A mammalian temperature-sensitive mutation affecting G1 progression results from a single amino acid substitution in asparagine synthetase

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ABSTRACT

ts11 is a temperature-sensitive (ts) mutant isolated from the BHK-21 Syrian hamster cell line that is blocked in the G1 phase of the cell cycle at the non-permissive temperature (39.5°C). We previously showed that the human gene encoding asparagine synthetase (AS) transformed ts11 cells to a ts⁺ phenotype and that ts11 cells were auxotrophic for asparagine at 39.5°C. We show here that ts11 cells exhibit a ts phenotype for AS activity, and that the ts11 AS was much more heat-labile than the wt enzyme. We have isolated AS cDNAs from wt BHK and ts11 cells and found that wt, but not ts11 AS cDNAs were capable of transformation. The deduced amino acid sequence of Syrian hamster AS showed 95% identity to the human protein as well as the same number of residues. The inability of the ts11 AS cDNAs to transform was due to a single base change, a C to T transition, that would result in the substitution of leucine with phenylalanine at a residue located in the C-terminal fourth of the enzyme. Thus the ts11 mutation identifies a mutated, thermolabile AS.

INTRODUCTION

Temperature-sensitive (ts) mutants are valuable tools in the genetic dissection and biochemical characterization of cellular processes (1,2). We previously reported (3,4,5) that ts11, a ts G1 cell cycle mutant isolated from the BHK-21 Syrian hamster cell line, was transformed to ts⁺ at high frequency with human DNA encoding asparagine synthetase (AS), and that ts11 cells were capable of sustained growth at the non-permissive temperature when the culture medium was supplemented with asparagine. These studies indicated that ts11 cells contained a defect in the biosynthetic pathway for asparagine production at the non-permissive temperature (39.5°C). In mammalian cells, asparagine is synthesized by the amidation of aspartate by asparagine synthetase, whose ability to utilize the amide of glutamine is also found in other amidotransferases such as glutamine phosphoribosyl-pyrophosphate amidotransferase (6),

CTP synthetase (7), and GMP synthetase (8). As such it is considered a non-essential amino acid which does not have to be exogenously supplied to cultured cells.

Although our previous studies (5) strongly suggested that ts11 cells encoded a ts asparagine synthetase, it was theoretically possible that the primary defect of ts11 cells lied elsewhere, and was suppressed or bypassed by the introduction of a constitutively expressed AS gene or by supplying the cells with asparagine. Indeed asparaginyl-tRNA synthetase mutants (9) exist that are capable of growth at the non-permissive temperature in the presence of high levels of asparagine in the medium. We therefore set out to identify the ts lesion in ts11 cells by comparing the wt and ts11 AS cDNAs. Although it is generally assumed that the defect in ts mutant cells results from single amino acid substitutions in a protein, in animal cells experimental evidence substantiating this notion has been scarce (10). Here we present evidence that ts11 cells produce a heat-labile AS, and that ts11 cDNAs encode a mutated AS which is incapable of complementing the ts11 defect at the non-permissive temperature.

MATERIALS AND METHODS

Cell culture

Cells were cultured in Dulbecco modified Eagle's medium (DMEM) containing 10% calf serum. The ts11 and wt BHK cell lines were maintained at 33.5°C.

Transfection

Cells were transfected with DNA by the calcium phosphate coprecipitation method as described previously (5).

Construction and screening of cDNA library

Total cellular RNA of wt BHK and ts11 cells was isolated by the guanidinium-cesium chloride method. Poly(A)⁺ RNA was selected by oligo d(T)-cellulose (Boehringer-Mannheim, Indianapolis, ID) chromatography and used for the synthesis of cDNA. The cDNA library was constructed in λ gt10 vector using a kit from Amersham (Arlington Heights, IL), and screened with a 1.7 Kb HindIII/HindIII human AS cDNA probe (3) by

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hybridizing at 60°C in 5× SSPE, 5× Denhardt's, 0.1% SDS, 0.1 mg/ml calf thymus DNA overnight. The nitrocellulose filters were washed twice, 0.5 h each in 1× SSC, 0.1% SDS at 50°C and autoradiographed. The strongest hybridizing phages were purified and the cDNA inserts were subcloned in plasmid vectors.

