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Haplopappus gracilis Cell Strains Resistant to Pyrimidine Analogues

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Summary. Strains of *Haplopappus gracilis* (Nutt.) Gray cells resistant to 6-azauracil have been isolated from cultures of diploid cells. These strains are also resistant to 8-azaguanine, as is their parent. The variants are 100- to 125-fold more resistant to 6-azauracil than their parent, and they exhibit different spectra of cross resistance to other pyrimidine analogues. The phenotype of each variant is stable in the absence of selection. The majority of cells in cultures of the variants are diploid; all others examined were tetraploid. Initial rates of uptake of uracil are not reduced in the variants. Fluorouracil, to which two variants are resistant, is taken up by one of them as well as by the parent. Responses of the other two to fluorouracil are not correlated with decreased ability to accumulate this analogue.

Key words: Cultured plant cells – Mutants – Pyrimidine analogues – *Haplopappus gracilis*

Introduction

The full realization of the potential of plant somatic cell genetics ultimately will depend upon the availability of a variety of cell mutants that express phenotypes that are easy to score in culture (Maliga 1976; Widholm 1977). Among the variant phenotypes so far described are resistance to the purine analogue 8-azaguanine (AG) (Bright and Northcote 1975; Horsch and Jones 1978; Lescure 1973) and to two pyrimidine analogues, 5-bromodeoxyuridine (BrdUdr) (Bright and Northcote 1974; Maliga et al. 1973; Marton and Maliga 1975; Ohyama 1974, 1976) or 5-fluorouracil (FU) (Sung 1976).

In this paper we describe the isolation and phenotypic characterization of variants of *Haplopappus gracilis* that were selected by virtue of their resistance to the pyrimidine analogue 6-azauracil (AU). Although the ability to culture protoplasts or regenerate plants of this species from cultured cells has not been fully developed, the ease of culturing the cells (Eriksson 1965), the low chromosome number of the species (2n = 4; Jackson 1973) and the high karyotypic stability of cultured cells of the species (Horsch and Jones 1978; Singh and Harvey 1975; Singh et al. 1975) make it well suited for many types of experiments. Responses of plant cells to pyrimidine analogues other than BrdUdr have not been extensively studied, but research with whole plant tissues (Bressan et al. 1978; Cox et al. 1973; Key 1966; Ross 1964a,b; Wolcott and Ross 1966, 1967) and other biological systems (e.g. in yeast: Grenson 1969; Jund and Lacroute 1970; Lacroute 1968) (extensively reviewed in Henderson and Paterson 1973 and Langen 1975) suggests that selection for alteration in pyrimidine metabolism might provide a diverse collection of mutations. That such is the case is indicated by the nature of the variants described below.

Materials and Methods

Cell Strains

Wild type strains were initiated from root tissue of *H. gracilis* plants as described previously (Horsch and Jones 1978). These plants were diploid (2n = 4) and of the standard chromosomal race as distinguished by Jackson (1973). Strains discussed in this paper are labeled AG7, AU1, AU3, AUc(2) and SR17-1. AG7 is a strain that exhibits resistance to 8-azaguanine (Horsch and Jones 1978) and has been in continuous culture for approximately two years. AG7 was used as the parent for selecting AU resistant variants described below [AU1, AU3 and AUc(2)]. SR17-1 is a wild type strain derived from a single cell of strain SR17 and has been in culture for about one year. SR17-1 is unrelated to the AG7 family. It was included in this study to provide a comparison of responses of AG7 and a fully wild type strain to the analogues. (The original parent of AG7 has become aneuploid and was not included in this work. SR17-1 differs only slightly from the AG7 parent in its sensitivity to AG.)

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Culture Conditions

Cultures were initiated and maintained on Eriksson's medium (Eriksson 1965) modified by the omission of kinetin. Cultures were grown at 28° ± 1°C in the dark. Medium for callus growth was solidified with 1% agar (Difco). Callus was maintained by transferring small pieces from 2-6 week old cultures to fresh medium. Suspension cultures in 125 ml or 500 ml Erlenmeyer flasks containing 25 ml or 100 ml of liquid medium, respectively, were incubated on a gyrotory shaker (140 rpm). Suspension cultures were routinely subcultured every 5-7 days by transferring 5 ml of cells from a growing culture into 20 ml of fresh medium in a 125 ml Erlenmeyer flask.

