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Recessive Constitutive Mutant Chinese Hamster Ovary Cells (CHO-K1) with an Altered A System for Amino Acid Transport and the Mechanism of Gene Regulation of the A System

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Chinese hamster ovary cells (CHO-K1) starved for 24 h for amino acids show a severalfold increase in velocity of proline transport through the A system (V_{max} is five times that of unstarved cells). This increase is inhibited by cycloheximide, actinomycin D, *N*-methyl-α-amino isobutyric acid (MeAIB, a non-metabolizable specific A system amino acid analog), and by other amino acids that are generally transported by the A system. However, transport by the A system is not a prerequisite for this repression, and all compounds that have affinity for the A system do not necessarily act as "co-repressors." The addition of proline, MeAIB, or other amino acids, as described above, to derepressed cells results in a rapid decrease in A system activity. As shown with proline and MeAIB, this decrease in activity is in part due to a rapid *trans*-inhibition and a slow, irreversible inactivation of the A system. Neither process is inhibited by cycloheximide or actinomycin D. Alanine antagonizes the growth of CHO-K1 pro$^-$ cells by preventing proline transport, and alanine-resistant mutants (ala^r) have been isolated (Moffett et al., Somatic Cell Genet. **9**:189–213, 1983). ala^{r}2 and ala^{r}4 are partial and full constitutive mutants for the A system and have two and six times the V_{max} for proline uptake by the A system, respectively. The A system in ala^{r}4 is also immune to the co-repressor-induced inactivation. Both ala^{r}2 and ala^{r}4 phenotypes are recessive. Ala^{r}3 shows an increase in V_{max} and K_m for proline transport through the A system, and this phenotype is codominant. All three mutants have a pleiotropic effect, producing increases in activity of the ASC and P systems of amino acid transport. This increase is not due to an increase in the Na^+ gradient. The ASC and P phenotypes behave similarly to the A system in hybrids. A model has been proposed incorporating these results.

The A system of amino acid transport is Na^+ dependent and transports mainly the small, polar, straight-chain amino acids such as alanine, the non-metabolizable analogs 2-aminoisobutyric acid and *N*-methyl-α-amino isobutyric acid (MeAIB), and also proline. This system is ubiquitous among the vertebrates (6, 14, 22, 26, 28; see references in 2, 3, and 12). Of the three Na^+-dependent transport systems (A, ASC, and P) present in Chinese hamster ovary cells, CHO-K1, the cell line with which we will be concerned, the A system, appears to be the only one whose activity is repressible (20).

When cells are placed under conditions in which they are starved for amino acids, a severalfold increase in velocity (V_{max}) of transport of amino acids through the A system occurs. This increase has been shown to be inhibited by cycloheximide and actinomycin D (9–13, 15). In general, amino acids that are transported through the A system have been shown to be the most effective in the repression of this system (12). However, this specificity has been disputed (13). When derepressed cells are given an A system amino acid, a rapid decline in A system activity occurs (9–13). With some cell lines, a portion of this decline in activity is sensitive to cycloheximide and actinomycin D (10).

This laboratory has been employing a mutational approach to elucidate the mechanism of gene control of the A system in CHO-K1 pro$^-$ cells. It has been shown previously that incubation of CHO-K1 pro$^-$ cells under starvation conditions results in a severalfold increase in the velocity of proline transport through the A system (20). Utilizing the antagonism between proline and other A system amino acids on the growth of CHO-K1 pro$^-$ cells (4), a selection system has been developed for the isolation of mutants that have elevated levels of amino acid transport (20). Two alanine-resistant mutants, ala^{r}2 and ala^{r}3, have been characterized that have elevated rates of proline transport through the A system. Both mutants also show a disproportionate increase in amino acid transport through the ASC and P systems. Mutants resistant to MeAIB and having increased velocity of transport of proline through the ASC system have also been isolated (7).

In this study, we describe the requirements for the repression and derepression of the A system in CHO-K1 and we examine the effect of derepression of the A system in mutants ala^{r}2 and ala^{r}3. In addition, we characterize a new alanine-resistant mutant, ala^{r}4, that was initially found to have a sixfold increase in the velocity of proline transport. Cell-cell hybridization studies have also been conducted with all three mutants to determine the dominance or recessiveness of the respective phenotypes. Our results have led us to propose as a guide for future experiments a model for the regulation of the A system based upon negative control of transcription and a post-translational modification of an A system carrier protein. The model also offers an explanation of the pleiotropic effect of these mutations on the ASC and P systems of amino acid transport.

MATERIALS AND METHODS

Cell cultures. The Chinese hamster ovary cell line CHO-K1 pro$^-$ was obtained from the American Type Culture Collection (CCL 61). This cell line and alanine-resistant mutants ala^{r}2 and ala^{r}3 have been previously characterized (20). Ala^{r}4 was isolated by the same procedure as described

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for ala^{r}2 and ala^{r}3. CHO-K1 pro$^-$ oua^r HPRT$^-$ was isolated from CHO-K1 pro$^-$ in a stepwise fashion by the usual procedure.

Media and cultural conditions. All cell lines were routinely grown as monolayers in MEMCHO-4 as previously described (20). MEMCHO-4 hypoxanthine-aminopterin-thymidine medium containing 1 mM ouabain was employed in hybrid selection. Quantitative growth experiments were as previously described (4).

Transport studies. We performed transport experiments using cells grown in a monolayer at the bottom of scintillation vials in MEMCHO-4 as previously described (20).

For extended starvation periods involved in derepression studies, cells were incubated in derepression buffer (137 mM NaCl, 2.68 mM KCl, 6.49 mM K_2HPO_4, 0.1% glucose; 1mM $MgCl_2$, brought to pH 7.3 with KH_2PO_4 and containing 4% dialyzed fetal calf serum). These derepression experiments have also been conducted in the absence of serum with cells attached to plastic with similar results. Other modifications of conditions employed in a study of the regulation of the A system will be described below.

To compare the velocity of proline transport in cell-cell hybrids with that of the parental cultures, we determined the water volume by measuring the uptake of [3-*O*-*methyl*-^{3}H]glucose (16). Protein was determined by the method of Lowry et al. (18) as modified by Oyama and Eagle (23). Results obtained with CHO-K1 were checked by measuring the uptake of [^{14}C]urea (8). The values obtained by these two methods were not significantly different. By these methods, we found that the water volume of CHO-K1 grown under standard conditions was 1.17×10^{-12} liter per cell or 3.1 μl per mg of protein. This water volume did not differ significantly from that reported previously (2). We found that the water volume in the hybrids varied from 2.76 to 3.84 μl per mg of protein, with a mean value of 3.45. We used the latter value in calculating the velocity of proline transported through the A system in the hybrids.