Plasmid construction and DNA sequencing

Nested deletion mutants and restriction fragment subclones of the cDNAs were constructed in Bluescript (Stratagene, San Diego, CA) and pGEM (Promega, Madison, WI) vectors. DNA sequence was analyzed by the dideoxy method (11) using a kit from United States Biochemicals (Cleveland, Ohio).

Enzyme Assay

Asparagine synthetase activity was assayed according to Luehr and Schuster (12). Pelleted tissue culture cells were resuspended in 50 mM Tris-Cl/1 mM dithiothreitol/1mM EDTA, pH 7.4, and lysed by freeze-thawing three times with dry ice. The lysate was spun in a microfuge for 20 min. and the supernatant was used for the enzyme assay. Protein concentration of the cell extract was determined using a Bio-Rad (Richmond, CA) protein assay reagent.

RESULTS

Asparagine synthetase activity in ts11 and wt BHK cells

To determine if the ts11 mutant indeed had a defective asparagine biosynthetic activity at 39.5°C, we compared the AS activity of protein extracts from ts11 and wt BHK cells that had been incubated for various times at 39.5°C. As shown in Fig. 1, when asynchronous, growing populations of wt BHK cells were shifted from 33.5°C to 39.5°C, AS activity increased two- to three-fold after 4 to 6 h and remained at this high level for at least 20 h. In contrast, when ts11 cells were shifted to 39.5°C, AS activity increased about two-fold during the first 4 to 6 h, but declined to a level lower than that detected at the time of shift-up over the next 20 h. Thus, it appears that ts11 exhibits a reduced asparagine biosynthetic activity at the non-permissive temperature compared to its wt counterpart.

The increase in AS activity in BHK cells following the temperature switch (Fig. 1) probably reflects in part a somewhat higher level of AS mRNA detected in the random population cultured at 39.5°C than that at 33.5°C (data not shown). On the other hand, exposure of ts11 cells to 39.5°C results in a drastic up-regulation of the levels of AS mRNA (5), and presumably a higher rate of synthesis of AS protein. To rule out the contribution of increased AS gene expression to the total levels of AS activity measured at 39.5°C, the translation inhibitor cycloheximide was added (10 µg/ml) to the medium at the time of shift-up. Under these conditions AS activity remained stable for 12–16 h in wt BHK but decreased by 80% over the same period in ts11 cells (Fig. 1). We also compared the AS activity of wt BHK and ts11 cytoplasmic extracts incubated at different temperatures. As shown in Fig. 2, the AS activity of both wt BHK and ts11 cell extracts remained stable at 33.5°C for a period of at least 5 h. However, while the AS activity of wt BHK extracts had a half-life at 39.5°C of about 3 h, the half-life of AS from ts11 was much shorter, on the order of 15 minutes. These results indicate that ts11 cells produce an AS that is much more heat-labile than the wt form, but the reason for the apparent difference between the *in vivo* and *in vitro* half-lives of AS activity at 39.5°C for both cell lines (compare Figs. 1 and 2) is not clear.

Fig 1. Asparagine synthetase (AS) activity in wt BHK and ts11 cells at 39.5°C. wt BHK (squares) or ts11 (circle) cells were plated onto 100-mm dishes (1.5×10^6 cells/dish) in fresh medium containing 10% calf serum, incubated at 33.5°C for 20 h, and then shifted to 39.5°C with (closed symbols) or without (open symbols) cycloheximide (10 µg/ml) added at the time of shift-up (time zero). At various times after the temperature switch, cells were harvested and assayed for AS activity as described in Materials and Methods. The enzyme reaction was carried out at 36°C for 1 h and the activity in each sample was normalized for the same amount of protein. Each point represents the ratio between the AS activity for that time point (A_t) and that of time zero (A_0) in each cell line.

