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Genetics

Wavelengths effective in induction of malignant melanoma

(*Xiphophorus* fishes/ultraviolet radiation/visible light/suppressor genes/ozone depletion)

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ABSTRACT **It is generally agreed that sunlight exposure is one of the etiologic agents in malignant melanoma of fair-skinned individuals. However, the wavelengths responsible for tumorigenesis are not known, although DNA is assumed to be the target because individuals defective in the repair of UV damage to DNA are several thousandfold more prone to the disease than the average population. Heavily pigmented backcross hybrids of the genus *Xiphophorus* (platyfish and swordtails) are very sensitive to melanoma induction by single exposures to UV. We irradiated groups of five 6-day-old fish with narrow wavelength bands at 302, 313, 365, 405, and 436 nm and scored the irradiated animals for melanomas 4 months later. We used several exposures at each wavelength to obtain estimates of the sensitivity for melanoma induction as a function of exposure and wavelength. The action spectrum (sensitivity per incident photon as a function of wavelength) for melanoma induction shows appreciable sensitivity at 365, 405, and probably 436 nm, suggesting that wavelengths not absorbed directly in DNA are effective in induction. We interpret the results as indicating that light energy absorbed in melanin is effective in inducing melanomas in this animal model and that, in natural sunlight, 90–95% of melanoma induction may be attributed to wavelengths > 320 nm—the UV-A and visible spectral regions.**

The incidence of malignant melanoma has been increasing for several years at a rate of ≈5% per year among fair-skinned individuals in North America and Europe, probably due to changes in lifestyle. The disease has a complex etiology. Although sunlight exposure is implicated, melanoma is not associated with chronic exposure nor is it located primarily on highly exposed areas of the body (1, 2). Because individuals with the DNA repair-deficient disease xeroderma pigmentosum are several thousandfold more susceptible than unaffected individuals (3), sunlight damages to DNA are thought to be initiating carcinogenic events. However, the wavelengths effective in melanoma induction are not known. The wavelengths in sunlight between 280 and 320 nm (UV-B) are more strongly absorbed by DNA than are the longer UV-A wavelengths. On the other hand, the melanin in melanocytes absorbs UV at all wavelengths and energy absorbed by this pigment might affect DNA by energy or free-radical transfer to DNA (4). Useful animal models for determining melanoma induction as a function of wavelength—the action spectrum—are the hybrid offspring from intra- and interspecific crosses between pigmented and nonpigmented fishes of the genus *Xiphophorus* (5). The F_1 hybrids show atypical, extended pigmentation, while backcross hybrids, BC_1, between these F_1 fish and the nonpigmented parent show approximate Mendelian segregation of the color pattern; 50% have no pigment, 25% are speckled, and 25% are heavily pigmented. The latter group are susceptible to melanoma induction by a single, relatively small exposure to UV. Classical linkage analyses have suggested that this susceptibility reflects the loss of a tumor suppressor gene and the enhanced expression of a dominant oncogene (6). The candidate tumor gene was recently isolated and sequenced (7).

The system probably is more complex. Thus, Vielkind *et al.* (6) stress the anomaly that F_1 hybrids rarely develop neoplasia after treatment with high concentrations of mutagens and carcinogens, even though they are hemizygous for the tumor suppressor gene, while the similar, hemizygous BC_1 hybrids develop a broad spectrum of tumors after equivalent exposures. Adding to the complexity, it was recently suggested that a promoter gene controlling transcription of the oncogene may have been accidentally acquired during evolution (8).

We have irradiated fish from these backcrosses with narrow wavelength bands from 302 to 436 nm; the exposure response data show that all the wavelengths tested induced melanomas. The action spectrum has significant values in both the UV-A and visible spectral regions. The high sensitivity of the BC_1 hybrids to melanoma induction is demonstrated by the fact that, at 302 nm, where DNA is affected directly by UV, the number of cyclobutane pyrimidine dimers per melanoma-inducing exposure is ≈2 per Mb.

