Maryland.

Upon returning to Colby in the fall of 2002, André began his thesis research with Dr. Millard. He will graduate with honors in Chemistry, with a concentration in Biochemistry, and will return to the NIH before entering a graduate program in the biomedical sciences.
Acknowledgements

First and foremost, thanks to Dr. Julie T. Millard. Mere words are ill-equipped to articulate my level of respect and admiration. I will be forever indebted for the support you have given throughout my tenure in the lab, both personal and professional. But for you and the Chemistry Department, where would I be today?

Similarly, I would be remiss in not thanking Dr. David M. Bodine, my mentor at the NIH, for “taking a chance on an unknown kid.” Your generosity of spirit and faith in my abilities were at first overwhelming, but the experience has, without a doubt, set me all the more steadily on the path, with a clearer eye toward the future.

To Alexis “Dr. Professor Wonderful, Ph.D.” Bond, the LUMO to my HOMO, how unbearable would the trenches have been without a comrade? Are we done yet?

Donna, I still love you, even though you didn’t get the “chemistry gene.” You’re going to be an amazing accountant one day. And who needed all those puppets, anyway?

To Serena, Anna, Beth, and Madeleine, thank you for becoming my “chosen family.” Me, running with a group of Carhartt-wearing, tree-hugging, vegetarian chicks? How queer! Vive la différence! <waving tiny flag>

And finally, immeasurable thanks to my parents, for the unwavering love and support that only a mother and father can give to a child. Of course, my being the “perfect” son made it easy for you. I think that’s how it goes, right? <shrug>
# Table of Contents

Approval  
Epigraph  
Vitae  
Acknowledgements  
Table of Contents  
List of Figures  
List of Tables  
Abstract  
Introduction  
Materials & Methods  
Results  
Discussion  
Conclusions  
References
List of Figures

Figure 1. Metabolism of 1,3-butadiene (BD).

Figure 2. DEB cross-link between N7 positions of deoxyguanosine residues.

Figure 3. Nucleosomal core particles

Figure 4. Acetylation of a lysyl side chain.

Figure 5. Strand cleavage of a DEB lesion upon heating.

Figure 6. Primer regions chosen from within the chicken β-globin domain.

Figure 7. 1.5% agarose gel of cytochrome b gene region amplification (stained with EtBr).

Figure 8. 1.5% agarose gel of β1 and β2 gene region amplification (stained with EtBr).

Figure 9. 1.5% agarose gel of β3 and β4 gene region amplification (stained with EtBr).

Figure 10. Phosphorimage of “open” β3 region.

Figure 11. Phosphorimage of “closed” β2 region.

Figure 12. Lesion frequency versus log [DEB].
List of Tables

Table 1. β2 and β3 sequences.

Table 2. Primer sequences for amplification of a 407 bp region of the cytochrome b gene and four β-globin regions.

Table 3. Calculated lesion frequency in "open" versus "closed" regions due to addition of varying strengths of DEB.
Abstract

In industrial polymer and synthetic rubber production facilities, workers are exposed to 1,3-butadiene. This compound is converted *in vivo* to 1,2,3,4-diepoxybutane (DEB) and has been linked to increased incidences of cancer in these individuals. Carcinogenesis has been attributed to formation of DEB induced DNA interstrand cross-links. Previous studies have demonstrated that DEB cross-links deoxyguanosine residues within 5'-GNC sequences in synthetic DNA, in restriction fragments, and in defined sequence nucleosomes. The current study utilized the polymerase chain reaction (PCR) to examine DEB damage frequencies within nuclear genes, found within "open" regions of chromatin, as compared to regions of unexpressed sequence that reside in tightly packed, "closed" chromatin, to more closely model DEB reactivity *in vivo*. These initial studies have been performed in chicken liver homogenates. Preliminarily, we have found a dose-dependent DEB lesion-forming response within "open" chromatin. DEB appears to have little-to-no effect upon regions of "closed" chromatin.
Introduction

1,3-Butadiene (BD) is an important monomer used in polymer synthesis and rubber production (Morrow, 1990). Industrial exposure to this colorless gas has been linked to increased incidences of cancer in individuals (Delzell et al., 1996). BD has also been found in automobile exhaust and cigarette smoke (Pelz et al., 1990; Brunnemann et al., 1990). With possible genotoxic and reproductive toxic effects, it is a Priority Substance under Canada’s Environmental Protection Act (Hughes et al., 2001), and is on the Original List of Hazardous Air Pollutants, published by the Environmental Protection Agency of the United States (EPA Air Toxics Website 2003).

