MOLECULAR AND CELLULAR BIOLOGY, Sept. 1985, p. 2381–2388
0270-7306/85/092381-08$02.00/0

Vol. 5, No. 9

Amplification of the Gene for Histidyl-tRNA Synthetase in Histidinol-Resistant Chinese Hamster Ovary Cells

FLORENCE W. L. TSUI,[1,2]†* IRENE L. ANDRULIS,[1,2]† HELIOS MURIALDO,[2] AND LOUIS SIMINOVITCH[1,2]†

Department of Genetics, The Hospital for Sick Children, Toronto, Ontario, Canada M5G 1X8,[1] and Department of Medical Genetics, University of Toronto, Toronto, Ontario, Canada M5S 1A8[2]

Received 29 February 1985/Accepted 21 June 1985

Histidinol-resistant (HisOH^R) mutants with up to a 30-fold increase in histidyl-tRNA synthetase activity have been isolated by stepwise adaptation of wild-type Chinese hamster ovary (CHO) cells to increasing amounts of histidinol in the medium. Immunoprecipitation of [^{35}S]methionine-labeled cell lysates with antibodies to histidyl-tRNA synthetase showed increased synthesis of the enzyme in histidinol-resistant cells. The histidinol-resistant cell lines had an increase in translatable polyadenylated mRNA for histidyl-tRNA synthetase. A cDNA for CHO histidyl-tRNA synthetase has been cloned, using these histidyl-tRNA synthetase-overproducing mutants as the source of mRNA. Southern blot analysis of wild-type and histidinol-resistant cells with this cDNA showed that the histidyl-tRNA synthetase DNA bands were amplified in the resistant cells. These HisOH^R cells owed their resistance to histidinol to amplification of the gene for histidyl-tRNA synthetase.

The proper functioning of aminoacyl-tRNA synthetases is critical to protein synthesis since these molecules catalyze the attachment of amino acids to the correct tRNAs. One approach to the examination of problems in this area is to isolate mutants in the synthetases and to use such mutants to study specific facets of protein synthesis. Some success has been achieved in this respect in mammalian cells, where several investigators have isolated temperature-sensitive mutants of Chinese hamster ovary (CHO) cells with defects in leucyl- (20), asparaginyl- (2, 22), glutamyl- (1), and arginyl- (1) tRNA synthetases. These mutations have generally been shown to be due to structural changes in the relevant enzyme (2, 3, 21). The findings in bacteria that the amino alcohols methioninol, tyrosinol, isoleucinol, and leucinol competitively inhibit the activation of the corresponding amino acids provided an alternative approach to the possible isolation of aminoacyl-tRNA synthetase mutations. Isolates resistant to these drugs might be particularly useful since, in principle, they could involve amplification of the target enzyme. The availability of amplification mutants is of interest, per se, but these mutants also provide excellent starting material for cloning the specific gene involved, as shown previously in several cases (16, 19). The feasibility of this approach in respect to the tRNA synthetases has already been demonstrated by Gerken and Arfin (7), who have shown that borrelidin, an inhibitor of threonyl-tRNA synthetase, can be used to isolate mutants which overproduce this enzyme.

Since histidinol has been shown to be a competitive inhibitor of histidyl-tRNA synthetase in animal cells (17), we have used this drug to isolate histidinol-resistant CHO cells. In this study we describe the isolation in CHO cells of first-step resistant isolates to histidinol, the further amplification of this resistance by continued growth in increasing concentrations of the drug, and the demonstration that the resistance of these mutants is not due to an alteration in the kinetic constants for histidyl-tRNA synthetase but to an increased level of activity of the enzyme. We then show that the above histidinol-resistant (HisOH^R) isolates contain increased amounts of a 52,000-dalton protein, characteristic of histidyl-tRNA synthetase, and the corresponding mRNA. This has allowed us to isolate a cDNA clone from this mRNA and to use this to demonstrate that the HisOH^R mutants have amplified the gene for histidyl-tRNA synthetase.

* Corresponding author.
† Present address: Research Department, Mount Sinai Hospital, Toronto, Ontario, Canada M5G 1X5.

MATERIALS AND METHODS

Cell lines and media. Histidinol-resistant mutants were obtained from three independent clones, WT1, WT2, and WT5, of the parental Pro$^-$ CHO lines (9). First step isolates were first obtained by selecting colonies after 10 days of growth in 0.15 mM histidinol in α medium (18) containing 1/10 the normal concentration of amino acids plus 7% dialyzed fetal calf serum. Single resistant clones from each parental line were then picked and grown in progressively increasing concentrations of histidinol to yield cell lines 2H1, 2H2, 1H3, and 5H5. The cells were maintained in the same medium as described above, except that the histidinol concentration was kept at 12 to 26 mM, depending on the degree of resistance of the individual line.

Determination of histidyl-tRNA synthetase activity and kinetic constants. Histidyl-tRNA synthetase activity was measured by esterification of radioactive histidine to tRNA^{His}. Briefly, a cell extract (final protein concentration, 0.5 mg/ml) was added to a reaction mixture which contained 100 mM Tris hydrochloride (pH 7.4), 5 mM ATP, 2.5 mM CTP, 40 mM KCl, 10 mM magnesium acetate, 0.4 mM dithiothreitol (DTT), yeast tRNA (2.5 mg/ml; Bethesda Research Laboratories, Inc. [BRL]), 0.09 mM histidine, and 0.01 mM [^{14}C]histidine (337 mCi/mmol; New England Nuclear Corp.). At different time points (0, 0.5, 1, and 2 min), portions of the reaction mixture were added to cold 10% (wt/vol) trichloroacetic acid to stop the reaction. The filters were counted by liquid scintillation counting. Protein concentrations were determined by the procedure of Lowry et al. (13), using bovine serum albumin as standard.

Kinetic constants were determined from double reciprocal plots of initial velocity measurements of histidyl-tRNA synthetase activity. For determination of inhibition by histidinol

with respect to histidine, the histidine concentration was varied from 0 to 10 μM and the histidinol concentration from 0 to 75 μM.

Determination of D_{10} values. The concentrations of histidinol required to reduce the relative plating efficiency of cells to 10% (D_{10}) were determined from dose-response curves of relative plating efficiencies of cells grown in minimal essential medium with 1/10 amino acid and 7% dialyzed fetal calf serum at various histidinol concentrations.

