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Genetics

Mutagenesis in S49 mouse lymphoma cells: Induction of resistance to ouabain, 6-thioguanine, and dibutyryl cyclic AMP

(protein kinase/nitrosoguanidine/ethyl methanesulfonate/ICR 191/x-rays)

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ABSTRACT **The effects of mutagens on three genetic markers—resistance to ouabain, 6-thioguanine, and dibutyryl cyclic AMP (Bt_2cAMP), were investigated in a mouse lymphoma cell line, S49. Nitrosoguanidine, ethyl methanesulfonate, ICR 191, and x-rays were used. Mutagen-specific responses were seen. Ouabain resistance was induced by nitrosoguanidine, but not by ICR 191. ICR 191 induced resistance to 6-thioguanine more efficiently than did nitrosoguanidine; the converse was true of resistance to Bt_2cAMP. The relative frequency of biochemically distinguishable subtypes of mutants resistant to Bt_2cAMP was characteristic of the mutagen used to generate them. The results can be interpreted as follows: nitrosoguanidine and ethyl methanesulfonate frequently, but ICR 191 and x-rays rarely, give rise to DNA base sequence changes that result in structurally altered but functional proteins. This type of change is required for induction of mutants resistant to ouabain and of certain classes of mutants resistant to Bt_2cAMP. Resistance to 6-thioguanine and other classes of mutants resistant to Bt_2cAMP can result from DNA base sequence changes that lead to extensive alteration of protein structure or expression; these changes are induced by ICR 191 or x-rays.**

Substantial evidence indicates that changes in DNA structure underlie at least some stable alterations in the phenotype of somatic cells (1). Nonetheless, this is an assumption that must be tested any new system. Adenosine 3′:5′-cyclic monophosphate (cAMP) resistance for S49 mouse lymphoma cells in tissue culture has many characteristics expected of a mutation. S49 wild-type cells stop growing and later die when they are incubated with the cAMP derivative N^6,$O^{2'}$-dibutyryl cAMP (Bt_2cAMP) (2). Resistant cells can be selected in a single step by cloning in soft agar containing Bt_2cAMP (3). Fluctuation analysis has shown that resistance occurs spontaneously at a rate of 2×10^{-7}per cell per generation (3). The variant phenotype is stable.

Compared to wild-type cells, variant cells have absolute or relative defects in their cAMP-activated protein kinase activity. Kinase, the only known intracellular receptor for cAMP in animal cells, is composed of regulatory and catalytic subunits in S49 cells and other tissues. Binding of cAMP to the regulatory subunit dissociates the holoenzyme. The free catalytic subunits thus activated can phosphorylate specific protein substrates, thereby altering their biologic activity. Clones of S49 cells resistant to Bt_2cAMP have one of three types of alterations in kinase activity (4). Types A, B, and C denote, respectively, cells with kinase that requires a 5- to 12-fold higher cAMP concentration for activation, cells with reduced levels of kinase activity, and cells with no detectable cAMP stimulated kinase activity. The thermal lability of the kinase activity from some type A mutants is different from that of wild-type cells: in distinct mutants it is decreased (5) or increased (I. Lemaire and P. Coffino, submitted for publication). Furthermore, the kinase of certain type A mutants has been found to consist of altered regulatory but normal catalytic subunits (5).

These studies provide evidence that at least some cells resistant to cAMP arise from mutations of DNA that result in the formation of structurally altered proteins. An increase in the incidence of variant cells after treatment with compounds that are mutagenic for prokaryotes would further strengthen this conclusion. Previous preliminary experiments (3) have shown that ICR 191 (6) increases the incidence of resistance to Bt_2cAMP in S49 cells. We have now systematically examined the mutagenic effects of ICR 191, which is predominantly a frame-shift mutagen in prokaryotes; *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (MNNG) and ethyl methanesulfonate (Et$MeSO_3$, EMS) which generate predominantly missense mutants by alkylation, and x-irradiation. In addition, we have compared the induction of Bt_2cAMP resistance with the induction of 6-thioguanine (S^6Gua, 6TG) and of ouabain resistance. Resistance to S^6Gua is among the most extensively studied of somatic genetic markers and is associated with a deficiency of hypoxanthine phosphoribosyl transferase (HPRT, IMP:pyrophosphate phosphoribosyltransferase, EC 2.4.2.8) activity. Ouabain resistance is imparted by a structural change in the Na^+/K^+-ATPase affecting the binding of ouabain or the response of the enzyme to bound ouabain (7, 8). In this report, we show that mutagens differ in the types of mutations they preferentially induce in somatic cells. Marker- and mutagen-specific responses can be understood by considering mechanisms of mutagen action previously demonstrated in prokaryotes and the nature of the biochemical changes that result in the various mutant phenotypes.

