

Animal model for ultraviolet radiation-induced melanoma: Platyfish–swordtail hybrid

(photoreactivation/antioncogenes/ozone depletion)

RICHARD B. SETLOW, AVRIL D. WOODHEAD, AND ELEANOR GRIST

Biology Department, Brookhaven National Laboratory, Upton, NY 11973

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ABSTRACT Sunlight exposure is strongly indicated as one of the important etiologic agents in human cutaneous malignant melanoma. However, because of the absence of good animal models, it has not been possible to estimate the wavelengths or wavelength regions involved. We have developed a useful animal model from crosses and backcrosses of platyfish (*Xiphophorus maculatus*) and swordtails (*Xiphophorus helleri*). Two strains of these fish are susceptible to invasive melanoma induction by exposure to filtered radiation from sunlamps in the wavelength ranges $\lambda > 290$ nm and $\lambda > 304$ nm. Multiple exposures on 5–20 consecutive days beginning on day 5 after birth or a single exposure of ≈ 200 J/(m²·day) of $\lambda > 304$ nm result in a tumor prevalence of 20% to 40% at 4 months of age compared with a background rate of 12% in one strain and 2% in another. Exposure of the fish to visible light after UV exposure reduces the prevalence to background. The melanomas are similar in many respects to mammalian melanomas, as judged by light and electron microscopy. The genetics of the crosses determined by others and the high sensitivity of the hybrids to melanoma induction indicate that the UV radiation probably inactivates the one tumor repressor gene (or a small number of tumor repressor genes) in the hybrid fish. The small size of the animals and their high susceptibility to melanoma induction make them ideal for action spectroscopy.

Agents that cause a decrease in stratospheric ozone would cause an increase in UV-B ($\lambda = 280$ –320 nm) intensities at the earth's surface without appreciably changing the longer UV or visible intensities of light. Melanoma among the white population of the United States and Europe is increasing dramatically as a function of time, probably as a result of changing lifestyles (1). The relation between latitude and melanoma mortality suggests that there is a correlation between the average solar radiation and mortality from malignant melanoma. However, it is not known which parts of the solar spectrum can plausibly be related to the increasing mortality because, although sunlight exposure seems to be an essential component in melanoma incidence, it is not the only one. Body areas most exposed to light are not the primary locations of melanomas as they are for basal and squamous cell carcinomas. There is good evidence that UV-B is tumorigenic in animals (2) and can cause neoplastic transformation *in vitro* (3). Four types of experiment indicate that light energy absorbed in DNA can cause cellular damage leading to tumors: (i) the tumorigenicity of fish cells as a result of UV-irradiation *in vitro* can be photoreactivated (4)—a process that monomerizes UV-induced cyclobutane pyrimidine dimers in DNA; (ii) the wavelengths effective in neoplastic transformation *in vitro* are those absorbed by DNA (5); (iii) transformation *in vitro* by UV is photoreactivable (6); and (iv) xeroderma pigmentosum individuals are

defective in the ability to repair UV damages in their DNA and are extraordinarily sensitive to cancer induction, including melanoma, by sunlight (7). Since melanin absorbs not only in the UV-B range but at all longer wavelengths and gives rise to free radicals, there is a possibility that the longer wavelengths might also be effective in melanoma induction by virtue of energy transfer to or free-radical attack on DNA.

As yet, animal models for light-induced melanoma have not been developed, although *Monodelphis domestica*, a small South American opossum, shows promise (8). Therefore, it has not been possible to determine which wavelengths are the damaging ones. This leaves a gap in our knowledge of the causation of this disease and in the knowledge to assist in making regulatory decisions about the agents that may affect the integrity of the ozone layer. A suitable, and perhaps the most convenient, animal model in which to investigate light effects upon melanoma induction is certain platyfish–swordtail hybrids (*maculatus* \times *helleri*) of the genus *Xiphophorus* that were introduced into cancer research over 50 years ago by Gordon (9), Kosswig (10), and Haussler (11). The hybrids have been used extensively in genetic studies by Kallman and his associate (12, 13) and were chosen by Anders and his colleagues as a model for the induction of melanoma by chemical carcinogens and x-rays (14, 15) because of their susceptibility to oncogenesis. The parental wild species are not susceptible to neoplasia, even after exposure to high doses of potential physical and chemical carcinogens. However, when these species are cross-bred in the laboratory, their hybrid offspring and succeeding backcross generations (to the parental swordtail) are sensitive to carcinogens, although to different degrees (16, 17), presumably as a result of crossing out most of the antioncogenes or melanocyte differentiation genes.

