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Isolation and Characterization of Interspecific Heat-Resistant Hybrids Between a Temperature-Sensitive Chinese Hamster Cell Asparaginyl-tRNA Synthetase Mutant and Normal Human Leukocytes: Assignment of Human *asnS* Gene to Chromosome 18

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Abstract—*We isolated interspecific somatic cell hybrids between human peripheral leukocytes and a temperature-sensitive CHO cell line with a thermolabile asparaginyl-tRNA synthetase. The hybrids were selected at 39° C so as to require the expression of the human gene complementing the deficient CHO enzyme. In vitro heat-inactivation profiles of cell-free extracts from temperature-resistant hybrid cells indicate the presence of two forms of asparaginyl-tRNA synthetase. One form is very resistant to thermal inactivation, like the normal human enzyme, while the other form is very thermolabile, like the altered enzyme from the CHO parent. Hybrids and temperature-sensitive segregants derived from them were analyzed for the expression of known human chromosomal marker enzymes. The strong correlation between the expression of the human form of asparaginyl-tRNA synthetase and the presence of human chromosome 18 in hybrids suggests that the human gene,* asnS, *which corrects the heat-sensitive phenotype of the CHO asparaginyl-tRNA synthetase mutant, is located on chromosome 18.*

INTRODUCTION

The process of protein synthesis in mammalian cells is a complicated one, involving more than 100 proteins and over 50 unique RNA species. Although the enzymology of the process is fairly well understood, relatively little is known

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about the organization or chromosomal locations of the genes encoding the various components of the protein synthetic apparatus. To date, chromosomal assignments have been made for only two aminoacyl-tRNA synthetases (1–3) and one ribosomal protein (2) in human cells and one aminoacyl-tRNA synthetase (4) and one ribosomal protein (5) in Chinese hamster cells. In order to better understand how the expression of this large group of functionally related genes is coordinated, more of the genes involved must be localized. Such a process will rely heavily on cell lines in which protein synthesis mutants can be easily selected.

Cell lines derived from Chinese hamsters have been particularly useful sources of protein synthesis mutants. Thus far mutants have been isolated with alterations affecting the ribosome (6–9), an elongation factor (10, 11), and numerous aminoacyl-tRNA synthetases (12–16). The majority of the aminoacyl-tRNA synthetase mutants have been isolated from the Chinese hamster ovary (CHO) cell line. The phenotypes of all of these mutants are recessive in intraspecific cell hybrids and most have a temperature-sensitive, conditionally lethal phenotype which can be suppressed by high concentrations of the cognate amino acid (12, 17).

In CHO cells, conditionally lethal, temperature-sensitive mutants with alterations in leucyl- or asparaginyl-tRNA synthetase (asnRS) arise at much higher frequencies than any other types of temperature-sensitive mutants. These observations led to the idea that the genes encoding these two enzymes were functionally haploid in CHO cells (18). It is now known that the gene encoding leucyl-tRNA synthetase is located in a region on the long arm of chromosome 2 that is physically haploid in CHO cells (4, 19). Whether an analogous situation exists for the gene encoding asparaginyl-tRNA synthetase is not known, since its chromosomal location has not been determined in Chinese hamster cells.

In order to further expand the now meager genetic map of genes encoding protein synthesis components in mammalian cells, we isolated interspecific hybrid cell lines between a temperature-sensitive CHO asparaginyl-tRNA synthetase mutant, Asn-5, and normal human leukocytes, in an attempt to assign the *asnS* gene to a specific human chromosome. The hybrids were isolated at a temperature, 39°C, which is nonpermissive for the CHO parent, to select clones in which the temperature-sensitive lesion of the CHO parent was complemented by the corresponding human gene. Several such temperature-resistant hybrids were isolated and all contained human chromosomal material. Studies on the in vitro thermostability of the asnRS activity from these hybrids revealed the presence of both a thermolabile asnRS activity, characteristic of the mutant CHO enzyme, and a thermostable asnRS activity, characteristic of the normal human enzyme. Thus, these hybrids appear to express both the mutant CHO *asnS* gene and the normal human *asnS* gene.

Temperature-resistant hybrids, and temperature-sensitive segregants selected from them, were analyzed for the presence of human isozymes that have been assigned to specific human chromosomes. All the temperature-resistant hybrids examined expressed human peptidase A (Pep A), which has been assigned to human chromosome 18. In addition, five of six temperature-sensitive segregants, which had lost both the ability to grow at 39°C and the thermostabile form of asnRS, lost human Pep A activity. This very strong correlation between the presence of human Pep A and the expression of the heat-stabile form of asnRS indicates that at least one gene required for the expression of the human asnRS is localized to chromosome 18. Our results are most compatible with the idea that this gene, *asnS*, encodes a polypeptide component of the enzyme.

MATERIALS AND METHODS

Cell Lines and Cell Culture. All cell lines were grown as monolayer cultures in Dulbecco's modified Eagle medium (DMEM) (Gibco, Grand Island, New York) supplemented with 2% fetal calf serum, 8% calf serum, 0.5 mM proline, 0.2 mM glycine, 37 μM adenosine, and 41 μM thymidine. When supplemental asparagine was added to cultures, the final concentration was 3 mM. Cultures were maintained in a humidified atmosphere of 5% CO_2, 95% air at the appropriate temperature. The temperature-sensitive Chinese hamster ovary (CHO) asparaginyl-tRNA synthetase mutant, Asn-5 (20) and its temperature-resistant parent, GAT^- (21), were kindly provided by Dr. S. Arfin, Department of Biological Chemistry, U.C., Irvine. Asn-5 has a mutation in the *asnS* gene resulting in the production of a thermolabile asparaginyl-tRNA synthetase. These cells are nonviable at 39°C in the absence of exogenous asparagine, but their temperature-sensitive phenotype is completely suppressed by the addition of high concentrations of asparagine to the culture medium.

