# Cysteine Auxotrophy of Human Leukemic Lymphoblasts Is Associated with Decreased Amounts of Intracellular Cystathionase Messenger Ribonucleic Acid†

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ABSTRACT: A series of human lymphoblastoid cell lines derived from nonleukemic donors are known to be cysteine prototrophs (*cys*$^+$), while several lymphoblastoid lines derived from leukemic donors are cysteine auxotrophs (*cys*$^-$). Compared to the *cys*$^+$ lines, the cysteine auxotrophic cell lines have been found to contain reduced amounts of cystathionase, the enzyme which catalyzes the conversion of cystathionine to cysteine, the terminal step in the cysteine biosynthetic pathway. We have developed a semiquantitative in vitro translation assay for the messenger ribonucleic acid (mRNA) which encodes cystathionase and have tested two pairs of *cys*$^-$, cystathionase$^-$ and *cys*$^+$, cystathionase$^+$ cell lines for their intracellular content of this RNA. There is a close correlation between the *cys*$^-$ phenotype, reduced intracellular cystathionase content, and the intracellular content of enzyme-specific messenger RNA. Thus, the absolute cysteine requirement of the human leukemic, *cys*$^-$, cystathionase$^-$ cell lines tested is likely due to an intracellular reduction in the functional messenger RNA coding for cystathionase. Among the possibilities that could account for these phenomena are (1) a reduction in the rate of transcription of the cystathionase mRNA precursor, (2) an alteration in the nuclear and/or cytoplasmic processing of the primary transcripts, or (3) an alteration in the mRNA structure, resulting in a decreased efficiency of translation.

Previous studies have shown that some leukemic cells derived from human peripheral blood have markedly different requirements for specific amino acids when compared to their nonleukemic counterparts (Ohnuma et al., 1971). It is also known that certain human and rodent hematopoietic tumor cell lines are auxotrophic for L-cysteine (*cys*$^-$) when compared to nontumored cell lines of similar origin (Livingston et al., 1976; Iglehart et al., 1977). Furthermore, extracts of these leukemic (*cys*$^-$) cell lines are characterized by markedly reduced levels of detectable cystathionase activity and immunoprecipitable $M_r$ 43 000 cystathionase when compared to their nonleukemic counterparts (Glode et al., 1980). As a result, the *cys*$^+$ lines are capable of normal growth in the absence of preformed cysteine when cystathionine is present in the medium, while *cys*$^-$ cells are growth-arrested under these conditions. Results of such experiments complement the in vivo observations that a reduction in murine thymocyte cystathionase activity occurs during thymic leukemogenesis whether induced by exogenous administration of a type C virus or arising spontaneously, as in the case of the AKR mouse (Livingston et al., 1976). Additional evidence suggests that extracts of many fresh human lymphoid and myeloid leukemic cell packs, obtained from peripheral blood or by bone marrow aspiration, contain less cystathionase enzyme activity than extracts of their normal mononuclear cell counterparts (Glode et al., 1979). Thus, there is a strong correlation between the presence of the leukemic phenotype and a decrease in intracellular cystathionase concentration. In an attempt to understand the molecular basis for the apparent linkage between these properties, we have begun to examine the biochemical basis for the cystathionase depletion phenomenon (Livingston et al., 1976; Iglehart et al., 1977; Glode et al., 1980). Biochemical studies of *cys*$^+$ and *cys*$^-$ human cell lines have suggested a defect in synthesis of this enzyme in the depleted cell populations (Glode et al., 1980; L. M. Glode and D. M. Livingston, unpublished observations). These observations have led us to ask whether the intracellular cystathionase depletion phenomenon might be due to a reduction in the intracellular concentration of the messenger RNA[1] encoding this protein. In this regard, we have developed a semiquantitative, in vitro translation assay for cystathionase messenger RNA derived from a variety of tissue and cultured cell sources. In testing representative *cys*$^+$, cystathionase$^+$, and *cys*$^-$, cystathionase$^-$ cell lines, we have found that the leukemic phenotype, cysteine auxotrophy, and cystathionase depletion all correlate with a major reduction in the intracellular concentration of functional, cystathionase messenger RNA. Thus, the absolute cysteine requirement of those leukemic, *cys*$^-$, cell lines tested is likely due, at least in part, to an intracellular reduction in the messenger RNA encoding this protein.

## Experimental Procedures

*Cells and Tissues.* Sprague–Dawley rat liver poly(A)-containing RNA was isolated from freshly killed animals. The origin characteristics and methods of cultivation of the various human lymphoid cell lines employed in these experiments have been reported elsewhere (Glode et al., 1980). In all experiments designed to quantitate the cystathionase messenger RNA content of cell lines, 8 L of cells were grown to a concentration of $1.5 \times 10^6$ cells/mL in plastic roller bottles and subsequently harvested at 1000*g* with a Szent-Gyorgyi continuous flow device attached to an SS-34 rotor at 4 °C.

*Preparation of Poly(A)-Containing RNA from Tissues and Cells.* All operations were performed at 4 °C unless otherwise noted, and all glassware was heat-baked. Fresh livers and

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[1] Abbreviations used: RNA, ribonucleic acid; mRNA, messenger RNA; poly(A), poly(adenylic acid); Tris, 2-amino-2-(hydroxymethyl)-1,3-propanediol; EDTA, ethylenediaminetetraacetic acid; $NaDodSO_4$, sodium dodecyl sulfate; $Cl_3CCOOH$, trichloroacetic acid.