Cell-cell hybridization. Crosses were made between each of the ala^r mutants and CHO-K1 pro$^-$ oua^r HPRT$^-$, using the polyethylene glycol procedure (5). After polyethylene glycol treatment, the cells were removed from the dishes by trypsinization, and 1/20 of the cells (10^5) were inoculated per 60-mm dish containing MEMCHO-4 HAT ouabain medium The medium was changed every 3 days, and five hybrid clones were picked from each cross. As a control in this experiment, hybrids of CHO-K1 × CHO-K1 oua^r HPRT$^-$ were also isolated. Three hybrids from each cross were characterized for water volume, growth inhibition by 12 mM alanine, transport of proline through the A system under repressed and derepressed conditions, and chromosome number.

Amino acid pools. Cells labeled with trace amounts of proline were extracted with hot 80% ethanol at time zero and at various periods during the starvation period. The ethanol extract was evaporated to dryness, the residue was reconstituted, and an aliquot of this solution was plated on cellulose thin-layer sheet (Eastman Kodak) and chromatographed as previously described (2). The area of the chromatogram containing proline, as determined with authentic proline, was scraped off and counted in a scintillation counter.

Determination of Na^+ and K^+. The Na^+ and K^+ contents of the cells were determined by a modification of a procedure previously described (19). Cells were inoculated into 100-mm dishes containing 10 ml of MEMCHO-4 at a density of 10^6 cells per dish. After 24 h, the cells were washed rapidly six times with 3-ml volumes of ice-cold 0.1 M $MgCl_2$. The cells were allowed to dry, covered with 4 ml of distilled water, and frozen and thawed twice. The supernatant was tested for Na^+ and K^+ with an Eppendorf Flame Photometer. All experiments were performed in triplicate. The Na^+ and K^+ concentrations were estimated based upon standard Na^+ and K^+ solution.

RESULTS

Characterization of the amino acid deprivation-derepression of the A system in CHO-K1. CHO-K1 was grown in scintillation vials and incubated in derepression buffer, and proline transport through the A system was determined every 4 h over a 24-h period. The velocity of proline transport through the A system increased steadily and leveled off in 20 h, leading to an increase in the initial velocity of 6.5 times that found with repressed cells (Fig. 1). In a 6-h derepression experiment (Table 1), we found that cycloheximide completely prevented the derepression and actinomycin D inhibited derepression by 55%. The presence of proline during this 6-h period as well as MeAIB, a nonmetabolizable amino acid analog, and alanine severely inhibited the derepressive effect, and the values observed were slightly less than the basal A system level (see below). On the other hand, the amino acids phenylalanine and lysine and an amino acid analog B-(±)-2-amino-bicyclo(2,2,1)-heptane-2-carboxylic acid that are transported mainly by systems other than the A system are weakly inhibitory, if at all. For the sake of simplicity in referral, we shall call those amino acids and amino acid analogs that inhibit derepression of the A system and repress the A system "co-repressors" and shall justify this nomenclature later.

We measured the effects of proline, cycloheximide, and actinomycin D on the derepressed levels of proline transport through the A system after a 12-h derepressive starvation regime (Fig. 2). Cycloheximide added at 12 h not only completely inhibited any further increase in A system activity but also maintained the 12-h derepressed level of activity for another 12 h, demonstrating the great stability of this derepressed transport system under these conditions. Actinomycin D added at 12 h showed a 56% inhibition of further derepression in 6 h. The addition of 2 mM proline at 12 h caused a rapid decrease in the activity of the A system which

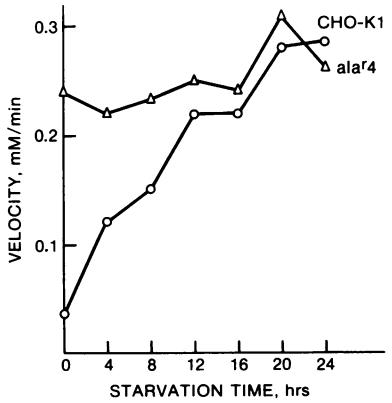

FIG. 1. Effect of starvation on the activity of proline transport through the A system in CHO-K1 and ala^{r}4. Cells were starved in derepression buffer. Uptake of proline at 0.05 mM through the A system was determined at intervals in triplicate (velocity in the absence of MeAIB minus velocity in the presence of 10 mM MeAIB [20]), and the average velocities in millimoles per liter per minute (mM/min) were calculated. Symbols: ○, CHO-K1; △, ala^{r}4.

TABLE 1. Effect of cycloheximide, actinomycin D, and amino acids on the derepression of the A system[a]

Conditions	Velocity (mmol/liter per min)	% Inhibition of derepression[c]	Velocity (mmol/liter per min) after 3 h starvation + cycloheximide[d]	% Inhibition of derepression[e]
Not starved	0.041 ± 0.007			
Starved				
No additions	0.10 ± 0.01	0	0.110 ± 0.004	0
Cycloheximide, 17.5 μg/ml	0.033 ± 0.002	>100		
Actinomycin D, 4 μg/ml	0.068 ± 0.003	55		
MeAIB	0.021 ± 0.001	>100	0.03 ± 0.01	100
Proline	0.028 ± 0.003	>100	0.04 ± 0.01	100
Alanine	0.035 ± 0.005	100	0.05 ± 0.01	100
Phenylalanine	0.105 ± 0.01	0	0.11 ± 0.01	0
BCH[b]	0.084 ± 0.002	38		
Lysine	0.10 ± 0.01	0	0.10 ± 0.01	0

[a] CHO-K1 cells were starved for 6 h in derepression buffer in the presence or absence of test compounds. Amino acids and analogs were added at a final concentration of 5 mM. Proline transport through the A system was then determined (see legend to Fig. 1 and the text).

[b] BCH, B-(±)-2-amino-bicyclo(2, 2, 1)-heptane-2-carboxylic acid.

[c] Percent inhibition = [1 − (velocity in 6 h with added test compound minus 0.041)/(0.10 minus 0.041)] × 100.

[d] Cells were starved for an additional 3 h in the presence of 17.5 μg of cycloheximide per ml to deplete the intracellular amino acid pool, and transport through the A system was then measured.

