

Analysis of Gene Regulation in Rabbit Corneal Epithelial Cells Induced by Ultraviolet Radiation

Jacqueline J. Stevens^{1*}, Christian Rogers¹, Carolyn B. Howard², Caronda Moore¹, and Lai-Man Chan³

¹Molecular Biology Research Laboratory, ²Breast Cancer Research Laboratory, ³Chemistry Research Laboratory, NIH-Center for Environmental Health, College of Science, Engineering and Technology, Jackson State University, 1400 J R Lynch Street, Jackson, Mississippi 39217, USA.

*Correspondence to Dr. Jacqueline J. Stevens. Email: jacqueline.j.stevens@jsums.edu

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Abstract: Ultraviolet (UV)-induced cataracts are becoming a major environmental health concern because of the possible decrease in the stratospheric ozone layer. Experiments were designed to isolate gene(s) affected by UV irradiation in rabbit cornea tissues using fluorescent differential display-reverse transcription-polymerase chain reaction (FDDRT-PCR). The epithelial cells were grown in standard medium for 2 or 4 hours post treatment. Cornea epithelial cells were irradiated with UVB for 20 minutes. RNA was extracted and amplified by reverse transcriptase-polymerase chain reaction using poly A⁺ specific anchoring primers and random arbitrary primers. Polyacrylamide gel electrophoresis revealed several differentially expressed genes in untreated versus UV irradiated cells. Complimentary DNA (cDNA) fragments resulting from fluorescent differentially expressed mRNAs were eluted from the gel and re-amplified. The re-amplified PCR products were cloned directly into the PCR-TRAP cloning system. These data showed that FDDRT-PCR is a useful technique to elucidate UV-regulated gene expressions. Future experiments will involve sequence analysis of cloned inserts. The identification of these genes through sequence analysis could lead to a better understanding of cataract formation via DNA damage and mechanisms of prevention.

Keywords: ultraviolet radiation, cornea, cataracts, differential display-reverse transcription-polymerase chain reaction, DNA damage, opacity, environment.

Introduction

The human eye and skin are the only tissues directly exposed to ultraviolet radiation (UV) and visible radiations. The full spectrum of UV radiation can be classified into three groups, based on wavelength, ultraviolet A (UVA) (400 – 315 nm), ultraviolet B (UVB) 315-280 nm, and ultraviolet C (UVC) (280-100 nm). UVB and UVA can reach the surface of the earth causing biochemical and physiological effects depending upon radiant exposure and wavelength [1]. Over 98% of solar UV radiation exposure is in the form of UVA. It penetrates the skin more deeply than UVB or UVC, but is less associated with DNA damage. UVB accounts for less than and 2% of our solar UV radiation exposure, as much of it is absorbed in the upper atmosphere. Although UVB is responsible for most of the DNA damage within skin cells that might lead to the promotion of cancers [1-2], however UVC is considered the most lethal form of UV radiation. The ozone layer

effectively blocks most UV. The risk of exposure to harmful UV radiation is gradually increasing due to continuous erosion of the stratospheric ozone layer [3-4]. Studies have shown that excess exposure to ultraviolet radiation can lead to severe abnormalities within radiation-exposed tissues. UV radiation has been shown to damage ocular tissues [5-6] and UVB is known to reach the cornea. Chronic exposure of eyes to UV is heavily implicated in the development of cataracts (for example, opacities of the lens), and can also cause phototoxic effects to the retina [7-9]. Age-related cataract is a multifactorial eye disease, the leading cause of blindness, and is becoming an increasing global problem. An estimated 1% decrease in ozone thickness will enhance the rate of cataracts by 0.7% [10]. Epidemiological studies have shown that cataract formation is associated with prolonged exposure to sunlight. DNA is a major target of UV-induced cellular damage. In addition, several risk factors for cataract formation include diabetes, alcohol, smoking, and

steroid use [11-13]. All three wavelengths of naturally occurring UV light, UVA, UVB, and UVC, may directly induce pyrimidine and thymine dimer formation [7,14], as well as DNA strand breaks and DNA-protein cross-linking [15]. It is thought that UV-induced skin cancer may largely result from such DNA damage [16, 17]. UV irradiation is known to induce programmed cell death or apoptosis in the cornea. Apoptosis is a mechanism associated with corneal cell death after UV irradiation [15, 18, 19]. Exposure of mammalian cells to UV radiation triggers an alteration in gene expression [16, 20]. The molecular mechanisms are still unclear and the effects on expression of UV-induced genes involved in DNA damage and cataract formation have not been determined.