MATERIALS AND METHODS

In previous work (5), and in preliminary experiments here, we used the classical cross between the southern platyfish *Xiphophorus maculatus* and the green swordtail, *Xiphophorus helleri*. However, the F_1 hybrids of this cross show very atypical sex ratios and many females are infertile (9). Therefore, to avoid these major problems, we changed to using crosses between the Monterey platyfish *Xiphophorus couchianus* as the male, nonpigmented parent, and the female southern platyfish, strain JP163B, that had prominent pigment spots on the flanks. The parental fish were obtained from Klaus Kallman (New York Zoological Society). The heavily pigmented 25% of the BC_1 generation proved very susceptible to melanoma induction by UV. Approximately 40 broods were used between August 1991 and April 1992, among which were 144 pigmented controls and 414 irradiated pigmented fish.

Irradiations. At 6 days, it was not apparent which fish would develop pigmentation; therefore, all were irradiated. Until about 10 days, the fishes' skin has no distinct layers, but prominent melanin-bearing cells lie on the surface of the myotomes. In preliminary experiments, animals from the *X. maculatus* × *X. helleri* cross were exposed as described (5), in shallow tanks, to radiation from two FS-40 lamps placed immediately overhead. The radiation was filtered by a thin Mylar-C film to absorb radiation at <304 nm. The exposure rate at the water surface was 570 J/m²·hr, as estimated with a UVX Ultraviolet Products (San Gabriel, CA) radiometer (UVX-30 sensor). After irradiation, the tops of the tanks were covered with cardboard and the sides were covered with yellow cellophane to minimize photoreactivation from the weak ambient light in the shaded greenhouse. In one experiment, UV-irradiated fish were exposed for 1.5 hr to daylight

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fluorescent lamps to attempt to photoreverse the melanoma induction.

Thereafter, for monochromatic irradiations, we used the BC_1 progeny from *X. maculatus* and *X. couchianus*; five fish in 1.6 ml of water in a 1-cm^2 quartz spectrometer cuvette were placed in the uniform light field behind the exit slit of a Bausch & Lomb grating monochromator (focal length, 500 mm; 1200 grooves per mm) illuminated with a 500-W mercury lamp. The fluence rate at the cuvette surface was measured with a calibrated photocell (10). During exposure (0.5–10 min), a small magnetic stirrer kept the fish moving randomly in the light beam: we estimate that each side of an animal received $\approx 1/\pi$ of the fluence incident on the cuvette. The wavelengths used were 302, 313 (with a Mylar-C filter to eliminate scattered radiation < 304 nm), 365 (with a glass filter to eliminate radiation < 320 nm), and 405 and 436 (with a plastic filter to eliminate wavelengths < 370 nm) nm. After irradiation, the animals were transferred for 2 weeks into covered 50-gallon tanks (0.19 m^3). Fish were examined monthly for tumors under a low-power microscope. Four months after irradiation, all fish were scored for melanomas and prepared for histological examination.

Histology. The fish were put into a beaker of aquarium water and shreds of ice were added until their respiratory movements ceased: 5 min later, the spinal cord was cut. The fish were placed in 10 times their volume of buffered formalin. Two days later, when the fragile melanoma was firm, it was finely dissected and transferred to fresh fixative. After a week, the tissue was embedded in wax; sections were cut at 6 μm and stained with hematoxylin and eosin.

RESULTS

Single Sunlamp Exposures. Single exposures to filtered sunlamp radiation (Fig. 1) result in significant numbers of melanomas above background with approximately single-hit kinetics. Subsequent exposure to visible radiation reverses this induction somewhat, but the reversal was not statistically significant, although it was significant in an earlier report in which more animals were used (5). Because photoreactivation is a repair system that works on UV-damaged DNA and was shown to monomerize cyclobutane pyrimidine dimers in these fish (11), the data are consistent with the interpretation that inactivation of replication or translation of a single suppressor gene in a target cell will result in melanoma induction. From the known action spectrum for erythema induction in humans (12), the spectral output of our filtered sunlamp, the transmission of 5 cm of tank water, and a knowledge of the angle between the incoming light and the sides of the fish, we estimated that the tumoricidal exposures in this experiment were less than a minimal erythemal dose.