Mutagenesis

Cells grown in suspension were exposed to 0.25% ethyl methanesulfonate (EMS; Sigma) by the addition of EMS directly to the cultures. The cultures were shaken for 3 hours, after which cells were collected on a filter, washed with growth medium, and transferred to fresh medium. This treatment results in 80-90% growth inhibition (Horsch and Jones 1978). To permit expression of the resistance phenotype in variant cells, EMS-treated cultures were allowed to recover and resume growth in fresh medium for 3 days before selections were begun.

Response Curves

Experiments in our laboratory have shown that responses of cells to AU depend strongly upon the growth state of cultures (data not presented). Therefore, unless otherwise stated, experiments were initiated with cells from 4 day old, exponentially growing cultures. Cells were collected on a sterile Miracloth filter and washed with fresh medium. Culture flasks (125 ml) containing growth medium were inoculated with 0.5 gm wet weight of cells, and the appropriate amount of filter-sterilized, concentrated analogue solution was added to each flask (final volume = 25 ml). Cultures were harvested after 6-8 days growth by collecting cells on a filter, washing them with distilled water and drying them to constant weight (18-24 hours) in an oven at 60°C before weighing. In our cultures, 1 gram dry weight is equivalent to approximately 15 grams wet weight or to about 3×10^8 cells.

Chromosome Examination

Cells from exponentially growing cultures were placed on a slide, hydrolyzed briefly in 15% HCl and covered with a few drops of aceto-orcein stain. After about 30 seconds, they were squashed under a coverslip, and chromosomes were examined and counted. All numbers reported here represent counts of chromosomes in at least 100 cells.

Pyrimidine Uptake

Mid-exponential phase cells were filtered through one layer of cheesecloth, collected on a Miracloth filter, washed with fresh growth medium and suspended in growth medium at a density of 50 mg wet weight/ml. Cells were incubated in a water bath shaker at 28° for about 30 minutes, after which ^{14}C-labeled pyrimidine solution was added to give the desired final concentration and specific activity. At appropriate times, duplicate 1 ml samples were diluted into 4 ml ice cold, nonradioactive pyrimidine (10 mM) in growth medium. These samples were allowed to stand for 10 min at 0°C, collected on filters, and washed 3 times with ice cold medium containing 10 mM pyrimidine. Filters with cells were placed into scintillation vials, dried overnight at 60°C, and radioactivity determined by liquid scintillation counting. Zero-time control counts were subtracted from all data values.

Results

Effect of Pyrimidine Analogues on H. gracilis Cells

Because the responses of *H. gracilis* cells to pyrimidine analogues have not been determined previously, a study of their effects on cells of this species was carried out. Several analogues were tested for ability to inhibit growth of cells of strain AG7 (Fig. 1). AU, FU, 6-azauridine (AUrd) and 2-thiouracil (TU) were all totally inhibitory at concentrations greater than 0.25 mM. 5-Azauracil or 6-azathymine did not inhibit growth at 0.25 mM (data not presented). AU was slightly more effective than the other analogues and, because of this and its relative stability and ease of handling, was chosen for use in selection experiments. AU similarly inhibited the growth of wild type strain SR17-1.

If AU exerts its toxicity by means of specific effects on endogenous pyrimidine metabolism, growth inhibition by AU should be reduced by the simultaneous presence of uracil or pyrimidine biosynthesis intermediates in the growth medium. AU toxicity also should be reversed by addition of uracil to cultures after exposure of cells to

