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A GENETIC ANALYSIS OF CYCLIC AMP METABOLISM:

DISCOVERY OF NOVEL S49 LYMPHOMA MUTANTS

by

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THESIS

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A GENETIC ANALYSIS OF CYCLIC AMP METABOLISM:

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by Michael R. Salomon

PREFACE

What follows is an account of investigations into cyclic AMP generation and degradation in somatic cells. I have attempted to apply the resolving power of genetics to the study of drug and hormone action.

The thesis is actually the sum of three parts. Part One is a paper submitted to Molecular Pharmacology which describes the isolation and initial characterization of novel variant lymphoma cells resistant to the cytocidal effects of cholera toxin.

Part Two contains ideation and speculation suggested by the newly discovered variants. Hopefully, these ideas will provide a framework for further investigations of cyclic AMP metabolism and the role of mutations in human disease.

Part Three is a description of another research project--a search for variants with enhanced response to adenylate cyclase effectors. The selection system used, its rationale, and the preliminary characterization of a new phenotype are discussed.

PART ONE: The discovery of novel S49 lymphoma variants with aberrant cyclic AMP metabolism

It is remarkable that a science which began with the consideration of games of chance should have become the most important object of human knowledge...The most important questions of life are, for the most part, really only problems of probability.

-- Laplace, Théorie Analytique des Probabilités

SUMMARY

S49 mouse lymphoma cells resistant to killing by cholera toxin (but sensitive to adenosine 3',5'-monophosphate) can be selected in a single step. The transition from cholera toxin sensitivity to resistance is stochastic and occurs at a rate of 1.1 x 10^{-5} per cell per generation. Chemical mutagens increase this rate. Screening of independently selected cholera toxin-resistant clones led to discovery of three novel variant phenotypes. Lesions in two of these phenotypes affect the guanine nucleotide regulatory component, called N, of adenylate cyclase (E.C. 4.6.1.1). We assessed the N protein in the variants by measuring the ability of membrane extracts to complement N-deficient S49 cyc^- membranes _in vitro_, and by radiolabelling peptide subunits of N in the presence of cholera toxin and (^{32}P)-NAD^+. Membranes of one variant phenotype, termed N^{par}, contain about 20 percent of the N activity seen in wild type (parental) S49 membranes and show reduced radiolabelling catalyzed by cholera toxin. N^{par} membrane extracts partially inhibit wild type extracts in complementing adenyate cyclase of cyc^- membranes. A second phenotype exhibits cholera toxin-specific radiolabelling of N subunits comparable to that seen in wild type, but its N protein exhibits very little activity in complementing the defect of cyc^-. Resistance to cholera toxin in the third phenotype is associated with normal N and adenylate cyclase activities, but extracts of these cells degrade adenosine 3',5'-monophosphate at a rate four times faster than wild type.

INTRODUCTION

Study of variant S49 mouse lymphoma cells has already increased our understanding of hormone-sensitive adenylate cyclase. Because S49 cells are killed by an elevation of intracellular adenosine 3',5'-monophosphate (cAMP),[1] it is possible to select variant clones resistant to cholera toxin or hormonal agonists that stimulate cAMP synthesis. The S49 cyc^- variant was isolated by virtue of its resistance to the cytocidal effect of a β-adrenergic agonist (1). The cyc^- phenotype led to discovery (2) and biochemical characterization (3-6) of a membrane protein, termed G/F (3) or N (5), that is required for stimulation of cAMP sythesis by hormones, guanine nucleotides, fluoride ion and cholera toxin. These agents do not stimulate adenylate cyclase in cyc^- membranes, which are deficient in N but contain hormone receptors and catalytic adenylate cyclase (2-4). Cyc^- membranes incubated with cholera toxin and (^{32}P)-NAD^+ do not show radiolabelling of the 42,000 and 52,000 dalton peptide subunits of N seen in membranes of wild type (parental) S49 cells (5). Adenylate cyclase in a second S49 variant, called UNC, can be stimulated by guanine nucleotides, fluoride ion and cholera toxin, but not by β-adrenergic amines or prostaglandins (7). The N protein of UNC appears functionally "uncoupled" from hormone recep-

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1 The abbreviations used are: cAMP, adenosine 3',5'-monophosphate; N or G/F, guanine nucleotide regulatory protein of adenylate cyclase; C, catalytic unit of adenylate cyclase; cyc^-, N-deficient S49 variiant; UNC, hormone-unresponsive, receptor "uncoupled" S49 variant; dbcAMP, N^6,$O^{2'}$-dibutyryl cAMP; MNNG, N-methyl-N'-nitro-N-nitrosoguanidine; SDS, sodium dodecyl sulfate; GTPγS, guanosine-5'-0-(3-thiotriphosphate); Hepes, N-2-hydroxyethyl piperazine-N'-2-ethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediamine tetraacetic acid; CT^r, cholera toxin resistant; PHP, pseudohypoparathyroidism.

tors, and the N peptides ADP-ribosylated by cholera toxin in UNC membranes differ in charge from the wild type peptides (8).

In a search for novel genetic lesions in adenylate cyclase, we investigated clones independently selected for resistance to the cytocidal action of cholera toxin. We found three novel variant phenotypes. Two of these will provide useful tools for elucidating the structure of N and its interactions with other components of hormone-sensitive adenylate cyclase. Resistance to cholera toxin in the third variant phenotype appears to result from increased capacity to degrade cAMP. This variant may provide new insight into regulation of cyclic nucleotide phosphodiesterase(s) (E.C. 3.1.4.17). In the present communication we report initial characterization of the new variants.

METHODS

Chemicals. All chemicals were obtained from commercial sources as described (6), except for ICR 191 and RO 20-1724, which were gifts from Drs. H. Creech and H. Sheppard, respectively. α-(^{32}P)-ATP and (^{32}P)-NAD^{+} were purchased from New England Nuclear.

Cells. Clonal sublines of mouse lymphoma line S49.1 were propagated as described (9) in Dulbecco's Modified Eagle's Medium with 3 grams/liter glucose and supplemented with 10% heat-inactivated horse serum. We used an established wild type subline (designated 24.3.2) and a previously characterized bromodioxyuridine-resistant cyc^{-} cell line, M3B1, which does not complement the original cyc^{-} line in cell hybrids (10).