MATERIALS AND METHODS

Materials. Media and chemicals came from the following sources: Dulbecco's modified Eagle's medium (high glucose), horse serum, and fetal calf serum—Grand Island Biological Co.; agarose—Seakem; MNNG—Aldrich; Et$MeSO_3$—Eastman Kodak; ICR 191—gift of H. J. Creech; S^6Gua—Calbiochem; [^{32}P]ATP—International Chemical and Nuclear Corp.; Bt_2cAMP, theophylline, ouabain, and histone F_{2b}—Sigma.

Cell Culture. S49.1 lymphoma cells were from the Salk Institute (9). The cells are pseudodiploid with a mean chromosome number of 40 (3, 9). Stationary suspension cultures were propagated in Dulbecco's modified Eagle's medium containing 10% heat-inactivated horse serum with no antibiotics. Growth

Abbreviations: cAMP, adenosine 3′:5′-cyclic monophosphate; Bt_2cAMP, N^6,$O^{2'}$dibutyryl adenosine 3′:5′-cyclic monophosphate; Et$MeSO_3$, ethyl methanesulfonate; S^6Gua, 6-thioguanine; HAT, hypoxanthine + aminopterin + thymidine medium; HPRT hypoxanthine phosphoribosyl transferase; MNNG, *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine.

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was at 37° in a humidified CO_2 incubator. The doubling time was 15–17 hr. Cultures were diluted three times per week to maintain cell densities within the limits for viability, about 5×10^4 cells per ml to 2.5×10^6 cells per ml. Tests for mycoplasma were negative. Cultures were reinitiated from stocks frozen in liquid nitrogen at 1–2 month intervals to assure a low and uniform background incidence of mutants. Cells were counted in a Coulter Counter, model Z_{BI}, or in a hemacytometer after staining the cells with trypan blue.

Cloning Procedure. Cells were cloned in growth medium made semisolid by the addition of agarose over a feeder layer of primary mouse embryo fibroblasts (10). Duplicate or triplicate samples were plated for each experimental point. Clones were counted on day 8, 9, or 10. The cloning efficiency was between 50 and 100%, but uniform within a given experiment. Clones were picked under a dissecting microscope at about day 10 using an Eppendorf pipet with a heat sterilized tip. The contents of the tip were resuspended in 0.4 ml of medium in a well of a Falcon Multiwell dish and were subsequently transferred to flasks for propagation or frozen at −70° directly in the multiwells after addition of dimethylsulfoxide and *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (Hepes) at pH 7.4 to final concentrations of 5% and 10 mM, respectively.

Mutagenic Treatment and Selection. ICR 191 was dissolved in water at 100 μg/ml, MNNG was dissolved in 5 mM sodium acetate at pH 4.8 as 200 μg/ml. Solutions were sterilized by filtration, and aliquots stored at −20°. All manipulations with ICR 191 were done in a semidark room. $EtMeSO_3$ was added directly to culture flasks. X-ray treatment was performed with a 250 kV peak Westinghouse Quadrocondex at 230 kV, 15 mA with 0.5 mm Cu and 1 mm Al filters with a dose rate of 989 rad/min. Exponentially growing cells were subjected to mutagenic treatment at a density of 5 to 8×10^5/ml. ICR 191 and $EtMeSO_3$ treatment was for 24 hr, MNNG treatment for 3 hr. Cells were then centrifuged and resuspended in fresh medium. Cells were maintained in suspension culture for 6–8 days and were then cloned at 1 to 2×10^6 cells per 6 cm dish in selective media. At the same time, 1 to 2×10^2 cells per dish were cloned in nonselective medium to determine their cloning efficiency. Mutation frequency was calculated as the cloning efficiency in selective medium divided by the cloning efficiency in nonselective medium. Mutation frequency was maximum for each marker after a post-treatment expression time of 6–8 days. The dependence of mutation frequency on expression time after exposure to mutagens will be described elsewhere (U. Friedrich and P. Coffino, manuscript in preparation).