[e] As in footnote *c*, but values employed for amino acid were those determined after the additional 3-h starvation.

in 6 h reached the repressed basal level. Cycloheximide and actinomycin D did not prevent this loss of activity but did inhibit the derepression when added at time zero as previously shown (Table 1). MeAIB, alanine, and serine had effects similar to that of proline on A system derepressed activity (data not shown).

Is *trans*-inhibition responsible for the derepression-repression phenomenon? Although the A system may be *trans*-inhibited by high concentrations of A system amino acid in some cell lines (11), evidence from a number of sources has made it clear that this phenomenon accounts for only a portion of the change in initial velocity observed during derepression and repression (12, 13). Our results with CHO-K1 support these findings.

For instance, when we labeled the internal amino acid pool with trace amounts of proline, serine, and alanine, we found no correlation during the starvation period between the rate of increase in activity of the A system and the rate of exit of these amino acids. In addition, starvation in the presence of cycloheximide for 3 h produced no increase, or at most a 5% increase, in A system activity as compared with cells starved in the absence of this inhibitor. Since these amino acids are transported through the A system and therefore might be implicated in the *trans*-inhibition of this system, and since their intracellular concentration probably reflects, in general, the state of the other amino acids as the result of the starvation procedure (13, 21), it appears that if *trans*-inhibition of proline transport through the A system occurs at all under these conditions, it can account for only a very minor portion of the increase observed, possibly reflecting the activity expressed as a result of starvation in the presence of cycloheximide.

In a previous experiment (Table 1), we had shown that the addition of 5 mM MeAIB or proline not only completely prevented the amino acid starvation-induced increase in A system activity but also reduced the basal level of activity of this transport system by 40 to 50%. In that experiment, to determine to what extent these compounds acted as co-repressors or as *trans*-inhibitors of the A system, we subjected these cells to a further 3-h starvation period in the presence of cycloheximide to deplete the cells of the internally concentrated amino acids and at the same time to prevent further derepression. We found that the activity of the A system cells treated with MeAIB or proline and subsequently starved for 3 h in the presence of cyclohexi-

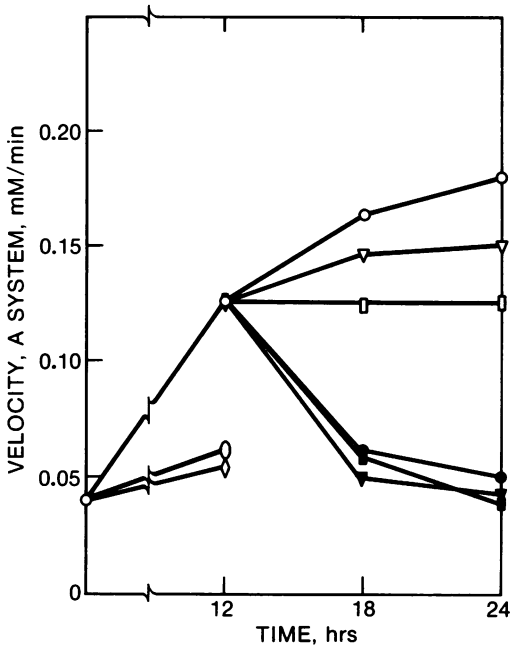

FIG. 2. Effects of proline, cycloheximide, and actinomycin D on the A transport system of partially derepressed CHO-K1 cells. Cells were initially starved in derepression buffer for 12 h, or in buffer with the addition at time zero of actinomycin D (○) or cycloheximide (◇). At 12 h, the partially derepressed cells were incubated for another 12 h in the same buffer (○), or in the same buffer with the addition of actinomycin D (▽), cycloheximide (□), proline (●), a mixture of proline and actinomycin D (▼), or proline plus cycloheximide (■). At the times indicated, transport of proline through the A system was determined in triplicate (see the legend to Fig. 1). Points represent the average of triplicate determination. Final concentrations employed: actinomycin D, 4 μg/ml; cycloheximide, 17.5 μg/ml; proline, 2 mM.

mide increased to the extent of bringing this activity only closer to the basal level and no further. Thus, the inhibitory effect of these added amino acids in preventing the starvation-induced increase in A system activity cannot be due to *trans*-inhibition.

We found a similar effect of *trans*-inhibition when A system amino acids were added to derepressed cells. In this experiment, cells were derepressed for 15 h as previously described. We measured the uptake of labeled 2 mM proline over a 6-h period with one set of vials and determined the initial velocity of proline transport with other cells incubated with unlabeled 2 mM proline. Cells were also incubated for an additional 6 h in derepression buffer. Derepressed cells given 2 mM proline showed a steep 40% drop in activity in the first 30 min and a steady, slow decline thereafter (Fig. 3). The steep drop in activity coincided with the rapid uptake of proline equal to 37 mmol/liter. A subsequent slow decline in activity occurred while the internal concentrations of proline remained fairly constant. These results suggest that this rapid decrease in activity is due to *trans*-inhibition. The subsequent slow decline in activity we attribute to a proline-induced inactivation of the A system carrier. That the rapid decrease in activity exhibited in 30 min is indeed mainly attributable to *trans*-inhibition is indicated by our finding that at least 70% of this decrease is reversible as a result of a subsequent starvation of these cells for 3 h in the presence of cycloheximide (from 0.075 ± 0.003 to 0.105 ± 0.006 mmol/liter per min; initial derepressed level, 0.12 ± 0.01 mM/min). In this same experiment, cells that were incubated for 6 h in the presence of proline and also starved in the manner described above recovered essentially the same amount of activity as we found for the cells treated for 30 min (from 0.046 ± 0.003 to 0.08 ± 0.01 mmol/liter per min). The similarity in recovered activity is exactly what one would expect if a large fraction of the initial rapid decrease in activity in 30 min were due to *trans*-inhibition and if the slow subsequent decline in activity were irreversible. Cycloheximide had no effect on the proline-induced decrease in A system activity, confirming our previous results.

In an experiment in which we substituted the non-metabolizable analog MeAIB as a co-repressor in place of proline, we obtained results similar to that presented above (Fig. 3), except that MeAIB appeared to be a more effective co-repressor since the irreversible A system activity remaining after 6 h of incubation in the presence of MeAIB was nine times less than the original basal A system activity. This sharp distinction between *trans*-inhibition and the secondary decline is strikingly shown in the analysis of an alanine-resistant mutant, ala^{r}4, as described below.

