Characterization of a Chinese Hamster-Human Hybrid Cell Line with Increased System L Amino Acid Transport Activity

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We have studied leucine transport in several Chinese hamster-human hybrid cell lines obtained by fusion of a temperature-sensitive line of Chinese hamster ovary cells, *ts025C1*, and normal human leukocytes. A hybrid cell line exhibiting a twofold increase in L-leucine uptake over that in the parental cell line was found. This hybrid cell line, 158CnpT-1, was temperature resistant, whereas the parental Chinese hamster ovary mutant, *ts025C1*, contained a temperature-sensitive leucyl-tRNA synthetase mutation. An examination of the different amino acid transport systems in this hybrid cell line revealed a specific increase of system L activity with no significant changes in systems A and ASC. The $V_{\max}$ for L-leucine uptake exhibited by the hybrid 158CnpT-1 was twice that in the CHO parental mutant, *ts025C1*. Cytogenetic analysis showed that the hybrid 158CnpT-1 contains four complete human chromosomes (numbers 4, 5, 10, and 21) and three interspecific chromosomal translocations in a total complement of 34 chromosomes. Biochemical and cytogenetic analysis of segregant clones obtained from hybrid 158CnpT-1 showed that the primary temperature resistance and high system L transport phenotypes can be segregated from this hybrid independently. The loss of the primary temperature resistance was associated with the loss of the human chromosome 5, as previously reported by other laboratories, whereas the loss of the high leucine transport phenotype, which is associated with a lesser degree of temperature resistance, was correlated with the loss of human chromosome 20.

Amino acid accumulation in mammalian cells is carried out by several distinct transport systems. The major components for neutral amino acid uptake in mammalian cells are systems A, ASC, and L, which were first identified in Ehrlich ascites tumor cells (5, 26). System A is Na^+ dependent and serves mainly for the uptake of amino acids with short, polar, or linear side chains. System L is Na^+ independent, shows reactivity toward branched-chain and aromatic amino acids, and exhibits rapid exchange between intracellular and extracellular substrates. System ASC is Na^+ dependent and shows a strong preference for alanine, serine, and cysteine. Similar systems have been characterized in a number of different cell lines (1, 4, 9, 13, 16, 27). Recently, transport systems A, ASC, and L have been characterized in Chinese hamster ovary (CHO) cells (2, 34). Studies on system L regulation has also been carried out in these cells (24, 33, 35-37) by using a temperature-sensitive leucyl-tRNA synthetase mutant, the CHO *tsH1* line. When this mutant is incubated at marginally permissive temperatures, a significant increase in leucine transport activity occurs, indicating that the cell responds to leucine limitation resulting from the defective leucyl-tRNA synthetase. Elevated leucine levels in the growth medium, however, allow CHO *tsH1* cells to grow at nonpermissive temperatures, indicating that increased uptake of leucine is associated with temperature resistance in this mutant. In addition, extra-high levels of leucine cause a repression of transport activity by system L.

We have initiated studies making use of somatic cell hybridization techniques to attempt to identify transport components in CHO and human cells. For these studies we have examined the transport properties of a series of Chinese hamster-human hybrid cell lines obtained by fusion of a temperature-sensitive leucyl-tRNA synthetase CHO mutant, *ts025C1*, with normal human leukocytes. The hybrid cells

were selected for growth at 38.5°C, a nonpermissive temperature for CHO *ts025C1*. These temperature-resistant hybrid cells were initially prepared by Giles et al. and were used to establish that the genetic locus that corrects the leucyl-tRNA synthetase defect is on human chromosome 5 (14). We became interested in the amino acid transport characteristics of these hybrids because we had found that an elevated system L transport phenotype could also contribute to the temperature resistance (33). In the present report, we describe the characterization of a temperature-resistant Chinese hamster-human hybrid cell line with increased system L transport and the assignment of the expression of this high system L transport activity to human chromosome 20.

MATERIALS AND METHODS

Cell lines and culture conditions. The cell line CHO *ts025C1* is a CHO temperature-sensitive mutant defective in leucyl-tRNA synthetase activity (14, 21). Hybrid cell lines 158CnpT-1, 158DnpT-1, and 158DpT-4 have been obtained by Sendai virus-mediated fusion of CHO *ts025C1* cells with normal human leukocytes and selection at a nonpermissive temperature (38.5°C). Hybrid cell lines 158CnpT-1 and 158DnpT-1 are temperature resistant as a result of the retention of human chromosome 5, and the cell line 158DpT-4 is a temperature-sensitive revertant that has lost human chromosome 5 after growth for several generations under nonselecting conditions (34°C) (14). The parental CHO mutant, *ts025C1*, and all of the Chinese hamster-human hybrid cell lines were the generous gifts of R. E. Giles, who obtained them during his studies in the laboratory of F. H. Ruddle at Yale University, New Haven, Conn. CHO-K1, a CHO cell line auxotrophic for proline (17), was obtained from L. H. Thompson of the Lawrence Livermore Laboratory, Livermore, Calif. CHO-Gat⁻ is a Chinese hamster cell line auxotrophic for glycine, adenosine, and thymidine (22). K1 × Gat⁻-1 is a nearly tetraploid (38 chromosomes)

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Chinese hamster cell line obtained by fusion of CHO-K1 and CHO-Gat⁻ (40). The cell lines CHO-Gat⁻ and K1 × Gat⁻-1 were generously provided by C. D. Whitfield of the Department of Biological Chemistry at the University of Michigan, Ann Arbor, Mich.

