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# Characterization of Cultured Tobacco Cell Lines Resistant to Ethionine, a Methionine Analog[1]

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## ABSTRACT

**Two cultured tobacco cell lines (*Nicotiana tabacum* L. cv Xanthi) were selected for resistance to growth inhibition by the methionine analog ethionine. Comparison of the free amino acid pool levels in these lines with those of the ethionine-sensitive parental line showed substantial accumulation of methionine (110×), threonine (18×), and lysine (5×). *In vitro* enzymic analysis of lysine-sensitive aspartate kinase activity showed the resistant lines to contain 16 times that found in the sensitive line. The lysine-sensitive enzymes from both resistant and sensitive lines coeluted from DEAE-cellulose and exhibited similar $K_m$ values. Both showed identical lysine plus *S*-adenosylmethionine inhibition profiles suggesting that the elevated activity in the resistant lines is not due to a structural change in the lysine-sensitive enzyme but possibly to the level of its expression.**

The aspartate family of amino acids share a common precursor in a multibranched biosynthetic pathway (Fig. 1). Regulatory control of this pathway in higher plants is primarily by feedback inhibition (4). Genetic variants, overproducing end-products of the pathway (*i.e.* methionine, threonine, isoleucine, and lysine), have been obtained in selection experiments directed against this regulatory mechanism.

Growth inhibition by the combination of lysine plus threonine was thought to be due to the feedback inhibition of aspartate kinase and homoserine dehydrogenase with subsequent starvation for methionine (3, 5, 7). Indeed, exogenously supplied methionine reversed the growth inhibition. Lysine plus threonine-resistant maize tissue culture lines were selected by Hibberd *et al.* (9), which overproduced both threonine and methionine. Resistance was attributed to an altered aspartate kinase showing reduced sensitivity to feedback inhibition by lysine. The resistance phenotype, selected in a different experiment, was subsequently shown to be inherited as a single dominant nuclear gene (8). Barley (2, 11, 18) and carrot (6, 11) lines, selected for resistance to lysine plus threonine, also exhibited increased levels of threonine and methionine, or threonine and isoleucine, respectively. In most cases, resistance was also attributed to reduced feedback control of aspartate kinase by lysine.

Growth inhibition by ethionine has been suggested to be the consequence of ethionine incorporation into protein and into the cellular SAM[3] pool in place of methionine (1). Therefore, overproduction of methionine could be expected to confer resistance to this analog by providing more competition for incorporation. Ethionine-resistant bacterial and fungal mutants have been of two types: altered amino acid uptake or defective methionine adenosyltransferase (10, 15, 20). Mutants with the altered transferase overproduced methionine. Selection of ethionine-resistant plant cell cultures has also yielded methionine overproducers (21, 22), but no enzymic characterization was reported. We describe here the selection of ethionine-resistant cultured tobacco cells that overproduce methionine and the partial characterization of the metabolic change responsible.

## MATERIALS AND METHODS

**Plant Material and Maintenance.** TX1 (*Nicotiana tabacum* L. cv Xanthi) was the parental cell line used in this study (23). All suspension cell lines were maintained at 27 to 28°C, with shaking, in basal Murashige and Skoog medium (17) containing 0.4 mg/l 2,4-dichlorophenoxyacetic acid. Cells were routinely subcultured every 4 d.

**Selection for Ethionine Resistance.** Flasks containing 100 ml culture medium plus 0.2 mM ethionine were inoculated with 1.0 g fresh weight cells and were incubated for up to 10 weeks. Flasks showing growth were subcultured into fresh medium with and without ethionine.

**Enzyme Extraction.** Four-day-old cell cultures were harvested by vacuum filtration and resuspended in cold buffer (1 g fresh weight/ml buffer) containing 50 mM Tris (pH 7.5), 1 mM EDTA, 10 mM 2-mercaptoethanol, and 10% (v/v) glycerol. Approximately 1 mg of DTT and PVPP for every 9 g fresh weight cells was added, and cells were disrupted by three passages through a French pressure cell (500 p.s.i.). Whole cells and debris were removed by centrifugation at 27,000*g* for 10 min. The supernatant was used as a crude enzyme preparation or brought to 40% saturation with a saturated solution of $(NH_4)_2SO_4$. Precipitated protein was collected by centrifugation at 27,000*g* for 10 min and resuspended in the above buffer. The preparation was desalted on Sephadex G-25 and the protein determined by the method of Lowry *et al.* (12) using BSA as standard.