We employed the fluorescent differential display polymerase chain reaction (FDD-PCR) technique to analyze changes in gene expression between untreated and UVB irradiated corneal epithelial cells. The mRNA differential display (DD) technique developed by Liang and Parde [21] allows the isolation of unknown genes without prior knowledge of their sequence, just on the basis of their cellular abundance. The technique also permits simultaneous identification of up-regulated and down-regulated genes between two groups of cells, tissues, or conditions. It has been successfully used to identify differentially expressed genes in numerous systems including ocular tissues [22-25]. UV light induces the expression of a wide variety of genes. The prevalence of cataracts approximately doubles with each decade after the age of thirty. However, the effects of UV exposure in corneal epithelial cells at the molecular level have not been elucidated. If we are able to delay the onset of age-related cataracts by ten years, the number of cataract operations could be decreased tremendously. The goals of this study were to identify differentially expressed genes in the corneal epithelial cells in response to UVB irradiation and to analyze the changes in gene expression related to DNA damage with subsequent cataract formation.

Materials and Methods

Cell Culture

Corneal Epithelial Cells

Eyes were purchased from eight to twelve weeks old New Zealand White rabbits. The method of Cubitt *et al.* [26] was used (with some modification) to culture the corneal epithelial cells. Briefly, corneas were dissected at the limbus, and digested overnight at 4°C by placing the cornea on top of 60 – 80µl of Dispase II (Boehringer Mannheim). The epithelial layer was gently dissected from the stroma using a small spatula, and the epithelial sheets were digested with 1 ml of trypsin (0.05)-EDTA (0.02) (Sigma) and was incubated at 37°C for 10 minutes. Trypsinization was stopped by adding soybean trypsin inhibitor (Sigma) (1.5ml). Single-cell suspensions were made by passing the suspensions four to five times through a syringe with a 23-gauge needle and 15% rabbit serum was added. The cells were centrifuged at 1000 rpm for 3 minutes and the

supernatant was discarded. The cells were seeded in three tissue culture flasks (Falcon Primaria positively charged 25 cm²) per two corneas. Three milliliters of media were added to each flask. Cells were cultured in media (Cascade Biologics) supplemented with rabbit corneal growth factors (Cascade Biologics) and gentamicin (Sigma) in a humidified incubator at 37°C with five percent CO₂. The epithelial cells were sub-cultured at a passage ratio of 1:3. The majority of the experiments were carried out using confluent cells from second and third passages, although primary cells and fourth passage cells were used in the indicated experiments. However, it should be noted that all cells had the characteristic epithelial cobblestone appearance, and the results were not dependent on passage number.

UVB Irradiation

The corneal epithelial cells were grown to 90% confluence. The confluent cells were incubated in fresh medium (4mls) at 37°C for 30 minutes before UV irradiation exposure. The corneal epithelial cells were cultured in Medium 500 and MEM containing fifteen percent rabbit serum, respectively. Two XX-15B lamps (Spectronics) were placed side by side and used as an UVB source. The UVB lamps had a maximum at 310 nm with a wavelength range of 280 – 365 nm. The radiant energy at the level of the cells was measured with a DRC-100H radiometer (Spectronics) with the sensor, DIX-300 for UVB. During UVB irradiation, the lids of the Petri dishes were removed and replaced with quartz plates. The UV dose was administered for 20 minutes for UVB exposure. The irradiated and untreated corneal epithelial cells were incubated at 37°C for 2 or 4 hours, after which they were harvested.

