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Evidence for coordinate regulation of the A system for amino acid transport and the mRNA for the α_1 subunit of the Na^+,K^+-ATPase gene in Chinese hamster ovary cells

(gene expression/repression/negative control/Na^+)

NAN-XIN QIAN, MARÇAL PASTOR-ANGLADA*, AND ELLIS ENGLESBERG†

Biochemistry and Molecular Biology Section, Department of Biological Sciences, University of California, Santa Barbara, Santa Barbara, CA 93106

Contributed by Ellis Englesberg, January 28, 1991

ABSTRACT **Previous work suggested that the structural gene for the A system transporter and the mRNA for the α subunit of the Na^+,K^+-ATPase in Chinese hamster ovary cells CHO-K1 [wild type (WT)] are coordinately controlled by regulatory gene R1. This conclusion was based on analysis of a mutant for the A system, ala^r4. This mutant had a constitutive level of A system transport activity equal to the level found in derepressed WT cells and a 4 times increase in abundance of the α_1 subunit of Na^+,K^+-ATPase mRNA over that found in repressed WT. The level of Na^+ per cell in ala^r4 was not significantly greater than that found in the WT. To further characterize the likely coregulation of both genes, we have studied the A system activity and Na^+,K^+-ATPase mRNA α_1-subunit levels in cells grown under various conditions that result in repression or derepression of the A system in the WT. System A activity increased up to 2–3 times the basal transport rate (repressed state) and Na^+,K^+-ATPase mRNA α_1-subunit levels showed a 3-fold increase after amino acid starvation (derepressed state). These changes occurred along with a decrease in intracellular Na^+ levels. *N*-Methyl-α-aminoisobutyric acid and β-alanine, previously shown to be corepressors for the A system, prevented to a similar extent A system derepression and Na^+,K^+-ATPase mRNA α_1-subunit accumulation. On the other hand, phenylalanine and lysine, amino acids that are not corepressors of the A system, failed to significantly prevent derepression of both genes. Hybrids between the WT and ala^r4 have the phenotype of the WT when grown under repressed conditions. These results give further support to the proposition that both the A system transporter and mRNA for the α_1 subunit of the Na^+,K^+-ATPase are coordinately controlled by regulatory gene R1 and elevated Na^+ concentrations are not involved. No Na^+,K^+-ATPase activity was detected in derepressed cells. Activity was restored by the addition of monensin. However, this activity was no greater than that obtained in repressed cells. Indications are that the reduced Na^+ content in derepressed cells inhibits Na^+,K^+-ATPase activity and that conditions that favored derepression do not allow for *de novo* synthesis of the Na^+,K^+-ATPase.**

Evidence has been presented suggesting that the activity of the A system of amino acid transport and the Na^+,K^+-ATPase is coordinately regulated at the transcriptional level by means of repression and negative control by the A system regulatory gene R1 (1).

The A system and the Na^+,K^+-ATPase are ubiquitous integral membrane proteins in mammalian cells that function as secondary and primary active transporters, respectively. The A system accepts as substrates most neutral amino acids but shows a strong preference for proline and small straight-chain amino acids such as alanine (2). It is Na^+ dependent and transports one Na^+ into the cells with each amino acid (3). This system is inducible by hormones (4–6), repressible by amino acids, which it generally transports (7–11), and present with increased activity in many transformed and malignant cells (6).

The Na^+,K^+-ATPase pumps three Na^+ out of the cells and two K^+ into the cells against the respective concentration gradients at the expense of one molecule of ATP. As a result, it establishes a Na^+ electrochemical gradient across the cell membrane, which provides the driving force for the A system of amino acid transport and other Na^+-dependent transporters. Its activity is stimulated by hormones (12–14) and transformed cells show a higher rate of Na^+ and K^+ transport (15). Both carriers, the A system and the Na^+,K^+-ATPase, are thus related mechanistically by sharing Na^+ as a substrate. It is therefore not surprising that the activities of both these systems are closely interrelated (16–22). Besides the concentration of Na^+ affecting the activity of the Na^+,K^+-ATPase, under certain conditions that lead to increases in Na^+ content there is an associated increase in the number of Na^+,K^+-ATPase molecules on the plasma membrane (16, 23–30) and an increase in the abundance of their corresponding mRNA (29, 30). This has led to the idea that the Na^+ concentration itself may have a pretranslational effect resulting in these reported increases (27, 31).