Evidence for mRNA accumulation and translation during derepression of the A system. As we have previously shown, the amino acid starvation-induced increase in A system activity is inhibited by actinomycin and cycloheximide. This suggested to us that active transcription of an mRNA and its translation is required for this phenomenon. To test this hypothesis further, we incubated cells in derepression buffer in the presence of cycloheximide for various periods of time to allow for the accumulation of the postulated mRNA for an A system carrier protein under conditions that prevented the translation of the accumulated mRNA. Cycloheximide was then removed, actinomycin D was added, and incubation was continued for an additional period of time to allow for mRNA translation and the eventual increase in A system activity. The latter was monitored by measuring the initial velocity of proline transport through the A system. Incubation in derepression buffer for 2, 4, and 6 h under the conditions described above (Fig. 4) resulted in a progressive increase in rate of transport through the A system.

The effect of mutation to ala^r on the repression-derepression phenomenon. Mutants have been isolated that are resistant to the alanine inhibition of proline transport in CHO-K1 pro$^-$ cells (20). Michaelis-Menten kinetic experiments have shown that ala^{r}2 has approximately a twofold increase in V_{max}, whereas ala^{r}3 has an increase in both K_m and V_{max} over that of the derepressed parental culture CHO-K1 (Table 2). We have recently characterized another alanine-resistant mutant, ala^{r}4. This mutant is shown to have a severalfold increase in proline transport through the A system at a proline concentration of 0.05 mM (Fig. 1 and 5), and Michaelis-Menten kinetics shows a V_{max} five times that of repressed CHO-K1, with no significant change in K_m. These mutants and CHO-K1 were compared for proline transport through the A system after incubation in derepression buffer for 24 h. The results (Fig. 5) show that although ala^{r}2 had a twofold increase in activity over that of the parental culture under repressed conditions, both mutant and CHO-K1 reached the same derepressed level at 24 h. On the other hand, ala^{r}3, which also had twice the A system activity under repressed conditions, upon derepression reached a level of A system activity twice that of CHO-K1. Michaelis-Menten kinetics of CHO-K1 derepressed for 24 h showed a V_{max} 5.3 times that of the repressed culture with no signifi-

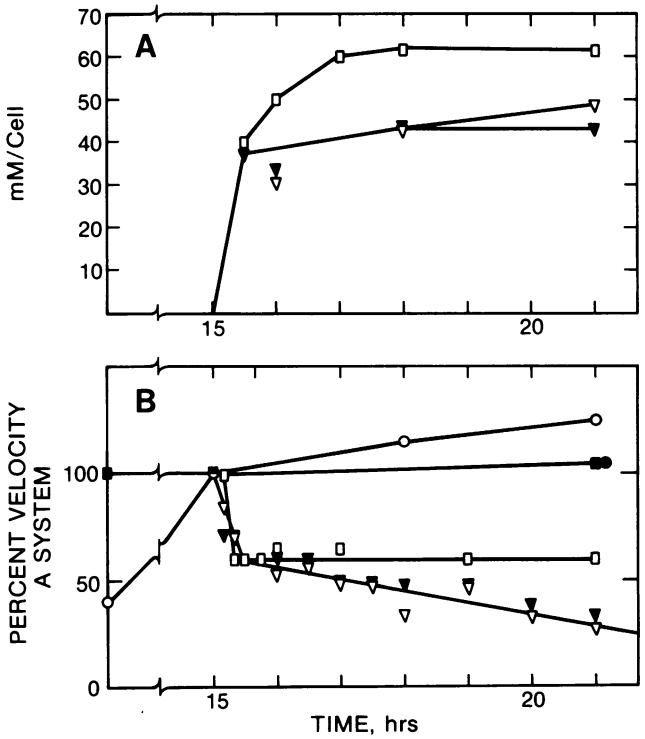

FIG. 3. Proline-induced inactivation of the A system in derepressed CHO-K1 and ala^{r}4. Cells were derepressed for 15 h as previously described. (A) One set of vials was given 2 mM [^{3}H]proline, and uptake was measured at various time intervals as indicated. Symbols: ▽, CHO-K1; ▼, CHO-K1 plus 17.5 μg of cycloheximide per ml; □, ala^{r}4. (B) A system activity was determined in another set of vials with cells incubated with the following conditions. CHO-K1: ○, no additions; ●, plus 17.5 μg of cycloheximide per ml; ▽, 2 mM proline; ▼, 2 mM proline plus 17.5 μg of cycloheximide per ml. ala^{r}4: ■, no addition; □, plus 2 mM proline.

cant change in K_m. The V_{max} of derepressed CHO-K1 did not differ significantly from that of ala^{r}4 grown under "repressible" conditions.

We were interested in determining whether the alanine-resistant mutants were changed in their sensitivity to the proline-induced *trans*-inhibition and inactivation of the A system carrier. As described with CHO-K1 (Fig. 3), ala^{r}4 was derepressed for 15 h, whereupon 2 mM proline was added and the activity of the A system was monitored. At 30 min, when ala^{r}4 had taken up ca. 40 mM proline (Fig. 3A), there was a sharp 40% drop in A system activity equal to that found with CHO-K1 (Fig. 3B). At this point, the curve for ala^{r}4 broke and activity remained unchanged for an additional 6 h. This is in contrast to what we found with CHO-K1, in which a steady, slow decline in activity continued for the length of the experiment. These results indicate that the A system of ala^{r}4 is still sensitive to *trans*-inhibition but is completely resistant to the proline-induced inactivation. These results demonstrate the sharp distinction between the two phenomena. In analogous experiments, we have found that the A system of ala^{r}2 and ala^{r}3 is still sensitive to both *trans*-inhibition and inactivation (data not shown).

Cell × cell hybridization experiments. Each of the ala^r mutants was crossed with CHO-K1 pro$^-$ oua^r HPRT$^-$. As a control, hybrids were also constructed from a cross between CHO-K1 pro$^-$ and CHO-K1 pro$^-$ oua^r HPRT$^-$. Selection was in MEMCHO-4 hypoxanthine-aminopterin-thymidine ouabain medium. Tests for resistance to 12 mM alanine and transport of 0.05 mM proline through the A system (Table 3) demonstrate conclusively that the partial constitutive phenotype and the full constitutive phenotype of ala^{r}2 and ala^{r}4, respectively, are recessive to the repressive wild-type allele. The phenotype of ala^{r}3, on the other hand, appears to be codominant to the wild-type phenotype with respect to both parameters that were measured.