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lymphoblast pools were weighed and immediately suspended in 5 volumes (v/w) of homogenization buffer (0.1 M Tris-HCl, pH 9.0, 0.1 M NaCl, and 1 mM EDTA). The tissue was homogenized for 30 s at top speed in a Sorvall OmniMixer at 0 °C, after which the homogenate was transferred to 30-mL Corex tubes; these were centrifuged at 9000 rpm in an SS-34 rotor at 4 °C for 10 min to remove cellular debris. The supernatant fraction was removed, and the pellet and lipid layer discarded. The supernatant fraction was made 10% in sodium dodecyl sulfate ($NaDodSO_4$) and subsequently extracted with an equal volume of phenol–chloroform–isoamyl alcohol (50:50:1) equilibrated with homogenization buffer at 25 °C until the interphase was clear. The aqueous fraction was removed, made 0.3 M in NaCl, mixed with 2.5 volumes of absolute ethanol, and allowed to precipitate at −20 °C overnight. Poly(A)-containing RNA was prepared by a batch procedure. The pellet was dissolved in high-salt binding buffer (0.5 M NaCl and 0.01 M Tris-HCl, pH 7.0) at a concentration of 20 mg/mL. Oligo(dT)–cellulose type T-2 (Collaborative Research, Waltham, MA) was added in quantities sufficient to bind all of the poly(A)-containing RNA (which constituted 2–4% of the total RNA) in the preparation. The suspension was then heated at 65 °C for 5 min, cooled, and gently shaken for 10 min, allowing the poly(A)-containing RNA to bind to the cellulose. The suspension was then spun at 500*g* to pellet the cellulose RNA complexes. The pellet was washed with 10 volumes of high-salt binding buffer and the RNA eluted with 3 pellet volumes of low-salt elution buffer (0.1 M NaCl and 0.01 Tris-HCl, pH 7.0). Any remaining poly(A)-containing RNA was eluted with an additional 2 volumes of this buffer. After pooling the two eluates, 0.1 volumes of 10 times concentrated high-salt binding buffer was added and the entire procedure repeated. The final poly(A)-containing RNA fraction was made 0.3 M in NaCl and ethanol precipitated. It was then resuspended in $dH_2O$ at a concentration of 3 mg/mL. At this point, the RNA was ready for further use.

In a typical experiment, 17–20 g of rat liver yielded ~0.39 g of total RNA from which ~7.8 mg of poly(A)-containing RNA was isolated. However, the RNA yield of various human lymphoblastoid cell lines was lower. In a typical experiment, 12 g of cells yielded ~80 mg of total RNA from which ~1.6 mg of poly(A)-containing RNA was isolated.

*Sedimentation Velocity Analysis of Poly(A)-Containing RNA Molecules in Nondenaturing Density Gradients.* In experiments designed to determine the sedimentation coefficient of the messenger RNA coding for cystathionase, poly-(A)-containing RNA prepared in the manner described above was incubated at 65 °C for 3 min and then subjected to centrifugation at 150000*g* in 5–20% sucrose gradients (total volume = 5 mL) prepared in 100 mM NaCl, 10 mM Tris-HCl, pH 7.4, 1 mM EDTA, and 0.1% $NaDodSO_4$. Gradients were poured in nitrocellulose tubes and were centrifuged in an SW 50.1 rotor at 20 °C for 4 h. Tritiated 16S and 23S ribosomal RNA markers were subjected to centrifugation in an identical manner in a parallel tube. After needle puncture, 15-drop fractions were collected in plastic, 1.5-mL microcentrifuge tubes from the bottom of each centrifuge tube. The RNA in each fraction was ethanol precipitated and dried and was then ready for further experimental manipulation.

*In Vitro Translation Assay for Cystathionase Messenger RNA.* Poly(A)-containing RNA preparations were translated in a micrococcal nuclease digested, rabbit reticulocyte lysate according to Pelham & Jackson (1976). Each reaction mixture was supplemented with [$^{35}$S]methionine [60 μL of [$^{35}$S]methionine (5 mCi/mL; sp activity > 400 Ci/mmol)/mL], and the translation reaction mixtures were incubated at 31 °C for 1 h. Incorporation of [$^{35}$S]methionine into trichloroacetic acid precipitable polypeptide was measured as described previously (Glode et al., 1980).

*Sera.* Rabbit antisera raised against murine liver cystathionase (Bikel et al., 1978b) and against human cystathionase (Glode et al., 1980) were utilized in immunoprecipitation experiments designed to identify or quantitate the messenger RNA encoding cystathionase.

*Immunoprecipitation Analysis of in Vitro Translation Reaction Mixtures.* In immunoprecipitation experiments designed to detect the translation products of liver cystathionase messenger RNA, synthetic reaction mixtures (50 μL) were added to 9 volumes of lysing buffer (20 mM Tris-HCl, pH 9.0, 137 mM NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 10% glycerol, and 1% NP40). These mixtures were divided in half, and 10 μL of normal rabbit serum and 50 μL of a 50% suspension of protein A–Sepharose beads (Pharmacia) were added to each. The tubes were agitated for 1 h at 4 °C when the bead–immune complexes were removed by centrifugation at 6000*g*. Then 10 μL of either normal rabbit serum or anti-mouse cystathionase serum and another 50 μL aliquot of protein A–Sepharose beads were added to each supernatant. After mixing for 1 h at 4 °C, the bead–immune complexes were washed and eluted as described previously (Glode et al., 1980).

In immunoprecipitation experiments designed to quantitate the translation products of cystathionase messenger RNA derived from *cys*$^+$ and *cys*$^-$ human lymphoblast cell lines, volumes of translation reactions predetermined to contain equivalent amounts of in vitro synthesized polypeptides were brought to a final volume of 5 mL with lysing buffer. Quantitation of newly synthesized protein was made by $Cl_3$-CCOOH precipitation of a standard aliquot of each reaction mixture. Then each mixture was divided in half, and 10 μL of normal rabbit serum, 10 μL of normal lamb serum, and 50 μL of a 50% (v/v) suspension of protein A–Sepharose beads were added to each. The tubes were mixed for 1 h at 4 °C and the bead–immune complexes removed by centrifugation at 6000*g*. This procedure was repeated twice, and, then, either 3 μL of normal rabbit serum (control) or 3 μL of rabbit anti-human cystathionase serum was added together with another aliquot of protein A–Sepharose beads for 12–14 h (with continuous mixing) at 4 °C, after which the bead–immune complexes were processed as described above.