Determination of Protein Kinase and Hypoxanthine-Guanine Phosphoribosyl Transferase Activity. Protein kinase activity of crude cell extracts was assayed as described (5). Transfer of ^{32}P from [γ-^{32}P]ATP to histone was measured in an incubation mixture containing the specified concentration of cAMP. Protein content of the cell extracts was determined by the method of Lowry *et al.* (11). Nonspecific activity measured in controls without cAMP was subtracted from total activity to reflect cAMP-dependent protein kinase activity. Hypoxanthine-guanine phosphoribosyl transferase activity was determined as described by Fenwick and Caskey (12).

RESULTS

Establishment of Conditions of Selection. Addition to cloning medium of Bt_2cAMP (0.5 mM) and theophylline (0.2 mM), an inhibitor of phosphodiesterase activity, killed virtually all of the wild-type cells. These were the lowest concentrations that did so. The incidence of false positive mutant colonies was low: among several hundred clones selected under the above conditions from several hundred million wild-type cells, only two clones proved to be sensitive to Bt_2cAMP when retested in suspension culture. Mutants had cloning efficiencies between 20 and 100% when replated in the same selective medium. Type C mutants cloned with higher efficiency than the others under these conditions, hence there may be a selection bias for them. The effect of different concentrations of S^6Gua on the efficiency of mutant colony recovery was determined by testing both a wild-type culture mutagenized with MNNG and a S^6Gua resistant culture that had been generated from a clone grown in soft agar containing 30 μg/ml of S^6Gua. Both recovery of mutants from the culture of mutagenized wild-type cells and the cloning efficiency of S^6Gua resistant cells dropped rapidly above 10 μg/ml. Thus, all selections were carried out at 10 μg/ml. Twelve resistant clones selected at this concentration and tested for HPRT activity were deficient in enzyme activity (<0.5% wild-type level). A concentration of 1 mM ouabain eliminated all wild-type cells (survival $<10^{-7}$) but allowed a measurable number of resistant clones to grow. Mutants resistant to 10 mM ouabain were not obtained by one-step selection. The phenotype of cultures propagated from several clones selected in 1 mM ouabain was confirmed by growing them with that concentration of drug in both suspension culture and as colonies in soft agar.

Table 1. Cell-density independence of mutant recovery

Cells plated per dish ($\times 10^5$)	Mutants/10^6 cells		
	S^6Gua	Bt_2cAMP	Ouabain
5	327	34	37
8	340	32	N.T.
10	358	27	45
20	285	33	46

Cultures for S^6Gua selection and Bt_2cAMP selection were mutagenized with ICR 191; the culture for ouabain selection was mutagenized with MNNG. After 8 days, the indicated number of cells were cloned, and mutant colonies counted after 10 days. Values are corrected for efficiency of plating, and were independently determined for each culture. N.T., not tested.

The use of high cell density enhances the convenience and efficiency of experimental determinations of mutant frequency. As cell density is increased beyond a certain point, however, the apparent efficiency of mutant recovery can change (13). To determine the range of cell density within which mutant frequency could be accurately assessed, we cloned different numbers of cells of mutagenized wild-type cultures in the three selective media (Table 1). The yield of resistant colonies did not vary significantly when plating densities between 5×10^5 and 2×10^6 cells per dish were used. Therefore, all subsequent selections were done at densities of 1 to 2×10^6 cells per dish.

Dose Response of Cell Killing and Induction of Mutants. The effect of four different mutagens on the colony-forming ability of S49 cells is shown in Fig. 1. The concentration dependence of killing varied considerably from one experiment to another. The results were, however, in the range reported for other cells (14). S49 cells were perhaps less sensitive to the killing action of MNNG and $EtMeSO_3$ and more sensitive to ICR 191. The sensitivity of S49 to x-rays was especially high, a property that has been found in other lymphoma cells (15).