Purification of histidyl-tRNA synthetase. Histidyl-tRNA synthetase was partially purified using two chromatographic steps. Histidinol-resistant cells were trypsinized, washed with phosphate-buffered saline, and lysed by vortexing in cold buffer A (50 mM Tris hydrochloride [pH 7.4], 1 mM DDT, 5 mM EDTA, 20% glycerol, 0.5 mM phenylmethylsulfonyl fluoride). The lysate was centrifuged at 10,000 × *g* for 15 min and loaded onto a DEAE-cellulose column (25 by 1 cm). The column was washed with buffer A to remove all unbound protein. Proteins bound to the column were eluted by a 0 to 0.5 M KCl gradient. Fractions collected were assayed immediately for histidyl-tRNA synthetase activity. A portion of each fraction was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and the gel was then stained with Coomassie blue. The proteins eluted from the 0.02 to 0.18 M fractions were pooled and loaded on a 1-ml P_{11} phosphocellulose column. The column was washed first with 0.1 M KCl–25 mM Tris hydrochloride (pH 7.5)–1 mM DTT–5 mM EDTA–20% glycerol–0.5 mM phenylmethylsulfonyl fluoride and then with 15 ml of 0.2 M KCl in the same buffer. A slow gradient was set up by mixing 20 ml of 0.2 M KCl and 20 ml of 0.3 M KCl. Fractions collected were assayed for histidyl-tRNA synthetase activity, and equal portions of each fraction were subjected to SDS-PAGE. The gel was stained with silver stain (Bio-Rad Laboratories). Fractions shown to be 95% pure for histidyl-tRNA synthetase were used for the preparation of antibody.

Preparation of rabbit anti-CHO histidyl-tRNA synthetase antibody. Anti-CHO histidyl-tRNA synthetase antibody was prepared by performing weekly subcutaneous injections of partially purified enzyme into a New Zealand White rabbit. Antibody was detected by immunoprecipitation of [^{35}S]methionine-labeled 52,000-dalton polypeptide.

[^{35}S]methionine labeling of CHO cells. Cells were grown to early confluence in 75-cm^2 tissue culture flasks. One day before radioactive labeling, the medium was removed and replaced by 10 ml of medium containing 10 μM methionine (final concentration) and 10% dialyzed fetal calf serum. The following day, the medium was replaced by 5 ml of the same medium supplemented with 50 μCi of [^{35}S]methionine per ml (800 to 1,200 Ci/mmol; New England Nuclear). After 15 h of incubation, the radioactive medium was removed, and the flasks were washed twice with phosphate-buffered saline. The cells were then trypsinized and lysed in buffer A. The lysates were centrifuged at 10,000 × *g* for 15 min at 4°C.

Immunoprecipitation of radioactive cell lysate. Radioactive cell extracts were preabsorbed once with normal human serum or normal rabbit serum. This was accomplished by the addition of 20 μl of normal serum, incubation at 4°C for 30 min, precipitation of the immune complexes with 200 μl of phosphate-buffered saline-washed Pansorbin (Formalin-fixed, *Staphylococcus* sp.-containing protein A; Calbiochem-Behring), and incubation at 4°C for 15 min, followed by centrifugation for 1 min at 10,000 × *g* (10). Either 10 μl of antiserum or normal serum was then added to the normal serum-cleared extracts, and the extracts were incubated at 4°C for 30 min. Subsequently, 100 μl of washed Pansorbin was added, and the suspension was incubated for 15 min and centrifuged for 1 min. The pellet was washed three times with 50 mM Tris hydrochloride (pH 7.5)–150 mM NaCl–5 mM EDTA–0.5% Nonidet P-40–0.5% sodium deoxycholate–0.1% SDS, with centrifugation after each wash, and solubilized by adding 40 μl of 0.065 M Tris hydrochloride (pH 6.8)–2% SDS–50 mM DTT, followed by immersion in a boiling water bath for 3 min. The suspension was centrifuged, and the supernatant was recovered. A 5-μl portion of the supernatant was removed for liquid scintillation counting. The remaining sample was subjected to SDS-PAGE (11). Radioactive bands were visualized by fluorography (4).

Preparation of mRNA. Total RNA was obtained from wild-type and histidinol-resistant cells by the procedure described by Chirgwin et al. (4a), with modifications. Cells were lysed by 4 M guanidinium thiocyanate. After shearing the DNA to reduce viscosity, the lysate was loaded onto 2.5 ml of 5.7 M CsCl. The samples were centrifuged at 35,000 rpm for 18 h at 15°C. The RNA pellet was rinsed with cold 70% ethanol, suspended in water, and ethanol precipitated at −20°C. Polyadenylated [poly(A)$^+$] RNA was isolated by oligodeoxythymidylate [oligo(dT)]-cellulose chromatography (BRL).

mRNA samples were translated in vitro by a rabbit reticulocyte lysate system (BRL), using [^{35}S]methionine to label the protein. The translatable products were analyzed by SDS-PAGE and fluorography.

Synthesis and cloning of cDNAs. Double-stranded cDNA was prepared as described by Lehrach et al. (12). Poly(A)$^+$ mRNA used for cDNA synthesis was purified by two cycles of oligo(dT) chromatography. The reaction mixture for the synthesis of the first-strand cDNA contained 50 mM Tris hydrochloride (pH 8.3), 10 mM $MgCl_2$, 100 mM KCl, 10 mM DTT, oligo(dT) (60 μg/ml; Sigma Chemical Co.), 1 mM each dATP, dGTP, and dTTP, 200 μM [^{3}H]dCTP (New England Nuclear), 50 U of AMV reverse transcriptase (Life Sciences, Inc.), and 5 μg of mRNA. After incubation for 1 h at 42°C, the mixture was diluted with an equal volume of 5 mM DTT–5 mM Tris hydrochloride (pH 8.3)–200 μM dCTP and incubated for an additional 1 h at 42°C. The cDNA mixture was then boiled for 2 min, chilled in ice-water, and brought to a final concentration of 10 mM dATP, dGTP, dCTP, and dTTP, 100 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; pH 6.9), and 70 mM KCl. Fifty units of the large fragment of polymerase I (BRL) was added, and the mixture was incubated for 6 h at 15°C. Double-stranded cDNA was purified by chromatography over Sephadex G150 in 20 mM NaCl. Excluded fractions were pooled, lyophilized, and ethanol precipitated.

Double-stranded cDNA was converted into duplex molecules by digestion with S1 nuclease (Sigma). The cDNAs were oligadeoxycytidylate tailed with terminal transferase (New England Nuclear), and they were annealed with oligodeoxyguanylate tailed *Pst*I-cut pBR322 (BRL). The recombinant plasmids were used to transfect *Escherichia coli* RR1, and the tetracycline-resistant and ampicillin-sensitive colonies were selected.