The hybrids had, on the average, 37 chromosomes, with an expected number of 40. It seems unlikely that the phenotype of the hybrids was due to the absence of a particular chromosome since three independently derived hybrids were selected for each mutant and the chances that the same chromosome was lost in each case is remote, assuming, of course, that chromosome loss in this case is random. We have no reason to doubt this assumption. To test this conclusion further we selected segregants from hybrids of ala^{r}2 and ala^{r}4. Ala^{r}2 × CHO (H2) and ala^{r}4 × CHO (H2) were plated out on selective medium MEM-4 plus 0.05 mM proline, 12 mM alanine, and the remaining nonessential amino acids at 0.1 mM. Ala^r segregants appeared at a frequency of 2.8×10^{-3} and 1.5×10^{-3} from ala^{r}2 × CHO (H2) and ala^{r}4 × CHO (H2), respectively. Since the spontaneous frequency to ala^r of CHO-K1 was 10^{-5}, it is unlikely that these variants were the result of mutation. Four segregants of each hybrid were characterized and found to have fewer chromosomes than the original hybrid and to have a

TABLE 2. Michaelis-Menten parameters for proline transport through the A system[a]

Mutant		V_{max} (mmol/liter per min)	K_m (mM)
ala^{r}2		1.42 (1.87–1.08)	0.73 (1.01–0.53)
ala^{r}3		1.72 (1.63–1.82)	1.14 (1.25–1.05)
ala^{r}4	(1)	3.69 (4.64–2.94)	0.77 (1.00–0.59)
	(2)	3.93 (4.31–3.62)	0.85 (0.91–0.65)
CHO-K1			
Repressed		0.75 (0.87–0.65)	0.80 (0.95–0.67)
Derepressed	(1)	3.67 (4.14–3.26)	0.75 (0.87–0.65)
	(2)	4.31 (4.8–3.86)	1.01 (1.16–0.77)

[a] Proline transport through the A system was measured in 1-min uptakes, in triplicate, in the presence and absence of 10 mM MeAIB as described in the text. The observed values were averaged and converted to millimoles of proline taken up per liter per minute. Velocities in the presence of MeAIB were subtracted from velocities obtained in the absence of MeAIB. The reciprocals of these values were plotted against the reciprocals of the proline concentrations. The data were analyzed by a linear weighted least-squares fitting and a jackknife error analysis computer program (20). Numbers in parentheses are the upper and lower confidence limits. The data for ala^{r}2 and ala^{r}3 and for derepressed CHO-K1 have been published previously (20) and are presented for comparison purposes. CHO-K1 was derepressed for 24 h as described in the text.

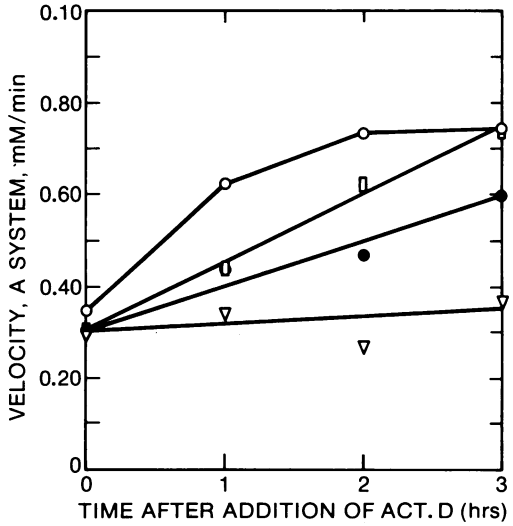

FIG. 4. CHO-K1 was grown in scintillation vials to 2×10^5 cells per vial. Cells were washed and incubated in derepression buffer plus 17.5 μg of cycloheximide per ml (final concentration). At time zero (▽), 2 (●), 4 (□), and 6 (○) h, vials were removed from the incubator, the cells were washed, and derepression buffer plus 4 μg of actinomycin D per ml (final concentration) was added. At time zero, and at 1, 2, and 3 h in the presence of actinomycin D, the cells were processed for A system activity as described in the legend to Fig. 1. Points represent averages of triplicate assays.

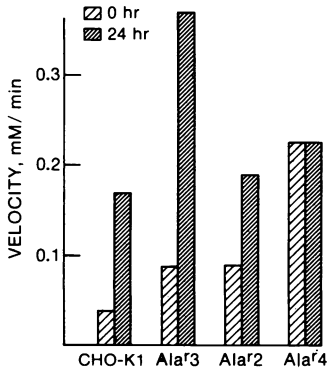

FIG. 5. Comparision of the repressed and derepressed levels of proline transport through the A system in CHO-K1 and in ala^r mutants. Cells were derepressed for 24 h as described in the legend to Fig. 1 and assayed before (▨) and after (■) derepression for the transport of 0.05 mM proline through the A system as described in the legend to Fig. 1.

TABLE 3. Cell-cell hybridization

Cells[a]	A system activity[b] (nmol/min per mg of protein)		% Growth[c]	Chromosome[d]
	Repressed	Derepressed		
CHO-K1 pro$^-$	0.14 ± 0.03	0.53 ± 0.05	3	20 ± 1
CHO-K1 pro$^-$ oua^r HPRT$^-$	0.20 ± 0.05	0.59 ± 0.03	30	20 ± 1
ala^{r}2	0.30 ± 0.03	0.56 ± 0.03	90	20 ± 1
ala^{r}3	0.28 ± 0.03	1.02 ± 0.03	85	20 ± 1
ala^{r}4	0.67 ± 0.03	0.68 ± 0.06	100	20 ± 1
CHO × CHO (H1)	0.21 ± 0.01	0.57 ± 0.02	4	38 (33–40)
CHO × CHO (H2)	0.24 ± 0.02	0.90 ± 0.04	4	37 (33–39)
CHO × CHO (H3)	0.22 ± 0.01	0.72 ± 0.01	3	37 (34–40)
ala^{r}2 × CHO (H1)	0.16 ± 0.03	0.73 ± 0.04	25	37 (33–40)
ala^{r}2 × CHO (H2)	0.18 ± 0.01	0.73 ± 0.1	3	40 (33–41)
ala^{r}2 × CHO (H3)	0.16 ± 0.01	0.79 ± 0.3	3	37 (33–39)
ala^{r}3 × CHO (H1)	0.34 ± 0.02	1.07 ± 0.09	100	36 (33–39)
ala^{r}3 × CHO (H2)	0.30 ± 0.02	0.79 ± 0.03	100	36 (34–40)
ala^{r}3 × CHO (H3)	0.28 ± 0.03	0.86 ± 0.03	100	37 (33–39)
ala^{r}4 × CHO (H1)	0.19 ± 0.03	0.59 ± 0.06	7	38 (33–40)
ala^{r}4 × CHO (H2)	0.20 ± 0.03	0.69 ± 0.05	6	37 (33–40)
ala^{r}4 × CHO (H3)	0.24 ± 0.03	0.83 ± 0.04	7	37 (33–39)

[a] CHO-K1 and each mutant were crossed to CHO-K1 pro$^-$ oua^r HPRT$^-$, and selection was on MEMCHO-4 HAT ouabain medium. Each hybrid represents the progeny from an independent hybridization event.

