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Assignment of Structural Gene for Asparagine Synthetase to Human Chromosome 7

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Abstract—*Somatic cell hybrids obtained from the fusion of human B lymphocytes and an asparagine synthetase-deficient Chinese hamster ovary cell line were isolated after growth in asparagine-free medium. The human and hamster forms of asparagine synthetase differ significantly in their rate of inactivation at 47.5° C. The asparagine synthetase activity expressed in the hybrids was inactivated at 47.5° C at the same rate as the human form of the enzyme. Karyotypic analysis and analysis for chromosome-specific enzyme markers showed that the structural gene for asparagine synthetase is located on chromosome 7 in humans. The heat-inactivation profile for asparagine synthetase in extracts of hybrids formed between human peripheral leukocytes and a hamster cell line expressing asparagine synthetase activity was intermediate between the two parental types when human chromosome 7 was present, but was identical to the hamster parent when chromosome 7 was absent.*

INTRODUCTION

Asparagine synthetase catalyzes the ATP-dependent synthesis of asparagine from aspartic acid and glutamine in higher eukaryotes (1). The enzyme is of interest for a number of reasons: the rate of synthesis of asparagine synthetase is regulated by dietary asparagine in whole animals (2, 3) and by the concentration of asparagine in the medium in cultured cells (4–6); certain animal and human tumors are responsive to chemotherapy with asparaginase because of a deficiency in asparagine synthetase activity (7–9); and enzyme activity is differentially expressed in various cell types (1) and during development (10, 11).

Waye and Stanners have isolated a mutant of Chinese hamster ovary cells which lacks asparagine synthetase activity and requires asparagine in the medium for growth (12). The phenotype of this mutant is recessive in intraspecific cell hybrids. This mutational auxotrophy should make it possible to isolate human-hamster hybrids able to grow in medium lacking asparagine because of the presence of the human gene for asparagine synthetase. In this report we describe the isolation and analysis of a number of such human-hamster hybrids. Our results indicate that these hybrids are producing the human asparagine synthetase and that the structural gene for asparagine synthetase is on chromosome 7.

MATERIALS AND METHODS

Cell Lines and Culture Conditions. The CHO cell lines used are GAT⁻ (13), N3, an asparagine auxotroph lacking detectable asparagine synthetase activity (12), obtained from Dr. C.P. Stanners, and Met-1, a temperature-sensitive methionyl-tRNA synthetase mutant (14). These cells were grown in either suspension or monolayer culture in α -MEM (K.C. Biologicals) supplemented with 10% fetal calf serum and 10 μ g/ml each of adenosine and thymidine. The human parental cells are 8866, an established human B cell line obtained from Dr. Gale Granger. The 8866 cells were grown in static suspension culture in RPMI-1640 medium containing 10% fetal calf serum. Cultures were maintained at 37°C in a humidified CO₂ incubator.

Cell Fusion. Fusion of 8866 cells and N3 cells was performed using polyethylene glycol as described by Brahe and Serra (15). The 6×10^5 8866 cells were added to 2×10^5 N3 cells in 60-mm plastic tissue culture dishes and the mixed culture was incubated for 4 h. The culture medium was then aspirated off and replaced with 1 ml of 50% PEG 1000 at 37°C. After 2 min of incubation, the PEG was removed by aspiration, the dishes were washed three times with Hanks' balanced salt solution, and incubated for 24 h with α -MEM. The cells were then split into 100-mm tissue culture dishes at a density of 10^5 cells/dish and incubated in asparagine-free α -MEM. The selective medium was changed every 4 days, and five primary hybrid clones were isolated 4 weeks after fusion. These clones were maintained in selective medium. A series of independent secondary hybrid subclones were isolated from each of the primary hybrids and maintained under the same selective conditions.

Hybrids were also formed between human peripheral blood leukocytes and the temperature-sensitive CHO mutant Met-1 using β -propiolactone-inactivated Sendai virus according to the procedure of Giles and Ruddle (16). Six million Met-1 cells were mixed with approximately 3×10^7 freshly

isolated leukocytes and treated with Sendai virus to induce cell fusion. The cells were then diluted into 100 ml of DMEM supplemented with 0.5 mM proline, 0.2 mM glycine, 37 μ M adenosine, and 41 μ M thymidine; distributed into 10 culture dishes (100 mm); and incubated overnight at 34°C. The following day the medium was removed and the dishes washed twice with medium to remove unfused leukocytes. The plates were then incubated at 39°C in medium containing a reduced methionine concentration (2×10^{-5} M, 1/10 the standard formulation). These conditions select for hybrids in which the human chromosome carrying the methionyl-tRNA synthetase structural gene has been retained. Colonies which appeared after 10–20 days were cloned and maintained at 39°C in medium containing 2×10^{-5} M methionine. These hybrid clones are designated by the prefix HHW.

