Somatic Cell Genetics

Edited by
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and
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PREFACE

This book represents a selected group of manuscripts from lecturers participating in the NATO/Gulbenkian Foundation sponsored course on Somatic Cell Genetics held May 31 to June 12, 1981 in the Hotel Montechoro in the Algarve of Portugal. The text will provide those students who could not attend the meeting with a current survey of important advances in the field of Somatic Cell Genetics. It is not possible to recapture here all the lectures, seminar discussions, student and faculty interactions, the ambience of the Algarve and the time devoted exclusively to scientific discussion. In summary, I feel that this book is good, but the scientists, the students, and the entire course were better.

Somatic Cell Genetics is a broad subject area and one which has contributed significantly to our understanding of the mammalian cell. Drs. Caskey, Buttin, Siminovitch, and Lechner elected in designing the course to focus on the results obtained with cultured animal cells.

Animal cells can alter their phenotype in culture, and thus we addressed the questions of mutation, chromosome alteration, and differentiation. Significant new advances were reported in the molecular delineation of mutational events at a wide variety of single gene loci (HPRT, AA-tRNA synthetases, protein biosynthesis, etc.) in hamster cells particularly. In some cases cells alter their phenotype by amplification of specific genes. The pioneer work related to methotrexate resistance has led this field. In still other cases such as the liver cell, differentiation events can be reproduced in culture but are not delineated at the molecular level.

The progress of cell hybridization, antibody-producing hybridomas, recombinant DNA cloning, and gene transfer have provided somatic cell geneticists with a new technology for achieving rapid advances. These advances include: establishment of a human gene map; molecular study and cellular expression of cloned genes; identification of unknown genes which have a phenotypic character expressed in a cultured cell (eg. neoplasia); and development of

specialized cells making specific antibodies. Each of these areas was discussed in the course and the manuscripts here described the most recent advances in the area.

The Somatic Cell Genetics course was made possible by our operating committee, faculty, and financial support from NATO and the Gulbenkian Foundation. It was made operative by the dedicated efforts of Dr. Maria C. Lechner and Mr. D. Christopher Robbins. Mr. Robbins is also to be credited for the careful attention to this text preparation. To these individuals we are grateful.

C.T. Caskey

January 1982

CONTENTS

PATHWAY MUTANTS IN MAMMALIAN CELLS AND THEIR CONTRIBUTION TO THE ANALYSIS OF DRUG RESISTANCE

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INTRODUCTION

The isolation by Beadle and Tatum (1) of Neurospora mutants with altered anabolic activity was a landmark in biology ; the mutant hunt which followed these pioneer experiments became an essential tool in the reconstruction of the main pathways which govern the metabolic activity of both prokaryotic and eukaryotic microorganisms. The analysis of metabolic steps in mammals and man did not rely as deeply on the genetic approach but it was largely guided by the information gained from these studies. Today most mammalian anabolic and catabolic pathways have been reconstructed; their enzymes have been extensively purified and controls exerted on their activity by a variety of regulatory effectors have been demonstrated. Yet an important part of the activity of somatic cell geneticists remains devoted to the isolation and characterization of pathway mutants. Their efforts are directed towards solving three main problems: (a) the nature of heritable variation in cultured mammalian cells, which was a source of considerable speculations, has been clarified in a limited number of systems only; (b) the availability of genetic markers necessary for the selection of cells which have acquired foreign genetic information remains a limiting factor in cell hybridization and gene transfer, and, more generally, in all rapidly expanding experiments of cellular engineering; (c) the physiological importance of an enzyme cannot be inferred from "in vitro" experiments: they do not take in account the network structure of most metabolic pathways and supply at best incomplete information on the influence which a variety of regulatory

effectors can exert on the activity of an enzyme. The turn off of this activity by a specific inhibitor or by a mutation is required, but most inhibitors have multiple targets, some of which may be unsuspected.