**Enzyme Assays.** Methionine adeonsyltransferase activity was assayed by the method of Mudd *et al.* (16). The reaction mixture contained 100 mM Tris (pH 8.0), 32 mM $MgSO_4$, 5 mM methionine, and 20 mM [$^{14}$C]ATP (0.4 $\mu$Ci) in 0.25 ml final volume. After incubation for 1 h at 28°C the reaction was terminated by adding 3 ml cold water. The reaction mixtures were then loaded onto 0.67 × 3.0 cm Dowex AG 50W-X2 cation-exchange columns (ammonium form). Unreacted methionine and ATP were eluted by washing with 10 ml cold water. Adsorbed SAM was

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[3] Abbreviations: SAM, *S*-adenosylmethionine; PVPP, polyvinylpolypyrrolidone; $I_{50}$, concentration of inhibitor producing 50% inhibition of growth.

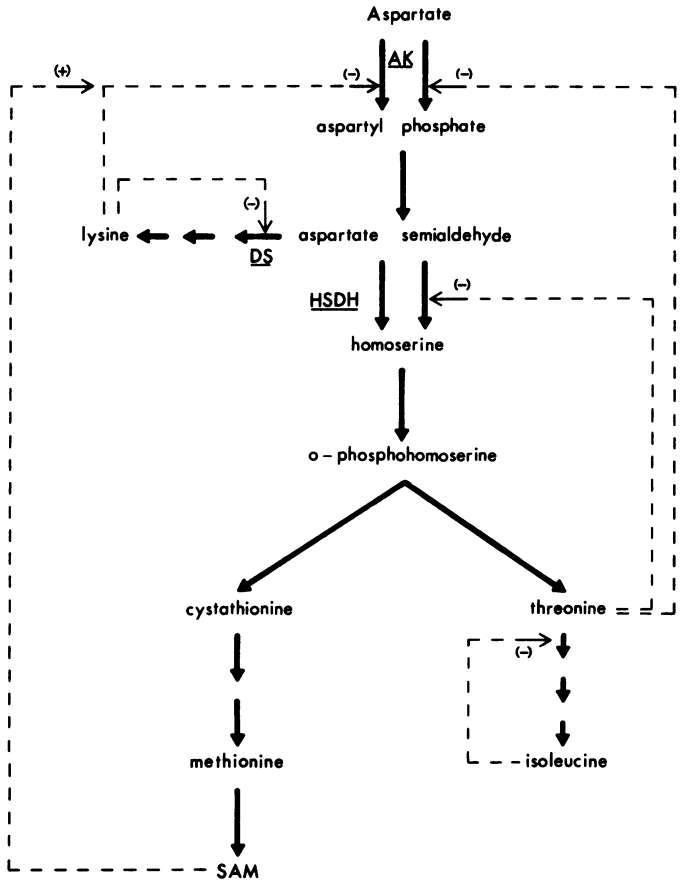


FIG. 1. Biosynthesis and regulatory control of the aspartate family of amino acids. AK, aspartate kinase; DS, dihydrodipicolinate synthase; HSDH, homoserine dehydrogenase.

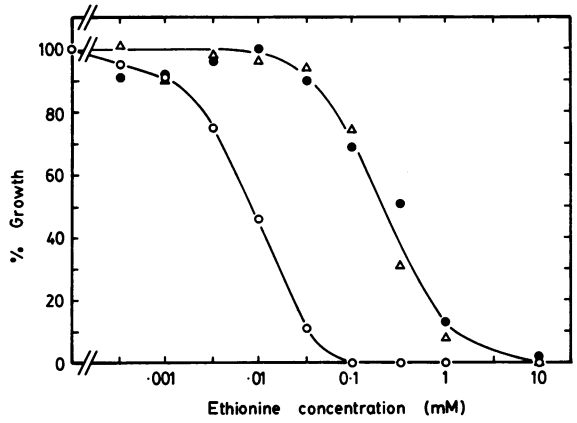


FIG. 2. Inhibition of tobacco cell growth by ethionine. Cells were harvested 10 d after inoculation with 0.25 g fresh weight, 4-d-old cells into 50 ml fresh medium containing ethionine. Fresh weight increases for control (no ethionine) TX1 (○), R11 (●), and R22 (△) cells were, 13.6, 10.5, and 11.5 g fresh weight, respectively.

eluted from the column with 10 ml $NH_4OH$ (30%). The $NH_4OH$ was evaporated, 15 ml Aquasol added, and the sample counted by scintillation spectrometry. Aspartate kinase and homoserine dehydrogenase activities were assayed as previously described (13). Lysine-sensitive aspartate kinase activity was specifically measured by including 10 mM threonine in the reaction mixture.