All cell lines were grown as monolayer cultures in Eagle minimal essential medium (KC Biological, Lenexa, Kans.) containing Earle salts, nonessential amino acids, 5% (vol/vol) fetal calf serum (KC Biological), and 2.2 g of sodium bicarbonate per liter. For growth of CHO-Gat⁻ cells, this medium was also supplemented with 37 μ M hypoxanthine, 21 μ M thymidine, and 21 μ M uridine. In the experiments for which low-leucine medium was required, we used leucine-free Dulbecco minimal essential medium (KC Biological) containing Earle salts and nonessential amino acids and supplemented with 5% (vol/vol) dialyzed fetal calf serum (KC Biological), 2.2 g of sodium bicarbonate per liter, and L-leucine to the appropriate final concentration. The temperature-sensitive and temperature-resistant cell lines were maintained at 34 and 39°C, respectively, in a humidified atmosphere at 5% CO₂-95% air.

Amino acid transport assays. All amino acid transport experiments were carried out with cells maintained as monolayer cultures in Costar cluster-trays containing 24 2-cm² wells. We used the cluster tray method as described by Gazzola et al. (12), with minor modifications, for the measurement of solute fluxes in adherent cultured cells. The sodium-containing phosphate buffer (PBS) was Dulbecco phosphate-buffered saline, pH 7.4, containing 137 mM NaCl, 2.7 mM KCl, 10.6 mM Na₂HPO₄, and 1.5 mM KH₂PO₄. In the sodium-free phosphate buffer (PBC), an equimolar amount of choline chloride replaced NaCl, and choline phosphate, prepared as previously described (34), replaced sodium phosphate. Before the experimental assays, the cells were depleted of endogenous amino acids by incubation for 40 min at 34°C in PBC or PBS buffer supplemented with 5.6 mM D-glucose, 0.49 mM MgCl₂, and 0.68 mM CaCl₂ (PBC-GMC and PBS-GMC) and then substituting the buffer for fresh buffer after the first 20 min of incubation. Protein was determined by the method of Zak and Cohen (41). Intracellular water was determined by the method of Kletzien et al. (19).

Analysis of intracellular amino acid levels. The total amino acid concentration of cell extracts was determined by a quantitative ninhydrin assay described by Rosen (29). Cells were grown in monolayer at 34°C in 75-cm² tissue culture flasks to subconfluency and then were quickly washed with ice-cold PBS buffer and extracted with 5 ml of 75% ethanol for 12 h at room temperature. This extract was evaporated to dryness with N₂ flux, and the residue was dissolved in 3 ml of sodium citrate buffer, pH 2.2 (19.6 g of sodium citrate · 2H₂O, 16.5 ml of concentrated HCl, and 5 ml of thiodiglycol in a final volume of 1 liter). Samples were centrifuged for 10 min at 2,000 × g, and 1-ml samples of supernatant fluid were run on a Beckman model 120C amino acid analyzer equipped with microbore column (60 cm [length] by 6 mm [inside diameter]) packed with 30 g of Beckman ion-exchange resin type W-2 and operated at 54°C. Sodium citrate buffers (Fisher Scientific Co., Pittsburgh, Pa.) were used at pH 3.25 for 31 min, then at pH 4.12 for 16 min, and finally at pH 6.0 for 108 min. Amino acids were determined by measuring the area under the peaks with a computer program. A commercial solution containing the twenty proteic amino acids (Pierce H) was used as standard.

Cytogenetic procedures. Human chromosomes were distinguished from hamster chromosomes in hybrid cells by

Giemsa alkaline differential staining (G-11) and were specifically identified by banding patterns produced by the trypsin-Giemsa method. Metaphase chromosome preparations, G-banding, and G-11 staining were carried out by described procedures (10, 11, 20).

Electrophoretic analysis of human isozyme markers. Electrophoretic analysis of cytoplasmic superoxide dismutase activity (EC 1.15.1.1), specific marker for human chromosome 21, was performed on starch gel by the method of Harris and Hopkinson (18). Electrophoretic analysis of adenosine deaminase (ADA) activity (EC 3.5.4.4), specific marker for human chromosome 20, was carried out on cellulose acetate gel by the procedure of Grunbaum (15), modified as follows: electrophoresis was performed on Sartorius cellulose acetate membranes 12200 (Sartorius Filters Inc., Hayward, Calif.) with Sartophor electrophoresis equipment (Sartorius). Human and Chinese hamster ADA isozymes were separated in a electrophoresis buffer containing 6.6 mM Tris, 6.6 mM maleic acid, 0.58 mM EDTA-Na₄, and 0.67 mM MgCl₂, adjusted with NaOH to pH 7.4. Electrophoresis was carried out at 350 V at room temperature for 1 h. Staining of the gel for enzymatic activity was performed by the method of Harris and Hopkinson (18).

