Assessment of λ transgenic fish as a new model for UV mutagenesis

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Abstract

The λ transgenic medaka, a fish model carrying the *cII* target gene, was used to evaluate ultraviolet radiation (UV)-induced mutagenesis. Mutant frequency induction (MF) was assessed 21 days after treatment of embryos with either UVB or UVA, and a 24 hour light or dark interval. Early (stage 6), middle (stage 25) or late stage (stage 38) embryos were exposed to 0, or 400 J/m^2 UVB (280-320 nm). Stage 25 and 38 embryos were also exposed to 0, 400, 800, or 1000 J/m^2 UVB. Additional embryos were treated with 0, 400, 800 or 1600 J/m^2 UVA (321-400 nm). MFs were obtained using the *cII* mutation assay in which the *cII* gene was recovered from genomic DNA and transferred into indicator bacteria to distinguish mutant and non-mutant genes. Significant mortality, deformities and MF inductions were observed only in stage 25 embryos exposed to UVB. In the 400 J/m^2 UVB + dark treatment, 68% of embryos died, 33% showed distinctive abnormalities and the MF was induced ~3-fold above controls. The survival in 400 J/m^2 + light treatment was comparable to untreated controls, whereas the MF was induced ~2-fold. Similar relationships between survival, and MFs with light or dark treatment were observed in Experiment 2. UVB + dark treatments exhibited significant mortality and up to 12-fold MF induction. MFs were elevated up to 3.7-fold with increasing UVB + light exposure, indicating photoreactivated repair did not fully reduce UVB-induced effects. Results show this to be a suitable animal model for UV mutagenesis as it is highly responsive to UVB radiation with survival, development and MF induction dependent upon embryonic stage, dose and photoreactivated repair.
Keywords: transgenic medaka, UVB and UVA radiation, mutation, photoreactivated DNA repair.

1 Introduction

The spectrum of solar ultraviolet radiation (UV) at the surface of the earth is comprised of both UVA (315-400 nm) and UVB (290-315 nm) radiation. Epidemiological and laboratory studies provide strong evidence linking UV exposure with the induction of skin cancer [1]. Although it remains unclear whether UVB or UVA, or a combination of both, or interactions between the wavebands is the main cause of skin cancer in humans [2], it is understood that both UVB and UVA causes direct or indirect damage to DNA. UVA is photocarcinogenic and is involved with photoaging, but it is absorbed weakly in DNA and protein. UVA is responsible for the indirect production of reactive oxygen species responsible for secondary oxidative damage to DNA [3]. UVB encompasses the DNA- and protein-absorption spectra in the range responsible for skin cancer through direct photochemical damage to DNA [4].

Transgenic animal models, based both in fish and rodent species, provide unprecedented opportunities for studies of comparative in vivo mutagenesis [5, 6, 7]. Analysis of mutations in these different models entails a similar general approach. The transgenic models carry specific genes that serve as mutational targets harbored within prokaryotic shuttle vectors. After treatment with a mutagen, and allowing sufficient time for the mutations to manifest, genomic DNA is isolated from the tissues of interest. The vectors are then separated from the animal's genomic DNA, and shuttled into indicator bacteria that facilitate distinguishing and quantifying mutant and nonmutant genes. This approach provides numerous practical benefits, including the ability to analyze mutations directly at the level of the DNA, at low frequencies, with high precision, and in a variety of tissues within a whole animal model. In addition, the recovered target gene can be directly sequenced to provide insights into possible mechanisms of mutagenesis.

The suitability of transgenic mutation models for study of UV-induced mutagenesis has been shown using mice models [8, 9]. The responsiveness of the recoverable mutation target gene to UV exposure in these studies provided the basis for continued and expanded use of the approach. In this study, we evaluate the efficacy of the λ transgenic medaka [7] as a new in vivo model for UV-induced mutagenesis. We compared induction of the frequency of mutant *cII* target genes recovered from fish after treatment of different embryonic stages to varying doses of UVB and UVA, and treatment post exposure with either a 24 hour light or dark interval.

2 Methods

2.1 Animals

λ Transgenic medaka that carry the λ bacteriophage vector harboring the *cII* mutation target gene (homozygotes, ~150 λ copies/diploid genome) were obtained from in-house stocks maintained at the Aquatic Biotechnology and Environmental Laboratory (ABEL), University of Georgia. The Institutional

Animal Care and Use Committee approved the animal use protocol for this study.