Differential colony hybridization. Tetracycline-resistant transformants were streaked onto a duplicate set of nitrocellulose filters, amplified, lysed in situ, and screened by differential hybridization (23). One set of filters was probed with ^{32}P-labeled, nick-translated single-stranded cDNA from the resistant line and another set with ^{32}P-labeled single-stranded cDNA from the wild-type cells.

TABLE 1. Properties of histidinol-resistant and wild-type CHO cell lines

Cell line	Selective concn of histidinol (mM)	Sp act (pmol/min per mg)	Increase (×)[a]	D_{10} (mM histidinol)	K_m (μM)	K_i (μM)
wt2	0	262.5		0.057	15	11
wt5	0	155.4		0.058	35	10
2H1	3.2	3,700.0	14.2			
	4.8	5,660.0	21.6			
	14.0	7,692.0	29.0	17.0	28	12
2H2	12.0	7,010.0	26.7			
5H5	8.0	2,651.0	17.0	9.0	10	11
1H3	10.0	3,676.0	23.7	15.5		

[a] Fold increase over that of appropriate parental lines.

Hybrid selection. Purified recombinant plasmid (10 μg) was bound to a 3-mm^2 nitrocellulose filter for hybrid selection of mRNA. The hybridization mix contained 65% formamide, 20 mM PIPES (piperazine-*N*,*N*′-bis[2-ethanesulfonic acid]) at pH 6.4, 0.04 M NaCl, 0.2% SDS, and 60 μg of total RNA from histidinol-resistant cells. Each filter was hybridized individually in a 50-μl volume at 50°C for 3 h. They were then washed 10 times in 65°C with 10 mM Tris hydrochloride (pH 7.6)–0.15 M NaCl–1 mM EDTA–0.5% SDS and twice in the same buffer without SDS. The mRNA selected was eluted by boiling the filter in 300 μl of water with 30 μg of calf liver tRNA (Boehringer Mannheim Biochemicals) for 1 min and quick-freezing in a dry ice-ethanol bath. The sample was thawed in ice, and the filter was removed. After phenol extraction and ethanol precipitation, the mRNA was lyophilized and dissolved in 5 μl of water and translated in a rabbit reticulocyte lysate system (BRL). The translatable products were examined with and without immunoprecipitation by SDS-PAGE and fluorography.

Southern blot analysis of genomic DNA. Genomic DNA from histidinol-resistant and wild-type cells was digested with restriction enzymes (New England BioLabs, Inc.; BRL; or IBI) and separated on 0.6% agarose gels. The DNA was transferred to nitrocellulose filters (S & S Bass) by Southern blotting. The nitrocellulose filters were prehybridized overnight at 65°C in 1× SCP (0.1 M NaCl, 0.03 M Na_2HPO_4, 0.01 M EDTA) adjusted to pH 6.2 with HCl–10× Denhardt solution–1% Sarkosyl(Sigma Chemical Co.) –denatured salmon sperm DNA (50 μg/ml). Hybridization was carried out at 65°C for 18 h in the same solution supplemented with 10% dextran sulfate and nick-translated probe (10^6 cpm/ml). Filters were rinsed in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.1% SDS and washed for 2 h in two changes of 0.1× SSC–0.1% SDS at 65°C. Autoradiography of the filters was carried out at −70°C with XAR-5 Kodak film and an intensifying screen for 1 to 3 days.

RESULTS

Selection and analysis of mutants. First-step mutants selected in the presence of histidinol were found to be about two to four times more resistant to the drug. Preliminary analysis of such isolates indicated that their K_ms and K_is were similar to those of wild-type cells and that their resistance could be attributed to an increased level of activity of histidyl-tRNA synthetase.

By growing the first-step mutants in gradually increasing concentrations of histidinol, more highly histidinol-resistant isolates were obtained. The properties of these isolates (2H1, 5H5, and 1H3) are presented in Table 1. As indicated by the survival curves (Fig. 1), the amplified cell lines were much more resistant than the parental cells with respect to plating efficiency. This is reflected in their D_{10}s, the resistant lines showing increases of about 160- to 290-fold over the wild type lines in this parameter. As expected, these increases in resistance were paralleled by elevations in enzyme activity, although the elevated level of resistance was much greater than that of the enzyme.

Comparisons of K_m and K_i were made for two of the lines, and no change was seen in these parameters (Table 1). Thus, these results indicated that the highly histidinol-resistant cells had probably amplified the gene for histidyl-tRNA synthetase. Experiments were then conducted to obtain further support for the latter view.

Analysis of the synthesis of histidyl-tRNA synthetase in histidinol-resistant and parental CHO cells. The three independently selected histidinol-resistant mutants as well as the wild-type parental cells were labeled with [^{35}S]methionine, and the newly synthesized cellular proteins were analyzed by SDS-PAGE. The results were similar in all isolates, and we describe below (Fig. 2) the data obtained for one mutant. A radioactive polypeptide of 52,000 daltons was very prominent in the resistant cell lysate (Fig. 2A, lane 2) compared to that of a wild-type cell lysate (lane 1). Mathews and Bernstein (14) have reported that sera from 25% of polymyositis patients contain an autoantibody to histidyl-tRNA synthetase. In vivo labeling of HeLa cells followed by immunoprecipitation with patient serum, SDS-PAGE, and fluorography revealed a polypeptide of 50,000 daltons, providing evidence that this polypeptide is a subunit of histidyl-tRNA synthetase (14). It seemed probable, therefore, that the 52K polypeptide observed in the labeling experiment

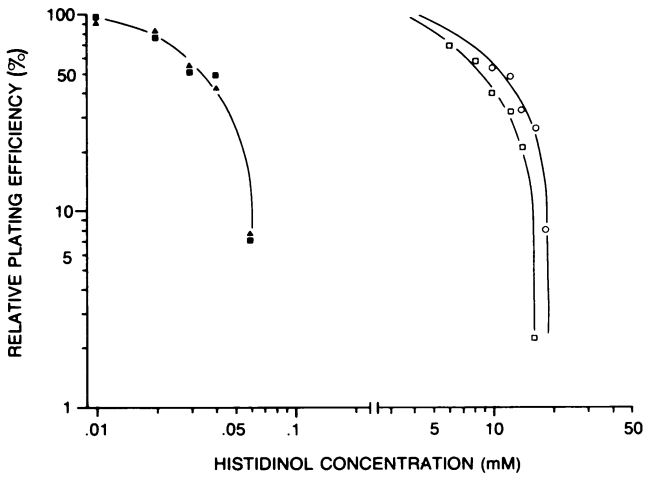

FIG. 1. Relative plating efficiencies in the presence of histidinol. Symbols: ▲, WT2 cells; ■, WT5 cells; ○, 2H2 cells; □, 1H3 cells.

