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Cell Variants Showing Differential Susceptibility to Ultraviolet Light–Induced Transformation

Abstract. *Six variant clones isolated from a subclone of BALB/3T3-A31 clone were classified into three groups according to their different susceptibilities to cell transformation by ultraviolet light irradiation: highly susceptible, intermediately susceptible, and resistant. All variant clones showed similar susceptibility to cytotoxic effects induced by ultraviolet light.*

Many hypotheses have been proposed to account for cell transformation due to chemical carcinogens or radiation. However, neither metabolism nor cellular target molecules have been unequivocally identified as factors directly involved in the induction of cell transformation. To define the factors involved, it would be very useful to isolate the cell variants that show different susceptibilities to transformation. Characterization of such variants may lead to the identification of the crucial processes leading to cell transformation.

One of the advantages of using established cell lines as target cells for a transformation assay system is the availability of cloned lines. Isolation, characterization, and use of mammalian cell variants are possible only with established lines unless the mutated cells are available as biopsies of animals or of humans. Cells are available from patients who are predisposed genetically to cancer. However, the transformation assay systems used for human cells are still far from being quantitative and rapid (*1*). There have also been reports of animal strains having different susceptibilities to polycyclic hydrocarbon–induced carcinogenesis (*2-4*). Differences in susceptibility between strains, however, have been ascribed to the differences in genetic factors that control carcinogen metabolism (*2-4*).

We now report the isolation of cell variants from a BALB/3T3-A31 clone showing different susceptibilities to ultraviolet (UV) light–induced transformation. To avoid isolating variants having either altered membrane permeability to chemical carcinogens or altered carcinogen-metabolizing activity, UV irradiation was used as the transforming agent in these experiments.

Malignant transformation was assayed by scoring transformed foci, as reported previously for A31-714 cells (*5, 6*). Actively growing cells were plated at 10^4 cells per 60-mm plastic dish for the transformation assay and 100 to 500 cells per dish for assay of plating efficiency. Eighteen hours after plating, the medium was removed and the cells attached to dishes were exposed to UV light at room temperature. The flux of the 15T8GE germicidal lamp used as a source of UV light was 1.01 W/m^2 as measured by MGL method (*7, 8*). After irradiation, the cells were reincubated in fresh medium. Plating efficiency was determined 7 days later, and transformation was scored 26 to 27 days after irradiation, as described previously (*5, 6*). Only the transformed foci that showed clear alteration in growth pattern and cell arrangement (such as abundant mitoses, crisscrossed cell arrangement in the center and at the edge of focus, and dense staining in the cytoplasm) were scored. Scoring foci showing only partial alteration of morphology (such as high cell density but no clear pattern of crisscrossed cell arrangement at the edge) resulted in a 1.3- to 1.9-fold increase in the absolute frequency of transformation compared to scoring only the clearly transformed foci, but such analysis did not affect the differences in relative susceptibility to transformation observed among the variants.

Mass cultures of A31-1 cells were used as sources for the isolation of variants. The A31-1 line was isolated from BALB/3T3-A31 clone (*9*) which was obtained from Ikawa at the National Cancer Institute. The A31-1 cells of early passage (at least up to 50 cell generations after isolation were homogeneous in cell morphology and susceptibility to transformation by UV, 3-methylcholanthrene, and benzo [*a*]pyrene. The A31-1 cells of late passage (150 to 180 cell generations after isolation) consisted of a mixture of cells varying in morphology and having variable frequency of transformation by chemical carcinogens. More than 100 subclones were isolated from mass culture of A31-1 of late passage. Among them, 22 clones that showed homogeneity in morphology and high sensitivity to density-dependent inhibition of cell growth were tested for the susceptibility

Table 1. Grouping of variant cells according to their susceptibility to UV-induced transformation.