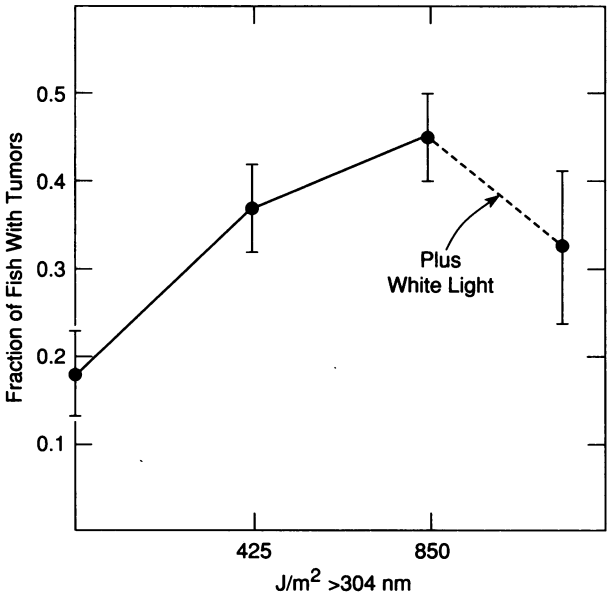

FIG. 1. Melanoma induction by filtered (λ > 304 nm) sunlamp radiation. Hybrids (*X. maculatus* × *X. helleri*) were irradiated from above at 5 days and scored at 4 months. Fluences on the fish are estimated to be $\approx 1/4$ of those shown for the water surface (5). Photoreactivation was by light from white fluorescent lamps for 1.5 hr. Error bars represent SD calculated from the numbers of fish with and without tumors.

Effects of Monochromatic Wavelengths. Table 1 summarizes the fish used at each wavelength and the resulting melanomas. To obtain sufficient numbers of fish at each wavelength and exposure level it was necessary to irradiate more than one brood, often months apart, and to pool the results. Because the photoreactivation data (ref. 5; Fig. 1) indicated that photons absorbed in DNA were effective in melanoma induction, we initially concentrated our attention on the UV-B region, 302 and 313 nm, and then gave large exposures at 365 nm. To our surprise, there was appreciable induction by this wavelength. The background melanoma prevalence, 0.24 (30/124), also was higher than expected. We realized then that our control animals were exposed to visible and UV-A in the shaded greenhouse and that this exposure could explain the high background. In a subsequent experiment, we kept 20 control fish under subdued yellow light for 2 months; the background then was 0.05 (1/20), marginally different from 0.24 [χ^2 (with Yates' correction for continuity) = 2.705; P = 0.100]. Unfortunately, our fish stopped breeding at this stage; hence, there was no opportunity to obtain further control data and we were only able to irradiate relatively small numbers of fish at two exposure levels at 436 nm.

Data Analysis. There is an increase in melanoma prevalence with exposure at all wavelengths (Fig. 2). Fig. 2 assumes that the appropriate values of background prevalence are 0.24 for the three shorter wavelengths and 0.05 for the two longer ones (Table 1). An alternative assumption is that the background prevalence is 0.22 for all wavelengths. Similar qualitative conclusions are drawn from this assumption (see Table 2) except for 436-nm exposures, for which there is no increase in prevalence with exposure. A simple expression for tumor prevalence, consistent with the apparent single-hit response, is

$$\text{Prevalence} = a + b(1 - e^{-kE}), \quad [1]$$

where E is the exposure in J/m^2 at the cuvette surface. At each wavelength, a represents the average prevalence at 0 exposure, b is the average maximum inducible prevalence ($a + b$ is the maximum prevalence), and k is the average cross-section for melanoma induction in m^2/J or m^2 per