Since the two first aspects of the utilization of somatic cell mutants will be considered elsewhere in this volume, this article will be primarily devoted to illustrate on two examples - arabinofuranosylcytosine (araCyt) resistance and coformycin resistance of Chinese hamster fibroblasts - the unique advantage of pathway mutants for physiological analysis. The pathways under investigation belong to those which generate nucleic acid precursors. These are very complex systems since both "de novo" biosynthetic pathways and the so-called "salvage" pathways for utilization of preformed bases and nucleosides can contribute to the replenishment of the nucleoside-triphosphates pools."Suicide" selection techniques have been exploited with great succes to isolate mutants lacking enzymes of the "de novo" biosynthetic pathways (2). Toxic nucleoside analogs are powerful tools for the direct and simple recovery of mutants bearing defects in the activity of "salvage" enzymes phosphorylating preformed bases and nucleosides. But they can also provide insights into the function and into the physiological regulation of a variety of enzymes involved in nucleotide metabolism, including those belonging to interconversion pathways. Moreover, as will be discussed, genetic analysis may contribute to a better understanding of the mechanisms which permit dividing cells to escape destruction by toxic analogs of common use in chemotherapy.

MUTATIONS ALTERING PYRIMIDINE METABOLISM: ARACYT RESISTANCE

Dose-dependent Pattern of Drug Resistance

When mutagenized Chinese hamster cells of the CCL39 line were plated in medium containing 5µg/ml of araCyt, all colony-forming cells tested for their resistance level were found able to form colonies at concentrations as high as 50µg/ml ("high resistance" phenotype). When the selective medium contained no more than 0.5µg/ml of araCyt, such mutants were indeed recovered, but a new class of resistant clones was observed : they are unable to form colonies when araCyt concentration reaches 1µg/ml ("low resistance"phenotype) (3).

Loss of dCyd-araCyt-Kinase Activity in "High Resistance" Mutants

All "high resistance" mutants examined were found deficient in araCyt-kinase activity (3). The same biochemical defect has been characterized in resistant clones selected in culture from different mammalian lines, and genetic and biochemical analysis

agree to identify the altered enzyme as a deoxycytidine-kinase. (designated below as "dCyd-AraCyt-kinase") (4,8). The observation that an important level of dCyd-kinase was preserved in araCyt-kinase deficient Chinese hamster fibroblasts, disclosed the presence in these cells of an active mitochondrial isozyme of dCyd-kinase, which does not utilize araCyt as a substrate (8).

Expansion of the dCTP Pool in "Low-Resistance" Mutants

The class of "low resistance" mutants is of greater complexity but they all (3,9) appear to share two additionnal phenotypic properties : their growth is not inhibited by an excess of thymidine (dThyd) and they have an expanded pool of dCTP. Mutants which exhibit the same pattern of low araCyt resistance and dCTP pool expansion can be isolated as resistant to the "dThd block" (3). Actually, the enlargment of the dCTP pool is expected to generate the resistance to both nucleosides (fig. 1): (a) dCTP act both as a competitor of araCTP at the level of nucleic acid polymerases and as a feed back inhibitor (10) of dCyd-araCyt-kinase, the first enzyme which converts the toxic nucleoside to its active form. Expansion of the dCTP pool is therefore expected to decrease the utilization of exogenous araCyt and to reduce incorporation of exogenous dCyd and this is indeed observed in the mutants.

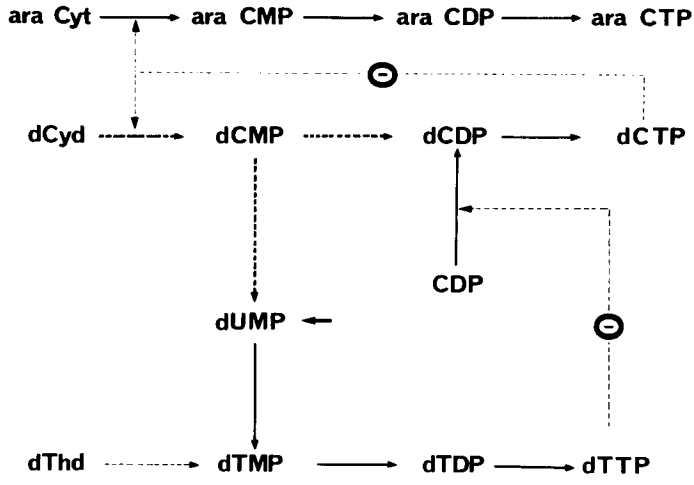

Fig.1. Pathways of pyrimidine deoxyribonucleotide and araCyt nucleotide synthesis in CCL39.
The ⊖ signs designate negative feedback controls.