Susceptibility to transformation	Subclone*	D_{37}† (erg/mm^2)	Transformation‡ frequency ($\times 10^5$)
Highly susceptible	A31-1-13	38	160 ± 28
Intermediately susceptible	A31-1-1	36	9 ± 0.2
	A31-1-2	34	8 ± 1.4
	A31-1-7	36	10 ± 2.0
Resistant	A31-1-8	40	< 1.6
	A31-1-15	41	1 ± 1.0

*Subclones isolated from BALB/3T3-A31-1 clone were plated at 100 and 300 cells for survival assay and 10^4 cells for transformation assay per 60-mm plastic dish containing 5 ml of Eagles minimum essential medium (Earle's salt solution) supplemented with 10 percent fetal bovine serum. Eighteen hours after cells were plated the medium was removed, and the cells attached to the substrate were exposed to UV at a dose of 75 ergs/mm^2 for transformation assay and at various doses ranging from 12.5 to 100 ergs/mm^2 for survival assay. After irradiation, the cells were reincubated in fresh medium. The dishes for assay of survival were maintained without medium change and fixed 7 days after irradiation. The cultures for transformation assay had a medium change twice a week and were fixed 25 or 26 days after UV exposure. †D_{37} was calculated from the survival curves. ‡Mean ± standard error for 12 to 27 plates, expressed as ratio of cells transformed to cells surviving UV dose of 75 ergs/mm^2.

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to transformation by UV at a dose of 75 erg/mm^2. Although 16 subclones showed unstable and variable susceptibility to transformation, 6 subclones showed constant susceptibility through more than 50 cell generations after isolation. These six subclones were classified into three groups: highly susceptible, intermediately susceptible, and resistant to UV transformation, respectively (Table 1). The susceptibility of intermediately susceptible variants was similar to that of the parent A31-1 line.

When the variants were exposed to doses of UV ranging from 25 to 100 erg/mm^2, a highly susceptible variant, A31-1-13, and an intermediately susceptible variant, A31-1-1, produced an increased number of transformed foci with the increased dose, whereas a resistant clone, A31-1-15, produced very few transformed foci at this dose range (Fig. 1). The dose-response curves for transformation indicate that A31-1-13 has a significantly higher susceptibility at all UV doses tested (Fig. 2B). On the other hand, the variants did not differ significantly in susceptibility to the killing effect of UV irradiation (Table 1 and Fig. 2A).

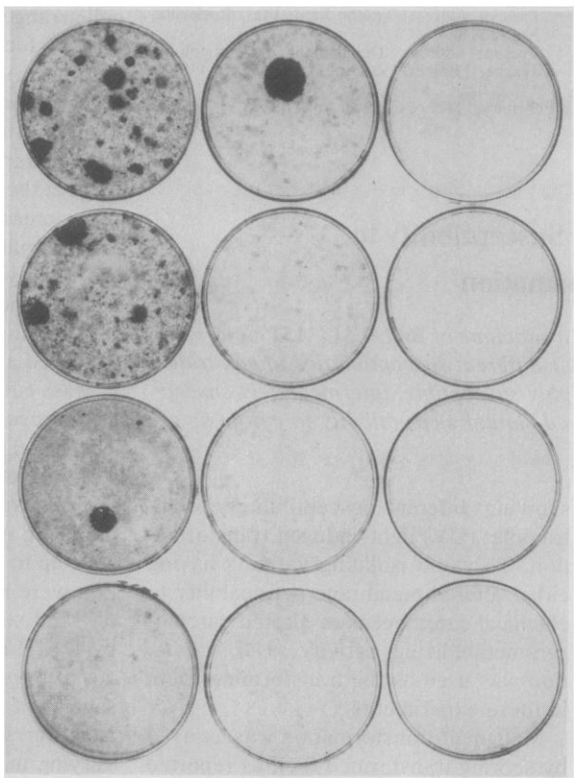

Fig. 1. Photograph, after fixation and Giemsa staining of dishes, showing the different frequencies of focus formation in (top) A31-1-13, (middle) A31-1-1, and (bottom) A31-1-15 cells after exposure to UV at doses of (left to right) 0, 25, 50, and 75 erg/mm^2, respectively.