We explored the responses of different hybrids to UV-irradiation and obtained two strains susceptible to UV-induced melanoma. The fish model is a legitimate one, since fish melanomas closely resemble the tumors that arise in human skin (18, 19). We are now in a position to determine the action spectrum for melanoma induction.

MATERIALS AND METHODS

Animals. Klaus D. Kallman (Osborn Laboratories, New York Zoological Society, Brooklyn, NY) gave us several pairs of swordtails *X. helleri*, dwarf swordtails *Xiphophorus couchianus*, and platyfish *X. maculatus* to start our breeding program. There were two strains of platyfish: one, designated 163A, had pigment on the dorsal fin (*X*-sd = spotted dorsal), while the second, designated 163B, had pigment on the flanks (*X*-sp = spot-sided).

For 2 years we generated seven different hybrid strains, first by artificial insemination and then by natural matings. Five strains were tested extensively for melanoma induction

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Abbreviation: UV-B, 280–320 nm.

by UV. These experiments involved $\approx 5,000$ fish. We obtained two susceptible crosses, one of which develops melanomas on the flanks and on the tail and the second of which develops melanomas on the dorsal fin. These two strains were used in all further experiments.

Strain 1. Platyfish 163B females were mated with male swordtails, and the female hybrids were then mated back to the male swordtail. The first backcross generation generally comprised (i) nonpigmented (white) fish, about 50% of the young; (ii) marbled (speckled) fish, about 25% of the brood; and (iii) fish with black pigment extending backward over the body from the region of the dorsal fin, about 25% of the young. However, this distribution of 50:25:25 was not invariable; in some broods the number of nonpigmented fish was as low as 25%, whereas in other broods from the same parents, the number of unpigmented hybrids was up to 70%. In the marbled offspring, the location and extent of the pigment varied, but all of the hybrids had large patches of intense black pigment on their flanks, extending to the tail. After exposure to UV light, tumors developed on the flanks and on the tail in areas where the pigmentation was the heaviest (Fig. 1). No tumors were observed in ≈ 600 white fish irradiated with various doses. The data given in the Tables that follow represent marbled and heavily pigmented animals. Our qualitative impression is that the susceptibility to melanoma induction is greater in the heavily pigmented animals.

The number of offspring in each brood ranged from 20 when the females were very young and very old to about 60 when they were in peak breeding condition. Because of this variability and the variability in the ratio of pigmented to nonpigmented young, the numbers available for each experiment were very different. Unirradiated animals were obtained from a number of broods.

Strain 2. This cross was specifically generated so that the pigmented areas were confined to the dorsal fin and also to the anal fin in males. The F_1 males, from a mating between female platyfish (163A) and male swordtails, were bred with the F_1 females from a mating between female platyfish (163A) and male dwarf swordtails *X. couchianus*. This localization of the tumor was particularly useful, as the tumor could be removed for tissue culture without killing the fish.

The fish were kept in a well-shaded greenhouse under a 14/10-hr light/dark cycle. We maintained breeding fish and young fish in large 50-gallon tanks with circulating water at $26^\circ\text{C} \pm 1^\circ\text{C}$. Irradiated fish were maintained in small 5-gallon tanks of still water. All fish were fed twice daily, once with brine shrimp and later with Nutrafin (Rolf C. Hagen Corp., Mansfield, MA). Breeding pairs were also given freeze-dried plankton and blood worms to ensure an excellent rate of growth.

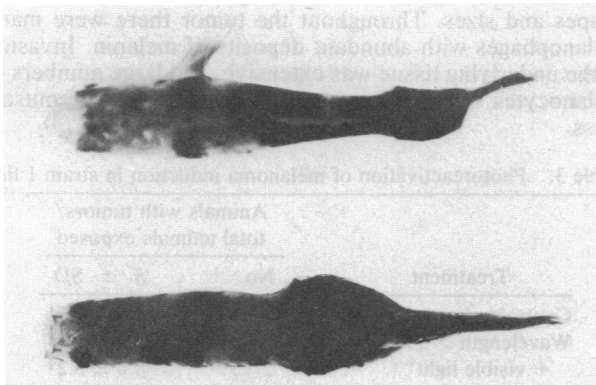


FIG. 1. Strain 1 hybrids with induced melanomas. The rapidly growing tumors had extensively invaded the musculature and visceral cavity of the fish.