[b] A system activity was determined by measuring the initial velocity of transport of 0.05 mM proline through the A system in triplicate as described in the legend to Fig. 1 and in the text. The activity given is based upon the following relationship: CHO-K1, 3.1 μl of cellular water per mg of cell protein; hybrids, 3.45 μl per mg of protein. Values are given with the standard deviation.

[c] The percent inhibition of growth was determined in a 6-day growth experiment in which the growth of each cell culture was compared in a modified MEMCHO-4 containing just 0.05 mM proline and 12 mM alanine and in MEMCHO-4. Growth in MEMCHO-4 was set as 100%.

[d] Values given were determined from at least 25 chromosome spreads and represent the modal number followed by the lowest and highest count.

transport and alanine resistance phenotype similar to that of the original ala^r parent (data not shown).

It has been shown previously that ala^{r}2 and ala^{r}3 have increases in velocity of proline transport through the ASC, P, and A systems. We found that ala^{r}4, besides having a fivefold increase in the initial velocity of proline transport through the A system, also showed an approximately five- to sixfold increase in the ASC and P systems, with no increase in the glutamine inhibitory fraction (20) or L systems. We measured the ASC and P systems in hybrids CHO × CHO (H3), ala^{r}4 × CHO (H2), ala^{r}3 × CHO (H3), and ala^{r}2 × CHO (H2), and found that these phenotypes behaved similarly to the A system in the respective hybrids. That is, they were recessive in ala^{r}4 and ala^{r}2 and codominant in ala^{r}3 (data not shown).

Characteristics of the co-repressor. We have shown above that amino acids or amino acid analogs that are transported by the A system are effective in inhibiting the amino acid starvation-induced increase in A system activity, whereas amino acids or amino acid analogs that are mainly transported by other systems are not. To determine whether affinity for the A system carrier is critical for repression, we ran a preliminary test of the effect of a large number of amino acids and amino acid analogs on this starvation-induced derepression as well as their effect on proline transport. A detailed report of this work will appear in a separate publication. We report here the results of experiments obtained with β-alanine, diaminobutyric acid (DAB), alanine, and proline. In this experiment, cells were starved for 24 h in derepression buffer in the absence and in the presence of 5 mM β-alanine, DAB, alanine, or proline. Cells were washed free of the compounds, and the cells were placed in derepression buffer in the presence of cycloheximide for 3 h, as previously described, to dilute out the concentrated test compound so as to exclude the possibility of *trans*-inhibition. The cells were then tested for proline transport through the A system. The effects of the test compounds at 5 mM were also assayed for direct inhibition of transport of 0.05 mM proline through the A system. The results (Table 4) demonstrated that β-alanine was a co-repressor that strongly inhibited the increase in A system activity as experienced by cells incubated in derepression buffer but had no inhibitory effect on proline transport through the A system. DAB, on the other hand, did inhibit transport of proline through the A system and was a very poor co-repressor. Both alanine and proline were effective co-repressors; alanine, and of course proline, were effective inhibitors of proline transport. Of further significance is the finding that DAB was able to antagonize the effect of alanine as a co-repressor (Table 5) while not inhibiting alanine uptake. DAB at 5 mM had no effect on the uptake of 1 mM alanine over a 3-h period (data not shown). There was a 4.5-fold increase in proline transport through the A system as a result of the starvation regime (Table 5). The addition of 5 mM DAB had no effect on this stimulation, whereas 1 or 2 mM alanine completely prevented this increase. However, the addition of 5 mM DAB completely neutralized the repressive effect of 1 mM alanine. Results of varying the concentration of alanine and DAB suggest that the interaction between these two compounds is probably competitive. The conclusions drawn from these experiments are obvious. The A system carrier is not directly involved in the repression of the A system. A compound that has no affinity for the A carrier as determined by inhibition analysis is an effective co-repressor of the A system (β-alanine). A compound that binds to the A carrier is not a co-repressor (e.g., DAB).

Pleiotropic effect of mutation to ala^r. As we pointed out above, all three ala^r mutants, besides having increases in initial velocity of transport through the A system, also showed increases in transport velocity through the ASC and P systems. We considered the possibility that the mutation had a primary effect leading to an increase in the Na^+

gradient, which in turn might have brought about the increased velocity of transport through the Na^+-dependent transport systems. Although this possibility did not seem likely since there was no effect on the Na^+-dependent glutamine inhibitory fraction (20), we measured the intracellular Na^+ and K^+ levels in CHO-K1 and in the three mutants with cells grown under similar conditions as employed for the measurement of amino acid transport. Instead of a decrease in intracellular Na^+, as one might expect to explain the increase in velocity of proline transport, we found that the Na^+ content of the mutants was significantly higher than that in CHO-K1 (Table 6). Ala^r4, which had the largest increase in velocity of proline transport, had the highest Na^+ content. These results are best explained on the basis that the increase in Na^+ content of the cells is the result of increases in Na^+-dependent transport activity.

DISCUSSION

The A system of amino acid transport in CHO-K1 provides us with a unique opportunity for the study of the mechanism of a substrate-mediated regulation of gene expression in higher eucaryotes, using a mutational approach. When CHO-K1 cells are incubated for a 15- to 24-h period in the absence of amino acids that are mainly transported through the A system, there occurs a large increase in the initial velocity of transport of amino acids through this system. Our studies have led us to conclude that this increase in A system activity is mainly the result of derepression of the A system involving the synthesis of specific mRNA and additional A system carrier protein. These conclusions are based upon the following observations. (i) The increase in activity, as measured by the increase in velocity of proline transport through the A system, is a result of an increase in V_{max} without any accompanying change in K_m; (ii) this derepression is inhibited by cycloheximide and actinomycin D; (iii) derepression in the presence of cycloheximide for various periods of time leads to increases in velocity of proline transport through the A system, as measured when cycloheximide is removed and substituted with actinomycin D, presumably as a result of specific mRNA synthesis and accumulation. This later experiment also indicates that there is no further mRNA synthesis required for the translation of the accumulated message.