(b) the dThd block is well known to be the consequence of dCTP depletion arising when excess dTTP inhibits CDP reductase; it is released by exogenous supply of dCyd and may be avoided by mutations leading to an abnormally high supply of endogenous dCTP.

Enzyme Alterations Leading to dCTP Pool Expansion

CDP-reductase alterations. The highly regulated ribonucleotide-reductase is an obvious possible target for mutations altering the rate of dCTP production. The properties of a mutant isolated from mouse fibroblasts through a multistep selection procedure are consistent with this expectation: this line which is both highly resistant to araCyt and resistant to dThd exhibits an increased level of CDP-reductase, a decreased sensitivity of this enzyme to dATP and an araCyt-kinase deficiency, but the contribution of these multiple alterations to the resistance pattern has not been clarified (11). Recently, mutants selected for their resistance to aphidicolin have been shown to be jointly resistant to araCyt and dThd and characterized as bearing a CDP-reductase desensitized to the inhibitory action of dATP (12).

CTP-synthetase alterations. When we examined however the properties of CDP-reductase in extracts from a set of "low resistance" mutants, we were unable to detect any alteration: the specific activity of the enzyme was identical in wild-type cells and in the mutants, as was its sensitivity to both dATP and dTTP (3).

Another clue came from the observation (Table 1) that in some "low resistance" mutants isolated as resistant to 0.5µg/ml

Table 1. Incorporation of cytidine and deoxycytidine by araCyt-resistant mutants of CCL39. (Incorporation of ^{3}H-labelled nucleosides into TCA precipitable material, expressed as percentage of the incorporation by the wild-type line)

Cell line	dCyd incorporation	Cyd incorporation	AraCyt dCyd kinase activity
CCL39(w.t.)	100	100	+
1A1	21	90	-
1A2	2	130	+
1A3	5	23	+
T1	7	19	+

of araCyt (1A3) or as resistant to 1mM dThd (T1), not only exogenous dCyd but also exogenous cytidine (Cyd) incorporation into macromolecules was severely reduced. This was not the case for other mutants such as 1A1, which exhibit the same pattern of resistance to both nucleosides, nor for a mutant (1A2) selected for resistance to 0.5µg/ml of araCyt but actually resistant to 50µg/ml of the drug and identified as an araCyt-dCyd-kinase deficient clone. The previous observations that dCTP pool expansion imposed a reduced incorporation of exogenous dCyd suggested that reduced Cyd incorporation in 1A3 and T1 might similarly be the consequence of an abnormally high pool of CTP. This hypothesis was supported by direct ribonucleoside-triphosphate pool measurements (Table 2-A), which also revealed that in 1A3 and T1, there is no increase of the intracellular concentration of UTP, the immediate precursor of CTP (13). These measurements strongly suggested that the primary biochemical defect in these lines was an altered regulation of CTP-synthetase activity or synthesis. In wild-type or mutant cell extracts, the level of CTP synthetase is actually identical; but the well known inhibition exerted on the activity of the enzyme by its product is considerably lower in T1 than in its CCL39 parent (fig.2): a 0.2 mM concentration of CTP completely inhibits the partially purified wild-type enzyme, but about 50% of the activity is preserved in the T1 mutant under these conditions. The desensitization of CTP-synthetase can indeed account for the expansion of the CTP pool and presumably of the dCTP pool derived from it. Therefore, it offers

Table 2. Triphosphate pool levels and araCyt resistance of wild-type, resistant, and revertant cell lines