Fluctuation analysis. Wild type cells were plated (200 cells/dish) on soft agar with a fibroblast feeder layer (9). After 10 days, colonies were picked randomly with an Eppendorf pipette and propagated for 10 more days to a population size of 5×10^6 cells. Each of the wild type subclones was treated for six hours with cholera toxin (1 μg/ml) plus RO 20-1724 (0.1 mM), a phosphodiesterase inhibitor, and then plated in soft agar containing cholera toxin plus RO 20-1724. Separate aliquots of each clone were plated in agar containing 0.5 mM $N^6,O^{2'}$-dibutyryl cAMP (dbcAMP). After 12 days, colonies were counted by hand under a microscope. Colonies were picked randomly and propagated to mass culture for characterization.

A reconstruction experiment (data not shown) was performed to test the hypothesis that the selection system kills all wild type cells but allows all cholera toxin-resistant cells to survive. Toxin-resistant (cyc^-) cells were mixed with wild type cells in known proportions and the mixtures subjected to cholera toxin selection. The number of cholera toxin-resistant colonies observed (corrected for cloning efficiency) agreed closely with the expected number.

Chemical mutagenesis. A wild type subclone (divided into three populations) was treated with MNNG (2 μg/ml) for four hours, ICR 191 (0.75 μg/ml) for 24 hours, or no mutagen. The cloning efficiency of mutagen-treated populations plated immediately after treatment was about 20%. After drug treatment, cells were suspended in conditioned medium and propagated for one week for expression of mutations. Then cholera toxin-resistant colonies were selected as described above. The number of toxin-resistant colonies was corrected for cloning efficiency and

for dbcAMP-resistant colonies, as described in the legend to Table 1.

Screening variants. Particulate extracts were prepared from 200 ml cultures ($0.8 - 1.2 \times 10^6$ cells/ml). The cells were washed once with isotonic buffer (5 mM Hepes, 150 mM NaCl, pH 7.5), resuspended at 4×10^7 cells/ml in membrane buffer (20 mM Hepes, 2 mM $MgCl_2$, 1 mM EDTA, pH 8.0) and placed on ice for 15 minutes. All further steps were performed at 4° C. The cells were sonicated (twice for five seconds) at a setting of 60 watts with a Biosonik IV sonicator. Lysates were centrifuged at 200 X *g* for 20 minutes. The resulting pellet was resuspended using a Dounce homogenizer into 0.75 ml membrane buffer containing 10% glycerol.

Cell particulates were treated with no addition, isoproterenol (0.1 mM) plus GTP (0.1 mM), NaF (5 mM), or $MnCl_2$ (10 mM) and adenylate cyclase was measured at 30° C using a modification (6) of the procedure of Salomon *et al*. (11). The rate of cAMP synthesis was constant during the 40 minute incubation. This procedure provided a semi-quantitative assay of the effector-stimulated adenylate cyclase activity in individual clones.

To test for growth inhibition caused by dbcAMP, variant cell populations were exposed for 14 hours to 0.5 mM dbcAMP and their size distributions determined using a Coulter counter, as described (12). Cells resistant to dbcAMP fail to show the characteristic decrease in size induced by dbcAMP in wild type S49 populations (12).

Intracellular cAMP. Cellular cAMP was measured by the competitive binding assay of Gilman (13) as described previously (1). Each sample

contained lysate from 4 x 10^5 cells. Samples that contained less than 0.2 pmol cAMP (the limit of sensitivity) are designated as "< 5" pmol per 10^7 cells in Table 2.

Adenylate cyclase. Partially purified S49 membranes, prepared as described previously (14), were incubated in the presence of various effectors under conditions (30° C, pH 8.0, 0.3 mM ATP with an ATP regenerating system) exactly as described previously (15), and cAMP was purified as described (11). Under these conditions, the rate of cAMP synthesis was constant during the time of incubation (20 minutes).

Phosphodiesterase. Whole cell lysates were prepared and their phosphodiesterase activity was measured as described previously (16), using the two-step batch assay of Thompson *et al.* (17), in which (^{3}H)-cAMP is converted to (^{3}H)-5'-AMP by cellular phosphodiesterase and then to (^{3}H)-adenosine by exogenous 5'-nucleotidase (supplied by *Crotalus atrox* venom). The concentration of substrate (cAMP) was 0.2 μM.

Complementation of cyc$^-$. *In vitro* complementation of recipient cyc$^-$ membranes was accomplished by adding Lubrol 12A9 extracts (100,000 X *g* supernatant fraction) from donor membranes of wild type and variant clones, using a modification (6,15) of the method of Ross and Gilman (2).

Complementation experiments using mixtures of extracts of two donor membranes (e.g., wild type and A3a, in Figure 1) were performed as follows: Donor membranes (2.5 mg protein per ml in membrane buffer) were solubilized by adding Lubrol 12A9 to a final concentration of 0.5%. After vigorous mixing and incubation on ice for 30 minutes, tubes were centrifuged (100,000 X *g* for 45 minutes). To destroy catalytic adenylate

cyclase activity, the supernatant fractions were incubated at 30° C for 10 minutes and then kept at 4° C for 4 hours. The indicated volumes (0-10 μl) of wild type and/or variant donor extracts were added to test tubes on ice, and mixed with heat-inactivated (10 minutes at 50° C) Lubrol-supernatant extracts of cyc^- membranes. The volume of cyc^- extract was adjusted (between 0 and 10 μl) so that the total amount of detergent and membrane protein was the same (equivalent to 10 μl) in all tubes. To each of the tubes containing donor extract was added 40 μl of a solution containing 15 μg cyc^- membrane protein, 0.375 mM α-(^{32}P)-ATP (0.3 μCi per tube), isoproterenol (0.1 mM), GTPγS (0.1 mM), and buffer, Mg^{2+}, and an ATP-regenerating system, as described (6,15). The mixtures were incubated for 40 minutes and cAMP purified (11).

Radiolabelling and gel electrophoresis. Membranes (1 mg/ml) were incubated for 30 minutes with 50 μg/ml cholera toxin (activated by treatment with dithiothreitol) and 100 μM (^{32}P)-NAD^+ (1 Ci/mmol) and other reagents exactly as described (6,15). Membranes were dissolved by adding SDS and β-mercaptoethanol to final concentrations of 2% and 5% (w/v), respectively, and subjected to discontinuous electrophoresis (18) in 10% polyacrylamide gels, as described (6). Staining, destaining and autoradiography were perfomed as described (6).