Materials. L-[4,5-³H]-leucine was obtained from New England Nuclear Corp., Boston, Mass. [methyl-³H]-2-aminoisobutyric acid and L-[2,3-³H]-alanine were from ICN Corp., Irvine, Calif. Unlabeled L-leucine, L-alanine, 2-methylaminobutyric acid (MeAIB), and 2-aminoisobutyric acid (AIB) were obtained from Sigma Chemical Co., St. Louis, Mo. 2-Aminobicyclo-[2,2,1]-heptane-2-carboxylic acid (BCH) was purchased from Calbiochem-Behring, La Jolla, Calif.

RESULTS

Leucine uptake in hamster-human hybrid cell lines. The initial rates of L-leucine uptake have been measured in several hamster-human hybrid cell lines of series 158 and compared with the parental mutant *ts025C1* and other appropriate control cell lines. The results are presented in Fig. 1.

We found that, when grown at 34°C, all of the cell lines tested showed very similar initial rates of leucine uptake, except the hybrid line 158CnpT-1, which exhibited a value more than twofold higher than any other cell line, as shown in the first bar of each set in Fig. 1. When shifted to 39°C for 6 h before the uptake assay, the parental CHO cell line, CHO *ts025C1*, showed a significant increase in leucine uptake compared with that after growth at 34°C. This temperature-dependent effect on leucine transport was similar to the system L derepression previously described for a different temperature-sensitive leucyl-tRNA synthetase CHO mutant, the CHO *tsH1* cell line. This adaptive response of leucine transport activity results when there is an insufficient supply of leucine for protein synthesis (24, 36). The same temperature-dependent effect on leucine uptake was exhibited by the temperature-sensitive hybrid 158DpT-4, which is also defective in the leucyl-tRNA synthetase. We did not observe system L derepression, however, when any of the CHO cell lines with normal leucyl-tRNA synthetase activities, such as CHO-K1, CHO Gat⁻, and K1 × Gat⁻-1, or the temperature-resistant hybrids 158DnpT-1 and 158CnpT-1 were shifted to 39°C. In these last two hybrid cell lines, the leucyl-tRNA synthetase defect of the CHO parental mutant was complemented by the presence of the human form of the enzyme (data not shown). All of the temperature-resistant cell lines exhibited approximately the same leucine transport activity

after growth at 39°C as was found after growth at 34°C (Fig. 1).

The intracellular water space for each of the cell lines was determined and was found to be very similar with a range between 5 and 6 $\mu\text{l}/\text{mg}$ of protein.

Effect of leucine concentration on leucine uptake in the hamster-human hybrid cell line 158CnpT-1 and the hamster line CHO *ts025C1*. It has been shown earlier that leucine limitation in the growth medium and growth at nonpermissive temperatures result in increased system L transport activities in the temperature-sensitive leucyl-tRNA synthetase mutant CHO *tsH1* (24, 36). We examined whether the limitation of leucine in the growth medium in cells cultured at 34°C and 39°C would give increased rates of leucine uptake in the hamster-human hybrid cell line 158CnpT-1 and in *ts025C1*, the CHO temperature-sensitive parental mutant.

Figure 2 shows that for cell line CHO *ts025C1*, growing the cells at 34°C and lowering the level of leucine in the growth medium produced increased rates of leucine uptake. The enhancement of leucine transport was more prominent when cells were incubated in low leucine concentrations at elevated temperatures since both of these conditions lead to a decreased leucine availability in cell lines containing a temperature-sensitive leucyl-tRNA synthetase. These results agree with the pattern of system L derepression reported previously for CHO *tsH1* cells (36). In contrast, the leucine uptake exhibited by the hybrid cell line 158CnpT-1 did not change significantly under either of these growth conditions, as has been found for a cell line with normal leucyl-tRNA synthetase activity.

We have also tested the stability of the high-transport phenotype in the 158CnpT-1 hybrid cell line by allowing it to grow for long periods under nonselective conditions (34°C).

FIG. 1. Initial rates of L-leucine uptake in hamster-human hybrid cell lines of series 158 and CHO cell lines. Cultures of cell lines CHO *ts025C1*, CHO K1, CHO Gat⁻, K1 x Gat⁻, and the three hamster-human hybrid lines 158CnpT-1, 158DnpT-1, and 158DpT-4 were seeded at 1.3×10^5 cells per well in 24-well tissue culture dishes and were grown for 22 h at 34°C. One half of the cultures was then shifted to 39°C for 6 h, and the other half was left at 34°C for 6 h. The 30-s uptake of 0.1 mM L-[³H]leucine in PBC was measured by the cluster tray assay method after the cells had been depleted of endogenous amino acids by 40 min of incubation in PBC-GMC buffer. The data are the averages of at least four determinations.

