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Increased levels of threonyl-tRNA synthetase in a borrelidin-resistant Chinese hamster ovary cell line

(drug-resistant cells/gene regulation/protein synthesis)

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ABSTRACT **The growth of Chinese hamster ovary cells in medium containing reduced concentrations of threonine is inhibited by borrelidin, a macrolide antibiotic. Borrelidin-resistant clones have been isolated after ethyl methanesulfonate mutagenesis. One clone, 1C-1, has a 3-fold increased level of threonyl-tRNA synthetase [L-threonine:tRNA^{Thr} ligase (AMP-forming), EC 6.1.1.3] as determined by both activity measurements and antiserum titrations. The levels of four other aminoacyl-tRNA synthetases and of tRNA^{Thr} are the same in strain 1C-1 and in the wild-type parent. The phenotype of increased threonyl-tRNA synthetase activity is recessive to wild type in cell hybrids.**

Aminoacyl-tRNAs are the products of the first committed step in protein biosynthesis. They may also be viewed as the final product of various cellular processes that provide the amino acid precursors of proteins. Thus, aminoacyl-tRNAs may serve an important role in coordinating certain aspects of cellular metabolism and protein synthesis (1, 2). Consequently, the regulation of the enzymes catalyzing the formation of aminoacyl-tRNAs is of interest.

There is evidence for at least two modes of regulation of aminoacyl-tRNA synthetases in bacteria and yeast. Metabolic regulation (3–5) affects the level of most, if not all, synthetases. In this type of control the levels of the synthetases are correlated with the growth rates of the cultures. There is also evidence for the specific regulation of individual synthetases, the most compelling of which comes from the isolation of regulatory mutants affecting the levels of single aminoacyl-tRNA synthetases. Bacterial cells containing regulatory mutations affecting the levels of threonyl- and seryl-tRNA synthetases have been isolated by selecting for resistance to the analogs borrelidin and serine hydroxamate, respectively (6–8). In an alternate approach, revertants of mutants containing temperature-labile aminoacyl-tRNA synthetases able to grow at the previously restrictive temperature have been isolated (9–12). Among these revertants are those that still contain the thermolabile enzyme but at concentrations 4- to 8-fold above those in the parental cell lines.

Evidence for regulation of the levels of specific aminoacyl-tRNA synthetases in mammalian cells is meager. Molnar *et al.* (13) isolated revertants of Chinese hamster ovary (CHO) strain *ts*H1, which contains a thermolabile leucyl-tRNA synthetase (14). One revertant clone contained twice the leucyl-tRNA synthetase activity of *ts*H1 but with a thermal inactivation profile identical to that of *ts*H1. Stanners and coworkers (15) studied the recovery of protein synthetic capacity in CHO cell lines containing temperature-sensitive aminoacyl-tRNA synthetases after exposure to highly restrictive conditions and suggested that the synthetases are under precise and specific control.

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As a prelude to studies of the regulation of aminoacyl-tRNA synthetases in mammalian cells, we wished to see if the levels of specific synthetases could be altered by mutation. In this report we describe the isolation and some properties of a borrelidin-resistant variant of CHO cells containing an increased level of threonyl-tRNA synthetase [L-threonine:tRNA^{Thr} ligase (AMP-forming), EC 6.1.1.3].

MATERIALS AND METHODS

Cells and Culture Conditions. The CHO cell line designated GAT$^-$, a mutant line auxotrophic for glycine, adenosine, and thymidine (16), was the parent cell line. Cells were maintained in monolayer culture or in suspension in α minimal essential medium (K. C. Biologicals, Lenexa, KS) containing 10% fetal calf serum and 10 μg/ml each of adenosine and thymidine.

Selection of Borrelidin-Resistant Cells. Subconfluent monolayer cultures were treated with 2 μl of ethyl methanesulfonate per ml for 1 hr as described (17). After 3–4 days of growth for phenotypic expression, cells were plated into 100-mm tissue culture dishes, allowed to attach, and then placed in α minimal essential medium modified to contain 20 μM threonine and 25 ng of borrelidin per ml. After 12–15 days, surviving colonies were cloned and retested for their ability to grow in the modified medium.