RNA Isolation

Total RNA was isolated from untreated (UT) and UVB exposed corneal epithelial cells using the RNApure reagent (GenHunter, Nashville, TN). The RNA samples were treated with RNase-free DNase I and incubated for 30 minutes to eliminate chromosomal DNA contamination prior to proceeding to the differential display system. The concentration of the RNA was determined spectrophotometrically (absorbency at 260 nm and A₂₆₀/A₂₈₀ ratios of 1.7 or higher) [27]. The size distribution and the integrity of the purified total RNA was analyzed by denaturing formaldehyde agarose gel electrophoresis.

Fluorescent Differential Display-Reverse Transcription-Polymerase Chain Reaction (FDDRT-PCR)

Fluorescent Differential Display (FDD) using rhodamine (GenHunter, Nashville, TN) was performed according to the manufacturer's instructions. The RNA was quantitated as previously described and diluted to the appropriate amount with diethylpyrocarbonate water (DEPC-H₂O). The mRNA Differential Display method was performed routinely by using 0.1µg of total RNA for reverse transcription reaction. Three reverse transcription reactions for each RNA sample was carried out in thin-

walled PCR tubes, and each containing one of the three different rhodamine labeled anchored oligo dT primers (RH-T₁₁M, where M was G) were prepared for each RNA sample isolated from untreated and UV-B induced corneal epithelial cells. Each reaction mix contained RNase-free H₂O (9.4μl), 5X reverse transcriptase buffer [125mM Tris-HCl, pH 8.3, 188mM KCl, 7.5mM MgCl₂, and 25mM DTT] (4μl), dNTPs [250μM] (1.6μl), RNA [0.1μg/μl] (2μl), and RH-T₁₁M primer (2μl). The thermocycler was programmed as follows: 65°C for five minutes, 37°C for 60 minutes, 75°C for five minutes, followed by a cooling at 4°C. One microliter of MMLV reverse transcriptase (100units/μl) was added to each tube 10 minutes after incubation at 37°C. Throughout the FDD experiments, control RNA (positive control) isolated from transformed rat embryo fibroblasts provided by the kit (GenHunter Corporation) was run simultaneous to compare the efficiency of the system. The fluorescent dyes were light sensitive and were kept in the dark until used.

Following the reverse transcription of the RNA, the resulting cDNAs were PCR amplified using various combinations of the rhodamine labeled arbitrary anchored oligo dT primer and an arbitrary decamer. The PCR reactions consisted dH₂O (10.2μl), 10X PCR buffer (2μl), dNTP mix (1.6μl), H-AP primer [2μM] (2μl), rhodamine labeled RH-T₁₁M primer (2μl), RT-mix (2 μl) and Taq DNA polymerase (0.2μl) for a total volume of 20μl. The thermocycler was programmed as follows: 94°C for 30 seconds, 40°C for 2 minutes, 72°C for 60 seconds for 40 cycles, followed by 72°C for 5 minutes, and stored at 4°C until further use.

6% Denaturing Polyacrylamide Electrophoresis

PCR products were resolved in parallel lanes on a 6% denaturing polyacrylamide gel in TBE buffer. Gels were pre-run for 30 minutes and 3.5μl of each sample with 2μl of FDD loading dye were mixed and incubated at 80°C for two minutes immediately before loading onto 6% DNA sequencing gels. The gels were electrophoresed for 2-3 hours at 60 watts constant power, and scanned on a Fluorescence Imager (Hitachi FMBIO[®] II) using 585 nm filters, following manufacturer instructions.

Selection of Differentially Expressed Bands, Re-amplification of cDNAs and Cloning System

Differentially expressed bands were excised from the gels, boiled for 30 minutes and re-amplified with the same primer combinations and PCR conditions, except unlabeled H-T₁₁M anchor primer was used instead of the rhodamine labelled primer. The re-amplification PCR reactions consisted of dH₂O (23.3μl), 10X PCR buffer (4μl), NTP mix (0.3μl), H-AP primer [2uM] (4μl), H-T₁₁M [2uM] (4μl), cDNA template (from the gel) (4μl) and Taq DNA polymerase (Qiagen) (0.4μl) for a total volume of 40μl. The re-amplified PCR products were run on 1.5% agarose gels stained with ethidium bromide before cloning. These products were cloned into the PCR-TRAP[®] cloning system (GenHunter, Nashville, TN) according to manual protocol. The size standards

used for colony PCR screening was NEB 100 base pair (bp) ladder (Gibco-BRL, Gaithersburg, MD). The clones selected for sequencing were restreaked onto Luria Broth plates containing tetracycline (LB-tet) for single-colony isolation.