Asparagine Synthetase Assay. Monolayer cultures were harvested and extracts prepared as previously described (17). Asparagine synthetase activity was measured by following the conversion of [14 C]aspartic acid to [14 C]asparagine as described (17).

Heat Inactivation of Asparagine Synthetase in Vitro. Cells were lysed in buffer containing 50 mM Tris, pH 7.5, 5 mM EDTA, 1 mM dithiothreitol, and 20% glycerol by freeze-thawing. The lysate was centrifuged at 25,000 *g* for 15 min and the protein concentration of the supernatants adjusted to 3 mg/ml. The supernatants were heated at 47.5°C. Aliquots were removed at

Fig. 1. Representative G-banded karyotype of human B-cell line 8866.

various times, placed on ice, and then assayed for asparagine synthetase activity at 37°C.

Chromosome Analysis. The human chromosome content of hybrids was determined by both karyotypic analysis and isoenzyme analysis. Fixed metaphase preparations of hybrid clones were analyzed for human chromosomes by Giemsa-11 staining (18) and G-banding with trypsin (19). Because 8866 is an established cell line, it was important to demonstrate that it contained a normal human chromosome complement. Identification of the modal karyotype was done by analysis of photographs from 20 different fresh metaphase spreads after G-banding. A representative karyotype is shown in Fig. 1. Line 8866 appears to have a normal human karyotype by this cytogenetic criterion.

Fig. 2. Heat inactivation of asparagine synthetase from human and hamster cell lines. Extracts (3 mg of protein per ml) were heated at 47.5°C for the times indicated. Samples were chilled in an ice bath and then assayed for remaining asparagine synthetase activity. ●, 8866 cells; ○, Gat⁻ cells.

The hybrid clones were also analyzed for chromosome-specific enzyme markers by electrophoretic separation and conventional histochemical staining. The following enzymes were tested as chromosome-specific markers. Chromosome 1: PGM 1 (20); 2: MDH 1 (21); 3: ACY-1 (22); 4: PGM 2 (20); 5: HEXB (21); 6: ME 1/SOD 2 (20); 7: GUSB (23); 8: GSR (24); 9: ACO 1 (25); 10: GOT 1 (20); 11: LDHA (20); 12: LDHB (20); 14: NP (20); 15: MPI (20); 16: PGP (26); 18: PEPA (20); 19: GPI (20); 20: ADA (20); 21: SOD 1 (20); 22: ACO 2 (25).

RESULTS

Asparagine Synthetase Activity in Hybrids. Five independent primary hybrids capable of prolonged growth in asparagine-free medium were

Fig. 3. Half-time for inactivation of asparagine synthetase from parental and hybrid cell lines. Matched extracts from human, hamster, and human-hamster cell hybrids were heated at 47.5°C for varying periods of time, chilled in an ice bath, and then assayed for remaining asparagine synthetase activity. The half-life for enzyme inactivation was determined from a plot of percent enzyme activity remaining vs. time at 47.5°C. Each point represents data from an individual experiment.

obtained from three independent fusions of the CHO cell line N3 and the human B-cell line 8866. These clones presumably have retained the human gene for asparagine synthetase. In order to confirm that the asparagine synthetase in the hybrid cells is of the human variety, we compared the heat sensitivity of this enzyme in human, hamster, and hybrid cells. There is a clear difference in the rate of inactivation at 47.5°C of asparagine synthetase from hamster cells and from human cells (Fig. 2). The enzyme from GAT⁻ cells was inactivated with a half-life of approximately 2 min, while the human enzyme was considerably more resistant (half-life approximately 7 min).

The kinetics of heat inactivation of asparagine synthetase was measured in each of the primary hybrids. The results are shown in Fig. 3. The asparagine synthetase of each of the hybrids is relatively stable at 47.5°C and the half-lives of inactivation are like that of the human enzyme. Similar results were obtained with the secondary hybrids tested. Thus, it is likely that these hybrids have retained and are expressing the human structural gene for asparagine synthetase.

