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CELLULAR HEREDITY IN HAPLOID CULTURES
OF SOMATIC CELLS

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SUMMARY

During the past year we have continued our studies of the relation of ultraviolet mutagenesis to DNA repair in cultures of the haploid frog cell line ICR 2A. Our method of irradiation of cells in suspension was improved by construction of an improved detector with major sensitivity to the 254 nm Hg resonance line, to give better estimates of actual exposure of the cells. Using this method, dose-response and dose-fractionation studies on irradiation of ouabain resistance were carried out. The UV induction of this phenotype in the ICR 2A cell line was found to be less than that necessary for adequate analysis of dose-response curves. Cell fusion experiments using frog and mouse cells revealed an enhancement of mutagenesis in the mouse parent that will be explored in further work.

BACKGROUND: UV SURVIVAL AND DNA REPAIR IN ICR 2A

The properties of our haploid frog cell line with regard to single cell survival after UV and the modes of DNA repair that they express have been described (Freed et al., 1979). These cells readily photoreverse pyrimidine dimers, but excision does not appear to provide a quantitatively important pathway since few dimers are lost during incubation in the dark and "unscheduled DNA synthesis" is saturated at low doses. Survival curves for the haploid cells and their diploid derivatives were indistinguishable, save that exponentially growing diploids showed an increased Dq value (width of shoulder at low fluence). Details will be found in the attached reprint.

These repair properties make it possible to test whether UV induction of variant phenotypes is photoreversible and thus likely to reflect a direct effect on DNA. Using ouabain resistance and thioguanine resistance as the induced phenotypes, we sought to test whether photoreversal prior to mutation expression reduced the yield of resistant colonies. Experiments of this kind are described in the attached manuscript, which proposes that photoreversibility might be a general test for the genetic origin of UV-induced cell culture variants. A problem is that neither of these phenotypes is induced in sufficient numbers by UV (maximum increase in frequency less than 10-fold) to demonstrate the effect satisfactorily.

IRRADIATION AND DOSIMETRY

Improvements in our UV irradiation protocol now provide more satisfactory data. Figure 1, showing a typical ICR 2A survival curve by the new method, may be compared to figures 2 and 3 in the attached reprint. The improvements stem from a suspension irradiation procedure and better dosimetry.

Suspension irradiation is carried out by dispersing 1×10^7 cells in 10 ml of calcium- and magnesium-free phosphate buffered saline plus EDTA; the suspension is stirred in a UV-transparent petri dish during exposure from below. 254 nm radiation is obtained from a low pressure Hg discharge tube, air-cooled to reduce generation of wavelengths other than the 254 nm resonance line, and is passed through a Corning 7-54 filter to reduce contaminating visible light. Samples of 100 to 200 microliters are removed before exposure and at intervals during the irradiation. The samples are diluted in growth medium in petri dishes to give inocula of a size determined by the expected surviving fraction. After formation of colonies, the dishes are stained, colonies counted and surviving fraction calculated.

This suspension irradiation procedure demands that the average exposure of the cells be calculated from the incident fluence by correcting for the sieve effect, *i.e.*, shielding of cells by cell intervening in the beam. Such a correction can be calculated from measurements of the apparent transmittance of the cell suspension at the wavelength of interest (Morowitz, 1950). When we attempted to make such measurements with our Tektronix J-16 digital photometer, we encountered a problem of detector response, leading to overestimation of the transmittance of cell suspensions. Although germicidal lamps are often treated as sources of nearly pure 254 nm radiation, there is significant emission of other longer wavelengths.

The problem comes from the response of the J-6504 silicon photodiode sensor, which falls rapidly with decreasing wavelength in the UV-B range. Thus, response is biased in favor of the longer Hg wavelengths and against the 254 nm major output. Since cells are strongly absorbing at 254 nm but reasonably transparent above 280 nm, we could not make valid estimates of the sieve effect.

To deal with this, we modified the photometer probe to respond only to absorbed wavelengths. A short-wave UV/visible transducer was attached to the probe (figure 2). Radiation passes a 7-54 visible-blocking filter to remove fluorescent light or stray visible and strikes a layer of willemite powder phosphor ($\text{Zn}_2\text{SiO}_4\text{:Mn}$) which produces visible light that is measured by the photodiode. With this detector, it can be calculated that 90% of the response to an Hg low pressure discharge will be to the 254 nm line, 10% to 313 nm. This was confirmed by experiments with a glass filter, which blocks 254 nm but passes 313 nm. Linearity of response, measured by an inverse square method, yielded a mean deviation of 1.4% (figure 2). Absolute calibration by chemical actinometry gave a sensitivity of 3.6 ergs/min/mm² per photometer scale unit. Since our work involves fluences near 50 ergs/min/mm², resolution is satisfactory both for fluence measurement and for determination of the Morowitz correction.