Fig. 2 shows the frequencies of Bt_2cAMP and S^6Gua resistant mutants obtained with different concentrations of each of three mutagens. The results have been plotted by using a scale for

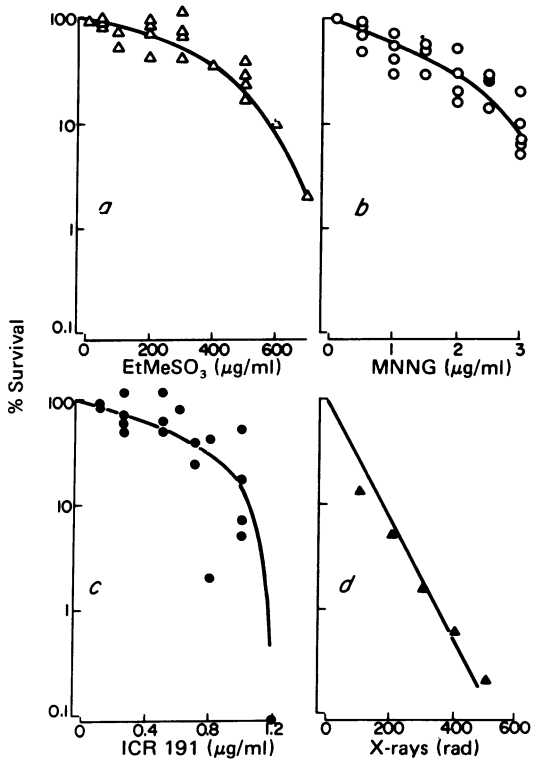

FIG 1. Survival of S49 cells after treatment with different doses of mutagens. Each point represents the mean number of samples that were plated in triplicate immediately after mutagenesis. All dilutions were made with respect to cell numbers in the untreated control culture so that the growth inhibitory effect of the mutagens is included in the value for killing. The curves for $EtMeSO_3$, MNNG, and ICR 191 were computed from data derived from five different experiments. (a) $EtMeSO_3$, (b) MNNG, (c) ICR 191, and (d) x-rays.

each mutagen that makes the cytolytic effect of mutagens roughly comparable at each value of the abscissa. X-rays induced almost no mutants at the survival levels (>5%) examined in this figure (data not shown). Mutagenesis was dose dependent. ICR 191 was a far more effective mutagen for S^6Gua resistance than for Bt_2cAMP resistance. The ratio of S^6Gua to Bt_2cAMP resistant mutants generated by ICR 191 (1.0 μg/ml) was consistently (four experiments) 8–10:1. Conversely, in the experiment shown, MNNG was a more efficient mutagen for Bt_2cAMP resistance; the ratio was about 1:2 when MNNG was used at a concentration of 2.5 μg/ml (two experiments). However, in one experiment, this ratio was as high as 4:1. The ratio produced by 800 μg/ml of $EtMeSO_3$ was about 1:1.

No induction of ouabain resistance by ICR 191 was observed in a series of experiments using that mutagen at concentrations up to 1 μg/ml and post-treatment expression times of 0–14 days. MNNG, however, generated mutants resistant to ouabain in a dose-dependent manner. In typical experiments, MNNG (2.5 μg/ml) increased the incidence of mutants from $<1/10^6$ to $20–60/10^6$. The effect of $EtMeSO_3$ on this marker was not examined.

