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Isolation of Chinese Hamster Ovary Cells That Overproduce Asparaginyl-tRNA Synthetase

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Temperature-resistant revertants, derived from the temperature-sensitive CHO asparaginyl-tRNA synthetase mutant, Asn-5, were isolated and characterized. Several lines of evidence indicate that the temperature-resistant phenotype of the revertants is due to their overproducing the same altered enzyme present in the Asn-5 parent.

Conditionally lethal, temperature-sensitive mutants with alterations in the gene encoding asparaginyl-tRNA synthetase (asnRS) can be isolated from two different Chinese hamster cell lines, Chinese hamster ovary (CHO) and V-79 Chinese hamster lung (CHL) cells, at very high frequencies (8, 12). In addition, there is a simple counterselective system, growth at the nonpermissive temperature, to isolate revertants in which the temperature-sensitive phenotype of mutants has been suppressed. The ease with which the gene, *asnS*, encoding this enzyme can be manipulated makes it one of the most amenable to detailed studies on mutation and reversion at the DNA level. For this reason, as well as a general interest in genes encoding protein synthesis components, we have begun attempts to clone the genomic sequences encoding asnRS.

We recently reported the isolation of cell lines derived from a CHO asnRS mutant into which we introduced the normal human *asnS* gene and in which less than 0.02% of the DNA is derived from the human genome (5). We have since prepared a complete lambda genomic DNA library from this cell line and have isolated a group of 160 recombinant phage containing human DNA, which should represent all of the human sequences present in this cell line, including those encoding asnRS. To aid in screening this group of recombinants, we were interested in isolating Chinese hamster cell lines with elevated levels of asnRS, and hopefully elevated levels of asnRS mRNA, from which we could prepare a cDNA library enriched for these sequences.

Isolation of temperature-resistant revertants from a temperature-sensitive asnRS mutant. The lack of availability of specific inhibitors of asnRS precluded the selection of cell lines that overproduced this enzyme based upon increased resistance to substrate analogs (1, 3, 11). As an alternate approach to obtaining such cell lines, we isolated temperature-resistant revertants from the CHO asnRS mutant Asn-5 (2, 8), which is nonviable at 39°C in medium lacking asparagine but has a normal generation time at 34°C. It seemed possible that variants of Asn-5 which greatly overproduced the defective enzyme were viable at 39°C and could be recovered as temperature-resistant revertants. To increase the likelihood of isolating cell lines which overproduced asnRS as a result of their having amplified the *asnS* gene, we pretreated cultures of Asn-5 cells with the phorbol ester 12-*O*-tetradecanoyl-phorbol-13-acetate (TPA), which has been shown to dramatically increase the frequency with which the gene encoding dihydrofolate reductase is amplified in mouse cells (10). Cultures of Asn-5 were grown for seven generations at 34°C in the presence or absence of 0.1 μM TPA. Cells from the two cultures were then seeded into 10 100-mm plates at a density of 10^5 cells per plate in medium lacking asparagine and transferred to 39°C. Forty-four colonies were obtained from a total of 10^6 cells from the TPA-treated cultures, a frequency of 4.4×10^{-5}. The frequency of temperature-resistant variants in untreated cultures of Asn-5 was 2.2×10^{-7}. Thus, the TPA treatment resulted in a 200-fold increase in the frequency of temperature-resistant colonies. Several independent, TPA-induced temperature-resistant revertants were isolated, maintained under selective conditions (39°C in medium lacking asparagine), and examined biochemically as described below.