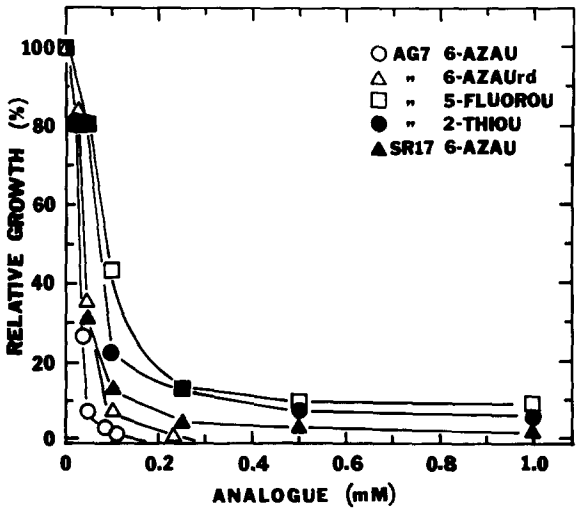

Fig. 1. Responses of *H. gracilis* cells to pyrimidine analogues. Relative growth was calculated by subtracting inoculum dry weight from each final dry weight, dividing net increase in dry weight for a particular treatment by the final net dry weight of controls containing no analogue, and multiplying by 100. Points represent means of triplicate samples

AU. Both these predictions were confirmed. As shown in Figure 2, the presence of uracil in cultures greatly relieved the toxic effects of AU in a concentration dependent manner. The effects of AU were obviated completely, even at concentrations that are normally totally inhibitory, by the simultaneous presence of uracil at 1-1.5 times the concentration of AU. As shown in Table 1, uridine also could compete out the effects of AU, and orotic acid and dihydroorotic acid were partially effective. Thymine and thymidine were ineffective.

Growth inhibition by 0.25 mM AU was totally reversed by the addition of twice that concentration of uracil to cultures even after 6 hours exposure to the drug (data not presented). Exposures to AU for longer times produced effects that could not be immediately reversed by addition of uracil.

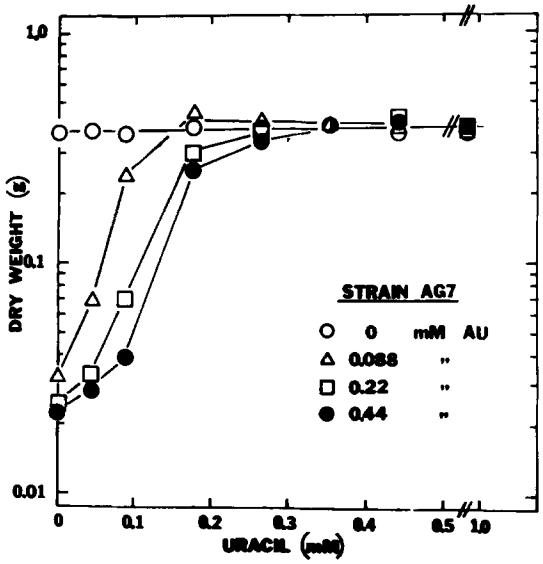

Fig. 2. Competitive effect of uracil against AU toxicity. AU was added to cultures at the final concentrations indicated in the figure. Simultaneously, uracil was added at the final concentrations given on the abscissa. Means of triplicate samples

Table 1. Effects of pyrimidines on AU toxicity

Pyrimidine	No AU		0.25 mM AU	
	0.25 mM	0.50 mM	0.25 mM	0.50 mM
None	100%[a]		0[b]	
Orotate	94 ± 1	95 ± 3	38 ± 1	53 ± 6
Dihydroorotate	98 ± 1	97 ± 1	35 ± 1	55 ± 2
Uracil	93 ± 1	92 ± 1	63 ± 1	99 ± 2
Uridine	91 ± 2	92 ± 2	48 ± 2	93 ± 3
Thymine	96 ± 1	92 ± 2	2 ± 1	2 ± 1
Thymidine	98 ± 1	96 ± 1	0 ± 1	0 ± 1

[a] Control (no AU, no pyrimidine): 0.397 gm dry weight = 100% ± 2% (S.E.)

[b] Total dry weight: 0.029 ± 0.002 (S.E.) gm. This value was subtracted from all others before calculating % net increase relative to control cultures

Selection of AU-resistant Variants

Exponential phase cultures of AG7 were exposed to EMS as described above. Then, cells were plated onto agar plates containing 2.65 mM AU. Preliminary experiments had shown that this concentration of AU and the following plating method led to growth of a few distinct colonies and total absence of 'background' growth. No attempt was made to verify or quantify the mutagenicity of EMS in these experiments, because nothing was known about the expression of phenotypes in the expected variants.