The magnitude of the observed increases in the incidence of each class of mutant virtually precludes the possibility that these results could be due to selection artefact rather than *de novo* generation of mutants. Significant mutagenic efficacy for Bt_2cAMP and S^6Gua resistance was demonstrated (Fig. 2) using conditions of $EtMeSO_3$, MNNG, and ICR 191 treatment that resulted in 5% or greater cell survival (Fig. 1). For all of these six combinations of mutagen with marker, the absolute number

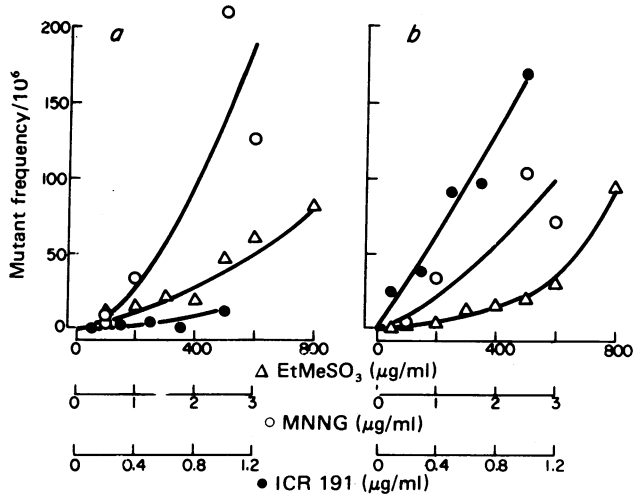

FIG. 2. Dose response curves for induction of mutants resistant to Bt_2cAMP and S^6Gua. Ater an expression time of 8 days, 1 to 2×10^6 cells per dish were cloned in medium selective for Bt_2cAMP (a) or S^6Gua (b) resistant mutants. Then 10^2 cells per dish were plated in nonselective medium for measurement of cloning efficiency. Values have been corrected for the incidence of spontaneous mutants. The incidence was $6/10^6$ for Bt_2cAMP resistance and $0/10^6$ for S^6Gua resistance.

of mutants was increased by treatment. The same was true for the combination of MNNG and ouabain resistance, but not for ICR 191 and ouabain resistance.

There was little distortion of the incidence of mutants by differential growth after mutagenesis. This was shown by testing several Bt_2cAMP resistant and S^6Gua resistant clones to determine their cloning efficiency after treatment with ICR 191, MNNG, and EMS. All were slightly (at most 2-fold) more resistant than wild-type cells to killing (data not shown). The growth rate and the time required for cultures to resume exponential growth after treatment with mutagens did not differ significantly for wild-type and mutant cells.

Classification of Bt_2cAMP Resistant Mutants. All previously reported mutants resistant to Bt_2cAMP contain an altered cAMP dependent protein kinase. This alteration, tested by measuring the ability of crude cell extracts to phosphorylate histone in the presence of various concentrations of cAMP, can assume several forms, as has been shown for spontaneously occurring mutants (4). Representative examples of Bt_2cAMP resistant mutants generated by each mutagen were analyzed in this way (Fig. 3). The three types, described earlier (4) and referred to in the introduction as types A, B, and C, are also found after mutagenesis (Fig. 3a–c). In addition, a new type of altered kinase activity is found that resembles type A in its increased apparent K_a for cAMP, but differs from that class in its even greater displacement from the wild type of its apparent K_a. These mutants (Fig. 3d) are termed type D. The dependence of protein kinase activity on cAMP concentration was determined, as in Fig. 3, by using extracts of 12 to 55 mutants induced by each mutagen. Each was classified with respect to type (Table 2). The spectra of mutants produced by the different mutagens are clearly distinguishable. Type D mutants are by far the most frequent class with $EtMeSO_3$ and MNNG whereas both types A and D are rare or absent with ICR 191 and x-rays. These two latter mutagens induce mainly or exclusively type B mutants. Only ICR 191 induces unequivocal type C mutants; these are absent in $EtMeSO_3$ and x-ray treated cultures. The one type C clone produced by MNNG mutagenesis (Fig. 3c) was not completely lacking in cAMP dependent kinase