## RESULTS AND DISCUSSION

**Selection for Resistance to Ethionine.** Two cell lines (R11 and R22), resistant to the growth inhibitory effects of ethionine, were obtained in selection experiments from TX1 cells at a frequency of approximately 1 in $1.8 \times 10^7$ cells. As shown in Figure 2, both R11 and R22 required 27-fold higher levels of ethionine to produce equivalent growth inhibition as observed for the parental line, TX1. $I_{50}$ values were 8 μM for TX1 and 217 μM for both R11 and R22. Resistance to ethionine was stably maintained after at least 25 cell generations away from the analog. The data for only one of these lines, R11, will be presented, since similar

Table I. *Variation in the Free Amino Acid Profiles of Ethionine-Resistant and -Sensitve Tobacco Suspension Cultures*

All other amino acids showed less than a 3-fold change in relative concentrations for R11 and TX1.

| Amino Acid | TX1 | RX1 | (R11/TX1) |
|---|---|---|---|
| Met | 5[a] | 511 | 110 |
| Thr | 370 | 6768 | 18 |
| Ile | 27 | 146 | 5 |
| Lys | 47 | 214 | 5 |
| Gly | 328 | 1369 | 4 |
| Ser | 286 | 1466 | 5 |
| Arg | 50 | 225 | 5 |
| Leu | 88 | 270 | 3 |

[a] Expressed as nmol/g fresh weight.

Table II. *Effect of the Addition of Aspartate Family Amino Acids on the Growth Inhibition of Tobacco Suspension Cells by Ethionine*

Cell weight was determined in duplicate flasks, containing 50 ml medium with the indicated addition, 10 d after inoculation with 0.25 g fresh weight TX1 cells.

| Addition | g fresh wt | % of Control |
|---|---|---|
| None | 16.8 | (100) |
| 0.2 mM Eth | 0 | 0 |
| 0.2 mM Eth + 1 mM Lys | 0 | 0 |
| 0.2 mM Eth + 1 mM Met | 8.4 | 50 |
| 0.2 mM Eth + 1 mM Thr | 0 | 0 |
| 0.2 mM Eth + 1 mM Ile | 0 | 0 |

Table III. *Comparison of Two Aspartate Pathway Enzymes in Ethionine-Sensitive and -Resistant Cultured Tobacco Cells*

| Cell Line | Addition | Methionine Adenosyl-Transferase | Homoserine Dehydrogenase | |
|---|---|---|---|---|
| | | | NAD | NADP |
| TX1 | None | 20.5[a] | 2.8[b] | 1.3 |
| | 10 mM Thr | | 2.6 | 1.3 |
| R11 | None | 18.6 | 3.0 | 1.4 |
| | 10 mM Thr | | 2.6 | 1.3 |

[a] Methionine adenosyltransferase expressed as nmol/h·mg protein.
[b] Homoserine dehydrogenase expressed as μmol/min·mg protein.

Table IV. *Feedback Inhibition of Aspartate Kinase in Ethionine-Sensitive and -Resistant Cultured Tobacco Cells*

| Addition | Inhibition | |
|---|---|---|
| | TX1 | R11 |
| | % | |
| None | 0[a] | 0[b] |
| 10 mM Lys | 55 | 92 |
| 10 mM Thr | 48 | 10 |
| 10 mM Lys + Thr | 97 | 100 |

[a] Control activity = 1.46 nmol/h· mg protein.
[b] Control activity = 13.66 nmol/h·mg protein.

