

Isolation of Human cDNAs for Asparagine Synthetase and Expression in Jensen Rat Sarcoma Cells

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Asparagine synthetase cDNAs containing the complete coding region were isolated from a human fibroblast cDNA library. DNA sequence analysis of the clones showed that the message contained one open reading frame encoding a protein of 64,400 M_r , 184 nucleotides of 5' untranslated region, and 120 nucleotides of 3' noncoding sequence. Plasmids containing the asparagine synthetase cDNAs were used in DNA-mediated transfer of genes into asparagine-requiring Jensen rat sarcoma cells. The cDNAs containing the entire protein-coding sequence expressed asparagine synthetase activity and were capable of conferring asparagine prototrophy on the Jensen rat sarcoma cells. However, cDNAs which lacked sequence for as few as 20 amino acids at the amino terminal could not rescue the cells from auxotrophy. The transferant cell lines contained multiple copies of the human asparagine synthetase cDNAs and produced human asparagine synthetase mRNA and asparagine synthetase protein. Several transferants with numerous copies of the cDNAs exhibited only basal levels of enzyme activity. Treatment of these transferant cell lines with 5-azacytidine greatly increased the expression of asparagine synthetase mRNA, protein, and activity.

Asparagine synthetase (AS) is a housekeeping enzyme responsible for the biosynthesis of asparagine from glutamine and aspartic acid. The regulation of AS has been studied in cultured mammalian cells (for a review, see reference 1), and overproducing cell lines, some with amplified copies of the gene, have been isolated in response to drug selection (2, 3, 6, 11). Most mammalian cells express AS and regulate the level of activity in response to the concentration of asparagine in the medium and the extent of tRNA aminoacylation (4, 7). In contrast to normal cells, some tumor cells exhibit little or no AS activity and rely on the surrounding medium as a source of exogenous asparagine. This property has been exploited in the treatment of certain cancers, since the tumor cells can be selectively killed by the chemotherapeutic drug asparaginase which hydrolyzes circulating asparagine (9, 24). One of the most sensitive human malignancies was found to be childhood acute lymphoblastic leukemia, and asparaginase is now routinely used in induction and consolidation protocols for patients with this disease (23). In mouse model systems, it was found that asparaginase-sensitive tumor cells could be made asparaginase-resistant by repeated subculturing of these cells in sublethal concentrations of the drug. The resistant cells were found to have regained AS activity and in some cases had elevated levels of enzyme activity up to 60-fold higher than that in normal cells (13, 24). The mechanism responsible for the lack of activity of AS in the sensitive leukemic cells and for the overproduction in the asparaginase-resistant variants is of clinical importance for the treatment of acute lymphoblastic leukemia patients both initially and during relapse.

To investigate the molecular basis for the sensitivity to asparaginase in human acute lymphoblastic leukemia cells and the correlation with the lack of AS expression, we isolated cDNA clones for the human AS gene by using as a

probe the CHO cDNAs we had isolated previously (25). We describe here the selection of these cDNAs from the human fibroblast cDNA library of Okayama and Berg (22) and their characterization by restriction mapping and DNA sequencing. The longest cDNAs contained the complete coding sequence for AS, as shown by the functional expression of an AS cDNA after calcium phosphate-mediated gene transfer into asparagine-requiring Jensen rat sarcoma cells.

MATERIALS AND METHODS

Screening of a human cDNA library. We have previously described the cloning of partial cDNAs for CHO AS (25). These were used to probe the human fibroblast cDNA library constructed by Okayama and Berg (22). Approximately 5×10^5 ampicillin-resistant (50 $\mu\text{g/ml}$) colonies were transferred to nitrocellulose filters and amplified overnight at 37°C on LB plates containing spectinomycin (250 $\mu\text{g/ml}$). Colonies were lysed in situ, denatured, and fixed to the filters as described previously (25). The filters were prewashed at 25°C in $3 \times \text{SSC}$ ($1 \times \text{SSC}$ is 0.15 M NaCl plus 0.015 M sodium citrate), prehybridized for 4 h at 60°C in solution 1 ($5 \times \text{SSPE}$ [20], $5 \times \text{Denhardt}$ solution, 0.1% sodium dodecyl sulfate (SDS), 100 μg of salmon sperm DNA per ml) containing 1 μg of poly(A) per ml, and hybridized at 60°C overnight in the same solution containing nick-translated (26) inserts of CHO AS cDNAs p12B and p11B (25). Filters were washed twice in $0.5 \times \text{SSC}$ -0.1% SDS at 60°C and exposed to Kodak XAR-5 film with an intensifying screen at -70°C. Colonies which produced strong signals were picked into 1 ml of LB broth containing ampicillin (50 $\mu\text{g/ml}$), grown for several hours, plated, and rescreened. After three rounds of screening, clonal isolates were obtained and DNA minipreps (16) were prepared from these. The plasmids containing the longest cDNAs were grown up as large-scale preps and characterized further.

Southern and slot blot analysis. High-molecular-weight

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DNA was prepared as described previously (5), and plasmid DNA was isolated from minipreps. Restriction enzymes were obtained from Bethesda Research Laboratories, Inc., and New England BioLabs, Inc., and used under conditions recommended by the supplier. The DNAs (10 μ g) were digested with restriction enzymes, run on 0.8% agarose gels, blotted by Southern transfer (27) as modified by Wahl et al. (31), and probed with nick-translated pH131 DNA as previously described (25).

Quantitation of the amounts of DNA and RNA was done by slot blotting (15) of serial dilutions of DNA and RNA and densitometric analysis. The amounts were calculated relative to that of the rat argininosuccinate lyase gene sequences. The intensities of the signals for endogenous rat AS DNA sequences were comparable to those of argininosuccinate lyase DNA in the recipient Jensen rat sarcoma cell line and spontaneous revertants of this line. Since the recipient cells did not express endogenous AS mRNA, the amount of RNA in the various cell lines was also normalized to that of rat argininosuccinate lyase.

RNA analysis. Total RNA was isolated as described previously (25). Total RNA (10 μ g) was denatured with formaldehyde and formamide, run on 1% agarose gels containing formaldehyde, and transferred to nitrocellulose (20). Hybridizations were performed overnight at 42°C in solution 1 containing 50% formamide with nick-translated inserts of human cDNAs as probes.

DNA sequencing. Restriction fragments of the cDNA inserts were subcloned into the vector pUC19 (33). The sequence was determined by the dideoxynucleotide chain termination technique directly from double-stranded plasmid DNA prepared from a modified alkaline lysis miniprep procedure (16). Sequencing was done in both directions by using the M13 17-mer primer and the M13 reverse primer. (For the subcloning and sequencing strategy, see Fig. 3).