FIG. 2. Effect of growth temperature and leucine concentration on leucine uptake by the hamster-human hybrid line 158CnpT-1 and the hamster line CHO *ts025C1*. Cultures of the hamster cell line CHO *ts025C1* (A) and the hamster-human hybrid line 158CnpT-1 (B) were seeded in 24-well culture dishes at 1.3×10^5 cells per well and were divided into four groups: ○, Cells grown for 24 h at 34°C in normal medium (0.40 mM leucine); □, cells grown 24 h at 34°C in low-leucine medium (0.08 mM leucine); ●, cells grown for the first 12 h at 34°C and for the last 12 h at 39°C in normal medium (0.40 mM leucine); ■, cells grown for the first 12 h at 34°C and for the last 12 h at 39°C in low-leucine medium (0.08 mM leucine). The uptake of 0.1 mM L-[³H]leucine in PBC buffer was measured for each group of cells by the cluster tray method after the cells had been depleted of endogenous amino acids by 40 min of incubation in PBC-GMC buffer. Each point represents the average of four determinations.

We found that after 3 months of continuous growth at 34°C, the hybrid exhibited the same high transport as that in cultures that had been kept at 39°C (data not shown).

Growth of the hamster-human hybrid cell line 158CnpT-1 and the hamster line CHO *ts025C1* at 34 and 39°C. Figure 3 shows the growth of 158CnpT-1, the hamster-human hybrid, at 34 and 39°C in medium containing 0.05 mM L-leucine compared with the growth of CHO *ts025C1*. Both cell lines grew well at 34°C, but CHO *ts025C1* cells could not grow at 39°C, whereas the growth of the hybrid 158CnpT-1 was vigorous at this temperature.

Transport system specificity of the increased leucine transport in the hamster-human hybrid line 158CnpT-1. The activities of transport systems A, ASC, and L in the hamster-human hybrid cell line 158CnpT-1 were measured after growth either for 28 h at 34°C or for 22 h at 34°C and 6 h more at 39°C. These results were compared with those for CHO *ts025C1* cells grown under the same conditions. The results are shown in Table 1. As reported previously (34), we operationally define the activities of the neutral amino acid transport systems by the following tests: system A, the portion of the sodium-dependent uptake of 0.2 mM AIB that is inhibited by 25 mM MeAIB; system ASC, the portion of the sodium-dependent uptake of 0.2 mM L-alanine that is not inhibited by 25 mM MeAIB; and system L, the portion of the sodium-independent uptake of 0.2 mM L-leucine that is inhibited by 10 mM BCH. It should be noted that this operational definition of the ASC system includes all Na⁺-

FIG. 3. Growth of the hamster-human hybrid line 158CnpT-1 and the hamster line CHO *ts025C1* at 34 and 39°C. Cultures of the hamster-human hybrid cell line 158CnpT-1 (○) and the hamster line CHO *ts025C1* (●) were seeded at 10^4 cells per well in 24-well culture dishes. One half of the dishes was incubated at 34°C (A) and the other half at 39°C (B). Growth of the cultures was measured by cell protein according to the procedure of Zak and Cohen (41). The data are the means of four determinations.

dependent transport components of alanine uptake not subject to inhibition by MeAIB.

We found that only system L, defined in this way, was increased significantly in the hamster-human hybrid line 158CnpT-1 grown at 34°C, compared with CHO *ts025C1* cells grown under the same conditions. Table 1 also shows that CHO *ts025C1* cells grown at 39°C exhibit increased system L uptake similar to that described previously for CHO *tsH1* cells (36).

Kinetic parameters of the sodium-independent L-leucine transport in the hamster-human hybrid cell line 158CnpT-1 and the hamster line CHO *ts025C1* grown at 34°C. To characterize kinetically the high-system L transport activity exhibited by the hybrid 158CnpT-1, we measured the effect of the leucine concentration on the initial rate of the sodium-independent leucine uptake in this hybrid cell line and in CHO *ts025C1* cells. Table 2 shows that the increased system L transport in the hamster-human hybrid cell line 158CnpT-1

grown at 34°C was accompanied by a more than twofold increase in the V_{max} of the sodium-independent L-leucine uptake and a twofold increase in the apparent K_m , compared with the kinetic values exhibited by the CHO parental cell line, *ts025C1*, grown under the same conditions. Since the levels of leucine in the growth medium are significantly higher than the K_m value obtained for the hybrid, the transport capacity for leucine is saturated.