*One-Dimensional Gel Electrophoresis.* Gel electrophoresis was performed in 12% $NaDodSO_4$–polyacrylamide slab gels (15 × 20 × 0.15 cm) with 4% polyacrylamide stacking gels according to Laemmli (1970). Destained gels were subjected to autofluorography (Bonner & Laskey, 1974), and, where required, the films were subjected to densitometric analysis.

*Two-Dimensional Gel Electrophoresis.* Two-dimensional gel electrophoresis was performed by the nonequilibrium technique, as described by Crawford & O'Farrell (1979). A 12.5% polyacrylamide slab running gel was utilized in the second dimension. Gels were stained with Coomassie brillant blue and destained as described above.

## Results

*In Vitro Translation of Rat and Human Cystathionase mRNA.* Poly(A)-containing RNA, prepared from rat tissues or from human cells as described under Experimental Procedures, was translated in vitro. Labeled rat cystathionase was precipitated with anti-mouse cystathionase serum from rat mRNA programed translation mixtures (Bikel et al., 1978b) and subjected to electrophoresis in a 12% NaDod-

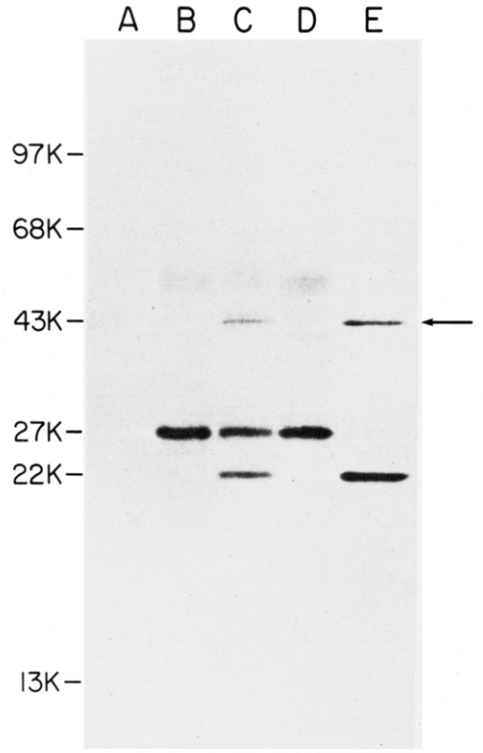


FIGURE 1: Immunoprecipitation of rat liver cystathionase synthesized in vitro. Rat liver poly(A)-containing RNA was translated in vitro in the presence of [$^{35}$S]methionine. The synthetic products (1.5 × $10^6$ cpm) were immunoprecipitated with rabbit anti-murine cystathionase serum as described below. Immune complexes were electrophoresed in a 12% $NaDodSO_4$–polyacrylamide gel, as described under Experimental Procedures, and the dried, fluorographed gel was exposed to Kodak XR-1 X-ray film. The autofluorogram of that gel is shown above. (Slot A) Products of a reaction mixture (50 μL) without added RNA, immunoprecipitated with 5 μL of anti-cystathionase serum. (Slot B) Products of a reaction mixture (50 μL) containing 1 μg of rat liver of poly(A)-containing RNA immunoprecipitated with 5 μL of preimmune serum. (Slot C) Products of a reaction mixture (50 μL) containing 1 μg of anti-cystathionase serum. (Slot D) Products of a reaction mixture (50 μL) containing 1 μg of rat liver poly(A)-containing RNA pretreated with 5 μL of preimmune serum, immunoprecipitated with 5 μL of preimmune serum. (Slot E) Products of a reaction mixture (50 μL) containing 1 μg of rat liver poly(A)-containing RNA pretreated with 5 μL of preimmune serum, immunoprecipitated with 5 μL of anti-cystathionase serum.

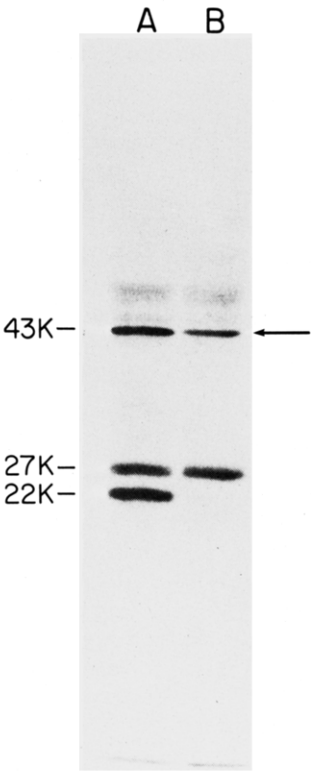


FIGURE 2: Immunoprecipitation of in vitro synthesized rat liver cystathionase by two different anti-mouse–rat serum lots. Poly-(A)-containing RNA isolated from rat liver was translated in vitro in the presence of [$^{35}$S]methionine. The synthetic products (1.5 × $10^6$ cpm) were immunoprecipitated with two different lots of rabbit anti-murine cystathionase serum as described below. Immune complexes were subjected to electrophoresis in a 12% $NaDodSO_4$–polyacrylamide gel. The autofluorogram of that gel is shown above. (Slot A) Products of a reaction mixture (50 μL) containing 1 μg of rat liver poly(A)-containing RNA, immunoprecipitated with 5 μL of anti-cystathionase serum, lot 1. (Slot B) Products of a reaction mixture (50 μL) containing 1 μg of rat liver poly(α)-containing RNA, immunoprecipitated with 5 μL of anti-cystathionase serum, lot 2.