Typical transmittance measurements of cell suspensions made with this instrument are shown in figure 3. With samples of 1×10^7 cells per dish, 254 nm transmittances fall between 40% and 60%; average exposure of the cells is about 75% of incident fluence, convenient for our work.

OUABAIN RESISTANCE AND UV, DOSE FRACTIONATION

The purpose of these experiments was to determine the curve for induction of ouabain-resistant mutants as a function of increasing UV exposure to describe alterations in the curve for fractionated doses and to determine the effects of varying the time between doses. Our method was to irradiate samples of 10^7 cells by the suspension method described above, to return the cells to growth medium for the chosen delay period, then to carry out a second irradiation by the same procedure. The samples were then transferred to 150 cm² culture flasks in growth medium and propagated for 7 days to allow expression of the resistant phenotype. This expression time had been found to produce full yield of nitrosoguanidine-induced mutants in prior experiments. The cells were then subcultured to 75 cm² flasks in medium supplemented with 1 mM ouabain, incubated in the dark for 14 days and then fixed and stained to score surviving colonies.

In prior experiments, we had found that these conditions were satisfactory for detection of UV or nitrosoguanidine induced mutants. Recovered single-step mutants showed an extreme resistant phenotype, as would be anticipated for haploid cells expressing a single altered allele coding for Na^+/K^+ -dependent ATPase, the target gene product in this selective system. Recovery of added mutants appeared to be independent of cell density, up to the limit set by the tendency of dense monolayer cultures to "peel" from the growth surface during selection in ouabain-containing medium, carrying away resistant cell colonies.

The results of these assays showed a restricted induction of ouabain resistant clones when UV was used as a mutagen. With single doses up to 10 Jm^{-2} (average surviving fraction 0.04), no more than 10 colonies per assay were

recovered. The limits of expectation at 5% level of significance were therefore so large that neither dependence on dose nor dependence on fractionation could be established. These results were consistent with our earlier studies, using single doses; however, we did not observe the increased mutant yield we had expected at higher doses of UV.

This low yield after UV treatment of ICR 2A contrasts with the results of treatment with the alkylating agent nitrosoguanidine. In such experiments, a maximal enhancement of mutant frequency (at least 100-fold) was obtained at a surviving fraction of about 0.4.

It is not clear whether the low yield reflects specific properties of the frog gene for Na^+/K^+ -dependent ATPase, or a problem in detecting the mutant phenotype. Exposure of mammalian cells to comparable UV fluence (resulting in equivalent pyrimidine dimer formation) yields larger numbers of mutants in a dose-dependent fashion (Chan and Little, 1978; Chang et al., 1978a, 1978b). Since our recovery of added mutants in reconstruction experiments is adequate, we tend to favor the view that the phenomenon reflects insensitivity of the gene itself to UV mutagenesis. It should be noted that in order for ouabain resistance to be expressed, there must be a base substitution in one or more of the critical codons that specify amino acids defining the ouabain-binding site on the protein. Therefore, this kind of mutation might be very sensitive to sequence differences defining mutagen specificity at the molecular level.

GENE TRANSFER EXPERIMENTS

We have used cell fusion and DNA uptake methods to explore transfer of frog genes to mammalian cells. Since interspecific transfers among mammals are feasible, we wished to determine if genetic material from more distantly related vertebrates could be handled in the same way. DNA-mediated gene transfer (Bacchetti and Graham, 1977; Wigler et al., 1977, 1978) is carried out by exposing cells to DNA co-precipitated with CaPO_4 which is taken up by phagocytosis. A selective system is then applied to isolate rare clones that have replicated, transcribed and expressed a particular DNA sequence.

Our experiments sought to detect transfer of frog genes for thymidine kinase (TK) or photoreversal enzyme (PRE) to mammalian recipient cells. Pilot experiments were carried out to transform mouse LM/TK⁻ cells with the purified 3.4 kilobase restriction enzyme fragment that codes for herpes simplex viral TK (Wigler et al., 1977). We were able to obtain transformants by this technique and at lower frequency by using high molecular weight DNA from mouse liver (Wigler et al., 1978). However, similarly prepared DNA from wild-type ICR 2A frog cells failed to elicit transformed colonies from 2×10^8 LM/TK⁻ cells treated.