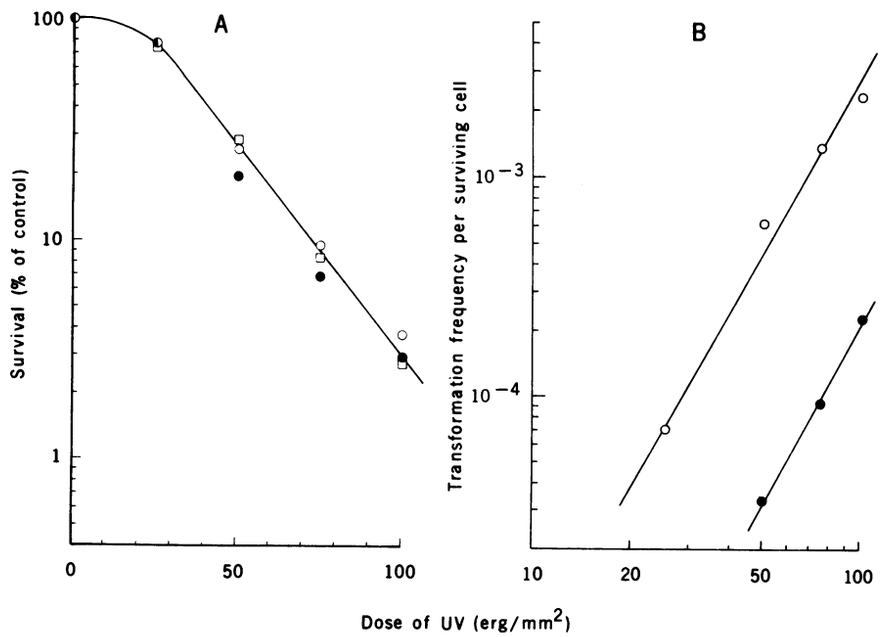

Fig. 2. Dose-response curves for (A) cytotoxic effects and (B) transformation produced by UV irradiation in (○) A31-1-13, (●) A31-1-1, and (□) A31-1-15 cells. Each value is the average of counts on 14 to 27 plates. See footnote to Table 1.

The transformed cell lines were isolated from the centers of individual transformed foci 28 days after UV irradiation and, within 2 weeks after the isolation, were tested for their saturation density and ability to grow in soft agar. All of them showed transformed morphology that was stable through successive passages of cultures. The saturation density of transformed lines was three to six times that of untransformed cells. All transformed lines grew well and formed colonies in soft agar. When the transformed lines were injected subcutaneously into nude mice at the dose of 10^5 cells per animal, progressively growing tumors were produced at the site of injection by all the transformed lines (unpublished data). There have been no significant differences in the morphology, saturation density, or colony-forming ability of the transformed lines due to differences in the original subclones from which the transformed lines were derived. These results indicate that the cells comprising the foci of clearly altered morphology were malignantly transformed and that there was no marked difference in transformed phenotype between transformed cells derived from different variants.

Because the variants discussed in this report differ in their response to the transforming effects of UV irradiation, they provide a system for studying parameters controlling the transformation response as issues clearly separate from those of carcinogen metabolism. That there was no difference in susceptibility to the killing effect by UV irradiation between variants suggests that the variants act similarly to repair the damage induced by UV irradiation, although some type of repair system that accounts for the induction of transformation, but not for the recovery from the killing effect, may be different between variants. It would be interesting to know whether the variants showing differential susceptibility to the induction of mutation by UV irradiation also show differential susceptibility to transformation by other agents such as tumor viruses. Preliminary results indicate that the differential susceptibility of the variants to transformation when UV is the transforming

agent is also observed when chemical carcinogens are used as the transforming agents (*10*). Therefore, studies of the function of genes responsible for differential susceptibility—which are segregated, depressed, or induced in the variant cells—will make it possible to identify crucial factors in the neoplastic transformation by physical and chemical agents. The isolation of variants reported here also suggests a possible way to develop a better system for rapid assay of environmental carcinogens.

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(−)Pentobarbital Opens Ion Channels of Long Duration in Cultured Mouse Spinal Neurons

Abstract. *Intracellular recordings from voltage-clamped mouse spinal neurons in tissue culture were used to study the membrane mechanisms underlying inhibitory responses to γ-aminobutyric acid and the (−) isomer of pentobarbital. Fluctuation analysis suggested that both substances activated ion channels in the membranes. However, the channels activated by pentobarbital remained open five times longer than those activated by γ-aminobutyric acid.*