Transfection of asparagine-requiring rat cells. Jensen rat sarcoma cells, which require asparagine for growth, were grown in complete alpha medium (28) containing 10% fetal calf serum (medium A). The AS cDNAs in the pcD plasmid or plasmids containing RSVneo insert sequences were precipitated with calcium phosphate and added to the medium as previously described for genomic DNA (5). After 18 h, the medium was replaced with fresh medium A. To detect cells expressing AS, cells were plated in selective medium B (medium A lacking asparagine and containing 10% dialyzed fetal calf serum). To determine the frequency of neomycin-resistant colonies, cells were plated in medium A containing 200 μ g of G418 per ml. The medium was changed after 5 days, and colonies were stained after 10 days of selection or picked and maintained in selective medium.

Determination of AS activity and protein. AS activity was assayed in cell extracts as described by Gantt et al. (11). One unit of activity catalyzes the formation of 1 nmol of asparagine per h. Protein concentrations were determined by the method of Lowry et al. (19) by using crystalline bovine serum albumin as a standard. Proteins were separated by SDS-polyacrylamide gel electrophoresis (18), and Western immunoblotting was performed as described by Towbin et al. (30) by using antisera raised against CHO AS as previously reported (25). The antisera cross-reacts with human AS, as shown for a human fibrosarcoma cell line. This cell line, which overproduces AS activity, was obtained by growing HT1080 cells in gradually increasing concentrations of albizzin (Andrulis et al., manuscript in preparation). This procedure was previously developed to obtain CHO cell mutants with amplified copies of the AS gene (2).

RESULTS

Isolation and characterization of human cDNAs for AS. To isolate full-length human cDNAs we used two nonoverlapping CHO AS cDNAs to probe the human fibroblast cDNA library of Okayama and Berg. From approximately 5×10^5 ampicillin-resistant colonies, we obtained 27 positive colonies by using a mixture of the probes. All of these hybridized with one of the CHO cDNAs (p11B), but only the longest human cDNAs (pH131 and pH181) hybridized with p12B. Restriction enzyme analysis showed that all of the cDNAs contained the same sites at the 3' end but differed in length (Fig. 1). Because of the method of the construction of the library, we concluded that p11B was complementary to the 3' region of the mRNA and p12B was complementary to the 5' end. This was confirmed by DNA sequence analysis of both the human (see below) and CHO cDNAs (Ray et al.; manuscript in preparation).

Using these clones, we were able to determine the size of the mRNA for AS by Northern blot analysis of RNA from the human fibrosarcoma cell line HT1080 (Fig. 2). A major species of AS mRNA was detected as a band migrating at 2 kilobases (kb). Of the three longest cDNA clones isolated from the initial screening, only one cDNA (pH131) was obtained which approached the size of AS mRNA; however, gel analysis indicated that the cDNA insert was approximately 200 base pairs short of full length. To obtain longer clones, a 5' fragment of this plasmid (pAS4) was used to rescreen the library, and two clones (pH57 and pH60) were isolated with inserts larger than pH131. A comparison of the restriction map and the orientation of the cDNA inserts in the pcD vector is shown in Fig. 1. The largest of the plasmids, pH57, had a 2,004-base-pair insert excluding the poly(A) tail and appeared by gel analysis to be full length.

Sequence of human cDNA for AS. To analyze the coding region contained in the AS cDNAs, we determined the sequence of the cDNA inserts by the strategy shown in Fig. 3 and examined the sequence for open reading frames (Fig. 4). The longest of these began at the first ATG and gave a predicted protein of 561 amino acids with a molecular weight of 64,400, which is in the size range of the protein purified by several groups (10, 12, 25). DNA sequencing indicated that the message contained at least 184 nucleotides of 5' untranslated region and 120 nucleotides of 3' noncoding sequences ending in a potential poly(A) addition site.

To confirm that we had isolated cDNAs containing the complete coding region for AS and to determine which sequences were necessary for expression, we used these plasmids for DNA-mediated gene transfer into cells which lack AS activity.

Transfection of asparagine-requiring Jensen rat sarcoma cells with human cDNAs. Several mammalian cell lines which are auxotrophic for asparagine (AS⁻) have been isolated and found to express no AS activity (21, 32). Since some cell lines have proven to be poor recipients in DNA-mediated gene transfer experiments, we tested the CHO N3 and rat Jensen sarcoma AS⁻ cell lines for the ability to take up and express a foreign gene by using the bacterial neomycin resistance gene carried on RSVneo. We found that Jensen rat sarcoma cells were better recipients (ca. 10-fold) than CHO AS⁻ cells were (Table 1), and we used this line in subsequent transfection experiments.

Plasmids containing AS cDNA inserts of various lengths were coprecipitated with calcium phosphate and added to Jensen rat sarcoma cells, which were then selected for the AS⁺ phenotype in medium lacking asparagine. The results of