Two different (but not independently derived) cultures were treated with EMS. From each culture, approximately 10^6 cells were plated onto each of 12 selection plates, which were sealed and incubated in the dark at 28 ± 1°C. A total of about 25 distinct colonies grew on plates from each mutagenized culture (0-10 colonies per plate). Under these conditions, nearly all the 10^6 cells per plate would have formed colonies on medium without the analogue. Strains AU1 and AUc(2) were derived from vigorously growing colonies that arose on different plates from one EMS-treated culture. AU3 originated as a vigorously growing colony derived from the other culture. Following their selection at 2.65 mM AU, these strains were transferred to medium containing no drug and have since been grown without selection (approximately 15 months; see below).

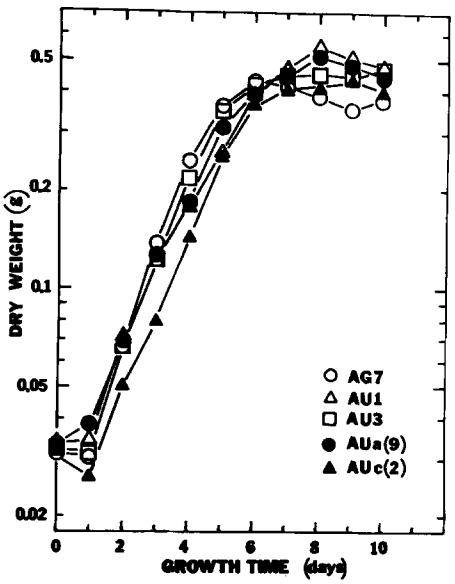

Fig. 3. Growth of parental and variant strains in Eriksson's medium. Means of triplicate samples

Growth of Parental and AU-resistant Cells

In Fig. 3 are shown growth curves of AG7 and AU-resistant variants cultured in Eriksson's medium without AU. Clearly, the variants grow as well as the parent strain both in terms of culture doubling time and of final yield in the cultures. [AUa(9) is a fourth variant that behaves similarly to AU3. It was not included in the remaining experiments.]

Resistance to AU

In Figure 4 are shown the results of an experiment in which the relative resistances of AG7, AU1, AU3 and AUc(2) to AU were determined. All three variants were totally resistant to 1 mM AU, while AG7 was completely inhibited in growth at about 0.2 mM. AUc(2) was slightly but consistently more sensitive to the analogue at the higher concentrations than were AU1 and AU3, which exhibited nearly identical responses. Concentrations required to inhibit growth by 50% were $\sim$ 0.03 mM for AG7 (Fig. 1), $\sim$ 3 mM for AUc(2) and $\sim$ 3.8 mM for AU1 and AU3.

Stability of the AU-resistance Phenotype

The three variants initially were selected for resistance to 2.65 mM AU. Data shown in Fig. 4 were derived from an experiment performed after these strains had been in continuous culture without selection for about 14 months. This represents about 95 consecutive cell generations [9 transfers ($\sim$ 30 doublings) on agar slants and 28 transfers ($\sim$ 65 doublings) in liquid medium]. After this time, AU1 and AU3 were still totally resistant to 2.65 mM AU, and AUc(2) was only slightly inhibited by this concentration of drug.

Karyotypic Stability of AU-resistant Variants

After cells had grown for $\sim$ 14 months, karyotypic analyses showed that cultures of AU1 and AU3 consisted of > 90% diploid cells (AU1: 91/100 mitotic cells diploid; 9/100 cells tetraploid. AU3: 95/100 cells diploid; 5/100 tetraploid). The AUc(2) cultures used in this study contained about 68% diploid cells (135/200) and 32% tetraploid cells (65/200). Cultures of the AG7 parental strain contained 95% diploid and 5% tetraploid cells (190/200 and 10/200 total cells, respectively). Chromosomes in all these cells were morphologically normal, and all cells examined were euploid.