A prior study of the regulation of the A system supported a model of regulation in which regulatory gene R1 produces an apo-repressor, apo-inactivator (apo-ri) and, in the presence of amino acid corepressor, coinactivators (co-ri) that are generally transported by this system, the apo-ri is converted into a repressor inactivator (ri). The ri is believed to act by inhibiting transcription of the mRNA for the A system transporter and also, probably indirectly, inactivating the transporter itself (7). This model has been modified to incorporate our recent findings, suggesting that the A system and the Na^+,K^+-ATPase are coordinately regulated by gene R1 (Fig. 1). Since we have no evidence as to whether or not the ri affects the activity of the Na^+,K^+-ATPase, we have omitted this possibility from the model.

The evidence for this proposed coordinate regulation of both these transport systems is based on the analysis of a mutant, ala^r4 (1, 7). This mutant is constitutive for the A system. Its A system activity, measured under conditions that are repressible for the wild type (WT) (growth in the presence of system A amino acids), is 5 times that of the WT and is equal to that of the WT when the latter is derepressed for the A system (growth under conditions of amino acid

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Abbreviations: WT, wild type; MeAIB, *N*-methyl-α-aminoisobutyric acid; ri, repressor inactivator; apo-ri, apo-repressor, apo-inactivator, ri; co-ri, corepressor, coinactivator.

*Present address: Biochemistry and Molecular Biology Unit, Department of Biochemistry and Physiology, University of Barcelona, Barcelona, Spain.
†To whom reprint requests should be addressed.

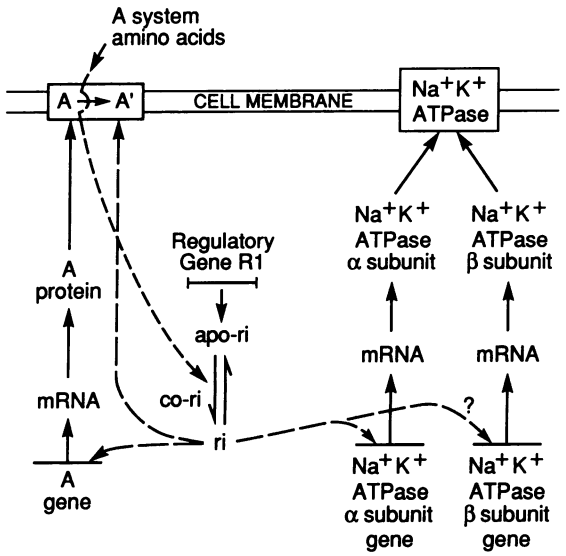

FIG. 1. Model for coregulation of system A and the Na^+,K^+-ATPase. Regulatory gene R1 produces an inactive repressor, inactivator (apo-ri). In the presence of a co-ri, which is usually a substrate for the A system, the apo-ri is converted into an active ri. The ri represses the gene for the A system transporter and the gene for the α_1 subunit of the Na^+,K^+-ATPase and also inactivates A system transport. Since we have no evidence as to whether or not the ri affects the activity of the Na^+,K^+-ATPase, we have omitted this possibility from the model.