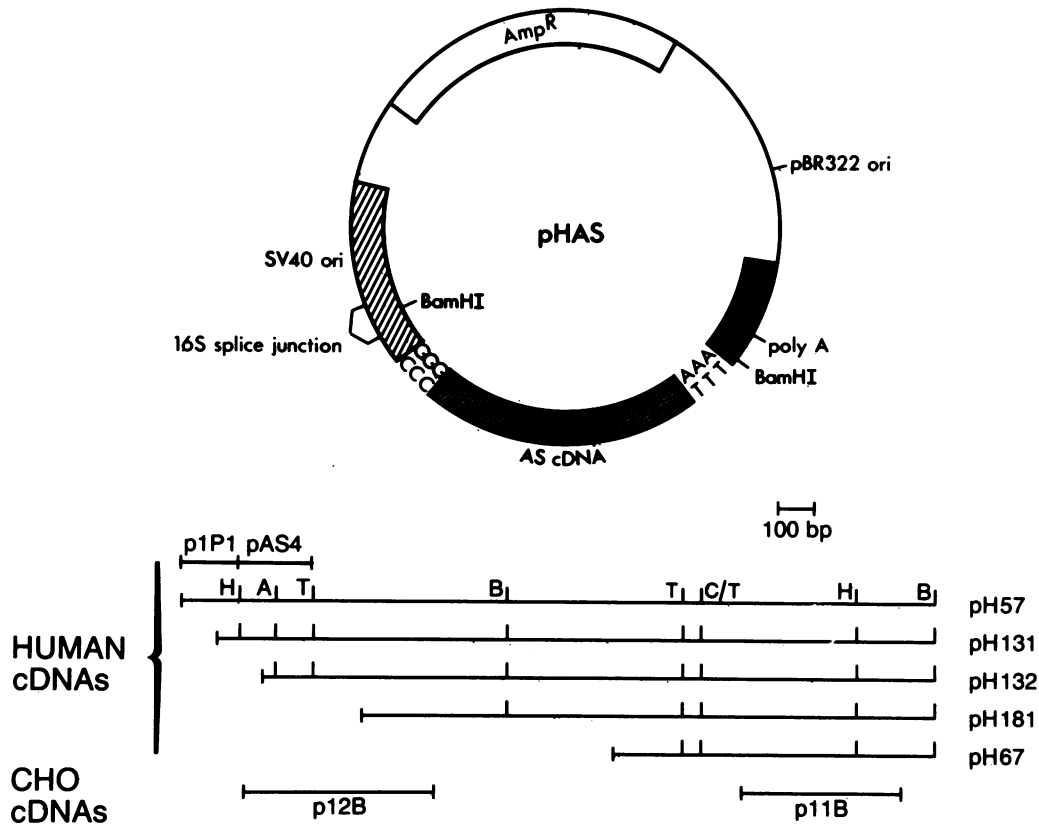


FIG. 1. Human AS cDNAs contained in the pcD expression vector were isolated by using the CHO cDNAs p12B and p11B as probes. The human AS cDNAs are overlapping, as determined by restriction analysis with *Hind*III (H), *Ava*II (A), *Taq*I (T), *Bam*HI (B), and *Cl*aI (C). All share the same 3' end, as detected by the p11B probe. SV40 ori, Simian virus 40 origin.

these experiments are presented in Table 1. As expected, transfection with a cDNA containing the complete open reading frame (pH131) consistently produced AS⁺ colonies at a frequency 7- to 50-fold over the background level, while a plasmid (pH67) containing only half of the open reading

frame gave no increase in AS⁺ colonies over the background. Transfections with plasmids missing the amino-terminal region of the open reading frame gave unexpected results. Both plasmids pH132 and pH181, which were missing the first 20 and 129 amino acids, respectively, produced a significant increase in AS⁺ colonies over the background.

While there are many possible interpretations of these results, one possibility is that the AS⁺ colonies arising from the selection are not true DNA transferants but are cells in which the native AS gene has reverted to AS⁺. To test this possibility, we analyzed a number of AS⁺ colonies derived from the transfection experiments for the presence and expression of human AS sequences.

Detection of human cDNA sequences in transferant cells.

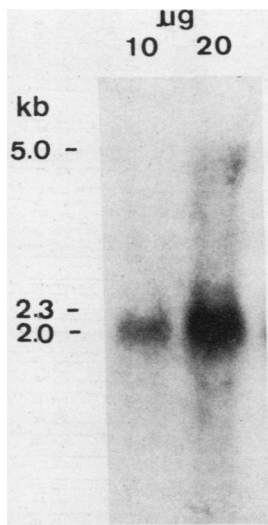


FIG. 2. Northern blot analysis of human HT1080 cells detected a predominant single species of approximately 2 kb of mRNA for AS.

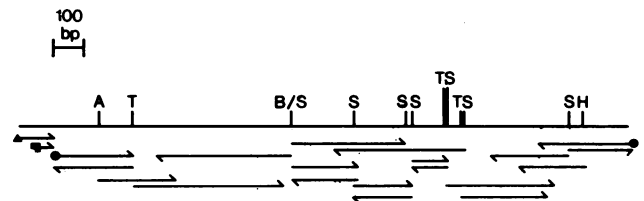


FIG. 3. Subcloning and sequencing strategy for human AS cDNAs. *Ava*II (A)-, *Taq*I (T)-, *Bam*HI (B)-, *Sau*3A (S)-, and *Hind*III (H)-digested cDNAs were subcloned into pUC19 and sequenced as described in Materials and Methods. Plasmids pH57 (▲), pH60 (■), and pH131 (●) were sequenced at the ends from restriction sites present in the vector.

GGG GGA AAC TTC CCG CAC GCG TTA CAG GAG CCA GGT C66 TAT AAG CGC CAC GGC TCG CCG CCC GTC AAG CTG TCF ACA TCC CTG GCC TCA GCC CGC CAC ATC ACC CTG ACC TGC TTA CGC
 CCA GAT TTT CTT CAA TCA CAT CTG AAT AAA TCA CTT GAA GAA AGC TTA TAG CTT CAT TGC ACC ATG TGT GGC GCG CTG TTT GGC AGT GAT GAT TGC CTT TCT GTT CAG TGT CTG
 Met Cys Gly Ile Trp Ala Leu Phe Gly Ser Asp Cys Leu Ser Val Gln Cys Leu
 >7

pH132
 AGT GCT ATG AAG ATT GCA CAC AGA GGT CCA GAT GCA TTC CGT TTT GAG AAT GTC AAT GGA TAC ACC AAC TGC TGC TTT GGA TTT CAC CGG TTG GCG GTA GTT GAC CCG CTG TTT GGA ATG
 Ser Ala Met Lys Ile Ala His Arg Gly Pro Asp Ala Phe Arg Phe Glu Asn Val Asn Gly Tyr Thr Asn Cys Cys Phe Gly Phe His Arg Leu Ala Val Asp Pro Leu Phe Gly Met
 177

CAG CCA ATT CGA GTG AAG AAA TAT CCG TAT TTG TGG CTC TGT TAC AAT GGT GAA ATC TAC AAC CAT AAG AAG ATG CAA CAG CAT TTT GAA TTT GAA TAC CAG ACC AAA GTG GAT GGT GAG
 Gln Pro Ile Arg Val Lys Lys Tyr Pro Tyr Leu Trp Leu Cys Tyr Asn Gly Glu Ile Tyr Asn His Lys Met Gln Gln His Phe Glu Tyr Gln Thr Lys Val Asp Gly Glu
 297

pH131
 ATA ATC CTT CAT CTT TAT GAC AAA GGA GGA ATT GAG CAA ACA ATT TGT ATG TTG GAT GGT GTG TTT GCA TTT GTT TTA CTG GAT ACT GCC AAT AAG AAA GTG TTC CTG GGT AGA GAT ACA
 Ile Ile Leu His Leu Tyr Asp Lys Gly Ile Glu Gln Thr Ile Cys Met Leu Asp Gly Val Phe Ala Phe Val Leu Leu Asp Thr Ala Asn Lys Lys Val Phe Leu Gly Arg Asp Thr
 417

