MOLECULAR AND CELLULAR BIOLOGY, Nov. 1983, p. 1937–1942
0270-7306/83/111937-06$02.00/0

Vol. 3, No. 11

Properties of Asparagine Synthetase in Asparagine-Independent Variants of Jensen Rat Sarcoma Cells Induced by 5-Azacytidine

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Jensen rat sarcoma cells in culture require L-asparagine for growth and lack detectable levels of asparagine synthetase. Cultures exposed for 24 h to graded concentrations of 5-azacytidine give rise to asparagine-independent variants in high frequency. These prototrophs are stable phenotypically whether maintained in the presence or absence of L-asparagine. Asparagine synthetase activity in several variant clones was uniform in thermolability and several kinetic parameters, as well as in immunological properties. Parental Jensen rat sarcoma cells contained no detectable immunologically cross-reacting material. Our data suggest that transitions between asparagine dependence and independence in these cells are mediated by stable shifts in gene expression rather than by structural gene mutations.

Mammalian cells do not, in general, require L-asparagine in culture, since aspartic acid can be converted to asparagine by an ATP-dependent glutamine amidotransferase, asparagine synthetase (7, 11, 17). However, a number of human and rodent cell lines of malignant origin have little or no measurable asparagine synthetase activity. These cells are asparagine auxotrophs and are sensitive to therapy with L-asparaginase (5, 20). Cells which lack asparagine synthetase activity also arise spontaneously from wild-type Chinese hamster cells, and the frequency can be enhanced 10-fold by treatment with ethyl methanesulfonate (8, 25). Fluctuation tests have been used to measure reversion of asparagine auxotrophs to the prototrophic state. Spontaneous rates range from 10^{-6} to 10^{-7} per cell per generation and can be increased somewhat by exposure to ethyl methanesulfonate or nitrosoguanidine (3, 25).

The basis for the loss and reappearance of asparagine synthetase activity in these cells is unknown. Although in past studies mutational changes have been invoked, asparagine dependence might also arise from failure of the normal wild-type gene for asparagine synthetase to be expressed. There is a generally inverse relationship between DNA methylation and gene expression (16, 19, 22), and methylation at one or more control sites could provide an effective block in asparagine-dependent cells. This possibility can be explored by treatment with the nucleoside analog 5-azacytidine (5-Aza-C). Cells exposed to this agent exhibit hypomethylation of newly synthesized DNA (13), and undergo a wide variety of heritable variations at the phenotypic level (2, 4, 12, 18, 23). Of particular interest is the massive induction of thymidine kinase-positive cells which results from 5-Aza-C treatment of enzyme-deficient Chinese hamster cells (9).

In the experiments described, we showed similarly that Jensen rat sarcoma cells, which are auxotrophic for asparagine, can be converted in high frequency to asparagine independence by exposure to 5-Aza-C. The resulting prototrophs are stable phenotypically whether propagated in the presence or absence of asparagine in the culture medium. We have examined a number of properties of the asparagine synthetase that are expressed in these clonal populations. These results are consistent with a model in which a normal asparagine synthetase structural gene is not expressed in the parental Jensen rat sarcoma line, but is reactivated by 5-Aza-C-induced hypomethylation in the asparagine-independent variants.

MATERIALS AND METHODS

Cell culture. All experiments were performed with Jensen rat sarcoma cells (14) obtained from the American Type Culture Collection, Rockville, Md.; these cells are referred to hereafter as the JR 45 line. Stock populations were maintained as monolayers in prescription bottles in a basal medium containing 10% fetal bovine serum and 90% Dulbecco modified Eagle medium (with 4.5 mg of glucose per ml and pyruvate omitted). Before use, L-asparagine (50 μg/ml), sodium penicillin G (60 μg/ml), streptomycin