starvation or in the absence of A system amino acids). This increase in A system activity is associated, in ala^r4, with an increase in abundance of the Na^+,K^+-ATPase and mRNA for the α_1 subunit of this enzyme (1). The A system transporter in ala^r4 is not inactivated by A system amino acids as it is in the WT and this mutant is not further derepressible when incubated in amino acid-free medium. This phenotype of ala^r4 is explained on the basis that this mutant has suffered a mutation in regulatory gene R1 so that it fails to produce a product that can be converted into a ri by co-ri and thus it has an increased, constitutive level of the A system and an increase in the abundance of the Na^+ pump and its corresponding mRNA. Since we could not detect any message for the β subunit (see below) in our experiments, the possible effect of gene R1 on the expression of the gene for the β subunit remains to be determined. Although, as mentioned above, previous work had implicated an increase in intracellular Na^+ as being the causal factor triggering the increase in abundance of the Na^+,K^+-ATPase, the fact that the intracellular Na^+ concentration in mutant ala^r4 is similar to that of the WT placed doubt on this proposed relationship (1).

As an attempt to further explore the postulated role of regulatory gene R1 in jointly controlling the expression of genes for the α_1 subunit of the Na^+,K^+-ATPase and the A system transporter, we studied the effect of derepression of the A system by amino acid starvation on the activity of the Na^+,K^+-ATPase and the corresponding mRNA for the α_1 subunit of this enzyme. According to the model, derepression of the A system by amino acid starvation converts the ri into the apo-ri form and this results in derepression of the A system and should also lead to derepression of the Na^+,K^+-ATPase. The results of our experiments firmly support the model for the coordinate regulation of both the A system and the Na^+,K^+-ATPase by the regulatory gene R1, as indicated in the model (Fig. 1).

MATERIALS AND METHODS

Cell Cultures, Medium, and Cultural Conditions. CHO-K1 (WT), ala^r4, and WT–ala^r4 hybrid H3, as well as the medium used in this study, MEMCHO-4, and the culture conditions used have been described (7). Hybrid H4 was isolated previously (7) but was not analyzed at that time.

System A Activity. System A activity was measured by the tray technique (4). To derepress the A system, cells were starved for amino acids as described (7), except that the experiment was conducted using 24-well trays. Briefly 1×10^4 cells were added in 1 ml of MEMCHO-4 per well. The cells were incubated for 48 hr, washed with phosphate-buffered saline and 1 ml of starvation buffer (137 mM NaCl/2.68 mM KCl/6.49 mM K_2HPO_4/0.1% glucose/1 mM $MgCl_2$, brought to pH 7.3 with KH_2PO_4 and containing 4% dialyzed fetal calf serum) was added per well. The cells were then incubated at 37°C for another 24 hr in either the absence or presence of 5 mM amino acids during the starvation period as follows: *N*-methyl-α-aminoisobutyric acid (MeAIB), a substrate and co-ri for the A system; β-alanine, not a substrate, but a potent co-r for system A; phenylalanine, a substrate but a poor co-ri for the A system; lysine, neither a substrate nor a co-ri for the A system. Subsequently, the cells were assayed for amino acid transport as described (7). System A activity is the fraction of L-proline uptake inhibited by saturating concentrations of MeAIB. Before transport assays, cells were incubated in the presence of cycloheximide in an amino acid-free medium for 20 min, to avoid further derepression of the A system and trans-inhibition by intracellular amino acids.

Na^+,K^+-ATPase Activity of Intact Cells. The activity of the Na^+,K^+-ATPase in intact cells was measured as the fraction of Rb^+ uptake inhibitable by ouabain (1). Exponentially growing cells were inoculated into 2 ml of MEMCHO-4 in 35-mm dishes at a density of 5×10^4 cells per dish. After 48 hr of incubation, the medium was aspirated and replaced by either 1 ml of fresh medium or starvation buffer and the cells were then incubated at 37°C for another 24 hr. For both sets of dishes, starved and unstarved cells, ^{86}Rb uptake was measured either in the absence or in the presence of 1 mM ouabain. This Na^+,K^+-ATPase inhibitor was added 30 min before the uptake measurements (1). To measure the activity of the Na^+,K^+-ATPase under conditions in which the Na^+ concentration of the cells was not limiting we used the ionophore monensin, which catalyzes a Na^+/H^+ exchange with a stoichiometry of 1:1 and therefore dissipates the sodium chemical gradient and increases the intercellular sodium content. In these experiments, 15 μM monensin was added 5 min before the uptake measurements.