DNA from ICR 2A cells and from frog liver was also applied to Chinese hamster CHO-K1 cells in an attempt to transfer PRE activity. To select recipients expressing this enzyme, the cultures were exposed to successive cycles of UV radiation and photoreversing illumination and the survivors were tested for alterations in survival curve and for dose-modifying effect of exposure to visible light. No significant alterations were detected.

Thus, any transfer of these frog genes was too rare for us to observe. It is possible that there is a block to expression of introduced DNA sequences when a sufficiently great evolutionary distance separates donor and recipient.

In related experiments, a cell fusion method was employed to combine frog and mouse genomes. The fusion method, using polyethylene glycol (PEG) as the fusogen, in our hands yields viable hybrids in intraspecific frog cell crosses employing our TK⁻ OUA^R "universal hybridizer" test strain from ICR 2A. Using this procedure, the revertable TK⁻ mouse cell strain B82 was fused with wild-type KR 2A frog cells. The cultures were selected at 37°C (to eliminate unfused frog parents) in HAT medium containing 1 mM thymidine (Hi-HAT), to eliminate unfused B82 mouse cells and permit survival only of cells expressing TK_B, the frog thermostable cytosol deoxypyrimidine kinase (Freed and Hames, 1976). The frequency of Hi-HAT resistant colonies rose from about 1×10^{-8} to 2.5×10^{-7} when the B82 cells were treated with PEG in the presence of ICR 2A cells. The effect required both ICR 2A cells and exposure to PEG. Representative resistant clones were isolated, expanded and shown to express a thymidine kinase with the electrophoretic mobility of the mouse cytosol enzyme. No amphibian chromosomes were detected cytogenetically. Thus, the evidence suggests that these strains are (mouse) revertants.

Why is TK reversion in the mouse cells enhanced by fusion with frog cells? Two classes of explanation seem worth considering. First, fusion with frog cells, by introduction of frog chromosomes that are subsequently degraded, may have induced a mutagenic repair phenomenon resembling radiation-enhanced-reactivation. Alternatively, introduction of the functional frog gene product, TK_B, may have permitted DNA synthesis in the presence of an imbalanced deoxynucleotide pool, a known mutagenic influence. In future experiments to detect the first class of effects, we wish to use purified DNA to avoid the possibility of effects of the second class.

STATISTICAL EVALUATION METHODS

The purpose of this work was to facilitate hypothesis testing in the interpretation of single cell survival and mutagenic response data. A particular aim was to provide objective methods for ascertainment of the best-fit curve and the confidence limits of the coefficients that describe it. At present, the survival curve analysis methods are complete, and curve-fitting methods for mutagenic response data are under development.

Single cell survival data in the form of colony counts per plate are provided to the ICR Biomathematics Laboratory for entry into the computer procedure. Their program calculates the surviving fractions at each dose, determines the best fit to the standard survival equation and displays the curve with proper coordinates, data points and values of D_0 and D_q together with their confidence limits. Hard copies are printed. To compare two sets of survival data, the separately estimated values of the curve coefficients are assigned in various combinations to test whether these give significantly better fit than if the same values were used for both populations. Significance is tested by the sampled permutation procedure described in the attached reprint (Freed et al., 1979).

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STAFF

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PUBLICATIONS

The following have appeared since our last annual report:

- 1978 Freed, Jerome J., Frank A. Angelsanto and Ronald H. Hoess Photo-reversal as a tool for study of mutagenesis in haploid frog cell cultures. J. Cell Biol. 79:390 (abstract).
- 1979 Freed, Jerome J., Ronald H. Hoess, Frank A. Angelsanto and Holman C. Massey, Jr. Survival and DNA repair in ultraviolet-irradiated haploid and diploid frog cells. Mutation Res. 62:325-339.

Figure 1. Haploid frog cell UV survival curve from a single experiment by the suspension method described in the text.

Figure 2. 254 nm-specific transducer attachment to a Tektronix radiometric probe and test of its linearity. D is the distance between the transducer and a 1 cm diameter source of 254 nm UV; E represents arbitrary units of photometer response.

Figure 3. Transmission of cell suspensions, in the irradiation apparatus, as a function of cell density, as measured with the 254 nm-specific transducer.