Enzyme Assays. Cell extracts were prepared by suspending washed cells at 10^7 per ml in a solution containing 10 mM Tris·HCl at pH 7.5, 50 mM KCl, 1 mM $MgCl_2$, 0.1 mM dithiothreitol, 20% (vol/vol) glycerol, and 1% Nonidet P-40. After incubation for 30 min at 0°C, the suspension was centrifuged at 25,000 × *g* for 20 min. The assay mixtures for threonyl-, leucyl-, tyrosyl-, and histidyl-tRNA synthetases contained, in a total volume of 250 μl: 0.1 M Tris·HCl at pH 7.5, 5 mM Na_2ATP, 2.5 mM Na_2CTP, 40 mM KCl, 10 mM $MgCl_2$, 0.4 mM dithiothreitol, 0.4 mg of tRNA, 75 μM ^{14}C- or ^{3}H-labeled amino acid, and 50–200 μg of extract protein. The reaction mixtures were incubated at 30°C. All reaction velocities were determined under linear assay conditions by transferring 50-μl samples directly from the reaction vessel into 2.5 ml of cold 10% trichloroacetic acid at 0-, 1.5-, 3-, and 5-min intervals. The precipitates were collected on glass-fiber discs, washed with 10% trichloroacetic acid, then with ethanol, and dried, and their radioactivities were measured. Asparaginyl-tRNA synthetase was assayed as described (18). Protein was determined by the method of Lowry *et al.* (19) with bovine serum albumin as standard.

tRNA Extraction and Analysis. RNA was extracted as described (20) and bulk tRNA was partially purified by chromatography on DEAE-cellulose (21). The aminoacylation of tRNA^{Thr} was performed essentially as described above for the assay of threonyl-tRNA synthetase activity, except that the tRNA concentration was varied from 0.8 to 5.0 A_{260} units/400

Abbreviation: CHO, Chinese hamster ovary.

μl of reaction mixture. The synthetase preparation used for these experiments was a 50–60% saturated ammonium sulfate fraction of the 105,000 × *g* supernatant fluid of a rat liver homogenate prepared as described by Dignam *et al.* (22).

Immunochemical Procedures. Rabbit antiserum was prepared against highly purified rat liver threonyl-tRNA synthetase (22), kindly supplied by Murray Deutscher of the University of Connecticut. Immunotitration of threonyl-tRNA synthetase in cell extracts was performed by holding the volume of extract constant and varying the amount of antiserum. Samples of cell extracts were incubated with various amounts of antiserum for 90 min at 25°C. The antigen–antibody complex was then adsorbed to a suspension of fixed *Staphylococcus aureus* cells. After a 30-min incubation at 4°C, the suspension was centrifuged for 30 sec in an Eppendorf microcentrifuge and the enzyme activity remaining in the supernatant fluid was determined. Control incubations were conducted with preimmune rabbit serum.

Chemicals. Radioactive amino acids were purchased from Amersham or New England Nuclear. Borrelidin (National Service Center no. 216128) was kindly supplied by the Developmental Therapeutics Program, Division of Cancer Treatment, National Cancer Institute.

RESULTS

Effects of Borrelidin on the Growth of CHO Cells. The inhibitory effect of borrelidin on the growth of CHO cell line GAT$^-$ is shown in Fig. 1. Cell growth was strongly inhibited when the threonine concentration of the medium was decreased to 20 μM (1/20th the normal concentration), a concentration that did not limit the growth rate of the cells in the absence of the drug. The concentration of borrelidin required to inhibit