Mapping the Structural Gene for Asparagine Synthetase. Enzyme markers specific for 20 human chromosomes were examined in the primary

Fig. 4. Electrophoretic patterns of β -glucuronidase from parental and some secondary hybrid cell lines. From left to right, extracts from: GAT⁻, subclones 6AF1/21, 4AF1/3, 1CF2/6, 1BF2/1, 1BF2/5, 6AF1/16, 1CF2/104, and human liver.

clones. The only human enzyme which was consistently present in all clones was β -glucuronidase, the gene for which is known to be located on human chromosome 7 (27).

Karyotypic analyses of the primary clones, using alkaline Giemsa staining to distinguish human from hamster chromosomes, revealed that within each clone there was considerable heterogeneity with respect to which human chromosomes were present, although all the clones contained only a few human chromosomes. Subclones were therefore derived from each primary clone. Thirty-four of these subclones were tested for the presence of human β -glucuronidase. A representative gel is shown in Fig. 4. Thirty of the subclones expressed the human form of β -glucuronidase. Karyotypic studies were done on several subclones derived from each of the primary clones. Subclones derived from 4AF1 contained an intact human chromosome 7 as the only human chromosome (Fig. 5); subclones derived from 6AF1 also contained an intact human chromosome 7 and also chromosomes 3, 6, and 20. Subclones derived from 1CF2 contained a portion of human chromosome 7 (apparently including the centromere and long arm) translocated to a piece of hamster chromosome (Figs. 6 and 7). Subclones derived from 1EF2 contained only a small human centric chromosomal fragment (Fig. 8). These latter subclones were negative for human β -glucuronidase, but expressed an as-

Fig. 5. Giemsa-stained metaphase chromosome spread from secondary hybrid clone 4AF1/106 showing a single intact human chromosome 7.

Fig. 6. Metaphase chromosome spread from secondary hybrid clone 1CF2/5. (A) G11 stained, showing hamster-human translocation. (B) Trypsin banded, showing that human material present in translocation has banding pattern identical to that in 7p12-7q31.

paragine synthetase activity whose heat sensitivity was the same as that of the human enzyme. Subclones derived from 1BF2 apparently contained a human chromosome 7 from which the short arm was deleted.

In order to make certain that the heat stable form of asparagine synthetase segregates with human chromosome 7, tertiary subclones were

Fig. 7. (A) Schematic representation of human chromosome 7. (B) Trypsin-banded intact chromosome 7. (C) Trypsin-banded human-hamster translocation from 1CF2/5. (D) G11-stained human-hamster translocation from 1CF2/5.

Fig. 8. G11-stained metaphase chromosome spread from secondary hybrid clone 1EF2/3 showing a centric chromosomal fragment as the only human chromosomal material.

selected from several of the secondary subclones that were maintained in nonselective medium (containing asparagine) for more than 20 passages. Table 1 summarizes the results of an analysis of these subclones. In every case those segregants which now showed a requirement for asparagine for growth and which lacked any measurable asparagine synthetase activity also lacked human chromosome 7 or fragments of it as determined cytogenetically or by expression of the human form of β -glucuronidase. Those subclones which retained the ability to grow in medium lacking asparagine also expressed a heat stable form of asparagine synthetase and had retained human chromosome 7 or the fragment of chromosome 7 which was present in the subclones from which they were derived.

Further confirmation of the assignment of the structural gene for asparagine synthetase to human chromosome 7 is provided by the analysis of interspecific hybrids formed between human peripheral blood leukocytes and the temperature-sensitive CHO mutant Met-1. A series of such hybrids have been isolated and characterized in a study of the chromosomal assignment of the human methionyl-tRNA synthetase gene (R.E. Cirullo and J.J. Was-

Table 1. Syntenic Relationship of Human Asparagine Synthetase and Chromosome 7 in Human-Hamster Hybrids^a

Clone	Heat-stable asparagine synthetase Human chromosome 7			
	+ /	- / -	+ / -	- / +
Primary ^b	6	1	0	0
Secondary ^c	30	0	0	0
Tertiary	11	7	0	0

^aThe presence of human chromosome 7 was determined by β -glucuronidase and karyotype analysis.

^bThe five 8866 $\times$ N3 hybrids, HHW 151, and HHW 271.