Table 1. Pigmented fish used in monochromatic irradiations

λ, nm	No. of exposure levels	Fish exposed	Fish with melanomas
Control*	—	124	30
302	4	123	37
313	4	124	46
365	6	85	38
Control†	—	20	1
405	4	61	18
436	2	21	5

*Controls in ambient light in shaded greenhouse for 313 nm and for seven of nine irradiations at 302 nm, seven of nine irradiations at 365 nm, and two of five irradiations at 405 nm.

†Controls in covered tanks for 2 months at 436 nm and for three of five irradiations at 405 nm.

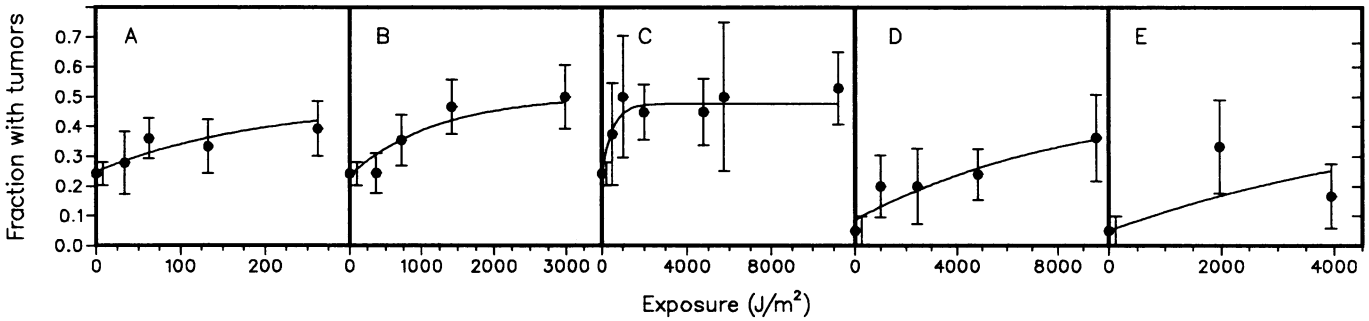

FIG. 2. Melanomas in BC_1 pigmented fish (*X. maculatus* × *X. couchianus*) induced by monochromatic radiations. Six-day-old fish in spectrophotometer cuvettes were irradiated for 0.5–10 min and scored for melanomas at 4 months. Each experimental point represents the pooled data of several different irradiations. The unirradiated prevalences were taken to be 0.24 for 302 (*A*), 313 (*B*), and 365 (*C*) nm, and 0.05 for 405 (*D*) and 436 (*E*) nm. Observed prevalences for the few irradiations carried out at 302 and 365 nm using experimental conditions corresponding to the lower background (Table 1) were corrected to the expectation for the higher background. Those at 405 nm carried out under high background conditions were corrected to the expectation for the lower background. Curves are the best fits of Eq. 1 to the weighted data points (Table 2).

photon. We used an iterative, nonlinear, least-squares procedure to determine the best stable values of the parameters. Except for 365 nm, there were insufficient data to evaluate all the parameters. Hence, to obtain reasonable fits to the equation, we fixed some parameters so that $a + b \cong 0.5$. Table 2 gives the calculated parameters for the two assumptions about background and the relative values of k corrected for decreasing photon energy with increasing wavelength. The action spectrum for melanoma induction, normalized to 1.00 at 302 nm, is given in Fig. 3 along with data from the literature on action spectra for cytotoxicity and mutagenicity of human cells in culture (13–17). The mutagenicity spectrum at wavelengths > 302 nm looks similar to that for a direct effect of UV on DNA (18) or for the production of cyclobutane pyrimidine dimers in the DNA of human skin (19). The cytotoxicity spectrum does not fall off as rapidly at long wavelengths, presumably because effects of UV on endogenous sensitizers may affect cellular components that can result in cell death. The action spectrum for human erythema (12) falls in between that for cytotoxicity and mutagenicity.