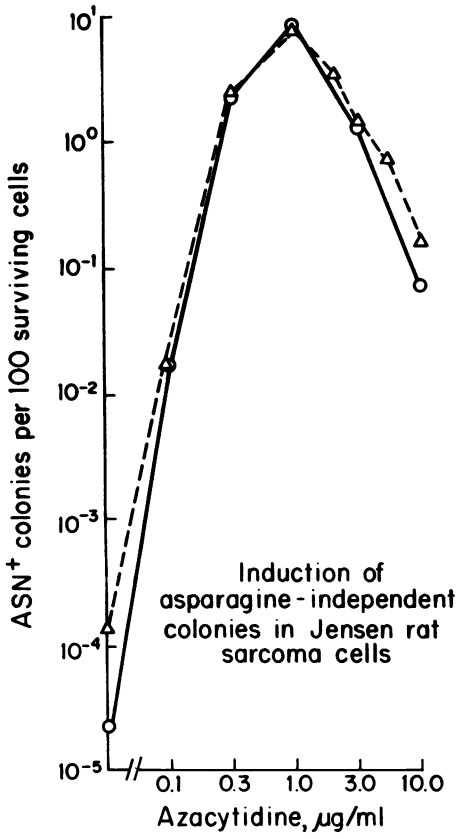

FIG. 1. Induction of asparagine-independent variants by 5-Aza-C in stock JR 45 cell populations. Two replicate experiments (○, △) are shown. Each point shown represents the average colony count for six or more petri dish cultures, corrected for relative plating efficiency.

(50 μg/ml), and glutamine (100 μg/ml) were added. FU5AH rat cells (21) were grown in the same medium. Plating experiments in the presence or absence of asparagine were performed in 60-mm petri dishes that were maintained in a humidified CO_2 incubator with fluid changes once or twice a week. When colonies were well formed, experiments were terminated by staining the cultures for 30 min in a saturated solution of crystal violet in 0.85% NaCl, after which the dishes were washed in tap water and air dried. For induction experiments, stock solutions of 5-Aza-C (Sigma Chemical Co.) were prepared at 1,000 μg/ml in Dulbecco nutrient. Since aqueous solutions of 5-Aza-C are unstable, a fresh stock of 5-Aza-C was made up for each experiment and added directly to the culture medium.

Asparagine synthetase assay. Asparagine synthetase activity was measured by following the conversion of [^{14}C]aspartic acid to [^{14}C]asparagine as described previously (7). Units are expressed as picomoles of asparagine formed per minute per milligram of protein. The thermolability of the enzyme from FU5AH cells and prototrophic clones was determined as follows. Cells were lysed by freeze-thawing in buffer containing 50 mM Tris (pH 7.5)–5 mM EDTA–1 mM dithiothreitol–20% glycerol (pH 7.5) and centrifuged at 30,000 × *g* for 20 min. The supernatant was adjusted to a protein concentration of 3 mg per ml. Portions of the supernatant were heated at 47.5°C for various times and then plunged into ice. After completion of the experiment, the remaining enzyme activity was determined.

Immunochemical procedures. The preparation and properties of anti-asparagine synthetase antibody have been described previously (6). Immunotitration of asparagine synthetase in extracts of FU5AH cells and of the asparagine-independent variants was performed as described previously (6). The presence of immunologically cross-reactive material in JR 45 was assayed by the ability of extracts of these cells to inhibit the immunoprecipitation of asparagine synthetase.

RESULTS

Induction of asparagine prototrophs with 5-azacytidine. Our experiments followed a protocol previously established for induction of thymidine kinase-positive cells with 5-Aza-C in Chinese hamster cells (9). Mass populations of JR 45 cells in log phase were treated for 24 h with graded concentrations of 5-Aza-C in basal medium containing L-asparagine. The cultures were then rinsed twice in the same medium without 5-Aza-C and incubated for a further 2-day recovery period. For assay, each population was trypsinized, and percent survival was determined by colony formation in groups of petri dishes containing basal medium with L-asparagine. The frequency of prototrophic variants was established from colonies formed by graded numbers of the same cells in asparagine-free medium.

Two replicate experiments performed with JR

TABLE 1. Plating efficiency in stock JR 45 cells and in asparagine-independent variants induced by 5-Aza-C[a]

Cell line	Maintenance medium	Plating efficiency	
		+Asn	−Asn
JR 45	+Asn	76.7	2.3×10^{-5}
1522-1	−Asn	73.7	90.0
	+Asn	82.7	91.0
1522-2	−Asn	49.0	47.3
	+Asn	52.3	48.3
1522-3	−Asn	82.7	80.0
	+Asn	78.7	77.7

[a] Stock JR 45 (asparagine-requiring) cells and asparagine-independent clones 1522-1, 1522-2, and 1522-3 were maintained for two or more passages in basal medium with (+Asn) or without (−Asn) asparagine as noted. Plating efficiencies were then determined for each population in the presence and absence of asparagine, respectively. Values shown represent average colony counts per 100 cells plated (3 to 6 petri dish cultures in each series).