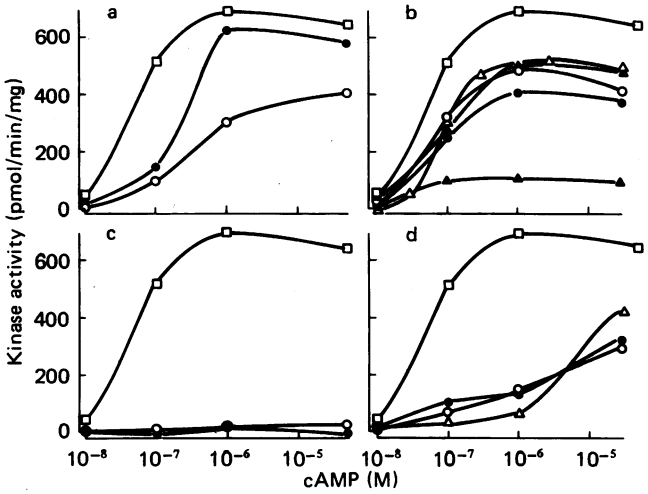

FIG. 3. Illustration of types of cAMP-dependent protein kinase activities in Bt_2cAMP resistant mutants of S49 cells. Shown in a, b, c, and d, respectively, are representative examples of mutant protein kinase of type A, B, C, and D. Mutants were generated by MNNG (○), $EtMeSO_3$ (△), ICR 191 (●) or x-rays (▲). A wild-type control (□) is included in each panel.

activity and should perhaps, therefore, be classified as a type B clone with extremely low activity.

DISCUSSION

Four different mutagens were tested for their effect on three different genetic markers in S49 cells. These agents raised mutant frequencies from $5/10^6$ for Bt_2cAMP resistance and $<0.5/10^6$ for S^6Gua and ouabain resistance to as much as $20–300/10^6$, $100–800/10^6$, and $60/10^6$, respectively, depending on the mutagen and dose used. These observations support the hypothesis that stable, heritable resistance to each of three drugs in S49 cells results from alterations in DNA base sequence. A series of different mutagens that interact with DNA in different ways and cause different types of mutation at the level of DNA sequence ought to produce, not only an increase in the frequency of somatic cell mutants, but also should cause predictable changes in the mutant gene product and phenotype, if the relation between gene product and phenotype is well enough understood. If it is assumed that mutagens increase the frequency of animal cell heritable variants by mechanisms similar to those established in prokaryotes, then the specificities of response to mutagens in S49 cells can be understood as follows: Base pair substitution mutagens (MNNG and $EtMeSO_3$) are able to produce structurally altered but functional proteins. This kind of mutation is required for resistance to ouabain and certain types of resistance of Bt_2cAMP. Mutagens that produce frame-shift or other mutations that result in extensive change in, or loss of, gene products (ICR 191 and x-ray) can lead to S^6Gua resistance and other types of resistance to Bt_2cAMP.

$EtMeSO_3$ and MNNG act as mutagens in microorganisms by alkylating bases, and thereby produce primarily transition type miscoding at the site of the altered base (16, 17). MNNG may also act as a frame-shift mutagen in *Salmonella* (18). ICR 191 has been shown to produce mainly frame-shift mutations in bacteria and fungi (19, 20), although it is also capable of inducing base pair substitutions by alkylation (20). X-rays can induce point mutations but can also produce large chromosome aberrations, chromosome breakage and chromosome loss. Each of these agents is capable of increasing the incidence of a variety of somatic cell mutants. Mutagen-specific responses for forward (17, 21, 22) and back (23, 24) mutation have been reported; in particular, Artlett *et al.* (25) have compared the mutagenic potency of several agents for induction of HPRT deficient and ouabain resistant mutants.

Table 2. Classification of Bt_2cAMP resistant mutants induced by different mutagens

Protein kinase type	Inducing mutagen			
	MNNG	$EtMeSO_3$	ICR 191	x-rays
A	0 (0)	0 (0)	6 (12)	0 (0)
B	13 (24)	3 (25)	36 (72)	12 (100)
C	1 (2)	0 (0)	6 (12)	0 (0)
D	41 (75)	9 (75)	2 (4)	0 (0)
Total	55 (100)	12 (100)	50 (100)	12 (100)

The data shown are the number of kinase mutants of a given type induced by the mutagen. The values in parentheses are the percentage of clones of this kinase type among the clones generated by a mutagen. The single MNNG derived type C clone may be type B (see *text*).