Cross-resistance of Variants to Other Uracil Analogues

In Figures 5-7 are shown the responses of AG7 and the three variants to AUdr, TU and FU, respectively. AU3 and AUc(2) were much more resistant to AUdr than was AU1 [50% inhibitory concentrations: AG7 $\sim$ 0.04 mM; AU1 $\sim$ 0.4 mM; AU3 $\sim$ 2.3 mM; AUc(2) > 3 mM]. In contrast, AU1 and AU3 responded nearly identically to TU, while AUc(2) was much more sensitive to this drug [50% inhibitory concentrations: AG7 $\sim$ 0.2 mM; AU1 and AU3 $\sim$ 1.8 mM; AUc(2) $\sim$ 0.7 mM] AU1 and AU3 also responded similarly to FU, but AUc(2) was much less resistant to this analogue [50% inhibitory concentrations: AG7 $\sim$ 0.1 mM; AU1 $\sim$ 0.8 mM; AU3 $\sim$ 0.7 mM; and AUc(2) $\sim$ 0.2 mM].

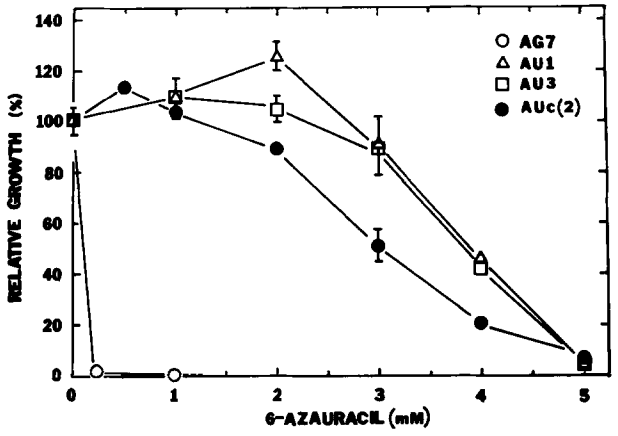

Fig. 4. Responses of parent and variant strains to 6-azauracil. Means and S.E. (where larger than symbols) of triplicate samples

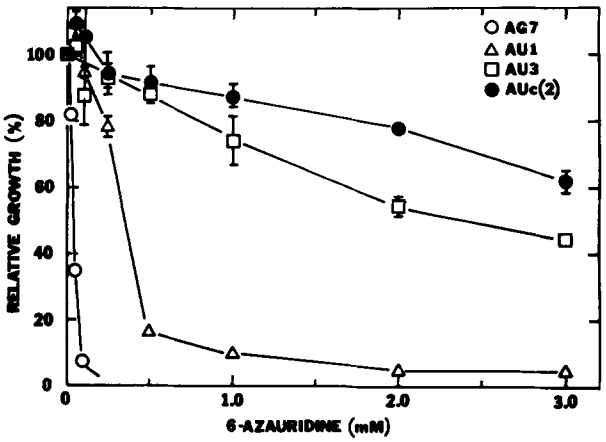

Fig. 5. Responses of parent and variant strains to 6-azauridine. Means and S.E. (where larger than symbols) of triplicate samples

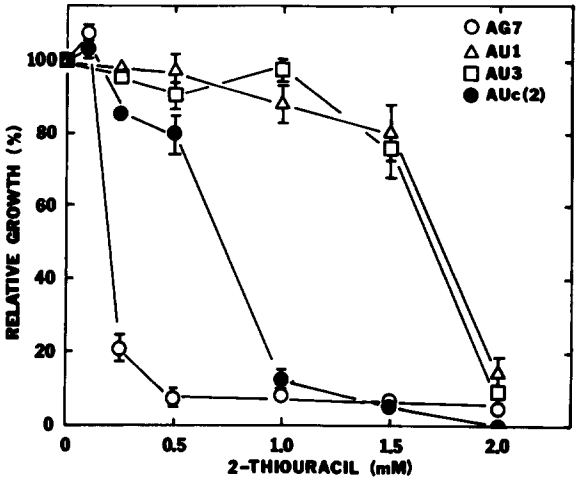

Fig. 6. Responses of parent and variant strains to 2-thiouracil. Means and S.E. (where larger than symbols) of triplicate samples