TAT GGA GTC AGA CCT TTG TTT AAA GCA ATG ACA GAA GAT GGA TTT TTG GCT GTA TGT TCA GAA GCT AAA GGT CTT GTT ACA TTG AAG CAC TCC GCG ACT CCC TTT TTA AAA GTG GAG CCT
 Tyr Gly Val Arg Pro Leu Phe Lys Ala Met Thr Glu Asp Gly Phe Leu Ala Val Cys Ser Glu Ala Lys Lys Ser Ala Thr Pro Phe Leu Lys Val Glu Pro
 537

TTT CTT CCT GGA CAC TAT GAA GTT TTG GAT TTA AAG CCA AAT GGC AAA GTT GCA TCC GTG GAA ATG GTT AAA TAT CAT CAC TGT CGG GAT GTA CCC CTG CAC GCC CTC TAT GAC AAT GTG
 Phe Leu Pro Gly His Tyr Glu Val Leu Asp Leu Lys Pro Asn Gly Lys Val Ala Ser Val Glu Met Val Lys Tyr His His Cys Arg Asp Val Pro Leu His Ala Leu Tyr Asp Asn Val
 657

GAG AAA CTC TTT CCA GGT TTT GAG ATA GAA ACT GTG AAG AAC AAC CTC AGG ATC CTT TTT AAT AAT GCT GTA AAG AAA CGT TTG ATG ACA GAC AGA AGG ATT GGC TGC CTT TTA TCA GGG
 Glu Lys Leu Phe Pro Gly Phe Glu Ile Glu Thr Val Lys Asn Asn Leu Arg Ile Leu Phe Asn Asn Ala Val Lys Lys Arg Leu Met Thr Asp Arg Arg Ile Gly Cys Leu Leu Ser Gly
 777

GGC TTG GAC TCC AGC TTG GTT GCT GCC ACT CTG TTG AAG CAG CTG AAA GAA GGC CAA GTA CAG TAT CCT CTC CAG ACA TTT GCA ATT GGC ATG GAA AGC GCC GAT TTA CTG GCT GCT
 Gly Leu Asp Ser Ser Leu Val Ala Ala Thr Leu Leu Lys Glu Ala Gln Val Gln Tyr Pro Leu Gln Thr Phe Ala Ile Gly Met Glu Asp Ser Pro Asp Leu Leu Ala Ala
 897

AGA AAG GTG GCA GAT CAT ATT GGA AGT GAA CAT TAT GAA GTC CTT TTT AAC TCT GAG GAA GGC ATT CAG GCT CTG GAT GAA GTC ATA TTT TCC TTG GAA ACT TAT GAC ATT ACA ACA GTT
 Arg Lys Val Ala Asp His Ile Gly Ser Glu His Tyr Glu Val Leu Phe Asn Ser Glu Glu Ile Gln Ala Leu Asp Glu Val Ile Phe Ser Leu Glu Thr Tyr Asp Ile Thr Thr Val
 1017

CGT GCT TCA GTA GGT ATG TAT TTA ATT TCC AAG TAT ATT CCG AAG AAC ACA GAT AGC GTG GTG ATC TTC TCT GGA GAA GGA TCA GAT GAA CTT AGC CAG GGT TAC ATA TAT TTT CAC AAG
 Arg Ala Ser Val Gly Met Tyr Leu Ile Ser Lys Tyr Ile Arg Lys Asn Thr Asp Ser Val Val Ile Phe Ser Gly Glu Gly Ser Asp Glu Leu Thr Gln Gly Tyr Ile Tyr Phe His Lys
 1137

GCT CCT TCT CCT GAA AAA GGC GAG GAG AGT GAG AGG CTT CTG AGG GAA CTC TAT TTG TTT GAT GTT CTC CGC GCA GAT CGA ACT GCT GCC CAT GGT CTT GAA CTG AGA GTC CCA
 Ala Pro Ser Pro Glu Lys Ala Glu Glu Ser Glu Arg Leu Leu Arg Glu Tyr Leu Phe Asp Val Leu Arg Ala Asp Arg Thr Thr Ala Ala His Gly Leu Glu Arg Val Pro
 1257

TTT CTA GAT CAT CGA TTT TCC TAT TAG TTG TCT CTG CCA CCA GAA ATG AGA ATT CCA AAG AAT GGG ATA GAA AAA CAT CTC CTG AGA GAG ACG TTT GAG GAT TCC AAT CTG ATA CCC
 Phe Leu Asp His Arg Phe Ser Tyr Tyr Leu Ser Leu Pro Glu Met Arg Ile Pro Lys Asn Gly Ile Glu Lys His Leu Arg Glu Thr Phe Glu Asp Ser Asn Leu Ile Pro
 1377

AAA GAG ATT CTC TGG CGA CCA AAA GAA GCC TTC AGT GAT GGA ATA ACT TCA GTT AAG AAT TCC TGG TTT AAG ATT TTA CAG GAA TAC GTT GAA CAT CAG GTT GAT GAT GCA ATG Met Ala
 Lys Glu Ile Leu Trp Arg Pro Lys Glu Ala Phe Ser Asp Gly Ile Thr Ser Val Lys Asn Ser Trp Phe Lys Ile Leu Gln Glu Tyr Val Glu His Gln Val Asp Asp Ala Met Met Ala
 1497

AAT GCA GCC CAG AAA TTT CCC TTC AAT ACT CCT AAA ACC AAA GAA GGA TAT TAC TAC CGT CAA GTC TTT GAA CGC CAT TAC CCA GGC GGT GAC TGG CTG AGC CAT TAC TGG ATG CCC
 Asn Ala Ala Gln Lys Phe Pro Phe Asn Thr Pro Lys Thr Lys Glu Gly Tyr Tyr Arg Gln Val Phe Glu Arg His Tyr Pro Gly Arg Ala Asp Trp Leu Ser His Tyr Trp Met Pro
 1617

AAG TGG ATC AAT GCC ACT GAC CCT TCT GCC CGC AGC CTG ACC CAC TAC AAG TCA GCT GTC AAA GCT TAG GTG GTC TTT ATG CTG TAA TGT GAA AGC AAA TAT TTC TTC GTG TTG GAT GGG
 Lys Trp Ile Asn Ala Thr Asp Pro Ser Ala Arg Thr Leu Thr His Tyr Lys Ser Ala Val Lys Ala ---
 1737

GAC TGT GGG TAG ATA GGG GAA CAA TGA GAG TCA ACT CAG GCT AAC TTG GGT TTG AAA ATA AAA TTC CTA AAT TTA AAA AAA AAA

FIG. 4. Complete cDNA sequence of AS from pH57 and the predicted amino acid sequence of the protein initiating at the first ATG (numbering of the cDNA begins here). The 5' ends of the various cDNAs are indicated above the sequence. The message contains a polyadenylation signal (AATAAA) at the 3' end.