The demands of producing large numbers of hybrids were compounded by the abnormal sex ratios in the F_1 generation, particularly in strain 1, where males greatly outnumbered females. Other workers found that, depending upon the cross involved, the sex ratio in the offspring might be unity, females might outnumber males by 3:1, or the progeny might be entirely male (20). However, once we had sufficient numbers of F_1 females, generating the sensitive backcross was not difficult because these females produced large broods of 60–80 young each month.

UV-Irradiations. Twenty fish in 5 cm of water were irradiated in 5-gallon (0.019 m^3) tanks covered on three sides and on the bottom with cardboard and on the fourth side with yellow cellophane to minimize photoreactivation. They were exposed to UV radiation from above. Irradiations were begun when the fish were 5 days old (2–3 mm in length) while their color pattern was developing, at a stage when a large number of melanoblasts are dividing. Brine shrimp were present throughout radiation, which ensured that the fish moved freely throughout the tank.

Animals were exposed to light from two Westinghouse FS-40 sunlamps, filtered by a thin acetate film ($\lambda > 290 \text{ nm}$), a thin Mylar film ($\lambda > 304 \text{ nm}$), or a thick plastic sheet ($\lambda > 360 \text{ nm}$). The respective exposure rates at the water surface in $\text{J}/(\text{m}^2\text{-hr})$ were estimated with a UVX Ultraviolet Products radiometer (San Gabriel, CA) as 900 (UVX-30 Sensor), 570 (UVX-30 Sensor), and 18 (UVX-36 Sensor). Exposures through a thick plastic sheet for 6 hr/day for 20 days gave results similar to unirradiated animals, and these data have been combined as controls. The filters and the dosimetry were checked every 2 months. The dose rates at the fish's surface were $\approx 1/4$ of those given in the Tables because the transmission of 2.5 cm of tank water, the average position of the fish, was ≈ 0.4 , and the incident radiation was not perpendicular to the fish surfaces.

At the end of the exposures, the fish were held for a further week in these tanks, whose top side was covered with cardboard. The fish were then transferred to holding tanks in the main aquarium and were examined for melanomas 1 month after exposure and thereafter every month. In our initial experiments we kept the fish for 6 months after exposure, but most of the UV-induced tumors had already appeared by 4 months. For example, at 4 months, after $1700 \text{ J}/(\text{m}^2\text{-day})$ of $\lambda > 304 \text{ nm}$ for 20 days, 10 of 37 fish had melanomas, and by 6 months there were 13 of 37. In unirradiated animals there were 0 of 26 at 4 months and 4 of 26 at 6 months. Therefore, we adopted 4 months as our end point in further experiments.

Histology and Electron Microscopy. The fish melanoma is fragile. To minimize distortion of it, the fish were kept separately for 1 day in clean aquarium water. They were killed by gradually lowering the water temperature, then the body was cut off at the level of the dorsal fin, and the water was replaced with fixative. After 1 week in fixative, the tumor was firm and could be dissected away cleanly. Tumors were sectioned at $6 \mu\text{m}$ and stained with hematoxylin/eosin.

For electron microscopy, the fish were fixed in 4% paraformaldehyde and 1% glutaraldehyde in phosphate buffer at pH 7.2. The tumor was finely dissected and washed with the buffer and then fixed in 2% buffered osmium oxide for 2 hr. The tissue was dehydrated through graded acetones and embedded in Epon. Ultrathin sections were stained with uranyl acetate and lead citrate.

RESULTS

Induction of the Melanoma. Strain 1 hybrids. Initial experiments showed that exposure to $3400 \text{ J}/(\text{m}^2\text{-day})$ of radiation of $\lambda > 340 \text{ nm}$ for 10–20 days results in sunburning. Hence, the maximum exposure used was $1700 \text{ J}/(\text{m}^2\text{-day})$. Since we

thought that repeated exposures would give higher tumor yields than single exposures, we started our experiments by giving exposures for 20 consecutive days beginning on day 5 when the animals were large enough to handle easily. Exposures beginning on day 15 or 20 gave somewhat lower tumor yields (data not shown), so we chose day 5 as the starting day. Once melanomas had appeared, there were no differences in their rate of growth in the exposed or control groups.