Accumulation and exodus of L-leucine and L-alanine in the hamster-human hybrid cell line 158CnpT-1 and the hamster line CHO *ts025C1*. Cultures of the hamster-human hybrid line 158CnpT-1 and the hamster line CHO *ts025C1* grown at 34°C for 24 h were incubated either in PBC containing L-[3 H]-leucine at 0.5 mM or in PBS containing L-[3 H]-alanine at 2.0 mM. The cells were then placed in amino acid-free buffer, and the loss of labeled amino acid was measured. As shown in Fig. 4, the hamster-human hybrid 158CnpT-1 accumulated twofold higher levels of L-leucine than CHO *ts025C1* cells, whereas L-alanine accumulation was similar in these two cell lines. The exodus of L-alanine followed a similar pattern in the two cell lines. The exodus of L-leucine from both cell lines reached a plateau after 10 min of incubation in amino acid-free buffer, but the final level of radioactive leucine retained was higher in the 158CnpT-1 hybrid cell line than in the CHO *ts025C1* line. These results demonstrate that the higher leucine transport activity exhibited by the hamster-human hybrid 158CnpT-1 results in a higher steady-state level of intracellular leucine.

Analysis of intracellular levels of free amino acids in the hamster-human hybrid line 158CnpT-1 and the hamster line CHO *ts025C1*. To investigate further the effect of the high system L activity in the hamster-human hybrid cell line 158CnpT-1 on the intracellular levels of system L amino acids, we determined the intracellular amino acid levels in the 158CnpT-1 cells and CHO *ts025C1* cells grown at 34°C as described above. The data (Table 3) show that in the hamster-human hybrid line 158CnpT-1 there were increases of about twofold in the levels of those amino acids that are mainly transported by system L, such as isoleucine, leucine, phenylalanine, valine, and histidine, when compared with those in CHO *ts025C1* cells. No significant differences were

TABLE 1. Activities of transport systems A, ASC, and L in the hamster-human hybrid cell line 158CnpT-1 and the hamster line CHO *ts025C1*^a

Cell line	Temp (°C)	Uptake rate (nmol/mg per min)		
		System A	System ASC	System L
158CnpT-1	34	1.25 ± 0.15	4.50 ± 0.45	14.84 ± 1.16
	39	1.47 ± 0.12	4.00 ± 0.44	12.86 ± 0.48
CHO <i>ts025C1</i>	34	1.71 ± 0.17	4.92 ± 0.40	5.20 ± 0.52
	39	1.64 ± 0.21	6.10 ± 0.61	13.98 ± 0.66

^a Cultures of the hamster-human hybrid line 158CnpT-1 and the hamster line CHO *ts025C1* were seeded in 24-well culture dishes at 1.3×10^5 cells per well and were grown for 22 h at 34°C. One half of the cultures was then shifted to 39°C for 6 h, and the rest of a second set was left at 34°C for the same period. Systems A, ASC, and L were operationally defined as follows: system A, the portion of the sodium-dependent uptake of 0.2 mM AIB inhibitable by 25 mM MeAIB; system ASC, the portion of the sodium-dependent uptake of 0.2 mM L-alanine noninhibitable by 25 mM MeAIB; system L, the portion of sodium-independent uptake of 0.2 mM L-leucine inhibitable by 10 mM BCH. Transport assays were carried out by the cluster tray method. The data represent the averages of four determinations.

observed in the levels of those amino acids mainly transported by systems A and ASC, such as glycine, alanine, serine, and threonine.

Cytogenetic characterization of the hamster-human hybrid cell line 158CnpT-1. Conventional chromosomal analysis has shown that 34 chromosomes are present in the hamster-human hybrid line 158CnpT-1. The use of the alkaline Giemsa (G-11) differential staining techniques (Fig. 5) revealed the presence of four complete human chromosomes as well as three portions of human chromosomes involved in interspecific translocations within hamster chromosomes. Studies with trypsin-Giemsa (G-banding) techniques (Fig. 6) allowed us to identify the intact human chromosomes as numbers 4, 5, 20, and 21. Human isozyme marker analyses were also performed, as discussed below, to provide additional identification of the human chromosomal material present in the hybrid cell line 158CnpT-1.

Selection and characterization of segregants from the hamster-human hybrid cell line 158CnpT-1. To test whether the high leucine transport and the primary temperature resistance phenotypes exhibited by the hybrid line 158CnpT-1 were conferred by two independent human chromosome-coded factors, we allowed human chromosomes to segregate from hybrid 158CnpT-1. The segregation was facilitated by treatment with sublethal concentrations of colcemid and growth under permissive conditions (34°C) in medium containing elevated concentrations of leucine (2 mM) supplemented with 10% fetal bovine serum. We found that treatment with low concentrations of colcemid (0.02 µg/ml) increased the frequency of segregation of human chromosomes from the hamster-human hybrid cells. The yield of segregants without this treatment was extremely low. This use of colcemid was based on reports that it can cause chromosomal nondisjunction in Chinese hamster cells (6). Similar increases in the segregation frequency of human chromosomes from hamster-human hybrid cells by using colcemid have been obtained by R. E. Giles (personal communication).