^cDerived from the 8866 $\times$ N3 primary clones, but not including subclones derived from 1EF2 containing the small human centric chromosomal fragment.

muth, manuscript in preparation). Two of these hybrids, HHW 151 and HHW 271, are relevant to these studies. HHW 151 contains chromosomes 7 and 12 as the only human chromosomal material, while HHW 271 contains only human chromosome 12 (Fig. 9). In vitro heat inactivation studies were performed on the asparagine synthetase from both hybrids, from Met-1, and from 8866. If either or both hybrids are expressing both the human and hamster forms of asparagine synthetase, then one would expect either a biphasic heat-inactivation curve or a linear rate of inactivation intermediate between the two parental lines. Asparagine synthetase is a dimer of identical subunits (6) and the expected heat inactivation curves will be dependent upon the relative rate of synthesis of the human and hamster forms of asparagine synthetase in the hybrids, the ability of the human and hamster subunits to associate with each other, and the effect of at least one heat-labile subunit on the rate of heat inactivation of the active dimer.

Figure 10 shows that the decay of asparagine synthetase activity in extracts of hybrid HHW 151 is intermediate to that of the parental lines, while that of extracts from HHW 271 is identical to that of the hamster parent. This result suggests that the asparagine synthetase activity in HHW 151 represents contributions from both the human and hamster forms while that in HHW 271 is due only to the hamster form of the enzyme. These results are consistent with the assignment of the structural gene for asparagine synthetase to human chromosome 7.

DISCUSSION

We have taken advantage of the asparagine auxotrophic mutant N3, derived from CHO cell line GAT⁻, to isolate human-hamster hybrid clones

Fig. 9. G-banded metaphase chromosome spread from hybrid HHW 151 (A) and HHW 271 (B). The arrows indicate human chromosomes 7 and 12 in HHW 151 and human chromosome 12 in HHW 271.

retaining and expressing the human structural gene for asparagine synthetase. Growth of the hybrids in asparagine-free medium provides strong selective pressure for the retention of the human gene. The asparagine synthetase activity in all 5 primary hybrid clones isolated resembles the human variety in its heat sensitivity. Electrophoretic mobility has been the most commonly used method for distinguishing human and rodent forms of an enzyme in interspecific hybrids, but differences in heat sensitivity have been used previously, as in the mapping of the gene for human acid α -glucosidase (28).

All five primary clones isolated contained only a small number of human chromosomes, and analysis of enzyme markers specific for each of the human chromosomes showed that the only chromosome present in all five hybrids is chromosome 7. Isozymic analysis of secondary subclones showed concordance between the presence of human β -glucuronidase and human asparagine

Fig. 9. Continued.

synthetase in 30 of 34 hybrids. Karyotypic analysis of the discordant hybrids showed the presence of only a small centric fragment of human chromosome. These findings indicate that the structural gene for asparagine synthetase is on human chromosome 7. A tentative regional assignment, based on karyotypic analysis of secondary subclones, would include the region around the centromere. The fact that three of five primary subclones obtained from the fusion of 8866 and N3 contained rearranged or deleted chromosomes 7 was surprising. However, Fournier and Moran have recently suggested that rearrangement of donor chromosomes is particularly high in certain genetic backgrounds, including CHO (29).

The assignment of the asparagine synthetase structural gene to human chromosome 7 is strengthened by an analysis of the *in vitro* heat inactivation of asparagine synthetase activity in independently derived hybrids formed between human peripheral leukocytes and the CHO cell mutant Met-1 (14). Two of these hybrids were of particular interest in this work since one (HHW

Fig. 10. Heat inactivation of asparagine synthetase from parental and hybrid cell lines. ●, 8866 cells; ○, Met-1 cells; △, HHW 151 cells; X, HHW 271 cells.

151) has retained human chromosomes 7 and 12 while the other (HHW 271) has retained only human chromosome 12. Heat inactivation of the enzyme from HHW 151 is intermediate between that of the parental cells while the heat inactivation of the enzyme from HHW 271 is identical to that of the hamster parent.

It is interesting to note that the structural gene for asparaginyl-tRNA synthetase has recently been mapped to human chromosome 18 (30). Thus, there is no linkage between the structural gene for the enzyme responsible for asparagine biosynthesis and the structural gene for the enzyme responsible for its utilization in protein biosynthesis.

The series of hybrids described here, some of which contain an intact chromosome 7 as the only human chromosome and others of which contain only portions of chromosome 7, should be useful for the regional mapping of genes known to be located on human chromosome 7. These include the structural genes for β -glucuronidase (27), argininosuccinate lyase (31), mitochondrial malate dehydrogenase (32), and pro α 2(I) collagen (33). In

addition, these hybrids should also prove useful for the identification and isolation of cloned human DNA segments specific for various regions of chromosome 7.

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