2.2 UVB and UVA irradiance

The UV spectral irradiance was administered by Phillips TL40W/125RS J2 or Phillips TLK 40 W/05 medical lamps (Phillips, USA) suspended in a horizontal rack above the petri dishes holding the embryos. The lamps, fitted into standard fluorescent lamp housings with reflectors directing the radiation downward, were powered by custom built power supplies using 110V AC from the main power supply. The total UV irradiance was measured at various point along the length of bulbs, at the midpoint between the two bulbs at a distance of 23cm. The UV irradiance was measured with a *SPEX* 1680 monochromator with a NM-26-3 integrating sphere as the entrance optics and a Phillips photomultiplier tube detector. This measurement system was calibrated to a UV standard with a known output traceable to the National Institute of Science and Technology (NIST) standard. Scans were taken on the bulbs and irradiance values were determined by transfer of the irradiance calibration to the output of the lamps. From this data, dose values were calculated and used for the exposures. The TL40W/125RS J2 lamps emit ultraviolet radiation primarily in the UVB (280 nm to 315 nm) range, and the TLK 40W/05 lamps emit ultraviolet radiation primarily in the UVA (321 nm-400 nm) part of the spectrum.

2.3 UVB exposures

2.3.1 Responses of different embryonic stages to 400 J/m^2 UVB

λ transgenic medaka embryos were collected (1-2 h post fertilization, pf) on a schedule to facilitate treatment of different embryonic stages simultaneously. Embryos corresponding to late-stage [10] embryos (stage 38, 8 days post fertilization, pf) were collected first, seven days prior to treatment, middle stage embryos (stage 25, 2 days pf) were collected five days prior, and early stage embryos (stage 6, 8 days pf) were collected on the day of treatment. Embryos were examined microscopically to ensure successful fertilization, and normal development.

To evaluate responses of different embryonic stages to a single dose of 400 J/m^2 UVB, embryos were subjected to four treatments consisting of either a UVB + light, UVB + dark, - UVB + light, or - UVB + dark (total 12 treatments). Twelve embryos were placed in black petri dishes (2 replicates/treatment) lined with wetted absorbent paper. Embryos in the + dark treatment were placed in a Petri dish containing water within a light proof box for 24 h. Embryos in the + light treatments were placed under a wide spectrum visible lighting for 24 h. After the interval embryos were placed in standard culture conditions with daily inspections to count and remove dead individuals. To facilitate manifestation of induced mutations, and to enhance recovery of the target gene from individuals, embryos were allowed to hatch, the larval fish (fry) were grown for 21 days post-treatment, flash frozen and stored at -80º C until DNA isolation.

2.3.2 UVB dose responses of stage 25, and 38 embryos

To evaluate responses of embryos to varying UVB doses, stage 25 and 38 embryos were treated with single doses of 400 + light, 400 + dark, 800 + light, 800 + dark, 1000 + light, 1000 + dark J/m^2 UVB. Embryos receiving -UVB + light, or - UVB + dark served as controls.

2.4 Stage 25 embryos UVB dose response

To evaluate the responses of embryos following exposure to UVA, stage 25 embryos were treated with either 0, 400, 800, or 1600 J/m^2 UVA, and with corresponding treatments of either a 24 h light or dark interval using similar procedures described above.

2.5 *cII* mutation assay

Mutations in a subset of fish selected from each treatment were analyzed using a positive-selection assay based on the *cII* gene as the mutation target gene [11]. The assay is based on the role of the *cII* protein in the commitment of bacteriophage λ to the lysogenic cycle in *E. coli*. Selection of mutant λ*cII*$^-$ is facilitated by using a specialized *E. coli* strain (G1250, *hfl*$^-$*)* that extends the longevity of the *cII* product. Briefly, genomic DNA isolated from an individual fish was mixed with *in vitro* packaging extracts, which excised the intact λLIZ vector sequence from the host animal's genomic DNA and simultaneously packaged the vector into viable bacteriophage. The packaged phage particles were then allowed to infect and lyse the *E. coli* host. To select λ*cII*$^-$ mutants, the packaged phage was mixed with *E. coli* cells, plated, and incubated at 24°C for 40 h. The phage with wild-type *cII* became lysogenic and were indistinguishable in the *E. coli* lawn, whereas phage that carry a mutation in the *cII gene* formed plaques in the bacterial lawn when incubated at 24°C. Mutant frequencies (MFs) were calculated by dividing the total number of *cII* mutant plaque forming units (PFUs) on the selective screening plates by the estimated total λ$^+$ and *cII* phage on the titer plates.

2.6 Statistical analyzes

Significant differences in the mean MF of the treatments were compared with the generalized Cochran-Armitage test [12] using the COCHARM analytical program (Proctor and Gamble, Cincinnati, OH).

3 Results and discussion

3.1 UVB-induced responses

3.1.1 Exp. 1: Stage 6, 25, and 38 responses to 400 J/m^2 UVB

To evaluate the responsiveness of embryos to UVB, embryos at different stages of development were treated with a single exposure of 400 J/m^2 UVB. Survival of fry 21 days post treatment was 70-97% among all groups, with the exception

of the 400 J/m^2 UVB + dark treatment for stage 25, in which 68% of the fish died prior to, or soon after, hatching. Thirty-three percent (8/24) of the embryos shared striking features of abnormal development including, highly characteristic malformed "hooked" tails, and mortality within 1 day after hatching (Fig. 1).