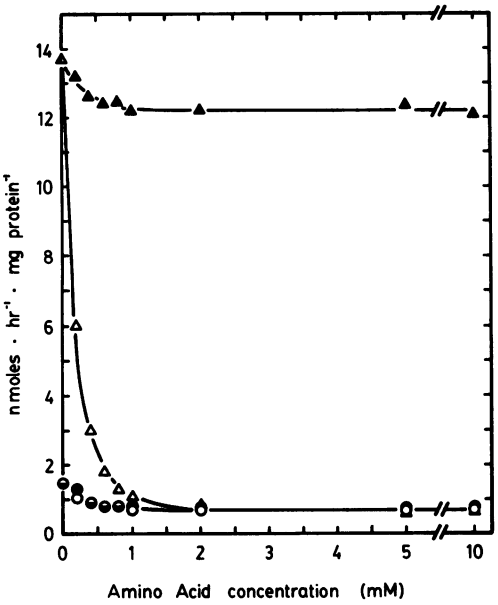


FIG. 3. Inhibition profiles of aspartate kinase activity from ethionine-sensitive (O) and -resistant (Δ) cultured tobacco cells for lysine (Δ, O) and threonine (▲, ●).

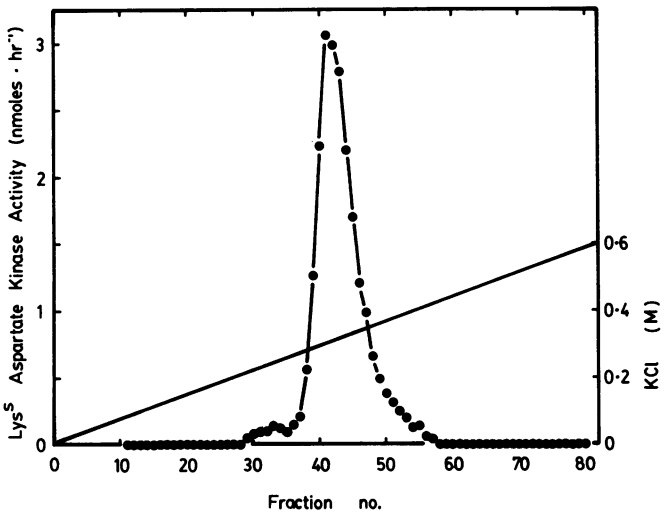


FIG. 4. DEAE-cellulose column chromatography of a mixture of lysine-sensitive aspartate kinase preparations from ethionine-sensitive and -resistant cultured tobacco cells. Twenty-five units of enzyme activity prepared from TX1 cells were mixed with 25 units from R11 cells and loaded onto a 28 × 1.5 cm column. After washing with 40 ml buffer, the column was eluted with a linear 0 to 0.6 M KCl gradient (150 ml). Collected fractions were assayed for lysine-sensitive aspartate kinase.

results were obtained in experiments utilizing R22.

An amino acid analysis of R11 revealed an increase in the cellular levels of several free amino acid pools over those of TX1 (Table I). The increases were most notable for methionine and threonine which showed levels 110 and 18 times those of TX1, respectively. As shown in Table II the growth of TX1 cells was completely inhibited by 0.2 mM ethionine, and the inhibition could be partially reversed by the addition of methionine to the growth medium. These data suggest that resistance to the growth inhibition of ethionine exhibited by R11 is due to the overproduction of methionine.

**Regulatory Enzymes.** Overproduction of methionine suggested a possible alteration in one of the regulatory control enzymes in