$SO_4$–polyacrylamide gel. The autofluorogram of that gel (Figure 1) reveals the presence of three polypeptides: $M_r$ 43 000, $M_r$ 27 000,and $M_r$ 22 000. The $M_r$ 43 000 band comigrated with authentic, purified rat cystathionase, and purified rat liver cystathionase competed with this molecule for binding to specific anti-cystathionase antibody (data not shown). The $M_r$ 27 000 band was also present in the nonimmune serum precipitate and was, therefore, precipitated in a nonspecific fashion. The $M_r$ 22 000 band was immunoprecipitated by some but not all pools of rabbit anti-murine cystathionase serum (Figure 2).

When an anti-cystathionase immunoprecipitate of a reaction mixture programed with rat liver poly(A) RNA was subjected to two-dimensional gel electrophoresis, the $M_r$ 43 000 species comigrated with purified rat liver cystathionase in both the isoelectric and molecular weight dimensions (Figure 3). Therefore, we conclude that the labeled $M_r$ 43 000 polypeptide shares similar immunologic and biochemical characteristics with authentic rat cystathionase and is likely intact rat cystathionase.

When a preparation of poly(A)-containing RNA derived from rat liver was subjected to ultracentrifugation in a nondenaturing sucrose gradient and the separated RNA species translated in vitro, the result shown in Figure 4 was obtained. $M_r$ 43 000 rat cystathionase mRNA migrated as a 13–15S structure. Of additional interest was the finding that the mRNA encoding the $M_r$ 22 000 protein migrated more slowly than cystathionase mRNA and had an approximate *s* value of 10–12S (Figure 4). Because it was nonspecifically precipitated, the $M_r$ 27 000 band was not of concern here. Total [$^{35}$S]methionine incorporated into newly synthesized protein in response to mRNA from fractions across the gradient was measured and compared with the amount of [$^{35}$S]methionine incorporated in response to the RNA in fractions 14 and 15, which contain the cystathionase messenger RNA. From these data, a rough estimate of the degree of purification of the cystathionase messenger RNA was obtained, i.e., ≥19-fold.

Having the ability to identify functional cystathionase mRNA in a clear and reproducible fashion from rodent tissue which is among the richest source of the enzyme (Mudd et al., 1965), we attempted to use this approach to assess the relative cystathionase mRNA concentrations in *cys*$^-$ and *cys*$^+$ human lymphoid cell lines. Total poly(A)-containing RNA was isolated from two human *cys*$^+$ lymphoid lines (NC37 and SB) grown in parallel. Immunoprecipitation of [$^{35}$S]-methionine-labeled protein, synthesized in reaction mixtures programed by these RNA preparations, was then performed. In these experiments, rabbit anti-human cystathionase serum,

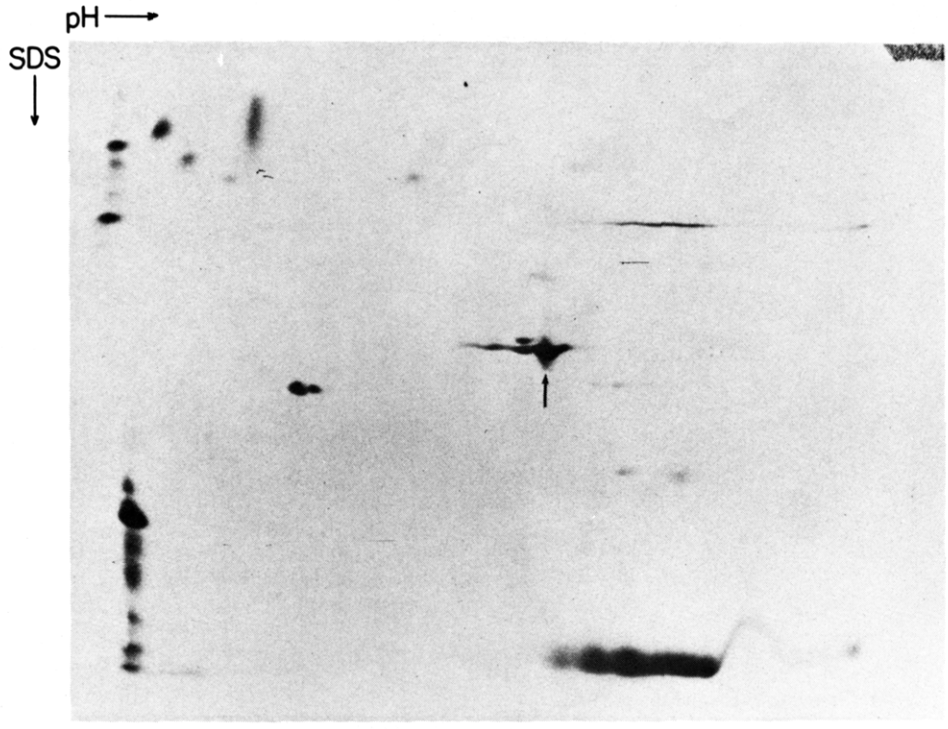


FIGURE 3: Two-dimensional gel electrophoresis of in vitro synthesized rat liver cystathionase. Rat liver poly(A)-containing RNA was translated in vitro in the presence of [$^{35}$S]methionine and the synthetic products ($6.0 \times 10^6$ cpm) immunoprecipitated with 5 $\mu$L of rabbit anti-murine cystathionase serum and subjected to two-dimensional gel electrophoresis as described under Experimental Procedures. An autofluorogram of that gel is shown above. The arrow indicates the location of purified rat liver cystathionase stained with Coomassie brillant blue and present in the same gel.