Histology of the Melanomas. Fish skin has two types of melanin-containing cells. First, the melanocytes, which are small (10–100 μm), dendritic or spindle-shaped cells that actively produce melanin and are capable of division; they occur in the deeper layers of the stratum compactum of the dermis. Second, the dense black melanophores, which are considered to be the final stage of differentiation of the pigment cell; these are found in the upper stratum spongiosum of the dermis and mediate the color response of the fish (20). Melanophores are either micromelanophores (up to 300 μm) or macromelanophores (300–500 μm) and are rounded or asteroid-shaped. Melanophores do not occur in the skin of humans.

Table 2. Values of parameters in Eq. 1 calculated from data in Fig. 2

λ, nm	*a*	*b*	*k*, m²/J × 10⁴	Relative *k* From Fig. 2	Relative *k* Alternative*
302	0.248	0.236†	50 (22)	1.00	1.00
313	0.235	0.270†	8.2 (3.6)	0.16	0.18
365	0.242	0.235	19 (11)	0.32	0.19
405	0.087	0.410†	1.1 (0.4)	0.017	0.021
436	0.050†	0.427†	1.6 (1.1)	0.023	0.000

Relative values of k are normalized to 1.00 at 302 nm and are quantum corrected. Numbers in parentheses represent SE.
*Less likely alternative assuming the same control values at all wavelengths.
†Parameter fixed.

In our previous work where *X. helleri* was the male parent, the induced tumors showed a progression described by Sobel *et al.* (21); at first, the transformed melanocytes divided rapidly and quickly progressed to terminal differentiation so that early-stage tumors were characterized by an abundance of dense black macromelanophores. Later, as the tumor became more aggressive and invasive, most melanocytes did not differentiate and the tumor was characterized by a predominance of small, dividing melanocytes with little pigment.

In this experiment, we saw few such mixed tumors. The tumors either had heavily pigmented macromelanophores or

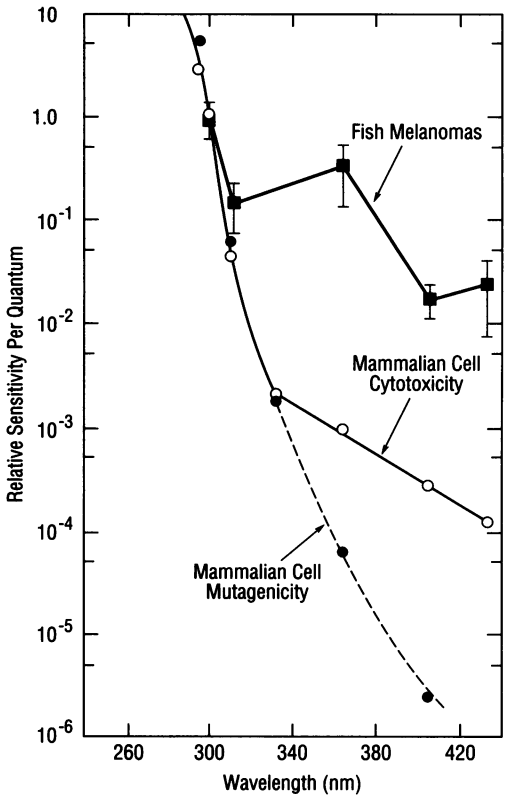

FIG. 3. Action spectrum for melanoma induction. Values of k (±SE) obtained from data in Fig. 2 (Table 2) on an exponential scale versus wavelength are normalized to 1.00 at 302 nm. Spectra for mammalian cell mutagenicity and cytotoxicity are geometric means of data in the literature (13–17). Mutation values of zero, observed in some experiments, were taken as one-half of the detection limit given by the authors. ○, Cytotoxicity; ●, mutagenicity; ■, melanoma induction.