Northern Blot Analysis

For northern blots, total RNAs (5 g) isolated from untreated and UVB irradiated corneal epithelial cells were fractionated on 1.2% agarose gel and transferred onto Hybond-N+ membrane (Amersham) using standard procedures (30). The membrane was hybridized with ³²P dCTP-labeled cDNA probe (JS6) at 42°C for 16 hours and extensively washed with 2xSSPE/0.1% SDS, 1xSSPE/0.1% SDS and 0.1xSSPE/0.1% at 42°C. Autoradiography was performed at -70°C overnight.

Results

By phase contrast microscopy, the morphology of the cornea epithelial cells appeared changed after UVB exposure. These cells were visualized at a 40X magnification. The untreated corneal epithelial cells (Figure 1A) were healthy and attached with a cobblestone appearance. These cells have been growing for 3-4 days and a few cells have shown signs of apoptosis. After exposing the cells to UVB for 20 minutes (Figure 1B), the cells became separated and elongated due to a loss of membrane integrity. This event may be a result of primary necrosis or secondary apoptosis [28]. Four cells exhibited apoptosis (Figure 1B). Corneal epithelial cells were exposed for 45 minutes (Figure 1C), which resulted in more separation with some rounding of the cells. Some cells were detached and formed dark bodies within the cells. This may be due to necrosis and other forms of DNA damage such as single strand breaks. Twenty-five cells exhibited apoptosis (Figure 1C).

The FDD allowed for parallel analysis of four RNA populations. The RNA populations compared were untreated versus UVB irradiated corneal epithelial cells with 2 hours post-treatment and untreated versus UVB irradiated corneal epithelial cells with 4 hours post-treatment. The FDD analysis of the untreated and UVB irradiated corneal epithelial cells indicated a number of similarities in gene expression between untreated and UVB irradiated cells (Figure 2). A large number cDNAs were present in both untreated and UVB irradiated corneal epithelial cells, however, the majority of these genes were not affected by UV radiation exposure. As a result, these cDNAs represent the house keeping genes found in corneal epithelial cells. Figure 2 represents a typical fluorescent image of differentially expressed cDNAs. Several differentially expressed bands were detected in the differential display gels. The eleven differentially expressed bands with the strongest intensities and best resolutions were excised from the gel and re-amplified using the same primer set. Eight of the differentially expressed bands were down-regulated and the other three differentially expressed bands were up-regulated in response to UVB exposure.

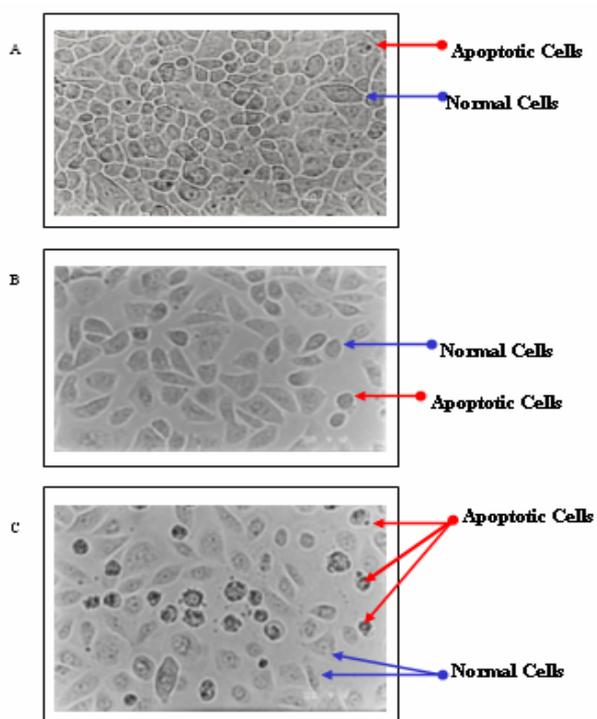


Figure 1: Morphology of Corneal Epithelial Cells by Phase Contrast Microscopy. Morphologic changes in corneal epithelial cells exposed to UVB by phase contrast microscopy (40X magnification).