Xenobiotics, such as industrial BD, or components of cigarette smoke or automobile exhaust, must be processed and excreted upon entering the body. This function falls largely to the liver. Enzymatic oxidation, reduction, hydrolysis, or conjugation reactions make organic compounds more water-soluble, allowing them to pass from the body as waste (Gringauz, 1997).

One way in which BD is cleared from the body involves oxidation by cytochrome P450 (Duescher and Elfarra, 1994). The 1,3-butadiene monoepoxide, 3,4-epoxy-1-butene (EB), and the 1,3-butadiene diepoxide, 1,2,3,4-diepoxybutane (DEB), are products of sequential oxidations of BD (Figure 1; Malvoisin and Roberfroid, 1982). However, it is precisely this oxidation that becomes detrimental to the organism. EB has been shown to induce chromosomal point mutations, while DEB has been found to induce point mutations, deletions, and other chromosomal alterations (Recio et al., 2001).

These epoxides are capable of alkylating deoxyribonucleic acid (DNA), forming a covalent attachment at the N7 position of deoxyguanosine (dG) residues. The diepoxide may
form two such attachments, producing di(guanin-7-yl) derivatives (Figure 2; Brookes and Lawley, 1961). The increased cytotoxicity of bifunctional alkylators (e.g., DEB), versus monofunctional agents, was originally attributed to the formation of interstrand cross-links at 5'-GC sites, which possess the minimal N7-to-N7 distance in B-DNA (Brookes and Lawley, 1967). However, DEB was later shown to form cross-links in short oligomers at 5'-GNC sites, where N is any base (Millard and White, 1993).

However, nuclear DNA does not exist as short oligomers in a free, linear state in vivo. A meter of linear double-stranded DNA must fit into the cell’s nucleus, only a few microns in
diameter (Elgin, 1995). This requires tight packaging into a higher order structure: the chromosome.

In brief, a chromosome is a condensed, though complex, structure. It is composed of double-helical DNA, first wrapped about core structural proteins, called histones. The histone core particles are then further twisted and looped about one another, with the aid of various non-histone structural scaffold proteins, until the final compacted structure is formed.

The initial structural element, the DNA-wrapped histone core, is of particular importance and relevance to us. The four histone proteins, H2A, H2B, H3, and H4, form an octameric core, about which 146 base pairs (bp) of linear DNA is wrapped. These octamers, or core particles, are linked to one another with a fifth histone protein, H1, along an additional length of DNA. These core particle-plus-linker structures are known as chromatosomes. Nucleosomes, the third level of organization, contain approximately 200 bp of DNA, plus the chromatosome histones (Wagner et al., 1993).

The alternating nucleosome – linker – nucleosome configuration (Figure 3) is often referred to as "beads on a string," or the "10 nm fiber," for the approximate thickness of a

![Figure 3. Nucleosomal core particles (Nelson and Cox, 2000).](image)
nucleosomal particle. This "10 nm fiber" is further packaged into a proposed solenoidal structure, dubbed the "30 nm fiber," which is further looped, folded, and packed about a non-histone protein scaffold, until it reaches the final condensed chromosomal structure (Wagner et al., 1993).

It was suggested in early studies that such chromosomal structures impact gene expression. Weintraub and Groudine (1976) noted that specific globin genes were preferentially digested by deoxyribonuclease I (DNase I) treatment of nuclei from chicken erythroid cells. However, this digestion did not occur in nuclei obtained from non-erythroid tissues, where globin genes are not actively expressed. These findings indicate that actively transcribed genes are likely associated with histones arrayed in a conformation such that the DNA is particularly DNase I sensitive. In 1978, Vidali et al. confirmed an increased degree of acetylation of histones H3 and H4 in regions of increased DNase I sensitivity, thus linking transcriptional activity to histone acetylation.