Biochemical characterization of temperature-resistant revertants. Cultures of two temperature-resistant cell lines, UCW 1037 and UCW 1039, were grown for four generations under conditions that were either restrictive or permissive for the Asn-5 parent, and then cell extracts were prepared and assayed for asnRS activity as described previously (4). Table 1 shows the specific activity of asnRS in extracts from Asn-5, GAT^- (the wild-type cell line from which Asn-5 was derived), and the two temperature-resistant cell lines, UCW 1037 and UCW 1039. In UCW 1037 and UCW 1039, the activity of asnRS is elevated 27-fold and 13-fold, respectively, above the level observed in the Asn-5 parent when the extracts are prepared from cells grown at 34°C. The specific activity of this enzyme in the two temperature-resistant variants is also significantly higher than the activity of the normal enzyme in extracts from the wild-type cell line, GAT^-. The activity of asnRS, however, is greatly reduced in extracts prepared from the temperature-resistant cells that have been grown at 39°C, indicating that the enzyme produced is thermolabile in vivo. It is not possible to determine the activity of asnRS in extracts of Asn-5 cells grown at 39°C since the cells lyse within 24 h after transfer to this temperature. Ten other independent temperature-resistant lines have been examined, and all had increased asnRS activity which ranged from 5 to 32 times that of the Asn-5 parent (data not shown).

The asnRS extracts from Asn-5 and wild-type cells can easily be distinguished in vitro based upon large differences in sensitivity to thermal inactivation (2, 4). If the temperature-resistant phenotype in either UCW 1037 or UCW 1039 was due, at least in part, to their producing a more heat-stable enzyme, the increased thermal stability should be detectable in vitro. Cell extracts were prepared from Asn-5, UCW 1037, and UCW 1039 cells that had grown at 34°C in

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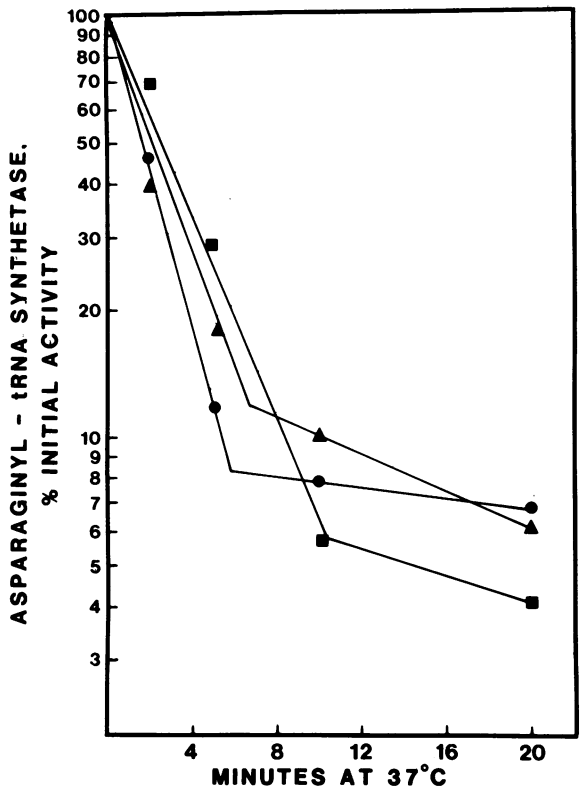

FIG. 1. In vitro thermostability of asnRS activity in cell extracts from Asn-5, UCW 1037, and UCW 1039. The preparation of extracts and the determination of asnRS activity were described previously (4). Cells were grown at 34°C in medium containing 3 mM asparagine for 7 days before harvesting and preparation of cell extracts. Extracts were incubated at 37°C for the indicated lengths of time, and then samples were removed and assayed for asnRS 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. Symbols: ▲, Asn-5; ■, UCW 1037; ●, UCW 1039.

medium containing asparagine. The stability of asnRS activity in the various extracts was then determined as described previously (4). The sensitivity of asnRS activity to heat inactivation at 37°C in vitro is virtually identical in all three cell lines, with the activities decaying in each case with a half-life of about 2 min (Fig. 1). As we have shown previously, the asnRS activity extracted from wild-type cells has a half-life of over 60 min under these same conditions (4). Thus, partial restoration of the thermal stability of asnRS does not appear to be responsible for the temperature-resistant phenotype of UCW 1037 or UCW 1039, and both cell lines apparently overproduce the same defective enzyme present in the Asn-5 parent. In further support of this idea, we found that the asnRSs extracted from Asn-5, UCW 1037, and UCW 1039 have very similar K_ms for asparagine, 2.02×10^{-4}, 1.86×10^{-4}, and 1.45×10^{-4} M, respectively, which are markedly higher than the K_m of the wild-type enzyme for asparagine, which is 2×10^{-5} M.