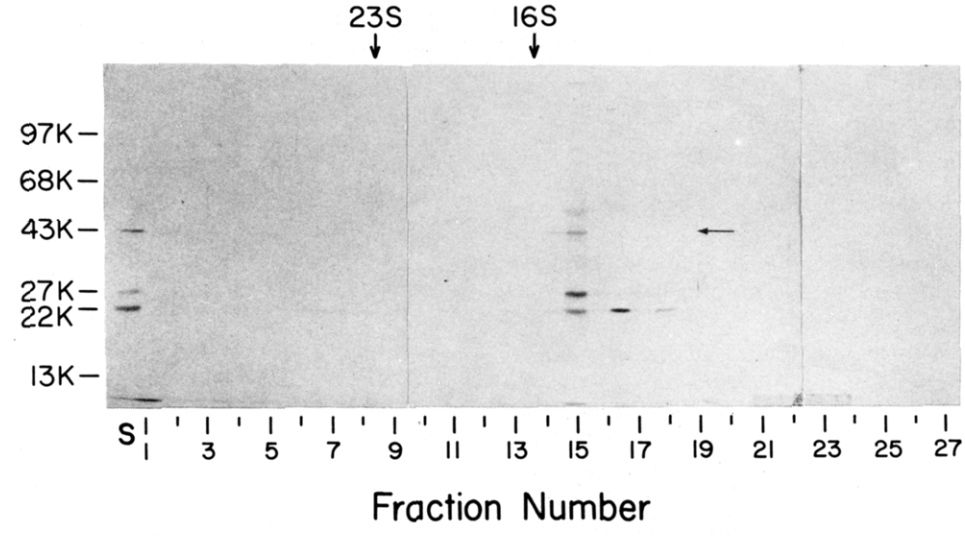


FIGURE 4: Analysis of the size of rat liver cystathionase messenger RNA by sedimentation velocity methods in nondenaturing gradient. Total poly(A)-containing RNA derived from rat liver was fractionated by zonal sedimentation in a sucrose gradient, and the fractions were translated in vitro. Each reaction mixture was treated with 5 $\mu$L of anti-murine cystathionase serum and subjected to $NaDodSO_4$ gel electrophoresis and autofluorography as described under Experimental Procedures. The autofluorogram of that experiment is shown above. Tritiated bacterial ribosomal RNA markers were centrifuged under identical conditions in a parallel gradient, and the positions of the 23S and 16S species are shown. Slot S contains in vitro synthesized [$^{35}$S]methionine-labeled cystathionase [$M_r$ 43 000 (43K)] from a reaction mixture programmed with unfractionated rat liver poly(A)-containing RNA. The arrow indicates the migration position of purified rat liver cystathionase, run in parallel.

which has been characterized previously, was used (Glode et al., 1980). The autofluorograms of gels of such immunoprecipitates (Figure 5, SB, left panel, slot B; NC37, right panel, slot B) reveal the presence of a newly synthesized polypeptide of $M_r$ 43 000 comigrating with highly purified human cystathionase, as well as a second minor band of $M_r$ 27 000. The $M_r$ 43 000 band was not identified in the preimmune serum slot. The $M_r$ 27 000 band was observed in immunoprecipitates derived from whole-cell lysates of both cystathionase$^+$ and cystathionase$^-$ human lymphoblastoid cell lines and has been shown to exhibit a wholly dissimilar [$^{35}$S]methionine-labeled tryptic peptide map when compared to the map of authentic human cystathionase derived from the same cell line (Glode et al., 1980) and is not a cystathionase gene product. Unlike the cases with the mouse and rat immunoprecipitation experiments, the $M_r$ 27 000 band here was not precipitated by preimmune rabbit serum. In some experiments with both *cys*$^+$ and *cys*$^-$ cells, a $M_r$ 22 000 band was observed in anti-cystathionase precipitates. In other experiments with the same reagents, it was absent while the $M_r$ 43 000 (major band) and

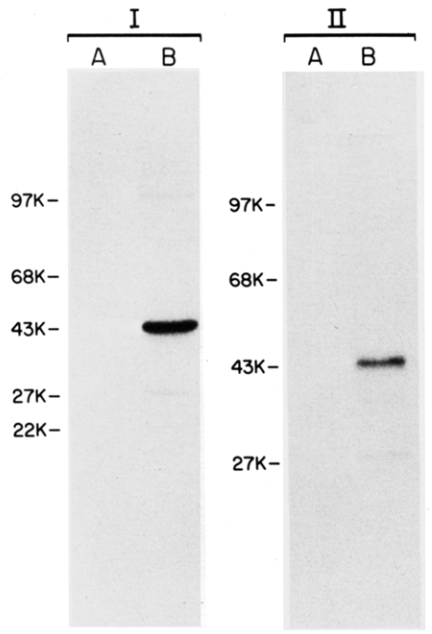


FIGURE 5: Identification of human lymphoblast cystathionase mRNA. Poly(A)-containing RNA isolated from the lymphoblast cell lines SB and NC37, both *cys*$^+$, was translated in vitro, and the products were immunoprecipitated with anti-human cystathionase serum as described below. Immune complexes were subjected to $NaDodSO_4$ gel electrophoresis and autofluorography as described under Experimental Procedures. The autofluorograms of those gels are shown above. The arrow indicates the migration position of purified human cystathionase. (Panel I) SB cells; (slot A) products of a reaction mixture (2.5 mL) containing 100 $\mu$g of human lymphoblast poly(A)-containing RNA immunoprecipitated with 3 $\mu$L of preimmune serum; (slot B) products of a reaction mixture (2.5 mL) containing 100 $\mu$g of human lymphoblast poly(A)-containing RNA immunoprecipitated with 3 $\mu$L of anti-cystathionase serum. (Panel II) NC37 cells: (slot A) products of a reaction mixture (2.5 mL) containing 100 $\mu$g of human lymphoblast poly(A)-containing RNA immunoprecipitated with 3 $\mu$L of preimmune serum; (slot B) products of a reaction mixture (2.5 mL) containing 100 $\mu$g of human lymphoblast poly(A)-containing RNA immunoprecipitated with 3 $\mu$L of anti-cystathionase serum.

$M_r$ 27 000 (minor band) proteins were consistently present. It is, likely, also unrelated to the $M_r$ 43 000 cystathionase protein.