- A. Untreated confluent corneal epithelial cells (no exposure to UVB); a few cells were sensitive to apoptotic death.
- B. Morphologic changes in corneal epithelial cells exposed to UVB (0.6J/cm²) for 20 minutes; four apoptotic cells were visualized.
- C. Morphologic changes in corneal epithelial cells exposed to UVB (0.6J/cm²) for 45 minutes; twenty-six apoptotic cells were visualized.

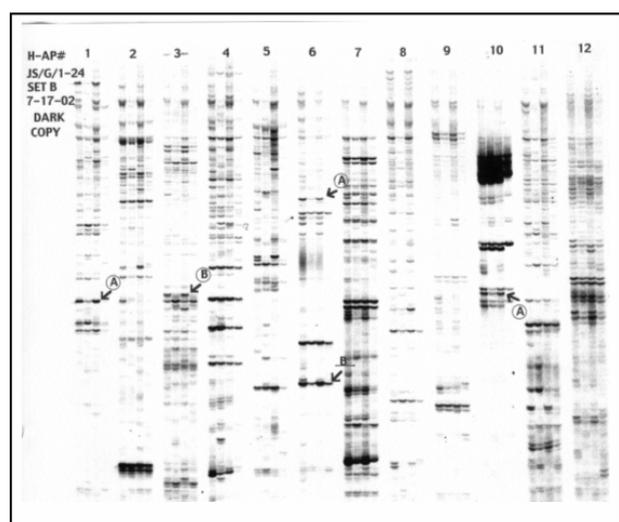


Figure 2: Fluorescent Differential Display. A typical fluorescent image of differentially expressed cDNAs using a 6% denaturing polyacrylamide electrophoresis. Differentially expressed bands obtained from untreated

or UVB irradiated cornea epithelial cells are marked by arrows.

The differentially expressed bands were electrophoresed on a 1.5% agarose gel and ranged in size from 200 base pairs (bp) to 800 base pairs (Figure 3).

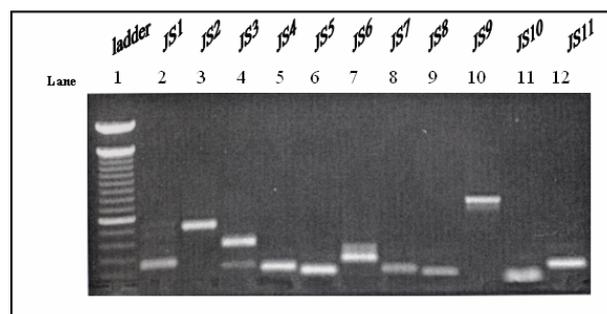


Figure 3: Re-amplification of differentially expressed genes. 1.5% agarose gel of the eleven differentially expressed bands chosen for re-amplification. Lane 1 is 100 bp ladder (Gibco-BRL) and lanes 2-12 are the selected differentially expressed bands (JS1-JS11).

The sizes of the differentially expressed bands chosen for reamplification were as follows: JS1, 300 bp; JS2, 550 bp; JS3, 400 bp; JS4, 300 bp; JS5, 280 bp; JS6, 300 bp; JS7, 280 bp; JS9, 200 bp; JS10, 800 bp; JS11, 200 bp; and JS12, 300 bp. The eleven re-amplified bands were cloned into the PCR-TRAP Cloning System. Four colonies for each band were checked for inserts by colony-PCR (Figure 4).