In the experiments described below to select temperature-sensitive segregants, a special medium which we have termed 0.1 × LF-MEM was used. Medium 0.1 × LF-MEM is Eagle's minimal essential medium with the following modifications: (1) lysine is omitted; (2) concentrations of all the rest of the amino acids in the standard formulation are reduced to 10% the normal amounts; and (3) glycine, proline, serine, adenosine, and thymidine are added to final concentrations of 0.2 mM, 0.1 mM, 0.5 mM, 37 μM, and 41 μM, respectively. In addition, this medium is supplemented with 8% dialyzed calf serum and 2% dialyzed fetal calf serum.

Asparaginyl-tRNA Synthetase Assay. Cells were harvested from 150-mm dishes by scraping with a rubber policeman. Cells were washed twice with 35 mM Tris HCl, pH 7.6, containing 0.25 M sucrose, then lysed by the

addition of 2 volumes of buffer A (10 mM Tris, 10 mM $MgCl_2$, 20 mM β-mercaptoethanol, 10^{-5} M phenylmethylsulfanyl fluoride, 15% v/v glycerol, pH 7.6) containing 1.5% NP-40. Nuclei and cell debris were removed by centrifugation at 30,000 *g* for 30 min. Prior to assaying, extracts were diluted with 3 volumes of buffer A lacking magnesium since high levels of magnesium were found to partially protect the mutant CHO enzyme from in vitro heat inactivation.

Reaction mixtures contained, in a final volume of 150 μl, 20 mM Tris, pH 7.6, 15 mM $MgCl_2$, 5 mM ATP, 0.35 mM CTP, 0.1 mM dithiothreitol, 200 μg yeast tRNA, 30 μM [^{14}C]asparagine (226 mCi/mmol, 0.1 μCi/ml), and 50–200 μg extract protein. Reaction mixtures were prewarmed to 30°C and assays were initiated by the addition of cell-free extract. Forty-microliter samples were removed at 0-, 2-, and 4-min intervals and injected into ice-cold 5% TCA. The TCA precipitates were collected under vacuum on glass fiber filters, washed twice with cold 5% TCA, once with cold 95% ethanol, and dried. Filters were placed in ACS scintillation fluor (Amersham) and radioactivity determined by liquid scintillation spectrometry. The amount of [^{14}C]asparaginyl-tRNA formed linearly with time was determined. Specific activities are expressed as pmol [^{14}C]asparaginyl-tRNA formed/min/mg extract protein.

In vitro Heat Inactivation of Asparaginyl-tRNA Synthetase. Cell-free extracts were prepared as described above, then incubated at 37°C in a constant temperature water bath. At various times replicate samples were removed and assayed for asparaginyl-tRNA synthetase activity as described above.

Protein Determinations. The protein concentration of cell-free extracts was determined using the method of Bradford (22) using bovine serum albumin as the standard.

In vivo Protein Synthesis. The cell lines to be assayed were dispersed into duplicate 16-mm wells of 24-well cluster dishes (10^5 cells/well) in 1 ml of DMEM containing 10^{-5} M lysine and incubated at 33°C or 39°C overnight. The following day L-[4,5-^{3}]lysine (68 mCi/mmol, Amersham) was added to each well at a final concentration of 5.3×10^{-5} M (4 μCi/ml). The pulse was terminated 1 and 2 hr later by the addition of 300 μl of 20% TCA to each well. Media was removed and each well was washed twice with 5% TCA to remove any remaining soluble radioactive material. Wells were then washed once with 2 ml of 0.9% NaCl and the cells, which remained fixed to the surface of the culture dishes up to this point, are solubilized by the addition of 400 μl of 0.1 N NaOH. Wells were washed once more with 400 μl of 0.1 N NaOH and the entire 800 μl was added to scintillation vials, neutralized with HCl, and radioactivity determined in ACS scintillation fluor (Amersham). The rate of incorporation of [^{3}H]lysine into TCA-insoluble material was then determined

for the various cell lines under various conditions, which are described in the text.

Cell Fusion and Isolation of Temperature-Resistant Human × Hamster Interspecific Hybrids. Interspecific hybrids between human peripheral blood leukocytes and the temperature-sensitive CHO mutant Asn-5 were formed using β-propionolactone-inactivated Sendai virus (Connaught Laboratories, Willowdale, Ontario, Canada) according to the methods of Giles and Ruddle (23). Six million Asn-5 cells were mixed with approximately 3×10^7 freshly isolated human leukocytes and treated with Sendai virus to induce cell fusion. Cells were diluted into 100 ml of DMEM supplemented with 3 mM asparagine, dispersed into 10 replicate 100-mm culture dishes, and incubated overnight at 33°C. The following day the medium was removed, plates were washed twice to remove the leukocytes, which do not attach to culture dishes, and medium lacking asparagine was added. Plates were then transferred to 39°C. Colonies that appeared 7–20 days later were cloned using sterile glass cloning rings and maintained at 39°C in absence of asparagine. All human × hamster hybrids were designated by the prefix HHW.