45 cells are shown in Fig. 1. Both show a sharp concentration-dependent rise in the incidence of asparagine-independent variants after exposure to 5-Aza-C. There is a well-defined mode for optimal induction of 1.0 μg of 5-Aza-C per ml, giving a one-step increase in variant frequency of 100,000 or more. Although this figure is corrected for relative survival, the relative plating efficiency of treated cells was 88.3, 62.1, and 37.1% at concentrations of 0.1, 1.0, and 10.0 μg/ml, respectively. Thus, the inductive response represents an overall population shift rather than selective proliferation of a small minority cell fraction.

Phenotypic stability of asparagine-independent variants. Several clones induced from JR 45 cells with 5-Aza-C were isolated in asparagine-free medium and grown to mass populations for further study. Each of these was divided into sublines that were maintained by serial subculture in basal medium with and without asparagine. Subsequent tests (Table 1) showed that cells from the two sublines were identical in plating properties, whether cultivated in the presence or absence of asparagine as a selective agent. Asparagine independence arising from inductive treatment thus appears to be a stable characteristic in these cells.

Asparagine synthetase activity in the JR 45 cells and asparagine-independent variants. Extracts of JR 45 cells failed to synthesize asparagine as measured by our conditions for the assay of asparagine synthetase. Since asparagine is required for growth, it may be concluded that the asparagine synthetase reaction is the primary biosynthetic pathway in these cells. Table 2 shows that the asparagine-independent variants obtained after 5-Aza-C pretreatment expressed substantial asparagine synthetase activity. Furthermore, the level of activity, like plating efficiency, was unaffected by growth of the variants in medium containing asparagine for up to 4 weeks. This is in contrast to the repression caused by asparagine in most established cell lines (1, 24).

TABLE 2. Asparagine synthetase activity in parental and asparagine-independent variants of Jensen rat sarcoma cells[a]

Cell line	Asparagine synthetase activity (U/mg of protein) in:[b]	
	Asn-free medium	Asn medium
JR 45		0
1522-1	100 ± 17	103 ± 11
1522-2	81 ± 20	76 ± 14
1522-3	125 ± 17	128 ± 20

[a] Asparagine synthetase activity was determined after cells were grown for 3 to 4 weeks in the indicated medium.

[b] Values shown represent the mean ± SE for at least 10 determinations.

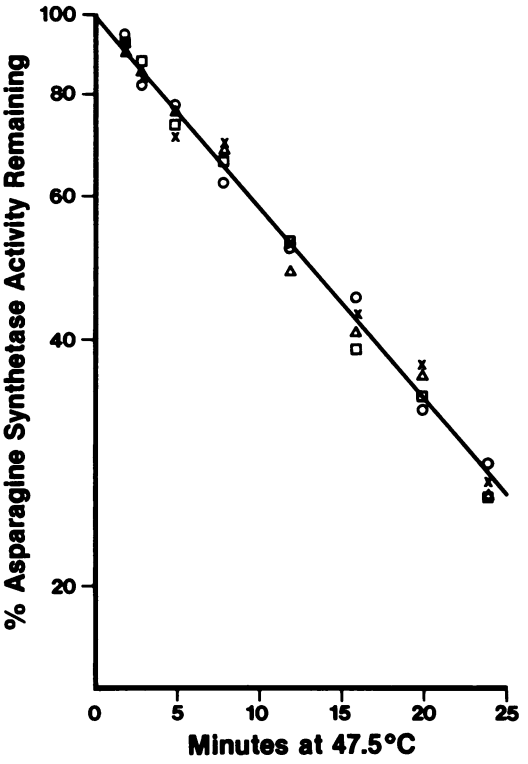

FIG. 2. Thermal stability of asparagine synthetase. Symbols: O, 1522-1; □, 1522-2; △, 1522-3; X, FU5AH.