*Comparison of Intracellular Content of Messenger RNA Coding for Cystathionase in Nonleukemic (Cys$^+$) and Leukemic (Cys$^-$) Human Lymphoblastoid Cell Lines.* The in vitro translation reaction system was used as a semiquantitative assay for functional cystathionase messenger RNA molecules in various mRNA preparations. In order to validate its usefulness, it was necessary to show that the response of the in vitro translation system to increasing messenger RNA input was linear. From the results of experiments in which the in vitro translation system was programmed with increasing amounts of total poly(A)-containing RNA from four human lymphoblastoid cell lines, NC37 (*cys*$^+$), CEM (*cys*$^-$), SB (*cys*$^+$), SB2 (*cys*$^-$) (Figure 6), this was the case. We chose to test each of the four messenger RNA populations at a concentration of 40 $\mu$g/mL of reaction mix for two reasons: (1) incorporation of radioactive amino acid was substantial at this point and (2) the response of the in vitro translation system to added mRNA in this range was still limiting and in the linear range and was consistent for each of the four mRNA pools to be tested. Thus, the possibility that more abundant messenger RNA species might compete with scarcer ones for free ribosomes (Alton & Lodish, 1977; Delvin & Emerson, 1979) was eliminated. The results of comparative immunoprecipitation of the products of in vitro translation of

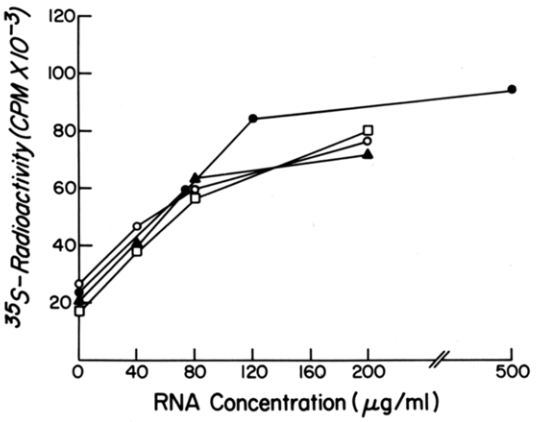


FIGURE 6: Response curve of reticulocyte lysate assay to increasing quantities of poly(A)-containing RNA derived from *cys*$^+$ and *cys*$^-$ human lymphoblastoid cell lines. Increasing quantities of poly-(A)-containing RNA from four cell lines were translated in vitro in replicated reaction mixtures. 3-$\mu$L samples of each reaction mixture were then removed, and the content of hot and insoluble radioactivity was measured as described under Experimental Procedures. SB RNA driven reaction (▲); SB2 RNA driven reaction (●); NC37 RNA driven reaction (□); CEM RNA driven reaction (○).

RNA (40 $\mu$g/mL) from each of these lines are shown in Figure 7.

SB (*cys*$^+$) and NC37 (*cys*$^+$) poly(A) RNA led to the appearance of a dark $M_r$ 43 000 band which was selectively precipitated by anti-cystathionase serum (Figure 7, slot B, left and right panels). This band was absent in reaction mixtures programmed by poly(A)-containing RNA derived from SB2 (*cys*$^-$) and CEM (*cys*$^-$) (Figure 7, slot D, left and right panels). A trace $M_r$ 27 000 band was observed in all four reaction mixtures after anti-cystathionase precipitation. Neither band was present in preimmune serum precipitates. In all of these experiments, anti-enzyme antibody was present in a 2–3-fold excess. Under these conditions, it was then possible to derive a minimum estimate of the relative differences in cystathionase messenger RNA concentration in paired experiments comparing SB with its isogenic *cys*$^-$ line SB2 and NC37 with CEM. This was obtained by densitometric analysis of the $M_r$ 43 000 band present in the autofluorograms of repetitive immunoprecipitation experiments of this type. The results indicate that both *cys*$^+$ cell lines contained at least 15–20-fold more functional cystathionase messenger RNA than their *cys*$^-$ counterparts.

## Discussion

In an attempt to more fully understand the relationship of the leukemic phenotype, cysteine auxotrophy, and the gross reduction in the intracellular content of cystationase in several human leukemic lymphoblast cell lines, we have developed an in vitro translation assay for functional cystathionase messenger RNA. As a means of developing the system, we first sought cystathionase mRNA activity from rodent liver, the richest known source of cystathionase protein (Mudd et al., 1965). Our results indicate that a specific 13–15S rat cystathionase messenger can be readily detected. The size is that expected for a monocistronic messenger RNA molecule encoding a protein of $M_r$ 43 000. Once able to detect this particular RNA species, we then adapted the in vitro translation assay to facilitate the detection of cystathionase messenger RNA in human lymphoblasts in an attempt to quantitate the population of functional messenger in continuous *cys*$^+$ and *cys*$^-$ lymphoblastoid cell lines.

The results of previous experiments in which we have measured the relative concentrations of cystathionase enzyme activity present in rat and human liver and in four nonleuk-