During the course of an experiment on strain 1 animals with radiation of $\lambda > 360$ nm, a group was inadvertently exposed on day 10 to 2340 J/m² of unfiltered UV. Seven of 16 fish developed melanomas by 4 months compared with 3 of 26 in fish exposed only to $\lambda > 360$ nm, suggesting that single exposures might be sufficient to induce tumors. Hence, we did experiments with 15-, 10-, 5-, and 1-day exposures (Table 1). For $\lambda > 290$ and $\lambda > 304$ nm, the percentage of fish that developed melanomas was the same within experimental error in all experiments. Exposure to the lower dose of $\lambda > 304$ nm for 1 day was about as effective as exposure for 15 to 20 days and also as effective as exposure to $\lambda > 290$ nm.

Strain 2 hybrids. Table 2 shows the number of melanomas that developed in strain 2 fish; the value for the group exposed for 20 days may be an underestimate because several fish became infected with a fungus and died, and we could not reliably score for the presence or absence of a tumor in these animals.

Photoreactivation. An experiment was made with strain 1 fish to see whether exposure to photoreactivating light (visible light) each day for 1.5 hr immediately after 15 days of irradiation with UV > 304 nm would reduce the number of melanomas. We found a decrease in the percentage of animals with tumors in the photoreactivated fish (Table 3). A similar result was obtained for a 1-day irradiation.

Histology of the Fish Melanoma. Unlike the mammalian epidermis, where the Malpighian cells die to form a cornified layer, the fish epidermis has living cells throughout. The pigment-containing cells lie in the dermis, and pigment is not transferred to the Malpighian cells as in higher animals, where it may act as a sunscreen. Melanomas became visible to the naked eye about 1 month after UV-exposure, growing on the flanks, on the caudal peduncle (strain 1), or on the

Table 2. Melanomas in strain 2 fish 4 months after UV-irradiation

Days exposed	Animals with tumors/ total animals exposed	
	No.	% \pm SD
0	1/50	2.0 \pm 2.0
7	23/79	29.1 \pm 5.1*
15	16/47	34.0 \pm 6.9*
20	18/90	20.0 \pm 4.2*

Exposure per day was 1700 J/m² at $\lambda > 304$ nm. Irradiations were begun on day 5 after birth.

* $P < 0.01$ vs. controls.

dorsal and anal fins (strain 2). Initially, the tumors on the dorsal fin and caudal peduncle were bilaterally symmetrical, but often the growth on one side later outstripped that on the other side. By about 12 months, the caudal fin, peduncle, and the dorsal fin were destroyed. The fish with tumors usually survived a further 6 months, but any minor change in their environment, such as a drop in temperature, triggered death.

The melanomas on the trunk and tail were made up of two regions. Nearest to the musculature, the tumor was firm and grayish-black in color. The outer layer was a dense black, slippery, and fragile; on contact with any surface it left behind a streak of broken cells and black, viscous fluid.

Histological sections of the growth confirmed that they were invasive melanomas (Fig. 2). At the outer edges of the tumor, where the junction of the dermis and epidermis could be seen, there was intense proliferation of the dermal melanophores and often an inflammatory reaction. The grayish-black interior of the tumor consisted of an ulcerating, swirling mass of interlacing spindle-like melanocytes and macromelanophores. [Fish melanomas differ from those of humans by the presence of such cells, which differ only in size and shape from melanocytes. The macromelanophores contain more pigment and are thought to be mature, older cells (18).] The amount of pigment in the cells varied from a fine stippling to heavy granules. The cells showed little uniformity, and there were many bizarre configurations. Scattered among the mass were single macrophages or groups of these cells laden with pigment that they had engulfed as it was released from disintegrating melanocytes. Fat cells were present throughout the tissue. Closer to the muscle, the cells became more uniformly and heavily pigmented; they appeared to be aligned almost parallel to the muscle striations. Chains of melanocytes had migrated into the underlying tissue, and muscle fibers were invaded and destroyed.

The melanomas on the fin did not show a clear demarcation into an inner and outer layer. However, the cells of the outermost area tended to be disorganized and of different shapes and sizes. Throughout the tumor there were many melanophages with abundant deposits of melanin. Invasion of the underlying tissue was extensive, and large numbers of melanocytes were seen among the myosepta on the muscle mass.

Table 3. Photoreactivation of melanoma induction in strain 1 fish

Treatment	Animals with tumors/ total animals exposed	
	No.	% \pm SD
Controls	10/79	12.7 \pm 3.7
Wavelength > 304 nm* + visible light [†]	6/22	27.3 \pm 9.5 [‡]
	2/25	8.0 \pm 5.2 [§]

*850 J/(m²-day) at $\lambda > 304$ nm for 15 days.

[†]1.5 hr per day of white fluorescent light after UV.