RESULTS

Does resistance to cholera toxin result from mutation?

Fluctuation analysis. In order to test the hypothesis that resistance to cholera toxin arises by a spontaneous, random event (e.g., a

mutation), we performed a fluctuation analysis (19) of the incidence of toxin-resistant cells in populations propagated from 16 independent wild type S49 clones (Table 1). The mean number of toxin-resistant (and dbcAMP-sensitive) colonies selected from these separate cell populations was much less than the variance in colony number, a result that is consistent with mutation as a cause of toxin resistance. The mutation rate, calculated by the median method (20), is 1.1×10^{-5} per cell per generation. To show that sampling error did not account for the extremely high variance, we selected toxin-resistant colonies from a single wild type population in 12 replicate plates; the ratio of variance to mean colony number was 0.65.

Effects of mutagens. If a phenotype results from mutation, its frequency should be increased by treating the parental population with chemical mutagens. Wild type cells were treated with MNNG (a putative base substitution mutagen [21]), ICR 191 (a putative frameshift mutagen [22]), or no mutagen, as described in METHODS. MNNG increased the proportion of toxin-resistant colonies from 52 to 342 per 10^5 cells, and ICR 191 increased the proportion from 52 to 230 per 10^5 cells. These results are consistent with the hypothesis that toxin resistance arises by mutation.

Screening variant clones

Cholera toxin-resistant colonies were picked randomly from agar plates corresponding to the independent wild type subclones used in the fluctuation analysis. This should enhance the probability of finding different variant phenotypes, because it guarantees that the variants

Table 1

FLUCTUATION ANALYSIS

A wild type cell line was cloned in soft agar. Sixteen colonies were picked and each was grown to about 5 x 10^6 cells. Two hundred cells were plated without selective agents in duplicate to determine cloning efficiency. The rest of the cells were treated with cholera toxin (1 µg/ml) plus RO 20-1724 (0.1 mM) for 6 hours. A known portion from each clone was plated with cholera toxin plus RO 20-1724 in triplicate (1 x 10^6 cells/dish). Another portion was plated with dbcAMP (0.5 mM) plus RO 20-1724 (0.1 mM) in duplicate (1 x 10^6 cells/dish). The mean number of dbcAMP-resistant colonies per one million cells was subtracted from the mean number of cholera toxin-resistant colonies per one million cells to give the number of cholera toxin-resistant, dbcAMP-sensitive colonies per one million cells. This number was corrected for cloning efficiency, which ranged from 56% to 100% for the sixteen independent clones. Mutation rate was calculated using the median method of Lea and Coulson (20).

Clone	CT^r colonies/ 10^6 cells	$\overline{X}$	$dbcAMP^r$ colonies/ 10^6 cells	$\overline{X}$	$\overline{X}$ CT^r, $dbcAMP^s$ colonies/10^6 cells
1	422,437,403	421	0,0	0	561
2	33, 44, 35	37	0,1	1	43
3	27, 35 22	28	0,0	0	36
4	293,275,315	294	140,160	150	164
5	239,216,200	218	0,0	0	254
6	102,127,109	113	0,0	0	161
7	72, 71	72	0,0	0	83
8	16, 13, 13	14	0,0	0	17
9	142,127,136	135	0,0	0	182
10	39, 33, 29	34	1,1	1	41
11	22, 25, 26	24	5,5	5	19
12	46, 56, 58	53	0,0	0	54
13	39, 44, 45	43	0,0	0	73
14	3, 5, 8	5	0,0	0	9
15	20, 13, 15	16	0,0	0	19
16	32, 47, 47	42	0,0	0	60

($\overline{X}$) mean number of CT^r, $dbcAMP^s$ colonies/10^6 cells = 111
(s) standard deviation = 140
(s^2) variance = 19,600
$s^2/\overline{X}$ = 177
degrees of freedom = 15
χ^2 = 2,813
$P < .005$

mutation rate by the median method = 1.1 x 10^{-5}/cell/generation

arose during at least 16 different mutational events. In contrast, variants isolated from a single clonal population may often be derived from a single "jackpot" mutation that occurred early in the life of the clone (19).

A total of 28 colonies from the 16 subclones were propagated to mass culture and adenylate cyclase in particulate extracts of each of these was assayed in the presence of GTP, GTP plus isoproterenol, NaF, or $MnCl_2$, as described in METHODS. Mn^{2+} ion caused an approximate doubling of adenylate cyclase activity in all 28 particulate extracts, as it did in similar extracts of wild type and cyc^- cells. Because Mn^{2+} appears to stimulate activity of the enzyme's catalytic (C) unit directly (3,10), this result probably indicates that C activity was unaffected in all clones tested.

Of all the extracts, 23 resembled cyc^- in showing no Mg^{2+}-dependent adenylate cyclase activity in the presence of GTP, isoproterenol plus GTP, or NaF. These will not be described in this report. Five clones clearly differed from cyc^-: Extracts of four clones exhibited substantial increases in adenylate cyclase activity in the presence of both NaF and isoproterenol plus GTP, while extracts of one clone (H21a) showed an approximate doubling of cAMP synthesis in response to isoproterenol plus GTP, but no response to NaF.

One of the five aberrant clones exhibited resistance to growth inhibition induced by dbcAMP (see METHODS). Because this was probably caused by a defect in cAMP-dependent protein kinase (E.C. 2.7.1.37) (23), this clone was not further analyzed. The other four clones exhibited a wild type response to dbcAMP, indicating that their cAMP-dependent

protein kinase is normal.

cAMP accumulation and synthesis

We first compared cells of the remaining four clones to wild type and cyc$^-$, with respect to cAMP accumulation in the presence of terbutaline, a β-adrenergic agonist, or cholera toxin plus an inhibitor of cAMP phosphodiesterase (Table 2). Three of the clones showed no response to terbutaline, and one showed a barely detectable rise in cAMP. Cholera toxin, the agent used in selecting these clones, caused a detectable rise in cAMP of three clones, but only to levels 5-10% of those measured in toxin-treated wild type cells. Clone H21a showed no response to either treatment.

For three of the clones, decreased adenylate cyclase activity in partially purified membrane fractions (Table 3) was associated with decreased cAMP measurements in intact cells. Adenylate cyclase in H21a membranes was slightly stimulated by guanine nucleotides and by isoproterenol, a β-adrenergic agonist. Isoproterenol and guanine nucleotides stimulated adenylate cyclase in A3a and T54b membranes, but the activities were only 20-30% of those observed in wild type.