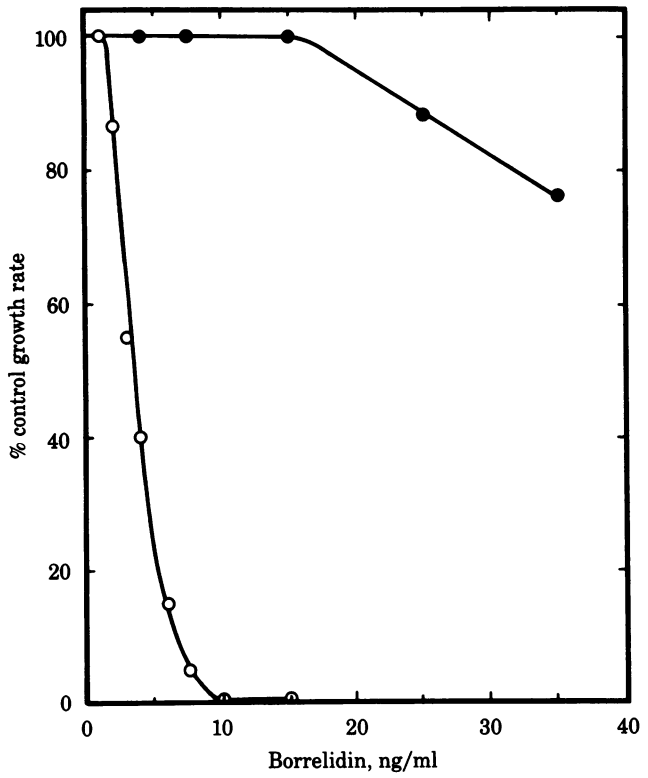

FIG. 1. Effect of borrelidin on the growth of wild-type GAT$^-$ cells. Growth rates were determined for cells growing in α minimal essential medium containing 20 μM (○) or 400 μM (●) threonine and the indicated concentrations of borrelidin.

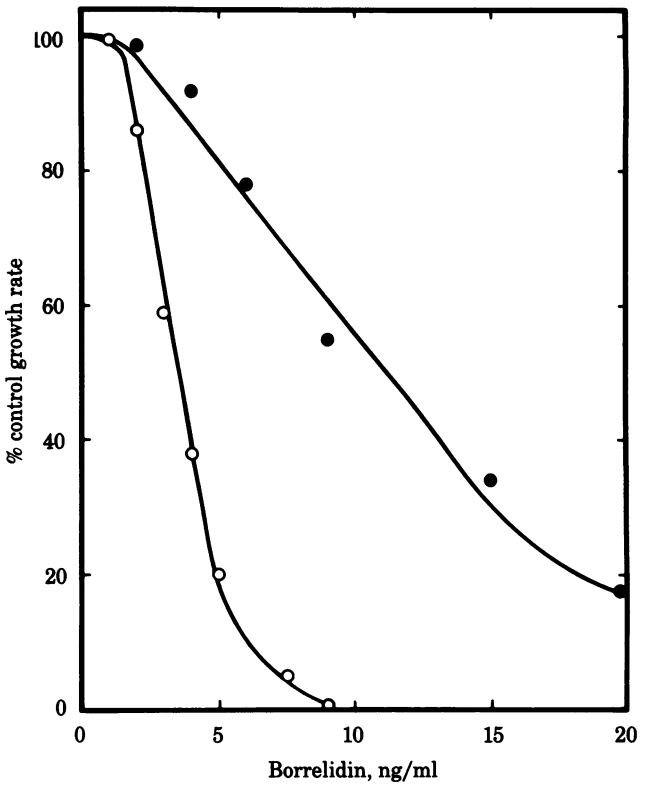

FIG. 2. Effect of borrelidin on the growth of 1C-1 (●) and wild-type (○) GAT$^-$ cells. Growth rates were determined for cells growing in α minimal essential medium containing 20 μM threonine and the indicated concentrations of borrelidin.

growth of these cells by 50% (EC_{50}) under these conditions was about 3.5 ng/ml. When GAT$^-$ cells were grown in complete α minimal essential medium containing 400 μM threonine, the cells were protected from borrelidin growth inhibition at concentrations up to 20 ng/ml.

Selection of Cells Resistant to Growth-Inhibitory Effects of Borrelidin. The frequency with which colonies resistant to borrelidin at 25 ng/ml appeared after selection was about 5×10^{-7}. Thirty clones were screened for threonyl-tRNA synthetase activity. Twenty-seven of these were indistinguishable from GAT$^-$ with respect to enzyme activity levels and sensitivity to borrelidin *in vitro*. These may represent permeability mutants. Three clones had a 3-fold increased level of threonyl-tRNA synthetase activity. One of these, strain 1C-1, is described in this paper. Fig. 2 shows the growth rate for GAT$^-$ and 1C-1 at different inhibitor concentrations. The EC_{50} for 1C-1 was about 12 ng/ml. This drug resistance has been maintained for more than 100 generations of growth in the absence of drug.