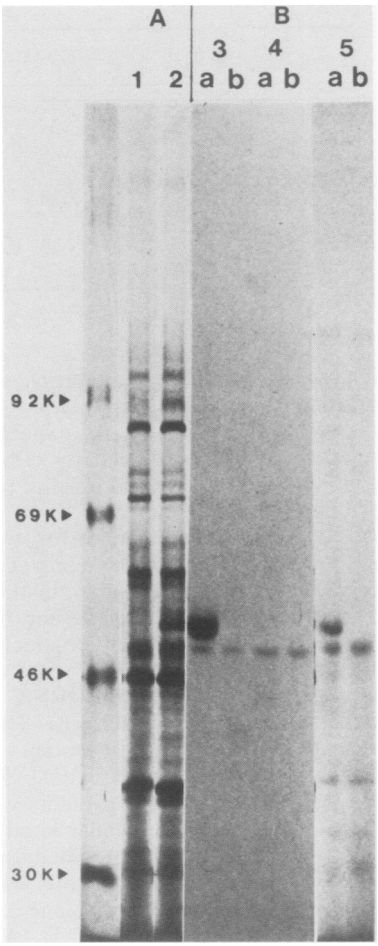

FIG. 2. Synthesis of histidyl-tRNA synthetase in 2H2 and WT2 cells. (A) [^{35}S]methionine-labeled cell lysate analyzed by SDS-PAGE. Lane 1, wild-type cell lysate; lane 2, resistant cell lysate. (B) Immunoprecipitation of labeled cell lysate. Lane 3, resistant cell lysate with (a) polymyositis patient autoantibody and (b) normal human serum; lane 4, wild-type cell lysate with (a) polymyositis patient autoantibody and (b) normal human serum; lane 5, same as in lane 4 except that 20-fold more lysate was used for immunoprecipitation.

described in Fig. 2 was equivalent to the polypeptide described by Mathews and Bernstein. We first checked whether the polymyositis patient autoantibody cross-reacts with CHO histidyl-tRNA synthetase and found that this human autoantibody can specifically inhibit the CHO histidyl-tRNA synthetase activity. We then examined the comparative ability of the autoantibody and normal serum to precipitate the labeled 52K polypeptide in mutant and parental cells. To this end, the [^{35}S]methionine-labeled cell extracts (500,000 cpm per sample) were precipitated with the autoantibody directed against histidyl-tRNA synthetase (a gift from M. Reichlin). As shown in Fig. 2B (lane 3a), the 52,000-dalton band of histidinol-resistant cell lysate was precipitated by the patient serum but not by the normal human serum (Fig. 2B, lane 3b). The autoantibody did not detect the 52K polypeptide when 500,000 cpm of wild-type extracts was used (Fig. 2B, lane 4a). However, the 52,000-dalton polypeptide was detected when much larger quantities (10^7 cpm) of wild-type extract were used (Fig. 2B, lane 5a).

The results described above for the human autoantibody have also been confirmed using a rabbit antibody to the enzyme partially purified as described above. We had previously determined that this antibody inactivates histidyl-tRNA synthetase activity (data not shown).

To obtain a more quantitative assessment of the amounts of protein made in parental and mutant extracts, we assayed the relative amounts of labeled precipitate in the two lines. This analysis showed that the histidyl-tRNA synthetase of wild-type CHO cells constituted 0.03% of the radioactivity in the soluble extract while that of the histidinol-resistant mutants was 0.9 to 1.2%, an increase of 30- to 40-fold. Since the methionine content of the enzyme is not known, the exact percentage of cellular protein which is histidyl-tRNA synthetase cannot be calculated. Using pulse-chase experiments, we found that enzyme turnover in the wild-type and histidinol-resistant cells (data not shown) was similar. It can be concluded that the difference in the amounts of immunoprecipitated enzyme indicates a major difference in the rate of synthesis of histidyl-tRNA synthetase, as would be expected for gene amplification.

Analysis of the level of histidyl-tRNA synthetase mRNA from histidinol-resistant and wild-type cells. Having demonstrated an increase in histidyl-tRNA synthetase in the HisOH^R cells, we then tested whether this was reflected in elevated mRNA levels. The products of poly(A)$^+$ mRNA in vitro translation of histidinol-resistant and wild-type cells were thus analyzed by immunoprecipitation and SDS-PAGE (Fig. 3). The amounts of [^{35}S]methionine incorporated into the translation products were equivalent when using poly(A)$^+$ mRNA derived from wild-type or histidinol-resistant cells. However, there were two bands, one at 52K and the other at 75K, which were much more prominent when we used HisOH^R mRNA (Fig. 3A). Furthermore, when these products were immunoprecipitated by the human polymyositis serum, a clear band was visible at 52K only in the samples from the resistant cells. No equivalent band was observed when using either the polymyositis serum and mRNA from wild-type cells or normal human serum with mRNA from resistant cells. Similar results were obtained with the rabbit antiserum to the enzyme and with the other two highly resistant lines (data not shown). The radioactivity in the immunoprecipitates from histidinol-resistant lines represented about 0.6% of the total trichloroacetic acid-precipitable in vitro translation products. This is about one-half of the percentage of the total protein synthesized, as determined by the [^{35}S]methionine-labeling method described earlier (Fig. 2). This discrepancy is probably due to degradation of mRNA after passage through the oligo(dT) column.

It was evident that another prominent polypeptide (75,000 daltons) could also be observed in the mRNA translation products from resistant cells (Fig. 3). Since this polypeptide was not precipitated by the specific antisera, it is probably not a precursor of histidyl-tRNA synthetase. It therefore represents an unrelated protein, which is overproduced during the process whereby CHO cells acquire resistance to histidinol. The 75K protein does not appear to accumulate cytoplasmically in vivo even if the cells were labeled for an hour, suggesting that it is unstable or rapidly exported.

Isolation of cDNAs for the genes for histidyl-tRNA synthetase and for the 75,000-dalton polypeptide. The demonstration of highly elevated mRNA levels for histidyl-tRNA synthetase and the 75,000-dalton polypeptide provided the opportunity for the isolation of the corresponding cDNAs. The procedure used for these isolations is described above.

The pBR322 recombinant plasmids containing double-stranded cDNA derived from HisOH^R poly(A)$^+$ mRNA (see above) were screened initially by differential colony hybridization.

Approximately 1,000 tetracycline-resistant transformants were streaked onto a duplicate set of nitrocellulose filters. We probed one set of filters with ^{32}P-labeled single-stranded cDNA from the resistant line and another with ^{32}P-labeled single-stranded cDNA from the wild-type parental line, and we screened for colonies hybridizing specifically to the cDNA probe from the resistant line. Two recombinants (Fig. 4) gave a strong positive signal with the cDNA probe from the resistant line and little or no signal with the cDNA probe from wild-type cells.