In contrast, K30a adenylate cyclase activities, unstimulated or in the presence of isoproterenol and guanine nucleotides, were comparable to those seen in wild type (Table 3). This raises the possibility that a genetic lesion separate from adenylate cyclase may account for the diminished accumulation of cAMP observed in intact toxin-treated K30a cells (Table 2) and for these cells' resistance to the cytocidal effect of cholera toxin.

Table 2

INTRACELLULAR CYCLIC AMP ACCUMULATION

Cell populations were treated with terbutaline (0.1 mM), cholera toxin (1 μg/ml) plus RO 20-1724 (0.1 mM), or no addition. Terbutaline treatment was for 12 minutes and cholera toxin treatment for 3.5 hours, since experiments in wild type cells found that cAMP peaked at these times for the respective drug treatment. The values in the table represent the mean ± one standard deviation of three determinations.

	pmol of cAMP/10^7 cells		
Cell line	No addition	Terbutaline	Cholera toxin + RO 20-1724
Wild type	< 5	594 ± 85	434 ± 40
A3a	< 5	14 ± 6	44 ± 2
T54b	< 5	< 5	23 ± 2
H21a	< 5	< 5	< 5
K30a	< 5	< 5	20 ± 5
cyc^-	< 5	< 5	< 5

Table 3

ADENYLATE CYCLASE ACTIVITY IN MEMBRANES

Adenylate cyclase activity was assayed in the presence of isoproterenol (0.1 mM) plus GTP (0.1 mM), isoproterenol (0.1 mM) plus GTPγS (0.1 mM), GTPγS (0.1 mM) alone, and $MnCl_2$ (10 mM). Table gives the mean value for adenylate cyclase activity derived from multiple determinations which were within 5% of the mean (except for mean values less than 15 picomoles, for which multiple determinations were within 2 picomoles of the mean). The amount of cAMP synthesis was directly proportional to the amount of protein added and to the time of incubation.

		pmol of cAMP per mg protein per min			
Source of membranes	No addition	Isoproterenol plus GTP	GTPγS	Isoproterenol plus GTPγS	$MnCl^2$
Wild type	13	288	279	405	21
A3a	4	51	71	71	11
T54b	5	57	90	124	14
H21a	2	13	12	15	9
K30a	12	280	155	297	20
cyc^-	3	3	4	4	9

$MnCl_2$ caused stimulation of adenylate cyclase in all the membranes tested, as in the screening assay.

In our laboratory, maximal adenylate cyclase activities in different membrane preparations can vary by as much as 30%. Accordingly, we measured adenylate cyclase in at least two sets of membranes prepared from each clone. In all cases the results (not shown) agreed with those in Table 3: i.e., H21a membranes showed slight cyclase stimulation by isoproterenol and guanine nucleotides, A3a and T54b membranes exhibited about 25% of wild type activity in response to all the effectors, and K30a adenylate cyclase activity was comparable to wild type.

In other experiments (not shown), we found that treatment with cholera toxin plus NAD^+ caused little stimulation of GTP-dependent cAMP synthesis in A3a and T54b membranes, in comparison with that observed (24) in wild type. The same treatment led to no detectable stimulation of adenylate cyclase in H21a membranes.

N activity and radiolabelled peptides

In vitro complementation. Ross and Gilman first showed (2,3) that detergent extracts of wild type S49 membranes, rendered devoid of intrinsic adenylate cyclase activity by heating at 37° C, can restore to N-deficient cyc^- membranes the capacity to make cAMP in response to isoproterenol, guanine nulceotides, and NaF. In order to determine whether N is the site of the lesion that leads to toxin resistance in the new clones, we added extracts from membranes of each clone to cyc^- membranes, and measured adenylate cyclase activity (Table 4). As a "blank" we used heat treated detergent extracts of cyc^- membranes, which do not complement cyc^- at all.

Table 4

RECONSTITUTION OF CYC$^-$ MEMBRANES WITH LUBROL EXTRACT

FROM WILD TYPE AND VARIANT MEMBRANES

Recipient cyc$^-$ membranes were reconstituted with the 100,000 X *g* supernatant fraction of detergent extracts of variant subclone membranes or of wild type membranes in the presence of GTP (0.1 mM) alone, isoproterenol (0.1 mM) plus GTPγS (0.1 mM), or NaF (5 mM). Each assay tube contained 30 μg cyc membrane and 10 μl Lubrol extract from donor membranes (3 mg protein/ml). These additions were incubated at 30° C for 20 minutes to allow functional coupling of membrane components, then α-(^{32}P)-ATP was added and the incubation was continued for an additional 20 minutes at 30° C. The rate of accumulation of cAMP was constant during the time of incubation. Wild type A and B membranes were made from the same subline (24.3.2) on two different days. Table gives mean value ± one standard deviation of three determinations.

	pmol of cAMP per tube		
Donor membranes	GTP	Isoproterenol + GTPγS	NaF
Wild type A	4 ± 1	169 ± 4	191 ± 6
Wild type B	5 ± 1	217 ± 5	250 ± 36
A3a	1 ± 1	31 ± 1	32 ± 2
T54b	2 ± 1	39 ± 1	52 ± 1
H21a	1 ± 1	4 ± 1	4 ± 1
K30a	4 ± 1	167 ± 6	191 ± 2
cyc$^-$	1 ± 1	2 ± 1	2 ± 1

The results of this in vitro complementation assay correlate with direct measurements of adenylate cyclase (Table 3) in membranes. Detergent extracts of H21a membranes are almost as ineffective as those of cyc^- in restoring responsiveness of cyc^- to NaF or to isoproterenol plus GTPγS (Table 4). A3a and T54b extracts are about 20% as effective as wild type extracts in restoring responsiveness to isoproterenol plus GTPγS and NaF. K30a extracts are as effective as wild type.