The amino-terminal tails of the nucleosomal core histones H3 and H4 are enriched in lysine and arginine. At physiological pH, these residues are positively charged, and have the potential to interact with the negatively charged phosphate backbone of DNA. Acetylation of the lysine residues reduces the overall positive charge, leading to a reduced electrostatic affinity of DNA for the histone (Figure 4; Hong et al., 1993).

![Figure 4. Acetylation of a lysyl side chain. Addition of an acetyl group to the physiologically protonated lysyl residue removes positive charge.](image-url)
Genes that are to be transcribed do not reside in an ever-acetylated histone environment. To allow DNA transcription, histone acetyltransferase complexes (HATs) are recruited to promoter regions in the transcriptionally repressive, unacetylated, "closed" chromatin. By acetylating the appropriate histones and creating a transcriptionally competent, "open" domain, the transcriptional machinery can then set to the task of replicating the target gene (Imhof and Wolffe, 1998).

This activity is checked by the histone deacetylases (HDACs), which function to remove acetyl groups, converting "open" chromatin to the "closed" conformation. In an erythroid tissue, for example, these HDACs would help prevent over-expression of globin genes beyond endogenous levels after sufficient transcription had occurred. In a non-erythroid tissue, the HDACs would help prevent expression of the globin genes altogether, by keeping the globin-containing regions "closed."

Chen and Townes (2000) illustrated the importance of an acetylated, "open" chromatin environment for active gene transcription using a virally transduced gene system. Once successfully integrated into a host genome, these genes are often silenced. By inhibiting histone deacetylase activity, viral gene expression was dramatically reactivated. Removal of the inhibitor lead to silencing of expression once again.

Given the higher order chromosomal structure, a more accurate description of in vivo DEB reactivity requires more than just a simple oligomeric model. Thus, Millard and Wilkes studied defined sequence nucleosomal core particles of the 5S RNA gene of *Xenopus borealis*. The same previously determined 5'-GNC binding preference was demonstrated in the core particles, as well as in the free DNA, uncomplexed to any proteins (2001). However, this work
did not take into consideration the rest of the dynamic chromatin environment that would be present in vivo.

To more closely model in vivo behavior, we investigated, in whole cells, the degree of DEB damage in regions of both "open" and "closed" chromatin. To this end, we utilized a quantitative polymerase chain reaction (QPCR) (Grimaldi et al., 1994).

By flanking a target gene region with two oligomeric DNA primers, the target region can be amplified exponentially via PCR. DEB lesions within the target region are thermally labile, causing DNA strand cleavage during heat cycling (Figure 5; Lawley and Brookes, 1967), which results in the amplification of shortened sequences. By using a $^{32}$P-labeled primer, we can evaluate incorporation of radiolabel into products, or amplicons, of various lengths. By subjecting samples to polyacrylamide gel electrophoresis (PAGE), we can separate amplicons of varying base pair lengths into discrete bands, and through phosphorimagery, can compare the band volumes. As DEB concentrations increase, we would expect to see concomitant decreases in $^{32}$P incorporation into full-length products, characterized by decreasing band volumes. Quantitative comparisons of DEB lesion frequency can then be made using one of the Poisson equations, which are used to analyze random events. In this case, we will use the equation, \( L = -\ln (A_d/A_c) \), where \( L \) is the lesion frequency per strand and
A and A are the amplification amounts from damaged and control templates, respectively (Grimaldi et al., 1994).

We initially screened four regions within the chicken β-globin domain, finally choosing two for our studies (Table 1). One region, which we have termed β2, resides in the “closed” condensed chromatin, upstream of the fourth DNase I-hypersensitive site (HS4). The second, β3, is approximately one nucleosome upstream (~200 bp) of the “open” HS4 site (Figure 6).

Both targets are approximately of equal bp length, have equal numbers of 5′-GNC sites, and are of similar GC % composition (Litt et al., 2001a).