Cloning and functional analysis of ts11 and wt BHK AS cDNAs

Syrian hamster cells as well as BHK cells produce two major species of AS mRNA, one of which is very similar in size to the single RNA species detected in human and mouse cells, while the other is slightly larger. This pattern of mRNA expression is conserved in ts11 cells at both permissive and non-permissive temperatures, suggesting that ts11 AS mRNAs are not improperly spliced or polyadenylated. To determine whether ts11 cells encoded a mutated AS, we cloned several wt BHK and ts11 AS cDNAs and compared their sequence. cDNAs were isolated from wt BHK and ts11 cDNA libraries as described in Materials and Methods. The largest wt BHK and ts11 clones gave identical restriction patterns for several enzymes except for the 5'- and 3'-end fragments (data not shown). The sequence of the wt BHK AS cDNA was determined (Fig. 3). The largest open reading frame encoded a 561 amino acid polypeptide that had 98% similarity to the human protein and the same number of residues

Fig 2. Inactivation of asparagine synthetase activity in wt BHK or ts11 cell extracts at 33.5°C and 39.5°C. Cell extracts for AS assay were prepared from wt BHK (square) and ts11 (circle) cultures as described in Materials and Methods. Extracts of similar protein concentration from each cell line were incubated at 33.5°C (open symbols) or 39.5°C (closed symbols). At the times indicated an aliquot of the extracts was removed and assayed for AS activity. The enzymatic reaction was carried out at 34°C for 1 h, and the ratio between the AS activity for each time point and that of time zero is plotted.

(4,5). The 3' sequences of two wt AS cDNAs were found to be identical to those of the two ts11 cDNAs. One of the wt and one of the ts11 cDNAs contained a poly(A) tract 12 nucleotides downstream of a poly(A) signal sequence, while the others were polyadenylated 18 nucleotides downstream of the poly(A) signal (Fig. 3). One of the ts11 cDNA clones contained an internal 144 bp sequence absent in other clones, located 25 bp upstream from the putative ATG initiation codon (Fig. 3). The 5' end of the AS mRNA was analyzed by primer extension using an oligonucleotide corresponding to cDNA sequences located downstream from the above-mentioned 144 nucleotides region and upstream from the ATG codon. All of the mRNAs from Syrian hamster primary fibroblasts, wt BHK, and ts11 cells gave an identical pattern of two main classes of heterogeneous cDNA products whose sizes differed by about 150 nt. (data not shown). These results indicated that there are multiple RNA start sites for the Syrian hamster AS gene, as is the case for the human gene (5). *In vitro* translation of the ts11 AS RNA synthesized *in vitro* by SP6 RNA polymerase from the cDNA containing the additional 5' sequences (see above) produced a protein of a molecular weight indistinguishable from that of the wt protein

Table 1. Transformation of ts11 cells by transfection with plasmids containing AS cDNAs^a

PLASMID(cDNA orientation)	ORIGIN of cDNA	NO. OF ts ⁺ COLONIES ^b
Mock		7
pCD-ts11-5A ^c (sense)	human fibroblasts	538
pCD-I (sense)	wt BHK	594
(anti-sense)		6
pCD-O (sense)	wt BHK	599
(anti-sense)		3
pCD-7 (sense)	ts 11	6
(anti-sense)		4
pCD-15 (sense)	ts 11	5
(anti-sense)		3
pCD-7 (sense)		4
+ 5' half of cDNA I ^d		
pCD-7 (sense)		59
+ 3' half of cDNA I ^e		
pCD-7 (anti-sense)		4
+ 3' half of cDNA I		
pCD-15 (sense)		7
+ 5' half of cDNA I		
pCD-15 (sense)		81
+ 3' half of cDNA I		
pCD-15 (anti-sense)		1
+ 3' half of cDNA I		

a. ts11 cells grown at 33.5°C were transfected with 1.8 µg of plasmid DNA per 100-mm plate (containing approximately 106 cells) by the calcium phosphate co-precipitation method. In co-transfections, 1.8 µg of each plasmid DNA were mixed with an equimolar amount of gel-purified restriction fragments of cDNA. One day later the cells were trypsinized, replated at 5 × 10⁵ cells per 100-mm plate and then shifted to 39.5°C one day later. Ts⁺ colonies were fixed and counted after 14 days of incubation at 39.5°C.