The activity of the A system is also inhibited by an apparent feedback mechanism which has been termed *trans*-inhibition (12). We have shown that *trans*-inhibition plays a minor role in the increase in activity produced upon derepression, in the A system amino acid inhibition of the derepression phenomenon, and in the A system amino acid-produced decrease in A system activity in derepressed cells. In the last case, when proline or certain other A system amino acids (see below for more details) were added to derepressed cells, further increases in A system activity ceased and we found a rapid decline in the rate of A system activity. With proline, which was studied in more detail, the decline in activity showed two distinct slopes. There was an initial reversible, rapid decrease in A system activity which coincided with the maximum uptake of proline, strongly suggesting that this decrease was due to *trans*-inhibition. The second, much slower decline that culminated with activity close to the basal level occurred after the proline pool had been saturated and appeared to be irreversible. We propose that this slow decline in activity was due to an amino acid-induced inactivation of the A carrier protein. MeAIB, a non-metabolizable analog, although apparently a more potent co-repressor, gave results similar to those with proline, suggesting that metabolism may not be necessary for some, if not all, co-repressors to exert their effect. A study of the effect of addition of 2 mM proline to ala^r4, a mutant fully derepressed for the A system, further distinguishes these two phenomena. There is a rapid decline in the A system activity by ala^r4 similar to that shown with CHO-K1. However, there is no further decrease in activity, thus demonstrating the ala^r4 insensitivity to the proline (co-repressor)-induced inactivation of the A system carrier; this clearly serves to separate the two phenomena.

Among the natural amino acids, those amino acids that are mainly transported by the A system appear to be the most effective in preventing the derepression of this system. An analysis of the specificity of this activity has shown conclusively that these compounds that are effective in preventing the derepression need not have any affinity for the A system

TABLE 4. Lack of correspondence between co-repressors and inhibitors of A system activity

Conditions	A system activity (mmol/liter per min)	% Inhibition of derepression[a]	% Inhibition of A system activity[b]
Not starved[c]	0.038 ± 0.007		
Starved[d]			
No additions	0.19 ± 0.02	100	
Proline	0.04 ± 0.01	99	100
Alanine	0.039 ± 0.004	100	100
β-Alanine	0.061 ± 0.003	85	0
DAB	0.18 ± 0.01	7	80

[a] Percent inhibition of derepression = [1 − (velocity of starved cells − 0.038)/(0.19 − 0.038)] × 100.

[b] Each of the test compounds was tested at 5 mM for its effect on A system activity as described in footnote *c*.

[c] Cells were grown in scintillation vials to 2×10^5 cells per vial, and transport of 0.05 mM proline through the A system was determined in triplicate at 1-min intervals as indicated in the legend to Fig. 1.

[d] Cells were starved for a 24-h period in derepression buffer in scintillation vials in the absence and in the presence of 5 mM concentrations of each of the test compounds. Cells were washed free of the test compounds and starved for an additional 3 h in derepression buffer in the presence of 17.5 μg of cycloheximide to dilute out the intracellular accumulated test compound under conditions that would prevent further derepression or inactivation of the A system carrier; they were then assayed for A system activity as indicated in footnote *c*.

TABLE 5. DAB as an anti-corepressor

Conditions	A system velocity (mmol/liter per min)
Not starved	0.05 ± 0.01
Starved (24 h)[a]	
No additions	0.18 ± 0.01
1 mM ala	0.06 ± 0.01
2 mM ala	0.05 ± 0.01
5 mM DAB	0.22 ± 0.05
1 mM ala + 5 mM DAB	0.18 ± 0.01
1 mM ala + 2 mM DAB	0.09 ± 0.02
2 mM ala + 5 mM DAB	0.089 ± 0.004

[a] Cells were incubated for 24 h in derepression buffer in scintillation vials in the presence and absence of the ingredients listed. Transport of 0.05 mM proline through the A system was determined at time zero and after the 24-h period in triplicate (see the legend to Fig. 1). Results are given as the average velocity with the standard deviation.

TABLE 6. Na^+ and K^+ contents of CHO-K1 and three ala^r mutants[a]

Cells	Na^+ concn (mM per cell)[b]	K^+ concn (mM per cell)[b]	Proline transport (mmol/liter per min)[c]
CHO-K1	58 ± 11	189 ± 14	0.061 ± 0.003
ala^r2	126 ± 12	124 ± 11	0.11 ± 0.003
ala^r3	100 ± 11	140 ± 23	0.10 ± 0.003
ala^r4	156 ± 11	157 ± 14	0.33 ± 0.004

[a] For details, see the text.
[b] Average of triplicate determinations with standard deviation.
[c] Total proline transport was measured in 1-min uptakes, in triplicate. Values are averages with standard deviation.

carrier, as presumed by other investigators (10). We have shown that β-alanine is a strong inhibitor of the derepression but does not have any inhibitory effect on proline transport through the A system. Also, not all compounds that bind to the A system carrier act as inhibitors of derepression. For example, DAB is an effective inhibitor of proline transport through the A system, yet it does not inhibit derepression. In fact, it behaves as an "anti-co-repressor," preventing alanine from acting as a co-repressor in this system. In this respect DAB behaves similarly to indole-acrylic acid and indole-proprionic acid in the tryptophan operon in *Escherichia coli*, where these compounds compete with tryptophan as a co-repressor of the tryptophan operon (24). It is reasonable to conclude from the experiments described above that the antagonism between alanine and DAB must be at some other site than the A system carrier protein (see model).

A study of the ala^r mutants helps shed some light on the mechanism of gene regulation of the A system. Evidence indicates that ala^r2 is a partial constitutive mutant for an A system carrier protein. First, kinetic studies have shown that ala^r2 has two times the V_{max} for proline transport through the A system when grown and tested under conditions that produce repression in the parental culture, i.e., growth in medium MEMCHO-4. There was no significant change in K_m. Second, upon incubation of ala^r2 under conditions that lead to derepression of CHO-K1, i.e., starvation for amino acids, the velocity of proline transport through the A system becomes equal to that found for the derepressed CHO-K1. Evidence has led us to propose that ala^r4 is a fully constitutive mutant for the A system. This is borne out by the fact that the A system in this mutant is fully derepressed under conditions that repress the parental culture. In addition, the A system activity in this mutant is not further elevated by placing it under conditions of amino acid starvation that serve to derepress the parental culture. Third, the increased activity is apparently due to the production of more A system carrier rather than to a change in the carrier molecule, as is evident by the large increase in V_{max} of proline transport through the A system (five times that for repressed CHO-K1) with no accompanying change in K_m. We found the phenotypes of both ala^r2 and ala^r4 to be recessive to the wild-type phenotype (repressibility is dominant to constitutivity, and alanine sensitivity is dominant to alanine resistance).