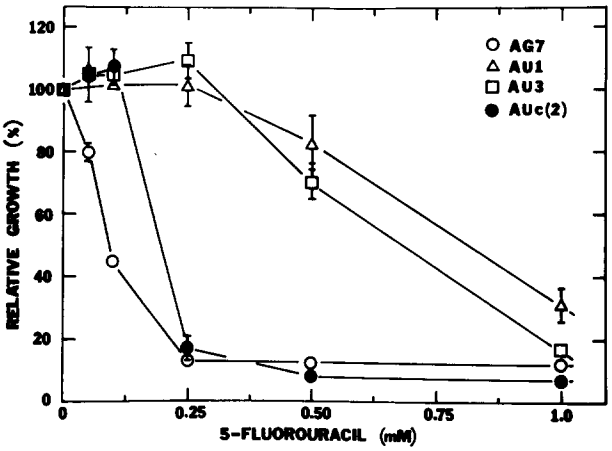

Fig. 7. Responses of parent and variant strains to 5-fluorouracil. Means and S.E. (where larger than symbols) of triplicate samples

Pyrimidine Uptake Experiments

The question immediately arises as to whether the AU-resistant variants are capable of taking up exogenous pyrimidines. That is, is the degree of resistance to an analogue correlated with ability to exclude the analogue? Two approaches to answering this question were used. In the first, we assayed the ability of strains to accumulate ^{14}C-labeled uracil (Fig. 8a). At 0.25 mM uracil, initial rates of uptake by the variants were no less than those exhibited by AG7. Uptake by the variants subsequently declined to a lower rate characteristic of the variant. In all cases, uptake continued for at least 6 hours. Similar results were obtained at a concentration of 0.1 mM uracil (data not presented).

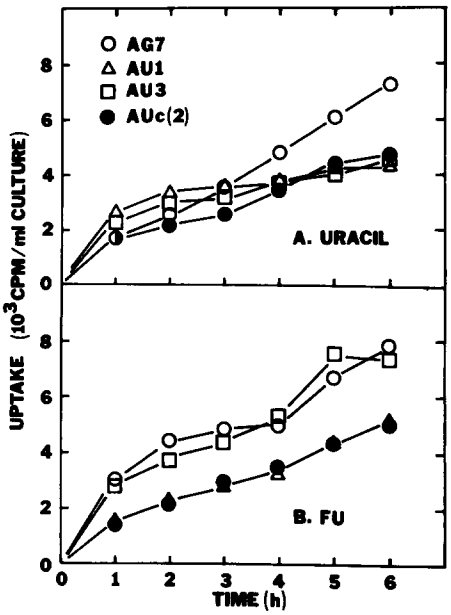

Fig. 8. Uptake of exogenous uracil or fluorouracil by parent and variant strains. Cells were prepared as given in Materials and Methods. Final concentration of pyrimidines was 0.25 mM. Specific activity of each was 0.8 mCi/mmol. Counting efficiency under these conditions was about 60%. Means of duplicate samples

The second approach was to determine the abilities of the four strains to accumulate ^{14}C-FU (Fig. 8b). (Radioactively labeled AU is not routinely available.) FU at a concentration of 0.25 mM was used because this concentration of analogue provides a clear discrimination between resistance and sensitivity responses among the strains used in this study (Fig. 7). All strains were capable of taking up the analogue, and changes in ability to accumulate FU were not correlated with degree of resistance to the drug. AU3, which is highly resistant to this concentration of FU, accumulated the drug in essentially the same way as its parental strain, AG7. AU1, which is totally resistant to this concentration of FU, and AUc(2), which is highly sensitive to this concentration, took up the analogue nearly identically.

Discussion

We have shown that cells of *H. gracilis* are highly sensitive to four pyrimidine analogues and have isolated variants that are highly resistant to 6-azauracil. In wild type cells, uracil, uridine and pyrimidine biosynthesis intermediates compete out or reverse the effects of AU. These results indicate that AU inhibits growth in this species by virtue of interaction with endogenous pyrimidine metabolism. This interaction probably occurs at a step at or before the synthesis of uridine monophosphate (UMP), because

thymine and thymidine had no effect on toxicity of AU. In most organisms AU is converted to AUMP ('lethal synthesis;' extensively reviewed by Henderson and Paterson 1973 and Langen 1975), which is a strong inhibitor of orotate 5'-monophosphate (OMP) decarboxylase, a key regulatory enzyme in pyrimidine metabolism. Inhibition of this enzyme accounts for most of the toxic effects of AU. Ross (1964b) has shown that a similar mechanism operates in plant tissues of some species. Our results are consistent with this mode of action of AU in *H. gracilis*.