Cell line	Triphosphate pool (nmol/10^6 cells) CTP	dCTP	AraCyt (µg/ml) resistance (LD90)
A.			
CCL39 (w.t.)	1.1	0.36	0.05
1A1	1.0	1.15	0.8
1A2	1.0	0.25	50
1A3	3.4	1.50	0.7
T1	3.7	1.60	0.7
B.			
TA43	3.5	1.50	0.8
TA43 rev1	1.0	0.45	0.08
TA43 rev2	1.2	0.55	0.10
TA43 rev3	1.0	0.40	0.08

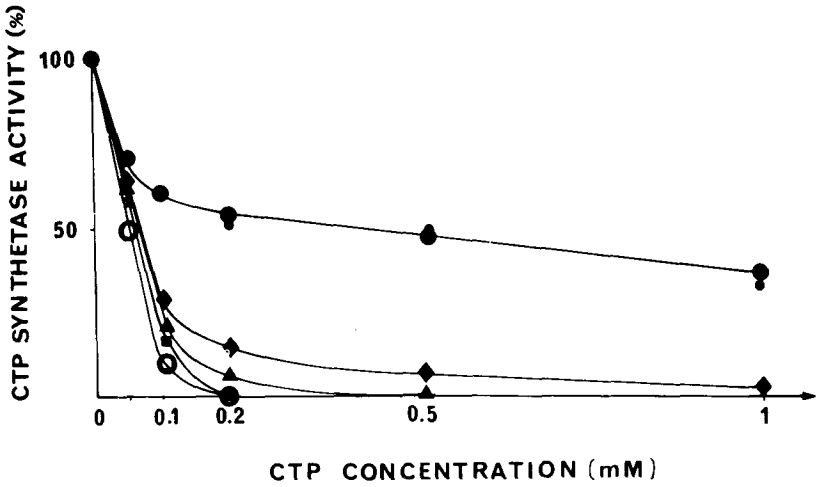

Fig.2. Inhibition of CTP-synthetase activity by CTP in wild-type, mutant and revertant lines; O: CCL39; ●: T1; •: TA43; ▲: TA43 rev_a; ◆: TA43 rev_b; ■: TA43 rev_c

a satisfactory explanation to the low resistance phenotype of the T1 mutant. It should be stressed however that one has to be cautious in drawing such a conclusion as long as it has not been unequivocally shown that reversion of the identified biochemical defect is sufficient to restore the wild-type resistance level. This word of caution is not gratuitous, as illustrated in this system by the properties of the 1A1 mutant line (3,13,14). In this low resistance mutant with a normal CTP pool we noticed that dCMP-deaminase is inactive. On a simple logical basis, this defect might account for the joint resistance to araCyt and dThd, since dCMP-deaminase activity diverts a potential source of dCTP towards the dTTP pool. But the same enzymatic deficiency was identified in a variety of sublines isolated from CCL39 on the basis of selections with no obvious relationship to dCMP metabolism. These independent lines preserve wild-type sensitivity to araCyt and dThd, indicating that dCMP deaminase deficiency is not responsible or not sufficient "per se" to determine the resistant phenotype.

A definitive proof that CTP-synthetase alteration is responsible for the resistant phenotype of T1 was brought by the isolation of revertants which simultaneously regained wild-type

sensitivity of the enzyme to CTP, and wild-type sensitivity to both araCyt and dThd (13).

This approach took advantage of two observations: (a) a clone (TA43) deficient in dCMP-deaminase was isolated by chance from the T1 line. The mechanism which generates at a surprisingly high frequency dCMP-deaminase-deficient variants from the CCL39 line is not known but its genetic or epigenetic character is irrelevant to this discussion. (b) In dCMP-deaminase deficient lines, expansion of CTP pool, which can be brought about either by a genetic alteration or by growing the cells in medium supplemented with CTP, inhibits the UDP-reductase pathway (14). This property makes dCMP-deaminase deficiency a conditionnally lethal trait, when coupled to a mutation expanding the CTP pool: in such double mutants, the two endogenous pathways which generate dUMP are shut off, as shown in fig.3, and cells rely on exogenous dThd or dUrd supply for dTTP synthesis and for their division. In medium devoid of these nucleosides, the double mutant dies, but surviving clones can be isolated; they are expected to include revertants which have recovered either an active dCMP-deaminase or a reduced CTP pool. This expectation was fullfilled: 3 clones, which do no longer manifest dThd auxotrophy were isolated from TA43; they have a wild-type CTP pool level: all three simultaneously recovered essentially wild-type sensitivity of CTP synthetase to CTP (fig.2), wild-type level of the dCTP pool and wild-type sensitivity to araCyt (Table 2) and to dThd (13).