Ala^r3 has been shown to have twice the velocity of proline (at 0.05 mM) transport through the A system, with an increase in both V_{max} and K_m. When ala^r3 is derepressed, its A system activity rises to two times that of the derepressed CHO-K1 and the other ala^r mutants. These results are compatible with the conclusions that ala^r3 is the result of a mutation in a structural gene leading to the production of an A system carrier that has twice the activity of the wild-type culture, so that upon derepression its activity rises to twice that of CHO-K1 and the other ala^r mutants. The change in K_m would appear to rule out gene amplification as the mechanism responsible for the increased activity (25). The increased A system activity of ala^r3 has been shown to be co-dominant to the parental phenotype, as expected of a structural gene mutation.

All three mutants have increased levels of transport of proline through the ASC and P systems, as well as the A system. We have shown that these pleiotropic effects cannot be explained by a primary effect producing an increased Na^+ gradient. We have also shown that the increases in ASC and P system activity behave similarly to the A system in cell-cell hybridization; that is, the increases in all three activities are recessive to that of CHO-K1 in ala^r2 and ala^r4, and co-dominant in ala^r3. These results, together with the fact that all three mutants differ significantly from each other and were isolated in a single-step selection event, strongly suggest that they are the result of a single mutational event (20).

The ability to isolate mutants in a single-step isolation procedure that proves to have a recessive phenotype is not so surprising in CHO-K1. Evidence from a number of sources has shown that a substantial portion of the genome of CHO-K1 is hemizygous in terms of gene activity (27).

We propose the following model as a guide to future experiments and to bring the results we have presented into clear focus (Fig. 6). It is the simplest model, and we realize that the mechanism of gene regulation of this system may turn out to be more complex. We propose that the A system carrier protein is under negative control of a regulatory gene, gene *R*. (Constitutivity is recessive to repressibility.) Gene *R* produces a protein molecule that acts as an apo-repressor-inactivator (Apo-RI). Amino acids that are generally transported by the A gene act as co-repressors-initiators. (Transport through the A system is not a strict requirement, as we have shown.) When CHO-K1 is grown in the presence of A system amino acids, these amino acids or products of these amino acids act as co-repressors-initiators and convert the Apo-RI into the repressor-inactivator (RI). (Since MeAIB, a non-metabolizable analog, is a potent co-repressor-initiator, we presume that amino acids structurally related to MeAIB

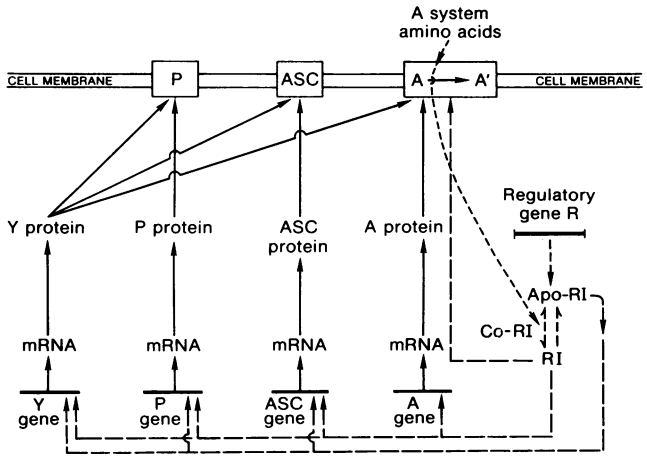

FIG. 6. Model for the regulation of the A system of amino acid transport and the regulatory link between the A, ASC, and P systems. See the text for further details.

may act directly as co-repressors-initiators.) This molecule has two functions. Not only does it repress the synthesis of mRNA from the *A* gene coding for an A system carrier protein, but it also acts as an inactivator, modulating the activity of the A system carrier itself. When cells are starved for A system amino acids, the RI resumes the form of an Apo-RI, releasing the *A* gene for the synthesis of an A protein mRNA, and the synthesis of new A carrier protein commences. We propose that ala^{r}2 and ala^{r}4 are mutant in the *R* gene. Ala^{r}2 Apo-RI can only respond poorly to the co-repressor, so these cells are partially constitutive. Ala^{r}4, on the other hand, probably produces an Apo-RI that does not react with the co-repressor (see below).

To explain the effects of ala^{r}2 and ala^{r}4 on the ASC and P systems, we propose that both the Apo-RI and RI have affinity for the "operator" sites of the genes coding for an ASC and P carrier protein. Thus, these operators would not be affected by conversion of Apo-RI to RI. Furthermore, we propose that the altered regulatory protein produced by ala^{r}2 and ala^{r}4 has lowered affinity for the "operator" sites on both genes ASC and P so that transcription of these genes takes place at a higher rate than in CHO-K1. We also propose that these carrier proteins are not sensitive to the inactivator function of this regulatory protein. There is precedence for two states of a regulatory protein having the same effect on a single regulatory site. In the *ara* operon in *E. coli*, the *araC* gene regulatory protein can exist in the form of a repressor and as an activator. Both of these molecules appear to autoregulate the expression of the *araC* gene, apparently to the same extent (17).

How do we explain the pleiotropic effect of ala^{r}3? Our evidence indicates that this mutant is a result of a structural gene mutation. We propose that this mutation occurs in a gene *Y* that produces a product shared by the A, ASC, and P systems. Thus, a mutation in this gene would lead to alterations in activity of these three systems, and one would expect such a mutation to be codominant. Transport systems sharing the same proteins have been previously described (1).

Gazzola et al. (10) have proposed a model in which an A system amino acid, in reacting with the A system carrier protein, emits a signal that stimulates the expression of an inactivator gene that inactivates the A system carrier. In addition, this signal is supposed to repress the transcription of the A system carrier protein mRNA. Our data show this model to be untenable, since we have shown that (i) the amino acids that repress the A system need not have any affinity for the A system carrier protein and (ii) amino acids that have affinity for the A system carrier need not be co-repressors.

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