TABLE 1. Transformation of AS⁻ cells with plasmids containing RSV neo or human cDNAs

Expt	Cell line	DNA	Selective medium	Colonies/ 2 × 10 ⁶ cells	Frequency (10 ⁻³)		
1	N3 (CHO)	None	G418	0	0.45		
		pRSVneo	G418	9			
		None	G418	0			
2	Jensen (rat)	pRSVneo	G418	65	3.3		
		None	G418	0			
		pRSVneo	G418	98			
3	Jensen	None	-Asn ^a	0	2.5		
		pH131	-Asn	51			
		None	-Asn	2		0.1	
		pH67	-Asn	2			
		pH181	-Asn	10			0.5
		pH131	-Asn	18			
None	-Asn	3	0.15				
pH132	-Asn	18					
4	Jensen	None		-Asn	3	0.9	
		pH132		-Asn	18		
		pH131		-Asn	22		

^a -Asn, Medium lacking asparagine.

DNA was isolated from AS⁺ Jensen rat cells which had received pH181, pH131, or pH132 as well as from a spontaneous revertant which had received no exogenous DNA. The DNAs were digested with *Bam*HI, which cuts the plasmids to give a vector fragment of 3 kb and insert fragments of 1.2 and 0.4 to 1.0 kb depending on the size of the insert. By using high-stringency washes (0.1 × SSPE-0.1% SDS at 65°C), no rat AS sequences were detected with the human cDNA as a probe. Only those transfectants which had received pH131 contained human AS sequences (Fig. 5). Some of the transfectants appeared to have more than one copy of the cDNA, as indicated by the intensity of the major *Bam*HI fragments (3, 1.2, and 0.8 kb) and by the multiple sites of integration, as suggested by the many minor bands of various sizes. Serial dilutions of the DNA samples were analyzed by slot blotting to quantitate

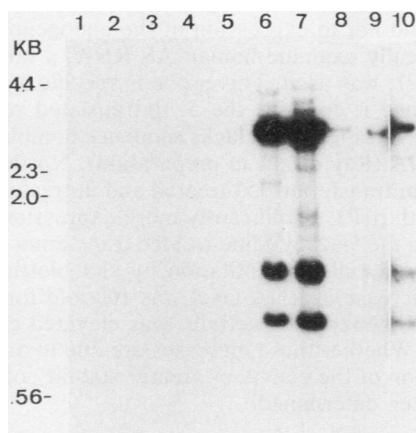


FIG. 5. Southern blot analysis of 10 µg of *Bam*HI-digested DNAs from AS⁻ Jensen rat sarcoma cells (lane 1); AS⁺ spontaneous revertant cells (lane 2); AS⁺ pseudotransferant cell lines C71 (lane 3) and C73 (lane 4) obtained after transfection with plasmid pH132; and AS⁺ transferant cell lines J41 (lane 5), J52 (lane 6), J53 (lane 7), and J54 (lane 8) obtained after transfection with plasmid pH131. Lanes 9 and 10 are longer exposures of lanes 5 and 8, respectively. The blot was processed as described in Materials and Methods, with total plasmid pH131 used as a probe.

TABLE 2. Determination of copy number of human AS cDNA sequences and level of AS mRNA in transferant lines

Cell line	Relative no. of AS copies ^a :		AS sp act (pmol/min per mg of protein) ^b
	Human ^c DNA	Human RNA	
Jensen rat (parental)	0	0	0
Transferants	31	16	9
J32			
J32 (treated) ^c	31	80	365
J33	16	8	237
J51	10	1-2	57
J53	64	6	11
J53 (treated) ^c	64	800	900
J56	4	6	52

^a Copy numbers were measured relative to the argininosuccinate lyase gene, as described in Materials and Methods.

^b The levels of specific activity in revertants were 167 to 63 pmol/min per mg of protein.

^c Treated with 5-azacytidine.

the cDNA copy numbers. Duplicate slot blots were probed with either the human AS cDNA or a probe for a single-copy rat liver gene (argininosuccinate lyase). The number of copies of the cDNA sequences varied between 4 and 64 for the various transferants, with most lines having numerous integrated copies of the cDNA (Table 2).

Transcription of human cDNAs in transferant cells. To determine the level of transcription of the transferred genes, RNA was isolated from the transferant cells and AS-specific RNA was quantitated by slot blot analysis. Since the sequences of the human and CHO cDNAs are highly homologous (Ray et al., in preparation), it was necessary to demonstrate that the human cDNA probes could be used to discriminate between transcripts of the transferred human gene and transcripts of the endogenous rat gene. To test for the level of endogenous rat gene transcription, RNAs from the recipient AS⁻ cells and spontaneous AS⁺ revertants of this line were used as controls. Under stringent hybridization conditions, no signal was observed for AS⁻ rat RNA

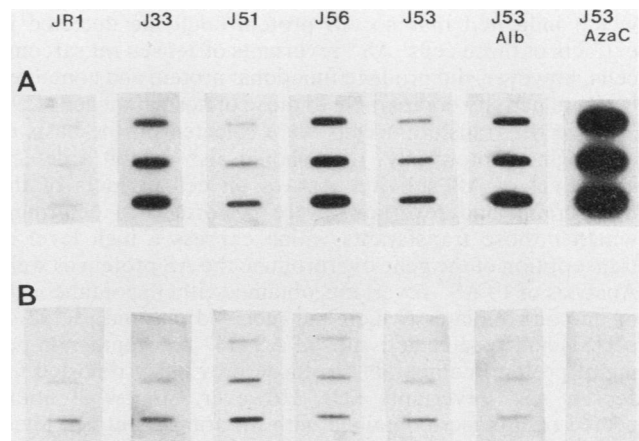


FIG. 6. Slot blot analysis of AS mRNA levels. Samples of 0.5, 1, and 2 µg of total RNA from JR1, a spontaneous revertant of Jensen sarcoma cells, and cDNA transferants J33, J51, J56, J53, J53 selected in albizziin (Alb), and 10-fold further dilutions of RNA from J53 treated with 5-azacytidine (AzaC) were probed with AS human cDNA (A) and compared with rat argininosuccinate lyase cDNA (B).