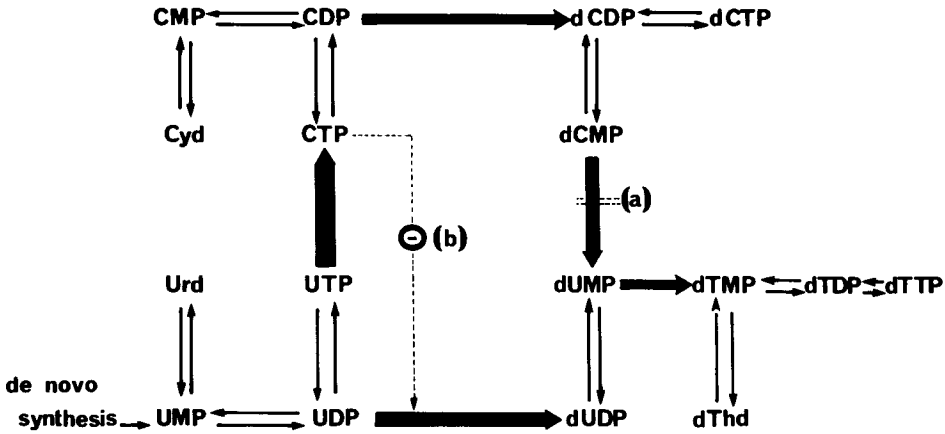

Fig.3. Pathways of pyrimidine nucleotide synthesis in CCL39.
(a): block imposed by dCMP-deaminase deficiency;
(b): block imposed by CTP overproduction.

The enzyme alteration responsible for dCTP pool expansion in other low resistance mutants such as 1A1 remains to be discovered. But a remarkable property of all "low resistance" lines which we examined is the semi-dominant expression of resistance in hybrids generated by fusion with sensitive cells. This is in striking contrast to the expression of araCyt resistance caused by araCyt dCyd-kinase deficiency, which is recessive in hybrids (3). Some possible implications of this property will be discussed below.

MUTATIONS ALTERING PURINE METABOLISM: COFORMYCIN RESISTANCE

A markedly different mechanism of resistance to a nucleoside analog was disclosed during the course of investigations on the regulation of cell proliferation by preformed purines and their analogs (15;16).

Adenine is not toxic to Chinese hamster fibroblasts (line GMA32, an araCyt-dCyd-kinase derivative of CCL39) growing in regular medium, unless it is added at 1mM concentrations or above. Coformycin, an adenosine analog known to inhibit adenosine-deaminase at concentrations as low as 0.02µg/ml, is not toxic either even if added at 2µg/ml. However, if adenine at 10^{-5} M is added to medium containing 0.5µg/ml of coformycin, the cells die. The mixture (HC + A) of coformycin at this high concentration (HC) and adenine (A) is not toxic to $APRT^-$ cells, indicating that adenine must be phosphorylated to AMP in order to contribute to lethality. Besides, the wild-type cells are rescued when the HC + A medium is supplemented with hypoxanthine, while $HGPRT^-$ cells are not: this suggests that, in HC + A medium, the cells die of IMP or GMP starvation, since they can synthesize adenylic nucleotides from adenine. This conclusion is supported by the observation that the very same concentration of adenine kills the cells and feed back inhibits the endogenous purine biosynthetic pathway. The simple model suggested by these results is that, when coformycin is present, the production of IMP from adenylic purines is decreased to a level unsufficient to sustain cell growth. Since two enzymes - adenosine-deaminase and AMP-deaminase - can divert adenylic purines to generate IMP, this further suggested that the drug - as such or via metabolic derivatives - inhibits both enzymes (fig. 4).