These two recombinants were shown to contain cDNAs specific to the 52K and 75K polypeptides, using hybrid selection and in vitro translation. The positive isolates, plus a control DNA derived from a pBR322 transformant which did not show any increased signal with cDNA from the resistant line, were first used to select potential specific

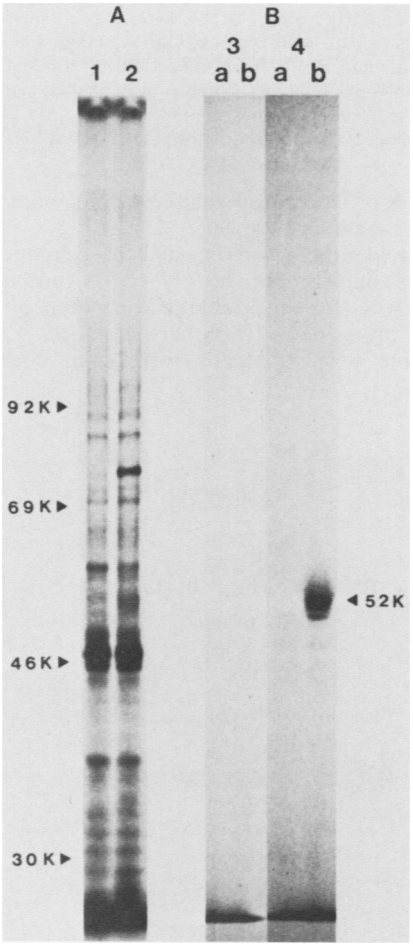

FIG. 3. In vitro translation of poly(A)$^+$ RNA from wild-type and HisOH^R cells. (A) SDS-PAGE analysis of total in vitro-translatable products of poly(A)$^+$ RNA from wild-type (lane 1) and resistant cells (lane 2). (B) Immunoprecipitation of in vitro-translatable products of wild-type cells with normal human serum (3a) and with polymyositis patient autoantibody (3b); immunoprecipitation of in vitro-translatable products of resistant cells with normal human serum (4a) and with polymyositis patient autoantibody (4b).

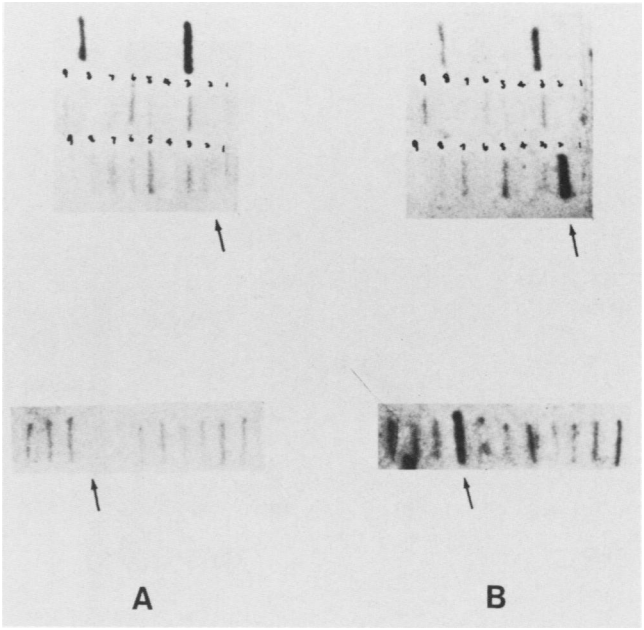

FIG. 4. Screening of recombinants containing cDNAs by differential hybridization. (A) Filters probed with ^{32}P-labeled single-stranded cDNA from wild-type parental cells. (B) Filters probed with ^{32}P-labeled single-stranded cDNA from histidinol-resistant cells.

mRNAs from extracts of histidinol-resistant cells (see above). The resultant mRNAs, separated from the DNA, were then tested for their ability to produce the 52K and 75K (Fig. 5) polypeptides by in vitro translation. The products were examined by SDS-PAGE before (lanes 2 to 5) and after immunoprecipitation with the autoantibody from the polymyositis patient (lane 8) and the rabbit antiserum against CHO histidyl-tRNA synthetase (lane 9). The gels portrayed in lanes 1 and 6 again showed the presence of the 52K and 75K translated products from in vitro translation of mRNA from the HisOH^R cells and indicated the specific precipitation of the 52K polypeptide by the polymyositis antibody. By comparing lanes 4 and 2, it can be seen that one of the RNAs isolated by hybrid selection with one of the recombinant DNAs produced the 52K histidyl-tRNA synthetase in the in vitro translation system. This was confirmed when the products of this RNA were immunoprecipitated by antiserum (lanes 8 and 9). We also performed control experiments by using anti-histidyl-tRNA synthetase (both human and rabbit antibodies) to precipitate the translation products in lanes 2, 3, and 5 and in all cases failed to precipitate the 52,000-dalton band. The second recombinant cDNA clone selected an RNA which produced the 75K polypeptide by in vitro translation (lane 2).

These results provide additional evidence that the HisOH^R isolates contain an amplified region of their genome and that the cDNA recombinant clones reflect increased copies of the gene for histidyl-tRNA synthetase and another gene coding for a 75K polypeptide.

We also determined the sizes of the inserts in the cDNA clones by preparing plasmid DNA samples, digesting them with *Pst*I, and analyzing them on a 1.2% agarose gel. From this analysis we found that the recombinant plasmid coding for histidyl-tRNA synthetase contains an insert of 250 base pairs, while the transformant coding for the 75,000-dalton