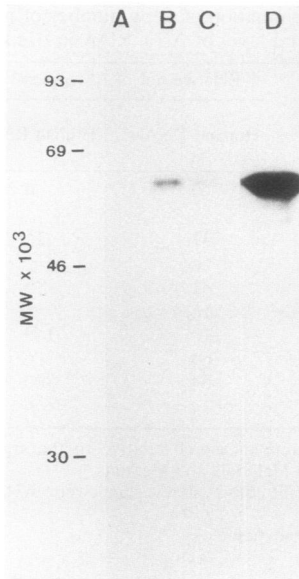


FIG. 7. Immunoblot of AS proteins from Jensen rat sarcoma cells (A), transferants J53 (B) and J32 (C), and a cell line which overproduces human AS (D), performed as described in Materials and Methods.

and a barely detectable level of cross-hybridization of the rat RNA was detectable in the spontaneous revertants when the total human AS cDNA was used as a probe (Fig. 6). On the other hand, the transferants exhibited significant levels of human AS RNA (Fig. 6). These levels were considerably greater than the amounts of cross-hybridization of the endogenous rat RNA detectable in the revertants. Transcription of the human AS sequences was demonstrated for all of the transferants, with levels varying among the cell lines (Table 2).

Activity of the human enzyme in Jensen rat sarcoma cells.

The parental Jensen rat sarcoma cells have been shown to be asparagine auxotrophs owing to a lack of AS enzymatic activity (29). This has been confirmed by SDS-polyacrylamide gel electrophoresis and Western blotting (Fig. 7), which indicated that no AS protein could be detected in extracts of these cells. AS⁺ revertants of Jensen rat sarcoma cells, however, did produce functional protein and contained levels of activity comparable to those of normal rat cells (29).

Since the transferant cells were selected on the basis of asparagine prototrophy, they should also exhibit a detectable level of AS activity. Assays on cell extracts of the transferants and revertants were performed to determine whether those transferants which express a high level of transcription of the gene overproduce the AS protein as well. Analysis of 10 AS⁺ revertants obtained either spontaneously or on control plates (which had received plasmid pH132 or pH181) had specific activities of AS (167 ± 63 pmol/min per mg of protein) comparable to those previously reported for Jensen AS⁺ revertants (29). However, somewhat unexpected results were obtained with the transferant cell lines. Various levels of AS specific activity (9 to 237 pmol/min per mg of protein) were exhibited by the transferant lines, with most expressing less activity than that observed for the revertant lines (Table 2). In fact, cell lines J32 and J53, which contained the most copies of the transfected cDNA, expressed the lowest levels of AS activity (only 7% of the revertant level). In general, enzymatic activity of AS in the

transferant cell lines did not correlate directly with the DNA and RNA copy numbers.

Even though the transferant cells had reduced levels of AS enzymatic activity, AS protein was detectable by immunoblotting (Fig. 7). The AS protein from the transferant lines was the same size as that from a human fibrosarcoma cell line which overexpresses AS activity (Fig. 7).

Analysis of AS expression in transferant cells. Although high levels of transcription were detected in some of the transferant cell lines (e.g., J33 and J56), the relatively low levels of specific activity of AS in the cells indicated that the human mRNA was not translated efficiently or that the human protein was poorly functional in the rat cell environment. Since the transferred DNA was integrated into the rat genome, the transcription of the human cDNAs would be affected by rat sequences as well as by the adjacent simian virus 40 sequences. The size and complexity of transcripts produced in the transferant cells were examined by Northern analysis. The AS mRNA from the J33 and J51 transferant cell lines was heterogeneous in size, producing a smear with no single prominent bands (data not shown). It was therefore likely that only a proportion of the transcripts from these lines was translated into functional protein.

Other cell lines (J53 and J32) contained numerous copies of the human cDNA, but RNA analysis indicated a low level of transcription of these sequences (Table 2). Since methylation of transfected DNA sequences has been observed in other systems and correlated with a lack of expression, we examined the methylation patterns of the transfected DNA. By comparing Southern blots of transferant DNA digested with *MspI* and *HpaII*, we found that in most of the cell lines the transfected DNA had become partially methylated. To test whether this affected the expression of AS, the J32 and J53 transferant cell lines were treated with 5-azacytidine (1 μ g/ml) to promote hypomethylation. The treated cells exhibited increases in AS activity of up to 80-fold over that of the untreated cells (Table 2) and up to 6-fold over the levels observed in spontaneous and 5-azacytidine-induced revertants (29) of the parental cell line.

The elevated level of activity of AS was correlated with an increased amount of mRNA for AS (Fig. 6). This elevation was due to specific transcription of the human cDNA sequences and not to expression of the endogenous rat gene. To specifically examine human AS RNA, a unique human probe, p1P1, was used. This probe is specific for the human cDNA, since it contains the 5' untranslated region of the human cDNA (Fig. 1) and lacks sequence homology with the CHO cDNA (Ray et al., in preparation). Northern blots of RNAs from transferant J53 treated and untreated cells were probed with p1P1. Significantly more expression of AS was detected in the 5-azacytidine-treated transferant cells than in the untreated cells. Quantitation by slot blotting indicated that the increase in RNA level was 100-fold for the treated cells and the enzymatic activity was elevated concordantly (Table 2). Whether these increases are due to an increase in transcription of the cDNA or greater stability of the mRNA has not been determined.

DISCUSSION

We isolated human cDNAs containing the entire coding region for AS by homology with our previously characterized CHO cDNAs (25). The human cDNAs were obtained from the Okayama and Berg library, which was constructed in the pcD vector such that the cDNAs contained the same

3' end and differed in the length of the 5' sequences. This library allowed us to isolate nearly full-length human AS cDNAs and orient the CHO cDNAs. Although a number of cDNAs were obtained, the frequency of detecting a full-length cDNA for human AS (pH57) was low (10^{-6}) but in the range of the frequencies for the isolation of nearly full-length cDNAs for other genes from this library (8, 14). We analyzed the human AS cDNAs by DNA sequencing and showed that the message encoded a protein of 561 amino acids if translation was initiated at the first AUG. The ACC sequence which preceded this AUG is in good agreement with the consensus translational start region identified by Kozak (17). We have recently sequenced the CHO cDNAs as well (Ray et al., in preparation) and have found that there is overall homology of 86% with the most extensive sequence homology in the translated portion. The homology at the protein level between human and CHO AS was predicted to be 95%.