b. Each number represents the mean of two experiments.

c. Vector plasmid pCD (14) contains the SV40 early promoter, SV40 late region splice and polyadenylation signal sequences for the appropriate expression of cDNA inserts in mammalian cells.

d. The 5' half (0.8 Kb) of cDNA I contains sequences extending from the 5' end (see also Fig. 3) of the cDNA I flanked by an EcoRI terminus to the unique SphI site (nucleotides 895–900 in Fig. 2) in the middle of the cDNA.

e. The 3' half (1.2 Kb) of cDNA I contains sequences extending from the unique SphI site in the middle to the 3' end of the cDNA I including the poly(A) tract (see also Fig. 3).

(data not shown), in line with the hypothesis that the additional 144 nucleotides were not translated and could have originated from an alternative splicing event (see Discussion).

Two of the AS cDNA clones of wt BHK and ts11 origin containing the entire coding region were placed in the mammalian expression plasmid vector pCD (13). Both of the wt cDNA plasmids transformed ts11 cells to ts⁺ with a frequency comparable to that of a human AS cDNA in the same vector (Table 1). In contrast, neither of the ts11 AS cDNA plasmids

Fig. 3 cDNA and predicted amino acid sequence of AS from wt BHK and ts11 cells. Shown on the third row in each block are the amino acids in the predicted sequence of the human AS (4,5) that are different from those of hamster AS. The 5' ends of various cDNAs are indicated above the sequence. cDNAs I and O were of wt BHK and 7 and 15 of ts11 origin. The ts11 mutation at nucleotide 1549 (C to T) was found in both cDNAs 7 and 15, and the predicted amino acid substitution (amino acid 414) is indicated. The underlined sequence in the 5' untranslated region was found in cDNA 7, but not in I, O, or 15. A polyadenylation signal sequence (AATAAA) in the 3' untranslated region is also underlined. A poly(A) tract was found in cDNAs I and 7 following nucleotide 2096 as shown in the figure. In cDNAs O and 15, the poly(A) tract was located after nucleotide 2090 (indicated by an arrow). The cDNA sequence has been submitted to the EMBL Data Library (Accession number X52130).

was capable of transformation. Thus, the AS cDNAs from ts11 cells contain changes that render them non-functional at 39.5°C, indicating that the primary defect of ts11 cells lies in the AS gene.

Identification of the ts mutation in ts11 AS cDNA

To identify the mutation in the ts11 AS cDNA, restriction fragments of wt cDNA were co-transfected onto ts11 cells together with the plasmids containing the entire ts11 cDNA. The transfected cells were then tested for ability to form colonies at 39.5°C in medium lacking asparagine. As shown in Table 1, transfection of the 3' 1.2 Kb but not of the 5' 0.8 Kb fragment of the wt cDNA together with either one of the two ts11 AS cDNA plasmids transformed ts11 cells to ts⁺ with a frequency

much higher than the background level. Thus, homologous recombination between the ts11 cDNA and the 3' portion of the wt cDNA could create a biologically active cDNA. When the ts11 cDNA was placed in the vector in the anti-sense orientation, no transformation was observed. Thus the ts11 mutation maps in the 3' half of the cDNA.

Sequence analysis of the 3' 1.2 Kb fragment of one of the ts11 AS cDNAs and comparison to the wt sequence revealed one single base change in the coding region, a C to T transition at nucleotide 1499 that would encode a phenylalanine (TTT) instead of leucine (CTT) (Fig. 3). Sequencing across the mutation site in the second ts11 cDNA clone revealed the same change.