![Figure 6](image-url)
In choosing target regions, we had to be mindful of the likely acetylation state of our homogenized tissue. The HDACs could well have deacetylated and “closed” any seemingly “open” regions after the livers were excised and the cells had died. However, HATs and HDACs are not non-specific, but belong to various families, whose members acetylate and deacetylate only specific targets (Strahl and Allis, 2000). Further, our nominal β3 region resides in HS4, a particularly hyperacetylated region (Litt et al., 2001b). These factors made it unlikely that this particular target region would have become totally deacetylated and “closed” prior to our studies. This is no guarantee that there was not some minimal loss of acetylation, however. To maintain full acetylation levels, live cells could be treated with HDAC inhibitors, such as sodium butyrate, phenylmethylsulfonyl fluoride (PMSF), and aprotinin (Litt et al., 2001b).

We know that chromatin structure and gene expression are inextricably linked, but their relationship remains to be thoroughly elucidated. All the exact mechanisms and factors involved have yet to be determined. Similarly, the more fundamental mechanisms of DEB carcinogenicity are not fully understood.

Living cells have intrinsic repair mechanisms to deal with xenobiotic insult. However, acetylation state of the region attacked by DEB may render these mechanisms more or less active or efficient. Lesions within specific genes, whose products normally code for key cellular proteins, may lead to cytotoxicity if not repaired.

Examining the similarities and differences in DEB damage within two clearly varied chromatin environments, such as these regions of the β-globin domain, may shed more light on the situation and help to dispel some of the current “black box” character.
Materials & Methods

Liver Homogenate – Razor-thin shavings (approximately 3-4 cm²) of frozen chicken liver (commercially obtained) were suspended in 2.5 mL iced 1X PBS (137 mM NaCl, 2.7 mM KCl, 11.9 mM phosphate, pH 7.4) and repeatedly passed through a 21 gauge needle, then brought to 10 mL total volume with iced 1X PBS. The sample was centrifuged at 1000 x g for 10 min at 4 °C (Beckman J2-HS centrifuge with a JA-18 rotor) to pellet extraneous material. The supernatant was split into 10 microfuge tubes, in 1 mL aliquots.

PCR – Primers (Integrated DNA Technologies; Table 2) were reconstituted in dH₂O at ~50 μM concentrations. PCR reagents were obtained from Applied Biosystems. A single amplification reaction required 5 μL 10X PCR Gold Buffer, 5 μL 10 mM dNTP mix, 7.4 μL 25 mM MgCl₂, 0.25 μL AmpliTaq Gold DNA Polymerase (5 U/μL), and 0.75 μL of each primer.

<table>
<thead>
<tr>
<th>Primer Sequences</th>
<th>Sequence 1</th>
<th>Sequence 2</th>
<th>Kilobase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytochrome b</td>
<td>5'-AAAAAGCTTCCATCCAACATCTCAGCATGATGAAA</td>
<td>5'-AAACTGCAGCCCCTCAGAATGATATTGTTGCTCCTCA</td>
<td></td>
</tr>
<tr>
<td>(Kocher et al., 1989)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>β1 sequences</td>
<td>5'-CACAGCAGTGCAAGCAGTTATT</td>
<td>5'-CACAAAGACCAGTCCTCCTCAAT</td>
<td>(7.342 kb)</td>
</tr>
<tr>
<td>(Litt et al., 2001b)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>β2 sequences</td>
<td>5'-GGGAACAAGTGCAAGACGCTAT</td>
<td>5'-TTTGCTGCCTCAGCGTAT</td>
<td>(10.350 kb)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>β3 sequences</td>
<td>5'-CTCTGTGCTGCACTCCCTTCAAT</td>
<td>5'-CCTTTTCGGAACCTCCTTCAAT</td>
<td>(21.365 kb)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>β4 sequences</td>
<td>5'-GCCCCACCACGCCGTG</td>
<td>5'-CATCACCAGCTGCCAAA</td>
<td>(27.649 kb)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 2. Primer sequences for amplification of a 407 bp region of the cytochrome b gene and four β-globin regions. β-globin primers are listed with their kilobase distance from an upstream start site within the domain. (Kocher et al., 1989; Litt et al., 2001b)
(Forward and Reverse), in a 50 µL total volume. An isolated DNA pellet was dissolved in the requisite amount of dH₂O to account for the 50 µL total reaction volume. Scaled master mixes were used for each round of reactions, combining all reagents (omitting radiolabeled primers, when applicable). Heat cycling for β-globin regions was performed on a GeneAmp PCR System 9700 with the following profile: 2 min hold at 50 °C; 10 min hold at 95 °C; 40 cycles of 95 °C for 15 sec, followed by 60 °C for 45 sec; 7 min hold at 60 °C; indefinite hold at 4 °C. Amplification of the cytochrome b region utilized the following heat cycling profile: 2 min hold at 93 °C; 35 cycles of 93 °C for 1 min, followed by 50 °C for 1 min, followed by 72 °C for 3.5 min; 10 min hold at 72 °C; indefinite hold at 4 °C.