We showed that the human AS cDNA pH131 contained the entire coding region by using the plasmids for DNA-mediated gene transfer into Jensen rat sarcoma cells. These cells are asparagine auxotrophs owing to a lack of AS activity (29). pH131, a cDNA which contained the entire open reading frame in an insert of 1.9 kb, was capable of rescuing the AS⁻ cells. To determine how much of the cDNA was necessary for function, two shorter cDNAs (pH132 and pH181) were also tested for the ability to confer the AS⁺ phenotype. If the first AUG in the cDNA is used, pH131 would produce AS protein of the correct size, pH132 would produce a protein lacking 20 amino acids from the amino terminal, and the protein from pH181 would be missing 129 amino acids. While pH181 would lack the proper initiation site, a fusion protein could be produced by initiation at an AUG site in the vector (22). On the other hand, if the second AUG is the correct initiation codon, both pH131 and pH132 would code for identical proteins and pH181 could code for a fusion protein. Since no transferants were obtained when either pH132 or pH181 were added to Jensen rat sarcoma cells, we conclude that the initial AUG is used and the first 20 amino acids are necessary for enzymatic activity of AS.

It should be noted that when pH132 and pH181 were used in gene transfer experiments, the frequency of AS⁺ colonies increased relative to that of the control. However, when the colonies were expanded under selective conditions and DNA was isolated from the cells, no human AS fragments were observed. One possibility for this result could be recombination between the cDNA sequences and the native gene. We would have detected these recombinant sequences on Southern blots probed with the human cDNAs if the integrated human cDNA contained as little as 100 base pairs. However, no human cDNA sequences were observed in these lines, and asparagine prototrophy was more likely the result of reversion. Why the frequency of reversion increased with the addition, but not the retention, of plasmid DNA remains to be elucidated. It was, in fact, somewhat surprising that no recombinant DNA was detected in the cells which had received pH132, since recipient cells generally take up large amounts of foreign DNA. It is likely that since no genomic carrier DNA was used and pH132 could not confer AS activity, the plasmid sequences were not at a selectable advantage.

The bona fide transferants contained integrated plasmid sequences with up to 64 copies of the human AS cDNA. Slot blot analysis indicated that although these sequences were transcribed, the amount of human AS RNA did not correlate directly with the DNA copy number. In addition, levels of

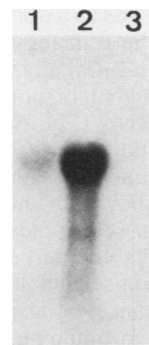


FIG. 8. Northern analysis of total RNA (9 μ g) from Jensen rat sarcoma cells (lane 3) and transferant cell line 53 (lane 1) showing increased transcription of human AS after treatment of transferant cells with 5-azacytidine (lane 2) by using a 5' portion (p1P1) of the human cDNA pH57 as a probe.

enzymatic activity were not proportional to either DNA or RNA copy number in all of the transferants. An explanation for this was provided by Northern blot analysis, which indicated that the transcripts from several lines were heterogeneous in size, suggesting improper initiation or processing of the transcripts. This may be owing to the random use of either the two splice acceptor sites (16S or 19S) (22) contained in the vector or the poly(A) addition sites in both the vector and the cDNA. If the 16S splice acceptor site is used, then the first AUG encountered would be in the cDNA and functional AS would be produced. However, if splicing to the 19S acceptor site occurs, an AUG upstream of the cDNA could be used to form a fusion protein which might not be enzymatically active. This possibility is supported by the finding of a greater amount of immunoreactive protein detected by Western blotting of cell extracts of the transferants compared with amounts from extracts of revertants which had similar or greater levels of enzymatic activity.

Another possibility is that some copies of the integrated plasmid are transcriptionally inactive. Two of the transferants, J32 and J53, contained multiple copies of the human cDNA but expressed very low levels of mRNA and barely detectable levels of AS activity. One explanation for the lack of transcription is that the integrated plasmid DNA is not expressed owing to methylation. This was tested by exposing these transferants to 5-azacytidine, a treatment which causes demethylation of the DNA. Southern blot analysis of *MspI* and *HpaII* digestions of DNA was used to confirm the differences in methylation patterns of treated and untreated cells (data not shown). Transferant cells treated with 5-azacytidine exhibited a large increase (100-fold) in transcription over untreated cells, suggesting that most of the plasmid DNA was inactivated by methylation. Treatment with 5-azacytidine could have activated the endogenous rat gene; however, the frequency of this event has been shown to affect only 5 to 10% of the treated cells (29). Nevertheless, we discounted this possibility by determining the level of human AS transcription in the 5-azacytidine-treated cells by using a probe specific for the human sequences (Fig. 8).

One of the objectives of this work was to determine whether we could obtain high levels of expression of AS in cells following DNA transfer, since cells which overproduce AS activity can be selected for on the basis of albizzin resistance. We have used this drug previously to isolate mutants of human and CHO cells (2) and have found that

albizziin resistance behaves as a dominant selectable marker in animal cells. When the transferant cell lines J53 and J32 were tested for growth in 1 mM albizziin, they were found to be resistant to this drug (data not shown) and exhibited elevated levels of AS RNA (Fig. 6). Therefore, it is likely that plasmid pH131 could be used for DNA-mediated gene transfer into other cell lines by using albizziin as the selective drug. This would have advantages in systems where cotransfer of a dominant selectable marker (albizziin resistance) with an unselectable marker is needed or where cotransfer of two different selectable markers (e.g., albizziin and methotrexate resistance) is desired.

Our primary interest in obtaining human cDNAs was to use these as probes to study the organization and expression of the gene in normal human cells and asparaginase-sensitive leukemic cells. Since asparaginase-sensitive cells tend to have little or no AS activity, these probes should be useful in determining how the gene is turned off. The human cDNAs will also be useful in determining the structure of the enzyme itself. The results presented here with two cDNAs which differ by 20 amino acids suggest that the extreme amino portion of the protein may be necessary for enzyme activity. For these studies, it is advantageous to have an expressible cDNA and a selection for the product. Studies involving site-directed mutagenesis and deletion analysis using transfer and selection should indicate other critical regions of the enzyme.