[‡] $P \approx 0.1$ vs. controls.

[§] $P \approx 0.1$ vs. $\lambda > 304$ nm.

Table 1. Melanomas 4 months after UV-irradiation (begun on day 5 after birth) of strain 1 hybrids

Exposure per day, J/m ²	Days exposed	Animals with tumors/ total animals exposed	
		No.	% \pm SD
Controls			
Unirradiated		7/53	13.2 \pm 4.6
Wavelengths > 360		3/26	11.5 \pm 6.3
	Total	10/79	12.7 \pm 3.7
UV-irradiation			
Wavelengths > 290			
150	15	4/12	33.3 \pm 13.5
300	15	3/16	18.8 \pm 9.8
	Total	7/28	25.0 \pm 8.1*
Wavelengths > 304			
850	1	3/16	18.8 \pm 9.8
850	5	8/20	40.0 \pm 11.0
850	10	4/20	20.0 \pm 8.9
850	15	6/22	27.3 \pm 9.5
850	20	9/37	24.3 \pm 7.0
1700	15	12/52	23.1 \pm 5.8
1700	20	10/37	27.0 \pm 7.3
	Total	52/204	25.5 \pm 3.1 [†]

* $0.1 < P < 0.2$ vs. controls.

[†] $P < 0.05$ vs. controls.

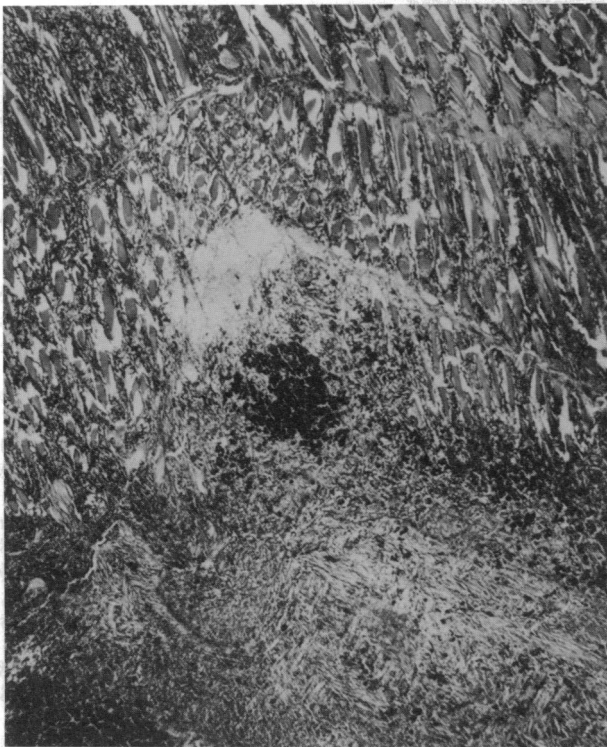


FIG. 2. Photomicrograph of an induced melanoma showing invasion of the tail muscles. The interior of the tumor (bottom right) shows a swirling mass of melanocytes and macromelanophores. ($\times 50$.)

John C. Harshbarger, Registry of Tumors in Lower Animals, Smithsonian Institution, Washington, DC, confirmed our finding that the tumor was "... a highly invasive melanoma. . . ." He observed, "... Neoplastic melanophores of the dermis and possibly the meninges but not the pigmented epithelium of the retina are proliferating over the entire specimen and are invading most tissues. They have extensively infiltrated skeletal muscle, gill arches, and cranial cavity but not the brain itself, and the visceral cavity but not the liver. . . ."

Electron Microscopy. Fig. 3 shows a view of a typical area near to the center of a tumor growing on the flanks of an irradiated fish. There were many pleomorphic melanocytes containing one or several prominent melanin granules; these cells had abundant mitochondria and a well-developed endoplasmic reticulum. The tumor was dominated by large, closely packed melanophages containing compound melanosomes; the melanosomes were enclosed in a single membrane, and there was abundant granular material within this membrane. The melanosomes were in two stages of development: they were partially melanized—the stage III of the progression described by Fitzpatrick and Freedberg (21)—or uniformly filled with electron-dense material, stage IV. We did not find any melanosomes in earlier stages of development. There were proportionately more melanophages at the outer edges of the tumor and in areas close to the muscle. Melanocytes had infiltrated the muscle, moving along the fasciculae between the muscle bundles. The melanoma was well vascularized.