**Primer Selection** – A 15 µL aliquot of each PCR product (control DNA, unreacted with DEB) was removed to a clean microfuge tube and mixed with 3 µL 6X agarose gel loading dye (0.025 g bromophenol blue, 4.0 g sucrose, 10 mL total volume with dH₂O). To determine PCR product length, a 4 µL aliquot of low molecular weight DNA mass ladder (Gibco) was added to 6 µL dH₂O and 2 µL loading dye. Samples and standard were run on a 1.5 % agarose gel (0.6 g agarose in 40 mL 1X TBE), under 1X TBE running buffer, at 70 V until the dye front traveled half-to-three-quarters of the length of the gel. Gels were stained by shaking for 15 min in approximately 100 mL running buffer with 20 µL ethidium bromide (EtBr, 10 mg/mL stock). Stained products were visualized on a UV light box.

**Cross-Linking Reactions** – 1,3-butadiene diepoxide (DEB) was used as obtained from Aldrich. Two stock solutions, 1/100 and 1/10 dilution, were made with dH₂O. Solutions should be made fresh for each experiment. Reaction mixtures consisted of 98 µL liver homogenate plus 2 µL DEB solution (either 1/100 dilution, 1/10 dilution, neat, or dH₂O for control). These DEB dilutions yielded final reaction concentrations of 2.5 mM, 25 mM, and
250 mM DEB. Reaction mixtures were incubated at 37 °C for 30 minutes. After incubation, an equal volume (100 µL) of buffered phenol/chloroform/isoamyl alcohol (pH 8.0) was added to the reaction and mixed with vortexing. Samples were centrifuged (15 min, 12,000 rpm, 4 °C) in an Eppendorf Centrifuge 5402. The aqueous layer was removed and transferred to a second microfuge tube containing 100 µL chloroform/isoamyl alcohol, then mixed and centrifuged as before. The aqueous layer was then transferred to another microfuge tube, containing 2 volumes (200 µL) ice-cold absolute ethanol with 1/10th volume (10 µL) 3 M sodium acetate (NaOAc, pH 5.2). The mixture was vortexed briefly and chilled at -40 °C for 15 min. Samples were then centrifuged as above. The ethanol was removed via micropipette, taking care not to disturb the DNA pellet. A volume of 500 µL ice-cold 70 % ethanol was added to each pellet. Solutions were mixed and chilled at -40 °C for 20 min, then centrifuged as before. The ethanol was again removed via micropipette, taking care not to disturb the DNA pellet. DNA pellets were vacuum dried (Labconco Centrivap Concentrator) and stored in the freezer until needed.

Radiolabeling of Primers – The nominal Forward primer of each pair was 5’-end radiolabeled with 32P. Each reaction used 2 µL 10X T4 Polynucleotide Kinase (PNK) Buffer (New England BioLabs), 2 µL γ-32P ATP (Amersham), twice the volume of primer required for amplification by PCR (due to loss during ethanol precipitation), and enough dH2O for a final volume of 20 µL. Reagents were pooled with a quick spin in a microcentrifuge, and each received 1 µL PNK (10 U/µL) (New England BioLabs). All reagents were mixed with a pipette and incubated at 37 °C for at least 30 minutes. Radiolabeled primers were ethanol precipitated by standard methods (Sambrook et al., 1989) and vacuum dried. For use, each
primer was dissolved in 1 μL dH₂O per intended PCR sample, with an extra 1 μL for use as a standard during electrophoresis (i.e., 5 μL dH₂O for 4 PCR samples plus 1 standard lane).