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LITERATURE CITED

1. Andrulis, I. L. 1985. Regulation and amplification of asparagine synthetase, p. 489-518. *In* M. M. Gottesman (ed.), *Molecular cell genetics*.
2. Andrulis, I. L., C. Duff, S. Evans-Blackler, R. Worton, and L. Siminovitch. 1983. Chromosomal alterations associated with overproduction of asparagine synthetase in albizziin-resistant Chinese hamster ovary cells. *Mol. Cell. Biol.* **3**:391-398.
3. Andrulis, I. L., S. Evans-Blackler, and L. Siminovitch. 1985. Characterization of single-step albizziin-resistant Chinese hamster ovary cell lines with elevated levels of asparagine synthetase activity. *J. Biol. Chem.* **260**:7523-7527.
4. Andrulis, I. L., G. W. Hatfield, and S. M. Arfin. 1979. Asparaginyl-tRNA aminoacylation levels and asparagine synthetase expression in cultured Chinese hamster ovary cells. *J. Biol. Chem.* **254**:10629-10633.
5. Andrulis, I. L., and L. Siminovitch. 1981. DNA-mediated gene transfer of β -aspartylhydroxamate resistance into Chinese hamster ovary cells. *Proc. Natl. Acad. Sci. USA* **78**:5724-5728.
6. Andrulis, I. L., and L. Siminovitch. 1982. Isolation and characterization of Chinese hamster ovary cell mutants resistant to the amino acid analog β -aspartyl hydroxamate. *Somatic Cell Genet.* **8**:533-545.
7. Arfin, S. M., D. R. Simpson, C. S. Chiang, I. L. Andrulis, and G. W. Hatfield. 1977. A role for asparaginyl-tRNA in the regulation of asparagine synthetase in a mammalian cell line. *Proc. Natl. Acad. Sci. USA* **74**:2367-2369.
8. Ayusawa, D., K. Takeishi, S. Kaneda, K. Shimizu, H. Koyama, and T. Seno. 1984. Isolation of functional cDNA clones for human thymidylate synthase. *J. Biol. Chem.* **259**:14361-14364.
9. Boyse, E. A., L. J. Old, H. A. Campbell, and L. T. Mashburn. 1967. Suppression of murine leukemias by L-asparaginase. *J. Exp. Med.* **125**:17-31.
10. Gantt, J. S., and S. M. Arfin. 1981. Elevated levels of asparagine synthetase activity in physiologically and genetically derepressed Chinese hamster ovary cells are due to increased rates of enzyme synthesis. *J. Biol. Chem.* **256**:7311-7315.
11. Gantt, J. S., C.-S. Chiang, G. W. Hatfield, and S. M. Arfin. 1980. Chinese hamster ovary cells resistant to β -aspartyl hydroxamate contain increased levels of asparagine synthetase. *J. Biol. Chem.* **255**:4808-4813.
12. Hongo, S., and T. Sato. 1981. Purification of rat liver asparagine synthetase by affinity chromatography on reactive blue 2-agarose. *Anal. Biochem.* **114**:163-166.
13. Horowitz, B., B. K. Madras, A. Meister, L. J. Old, E. A. Boyse, and E. Stockert. 1968. Asparagine synthetase activity of mouse leukemias. *Science* **160**:533-535.
14. Jolly, D. J., H. Okayama, P. Berg, A. C. Esty, D. Filipula, P. Bohler, G. G. Johnson, J. E. Shively, T. Hunkapillar, and T. Friedmann. 1983. Isolation and characterization of a full-length expressible cDNA for human hypoxanthine phosphoribosyltransferase. *Proc. Natl. Acad. Sci. USA* **80**:477-481.
15. Kafatos, F. C., C. W. Jones, and A. Efstratiadis. 1979. Determination of nucleic acid sequence homologies and relative concentrations by a dot hybridization procedure. *Nucleic Acids Res.* **7**:1541-1552.
16. Korneluk, R. G., F. Quan, and R. A. Gravel. 1985. Rapid and reliable dideoxy sequencing of double-stranded DNA. *Gene* **40**:317-323.
17. Kozak, M. 1984. Compilation and analysis of sequences upstream from the translational start site in eukaryotic mRNAs. *Nucleic Acids Res.* **12**:857-872.
18. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (London)* **227**:680-685.
19. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**:265-275.
20. Maniatis, T., E. F. Fritsch, and J. Sambrook. 1982. *Molecular cloning: a laboratory manual*. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
21. McCoy, T. A., M. Maxwell, and P. F. Kruse, Jr. 1959. The amino acid requirements of the Jensen sarcoma in vitro. *Cancer Res.* **19**:591-595.
22. Okayama, H., and P. Berg. 1983. A cDNA cloning vector that permits expression of cDNA inserts in mammalian cells. *Mol. Cell. Biol.* **3**:280-289.
23. Ortega, J. A., M. E. Nesbit, Jr., M. H. Donaldson, R. H. Hittle, J. Weiner, M. Karon, and D. Hammond. 1977. L-Asparaginase, vincristine, and prednisone for induction of first remission in acute lymphocytic leukemia. *Cancer Res.* **37**:535-540.
24. Patterson, M. K., Jr., and G. Orr. 1967. L-asparagine biosynthesis by nutritional variants of the Jensen sarcoma. *Biochem. Biophys. Res. Commun.* **26**:228-233.
25. Ray, P. N., L. Siminovitch, and I. L. Andrulis. 1984. Molecular cloning of a cDNA for Chinese hamster ovary asparagine synthetase. *Gene* **30**:1-9.
26. Rigby, P. W. J., M. Dieckmann, C. Rhodes, and P. Berg. 1977. Labeling deoxyribonucleic acid to high specific activity in vitro by nick translation with DNA polymerase I. *J. Mol. Biol.* **113**:237-251.
27. Southern, E. M. 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. *J. Mol. Biol.* **98**:503-517.
28. Stanners, C., G. Elicieri, and H. Green. 1971. Two types of ribosome in mouse-hamster hybrid cells. *Nature (London) New Biology* **230**:52-54.
29. Sugiyama, R. H., S. M. Arfin, and M. Harris. 1983. Properties of asparagine synthetase in asparagine-independent variants of Jensen rat sarcoma cells induced by 5-azacytidine. *Mol. Cell. Biol.* **3**:1937-1942.
30. Towbin, H., T. Staehelin, and J. Gordon. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellular

- sheets: procedure and some applications. Proc. Natl. Acad. Sci. USA **76**:4350–4354.
31. **Wahl, G. M., R. A. Padgett, and G. R. Stark.** 1979. Gene amplification causes overproduction of the first three enzymes of UMP in *N*-(phosphonacetyl)-L-aspartate-resistant hamster cells. J. Biol. Chem. **254**:8679–8689.
32. **Waye, M. M. Y., and C. P. Stanners.** 1979. Isolation and characterization of CHO cell mutants with altered asparagine synthetase. Somatic Cell Genet. **5**:625–639.
33. **Yanisch-Perron, C., T. Viera, and J. Messing.** 1985. Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. Gene **33**:103–119.