The segregation procedure was carried out as follows: cultures of hybrid cell line 158CnpT-1 were seeded in six-well tissue culture dishes (16 cm² per well) at a density of 10⁴ cells per cm² in medium containing 2 mM L-leucine and were incubated at 34°C for 24 hours. At this time, colcemid was added to the culture medium to a final concentration of 0.02

µg/ml. The cultures were then incubated in the presence of colcemid for 30 h at 34°C, washed with PBS buffer, and allowed to recover in fresh medium containing 2 mM L-leucine for 42 h at 34°C. The cultures were then subcloned,

FIG. 4. Accumulation and exodus of L-leucine and L-alanine in the hamster-human hybrid cell line 158CnpT-1 and the hamster line CHO ts025Cl. Cultures of the hamster-human hybrid cell line 158CnpT-1 (○) and the hamster line CHO ts025Cl (●) were seeded at 1.5×10^5 cells per well in 24-well culture dishes and were grown for 24 h at 34°C. (A) Cells were depleted of internal amino acids by 40 min of incubation at 34°C in PBC-GMC buffer and then were incubated at 37°C for 20 min in PBC buffer containing 0.5 mM L-[³H]leucine. At the end of the uptake period, the cells were washed once with amino acid-free PBC buffer and were incubated at 37°C in the same buffer. At the times indicated, the medium was then removed, the cells washed with ice-cold PBC-GMC buffer, and the labeled L-leucine remaining in the cells was determined by the cluster tray method. (B) Cells were depleted of internal amino acids by incubation at 34°C for 40 min in PBS-GMC buffer and then were incubated for 30 min at 37°C in PBS buffer containing 2.0 mM L-[³H]alanine. At the end of the uptake period, the cells were washed once with amino acid-free PBS buffer and were incubated at 37°C in the same buffer. At the times indicated, the buffer was then removed, and the residual labeled L-alanine was determined. Each point is the average of four determinations.

TABLE 2. Kinetic parameters of sodium-independent L-leucine uptake in the hamster-human hybrid cell line 158CnpT-1 and the hamster line CHO ts025Cl^a

Cell line	K_m (µM)	V_{max} (nmol/mg per min)	K_D (nmol/mg per mM)
158CnpT-1	39.77 ± 2.90	16.19 ± 0.60	6.91 ± 0.60
CHO ts025Cl	17.62 ± 2.00	6.65 ± 0.30	6.06 ± 0.52

^a Cultures of the hamster-human hybrid line 158CnpT-1 and the hamster line CHO ts025Cl were seeded in 24-well culture dishes at 1.3×10^5 cells per well and were grown for 24 h at 34°C. The initial rates of sodium-independent L-leucine uptake in a concentration range from 5 µM to 10 mM were measured by the cluster tray method. Initial transport velocities are expressed as nanomoles of amino acid taken up per milligram of cell protein per minute. Secondary analyses of initial velocity kinetic curves were carried out using a Fortran program applying the Gauss-Newton nonlinear least-square method to the data, described by the equation $\ln v = \ln(V_{max} [S]/K_m + [S]) + K_D [S]$, where v , S , V_{max} , and K_m have their usual meanings and K_D represents the first-order rate constant describing the nonsaturable uptake.

TABLE 3. Analysis of intracellular levels of free amino acids in the hamster line CHO *ts025C1* and the hamster-human hybrid line 158CnpT-1^a

Amino acid	Concn (nmol/mg of cell protein)	
	CHO <i>ts025C1</i>	158CnpT-1
Asp	19.3	18.3
Thr	47.7	51.8
Ser	95.3	107.8
Glu	151.6	180.3
Gly	34.4	27.8
Ala	43.2	38.6
Cys	2.0	3.8
Val	11.0	18.5
Met	5.7	7.8
Ile	8.7	18.3
Leu	10.0	21.4
Tyr	9.6	18.4
Phe	8.3	15.0
Lys	4.8	4.6
His	7.1	12.9
Arg	7.2	10.3

^a The intracellular amino acid concentrations in the hamster-human hybrid line 158CnpT-1 and the hamster line CHO *ts025C1* were determined in cells grown at 34°C as described in the text. The data represent the averages of three determinations.

and the clones were tested for leucine transport and temperature resistance of growth. They were also subjected to cytogenetic analysis. From these studies, the segregant clones were divided into four different classes according to a comparison of their transport and temperature resistance-sensitivity phenotypes with those of the parental hybrid cell line, 158CnpT-1, which exhibited high leucine transport and temperature resistance: class 1, low leucine transport and temperature resistance; class 2, low leucine transport and temperature sensitivity; class 3, high leucine transport and temperature sensitivity; class 4, extra-high leucine transport and temperature sensitivity (Fig. 7). Growth studies showed that the high leucine transport activity of classes 3 and 4 produced a slight but significantly greater temperature resistance than that shown by the CHO parental cell line *ts025C1* (data not shown).