Sequence analysis of the 5' 0.8 Kb region of the ts11 cDNA

showed no changes compared to the wt cDNA. Thus, the lesion in ts11 cells was localized in the coding region of AS mRNA as a single base substitution. Indeed, a hybrid AS cDNA consisting of the ts11 5' 0.8 Kb fragment fused to the wt 3' 1.2 Kb region transformed ts11 cells with a frequency comparable to that of the wt cDNA (not shown).

DISCUSSION

ts11 is a mammalian temperature-sensitive mutant that is blocked in the G1 phase of the cell cycle at the non-permissive temperature. Through DNA transfection and cloning we have isolated the human gene encoding asparagine synthetase as well as its cDNA and showed that they could transform ts11 cells to ts⁺ with high frequency. We show here that ts11 conditional auxotrophy for asparagine is due to a single amino acid substitution in the C-terminal fourth of its asparagine synthetase, resulting in defective enzymatic activity at the non-permissive temperature.

It is intriguing that the conservative substitution of leucine with phenylalanine at residue 414 would strongly decrease the thermostability of AS, considering that there are thirty different amino acid residues between the human and hamster AS including 18 conservative changes (Fig. 2). However, the mutation occurs in a region (amino acid 312–425) that is relatively more conserved than other regions between the two proteins. Perhaps the bulkier side chain of phenylalanine introduces a constraint on the protein in a region crucial for its structure and/or catalytic activity rendering the enzyme more sensitive to heat-inactivation.

AS can function as a glutaminase in the absence of ATP and aspartate. Studies using monoclonal antibodies against bovine AS suggested that distinct binding sites for glutamine and aspartyl-AMP reside on the enzyme surface (14). The deduced amino acid sequence of hamster and human AS shared four identical N-terminal residues with those of glutamine phosphoribosyl-pyrophosphate amidotransferase from *Escherichia coli* (6), *Saccharomyces cerevisiae* (15) and *Bacillus subtilis* (after processing) (16), and also of glucosamine-6-phosphate synthase from *E. coli* (17). In the case of amidophosphoribosyl transferase, which catalyzes the first reaction in the de novo pathway of purine biosynthesis, the N-terminal cysteine was shown to be the active site residue required for the glutamine amide transfer function of the enzyme (18,19). It should be of interest to determine how distinct functions of AS are affected by the ts11 mutation.

As discussed above, two main species of AS mRNAs are produced by hamster cells, and their difference in molecular weight could be accounted for by the presence or absence of the 144 nt insertion we found in some ts11 cDNAs. Probes including this region hybridized preferentially to the upper band of AS messages in Northern blots (data not shown). Furthermore, 5'-end analysis of AS mRNA by primer extension produced cDNA products whose sizes differed by about 150 nt from one another. Differential splicing of an identical primary transcript in the 5' untranslated region could give rise to two AS mRNAs that can both be translated into functional AS. The 3' and 5' ends of this 144 nt insertion do not contain sequences conforming to a 3' acceptor or 5' donor site, respectively (Fig. 2, 20). Thus the splicing of a common 5' donor site to two alternative 3' acceptor sites or of two alternative 5' donor sites to a common 3' acceptor site does not appear likely. Similarly, the 144 nt sequence is unlikely to represent an unspliced intron. The most likely hypothesis is that the two mRNAs result from alternative splicing

events that would splice or skip an exon corresponding to the 144 nucleotide stretch. The sequences immediately preceeding (CAG) or ending (AAG) the 144 nucleotides insertion (Fig. 3) are indeed a good match for a 5' exon-intron boundary (20). Interestingly, we have found within the second intron (601 nt in length) of the human AS gene (5) a region of 120 nt that shared 63% identity with the 144 nt sequence (data not shown). This finding further supports our hypothesis about the origin of the 144 nt sequence in the AS hamster mRNA. The mechanism by which a deficient production of asparagine leads to a G1 block, rather than to a generalized growth arrest remains to be elucidated.

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