DISCUSSION

We developed two strains of hybrid fish that are highly susceptible to UV-induced melanoma. We consider that these hybrid fish are a useful animal model for human melanoma, as was proposed by Sobel *et al.* (18, 19). The

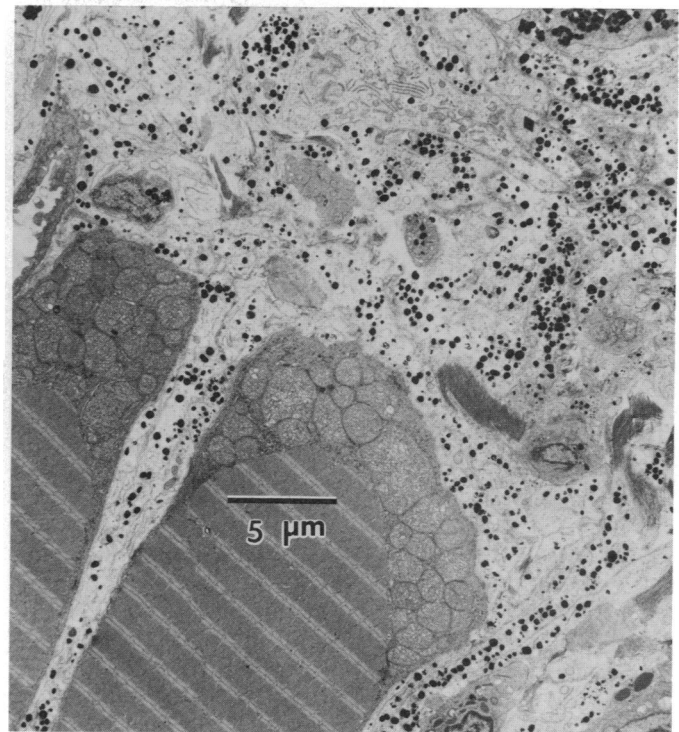


FIG. 3. Electrograph of a melanoma showing well-differentiated melanoma cells penetrating the fasciculae of the muscle bundles. At the lower left is a melanophage with abundant melanosomes.

xiphophorid and the human melanoma have considerable similarities: the principal difference between the two lies in the presence in the fish tumor of melanophores, which are the normal terminal stage of pigment-cell differentiation in lower vertebrates. Riehl *et al.* (22) made detailed comparisons of the ultrastructure of the malignant melanoma of fish and humans by freeze-etching techniques and by transmission electron microscopy: they concluded that the tumors reflect the same biological phenomena—indeed, the freeze-etched replicas were indistinguishable. Recent work showed that the immunological characteristics of piscine and mammalian (including human) melanomas are similar (23, 24). Also, the chemical nature and immunohistological localization of the gangliosides in fish melanoma correspond strikingly to that of the known gangliosides in human melanoma (25).

Our finding (Table 1) that the induction of melanoma over the range of daily doses and number of days so far used is independent of these parameters suggests that the total and daily doses are near a plateau level of a dose-response curve. The plateau, at ≈ 30 – 40% of animals with tumors, could arise from an approximately steady state of pyrimidine dimers at high doses because of their concomitant formation by wavelengths < 320 nm and their monomerization by photoreactivation with the longer wavelengths in the broad-band light sources we used. The plateau could also be the result of combining two groups of fish—marbled and heavily pigmented animals—in our calculations, with the latter having many tumors at lower doses and the numbers in the former rising slowly with dose in the exposure range used. The finding of photoreactivation (Table 3) indicates that DNA is the probable target for the melanoma-inducing effect of UV-B and that these wavelengths cause their effect by direct absorption in DNA.

The lowest total exposure that induced melanomas was 850 J/m^2 of radiation of $\lambda > 304$ nm at the water surface or ≈ 200 J/m^2 at the skin surface. This low dose should be compared with $\approx 10,000$ J/m^2 of $\lambda > 280$ nm for melanoma induction in

Monodelphis domestica (8) and to 25,000 J/m² of $\lambda > 280$ nm to stimulate the growth of transplanted melanomas in mice (26). The doses needed to abolish rejection of UV-induced tumors were also in the 100 kJ/m² range of radiation of $\lambda > 280$ nm (27). These comparisons indicate that the high susceptibility of the hybrid fish to melanoma induction by UV is not the result of the induction of a stimulatory factor nor the inhibition of an immunological rejection system for preexisting transformed cells in the hybrid fish but probably reflects the UV-inactivation of the small number of tumor suppressor or antioncogenes in the hybrid animals (28) as Anders and his colleagues found with chemical carcinogens (17).

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