Figure 1: UVB-induced deformed (left) and normal (right) embryos.

In addition to low survival and high incidence of abnormal development, the MF in fish from the 400 J/m^2 UVB + 24 h dark treatment was induced significantly, ~3-fold (9.5 X 10^{-5}), above that of control treatments (3.2 X 10^{-5}, -UVB + dark; 2.8 X 10^{-5}, -UVB + dark; Fig. 2). Stage 25 embryos in the UVB + light treatment also exhibited a significant ~2-fold induction of *cII* mutants compared to untreated controls. It is notable that the elevated MF was not associated with increased mortality as this group exhibited a survival rate similar to that of other treatments (87%). The MFs from the remaining UVB treatments were not elevated significantly above the controls, comparable to the historical spontaneous MF observed in the λ transgenic medaka [7, 13].

3.1.2 Exp. 2: Stage 25, and 38 responses to 400, 800, or 1000 J/m^2 UVB

To evaluate dose responses of embryos to UVB, stage 25 and 38 embryos were exposed to 400, 800 or 1000 J/m^2 UVB. Survival of stage 25 embryos in the second experiment was similarly dependent upon whether the embryos received a 24 h light or dark interval after UVB exposure. Eighty-three per cent of the fish in the 400 + light treatment survived, 92% in the 800 + light, and 54% in the 1000 J/m^2 UVB + light treatment. In sharp contrast, survival of fry 21 days post exposure in the UVB + dark treatments was extremely low, 4% survived in the 400 + dark, 0% in the 800 + dark, and 4% in the 1000 J/m^2 UVB + dark treatment. The few embryos that did hatch in these treatments did so 2-3 days later than those from other treatments. By comparison, as observed in the first experiment, survival of stage 38 embryos in all treatments was high, ranging from 83-96%. Further, MFs in all treatments were not significantly different from controls (data not shown).

Upon observing the limited number of fry available for mutation analyses from the UVB + dark treatments, live embryos that had not hatched by 14 days post treatment (presence of a beating heart) were analyzed to compare induced MF with fry from the UVB + light treatments. MFs in embryos from the 400 + dark, 800 + dark and 1000 J/m^2 + dark UVB treatments were induced significantly, up to 12-fold, above that of the – UVB + light control (2.8 X 10^{-5}) (Table 1). MFs of 21.3 X 10^{-5}, 33.4 X 10^{-5} and 26.7 X 10^{-5} were observed in the 400 + dark, 800 + dark, and 1000 J/m^2 + dark treatments, respectively. In

comparison, MFs in fry from UVB + light treatments were elevated with increasing UVB exposure, corresponding to a 2.3-fold induction in the 400 + light (5.3 X 10^{-5}), 3.1-fold in the 800 + light (7.1 X 10^{-5}), and 3.7-fold in the 1000 J/m^2 UVB + light (8.5 X 10^{-5}) treatments compared to the – UVB + light control (2.3 X 10^{-5}).

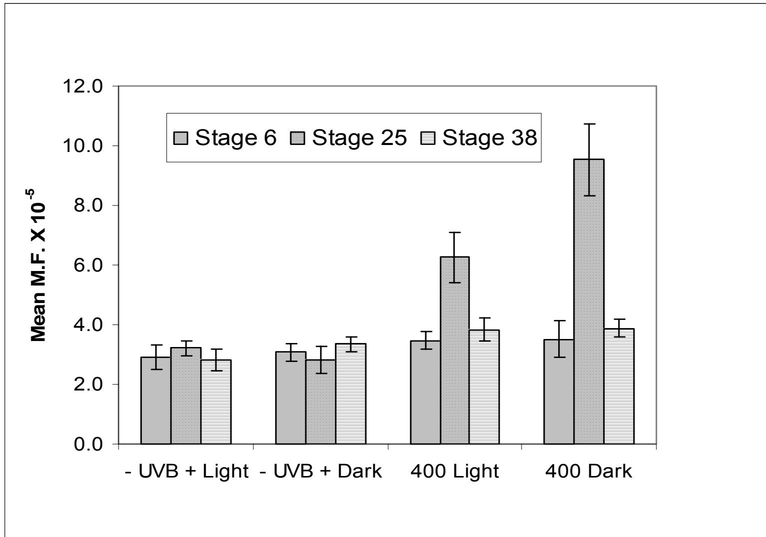

Figure 2: Mutant frequencies in embryos exposed to 0, 400 J/m2 UVB + light, or UVB + dark.