Properties of asparagine synthetase in asparagine-independent variants. If asparagine prototrophy in 5-Aza-C-induced variants is the result of the reactivation of a normal structural gene, then the properties of the enzyme in each of the variants should be the same. Conversely, if the change to asparagine independence represents different second site mutations in a defective structural gene, differences might be expected in physical, catalytic, or immunological properties or in a combination of these properties. A comparison of the thermolability (Fig. 2) and kinetic constants (Table 3) of the asparagine synthetase from three revertants shows that there are no significant differences in these properties. The results obtained with the revertants are very similar to those obtained with the FU5AH rat cell line and also agree with those determined by others for purified rat liver asparagine synthetase (10).

Immunotitration of extracts from these same three revertants with rabbit anti-asparagine synthetase antiserum (Fig. 3) shows that the amount of antiserum required to neutralize the enzyme activity is directly proportional to the activity of asparagine synthetase in these extracts and also

TABLE 3. K_m's for substrates of asparagine synthetase in asparagine-independent variants[a]

Cell line	K_m (mM) of:	
	Aspartate	Glutamine
1522-1	0.75 ± 0.02	0.80 ± 0.06
1522-2	0.78 ± 0.05	0.84 ± 0.06
1522-3	0.77 ± 0.05	0.84 ± 0.07
FU5AH	0.82 ± 0.04	0.90 ± 0.07

[a] Results are the mean ± SE of four determinations.

proportional to the amount required to neutralize activity in extracts of FU5AH cells. Thus, no difference was detected in immunological properties of the asparagine synthetase from the three revertants.

To test for the presence of immunologically cross-reacting material in the parental Jensen rat sarcoma line, the ability of extracts from JR 45 cells to inhibit the immunoprecipitation of asparagine synthetase from the revertant lines was measured. In a constant antigen experiment, 1 volume of revertant extract, 1 volume of revertant extract plus 3 volumes of extract from JR 45, and 3 volumes of revertant extract was titrated with anti-asparagine synthetase serum. If JR 45 extracts contain immunologically cross-reacting material, then the titration curve for the mixture should be displaced from that for the titration of 1 volume of revertant extract towards that for the titration of 3 volumes of revertant extract. The results of such an experiment are shown in Fig. 4. By this test, there was no detectable cross-reacting material in extracts of JR 45 cells.

DISCUSSION

Our experiments show that 5-Aza-C induces massive conversion of Jensen rat sarcoma cells to the prototrophic state and that the asparagine independence thus established is maintained stably without selective pressure or further exposure to the inductive agent. Quantitatively, induction of 5-Aza-C greatly exceeds the level of response expected for mutagen treatment, and, in fact, exposure of these cells to ethyl methanesulfonate or nitrosoguanidine is reported to give only a 10- to 20-fold increase over background (3, 25). Furthermore, 5-Aza-C in direct tests shows little or no ability to induce conventional gene mutations (14), although striking alterations in cellular phenotype that are heritable can clearly be produced (23). These observations point to changes in gene expression as the probable mechanism of action by 5-Aza-C and suggest that explanations involving mutations at structural or processing loci for asparagine synthetase are inadequate.

By several criteria, the properties of asparagine synthetase in 5-Aza-C-induced variants are those expected for the product of a normal, unaltered structural gene. Thus, the thermolability and kinetic constants of the asparagine synthetase in three revertant clones were essentially identical, as were the immunological properties when examined by immunotitration studies with rabbit anti-asparagine synthetase antiserum. These properties are also very similar to those of the asparagine synthetase from the FU5AH rat cell line and purified rat liver asparagine synthetase (10). Further tests failed to reveal detectable amounts of immunologically cross-reacting material in extracts from the parental Jensen rat sarcoma cells. The sensitivity of the assay employed is such that we would have detected expression of the asparagine synthetase gene at a level of 5% of the expression in the revertant cells. Formally, an explanation for these data might be made in terms of a structural gene mutation leading to early termination or to an enzyme so structurally altered as to be antigenically inactive or rapidly degraded. However,