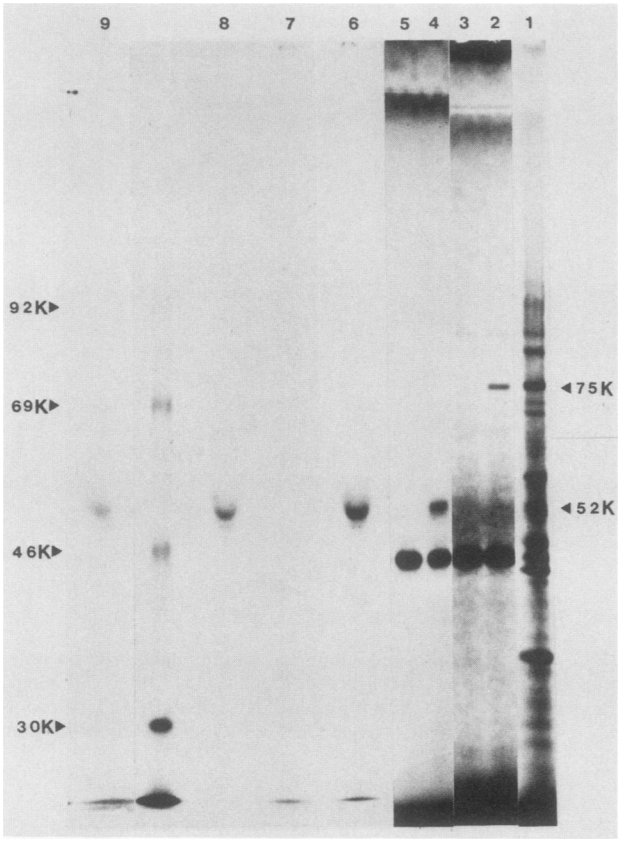

FIG. 5. In vitro translation of RNA isolated from hybrid selection using recombinants containing cDNAs from HisOH^R cells. Lanes: 1, total in vitro translation products; 2 and 4, translation products of RNA from hybrid selection with cDNA containing recombinants; 3 and 5, translation products of RNA from control plasmid; 6, immunoprecipitation of products shown in lane 1 by autoimmune serum; 7, immunoprecipitation of products shown in lane 5 by autoimmune serum; 8 and 9, products shown in lane 4 precipitated by autoimmune serum and rabbit anti-histidyl-tRNA synthetase, respectively.

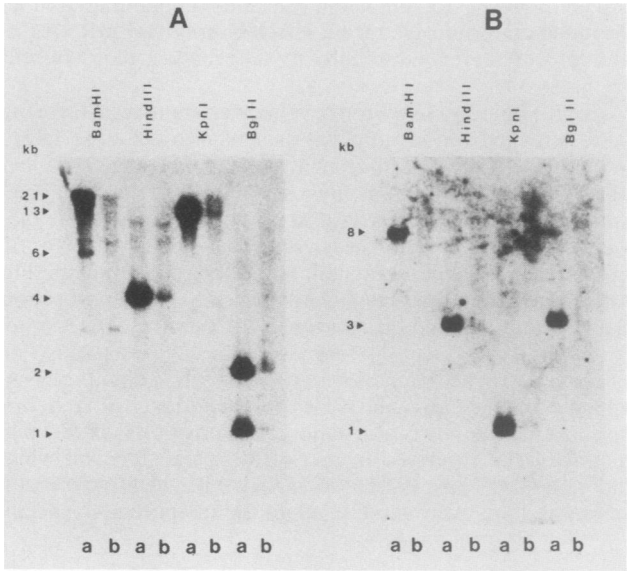

FIG. 6. Detection of genomic sequences of (A) 75,000-dalton polypeptide and (B) histidyl-tRNA synthetase. Genomic DNA of resistant cells (a) and wild-type cells (b) was digested with *Bam*HI, *Hin*dIII, *Kpn*I, and *Bgl*II, fractionated on a 0.6% agarose gel, and transferred to nitrocellulose. Filter A was probed with ^{32}P-labeled cDNA for the 75,000-dalton polypeptide, and filter B was probed with ^{32}P-labeled cDNA for the histidyl-tRNA synthetase.

polypeptide contains an insert of 350 base pairs. The frequency of recombinant transformants for both genes was found to be 1/1,000.

Amplification of the genes for histidyl-tRNA synthetase and the 75,000-dalton polypeptide in histidinol-resistant CHO lines. The conclusive demonstration that gene amplification had occured in HisOH^R cells was obtained by Southern and Northern blot analysis of resistant and wild-type cells. The comparative Southern blots of genomic DNA from histidinol-resistant and wild-type cells, after digestion with different restriction enzymes, are shown in Fig. 6. One set of such electrophoretograms was examined with the ^{32}P-labeled probe for histidyl-tRNA synthetase, and the duplicate gels were probed with the [^{32}P]cDNA recombinant for the 75,000-dalton polypeptide. In all cases, the genomic DNA derived from HisOH^R cells showed considerable amplification of hybridizable DNA compared to DNA derived from wild-type cells. The data in Fig. 6 were obtained with line 2H2, but similar results were found with the other two HisOH^R isolates (data not shown).

An estimate of the level of amplification of the two genes was obtained by comparing the intensities of signals from a serial dilution of DNA from the resistant cells and from a fixed amount of DNA from wild-type cells, both electrophoresed in the same agarose gel.

For this experiment, we digested the genomic DNA with *Xba*I for the analysis of the histidyl-tRNA synthetase (Fig. 7) and with *Eco*RI for the 75,000-dalton polypeptide (Fig. 8). The signal intensities of Southern blots of 5 μg of DNA derived from wild-type cells were then compared with

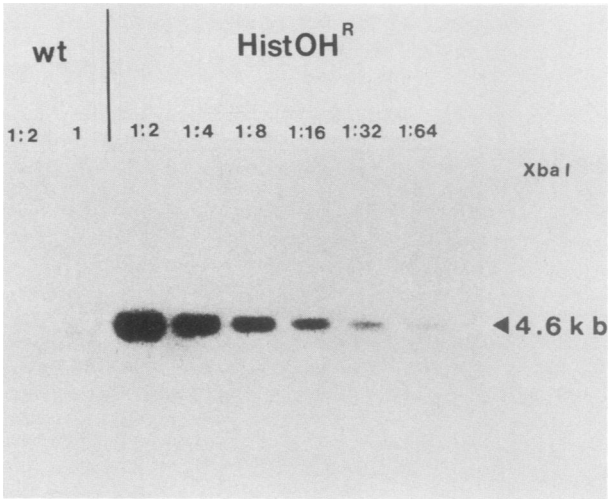

FIG. 7. Hybridization of cDNA for histidyl-tRNA synthetase to genomic DNA from histidinol-resistant cells and wild-type cells. Genomic DNA was serially diluted and digested with *Xba*I. The indicated dilutions of DNA were subjected to electrophoresis in 0.6% agarose gel. The DNA was transferred to nitrocellulose and hybridized with the 250-base-pair cDNA fragment for the histidyl-tRNA synthetase.

several dilutions of the DNA from the HisOH^R mutants. In both cases, the intensities were similar when the DNA from the resistant cells was diluted 1/32. This result is in agreement with the level of amplification determined from the enzyme analysis (Table 1).

We also examined the mRNA levels of resistant and wild-type isolates, using Northern analysis (Fig. 9), again by comparing the amounts of 1 μg of mRNA from wild-type cells with serial dilutions derived from 1 μg of mutant cell mRNA. Unexpectedly, the two cDNA probes identified an mRNA of similar molecular size (2.5 kilobases), but in both cases the extent of amplification was about 30-fold. The fact that the cDNA for histidyl-tRNA synthetase identifies an mRNA much longer than necessary to code for a 52,000-dalton polypeptide indicates that the mRNA for this gene has a relatively long 3′ or 5′ untranslated region.