**PAGE** – Radiolabeled PCR products were separated using polyacrylamide gels. A 10X Tris-borate/EDTA buffer was prepared as follows: 54 g Tris base, 27.5 g boric acid, 3.72 g disodium ethylenediaminetetraacetate (EDTA), in 500 mL dH₂O. Native 8% polyacrylamide gels were prepared according to Sambrook *et al.* (1989): 8 mL 40% (19:1) acrylamide:bisacrylamide, 4 mL 10X TBE, 28 mL dH₂O, 350 μL 20% ammonium persulfate (APS), 20 μL N,N,N',N'-tetramethylethylenediamine (TEMED). Gels were allowed to polymerize for at least one hour. Wells were blown out with running buffer (1X TBE) to remove air bubbles, and gels were pre-run for 1.5 hours at 250 V. PCR samples and primer standards were mixed with 6X loading dye (0.05% xylene cyanol in 50% glycerol); 10 μL dye into each 50 μL product and 5μL dye into each 1 μL primer standard. Samples were electrophoresed for 1.5 hours at 250 V. After electrophoresis, gels were transferred to 3MM chromatography paper (Whatman), covered with plastic wrap, and dried under vacuum (20 minutes of heating, approximately 1 hour total drying time) on a Drygel Sr. Slab Gel Dryer SE 1160 (Hoefer Scientific Instruments). Dried gels were exposed to a Molecular Imaging Screen-BI on a GS-505 Sample Exposure Platform (BioRad) for 4 hours. Bands were visualized and quantitated on a GS-505 Molecular Imager using Molecular Analyst software (version 2.1.2) (BioRad). Boxes of equal area were drawn around each band to be quantitated, then automatically integrated by the program.
Results

Isolation of DNA & Primer Selection - Chicken liver was homogenized in 1X PBS. Initial tests on the homogenate utilized a Chelex treatment (Walsh et al., 1991) to obtain DNA. After thermal cycling, the amplification products were visualized on a 1.5 % agarose gel with ethidium bromide (EtBr) staining. The β-globin primers failed to amplify this DNA. A confirmatory test, run with primers for a 407 bp region of the mitochondrial cytochrome b gene that had been successfully amplified previously (Kocher et al., 1989), failed as well.

DNA was then isolated from the homogenate via phenol/chloroform/isoamyl alcohol extraction, followed by ethanol precipitation. The β-globin and cytochrome b primers successfully amplified this DNA (Figures 7-9).

Figure 7. 1.5 % agarose gel of cytochrome b gene region amplification (stained with EtBr). Lanes 1, 6-8: Blank; Lanes 2-4: Cyt b; Lane 5: Mass Ladder
Next, test reactions were run on whole homogenate, using no DEB. DNA pellets were recovered as before, with each recovered pellet being used for a single amplification reaction. Bands were visualized again by agarose gel electrophoresis and EtBr staining. All four β-globin regions were amplified.

We next tested for incorporation of radiolabel during amplification. We were able to see successful amplification in three of the four samples. The 589 bp “closed” region, β1, did not amplify as expected. The two ~140 bp β2 and β3 fragments, “closed” and “open” regions, respectively, amplified, as did the 61 bp “open” region, β4.
QPCR – For quantitative comparison of lesion frequency in “open” versus “closed” chromatin, primers for regions β2 and β3 were chosen for further investigation, as they amplify regions of approximately the same length. We first tested the “open” β3 region. Upon visualization, we noted a decrease in full-length band intensity as the amount of DEB added to the reaction mixture was increased. Integrating the band volumes, we found a linear relationship between lesion frequency and log [DEB]. Additionally, there were a number of bands appearing below that of the unincorporated primer in each reaction well lane (Figure 10).

To remove these bands, which may have been degraded primer fragments and very small amplification side-products due to those fragments we decreased the extension time from
1 minute to 30 seconds, in hopes of only amplifying our major products in abundance, and used a shorter exposure time when phosphorimaging (4 hours instead of overnight). This shortened extension time proved to inhibit amplification altogether. To rescue amplification, we increased the extension time to 45 seconds. Applying this modified thermal profile and imaging time, we were able to successfully amplify full-length product, while still suppressing amplification and visualization of the extraneous bands.
Phosphorimage analysis of this gel gave similar results to those obtained previously. We found a linear, dose-dependent relationship between lesion frequency and the log [DEB]. However, samples did not travel cleanly through the gel. There was radiolabel trapped in the wells.