Threonyl-tRNA Synthetase Levels in Wild-Type and Resistant Cells. The specific activity of threonyl-tRNA synthetase

Table 1. Specific activity of several aminoacyl-tRNA synthetases in GAT$^-$ and 1C-1 cells

Amino acid	Specific activity, pmol/min per mg protein	
	GAT$^-$	1C-1
Asparagine	13.9 ± 1.7	14.1 ± 1.2
Histidine	10.0 ± 1.9	9.3 ± 2.0
Leucine	5.9 ± 0.6	6.0 ± 0.9
Threonine	23.0 ± 3.4	62.7 ± 8.1
Tyrosine	15.5 ± 1.3	17.1 ± 1.6

The data are mean ± SEM of at least three different experiments.

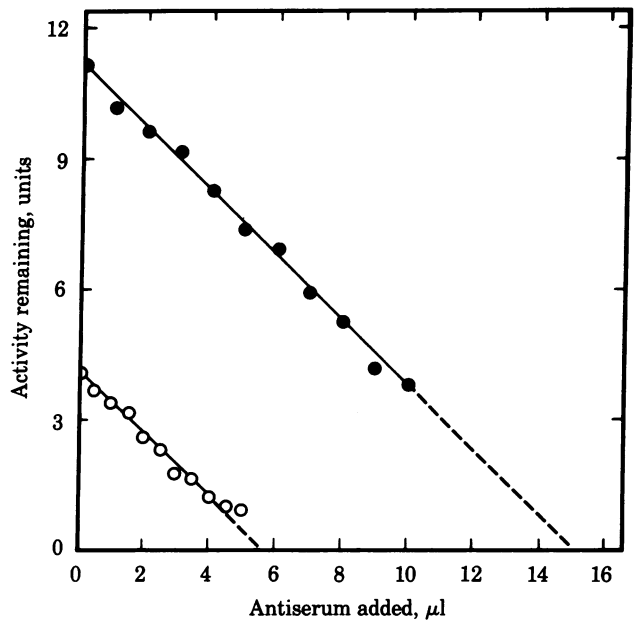

FIG. 3. Immunotitration of threonyl-tRNA synthetase from 1C-1 and wild-type cells. Samples (100 μl containing 180 μg of protein) of extracts from GAT⁻ (○) or 1C-1 (●) cells were mixed with the indicated volume of antiserum and incubated; nonprecipitated enzyme activity was assayed in the supernatant fluids.

in crude extracts from 1C-1 cells was about 3 times higher than that in extracts from the wild-type cells (Table 1). When the activities of four other aminoacyl-tRNA synthetases were assayed in these same extracts, no differences were seen (Table 1). Fig. 3 shows that the threonyl-tRNA synthetase content of these extracts as determined by antiserum titration is proportional to that determined by enzyme activity measurements. This result suggests that the difference in threonyl-tRNA synthetase levels between extracts from GAT⁻ and 1C-1 is due to

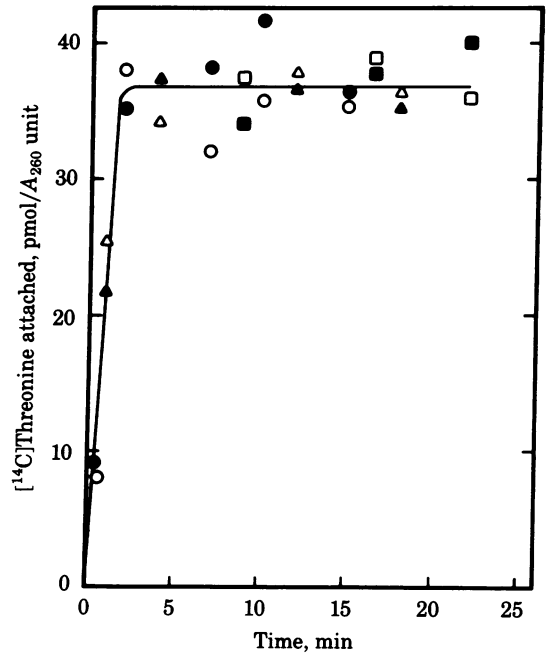

FIG. 4. Aminoacylation of $tRNA^{Thr}$ from 1C-1 and wild-type cells. Open symbols represent tRNA isolated from GAT⁻ cells, closed symbols represent tRNA isolated from wild-type cells. ○ and ●, 0.82 A_{260} unit; △ and ▲, 1.65 A_{260} units; □ and ■, 4.94 A_{260} units.