DISCUSSION

In this study we have shown that CHO cells highly resistant to histidinol contain elevated levels of histidyl-tRNA synthetase, with kinetic properties unchanged from that of the parental line. This is reflected in corresponding elevations in the amount of a 52,000-dalton protein, in mRNA coding for this protein, and in specific genomic DNA sequences. In addition, we found that HisOH^R cell lines exhibit karyotypic alterations involving chromosome 2, such as homogeneously staining regions (HSRs) and extra number 2 chromosomes (unpublished observations). All of these characteristics indicate that this system provides another example of the ability of cells to acquire drug resistance due to amplification of genetic material.

There are several aspects of this system which are of particular interest. Since in vitro ^{35}S labeling indicated that histidyl-tRNA synthetase was the major protein overproduced in the HisOH^R cell lines, we were surprised to find that the products of in vitro translation of mRNA from these cells showed that an additional protein of 75,000 daltons was also overexpressed. The 75K protein does not appear to accumulate cytoplasmically in vivo, suggesting that it is unstable or rapidly exported. This protein does not cross-react with antibody to histidyl-tRNA synthetase, and molecular studies using longer cDNA to the 52K polypeptide and cDNA to the 75K protein indicate that they are not related genes (unpublished observations). The finding that another gene is coamplified along with the gene for histidyl-tRNA synthetase is not unusual. Unselected genes are often coamplified with the gene of interest if they are located nearby and comprise part of the amplification unit. Padgett et al. (15) have shown that three transcriptionally active genes are coamplified along with the CAD gene (carbamylphosphate synthetase, aspartate transcarbamylase, and dihydroorotase) and Debatisse et al. (6) found several unrelated proteins coamplified in adenylate deaminase-overproducing cells.

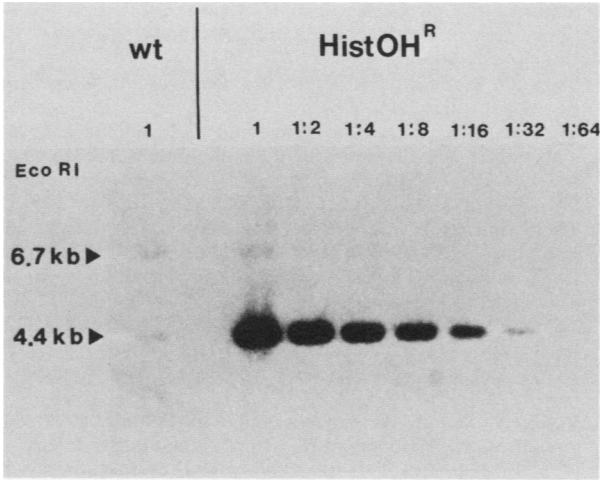

FIG. 8. Hybridization of cDNA for the 75,000-dalton polypeptide to genomic DNA from histidinol-resistant cells and wild-type cells. DNA was serially diluted and digested with *Eco*RI. The indicated dilutions of DNA were subjected to electrophoresis in a 0.6% agarose gel. The DNA was transferred to nitrocellulose and hybridized with the 350-base-pair cDNA fragment for the 75,000-dalton polypeptide.

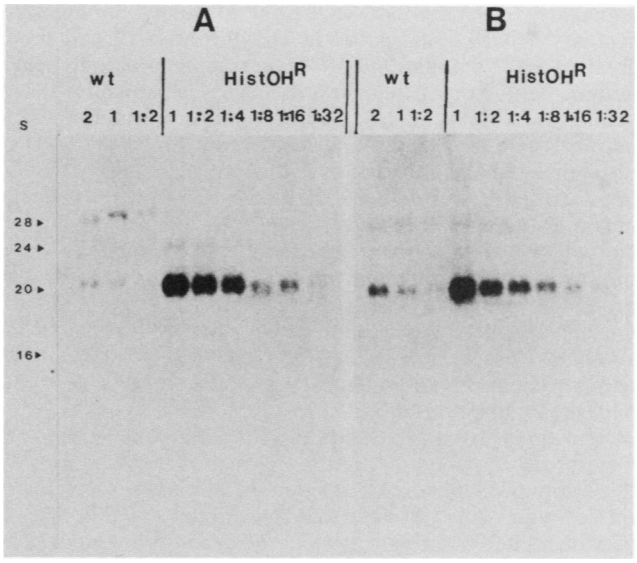

FIG. 9. Northern blot analysis of (A) mRNA for 75,000-dalton polypeptide and (B) mRNA for histidyl-tRNA synthetase from histidinol-resistant cells and wild-type cells. mRNA was serially diluted and subjected to electrophoresis in a 1.5% RNA-formaldehyde gel. The mRNA was transferred to nitrocellulose and hybridized with (A) cDNA for 75,000-dalton polypeptide and (B) cDNA for histidyl-tRNA synthetase.

In other experiments we have found that tyrosinol-resistant CHO cells also contain increased levels of the target enzyme, tyrosyl-tRNA synthetase, and an HSR (unpublished results). Other than the fact that gene amplification has also probably occurred in such cells, it is intriguing that the HSRs are found both in this case and in the HisOH^R cells on chromosome number 2 of the CHO cell. Although it will be necessary to prove the location of the genes for the two tRNA synthetases (e.g., by in situ hybridization), other amplified genes have been localized to HSRs (16, 19). Since the gene for leucyl-tRNA synthetase is also on chromosome 2, this would place at least 3 of the genes for the CHO aminoacyl-tRNA synthetases on one chromosome.

CHO cell lines which overproduce other aminoacyl-tRNA synthetases have been isolated previously. Overexpression of threonyl- and asparaginyl-tRNA synthetase activites has been shown to occur in cells resistant to borrelidin (7) and revertants of temperature-sensitive mutants (5), respectively, suggesting that these genes have been amplified in response to selective pressure. However, our experiments provide the first example of the cloning of a mammalian-cell

aminoacyl-tRNA synthetase. Unlike for many amplification systems in which increases in enzyme activity can be as great as 300- to 1,000-fold, we have not been able to select mutants with greater than 30-fold increases in histidyl-tRNA synthetase activity. It seems likely that too much enzyme may be toxic to the cell, and this in turn limits the level of resistance which can be achieved. Gerken and Arfin (7) obtained similar results with borrelidin resistance, where the maximal increase in threonyl-tRNA synthetase activity is 20-fold. In this case the cells produce an overabundance of threonyl-tRNA synthetase protein of up to 100-fold. The protein is phosphorylated (8), and this may modulate the enzyme activity. We do not know whether histidyl-tRNA synthetase is phosphorylated in CHO cells, but Mathews and Bernstein (14) have suggested that the HeLa cell enzyme may be a phosphoprotein.