Selection of Temperature-Sensitive Segregants. Cells from the temperature-resistant hybrids which had lost the human *asnS* gene, and as a result had become temperature-sensitive, were selected using a slight modification of the amino acid-suicide selection procedure described by Wasmuth and Caskey (13). Temperature-resistant hybrids were grown at 33°C for two weeks in medium with 3 mM asparagine to allow segregation of the human *asnS* locus to occur in the absence of selective pressure. Cells were then distributed into 100-mm culture dishes at a density of 10^6 cells per dish and incubated overnight at 33°C in medium with 3 mM asparagine. The following day the medium was removed, cells were washed twice with a balanced salts solution, 8 ml of 0.1 × LF-MEM were added, and cells were transferred to 39°C. Five hours later, the toxic lysine analog, *S*-2-aminoethyl-L-cysteine (thialysine), was added to the dishes to a final concentration of 1.5×10^{-3} M, and the plates were returned to 39°C. Twenty hours later the toxic medium was removed, the cells were washed twice with normal medium, and placed at 33°C in DMEM supplemented with 3 mM asparagine. Under these selective conditions, cells which are actively synthesizing protein at 39°C incorporate the toxic lysine analog into protein in place of lysine, resulting in cell death (13). However, segregants in the population that have lost the human *asnS* gene, which precludes their ability to synthesize protein at the elevated temperature, do not incorporate thialysine into protein and can be recovered when the cells are returned to 33°C in the absence of the toxic analog. Clones which survived this selective procedure were isolated 2–3 weeks later using glass cloning cylinders and were maintained at 33°C medium with asparagine. All of the cell lines isolated in this manner were subsequently tested to

determine if they were indeed temperature-sensitive by examining their ability to grow at 39°C in the absence of asparagine. Clones which showed the marked temperature-sensitive phenotype characteristic of the Asn-5 parent were saved and analyzed as described below.

Karyological Analyses. Differential staining of human and hamster chromosomes in metaphase preparations of hybrid cells was performed using the alkaline–Giemsa (G-11) procedure described by Friend et al. (24) with the modifications of Dana and Wasmuth (2). Trypsin–Giemsa banding (G-banding) of metaphase chromosome preparation was performed as previously described (32). Following G-banding and photography of metaphase chromosome preparations, slides were destained, then restained using the alkaline–Giemsa procedure as previously described (32). The photographed, G-banded chromosome spreads were then located again under the microscope after differential staining, and the human (light blue-staining) chromosomes were identified on the corresponding photographs of the G-banded spread. This allows unequivocal identification of human chromosomes in the photographs of G-banded chromosomes.

Electrophoretic Analysis of Human Isozyme Markers. Hybrid cells and temperature-sensitive segregants were analyzed for the presence of human isozyme markers specific for human chromosomes 10–22 following electrophoretic separation of the human and hamster forms of the various enzymes on starch gels or Cellogel. The enzyme markers analyzed, their chromosomal assignments, and references for the conditions of electrophoresis and staining of gels are given in Table 1. As discussed below, we restricted the isozyme analyses to the smaller human chromosomes because karyological analyses of

Table 1. Human Chromosomal Marker Enzymes

Enzyme	Abbreviation	Human chromosomal assignment	Reference
Glutamate-oxaloacetate transaminase	GOTS	10	25
Esterase A	EST A	11	25
Lactate dehydrogenase B	LDH B	12	25
Esterase D	EST D	13	25
Nucleoside phosphorylase	NP	14	25
Mannose phosphate isomerase	MPI	15	25
Phosphoglycollate phosphatase	PGP	16	26
Thymidine kinase	TK	17	27
Peptidase A	PEP A	18	25
Glucose phosphate isomerase	GPI	19	25
Adenosine deaminase	ADA	20	25
Superoxide dismutase 1	SOD 1	21	25
Aconitase (mitochondrial)	ACON M	22	28

the hybrids revealed that the only human chromosome consistently present in all hybrids was a small, submetacentric E group chromosome.

RESULTS

Isolation of Interspecific, Temperature-Resistant Hybrid Cell Lines. The temperature-sensitive CHO asnRS mutant, Asn-5, was fused to human peripheral leukocytes as described in the Materials and Methods section and hybrids were selected based upon their ability to grow at 39°C. This selection was, therefore, designed to isolate hybrid cell lines in which the defective CHO *asnS* gene was complemented by its normal human counterpart. Seventeen temperature-resistant colonies were obtained from 6×10^6 fused Asn-5 cells, a frequency of 2.8×10^{-6}. This frequency is approximately 12-fold higher than the spontaneous reversion frequency for the Asn-5 cell line, which is 2.2×10^{-7}. Seven of the heat-resistant clones, each from a separate culture dish to ensure they were independent, were isolated and grown into mass culture at 39°C for further analysis.

In order to determine if the temperature-resistant clones were indeed hybrids, we prepared metaphase chromosome spreads from each hybrid and examined them using the alkaline–Giemsa (G-11) staining technique (2, 24). This procedure distinguishes human from rodent chromosomes based upon differential staining characteristics at high temperature and high pH. In each hybrid examined, the presence of at least three human chromosomes was confirmed. The two hybrids containing the fewest human chromosomes, HHW 117 (4 human chromosomes) and HHW 119 (three human chromosomes) were examined biochemically, as described below.