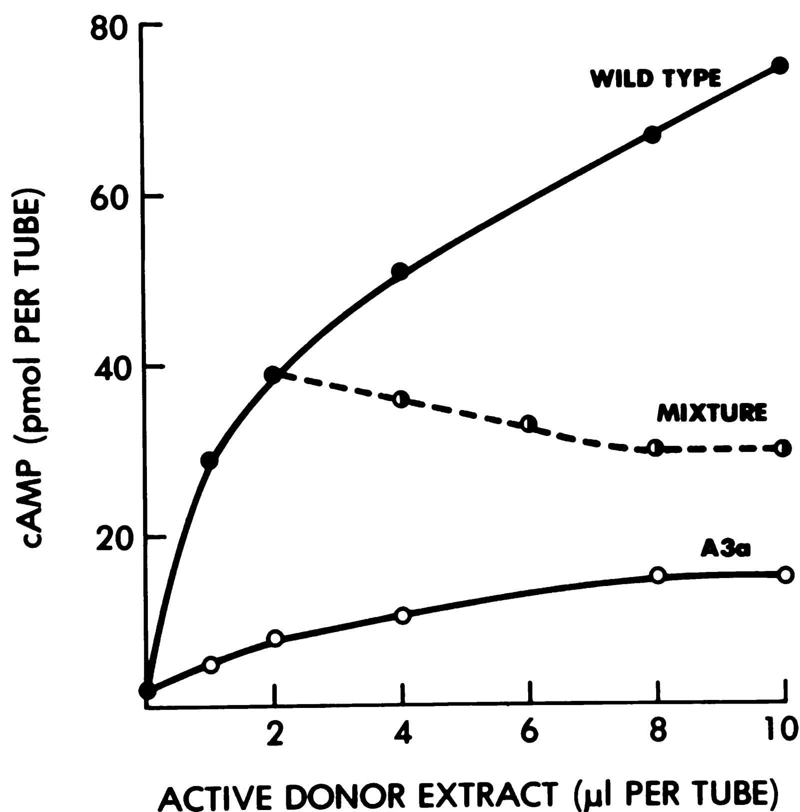
One possible lesion of A3a and T54b membranes is that they contain quantitatively diminished but functionally normal N. If so, increasing amounts of membrane extract from these variant membranes should be able to complement cyc^- as well as smaller amounts of wild type extract. The results of an experiment comparing different amounts of wild type and A3a extracts (Figure 1) suggest that this is not the case. Increasing amounts of wild type extract appeared not quite to saturate the capacity of a fixed amount of cyc^- membranes to make cAMP in response to isoproterenol plus GTPγS; in contrast, adenylate cyclase activity in mixtures of cyc^- and A3a extracts reached a plateau that was about 20% of the highest activity observed with wild type.

The plateau of activity with increasing amounts of A3a extract suggests that these extracts interact abnormally with cyc^-, in comparison with wild type. If this abnormal interaction were due to deficiency of a second component (distinct from N) in A3a extracts, mixtures of wild type and A3a extracts should produce at least additive effects on adenylate cyclase in the complementation assay. Instead, addition of increasing amounts of A3a extract to a constant amount of wild type appears to reduce the complementation of cyc^- produced by the wild

Figure 1

RECONSTITUTION OF CYC$^-$ RECIPIENT MEMBRANES WITH WILD TYPE AND/OR A3a MEMBRANE EXTRACTS

Recipient membranes (cyc$^-$) were reconstituted with Lubrol extracts (100,000 X *g* supernatant of wild type membranes (solid line, filled circles), A3a membranes (solid line, open circles), or both wild type membranes and A3a membranes mixed together (broken line, half filled circles). In the mixing experiment (broken line, half filled circles), 2, 4, 6 or 8 µl of A3a extract were added to 2 µl of wild type extract (see METHODS). Each assay tube contained 15 µg of cyc$^-$ protein (recipient) and isoproterenol (0.1 mM) plus GYPγS (0.1 mM). Activity is expressed as pmol cAMP produced per tube during 40 minutes of incubation at 30^0 C. Each point represents the mean of three determinations which were all within 5% of the mean. The mean cyclase activity produced by the addition of 4, 6, or 8 µl of A3a extract to 2 µl wild type extract was significantly different from the adenylate cyclase activity seen with 2 µl wild type extract alone ($p < 0.05$).



type extract (Figure 1, broken line). Thus the A3a extract behaves in the complementation assay as if its N were a partial agonist with diminished intrinsic complementing activity, but able to inhibit the complementing activity of wild type. An alternative possibility is that A3a extracts contain an inhibitory activity, potentially separable from N itself.

A result similar to that in Figure 1 was obtained using T54b extracts (not shown). Thus the A3a and T54b variants, which behave similarly also in assays of cAMP accumulation and adenylate cyclase in membranes, probably bear a similar lesion. We will term these variants "N^{par}" in order to indicate their partial defect in N and adenylate cyclase activities, and to suggest that their N proteins may be partial agonists in complementing cyc^-.

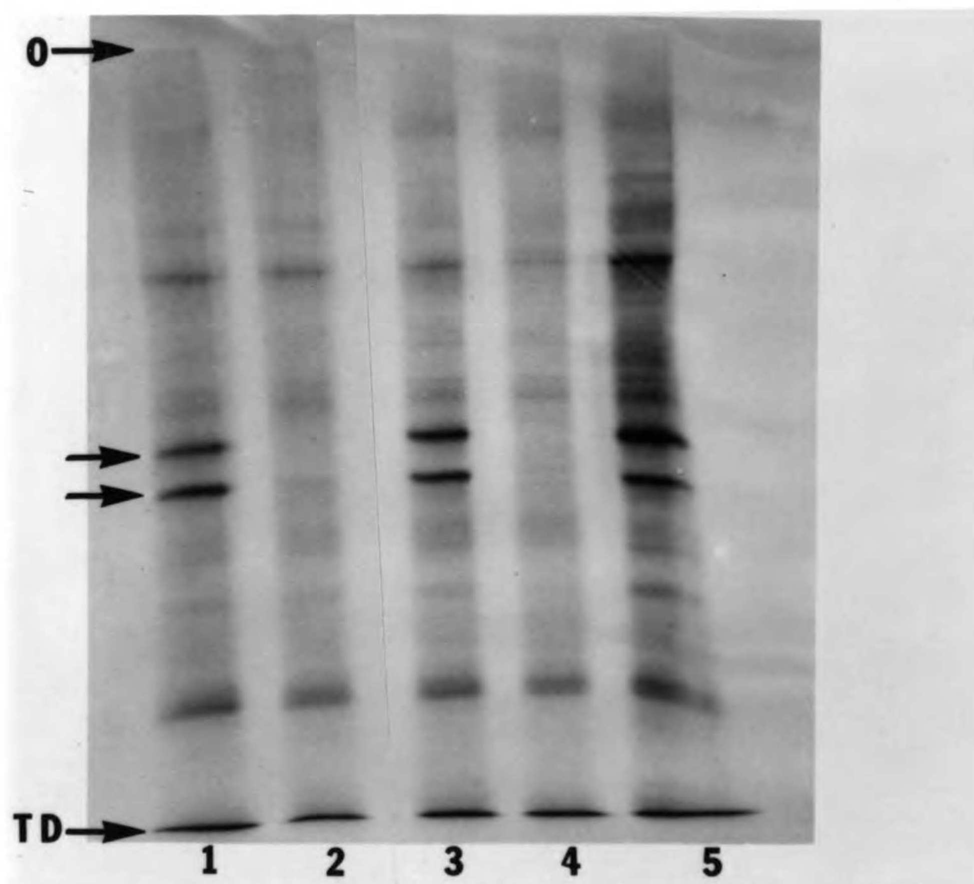
Radiolabelled N peptides. Incubation of wild type membranes with cholera toxin and (^{32}P)-NAD$^+$ leads to specific radiolabelling of two peptides, of 42,000 and 52,000 daltons; neither peptide is radiolabelled in cyc^- (5). The smaller of these two peptides is probably a subunit of the N protein, because a toxin-radiolabelled peptide of the same molecular weight comigrates in sucrose gradients and gel exclusion chromatography with N activity of another cell type, the human erythrocyte (6).