The variants we have isolated show several important characteristics. They were isolated from a parental strain that is resistant to AG and have retained AG resistance (data not presented); thus, they are doubly marked. (The AG resistance of the parent does not appear to be relevant to the isolation of AU resistant strains; we have recently isolated AU resistant variants using totally wild type parental strains.) The AU variants grow as well as parental cells in regular growth medium and are not incapacitated by the lesions they carry. In terms of 50% inhibitory concentrations, the variants are 100- to 125-fold more resistant to AU than are their parent. This high resistance is extremely stable in the absence of selection. The marked difference in sensitivity to AU between parental and variant cells allows the confident identification of such variants in selection experiments, a property that makes this phenotype ideally suited for use in genetic experiments.

The karyotypes of the variants also are highly stable. The only changes we have detected is the presence of tetraploid cells in cultures that consist mainly of diploid cells. Cultures of two of the variants contain only a few percent tetraploid cells. AUc(2) cultures contained a higher percentage of tetraploid cells than cultures of the other strains; we are cloning diploid and tetraploid populations of cells derived from AUc(2) cultures to determine whether ploidy affects expression of the AU resistance phenotype. Most importantly, of the 600 mitotic figures examined in this study, all were euploid. Although aneuploidy certainly occurs in *H. gracilis* cultures, we have found that euploidy (and primarily diploidy) can be maintained for many months of continuous growth of cultures.

Perhaps the most striking aspect of our variants is their clearly different spectra of cross resistance to other pyrimidine analogues. AU3 is highly resistant to all four analogues tested; AU1 is highly resistant to AU, FU and TU; and AUc(2) is highly resistant only to AU and AUdr. These results, which are highly reproducible, strongly indicate that these three variants arose from different mutational events either in the same gene, or, more likely, in different genes involved in the synthesis of different enzymes involved in pyrimidine metabolism. We propose that our variants carry lesions in a variety of biochemical steps and are in the process of determining which steps are affected in them.

In this paper, we have presented evidence that our variants are not transport mutants. Uracil and fluorouracil are accumulated by all of them, and initial rates of uracil accumulation in the variants are not lower than in parental cells, as would be expected of transport mutants. AU3, which is totally resistant to 0.25 mM fluorouracil, accumulates the drug at this concentration as well as its parental strain. Resistance to FU is not correlated with reduced ability to accumulate the analogue. AU1 and AUc(2), which show vastly different sensitivities to FU, accumulate it nearly identically. The decline in rates of uptake of uracil or FU by the variants with continued incubation possibly is due to the buildup of unmetabolized pyrimidine derivatives in the cells, which might reduce the transport capacities of the cells. This is known to occur in yeast: when uracil or uridine utilization is reduced by mutation, feedback inhibition by internal pyrimidines acting on the corresponding permease reduces the rate of uptake of uracil or uridine, respectively (Grenson 1969). The availability of our variants now permits a detailed analysis of these features in plant cells.

Several observations are consistent with the suggestion that our variants arose as a result of mutation (Siminovitch 1976): (1) their AU resistance is highly stable; (2) the frequency of such variants ($\sim 2 \times 10^{-6}$) is in the range expected of genetic variants; and (3) each of the three variants exhibits a different but highly stable spectrum of cross resistances to other analogues, although they all were selected in a single step from closely related cultures treated in the same way. Only a complex, ad hoc explanation could account for the latter characteristic on the basis of epigenetic changes forced by the selection procedure.

That our variants were isolated from cultures of diploid cells and have remained euploid suggests that resistance to the analogues is dominantly or semidominantly expressed, although we cannot exclude the possibility that our strains carry undetectable deletions or regions of heterozygosity that would allow the selection of hemi- and homozygous recessive strains. However, the simplest explanation for the characteristics of these strains is that they carry regulatory mutations in a pyrimidine synthesis enzyme or that they contain reduced specific activities of enzymes involved in lethal synthesis of toxic analogue derivatives.

Experiments are in progress to study the effects of a variety of mutagens on frequencies of these variants in cultures of wild type cells; the characteristics of AU resistance in *H. gracilis* make it especially well suited for answering definitive questions about the relationship between phenotype alteration and mutation in somatic eukaryotic cells growing in culture.

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