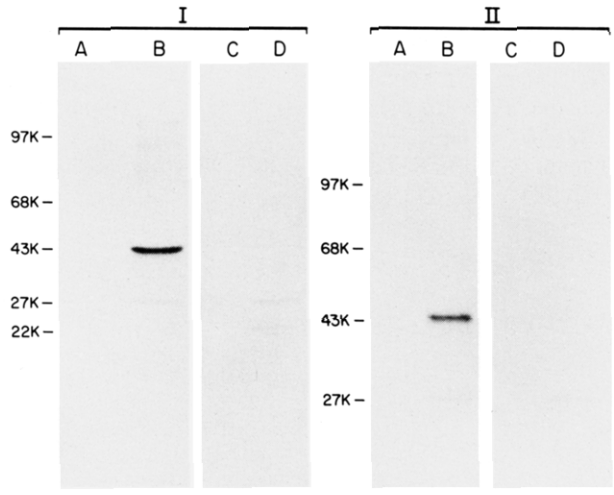


FIGURE 7: Analysis of functional cystathionase messenger RNA content of *cys*$^+$ and *cys*$^-$ lymphoblastoid cell lines. Aliquots of poly(A)-containing RNA from *cys*$^+$ and *cys*$^-$ cells were translated in vitro in the presence of [$^{35}$S]methionine (450 Ci/mmol, concentration 600 $\mu$Ci/mL). As described under Experimental Procedures, equivalent quantities of newly synthesized protein were treated with either rabbit anti-human cystathionase serum or preimmune serum. The following amounts of in vitro synthesis protein were incubated with each serum: (I) SB and SB2, $3.3 \times 10^7$ cpm; (II) NC37 and CEM, $3.2 \times 10^7$ cpm. Immune complexes were eluted from the protein A–Sepharose beads, and the entire bead–eluate suspension was subjected to electrophoresis and autofluorography as described under Experimental Procedures. Autofluorograms of such experiments performed with RNA derived from multiple *cys*$^+$ and *cys*$^-$ cell pairs are shown above. (Panel I) Slots A and B, SB. (Slot A) Products of a reaction mixture (2.5 mL) containing 100 $\mu$g of poly(A)-containing RNA immunoprecipitated with 3 $\mu$L of preimmune serum. (Slot B) Products of a reaction mixture (2.5 mL) containing 100 $\mu$g of poly(A)-containing RNA immunoprecipitated with 3 $\mu$L of anti-cystathionase serum. Slots C and D, SB2. (Slot C) Products of a reaction mixture (2.5 mL) containing 100 $\mu$g of poly(A)-containing RNA immunoprecipitated with 3 $\mu$L of preimmune serum. (Slot D) Products of a reaction mixture (2.5 mL) containing 100 $\mu$g of poly(A)-containing RNA immunoprecipitated with 3 $\mu$L of anti-cystathionase serum. (Panel II) Slots A and B, NC37. (Slot A) Products of a reaction mixture (2.5 mL) containing 100 $\mu$g of poly(A)-containing RNA immunoprecipitated with 3 $\mu$L of preimmune serum. (Slot B) Products of a reaction mixture (2.5 mL) containing 100 $\mu$g of poly(A)-containing RNA immunoprecipitated with 3 $\mu$L of anti-cystathionase serum. Slots C and D, CEM. (Slot C) Products of a reaction mixture (2.5 mL) containing 100 $\mu$g of poly(A)-containing RNA immunoprecipitated with 3 $\mu$L of preimmune serum. (Slot D) Products of a reaction mixture (2.5 mL) containing 100 $\mu$g of poly(A)-containing RNA immunoprecipitated with 3 $\mu$L of anti-cystathionase serum.

emic, human lymphoblastoid cell lines indicate that the concentration of cystathionase in the human cell lines is at least 100-fold less than that of liver tissue (Livingston et al., 1976; Iglehart et al., 1977; Bikel et al., 1978a; Glode et al., 1980). The experiments reported here indicate that two *cys*$^+$, human lymphoblastoid cells contain at least 100-fold less cystathionase messenger RNA than rat liver as well. The relative scarcity of the mRNA species in these cells necessitated the use of very large in vitro translation reaction mixtures (2.5 mL)—50 times the volume required to detect rat liver cystathionase mRNAs (0.05 mL). When we employed such large-scale reactions, cystathionase messenger RNA derived from nonleukemic human lymphoblasts was readily detectable by using immunoprecipitation of in vitro synthesized protein as an assay.

We have tested two nonleukemic, *cys*$^+$, cystathionase$^+$ human lymphoblastoid cell lines and two leukemic, *cys*$^-$, cystathionase$^-$ human lymphoblastoid cell lines for their intracellular content of functional cystathionase messenger RNA. The results indicate that the *cys*$^-$, cystathionase$^-$ phenotype of the leukemic cells is associated with a dramatic reduction in the intracellular concentration of the enzyme messenger RNA when compared to the *cys*$^+$ lines. Densitometric analysis by $M_r$ 43 000 cystathionase gel bands synthesized in response to limiting quantities of poly(A)-containing RNA revealed that the *cys*$^+$ cells contain at least 15–20-fold more enzyme messenger compared to their *cys*$^-$ counterparts. In keeping with these findings, we have previously reported that these two *cys*$^-$ cell lines both contain 10–20-fold less cystathionase catalytic activity (Livingston et al., 1976; Iglehart et al., 1977) and 16–20-fold less immunoprecipitable $M_r$ 43 000 cystathionase protein than do either of the *cys*$^+$ counterparts studied here (Glode et al., 1980).

Thus, in those cell lines tested, there was a close correlation between cysteine auxotrophy, a major reduction in intracellular cystathionase (Glode et al., 1980), and a depletion in the intracellular content of functional cystathionase messenger RNA. On the basis of these observations, we suggest that the absolute cysteine requirement of the *cys*$^-$ cell lines tested is due, at least in part, to an intracellular reduction of the messenger RNA encoding cystathionase. This may result from (1) a reduction in the rate of transcription of the cystathionase mRNA precursor, (2) an alteration in the nuclear and/or cytoplasmic processing of the primary transcript, and/or (3) a reduction in translational activity of the messenger due to a deleterious alteration in the mRNA sequence (e.g., a defect resulting in a messenger with an altered ribosome binding site). Albeit unlikely, it is also possible that *cys*$^-$ cells degrade cystathionase mRNA molecules at a significantly faster rate than *cys*$^+$ cells. This is not likely a result of enhanced nonspecific degradation of mRNA in *cys*$^-$ cells, since the yields of poly(A)-containing RNA and the quantitative effects of adding known amounts of mRNA upon reticulocyte protein synthesis were identical for poly(A)-containing RNA from all four lines tested.