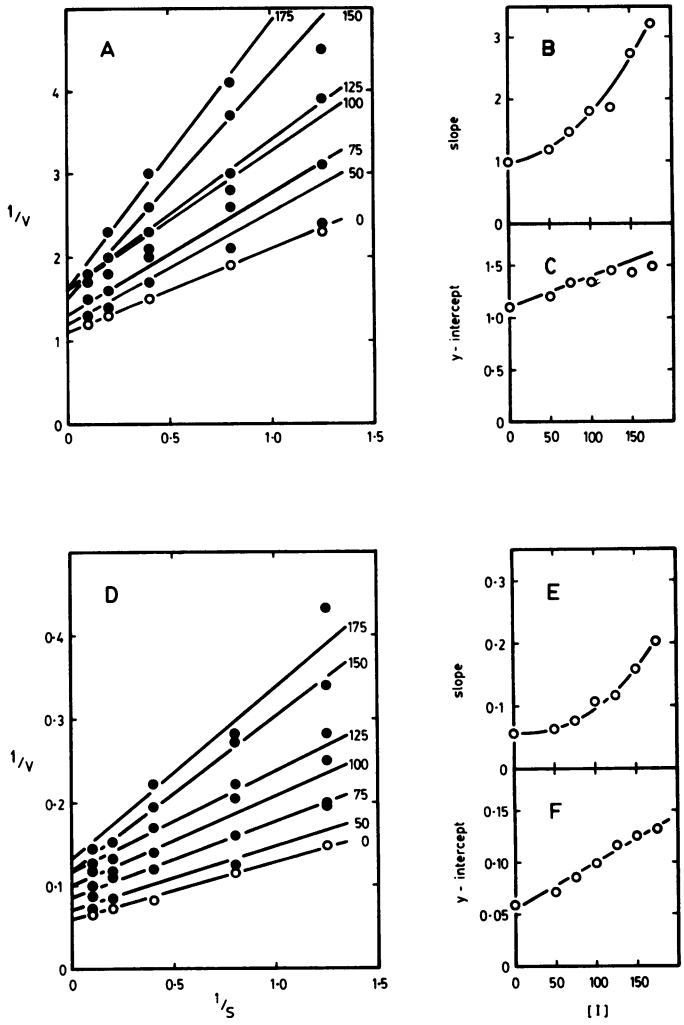


FIG. 5. Double reciprocal plots of lysine-sensitive aspartate kinase activity *versus* aspartate concentration and the changes in the slope and *y* intercept at various lysine concentrations for ethionine-sensitive (A, B, C) and -resistant (D, E, F) cultured tobacco cells.

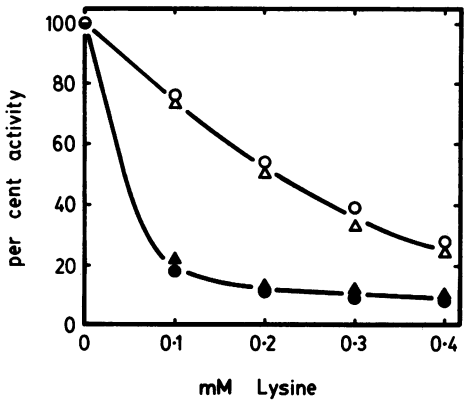


FIG. 6. Inhibition of lysine-sensitive aspartate kinase activity from ethionine-sensitive (O) and -resistant (Δ) cultured tobacco cells by lysine (O, Δ) or lysine plus 1 mM SAM (●, ▲).

the methionine biosynthetic pathway. Ethionine resistance and concomitant methionine overproduction in bacterial and fungal systems were previously found to have been due to the loss of activity of methionine adenosyltransferase (adenosine triphosphate ATP:L-methionine *S*-adenosyltransferase, EC 2.5.1.6) (10,

15, 20). Apparently, the regulatory control mechanism functioned to maintain an appropriate level of the SAM pool in the cell. However, as shown in Table III, the methionine adenosyltransferase activity in R11 was unaltered (91% the activity of TX1).

Homoserine dehydrogenase activity and its sensitivity to feedback inhibition by threonine was also found to be unaltered in the resistant cells (Table III).

Methionine overproduction in barley plants and seeds (2, 11, 18) and in cultured maize cells (9), selected for resistance to lysine plus threonine, was shown to have been the result of an altered feedback sensitivity of the lysine-sensitive aspartate kinase isozyme. Previous DEAE-cellulose column chromatographic separations of differentially feedback sensitive aspartate kinase activities have suggested at least two isozymes in soybean suspension cells (14) and carrot root tissue (19), and possibly three isozymes in barley (2).

Total aspartate kinase activity in TX1 was inhibited approximately 50% each by lysine and threonine (Table IV). The combination of the two amino acids resulted in almost complete inhibition of activity, suggesting the presence in tobacco of at least two isozymes, one sensitive to lysine and one to threonine. In R11, however, the per cent inhibition by these amino acids was quite different; 90% for lysine and 10% for threonine (Table IV).