Table 2. Specific activity of threonyl-tRNA synthetase in parental and hybrid cell lines

Cell line	Specific activity, pmol/min per mg protein
GAT⁻	23.0 ± 3.4
*ts*H1	28.3 ± 2.9
1C-1	61.7 ± 8.1
*ts*H1 × GAT⁻ hybrid 1	24.6 ± 1.6
*ts*H1 × GAT⁻ hybrid 2	27.1 ± 3.0
*ts*H1 × 1C-1 hybrid 1	28.2 ± 3.6
*ts*H1 × 1C-1 hybrid 2	27.0 ± 2.2

The data are mean ± SEM of at least three different experiments.

a difference in enzyme concentration and not to a difference in catalytic efficiency.

$tRNA^{Thr}$ Levels in Wild-Type and Resistant Cells. The level of $tRNA^{Thr}$ was determined by aminoacylating samples of total tRNA prepared from GAT⁻ and 1C-1 cells with [^{14}C]threonine. The results (Fig. 4) show that there was no difference between threonine acceptance activities of the tRNAs from these cell lines. Thus, the increased level of threonyl-tRNA synthetase in strain 1C-1 is not coordinated with an increase in $tRNA^{Thr}$ content.

Cell Hybridization Experiments. Tetraploid hybrid cells were formed from GAT⁻ or 1C-1 and cells that are wild type with respect to threonyl-tRNA synthetase levels by fusion mediated by polyethylene glycol 1000 (23). The wild-type strain used was a clone of *ts*H1 containing a dominant ouabain resistance marker and a recessive hypoxanthine phosphoribosyltransferase marker. Hybrid clones were selected in hypoxanthine/aminopterin/thymidine (HAT) (24) medium containing 1 mM ouabain and assayed as soon as sufficient cells were available. The specific activities of threonyl-tRNA synthetase in two 1C-1 × *ts*H1 hybrids, two GAT⁻ × *ts*H1 hybrids, *ts*H1, and GAT⁻ were the same (Table 2), indicating that the mutation responsible for increased threonyl-tRNA synthetase activity in 1C-1 is recessive to the wild-type allele.

DISCUSSION

The macrolide antibiotic borrelidin has been shown to be a specific inhibitor of threonyl-tRNA synthetase in bacteria (6, 7). We have found it also to be an extremely effective inhibitor of CHO cell growth. This inhibition is antagonized by threonine.

Among clones selected for resistance to borrelidin we have characterized one, strain 1C-1, which has a 3-fold higher level of threonyl-tRNA synthetase activity than the parental line. The levels of four other aminoacyl-tRNA synthetases and the threonine acceptance activity of total tRNA are unchanged. Thus, there is no evidence for an alteration affecting either the coordinate regulation of all aminoacyl-tRNA synthetases or of an individual synthetase and its cognate tRNA isoacceptor family.

Immunotitration of enzyme activity demonstrated that the increase in enzyme activity levels is due to an increase in the content of threonyl-tRNA synthetase molecules. This could result from changes in the rate of enzyme synthesis or degradation or both. Changes in rate of synthesis could result from an alteration in a regulatory element that modulates the rate of transcription of the threonyl-tRNA synthetase structural gene or from amplification of the structural gene, as has been found for other drug-resistant mutants (25, 26).

The fact that the mutation in strain 1C-1 is recessive to the wild-type allele suggests that the increased levels of threonyl-tRNA synthetase are not due to gene amplification or multiple

chromosomes, because cell hybrids formed by fusing a strain containing multiple structural genes with a wild-type strain would be expected to have intermediate activity. Furthermore, the recessive nature of this mutation suggests that GAT^- cells are functionally haploid at this putative regulatory locus, as has been suggested for a number of other loci in CHO cells (27). The results presented here show that resistance to borrelidin can result from an increased cellular content of threonyl-tRNA synthetase. However, it is not yet clear that strain 1C-1 is defective in a physiologically significant mechanism that normally regulates threonyl-tRNA synthetase levels in response to specific metabolic signals. Indeed, the existence of such mechanisms for regulating aminoacyl-tRNA synthetase levels in mammalian cells remains an open question (13, 15). Further analysis of strain 1C-1 and of other mammalian cell mutants containing altered levels of aminoacyl-tRNA synthetases, tRNAs, or both should help to answer this question.

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