TABLE 1. asnRS activity in cell extracts from wild-type, Asn-5, and temperature-resistant revertant cells[a]

Cell line	Growth conditions	asnRS sp act
GAT$^-$	34°C + Asn	314
Asn-5	34°C + Asn	129
UCW 1037	34°C + Asn	3,762
UCW 1037	39°C − Asn	2,087
UCW 1039	34°C + Asn	1,913
UCW 1039	39°C − Asn	1,250

[a] The specific activity of asnRS was determined as described previously (4). The cell lines were grown under the indicated conditions of temperature and supplemental asparagine for at least 1 week before harvesting. Each of the values listed represents the average of at least two independent determinations. The activities are expressed as picomoles of [^{14}C]asparaginyl-tRNA formed per minute per milligram of protein extract. The lower limit of asnRS specific activity that is detectable with this assay is ca. pmol/min per mg of protein extract.

Identification of a protein band on sodium dodecyl sulfate-polyacrylamide gels that is elevated in temperature-resistant revertants. If the elevated activity of asnRS in temperature-

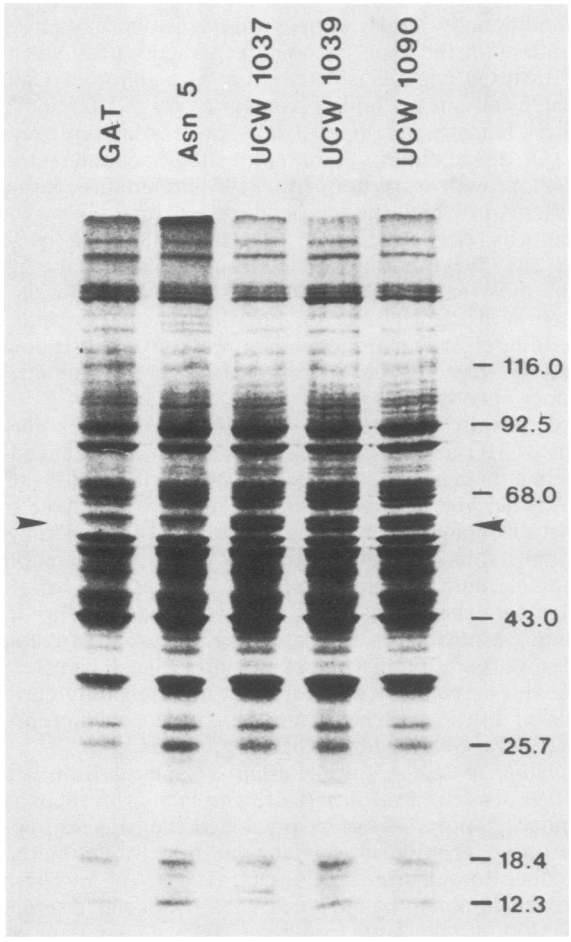

FIG. 2. Analysis of cell extracts from GAT$^-$, Asn-5, UCW 1037, UCW 1039, and UCW 1090 by polyacrylamide gel electrophoresis. Cells were grown for 3 days at 34°C in medium supplemented with 3 mM asparagine, 2×10^{-5} M methionine, and 5 μCi of [^{35}S]methionine per ml (13 Ci/mmol). Extracts were prepared and electrophoresed on 7 to 12% gradient polyacrylamide gels containing sodium dodecyl sulfate. The gels were dried and exposed to X-ray film at −70°C for 3 days with an intensifying screen. Each lane contains 10^6 cpm of trichloroacetic acid-precipitable ^{35}S-labeled protein extract from the indicated cells lines. Lane 1, GAT$^-$; lane 2, Asn-5; lane 3, UCW 1037; lane 4, UCW 1039; lane 5, UCW 1090. The band indicated by the arrows (molecular weight, 56,000) is the only one detectable that is consistently present in higher amounts in the temperature-resistant cell lines than in the GAT$^-$ and Asn-5 cells.