We treated membranes of the newly-isolated S49 variants with toxin and (^{32}P)-NAD$^+$ and subjected them to SDS polyacrylamide gel electrophoresis and autoradiography (Figure 2). K30a membranes exhibited radiolabelling, of both the 42,000 and 52,000 dalton peptides, comparable in intensity to wild type. The N^{par} membranes exhibit slight radiolabelling of the 42,000 dalton peptide. The 52,000 dalton band was not

Figure 2

AUTORADIOGRAM OF AN SDS SLAB GEL ELECTROPHORESIS OF LUBROL EXTRACTS OF MEMBRANES INCUBATED WITH (^{32}P)-NAD^{+} AND CHOLERA TOXIN

1 = K30a; 2 = T54b; 3 = H21a; 4 = cyc^{-}; 5 = wild type. 0 = origin, TD = position of tracking dye, top arrow = M_r 52,000, and bottom arrow = M_r 42,000. All of the membranes were treated with cholera toxin and radiolabelled as described in METHODS. In another experiment, the membranes were incubated with (^{32}P)-NAD^{+} without cholera toxin, and there was no radiolabelling of the 42,000 and 52,000 dalton peptides (not shown).



labelled in the N^{par} membranes. These results are in harmony with results in Tables 3 and 4 and Figure 1: K30a membranes have adenylate cyclase and N activities similar to wild type, and therefore should show toxin-dependent radiolabelling similar to wild type as well; the N^{par} variants exhibit diminished adenylate cyclase and N activities, and thus it is not surprising that they should show diminished radiolabelling of peptide subunits of N.

This experiment produced one surprising result: H21a membranes, which possess very little stimulable adenylate cyclase activity and no N activity capable of complementing cyc^-, exhibit toxin-dependent radiolabelling of the 42,000 and 52,000 dalton peptides similar in apparent intensity to that seen in wild type (Figure 2). The same result was seen with two other sets of membranes prepared from H21a (not shown). We have not attempted direct quantitation of the radioactivity associated with these bands. Visual inspection of autoradiograms from multiple experiments, comparing them to bands that are not toxin-dependent (5) and to Coomassie blue staining of the corresponding gels, fails to reveal any difference between the patterns of H21a and wild type. This result contrasts with the failure of cholera toxin plus NAD^+ to stimulate H21a adenylate cyclase.

Phosphodiesterase activity

With respect to wild type, K30a membranes appear completely normal in adenylate cyclase activity, ability of N in membrane extracts to complement cyc^- *in vitro*, and toxin-specific radiolabelling (Tables 2 and 4 and Figure 2). How then can we account for K30a's markedly diminished capacity to accumulate cAMP in intact cells (Table 2) and its ability

to survive the cholera toxin selection? One possibility is that K30a is a cAMP transport mutant, able to export cAMP into the extracellular medium at an increased rate. One such S49 variant, already described (25), differs from K30a in being resistant to the cytocidal effect of dbcAMP.

Another possibility is that phosphodiesterase in K30a degrades cAMP more rapidly within the cell, resulting in resistance to agents (e.g., cholera toxin) that act by stimulationg cAMP synthesis. This possibility would be consistent with normal sensitivity of K30a cells to dbcAMP, since dbcAMP and its monobutyryl derivative are not significantly degraded by phosphodiesterase.

We tested the second possibility by comparing cAMP phosphodiesterase activity in lysates of K30a and wild type S49 cells (Table 5). Lysates of logarithmically growing K30a cells contain four times as much phosphodiesterase activity as wild type cell lysates. Treatment of wild type cells for four hours with dbcAMP induces a three-fold increase in phosphodiesterase activity, as previously described (23,26). The dbcAMP-induced increase in K30a phosphodiesterase activity is less than that seen in wild type, both absolutely and in proportion to the basal activity in untreated cells (Table 5).

Because these phosphodiesterase activities were measured at 0.2 μM substrate concentration they probably reflect predominantly "low K_m" phosphodiesterase(s) previously assayed in S49 cells (26). We have not yet explored the many possible mechanisms by which phosphodiesterase activity could be increased in K30a.

Table 5

PHOSPHODIESTERASE ACTIVITY

The table gives the mean value of duplicate determinations, which were within 5% of the mean. Treated cells were incubated with 1 mM dbcAMP for four hours.

	pmol of cAMP/30 min/10^6 cells	
Cell type	No treatment	dbcAMP
Wild type	23	70
K30a	97	122

DISCUSSION

S49 cells that are resistant to cholera toxin (but sensitive to dibutyryl cyclic AMP) can be selected in a single step. On the basis of the fluctuation analysis and the ability of chemical mutagens to increase the frequency of toxin resistance, it is very likely that S49 variants with altered adenylate cyclase arise by mutation. The calculated mutation rate for cholera toxin resistance is high relative to rates determined for many markers studied in somatic cell lines. This may indicate a highly mutable locus, an X-linked locus, or that S49 cells are functionally haploid for a critical gene product (e.g., a peptide subunit of N). Interestingly, mutations that confer glucocorticoid resistance in S49 cells (27) occur at a rate almost as high as that calculated for cholera toxin resistance (3.5 vs. 11 x 10^{-6}/cell/generation), and there is evidence that S49 cells are functionally haploid for the glucocorticoid receptor (28).