Asparaginyl-tRNA Synthetase Activity in Hybrid Cells. In view of the fact that the temperature-resistant clones we isolated all contained human chromosomes, we suspected that their ability to grow at 39°C in the absence of asparagine was due to their expressing the human gene encoding asnRS. To examine this possibility, the activity of this enzyme was determined in extracts from two hybrid cell lines that had been grown under permissive conditions (33°C plus asparagine) and nonpermissive conditions (39°C minus asparagine) for the Asn-5 parent. At the low temperature, both the mutant CHO enzyme and the normal human enzyme should be present. At the elevated temperature, however, the CHO enzyme is inactivated, and only the human enzyme should be detected. AsnRS activity in extracts prepared from Asn-5 cells that have been grown at 39°C in medium without asparagine for as short a period as 12 h is undetectable (specific activity of less than 4). The results of these experiments are shown in Table 2. In both hybrids, the activity of asnRS is considerably higher in extracts prepared from cells that had been grown at 33°C. This is the expected result if both the mutant Asn-5 and normal human

Table 2. Asparaginyl-tRNA Synthetase Activity in Cell-Free Extracts from Hybrid, Mutant, and Wild-Type Cells[a]

Cell line	Growth conditions	Asparaginyl-tRNA synthetase specific activity
Asn-5	33°C + Asn	129 (±13)
GAT$^-$	33°C + Asn	314 (±10)
HeLa	39°C − Asn	362 (±23)
HHW 117	33°C + Asn	158 (±25)
HHW 117	39°C − Asn	77 (±28)
HHW 119	33°C + Asn	196 (±50)
HHW 119	39°C − Asn	107 (± 4)

[a]The specific activities of asparaginyl-tRNA synthetases were determined as described in Materials and Methods. The cell lines were grown under the indicated condition of temperature and asparagine supplementation for at least one week prior to being harvested. Each of the values listed represents the average of at least three independent determinations, with the standard derivations listed in parentheses. The activities are expressed as pmol [^{14}C]asparaginyl-tRNA formed/min/mg extract protein. The lower limit of asparaginyl-tRNA synthetase specific activity that is detectable using this assay is approximately 4.

enzymes are detectable in extracts prepared from hybrid cells grown at 33°C but only the human enzyme is detectable in extracts prepared from cells grown at 39°C. That both forms of AsnRS (mutant Chinese hamster and normal human) are present in hybrid cells grown at 33°C was confirmed in experiments described below. Although the activity of asnRS in hybrids grown at 39°C is considerably lower than the activity in wild-type CHO (GAT$^-$) cells or human (HeLa) cells, it is sufficient for normal growth and protein synthesis since these hybrids grow vigorously at the elevated temperature without exogenous asparagine.

Thermostability of Asparaginyl-tRNA Synthetase from Hybrid Cells. The asnRSs extracted from Asn-5 and human (HeLa) cells can easily be distinguished in vitro based upon large differences in their susceptibility to thermal inactivation. As shown in Fig. 1, the asnRS in cell-free extracts from Asn-5 is very unstable at 37°C, with a half-life of about 2 min. In contrast, the human asnRS is very stable under these conditions, with essentially no loss of activity even after 20 min of incubation at 37°C. Therefore, if the temperature-resistant hybrids express both forms of the enzyme when grown under permissive conditions, part of the asnRS activity in vitro should be very unstable, like the enzyme from Asn-5, and part of the activity should be very stable, like the human enzyme. In vitro heat-inactivation studies on the asnRS extracted from two hybrids, HHW 117 and HHW 119, were performed and the results are shown in Fig. 1. In extracts prepared from both of these cell lines, 30–40% of the total asnRS activity is inactivated in the first 2 min of incubation at 37°C, while the remaining activity is much more stable. The biphasic nature of the thermal inactivation of asnRS in these extracts provides

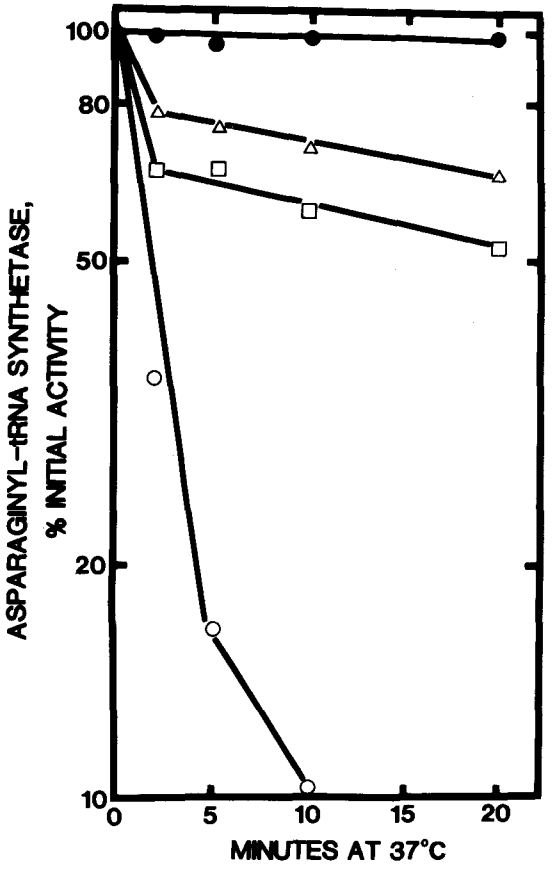

Fig. 1. In vitro thermostability of asparaginyl-tRNA synthetase activity in cell-free extracts of human cells, mutant Asn-5 cells, and hybrids. The preparation of extracts and determination of asparaginyl-tRNA synthetase activity are described in Materials and Methods. Cells were grown at 33°C in medium with 3 mM asparagine for 7 days prior to harvesting and preparation of extracts. Extracts were incubated at 37°C for the indicated lengths of time, then samples were removed and assayed for asparaginyl-tRNA synthetase activity at 30°C. The amount of activity without preincubation at 37°C is defined as 100% for each cell line. The results represent the average of three separate experiments for each cell line. (●) Human (HeLa) cells; (○) Asn-5; (△) HHW 117; (□) HHW 119.

a strong indication that there are indeed at least two forms of asnRS present, the thermolabile enzyme derived from the mutant hamster *asnS* gene and a stable enzyme which is derived from the human *asnS* gene.