In the present studies, mutagen specificity has been demonstrated in several ways: (*i*) ICR 191 did not induce ouabain resistance but MMNG did. (*ii*) ICR was a much better mutagen for resistance to S^6Gua than for resistance to Bt_2cAMP; MNNG tended to show the opposite effect, though not as consistently (Fig. 2). (*iii*) The frequency distribution of the types of kinase mutants induced by ICR 191 and x-rays was different; each of these was different from that induced by MNNG and $EtMeSO_3$, but the latter two were similar (Table 2).

Resistance to ouabain is presumably the result of a mutation of the gene coding for Na^+/K^+ATPase. In this case, the properties of the mutant enzyme require a specific change in the enzyme that can only result from a base pair substitution. Frame-shift or deletion mutations would likely result in reduced or absent activities, but would not confer on the enzyme the ability to generate essential Na^+/K^+ gradients in the presence of ouabain. Resistance to S^6Gua, however, can be produced by any mutation that sufficiently alters or deletes the single active gene coding for HPRT, an enzyme not usually required for cell viability (25). When S49 cells were selected for retention of some degree of HPRT activity by first cloning them in S^6Gua and then counterselecting them in HAT medium (26), the resultant mutants contained functioning HPRT with an altered requirement for substrate (making them resistant to both S^6Gua and HAT). This class of mutant could be obtained only after mutagenesis with $EtMeSO_3$ or MNNG but not with x-rays or ICR 191 (U. Friedrich and P. Coffino, submitted). These results suggest that x-rays and ICR 191 can seldom induce the small structural changes that lead to altered but not totally functionally deficient proteins, but that MNNG and $EtMeSO_3$ can readily do so.

The induction of mutations that result in resistance to Bt_2cAMP can be understood in terms of this analytic framework. All Bt_2cAMP resistant S49 cells we have examined so far have defects of the cAMP dependent protein kinase. These could arise from mutations that affect the structure or expression of either the regulatory or catalytic kinase subunit. cAMP dissociates regulatory from catalytic subunit, thereby activating the latter. This is a lethal event in S49 cells. Hence, mutations that lead solely to loss of functional regulatory subunits should not be obtained. Structural mutations in the regulatory subunit that increase its affinity for the catalytic subunit or reduce dissociability of holoenzyme by cAMP, however, are readily selected as type A and D kinase mutants. We postulate that, in principle, similar constraints limit the types of mutation that

can be found in both the regulatory subunit of protein kinase and the Na^+/K^+ ATPase.

The validity of this analysis is supported by similarity of mutagen specificity for the type D kinase mutants and ouabain resistant mutants; both are far more readily induced by MNNG than by ICR 191. The failure of MNNG to induce type A kinase mutants, however, is not explained. The hypothesis that some type A and D mutants are point mutants in the regulatory subunit of the kinase has been further strengthened by analyzing isotopically-labeled regulatory subunits of wild-type and mutant cells in two-dimensional polyacrylamide gel electropherograms: many of these mutants show differences in charge without apparent change in molecular weight (27).

Mutations that reduce the activity of the catalytic subunit of kinase should also produce resistance to Bt_2cAMP. Type B and C mutants may arise in this way. Recent evidence suggests that type C mutants are devoid of catalytic activity but synthesize detectable amounts of functionally and electrophoretically normal regulatory subunits (unpublished). By analogy with the inessential HPRT, all classes of catalytic subunit mutants that result in sufficient loss of activity should be expressed and selectable, including frame-shift and deletion mutations. Both ICR 191 and x-rays induce a preponderance of type B mutants. Only ICR 191 has been found to produce unequivocal type C mutants among the clones we have tested. These relations, while intriguing, will remain speculative until the various mutants have been characterized structurally.

Tests of suspected mutagens by using S49 cells as described here may be useful and informative for the following reasons: (*i*) specificity of mutagenic action has been demonstrated for known mutagens. Substances with unknown mutagenic action could be screened to determine their relative potency for three or more genetic markers. The chance of failure to detect mutagenic action should thus be diminished. (*ii*) The system is sensitive and technically simple; high mutant frequencies can be achieved and large numbers of cells (1 to 2×10^6 per plate) can be subjected to selection. (*iii*) As suspension cells, S49 can be grown easily and quickly from single cells to quantities sufficient for biochemical analyses. This facilitates detailed examination of mutants.

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