While most of the variant clones isolated in the present study appeared similar to cyc$^-$, we have discovered three novel phenotypes. The phenotypes are sufficiently well-defined to allow formulation of working hypotheses regarding their underlying molecular lesions:

Clone H21a. Isoproterenol and guanine nucleotides cause only slight stimulation of adenylate cyclase in H21a membranes, and detergent extracts of these membranes are unable to complement the cyc$^-$ lesion; yet cholera toxin catalyzes ADP-ribosylation of the 42,000 and 52,000 dalton peptides of H21a membranes to an extent similar to that seen in wild type. Because at least the smaller of these peptides is a subunit of

the N protein (6), this discrepancy must be explained. One possibility is that H21a membranes contain normal N proteins but lack an additional component required to complement cyc^-. This appears unlikely, because N activity migrates in a monodisperse fashion in several physicochemical separation procedures (4,6), and because of the recently reported purification from liver membranes of a homogeneous protein that can complement all the defects of cyc^- (29).

To us it appears likely that the H21a lesion will prove to be parallel to but different from that of the UNC phenotype. In the latter variant, a structural change in N (detected as a change in electrical charge of cholera toxin substrates) is associated with diminished ability of N to interact with hormone receptors (7,8). A structural lesion in the N of H21a membranes may cause it to be functionally "uncoupled" from the catalytic unit of adenylate cyclase, rather than from receptors. It will thus be interesting to determine whether N in H21a membranes can mediate guanine nucleotide regulation of receptors for β-adrenergic agonists, as it does in wild type (but not in UNC). As is the case with UNC, the putative N lesion in H21a could be due to a structural change in the gene for one of the N subunits, or to alteration of an additional cellular activity required for optimal N activity.

N^{par} variants. The simplest explanation of the A3a and T54b lesions is that they possess altered N proteins which are poor substrates for ADP-ribosylation by cholera toxin and which are incapable of maximally activating the cyclase's catalytic (C) unit. The observation that detergent extracts from N^{par} membranes impair activation of cyc^- by wild type N (Figure 1) suggests that N in the N^{par} variants can interact with a

saturable component of cyc^-, perhaps C itself, in a fashion that fails to stimulate cAMP synthesis maximally but prevents normal N from doing so. An alternative possibility is that N^{par} membranes contain an inhibitory activity which is physically distinct from N. Although not yet ruled out, this alternative explanation appears less likely because it does not account for the reduced ADP-ribosylation of N peptides in N^{par} membranes by cholera toxin.

K30a variant. Resistance to the cytocidal and cAMP-elevating actions of cholera toxin in K30a cells is apparently the result of increased cAMP degradation (Table 5). Potential molecular explanations for the increased phosphodiesterase activity in K30a include increased expression of one or more phosphodiesterase enzymes already present in the wild type parental cells, an increase in an activator or cofactor of phosphodiesterase, or a decrease in a phosphodiesterase inhibitor. Further characterization of the K30a variant may discriminate among these possibilities, and should increase our understanding of the regulation of phosphodiesterase activity in somatic cells.

Possible relevance to a human disease. Patients with pseudohypoparathyroidism (PHP), a heritable endocrine disease, exhibit defective responses to several hormones (e.g., parathormone and thyrotropin) that work by stimulating cAMP synthesis. This laboratory has recently reported (30) that N activity is substantially reduced in erythrocytes of most PHP patients, but not in all. Generalized genetic defects in N, other components of adenylate cyclase, or even in phosphodiesterase, could produce the PHP phenotype by causing decreased cAMP accumulation

in endocrine target cells. The novel S49 phenotypes reported here, added to the cyc$^-$ and UNC lesions previously defined, provide a challenging view of the many mechanisms by which mutations that affect cAMP metabolism could produce disease in man.

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PART TWO: What do the novel variants tell us about cyclic AMP metabolism in somatic cells and in human disease?

Limiting components in adenylate cyclase

The adenylate cyclase system of animal membranes is made up of at least three components: hormone receptor (R), catalytic adenylate cyclase (C), and the guanyl nucleotide regulatory protein (N). Wild type membranes have all three components, whereas cyc^- membranes are functionally deficient in N. In the *in vitro* complementation procedure (described in METHODS of Part One), N extract from donor membranes is added to recipient membranes. In a series of experiments not included in the paper, wild type membranes were used as recipients. The recipient membranes, therefore, had all three cyclase components present in their usual amount. The addition of donor extract from variant or wild type membranes allowed me to observe the effect of adding extra N.

Addition of donor extract from A3a (N^{par}) membranes to wild type recipient membranes had a predictable effect: adenylate cyclase activity in the reconstituted system was about half of that seen when an equivalent amount of cyc^- donor extract was added to wild type membranes. This result is consistent with the mixing experiment (Figure 2) reported in the paper and suggests partial agonism or an inhibitory component.

Addition of wild type donor extract to wild type membranes did not result in increased adenylate cyclase activity (relative to addition of cyc^- extract). Apparently, extra N does not result in additional fluoride or guanyl nucleotide induced cyclic AMP synthesis. The failure of extra N to increase the cyclic AMP generating capacity of wild type membranes suggests that the number of catalytic units in the membrane is limiting.

Accumulation of second messenger in intact cells

The N^{par} variants have less than 5% as much cAMP accumulation in response to effectors as wild type cells (Table 2), even though they have 20-30% as much adenylate cyclase activity (Table 3) and N activity (Table 4). Thus, a decrease in adenylate cyclase activity or in N activity does not lead to a proportional decrease in cAMP accumulation. Conversely, a four-fold elevation of basal phosphodiesterase activity in K30a, relative to wild type (Table 5), is associated with a 20-fold reduction in toxin-stimulated cAMP accumulation in intact K30a cells.

The disparity between changes in enzyme activity and changes in intracellular cAMP accumulation can be explained in two ways. It is possible that assays of enzyme activity in broken cell preparations are only approximately related to enzyme activities in the whole cell. According to this explanation, broken cell enzyme assays give a rough idea of enzyme activity in the cell but cannot be used for quantitative comparisons of whole cell enzyme activities.