It should be pointed out that even though the asnRS activity that remains in the hybrid extracts after the first 2 min of incubation at 37°C is much more stable than the mutant Asn-5 enzyme, it does appear to be somewhat less stable than the asnRS activity derived from HeLa cells. The possibility that

this result is due to the presence of a hybrid multimeric enzyme in HHW 117 and HHW 119 is discussed below.

Selection and Characterization of Temperature-Sensitive Segregants. It is well known that human chromosomes are preferentially lost from rodent-human hybrids if their retention is not required for cell survival (29). The segregation of the human chromosome carrying the *asnS* locus from hybrids would result in the loss of the ability to grow at 39°C in the absence of exogenous asparagine. If the loss of the temperature-resistant phenotype and the human *asnS* locus could be correlated with the loss of a specific human chromosome, a chromosomal assignment could be made for this gene. Temperature-sensitive segregants were selected using the cytotoxic lysine analog, *S*-2-aminoethyl-L-cysteine (thialysine) as described in the Materials and Methods section. When cells are exposed to this amino acid analog, they rapidly lose viability if they are actively synthesizing protein due to the incorporation of thialysine into proteins in place of lysine (13). At 39°C in the presence of thialysine, the temperature-resistant hybrids will be sensitive to the cytotoxic effects of thialysine since they synthesize protein actively at this temperature. In contrast, in segregants that have lost the human *asnS* locus, protein synthesis will cease at the elevated temperature (in the absence of exogenous asparagine), the cells will not incorporate the toxic analog into protein, and they can be recovered following removal of thialysine and transfer of culture to 33°C plus asparagine.

To ensure that every segregant we examined was independent, several of the primary heat-resistant hybrids were subcloned, at 39°C in the absence of asparagine. The subclones were then grown at 33°C for at least 10 generations to allow temperature-sensitive segregants to accumulate in the populations. Temperature-sensitive segregants were then selected from the various subclones as outlines above. From each subculture, a number of colonies that survived the selective procedure were isolated and grown into mass culture at 33°C. All of these cell lines were then examined to determine if they were temperature-sensitive by examining their viability at 39°C. A total of 40 bona fide temperature-sensitive segregants were identified. In each of these 40 segregants the temperature-sensitive phenotype was suppressed by including 3 mM asparagine in the culture medium, indicating that they had indeed lost the human *asnS* gene.

In order to quantitate the effect of asparagine on these cell lines, we examined their rates of protein synthesis in vivo under various culture conditions, as described in the Materials and Methods section. The rates of protein synthesis were determined 20 h after cells had been established in three different culture conditions: 33°C plus asparagine, 39°C plus asparagine, and 39°C minus asparagine. The first two sets of conditions are permissive for the mutant, Asn-5, while the latter condition is very restrictive.

Table 3. Effect of Temperature and Exogenous Asparagine on Rates of Protein Synthesis in Vivo in Hybrids, Segregants, Asn-5, and Human Cells[a]

Cell line	Rate of protein synthesis at 39°C + Asn / Rate of protein synthesis at 33°C + Asn	Rate of protein synthesis at 39°C − Asn / Rate of protein synthesis at 33°C + Asn
Asn-5	1.39	0.04
HeLa	1.40	1.18
HHW 119 clone 1	2.56	2.88
HHW 119 clone 2	2.69	2.69
HHW 119 clone 3	2.45	2.50
Segregant 1	1.80	0.10
Segregant 2	2.20	0.13
Segregant 3	2.17	0.06

[a] The rates of protein synthesis (incorporation of [^{3}H]lysine into protein) were determined at 33°C in medium plus asparagine, 39°C in medium plus asparagine, and 39°C in medium without asparagine, as described in Materials and Methods. The cpm of [^{3}H]lysine incorporated into protein per hour at 33°C in medium plus asparagine (conditions which are permissive for all the cell lines) ranged from 12,400 for segregant 3 to 40,320 for HeLa cells.

The results of these experiments for three independent temperature-sensitive segregants, their temperature-resistant parents, the Asn-5 mutant and HeLa cells are shown in Table 3. In the three temperature-resistant subclones and HeLa cells, the rates of protein synthesis are higher at 39°C than 33°C whether or not asparagine was present in the medium at the elevated temperature. In Asn-5 and the three temperature-sensitive segregants, the rates of protein synthesis are also higher at 39°C than at 33°C if asparagine is present at the elevated temperature. However, at 39°C in the absence of asparagine, the rates of protein synthesis in these four cell lines are reduced to about 10% the rates at 33°C. These results demonstrate that in the temperature-sensitive segregants, as in Asn-5, protein synthesis at 39°C is dependent upon exogenous asparagine, most likely because the segregants no longer express the human *asnS* gene. To confirm this, we examined the thermal inactivation of asnRS in cell-free extracts prepared from two segregants, one from an HHW 117 subclone and one from a HHW 119 subclone. As shown in Fig. 2, both segregants contain only the heat-sensitive form of asnRS characteristic of the Asn-5 parent. Thus, both segregants have lost the heat-stable form of asnRS that was present in their heat-resistant parents.