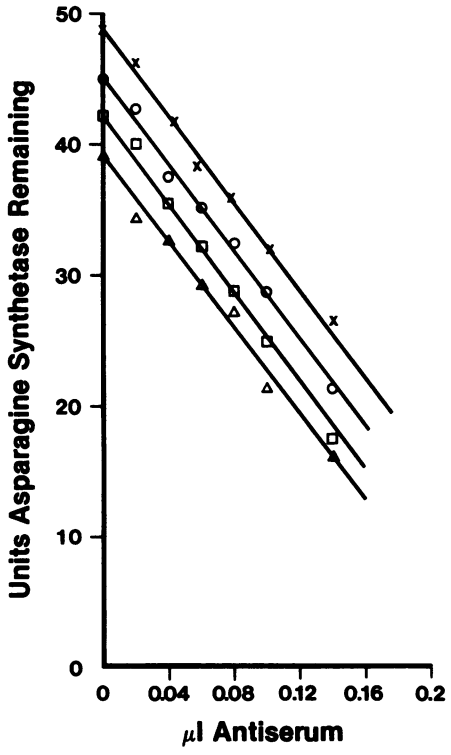

FIG. 3. Immunotitration of asparagine synthetase activity of asparagine-independent variants. Samples of cell extracts were mixed with the indicated volume of antiserum and incubated at 4°C for 30 min. After precipitation with fixed *S. aureus*, the activity remaining in the supernatant was assayed. Symbols: O, 1522-1; □, 1522-2; △, 1522-3; X, FU5AH.

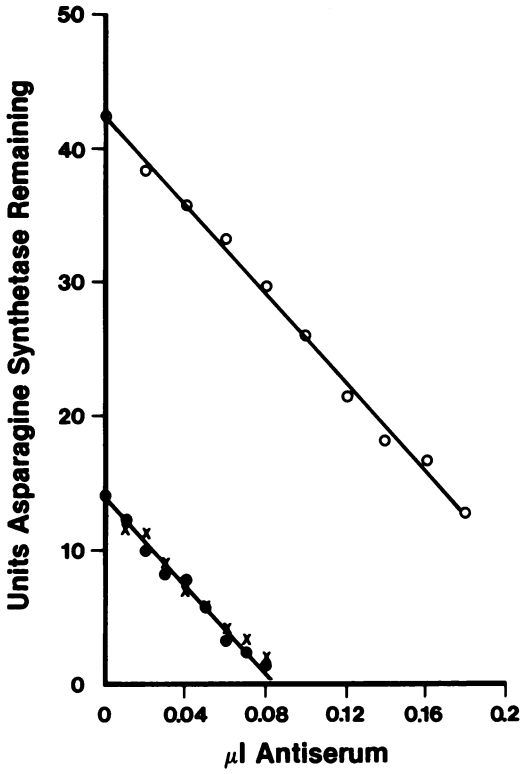

FIG. 4. Assay for immunologically cross-reacting material in JR 45 cells. Constant antigen immunotitration in which 3 volumes of extract from 1522-3 cells (○), 1 volume of extract from 1522-3 cells (●), or 1 volume of extract from 1522-3 cells plus 3 volumes of extract from JR 45 cells (X) were mixed with the indicated volume of antiserum.

this fails to account for the high-frequency induction by 5-Aza-C of variants with a functional asparagine synthetase, the properties of which are essentially identical in the three variants analyzed.

Taken as a whole, our data suggest that the structural gene for asparagine synthetase is inactive in auxotrophic cells and that it can be reexpressed in essentially normal form by spontaneous or inductive change. Potentiation by 5-Aza-C suggests that reappearance of activity is triggered by hypomethylation at sites in or near the asparagine synthetase gene. Direct evidence for this mechanism is already available from experiments on induction of the inactive herpes *tk* gene in transformed cells. Restriction enzyme analysis showed clearly that site-specific hypomethylation accompanies reappearance of herpes thymidine kinase after exposure of the cells to 5-Aza-C (2). Similar studies can be extended to the variants we have described when cloned probes for the asparagine synthetase gene become available.

ACKNOWLEDGMENTS

This work was supported by Public Health Service grants CA12130 (to M.H.) and GM26014 (to S.M.A.). R.H.S. is a postdoctoral fellow of The Leukemia Society of America.

We thank Patrick Link for excellent technical assistance.