An appreciation of how the depletion in functional cystathionase messenger RNA occurs in certain human leukemic cells may lead to greater insights into one or more mechanisms governing shifts in gene expression during neoplastic transformation. It may also generate some understanding of certain genetic regulatory mechanisms operating during differentiation. For example, the appearance of cystathionase is a late event in human liver development (Greengard, 1977). Although a different tissue, leukemic cell cystathionase messenger RNA depletion and the resultant depletion in cystathionase protein could, by analogy, reflect a block in normal hematopoietic cell development.

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# In Vitro Microtubule Assembly Regulation by Divalent Cations and Nucleotides†

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ABSTRACT: $Mg^{2+}$ and guanosine 5′-triphosphate (GTP) are usually used to assemble microtubule protein (tubulin + microtubule-associated proteins) or tubulin into microtubules in vitro. Recently, it has been shown that $Mn^{2+}$ will substitute for $Mg^{2+}$ in inducing pure tubulin + GTP to assemble into microtubules. We find that $Mn^{2+}$ also substitutes for $Mg^{2+}$ in inducing two-cycle calf microtubule protein (MTP) + GTP to assemble into microtubules. $Zn^{2+}$ or $Co^{2+}$ induces MTP + GTP to form sheets with more than 13 protofilaments. We find that $Co^{2+}$ also substitutes for $Zn^{2+}$ in inducing tubulin to form 200-nm tubules similar to those reported with $Zn^{2+}$. To learn whether metal-induced assembly is due to direct binding and/or a metal–GTP complex, metal-induced assembly of MTP and tubulin was studied in the presence of $Cr^{III}$GTP or taxol, two probes which promote assembly in the absence of added GTP. With CrGTP, $Mg^{2+}$ and ethylene glycol bis(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid (EGTA) were required for optimal assembly into microtubules, and $Mn^{2+}$ could substitute for $Mg^{2+}$. MTP incubated with $Zn^{2+}$ and CrGTP assembled into sheets, shorter than but similar to those induced by $Zn^{2+}$ + GTP. $Mg^{2+}$-induced microtubules and $Zn^{2+}$-induced sheets contained 0.45 mol of [8-$^3$H]-GDP/mol of tubulin if assembled from [8-$^3$H]GTP and a mixture of 0.25 mol of [8-$^3$H]CrGTP, 0.1 mol of [8-$^3$H]GDP, and 0.05 mol of [8-$^3$H]CrGDP/mol of tubulin if assembled from [8-$^3$H]CrGTP. $Zn^{2+}$ induced taxol-treated MTP to form sheets. Sheets were also induced from tubulin + $Zn^{2+}$ and either CrGTP or taxol. These studies suggest that the $Zn^{2+}$-induced structures are not due to a Zn–GTP complex and that $Mg^{2+}$ does not promote assembly only through a Mg–GTP complex.

$Mg^{2+}$ + guanosine 5′-triphosphate (GTP) promotes assembly of two-cycle microtubule protein (MTP)[1] into microtubules (22–24 nm in diameter and with 13–14 protofilaments) (Weisenberg, 1972; Pierson et al., 1978). Although it has been reported that $Mn^{2+}$ does not affect MTP polymerization (Wallin et al., 1977), we find that it substitutes for $Mg^{2+}$. Our results agree with those of Buttlaire et al. (1980), who showed that $Mn^{2+}$ will substitute for $Mg^{2+}$ in promoting pure tubulin to assemble into microtubules. $Zn^{2+}$ or $Co^{2+}$ induces MTP + GTP to form sheets with considerably more than 13 protofilaments (Larsson et al., 1976; Gaskin et al., 1976; Gaskin & Kress, 1977). $Zn^{2+}$ alters the alignment of protofilaments from a parallel to an antiparallel arrangement (Crepeau et al., 1978; Baker & Amos, 1978). We have previously shown that $Zn^{2+}$ induces 6S tubulin + GTP to assemble into protofilamentous structures which are consistent with a tubular structure of 200–250 nm (Gaskin & Kress, 1977) and report that $Co^{2+}$ will also substitute for $Zn^{2+}$ in promoting these structures. GTP is usually used in MTP assembly experiments, and, thus, metal may be coordinated directly and/or through a metal–GTP complex. To test whether a Mg–GTP or a Zn–GTP complex promotes microtubules or sheets of protofilaments, respectively, we studied assembly of calf MTP in the presence of two probes which can substitute for GTP. One probe was CrGTP, a stable metal–nucleotide complex that has been shown to promote assembly of MTP in the presence of 1 mM $Mg^{2+}$ and 1 mM EGTA (MacNeal & Purich, 1978). The other probe was taxol, a low molecular weight neutral plant product which has antitumor activity and which promotes assembly of MTP and tubulin in the presence of 0.5 mM $Mg^{2+}$ and 1 mM EGTA (Schiff et al., 1979). The effects of $Zn^{2+}$ on the assembly of tubulin in the absence of microtubule-associated proteins (MAPs) and in the presence of GTP, CrGTP, or taxol are also compared.

## Experimental Procedures

*Preparation of Protein.* MTP was prepared from calf brain by the recyclization procedure of Shelanski et al. (1973). The final pellet of microtubules was resuspended in 0.1 M 2-(*N*-morpholino)ethanesulfonic acid, pH 6.6 (Mes), and stored in liquid nitrogen. On the day of the experiment, 1–2 mL of protein was dialyzed for 3 h against 3 × 500 mL of 0.1 M Mes buffer (charged hourly) in a Crowe–Englander-type thin-film microdialyzer at 4 °C. The dialyzed sample was centrifuged at 120000*g* for 40 min at 4 °C, warmed to 37 °C for 30 min, and kept on ice for 10 min before the experiment was started. This MTP will not assemble into microtubules at 37 °C unless

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[1] Abbreviations used: $C_c$, critical concentration; CrGTP, chromium guanosine 5′-triphosphate; $Me_2SO$, dimethyl sulfoxide; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid; MAPs, microtubule-associated proteins; Mes, 2-(*N*-morpholino)ethanesulfonic acid; MTP, two-cycle microtubule protein (tubulin + microtubule-associated proteins).

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