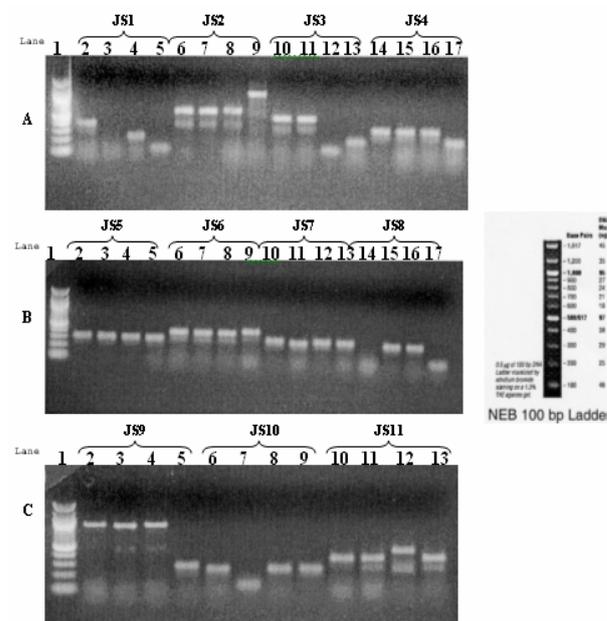


Figure 4: Cloning of reamplified PCR products. Four colonies for each differentially selected band reamplified were checked for inserts by colony PCR. 100 bp ladder (NEB) was used as a size standard in lanes 1A, 1B and 1C for gels. JS represents selected differentially bands from normal and UVB irradiated corneal epithelial cells. Gel A: Lanes 2-5 (Inserts from JS #1; Lanes 6-9 (Inserts from JS #2); Lanes 10-13 (Inserts from JS #3); Lanes 14-17 (Inserts from JS #4). Gel B: Lanes 2-5 (Inserts from JS #5; Lanes 6-9 (Inserts from JS #6); Lanes 10-13 (Inserts from JS #7); Lanes 14-17 (Inserts from JS #8).

Gel C: Lanes 2-5 (Inserts from JS #9; Lanes 6-9 (Inserts from JS #10); Lanes 10-13 (Inserts from JS #11).

Two colonies from each band showing the correct size were re-streaked on LB agar plates containing tetracycline. Differential expression of one of the eleven cDNAs was confirmed by Northern blot analysis (Figure 5) following standard procedures; as indicated in the material and methods section. The cDNA (JS#6) was used as a probe; a distinct band approximately 1.2 kb (Figure 5, lane 1) was detected in the untreated cells whereas, no band was detected in the UVB irradiated corneal epithelial cells (Figure 5, lane 2), suggesting a down-regulation of the gene in the corneal epithelial cells following UVB exposure.

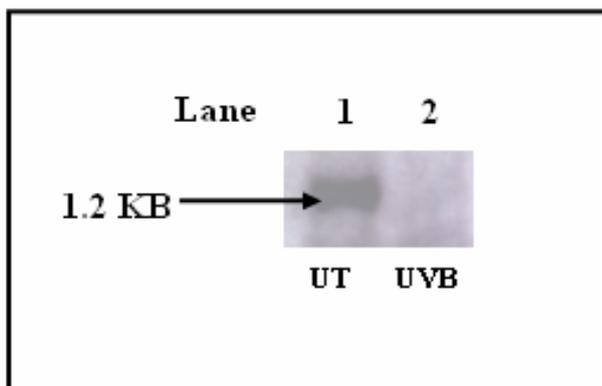


Figure 5: Down Regulation of Corneal Epithelial Cells after UVB Exposure. The differentially band, JS#6, was used as a probe; a 1.2 kb band was detected in the untreated (UT) cells (lane 1), whereas no band was detected in the UVB irradiated corneal epithelial cells (lane 2).

Discussion

The corneal epithelial cells were grown to confluency and irradiated for 20 minutes. Rogers *et al.* [29] have shown that these conditions have been effective in corneal epithelial cells. The cells were incubated for 2 or 4 hours post-treatment after which the cells were harvested to allow cellular recovery after UV irradiation. Some cells had undergone apoptosis following UVB exposure. Rogers *et al.* [29] have performed tunnel assays to study the induction of cell death due to broadband UVA and UVB to the cornea epithelial cells; furthermore, they have shown that UVA and UVB, at low dose, induced cell death in corneal epithelial cells.