Some polymyositis patients carry autoimmune antibody to histidyl-tRNA synthetase. Although several explanations for this finding can be advanced since the antibody is specific to this enzyme, it is possible that the etiology of this disease may be related to amplification of the gene for histidyl-tRNA synthetase. Experiments to test this hypothesis are in progress.

We are now using the cDNA probe for histidyl-tRNA synthetase to isolate full-length cDNAs from larger libraries to examine both the structure of the gene for histidyl-tRNA synthetase and the nature of the amplification process in $HisOH^R$ CHO cells. It should also be possible to isolate cDNA probes from the tyrosinol-resistant cells, to examine the structure of this gene, and then to compare the structure of two aminoacyl-tRNA synthetase genes in mammalian cells. Furthermore, similar comparative studies should be possible in near-diploid human cells, since we have also isolated histidinol-resistant mutants in the human HT1080 cell line.

ACKNOWLEDGMENTS

This work was supported by the Medical Research Council and National Cancer Institute of Canada. F.T. is a recipient of an Ontario Graduate Scholarship. I.L.A. was a recipient of a Public Health Service fellowship from the National Institutes of Health.

We thank Morris Reichlin and Edward Keystone for their gifts of autoimmune sera from polymyositis patients and L. C. Tsui for his advice in cDNA cloning.

LITERATURE CITED

1. **Adair, G. M., L. H. Thompson, and P. A. Lindl.** 1978. Six complementation classes of conditionally lethal protein synthesis mutants of CHO cells selected by ^{3}H-amino acid. Somatic Cell Genet. **4**:27–44.
2. **Andrulis, I. L., C. S. Chiang, S. M. Arfin, T. A. Miner, and G. W. Hatfield.** 1978. Biochemical characterization of a mutant asparaginyl-tRNA synthetase from Chinese hamster ovary cells. J. Biol. Chem. **253**:58–62.
3. **Ashman, C. R.** 1978. Mutations in the structural genes of CHO cell histidyl-, valyl- and leucyl-tRNA synthetase. Somatic Cell Genet. **4**:299–312.

4a. **Chirgwin, J. M., A. E. Przybyla, R. J. MacDonald, and W. J. Rutter.** 1979. Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. Biochemistry **18**:5294–5299.

4. **Bonner, W. M., and R. A. Laskey.** 1974. A film detection method for the tritium-labelled proteins and nucleic acids in polyacrylamide gels. J. Biochem. **46**:83–88.
5. **Cirullo, R. E., and J. J. Wasmuth.** 1984. Isolation of Chinese hamster ovary cells that overproduce asparaginyl-tRNA synthetase. Mol. Cell. Biol. **4**:1939–1941.
6. **Debatisse, M., B. R. de Saint Vincent, and G. Buttin.** 1984. Expression of several amplified genes in an adenylate-deaminase overproducing variant of Chinese hamster fibroblasts. EMBO J. **3**:3123–3127.
7. **Gerken, S. C., and S. M. Arfin.** 1984. Chinese hamster ovary cells resistant to borrelidin overproduce threonyl-tRNA synthetase. J. Biol. Chem. **259**:9202–9206.
8. **Gerken, S. C., and S. M. Arfin.** 1984. Threonyl-tRNA synthetase from Chinese hamster ovary cells is phosphorylated on serine. J. Biol. Chem. **259**:11160–11161.
9. **Gottesman, M. M.** 1985. Lineages of Chinese hamster cell lines, p. 883–885. *In* M. M. Gottesman (ed.), Molecular cell genetics. Wiley-Interscience, New York.
10. **Kessler, S. W.** 1976. Cell membrane antigen isolation with the staphylococcal protein antibody adsorbent. J. Immunol. **117**:1482–1490.
11. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
12. **Lehrach, H., A. M. Frischauf, D. Hanahan, J. Wozney, F. Fuller, and H. Boldtker.** 1979. Construction and characterization of proα 1 collagen complementary deoxyribonucleic acid clones. Biochemistry **18**:3146–3152.
13. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**:265–275.
14. **Mathews, M. B., and R. M. Bernstein.** 1983. Myositis autoantibody inhibits histidyl-tRNA synthetase: a model for autoimmunity. Nature (London) **304**:177–179.
15. **Padgett, R. A., G. M. Wahl, P. F. Coleman, and G. R. Stark.** 1979. *N*-(Phosphonacetyl)-L-aspartate-resistant hamster cells overaccumulate a single mRNA coding for the multifunctional protein that catalyzes the first steps of UMP synthesis. J. Biol. Chem. **254**:974–980.
16. **Schimke, R. T.** 1984. Gene amplification in cultured animal cells. Cell **37**:705–713.
17. **Scornik, O. A., M. L. S. Ledbetter, and J. S. Malter.** 1980. Role of amino-acylation of histidyl-tRNA in the regulation of protein degradation in Chinese hamster ovary cells. J. Biol. Chem. **255**:6322–6329.
18. **Stanners, C., G. Elicieri, and H. Green.** 1971. Two types of ribosome in mouse-hamster hybrid cells. Nature (London) New Biol. **230**:52–54.
19. **Stark, G. R., and G. M. Wahl.** 1984. Gene amplification. Annu. Rev. Biochem. **53**:447–491.
20. **Thompson, L. H., J. L. Harkins, and C. P. Stanners.** 1973. A mammalian cell mutant with a temperature sensitive leucyl-transfer RNA synthetase. Proc. Natl. Acad. Sci. U.S.A. **70**:3094–3098.
21. **Thompson, L. H., D. H. Lofgren, and G. M. Adair.** 1978. Evidence for structural gene alterations affecting aminoacyl-tRNA synthetases in CHO cell mutants and revertants. Somatic Cell Genet. **4**:423–435.
22. **Thompson, L. H., C. P. Stanners, and L. Siminovitch.** 1975. Selection by ^{3}H-amino acids of CHO-cell mutants with altered leucyl- and asparagyl-transfer RNA synthetases. Somatic Cell Genet. **1**:187–208.
23. **Wahl, G. M., R. A. Padgett, and G. R. Stark.** 1979. Gene amplification causes overproduction of the first three enzymes of UMP synthesis in *N*-(phosphonacetyl)-L-aspartate-resistant hamster cells. J. Biol. Chem. **254**:8679–8689.