Chromosomal Assignment of Human asnS *Gene.* In order to determine on which human chromosome the *asnS* gene is located, we analyzed hybrid clones and temperature-sensitive segregants for the presence of human enzyme markers specific for human chromosomes. Karyological analysis of six different primary hybrids, and eight different secondary subclones, using the alkaline–Giemsa staining procedure to distinguish human from hamster

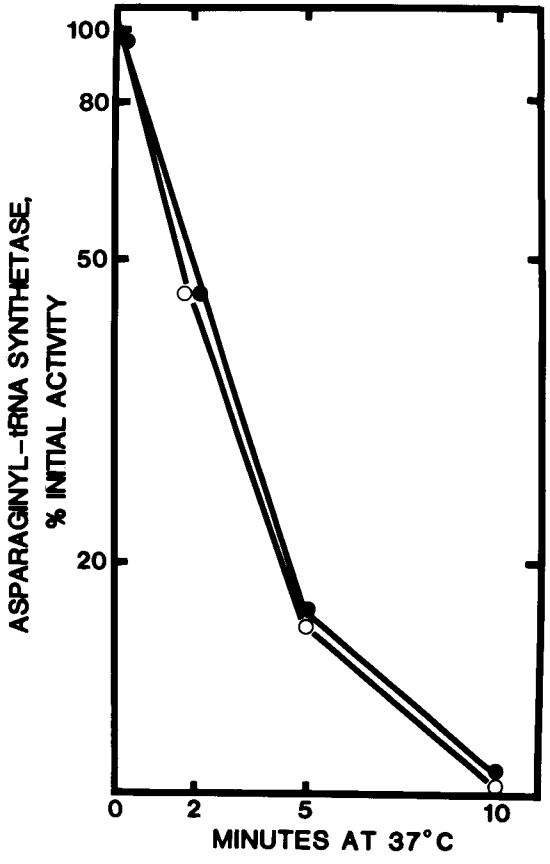

Fig. 2. In vitro thermostability of asparaginyl-tRNA synthetase activity in cell-free extracts of temperature-sensitive segregants. Experimental details are described in Materials and Methods and in the legend to Fig. 1. The results represent the average of three separate experiments for each cell line. (○) Temperature-sensitive segregant derived from HHW 117; (●) temperature-sensitive segregant derived from HHW 119.

chromosomes, revealed that all of these cell lines had retained at least one very small, submetacentric E group chromosome. No other human chromosomes were consistently present in all hybrids. Therefore, we restricted these isozyme analyses to human chromosomes 9–22. The Asn-5 cell line, like its parent, is deficient in formyl polyglutamate synthetase and therefore requires exogenous glycine, adenosine, and thymidine (GAT) for growth. The human gene which complements this defect in GAT-requiring cells is located on chromosome 9 (30). Therefore, the presence of human chromosome 9 was scored using a bioassay to determine the ability of the hybrids to grow in the absence of glycine, adenosine, and thymidine.

Table 4. Analysis of Human Chromosomal Marker Enzymes in Hybrids and Segregants[a]

Cell line	Human asnRS	Human chromosomes													
		9	10	11	12	13	14	15	16	17	18	19	20	21	22
HHW 119 clone 1	+	–	–	–	+	NS[b]	–	–	–	–	+	+	–	–	NS
TS Segregant	–	–	–	–	+	NS	–	–	–	ND[c]	–	+	–	–	NS
HHW 119 clone 2	+	–	–	–	+	NS	–	–	–	–	+	+	–	–	NS
TS Segregant	–	–	–	–	+	NS	–	–	–	ND	–	+	–	–	NS
HHW 119 clone 3	+	–	–	–	+	NS	–	–	–	–	+	+	–	–	NS
TS Segregant	–	–	–	–	+	NS	–	–	–	ND	+	+	–	–	NS
HHW 117 clone 1	+										+				
TS Segregant	–										–				
HHW 117 clone 2	+										+				
TS Segregant	–										–				
HHW 115 clone 1	+										+				
TS Segregant	–										–				
HHW 122	+										+				
HHW 124	+										+				
HHW 127	+										+				
HHW 128	+										+				

[a]The presence of the various human chromosomes was determined by analyzing cell-free extracts for the presence of human isozymes that have been assigned to specific chromosmes. These analyses and the marker enzymes utilized are described in Materials and Methods and Table 1.
[b]NS, no separation of hamster and human isozymes when analyzed.
[c]ND, not determined.

The initial marker analyses were performed on three subclones of HHW 119, which has the fewest human chromosomes (three) and temperature-sensitive segregants derived from these subclones. As shown in Table 4, the three subclones had the human enzymes corresponding to chromosomes 12, 18, and 19, which accounts for all three of the human chromosomes present. The three temperature-sensitive segregants all retained human chromosomes 12 and 19, but two of the three have lost human peptidase A (Pep A) activity (chromosome 18), suggesting this latter human chromosome contained the *asnS* locus. We next analyzed subclones and temperature-sensitive segregants derived from two other hybrids, HHW 115 and HHW 117, as well as four more primary hybrids, HHW 122, HHW 124, HHW 127, and HHW 128, for the presence of human Pep A. As shown in Table 4, all seven of the additional temperature-resistant hybrids expressed human Pep A, while all three of the additional temperature-sensitive segregants have lost human Pep A. A photograph of a starch gel, demonstrating the presence of the human form of Pep A in heat-resistant hybrids and its absence in a temperature-sensitive