LITERATURE CITED

1. **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. U.S.A. **74**:2367–2369.
2. **Clough, D.W., L. M. Kunkel, and R. S. Davidson.** 1982. 5-azacytidine-induced reactivation of a herpes simplex thymidine kinase gene. Science **216**:70–73.
3. **Colofiore, J., J. Morrow, and M. K. Patterson, Jr.** 1973. Asparagine-requiring tumor cell lines and their non-requiring variants: cytogenetics, biochemistry, and population dynamics. Genetics **75**:503–514.
4. **Compere, S. J., and R. D. Palmiter.** 1981. DNA methylation controls the inducibility of the mouse metallothionein-1 gene in lymphoid cells. Cell **25**:233–240.
5. **Cooney, D. A., and R. E. Handschumacher.** 1970. L-asparaginase and L-asparagine metabolism. Annu. Rev. Pharmacol. **10**:421–440.
6. **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.
7. **Gantt, J. S., C. S. Chiang, G. W. Hatfield, and S. M. Arfin.** 1980. Chinese hamster ovary cells resistant to β-aspartylhydroxamate contain increased levels of asparagine synthetase. J. Biol. Chem. **255**:4808–4813.
8. **Goldfarb, P. S. G., B. Carritt, M. L. Hooper, and C. Slack.** 1977. The isolation and characterization of asparagine-requiring mutants of Chinese hamster cells. Exp. Cell Res. **104**:357–367.
9. **Harris, M.** 1982. Induction of thymidine kinase in enzyme-deficient Chinese hamster cells. Cell **29**:483–492.
10. **Hongo, S., T. Matsumoto, and T. Sato.** 1978. Purification and properties of asparagine synthetase from rat liver. Biochim. Biophys. Acta **522**:258–266.
11. **Horowitz, B., and A. Meister.** 1972. Glutamine-dependent asparagine synthetase from leukemia cells. J. Biol. Chem. **247**:6708–6719.
12. **Ivarie, R. D., and J. A. Morris.** 1981. Induction of prolactin-deficient variants of GH_3 rat pituitary cells by ethyl methanesulfonate: reversion of 5-azacytidine, a DNA methylation inhibitor. Proc. Natl. Acad. Sci. U.S.A. **79**:2967–2970.
13. **Jones, P. A., and S. M. Taylor.** 1980. Cellular differentiation, cytidine analogs and DNA methylation. Cell **20**:85–93.
14. **Landolph, J. R., and P. A. Jones.** 1982. Mutagenicity of 5-azacytidine and related nucleosides in C3H/10T1/2 clone 8 and V79 cells. Cancer Res. **42**:817–823.
15. **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.
16. **McGhee, J. D., and G. D. Ginder.** 1979. Specific DNA methylation sites in the vicinity of chicken β-globin genes. Nature (London) **280**:419–420.
17. **Milman, H. A., and D. A. Cooney.** 1974. The distribution of L-asparagine synthetase in the principal organs of several mammalian and avian species. Biochem. J. **142**: 27–35.
18. **Mohandas, T., R. S. Sparkes, and L. J. Shapiro.** 1981. Reactivation of an inactive human X chromosome: evidence for X inactivation by DNA methylation. Science **211**:393–396.
19. **Naveh-Many, T., and H. Cedar.** 1980. Active gene sequences are undermethylated. Proc. Natl. Acad. Sci. U.S.A. **78**:4245–4250.
20. **Patterson, M. K., Jr., and G. R. Orr.** 1967. L-Asparagine

biosynthesis by nutritional variants of the Jensen sarcoma. Biochem. Biophys. Res. Commun. **26**:228–233.

21. **Schneider, J. A., and M. Weiss.** 1971. Expression of differentiated functions in hepatoma cell hybrids. I. Tyrosine aminotransferase in hepatomafibroblast hybrids. Proc. Natl. Acad. Sci. U.S.A. **68**:127–131.
22. **Sutter, D., and W. Doerfler.** 1980. Methylation of integrated adenovirus type 12 DNA sequences in transformed cells is inversely correlated with viral gene expression. Proc. Natl. Acad. Sci. U.S.A. **77**:253–256.
23. **Taylor, S. M., and P. A. Jones.** 1979. Multiple new phenotypes induced in 10T1/2 and 3T3 cells treated with 5-azacytidine. Cell **17**:771–779.
24. **Uren, J. R., W. P. Summers, and R. E. Handschumacher.** 1974. Enzymatic and nutritional evidence for two-stage expression of the asparagine synthetase locus in L5178Y murine leukemia mutants. Cancer Res. **34**:2940–2945.
25. **Waye, M. M. Y. and C. P. Stanners.** 1979. Isolation and characterization of CHO cell mutants with altered asparagine synthetase. Somat. Cell Genet. **5**:625–639.