were composed mainly of strands and whorls of long, spindle-like melanocytes (Fig. 4). Both types of melanoma appear highly aggressive and had infiltrated the muscle tissue down to the spinal cord. John C. Harshbarger (Registry of Tumors in Lower Animals, Smithsonian Institution, Washington, DC) (personal communication) considered for both, ". . . based on continued proliferation plus autonomy from host controls and behavioral and cytological atypia . . . an interpretation of neoplasia is defensible."

DISCUSSION

The relative sensitivities of melanoma induction at wavelengths > 313 nm are orders of magnitude greater than expected from spectra similar to the direct effect of light on DNA, as exemplified by the mutation spectrum in Fig. 3. Our conclusion is that light energy absorbed in melanin is effective in melanoma induction in fish. It is reasonable to extend this conclusion to humans. Although 302 nm is the most effective wavelength among those investigated, the amounts of UV-B present in sunlight at the earth's surface are >10 times less than the amounts of radiation between 320 and 436 nm (18). As a result, UV-B would account for only 5–10% of the melanoma-inducing effects of sunlight. Hence, sunscreens effective in the UV-B region, or those designed to minimize erythema, would not protect against melanoma induction by sunlight. Moreover, because melanoma-inducing effects at wavelengths > 320 nm also are several orders of magnitude greater than the human erythemal effects, sunscreens designed to minimize erythemal induction by UV-A may not afford significant protection against melanoma induction by sunlight. The difference in background melanoma prevalence between fish exposed to weak ambient light (0.24) compared to those exposed to subdued yellow light (0.05) is independent evidence indicating that wavelengths greater than UV-B are important in melanoma induction. Because ozone absorbs significantly only at wavelengths < 320 nm, our data indicate that depletion of stratospheric ozone will have only a minor effect on melanoma incidence from sunlight exposure.