A second explanation is that the mechanisms for reducing the amount of cAMP in the cell (phosphodiesterase and the export of cAMP out of the cell) are saturated by very large increases in cAMP, but are not saturated by the smaller increases in cAMP obtained in cells with partial adenylate cyclase activity. Similarly, increasing the amount of phosphodiesterase activity three- to four-fold can make it impossible for normal effector-stimulated adenylate cyclase to produce enough cAMP to saturate the cyclic AMP depleting mechanisms.

According to this analysis, phosphodiesterase activity in the cell constitutes a barrier (or threshold) which must be surmounted in order

to significantly elevate the intracellular concentration of cyclic AMP. Large increases in adenylate cyclase activity (such as those produced by drugs and hormones) generate sufficient second messenger to saturate phosphodiesterase in the cell. A spike effect is observed, with intracellular cyclic AMP concentrations reaching a maximum a few minutes after the addition of drug and then falling rapidly back to the basal level, mainly because of tachyphylaxis and phosphodiesterase activity.

The notion of a "phosphodiesterase barrier" may explain the apparent discrepancy between adenylate cyclase activity and cyclic AMP accumulation in individuals with pseudohypoparathyroidism (PHP). Patients with PHP fail to show a significant response to parathyroid hormone: upon administration of parathyroid hormone, PHP patients excrete less than 5% as much cyclic AMP in their urine as normals. Responsiveness to other hormones which act by increasing cyclic AMP is also impaired in PHP, although this is quite variable. Recently, Farfel _et al_. have shown that erythrocytes (30) and platelets (personal communication) from most PHP patients have only half as much N activity as blood cells from normal individuals. The question remains: How does a 50% decrease in N activity lead to a 95% decrease in accumulation of second messenger? One explanation is that the impaired cyclic AMP synthesis in these patients is not sufficient to overcome the putative phosphodiesterase barrier.

The observation that different hormone responses mediated by cyclic AMP are reduced to an unequal extent in PHP may reflect variations in the balance between cyclic AMP synthesis and degradation in hormone responsive tissues.

Finally, a subpopulation of PHP patients have apparently normal N activity, but still lack hormone responsiveness. I recently discovered a variant cell line, K30a, with a stable increase in phosphodiesterase activity resulting in impaired cyclic AMP accumulation. An increase in phosphodiesterase activity can have the same effect on cyclic AMP concentration as a decrease in adenylate cyclase activity. The possibility exists that some PHP patients have normal cyclic AMP synthesis, but degrade cyclic AMP at an abnormally high rate.

PART THREE: A search for variants with enhanced response to adenylate cyclase effectors

Generally, a selection protocol is used to isolate drug resistant clones. I am attempting, however, to uncover variants with enhanced sensitivity to β-adrenergic agonists, in order to learn more about the termination of drug action. The phenomenon of tachyphylaxis or refractoriness to β-agonists is observed in many somatic cell lines as well as clinically in the treatment of asthma. Treatment of an S49 cell population with a β-agonist leads to a hundred-fold increase in intracellular cAMP within 15 minutes. The concentration of cAMP drops rapidly, however, back to basal levels. The precipitous fall in cyclic AMP is due to tachyphylaxis: β-receptors become inoperative by a process which is dependent on the presence of N, but not cAMP (31).

S49 cells are growth arrested by elevating cAMP. β-agonists plus a phosphodiesterase inhibitor produce only temporary growth arrest (about 18 hours) in wild type cells because the elevation in intracellular cAMP is transient. Thus, after 18 hours of exposure to a β-agonist, wild type S49 cells are cycling normally and can be killed by treatment with S phase-specific cytotoxic agents for 24 hours. Cells which remain growth arrested for 42 hours are able to survive the round of selection.

I subjected D^- S49 cells (32) to five rounds of selection using terbutaline plus RO 20-1724 to arrest the cells and bromodeoxyuridine or cytosine arabinoside to kill cycling cells. Survivors were plated in HAT (hypoxanthine, aminopterin, thymidine) medium to kill cells which were deficient in thymidine kinase. I was able to isolate a large number of variant clones. The variants, which so far appear alike, are growth arrested by terbutaline plus RO 20-1724 for several days (and eventually die). Terbutaline alone does not irreversibly arrest growth. G_1 arrest

induced by dibutyryl cyclic AMP can be reversed by removal of dibutyryl cyclic AMP from the medium, indicating that the variants can escape from G_1 arrest. Membranes from the variants show reduced isoproterenol stimulation of adenylate cyclase if the cells were treated with a β-agonist prior to making membranes. This experiment indicates that the variants can down-regulate their β-receptors and adenylate cyclase.

The variants seem to be more sensitive to dibutyryl cyclic AMP than parental D^- cells as measured by growth arrest induced by low concentrations of dibutyryl cyclic AMP. Intracellular cyclic AMP concentrations in the variants after treatment with terbutaline (plus or minus a phosphodiesterase inhibitor) are higher than in parental cells. Most importantly, the variants exhibit some cyclic AMP elevation for more than 24 hours after treatment with terbutaline plus RO 20-1724.

The variants probably have reduced phosphodiesterase activity, increased adenylate cyclase activity, or decreased cAMP export. Reduced phosphodiesterase activity could be due to failure to induce phosphodiesterase activity, generally decreased phosphodiesterase activity, or abnormal sensitivity to the phosphodiesterase inhibitor used (RO 20-1724). Increased adenylate cyclase activity could be due to a change in the enzyme, the presence of a cofactor, the absence of an inhibitor, or increased genetic expression of the enzyme. A finding of increased genetic expression raises the fascinating possibility that adenylate cyclase expression could be functionally haploid in most S49 cells (see DISCUSSION of Part One) but diploid in the enhanced response variants. One would then predict that the frequency of cholera toxin resistance in the enhanced responders would be about the square of the mutation

rate for functionally haploid cells. Investigations are now underway to find out which of the remaining hypotheses is correct.

CONCLUSION

Genetic analysis does not have universal heuristic value, but it can serve two functions in scientific research. If a system is very well understood, analysis of mutants can often provide elegant support for the accepted hypotheses. On the otherhand, analysis of novel mutants can sometimes shed light on obscure biological processes by generating possible models and providing a means of hypothesis testing. The mutants I have isolated will require additional characterization , but I am hopeful that they will contribute to our understanding of cyclic AMP metabolism.

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