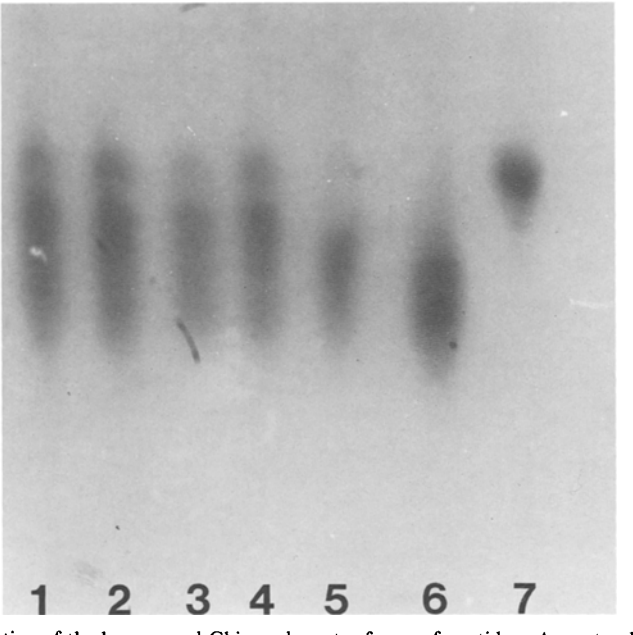

Fig. 3. Separation of the human and Chinese hamster forms of peptidase A on starch gels. The conditions used for the preparation of extracts, electrophoresis, and staining of gels are described in reference 25. Lanes 1–4, human Pep A positive hybrids (HHW 117 clone 2, HHW 119 clone 1, HHW 122, HHW 124, respectively). Lane 5, human Pep A-negative segregant derived from HHW 119 clone 1. Lane 6, Chinese hamster (Asn-5) cells. Lane 7, human (HeLa) cells. The origin was at the very top of the photograph and electrophoresis was from the cathode (top) to the anode (bottom).

segregant, is shown in Fig. 3. Thus, 10 out of 10 heat-resistant hybrids expressed human Pep A, whereas 5 out of 6 temperature-sensitive segregants have lost this activity. The presence of human chromosome 18 was also confirmed cytogenetically in HHW 117 and HHW 119 following trypsin–Giemsa banding (G-banding) of metaphase chromosome preparations, coupled with differential staining, as described in the Materials and Methods section. A photograph of a G-banded chromosome spread from HHW 119 is shown in Fig. 4. As shown in this photograph, only the three human

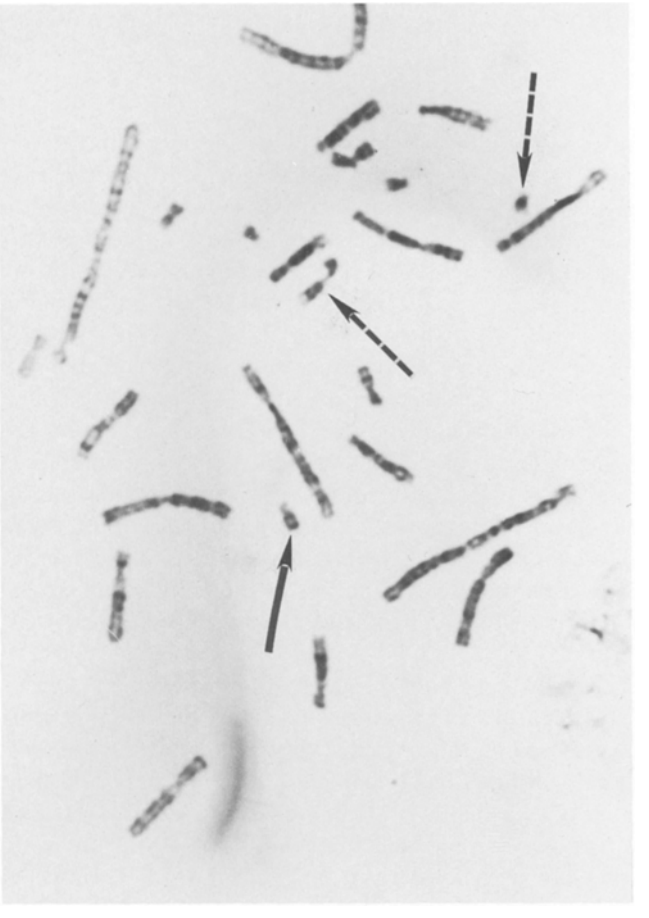

Fig. 4. Trypsin–Giemsa-banded metaphase chromosome spread from the hybrid HHW 119. Metaphase chromosome preparations were G-banded as described in Materials and Methods and photographed. After photography, the slides were destained, then differentially stained to allow unequivocal identification of the human chromosomes in photographs of the G-banded chromosome preparations. Human chromosomes 12 and 19 are indicated by dashed arrows, while human chromosome 18 is indicated by a solid arrow.

chromosomes, 12, 18, and 19 whose presence was indicated by the isozyme analyses, are present in metaphase chromosome preparations from this hybrid.

The very strong correlation between the temperature-resistant phenotype, the presence of a thermostabile (human) form of asnRS and human chromosome 18, indicates that the human gene, *asnS*, which corrects the temperature-sensitive phenotype of the CHO asnRS mutant, is located on chromosome 18. This assignment is strengthened by the observation that the three characteristics, temperature-resistance, thermostable asnRS activity, and human Pep A activity, segregated concordantly in 5 out of 6 cases.