Table 1: UVB-induced MFs analyzed in fry from UVB + light, and in embryos from UVB + dark treatments.

	Light (Fry)		Dark (Embryos)	
UVB (J/m^2)	**AVG MF x 10^{-5}** *(n)*	**+/- SEM**	**AVG MF x 10^{-5}** *(n)*	**+/- SEM**
0	**2.3** *(6)*	0.2	**2.8** *(6)*[a]	0.2
400	**5.3** *(7)*	0.9	**21.3** *(6)*	2.5
800	**7.1** *(7)*	0.7	**33.4** *(4)*	2.7
1000	**8.5** *(7)*	0.8	**26.7** *(5)*	3.5

[a] Analyses from fry

3.2 Exp. 3: Stage 25 and 38 responses to 400, 800, 1600 J/m^2 UVB

To evaluate the responses of embryos to UVA, stage 25 and 38 embryos were exposed to 400, 800 or 1600 J/m^2 UVA. Survival of embryos from the UVA treatments was comparable to that of unexposed controls, ranging from 71% in the UVA + light, 100% in the 800 + dark treatments, to 91% in the 1600 J/m^2 UVA + light, and 87% in the 1600 J/m^2 UVA + dark treatments. Similarly, the MFs of all treatments were not elevated, and were comparable to historical spontaneous MF observed in λ transgenic medaka tissues [5, 14].

4 Discussion

In this first application of a transgenic fish model to the study of UV-mutagenesis, we show that the λ transgenic medaka is highly responsive to UVB, but not UVB, with survival, normal development and MF induction dependent upon embryonic stage at exposure, dose and photoreactivated repair.

Based on results from preliminary studies in which we observed a significant 2-fold induction of mutant frequencies in the skin of λ transgenic medaka fish exposed to 1000 J/m^2 UVB (data not shown), we reasoned that embryos might have promise as practical additional test subjects for UV studies because of their amendability to experimental manipulation and sensitivity shown in toxicological studies. The pronounced sensitivity of embryos exposed to UVB at middle stages of development was unexpected. We observed remarkably high rates of mortality, incidence of abnormal development and significant MF induction in stage 25 embryos. Whereas it is apparent that medaka embryos at mid-stage of development exhibit a window of enhanced vulnerability to UVB-induced effects not observed in embryos at either early or late extremes of development, the mechanism of the differential responsiveness is unclear. Embryos were exposed at stages corresponding to approximately the 16-cell stage (stage 6, ~ 2 h pf), the 18-19 somite stage (stage 25, ~2 days pf), and at an advanced late development stage (8 days pf). Depending upon the persistence of the photoproducts, it is likely that direct DNA damage could be induced in the subsequent stages after exposure. Considering the variety of complex and dynamic processes taking place in a developing embryo, it is most likely that the mechanisms leading to or protecting against direct and indirect mutations will differ depending upon the stage at which the embryos are exposed.

Photoreactivation, or enzymatic removal (PER) of certain types of DNA lesions in the presence of visible light has been demonstrated in aquatic organisms [14, 15, 16]. Photoreactivation decreases the mutagenic and cytotoxic effects of UV exposure by reversing the formation of cyclobutane pyrimidine dimers (CPDs) and pyrimidine (6, 4) pyrimidone photoproducts. The results presented here showing reduction in both mortality and MFs in the UVB + light compared to the UVB + dark treated fish support findings of others showing that fish have an active and efficient PER. The UVB + light treatments showed a distinct dose response in MFs, albeit, increasing at a lower rate than that observed in the UVB + dark treatment. The dose response of MF induction in the

UVB + dark treatment was not linear, showing a distinct reduction at 1000 J/m^2. Similar suppression of MFs has been observed in other systems, including the epidermis of a transgenic mouse mutation model at doses above 500 J/m^2 [9].

A distinct benefit of this and other transgenic mutation assays is the ability to characterize the mutations recovered from the animals by direct sequencing. UVB-induced mutagenesis is characterized by the signature mutational spectra comprised of a predominance of G:C to AT transitions at dipyrimidine sites. This UVB-induced mutational spectra has been confirmed in the skin of the transgenic medaka (unpublished data). We are currently sequencing the *cII* mutants recovered from the fish to more fully characterize the mutational responses, including the relative proportion of clonal mutations likely present in these rapidly growing individuals. Additional analyses are underway to more fully characterize the UVB-induced MFs responses in the skin, as well as, in embryos exposed at lower doses.

In summary, fish are being increasingly recognized as powerful animal models in which they are demonstrating the value of the comparative approach whereby the differences, as well as, the similarities provide insights into important biological processes. Here we show that the λ transgenic medaka is capable of providing sensitive and reliable mutation data, suggesting it may be an invaluable alternative nonmammalian model of UV mutagenesis relevant to the human condition and to impacted natural populations.

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