Racemic pentobarbital is used clinically to induce general anesthesia. Studies of the cellular mechanisms underlying this pharmacologic action have revealed that the drug depresses neuronal excitability in a variety of ways (*1–4*). Two of these depressant effects appear to be stereospecific and are caused primarily by the (−) isomer of the drug (*5*). One of these actions involves an increase in Cl^- conductance (*3*, *5*), an effect that is blocked by drugs that also antagonize Cl^- conductance evoked by the putative neurotransmitter γ-aminobutyric acid (GABA) (*2*, *3*). It is thus possible that the effects of (−)pentobarbital are mediated through activation of GABA receptors (*2*, *3*). The recent application of fluctuation analysis and single-channel recording techniques to membrane responses induced by endogenous ligands revealed the statistical nature of the underlying elementary events and their associated electrical properties (*6*). We applied fluctuation analysis to membrane current responses evoked in cultured mouse spinal neurons by GABA and (−)pentobarbital, and we report that the drug opens ion channels whose conductance is similar to that of GABA-activated channels but which remain open five times as long.

We grew mouse spinal neurons in tissue culture (*3*) and, at the time of the experiment, replaced the normal maintenance medium with Hanks balanced salt solution containing 1 m*M* $CaCl_2$, 10 m*M* $MgCl_2$, and 0.5 μ*M* tetrodotoxin to eliminate all evoked synaptic activity and allow clearer study of the pharmacologic responses. Using the voltage clamp technique, we made intracellular recordings on the modified stage of an inverted phase microscope with two electrodes filled with 3*M* KCl. The recordings were made at room temperature (25° ± 1°C). The GABA was applied to individual cells by iontophoresis from pipettes containing 1*M* solutions, and the (−) isomer of pentobarbital was applied by pressure from pipettes containing 100 μ*M* solutions. Analysis of current fluctuations was performed with the aid of PDP 11/10 and 11/40 computers.

Fourteen cells were studied with GABA alone; two, with (−)pentobarbital alone; and seven, with both agonists. Application of GABA or (−)pentobarbital induced membrane current responses in all of the cells tested. The responses to GABA and (−)pentobarbital were accompanied by additional membrane current variance (Fig. 1). Variances of membrane current fluctuations during 30-second samples of baseline records and of agonist responses were averaged, and the former were subtracted from the latter to yield the variance induced by the drug. Agonist-induced variance was directly proportional to the amplitude of the current response over the 0.3- to 3.0-nA response range. Assuming that the variance reflects statistical fluctuation in the number of open ion channels about a mean number of open channels, the average conductance γ of a single channel can be estimated from the relation $\gamma = \sigma^2/[\Delta I(V_c - E_R)]$, where σ^2 is the variance associated with agonist-induced changes in membrane current ΔI, E_R is the reversal potential of the agonist response, and V_c is the holding potential under voltage clamp. Estimates of γ for GABA, 14.5 ± 7 pS (mean ± S.D., $N = 21$ cells), and (−)pentobarbital responses, 14.7 ± 9 pS ($N = 9$ cells), were not significantly different ($P > .90$, Student's *t*-test), indicating that both substances open channels of similar conductance.

Estimates of the average lifetime of open channels were obtained from the power spectral density of current fluctuations in the presence of the agonists. The spectra were calculated as the fast Fourier transform of 6144-point samples of membrane current, digitized at 5 msec per point. Spectral analysis of baseline fluctuations in all of the cells showed that, in the absence of agonist, power spectral density was proportional to the inverse of frequency *f* over the entire frequency range of the spectrum and over the membrane potential range used (−50 to −90 mV) (Fig. 2A). Similar $1/f$ spectra have been reported in a number of excitable tissues studied in the absence of chemical stimulation, but the physical basis of the $1/f$ spectrum remains unclear (*7*).

Spectra of baseline fluctuations immediately before or after agonist responses were subtracted from spectra obtained during agonist responses, giving difference spectra of agonist-induced fluctuations. We obtained 139 spectra of GABA responses in 21 cells and 75 spectra of (−)pentobarbital responses in nine cells. For holding potentials over the −50 to −90 mV range, the difference spectra can be described by a single Lorentzian term, $S(f)/S(0) = 1/[1 + (f/f_c)^2]$, where $S(f)$ is the spectral density as a function of frequency and f_c is the half-power frequency of the spectrum (Fig. 2B) (*6*). The Lorentzian character of the spectra suggests that both GABA and (−)pentobarbital open an array of channels whose lifetimes are exponentially distributed around an average duration τ, where $\tau = 1/2\pi f_c$ (*6*). The estimated mean lifetime of (−)pentobarbital-activated channels,

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