Our next gel tested the "closed" β2 region. This first gel failed to run, with all primers and amplification products retained in the wells. On our second attempt, we were able to visualize full-length products (Figure 11). However, once again, large portions of both the

![Phosphorimage of "closed" β2 region. Lanes 1, 6: Radiolabeled primer; Lanes 2, 7: Control (no DEB); Lanes 3, 8: DEB (2.5 mM); Lanes 4, 9: DEB (25 mM); Lanes 5, 10: DEB (250 mM); Lanes 6 through 10 used for quantitation.](image-url)
radiolabeled primers and the reaction products were retained in the wells. Nevertheless, we integrated the band volumes. Our results indicated that there was no inhibitory effect on amplification of our full-length product by DEB addition, although these are highly speculative data (Figure 12, Table 3).

![Lesion Frequency vs. log [DEB]](image)

**Figure 12.** Lesion frequency versus log [DEB]. Data for two trials of "open" region, and their average, are shown, along with the single "closed" region trial. Results for previously determined lesion frequency in mitochondrial DNA (mtDNA) are also included for comparison (Juskewitch and Millard, 2003). One standard deviation is illustrated by the error bars on the "open" trial average.

Two repetitions of the experiment with the "open" β3 region resulted in all bands co-migrating with the primer band.
Table 3. Calculated lesion frequency in "open" versus "closed" regions due to addition of varying concentrations of DEB.
Discussion

Chicken liver is a convenient source of commercially available DNA. Using chicken as our model organism also facilitated target gene selection. Published work on the chicken β-globin domain, done by Litt and colleagues (2001a,b), gave us access to primer sequences and a thermal cycling profile of proven functionality. Further, given that BD and DEB have been associated with hematopoietic cancers, a gene related to blood cells seemed an appropriate target.

Four sets of primers were chosen from regions of both "open" and "closed" chromatin within the chicken β-globin domain, to allow for comparisons of lesion frequency between regions of transcribed and non-transcribed sequence.

Shifting from the Chelex preparation to the organic extraction may provide the added benefit of stopping the DEB cross-linking reaction at discrete intervals when time-course studies are performed by removing excess, free DEB from the mixture. The previous work, utilizing the Chelex treatment, was not able to halt the DEB reaction at specific time points (Juskewitch and Millard, 2003).

The first test for incorporation of radiolabel failed because the tracking dye used was for agarose gels, and we did not run samples of the radiolabeled primers as standards. These issues were addressed in our successful second attempt at visualization, wherein all four regions amplified (Figure 10).

When we began experiencing incomplete runs, we looked to our reagents. As we had been using a TBE buffer previously made by members of the lab, we surmised that precipitation of buffer salts may have affected our acrylamide gels. We made a fresh stock of 10X TBE for future gel casting, and ran the DEB reactions again. Although able to visualize
full-length products (Figure 11), portions of both the radiolabeled primers and the reaction products were still retained in the wells. Since we were not completely hampered in visualizing a full-length product, we integrated the band volumes (Table 3, Figure 12). However, given the amount of material retained in the gel wells, these numbers are uncertain.

For the "closed" β2 region, results from the Poisson equation (Table 3), representing lesion frequency, hovered above and below zero. The calculated numbers themselves indicated that addition of DEB effectively increased amplification of the target region, albeit only nominally. We know this is not the case. Further trials are needed, in which all primers and products leave the wells and migrate through the gel properly.

As we were not certain what exactly confounded our second β2, "closed" region gel run, we began the experiment anew, with the β3, "open" region. The co-migration of these β3 samples indicated that although the gel ran properly, there were no amplified PCR products to visualize in the first place. Thinking that perhaps our reagents were responsible for the lack of amplification, we repeated the experiment with a new mixture of deoxynucleotide triphosphates (dNTPs) and fresh Taq polymerase. Once again, the gel ran properly, with nothing trapped in the wells, but each sample band co-migrated with the primers, indicating that PCR failed to amplify our target region.