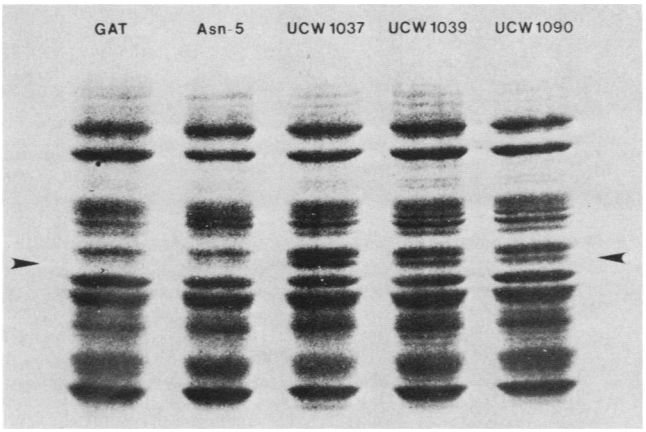

FIG 3. Analysis of cell extracts from GAT$^-$, Asn-5, UCW 1037, UCW 1039, and UCW 1090 by polyacrylamide gel electrophoresis. This figure is an enlargement of the region of the autoradiogram shown in Fig. 2 which contains protein bands of molecular weight from ca. 43,000 to 92,500.

resistant revertants is due to their overproducing the altered enzyme characteristic of the Asn-5 parent, polyacrylamide gel electrophoresis may detect the presence of a protein species whose concentration is higher in the revertants than the parent. Asn-5, UCW 1037, UCW 1039, UCW 1090 (another temperature-resistant revertant), and the wild-type GAT$^-$ cell line were grown at 34°C for 3 days in the presence of [^{35}S]methionine, at which time cells were harvested and cell extracts were prepared. A total of 10^6 acid-precipitable counts of ^{35}S-labeled proteins from each extract were loaded onto slab gels and electrophoresed through a 7 to 12% gradient polyacrylamide gel containing sodium dodecyl sulfate as described by Studier (7). Figures 2 and 3 show an autoradiogram of one such gel. All of the lanes show virtually identical patterns of protein bands except for a band at a position corresponding to a molecular weight of about 56,000 which is much more intense in the lanes containing samples from UCW 1037, UCW 1039, and UCW 1090 than in the lanes containing extracts from GAT$^-$ or Asn-5. Although there are a few minor differences in the pattern of protein bands among the different cell lines, the protein band indicated by the arrow in Fig. 2 and 3 is the only one we could detect which fits the criteria of being the same intensity in GAT$^-$ and Asn-5 but increased in all the cell lines with elevated levels of asnRS. Since asnRS has never been purified from mammalian sources, we have no idea what its subunit structure or molecular weight are. Thus, we cannot be certain that the protein of 56,000 molecular weight, which is present in significantly higher amounts in the temperature-resistant revertants than in either GAT$^-$ or Asn-5 cells, is related to the increased activity of asnRS. However, since the increase in the intensity of this band roughly corresponds to the increase in asnRS activity in the temperature-resistant revertants, it is attractive to speculate that this protein is at least a subunit of the native asnRS.

The results presented here strongly suggest that the temperature-resistant revertants we have characterized have the same defective asnRS produced by the temperature-sensitive parent, Asn-5 but that they contain it in greatly elevated amounts. Although the molecular mechanism(s) responsible for the overproduction of the enzyme has not as yet been defined, the enhancement by TPA of the frequency of temperature-resistant revertants is most consistent with amplification of the *asnS* gene (9, 10). It is interesting to note that while this work was in progress, Fuscoe et al. reported the isolation of temperature-resistant revertants from a temperature-sensitive Chinese hamster lung cell *hprt* mutant which arose as a result of amplification of the defective *hprt* gene (6).

The ultimate proof that the *asnS* gene is amplified in these cell lines will depend upon the isolation of recombinant DNA probes for this gene. In fact, our primary interest in isolating cell lines with elevated levels of asnRS, and presumably elevated levels of the corresponding mRNA, is to aid in cloning cDNA and genomic sequences encoding this enzyme.

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