Cytogenetic analysis of the segregants from the different classes, performed by using alkaline Giemsa staining (G-11) and G-banding procedures, showed a concordance between the loss of the primary temperature resistance phenotype and the loss of human chromosome 5, in agreement with the results obtained previously by Giles et al. (14) and by Dana et al. (7). The loss of the high-leucine-transport phenotype in classes 1 and 2 was accompanied by the loss of one of the small human chromosomes. On the basis of cytogenetic analysis, we tentatively assigned the high leucine transport to human chromosome 20. The analysis of leucine transport properties of several culture lines of human fibroblasts provided by the National Institute of General Medical Sciences Human Genetic Mutant Cell Repository (Camden, N.J.) and containing one, two, or three copies of human chromosome 21 showed no significant differences in the leucine transport values for any of the cell lines studied, indicating that the high leucine transport was not associated with the presence of chromosome 21 (data not shown). To provide further evidence of the identity of the small human chromosome responsible for the expression of the high-leucine-transport phenotype, several high- and low-leucine-transport clones, obtained from the segregation procedures,

were analyzed for the presence of human chromosome markers. We concentrated these studies on isozyme analyses of the specific markers for human chromosome 20, ADA, and 21, cytoplasmic form of superoxide dismutase (8, 23), which were the two small human chromosomes tentatively assigned to the hybrid cell line 158CnpT-1, (Fig. 5, 6). These analyses showed a strong correlation between the segregation of the high-leucine-transport phenotype and the human isozyme ADA, the specific marker of chromosome 20, suggesting that a genetic locus for the expression of high system L transport activity is present in this human chromosome (Table 4).

DISCUSSION

Somatic cell hybridization has proven to be an important approach for determining the chromosomal location of specific genes in mammalian cells. After interspecific cell fusion, a hybrid cell is initially obtained in which essentially the complete genomes of both parental cell lines are contained in a single nucleus. In subsequent cell divisions, chromosomes derived from one of the original cells are lost preferentially, resulting in hybrid cells that retain only a few chromosomes from one of the parental cell lines and essentially the complete complement of chromosomes from the other cell line. Human chromosomes are preferentially lost in fusions with CHO or mouse cells, and the use of adequate selective conditions allows the retention of specific human genes (14, 23, 28, 30, 31). Precise identification of chromosomes or portions of chromosomes is possible with the use of banding and differential staining techniques (3, 28, 31, 39) and isozyme marker analysis (25, 32). The chromosomal

FIG. 5. Metaphase spread of the hamster-human hybrid cell line 158CnpT-1 stained by the alkaline Giemsa method (G-11 staining). Metaphase chromosome slide preparations were differentially stained by the alkaline Giemsa procedure as described in the text. Human chromosomes stain pale blue and appear lighter in the black-and-white photograph than the hamster chromosomes, which stain magenta. The four human chromosomes present in this hybrid cell line are indicated by long arrows. The three hamster-human chromosomal translocations are indicated by short arrows.

assignment of a specific gene is inferred by concordance between the presence or absence of a specific chromosome and an identifiable phenotype in a series of hybrid clones. In this manner, a large number of genetic loci have already been assigned to specific human chromosomes as well as chromosomes for a number of other mammalian cell types (23, 30, 31, 38).

Our laboratory has been using the temperature-sensitive leucyl-tRNA synthetase mutant CHO *ts*H1 to study the regulation of amino acid transport system L. This transport system serves for the uptake of branched-chain and aromatic amino acids in Chinese hamster cells (34). It was shown that incubation of CHO *ts*H1 cells at 38.5°C, a marginally permissive temperature, leads to a partial inactivation of the leucyl-tRNA synthetase, and the resulting leucine limitation produces a slowing of growth. High leucine levels in the growth medium, which result in increased intracellular leucine pools, have been shown to reverse the temperature sensitivity of growth of the CHO *ts*H1 cells. When CHO *ts*H1 cultures are subjected to marginally permissive temperatures, the adaptive response of the cells leads to a significant increase in leucine transport. Leucine transport enhancements can also be observed when CHO *ts*H1 or wild-type CHO cells are grown at 34°C in a medium containing low levels of leucine (24, 36).

These early results suggested that somatic cell hybrid techniques with temperature-sensitive leucyl-tRNA synthetase mutants of Chinese hamster cells would be useful for the characterization of transport. Hybrid cells expressing higher leucine transport than normal would be expected to be more temperature resistant than the CHO temperature-sensitive mutants defective in leucyl-tRNA synthetase activity. Hy-

FIG. 6. Trypsin-Giemsa-banded metaphase chromosome spread from the hamster-human hybrid cell line 158CnpT-1. Metaphase chromosome slide preparations were trypsin-Giemsa banded as described in the text. Human chromosomes 4, 5, 20, and 21 are identified by arrows.