In this study, we used the FDD system to identify differentially expressed genes induced by UVB irradiation in rabbit corneal epithelial cells. DD has proven to be a powerful technique for the detection and isolation of differentially expressed genes. The DD technique provides a novel method for identifying those mRNAs that are induced or repressed at the gene level and is applicable in a variety of biological systems [30-32].

The DD has been used as an alternative to the conventional differential or subtraction hybridization techniques for detecting differences in gene expression between closely related populations of eukaryotic cells. The major obstacle of differential display is not the

technique itself however it is the post-differential display issue of discriminating between false positives and the truly differentially expressed mRNA. Therefore, to overcome this obstacle, and to achieve a reduced number of false positives, a variety of modified primers and altered cycling conditions have been implemented [32]. By generating reproducible cDNA expression data, it is possible to compare gene expression in two or more cell types, or developmental stages or tissues associated with diseases, and a technique to isolate unknown differentially expressed genes. For example, this method has been quite successful in identifying differentially expressed genes in normal versus tumor-derived human mammary epithelial cells [33], isolating the gene D2-2 that was over expressed in glioblastoma multiforme tissue as compared to normal brain tissue [34], as well as identifying and characterizing differentially expressed genes in various stages of prostate cancer development [35] and isolating light activated genes differentially expressed in *Coprinus congregatus* [36]. The differential display technique allows side-by-side comparison of two different cell populations and therefore helps to identify known genes as well as unidentified new genes. FDD methodology has many advantages in that it is based on PCR and DNA sequencing gel electrophoresis and is more sensitive and reproducible in screening new genes. Importantly, this technology has been revolutionized by the use of fluorescent detection of PCR products instead of radioactivity. The use of fluorescent dyes is significantly comparable to gamma [³²P] isotopic labelling [37]. The goals of this study were to identify differentially expressed genes in response to ultraviolet exposure in corneal epithelial cells. In this study, the FDD was used to identify and isolate differentially expressed genes that are associated with UVB response in corneal epithelial cells.

Total RNA isolated from untreated or UVB irradiated corneal epithelial cells was used as a template to synthesize cDNA. Three, one-base-anchored oligo-dT primers, labelled with 5' rhodamine served as the primers. The use of denaturing sequencing gels showed several distinct bands from normal and UVB irradiated cells. After the gel was electrophoresed and scanned, eleven differentially expressed bands were selected and one was used as a probe for gene expression of differential display. It was demonstrated by Northern blot analysis that the gene (JS6 as a probe) was differentially expressed found in untreated and UVB irradiated cornea epithelial cells. In this case some genes were either turned on or off to UVB exposure (Figures 2-6). Gene regulation is manifested after UV radiation. This manifestation is seen as a down regulation of the gene of interest and is associated with a 1.2kb down regulated gene (Figure 5). Furthermore, this gene may be associated with cataract development involved in damaging the eye tissue.

The findings of this study indicate that the fluorescent differential display-reverse transcription-polymerase chain (FDD-RT-PCR) reaction is a useful method for identifying genes that are differentially expressed in response to corneal epithelial cells exposed to UVB irradiation. The literature has not elucidated which genes are activated or deactivated in association

with cataract development due to UVB irradiation, which is the impetus for further ongoing studies in our laboratory. The genes differentially expressed in the untreated and UVB irradiated corneal epithelial cells will be used to study the mechanisms of action associated with cataract development. Therefore, we will address the identification through sequence analysis of genes activated or deactivated by UVB irradiation that may reveal molecular mechanisms underlying UVB exposure to cataract development. In addition, our laboratory also plans to use other cDNAs as probes to determine if there are other genes differentially expressed. Further, we would like to use the DD techniques on varying times of UVB exposure to the corneal epithelial cells and to determine if these genes are down-regulated or up-regulated, as well as exposure of cells to UVA. Therefore, the results of these studies should lead to a better understanding in the prevention of UVB-induced lens opacity (cataracts) with subsequent DNA damage to the cornea.

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