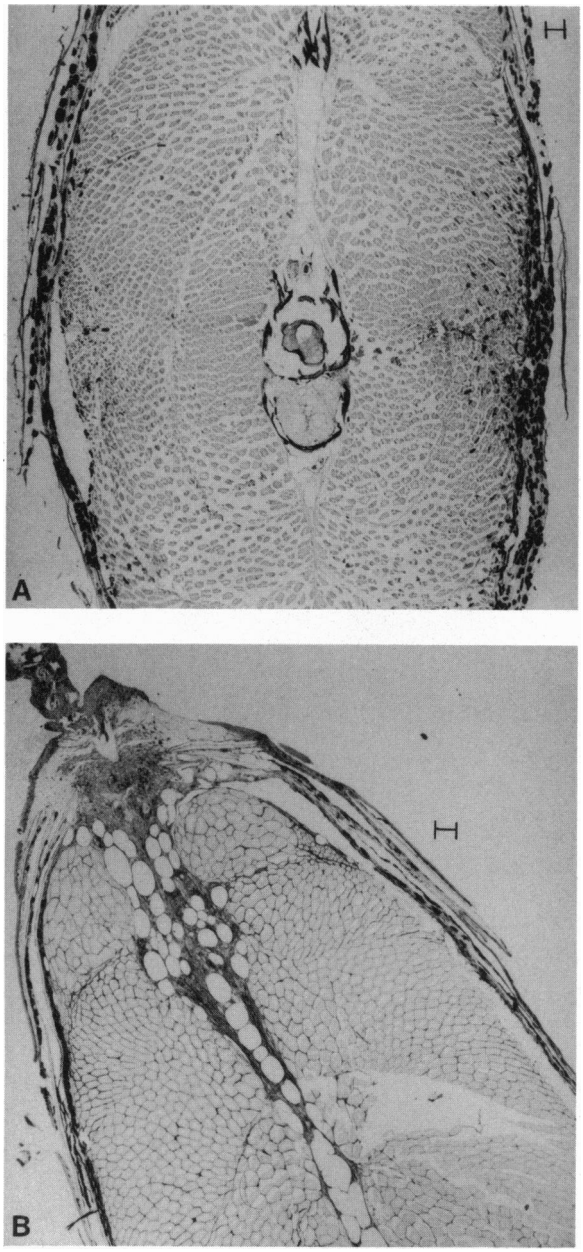

FIG. 4. Photomicrographs of induced melanomas. (*A*) Melanoma composed of macromelanophores. Melanin-laden cutaneous melanophores are invading muscle tissue along the fascial tracts. (*B*) Melanoma predominantly made up of melanocytes. Most of the dermal melanocytes are proliferating and invading nearly all normal tissues. Throughout the tumor, there are individual and groups of clustered macrophages full of released pigment. (Bar = 100 μm.)

The absolute sensitivity of the hybrid fish to melanoma induction at all wavelengths studied is significantly greater than for erythema induction in fair-skinned individuals. For example, the average minimal erythemal doses at 302 and 313 nm for a stationary person are 400 and 8000 J/m^2 (12), and the values of $1/k$ (Table 2) for moving fish are 200 and 1200 J/m^2, respectively. At 302 and 313 nm, where the major effect in fish seems to arise from the direct absorption of radiation in DNA, these exposures result in only ≈1.5 cyclobutane dimers per Mb of the DNA of exposed fish (11), whereas at 302 nm a minimal erythemal dose corresponds to 20–50 dimers per Mb (22). We interpret the high sensitivity as indicating that the tumors arise from inactivation of a suppressor gene in a pigment cell containing a single suppressor gene. Since single minute exposures result in melanomas, we reject the interpretation (23) that UV is acting as a promoter in these fish. Cell counts showed there were ≈35,000 melanin-containing cells in the fish at 6 days of age. Hence, the probability of a transformation is ≈1/35,000 = 3×10^{-5}. On this basis, we calculate that the probability of transforming a cell containing two suppressor genes would be ≈10^{-9}.

The maximum melanoma prevalence observed, 0.5, indicates to us that even though we are irradiating the apparently 25% homogeneous BC_1 population, they may be heterogeneous for other modifying factors, such as promoter genes. In this context, Vielkind *et al.* (6) suggest that other genetic disturbances are probably involved in hybrids from *X. couchianus*; they found that the second backcross from BC_1 hybrids bearing malignant melanoma to *X. couchianus* does not give the expected 100% offspring with malignant melanoma. Alternatively, the number of transformable pigment cells in 6-day-old fish may be much smaller than the number we counted. It is noteworthy that treatment of these hybrids at 5 weeks with four exposures to 1 mM methylnitrosourea resulted in melanomas in 38 of 52 treated animals (unpublished observations). Hence, the results of irradiating older animals may help clarify the reasons for tumor yields appreciably < 100%.

We cannot explain why the tumors were of two distinct types, though apparently equally aggressive, nor can we explain why so few fish exhibited tumor progression. We found no relation between the types of tumor and exposure wavelength, and control fish had both types. Our aquarium conditions were standard throughout with no major temperature changes.

However, there was a correlation between the sex of the fish and the number with melanomas in all groups that was related to their degree of pigmentation. Among the 25% of the heavily pigmented BC_1 hybrids, there were about twice as many males as females; hence, twice as many males as females had tumors. Earlier, Schartl *et al.* (24) suggested that male steroid hormones affected pigmentation, and Atz (25) found more atypical melanosis in males.

These experiments could not have been carried out without the skill and dedication of Richard Schultz, who bred and maintained the stocks of fish. We thank B. M. Sutherland for helpful discussions and comments. This work was supported by the Office of Health and Environmental Research of the U.S. Department of Energy.

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