To check this hypothesis, mutants able to grow in HC + A medium were sought for. These clones distribute into two classes (16):

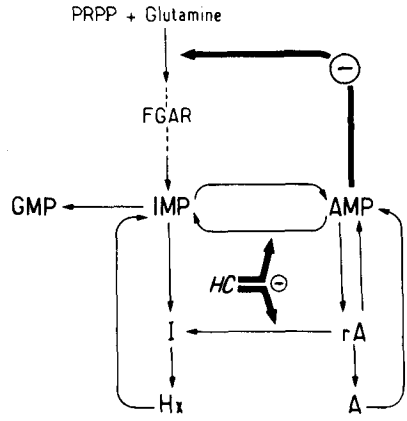

Fig.4. Pathways of purine nucleotide synthesis in hamster fibroblasts. The ⊖ signs designate negative controls exerted by AMP, or coformycin at 0.5µg/ml (HC).

Mutants with Altered Regulation of "de novo" Purine Biosynthesis

Class 1 mutants are characterized by their property to be unable to grow in the selective medium if azaserine - an inhibitor of "de novo" IMP synthesis - is further added. This indicates that the proliferation of these cells in the selective medium is possible because they somehow escape the feedback inhibition of the endogenous pathway. To check this point we examined the influence of adenine on the accumulation of N-formylglycine amide ribotide (FGAR) - which measures (17) the activity of the endogenous pathway of purine synthesis - in the parental line and in some of these mutants. As expected, 10^{-5}M adenine completely blocks purine synthesis in the parental cells but not in the class 1 mutants. The mechanisms for the release of inhibition are not known, but it is interesting that in one mutant, half inhibition is maintained. This is the situation predicted if the mutation alters one autosomal gene coding for the first enzyme of the "de novo" pathway, which - in wild-type cells - is feedback inhibited by AMP.

Mutants with Increased Activity of a Purine Interconversion Enzyme

In contrast to class 1 mutants, class 2 mutants grow in the selective HC + A medium independently of the activity of the endogenous pathway: selection in this medium supplemented with azaserine can be utilized to recover only mutants of this class. All class 2 mutants examined (3/3) had normal adenosine-deaminase activity but 7-13 fold the wild-type level of AMP-deaminase activity, suggesting that resistance is the manifestation of an increased AMP-deaminase activity, compensating for partial inhibition of this enzyme by coformycin. In agreement with this interpretation, we observed that coformycin slowly inhibits "in vivo" AMP-deaminase of both wild-type and mutant cells. Inhibition reaches a plateau, leaving in the mutant an AMP-deaminase activity comparable to the activity found in wild-type cells growing in the absence of the drug. Interestingly, the level of resistance

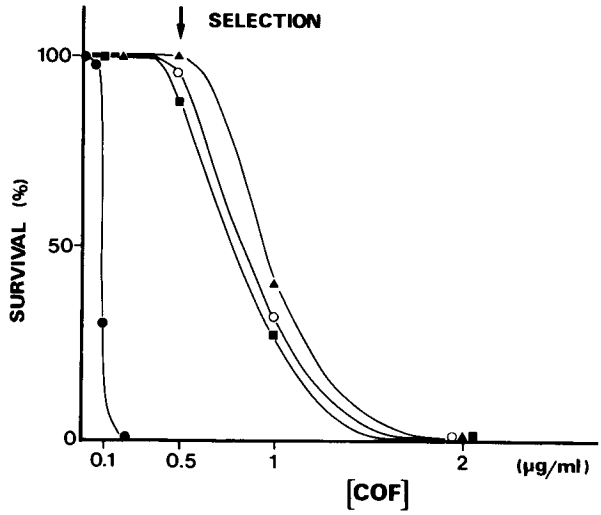

Fig.5. Plating efficiency of mutants, selected for their resistance to coformycin (0.5µg/ml) + adenine, in the same medium supplemented with increasing concentrations of the analog.