FIG. 7. Initial rates of L-leucine uptake in several segregants obtained from the high-leucine-transport, temperature-resistant, hamster-human hybrid cell line 158CnpT-1. Cultures of cell line CHO *ts*025C1, 158CnpT-1 hybrid, and segregants (S) from classes 1, 2, 3, and 4 (see text) were seeded at 1.3×10^5 cells per well in 24-well tissue culture dishes and were grown at for 22 h at 34°C. One half of the cultures was then shifted to 39°C for 6 h and the other half was left at 34°C for the same period of time. The 30-s uptake of 0.1 mM L-[3 H]leucine in PBC was measured by the cluster tray assay method after the cells had been depleted of endogenous amino acids by 40 min of incubation in PBC-GMC buffer. The data are given as nanomoles of leucine per milligram of protein per minute and are the averages of four determinations. The abbreviation ts and tr identify temperature-sensitive and temperature-resistant cell lines, respectively.

brids with increased leucine transport, therefore, might be selected along with hybrids retaining the human gene that can complement the deficient CHO enzyme under nonpermissive conditions. Because of this possibility, we became interested in examining the transport properties of a series of temperature-resistant hybrid cells that Giles et al. (14) obtained by fusion of a temperature-sensitive leucyl-tRNA synthetase CHO mutant cell line, *ts*025C1, and normal human leukocytes. We found that one of these hybrid cell lines, 158CnpT-1, exhibits more than twice as much leucine transport as the CHO parental cell line, *ts*025C1, and other control cell lines (Fig. 1). Studies on the system specificity of this high leucine uptake led to the conclusion that only system L transport is elevated in this hybrid cell line, and no significant changes in the activities of systems A and ASC were observed (Table 1). Kinetic parameters determined for sodium-independent L-leucine uptake showed significant increases in the hybrid 158CnpT-1 when compared with CHO *ts*025C1 (Table 2). These kinetic data are consistent with the idea that a human gene coding for a component that produces increased system L activity is present in the hybrid cell line 158CnpT-1. This hybrid line is able to accumulate higher leucine concentrations than *ts*025C1 cells when incubated in the presence of tritiated leucine, whereas the two cell lines accumulate alanine to the same extent. Analyses of intracellular amino acid levels in the hybrid cell line 158CnpT-1 show increased steady-state levels of the amino acids that are transported primarily by system L over those found in CHO *ts*025C1 cells (Table 3). Increased internal levels of leucine would be expected to contribute to the temperature resistance of the hybrid cell line 158CnpT-1.

Cytogenetic analysis by differential staining of hamster

TABLE 4. Analysis of human chromosomal marker enzymes ADA (chromosome 20) and superoxide dismutase 1 (chromosome 21) in clones and segregants obtained from the hamster-human hybrid cell line 158CnpT-1

Cell line	Leucine transport	Presence of enzyme	
		ADA (chromosome 20)	Superoxide dismutase 1 (chromosome 21)
158CnpT-1			
Original	High	+	+
Clone 35	High	+	+
Clone 67	High	+	+
Clone 75	High	+	+
Clone 89	High	+	+
Clone 95	High	+	+
Segregants			
11 (class 1)	Low	—	+
23 (class 1)	Low	—	+
38 (class 1)	Low	—	+
61 (class 1)	Low	—	+
62 (class 1)	Low	—	+
69 (class 1)	Low	—	+
70 (class 1)	Low	—	+
41 (class 2)	Low	—	+
14 (class 3)	High	+	+
15 (class 4)	Extra high	+	+

and human chromosomes, carried out by the Giemsa alkaline (G-11) method, indicates that four human chromosomes and three human-hamster interspecific chromosome translocations are present in the hybrid cell line 158CnpT-1 (Fig. 5). Trypsin-Giemsa staining (G-banding) techniques have allowed us to identify the four human chromosomes as numbers 4, 5, 20, and 21 (Fig. 6).

We allowed the human chromosomes to segregate further from the 158CnpT-1 hybrid line by treatment with sublethal concentrations of colcemid. The results showed that the major temperature resistance phenotype and high-leucine-transport phenotype segregated independently. The major loss of temperature resistance of growth correlated, by G-11 staining, with the loss of a medium-size human chromosome which was identified by G-banding techniques as human chromosome 5. These results were in agreement with those previously published by Giles et al. (14) and later by Dana et al. (7). The loss of the high leucine transport and lesser temperature resistance was accompanied by the loss of a small human chromosome, as evidenced by G-11 staining. These results strongly suggest that independent human factors are responsible for the expression of the primary temperature resistance and for the high leucine transport in the hybrid cell line 158CnpT-1. Isozyme analysis of the segregants missing one of the two small human chromosomes, 20 and 21, showed a strong correlation between the presence of the human form of ADA and high leucine transport, suggesting that human chromosome 20 was responsible for the high-leucine-transport phenotype (Table 4). We were also interested to find that segregants belonging to class 4, which exhibit an extra high leucine transport and slight but significant temperature resistance, contained two copies of human chromosome 20, as evidenced by cytogenetic analysis. These segregants also showed a greater density of the stained band for human ADA activity. These results permit the assignment of the locus for the expression of high system L transport activity to human chromosome

20. Chromosome sorting and DNA recombinant technologies will be used to attempt to isolate the human gene responsible for the elevated system L transport activity.

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