It is important to note that the level of total aspartate kinase activity in R11 was 9 times that observed for TX1. Lysine and threonine inhibition profiles of aspartate kinase from both TX1 and R11, shown in Figure 3, suggest that this difference was due solely to an increase in the level of activity for the lysine-sensitive isozyme in R11. A decline in total activity with increasing concentration of lysine or threonine should be due to the loss of activity of the corresponding feedback-sensitive isozyme, and the activity remaining at high levels should be due to the complementary isozyme. Therefore, the activity of the two isozymes can be calculated. By these criteria, the threonine-sensitive activities calculated for TX1 and R11 were 0.68 and 1.04 nmol/h·mg protein, respectively, and the calculated lysine-sensitive activities, were 0.77 and 12.22 nmol/h·mg protein, respectively. Clearly, the amount of the threonine-sensitive activity was essentially the same in both cell lines while the lysine-sensitive activity of the resistant line was elevated to 16-fold that of the sensitive line.

**Lysine-Sensitive Aspartate Kinase.** When enzyme preparations from TX1 and R11 were mixed and then assayed, the lysine-sensitive aspartate kinase activity was found to be additive (data not shown). This suggested that the increased level of enzyme activity in R11 was not due to the absence of an inhibitory regulator.

If, instead, the elevated enzyme activity was due to altered enzyme structure or to the induction of a second lysine-sensitive isozyme, one might expect detectable physical differences between the TX1 and R11 enzymes, as was observed for two lysine-sensitive isozymes in barley (2, 18). Mixed enzyme preparations from TX1 and R11, containing equivalent levels of enzyme activity, were chromatographed on DEAE-cellulose. As shown in Figure 4, the elution pattern resulted in a single sharp peak at approximately 0.3 M KCl. It was also found, as expected from the results seen earlier in Figure 3, that the $V_{max}$ for the R11 activity was roughly 19 times greater, on a per mg protein basis, than the $V_{max}$ for the TX1 activity (Fig. 5, A and D). However, the $K_m$ values of 1.15 and 0.83 mM for the R11 and TX1 activities, respectively, were not significantly different. Additionally, both R11 and TX1 lysine-sensitive activities exhibited S-parabolic I-linear noncompetitive inhibition by lysine (Fig. 5, B, C and E, F). Since the $K_m$ value is a reflection of substrate binding affinity, it appears that the elevated activity in R11 is not due to an alteration in the active site of the lysine-sensitive isozyme. Similar inhibition characteristics also suggest that the lysine binding site is not altered either. This was supported by the data shown in Figure 6, indicating that the sensitivities of the enzymes from TX1 and R11 to feedback inhibition by lysine or lysine plus SAM were identical. While not completely eliminating other possibilities, these data strongly suggest that the elevated lysine-sensitive aspartate kinase activity found in R11 was not due to either an alteration in the enzyme structure or the induction of a second lysine-sensitive isozyme.

As observed in bacterial (10) and fungal systems (15) the selection for cultured tobacco suspension cells resistant to growth inhibition by ethionine also resulted in the overproduction of methionine. However, in tobacco the cause of this phenotype is quite different. Instead of a drastically reduced capacity to synthesize SAM, the activity of a regulatory enzyme early in the methionine pathway has been considerably elevated. With increased levels of aspartate kinase present, as in R11, more aspartate would be processed into the pathway (see Fig. 1). Most of the carbon would be shunted toward methionine and threonine at the lysine branch point since lysine stringently regulates its own synthesis at dihydrodipicolinate synthase (4). The carbon would then be processed by the threonine-resistant homoserine dehydrogenase to methionine and threonine (4).

## CONCLUSION

Selection for resistance to growth inhibition by ethionine with microbes has routinely yielded mutants with a defective methionine adenosyltransferase, resulting in the accumulation of methionine. However, in selection experiments with cultured tobacco cells we have obtained lines with a different enzymic alteration conferring methionine overproduction: elevation of the lysine-sensitive aspartate kinase activity. The physiological effect of this modification is similar to that observed in lysine plus threonine-resistant barley (2, 11, 18), carrot (6, 11) and maize (9) lines, where a lysine-sensitive aspartate kinase showed reduced sensitivity to feedback inhibition by lysine.

We have shown evidence that the difference in the lysine-sensitive aspartate kinase activities of the ethionine-resistant and -sensitive cell lines was probably not due to a newly induced isozyme or an alteration in enzyme structure. The question remains, however, as to what kind of modification at the molecular level would give rise to the elevated isozyme levels. Two possibilities we are examining are gene amplification and derepression.

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