DISCUSSION

The interspecific temperature-resistant hybrid cell lines described in this report demonstrate that the temperature-sensitive phenotype of the CHO asparaginyl-tRNA synthetase mutant, Asn-5, can be corrected by a human gene, *asnS*. Several lines of evidence indicate that the human *asnS* locus encodes a polypeptide component of asparaginyl-tRNA synthetase: (1) The mutation in the Asn-5 mutant, which is complemented by the human *asnS* gene, is associated with alterations in its asparaginyl-tRNA synthetase, including an elevation in the K_m for asparagine and a very dramatic increase in the thermolability of the enzyme in vitro (20). Although not absolutely conclusive, these data strongly suggest the mutation in the *asnS* gene in Asn-5 results in a direct alteration in the asparaginyl-tRNA synthetase enzyme (2). The temperature-resistant interspecific hybrids examined above have, in addition to the heat-labile form of asnRS characteristic of the Asn-5 parent, a heat-stable form of asnRS, which accounts for their ability to grow at 39°C in the absence of asparagine. (3). The loss of the temperature-resistant phenotype from hybrids is associated with the loss of the heat-stable form of asnRS.

Based upon the very high frequency with which asnRS mutants can be isolated from CHO cells and the recessive nature of the temperature-sensitive phenotypes of these mutants, it seems very likely that the *asnS* gene in CHO cells is physically or functionally haploid (18). It is interesting that in another Chinese hamster cell line, V-79 Chinese hamster lung (CHL) cells, asnRS mutants also arise at a very high frequency (13). All of the asnRS mutants examined thus far, whether derived from CHO or CHL cells, belong to the same complementation group, so they have alterations in the same gene, *asnS*. Thus, it appears that the *asnS* gene has, by chance, been rendered haploid in two different Chinese hamster cell lines. It would be of interest, therefore, to know the chromosomal location of this gene in Chinese hamster cells and then to determine if there have been karyotypic alterations of this chromosome in

CHO and CHL cells, which might explain the apparent haploidy of the *asnS* locus in both cell lines. We are hopeful that the finding that the *asnS* gene and Pep A are linked in humans will help answer this question, since the conservation of linkage relationships between autosomal genes among mammalian species is not uncommon. Stallings and Siciliano reported evidence that the *Pep A* locus in Chinese hamster cells may be linked to chromosome 5, although the data were insufficient to make even a provisional assignment (31). Thus, initial efforts to localize the *asnS* gene in Chinese hamsters might focus on chromosome 5.

Although very little is known about the structure of the mammalian asparaginyl-tRNA synthetase, the molecular weight of the active enzyme has been estimated to be approximately 150,000 (20). It is likely, therefore, that the enzyme is composed of multiple subunits. Whether the subunits are identical or nonidentical is unknown. If the enzyme is composed of subunits which are nonidentical and are products of separate genes, only one of these subunits would be altered in the asnRS mutants. If this were the case, it would imply that the subunit of asnRS specified by the human *asnS* gene (which would obviously correspond to the mutant subunit produced by Asn-5) combines with the other, normal subunit of the Asn-5 parent to produce a functional interspecific enzyme. If the enzyme were composed of identical subunits, and was a dimer for example, the hybrid cells could theoretically contain three forms of the enzyme, hamster–hamster dimers, human-human dimers, and hamster–human mixed dimers. Such a possibility may explain our observation that, although the hybrids contain a heat-stable form of asnRS, it is inactivated in vitro slightly more rapidly than the enzyme from human cells (see Fig. 1). Thus, this result may be due to the presence of the human–hamster dimer form of the enzyme, which is somewhat more heat labile than the human–human dimer. In any case, it is obvious that the product of the human *asnS* gene functions very well in place of the mutant CHO *asnS* gene product since the rates of protein synthesis in the interspecific hybrids and wild-type CHO cells are very comparable at 39°C.

The assignment of the human *asnS* gene to chromosome 18 is strongly supported by the correlation between the temperature-resistant phenotype of hybrids and the presence of human Pep A activity, the gene for which has been assigned to chromosome 18. Only one discordant clone was found out of 16 examined. Thus, 10 of 10 temperature-resistant hybrids expressed human Pep A and 5 of 6 segregants, which lost the temperature-resistant phenotype, also lost human Pep A activity. Our lack of knowledge concerning the subunit structure of asnRS precludes calling *asnS* the structural gene for asnRS, since the enzyme could be composed of nonidentical subunits which are products of separate genes. However, the evidence strongly suggests that *asnS* does encode a polypeptide component of this enzyme. In addition, we cannot

completely rule out the possibility that more than one human gene is required to suppress the temperature-sensitive phenotype of the CHO Asn-5 mutant, since not all the hybrid clones were examined for the presence of every human chromosome.

Besides *Pep A* and *asnS*, only one other locus, a gene required for the expression of human chorionic gonadotropin, has been assigned to chromosome 18 (33). The human genes encoding only two other aminoacyl-tRNA synthetases have been given chromosomal assignments, tryptophanyl-tRNA synthetase to chromosome 14 (1) and leucyl-tRNA synthetase to chromosome 5 (2, 3). Thus, as in prokaryotic cells, there is no clustering of this group of functionally related genes. One other gene encoding a polypeptide product involved in protein synthesis, the gene for ribosomal protein S14, has also been assigned to chromosome 5 (2). It is also interesting to note that the gene encoding the enzyme involved in the biosynthesis of asparagine, asparagine synthetase, has recently been assigned to human chromosome 7 (S. Arfin, F. Arrendondo-Vega, and M. Smith, manuscript submitted for publication).

The large number of cell lines that have been isolated with different mutations in the *asnS* gene, including temperature-sensitive mutants and temperature-resistant revertants, makes this locus very amenable to detailed genetic analysis. In order to utilize this collection of mutant cell lines to examine the effects of various mutations on the function and expression of the *asnS* locus, we are attempting to clone this gene. The approach we are using to clone the *asnS* gene utilizes the hybrid cell lines described here together with a series of procedures that have been outlined previously as an approach to cloning the gene for leucyl-tRNA synthetase (32).

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