These results are daunting, but we are keenly aware of the oft-times finicky nature of PCR. Many variables affect successful amplification, and it can take time to ascertain exactly which step in the procedure or ingredient in the reaction mixture is causing difficulty.

Fresh reagents should be used in further experimentation. Making a fresh batch of liver homogenate may also prove useful. Although the aliquots of the original homogenate were only thawed as needed, stability issues play a significant role when storing cells in PBS.
suspension for extended periods at -20 °C. Samples may degrade within a matter of weeks, so fresh homogenates should be made often, if not each time reactions are to be run.

Once the details of the PCR are worked through on the liver homogenate DNA, future experiments will seek to further elucidate the action of DEB in vivo using cultured chicken tissues. At discrete time points, aliquots will be removed for analysis, enabling a time-course study of cellular response to DEB exposure.

In the liver homogenate, within “open” chromatin, we have seen decreases in full-length product amplification due to lesion formation by DEB, monitored by decreases in radiolabel incorporation. In the second phase, utilizing living, cultured cells, subsequent cellular repair of the DEB-induced lesions would be marked by increases in full-length product band intensity as compared to initially DEB-damaged baseline band intensity.

The “open” or “closed” nature of chromatin regions clearly has important implications. Carcinogenicity of BD, by way of its metabolite DEB, may be a function of the diepoxide’s ability to alkylate acetylated chromatin regions that normally code for important gene products. Cancers can arise from damage to tumor suppressor genes or activation of oncogenes, and perhaps an alkylaion event such as this could accomplish either, or both, of these tasks. If one of these key regions were particularly amenable to alkylation by DEB, it might help shed further light on the cytotoxic mode of action.

Purported “hot spots” for activity have been cited for some DNA damaging agents. For example, in the 5S RNA gene of Xenopus borealis, calicheamicin has been shown to bind and cleave nucleosomal DNA in a regular, periodic fashion, which is not explained by sequence alone. This preference arises when DNA is complexed to histones, but is not seen in free DNA (Kuduvalli et al., 1995). However, findings by Millard and Wilkes (2001) suggested no
significant difference in the alkylation of nucleosomal versus free DNA by DEB in this core particle. These studies did not take into account histone modification (e.g., acetylation), varied DNA sequence within the core particle, or linker DNA, which will likely bear influence on chromatin in vivo. Nor do we have a clear understanding of possible flanking sequence effects during binding. DEB may display a preference for binding to the N7 position of guanine in a 5'-GNC motif, but what of the upstream or downstream neighboring bases? This may add yet another layer of complexity, and these studies are ongoing.

The previous body of work has laid the foundation of our understanding of DEB reactivity, but the living cellular environment is far too complex to be modeled in any static way. Numerous questions yet remain.

Depending upon which stage of the cell cycle a target cell is in, it may react very differently to DEB exposure than a neighboring cell in a different stage. How does the mitotic cell compare to its quiescent neighbor? Will transcription factors or other regulatory elements out-compete DEB for binding to different sequences residing in "open" chromatin?

Histone involvement has been shown to be very important, but what of the various non-histone scaffolding proteins? Could these be contributing steric blocking effects, limiting DEB access to regions of "closed" chromatin? And what of natural DNA repair mechanisms? Are monoalkylated lesions more easily excised and repaired than inter- or intra-strand cross-links? Does one of these lesions tend to lead to mutations and chromosomal aberrations more so than its counterparts?
Conclusions

To effectively build upon our fundamental knowledge, and to begin to answer questions that remain, cultured cells need to be the next model system for DEB action in vivo. However, for now, based upon our study of liver homogenate in vitro, we make some preliminary conclusions. First, DEB alkylates “open” regions of chromatin in a dose-dependent manner, as evidenced by the linearly increasing lesion frequency, as compared to the log [DEB]. Second, DEB has little-to-no lesioning effect on “closed” regions of chromatin, with the caveat that these data were not completely conclusive, but only tended to suggest little-to-no lesioning effect. Confirmatory tests are required for a greater degree of certainty.
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