Na^+ and K^+ Determinations. The Na^+ and K^+ content of cells incubated under the conditions mentioned above was determined with an Eppendorf flame photometer as described (1).

RNA Blot Hybridization. Cells used for these experiments were grown in 150-mm dishes under analogous conditions as described above, so that the ratio of cell density to volume of medium was the same as that used to measure A system transport activity. Furthermore, when cells were derepressed for the A system by amino acid starvation, care was also taken to keep experimental conditions similar to those mentioned above. RNA was isolated by the guanidine monothiocyanate/LiCl procedure and hybridization conditions were as described (1), except that blots were hybridized only with the insert for the α_1 subunit of the Na^+,K^+-ATPase from plasmid pRB 5.1, kindly provided by R. Levinson (Yale University School of Medicine). All blots were stripped and then reprobed with ^{32}P-labeled oligo(dT) (18-mer; Pharmacia) as an internal control (32). Bands on the autoradiograms were quantitated with a scanning laser densitometer and normalized on a poly(A)$^+$ RNA abundance basis.

RESULTS AND DISCUSSION

Effect of Derepression on A System Activity and the Na^+,K^+-ATPase. We have previously shown that starving CHO-K1 cells for amino acids that are generally transported by the A system resulted in an increase in A system activity under conditions that required both mRNA and protein synthesis (7). Amino acids that were generally transported by this system, such as proline and MeAIB, acted as co-ri inhibiting the increase in A system activity and associated mRNA. Other amino acids not transported by this carrier failed to act as co-ri—for instance, lysine (9). However, this association between transport through the A system and corepression was proved to be not absolute. For instance, phenylalanine, although a substrate for the A system, is a poor co-ri, and β-alanine, although not a substrate for the A system, was shown to be a very effective co-r (9). Thus, during the starvation period, the introduction of proline, MeAIB, or β-alanine prevents the derepression of the A system, while phenylalanine and lysine do not.

To further test the model for the joint regulation of the A system and the Na^+,K^+-ATPase by regulatory gene R1 we subjected the cells to starvation–derepression in the presence and absence of these specific effectors. We incubated cells for 24 hr in starvation buffer without the addition of amino acid co-ri or in the presence of 5 mM MeAIB, β-alanine, phenylalanine, or lysine. MeAIB was chosen because it is a nonmetabolizable specific substrate and a co-ri of the A system. After this incubation, A system and Na^+,K^+-ATPase activity and mRNA for the α_1 subunit of the Na^+,K^+-ATPase were measured. After amino acid starvation, A system activity showed a 2.6 times increase over the repressed WT values (Table 1). Although there was significant Na^+,K^+-ATPase activity (ouabain-inhibitable ^{86}Rb uptake) present in the repressed (unstarved cells), we could not detect any activity in the derepressed (starved cells). To determine whether the absence of detectable activity might be due to the lowered Na^+ content of the cells (see below) we measured pump activity in the presence of monensin. The presence of the ionophore increased Na^+,K^+-ATPase activity as expected in the derepressed cells. This activity, however, is not significantly different from that obtained with repressed cells (Table 1). On the other hand, we found that starvation induced a significant increase in its abundance of the mRNA for the α_1 subunit of the Na^+,K^+-ATPase equivalent to 2.9 times that of the repressed WT (Fig. 2; Table 1). Furthermore, those amino acids reported to act as corepressors for the A system (MeAIB and β-alanine) significantly prevented both A system induction and the accumulation of mRNA for the Na^+,K^+-ATPase to the same extent. In contrast to the effect of MeAIB and β-alanine, both phenylalanine and lysine, which have little or no ri activity, had a negligible inhibitory effect on the derepression of both the A system and the Na^+,K^+-ATPase α_1-subunit mRNA (Fig. 2; Table 1). The ratios of A system activity and α_1-subunit mRNA abundance under these varied conditions of repression and derepression show close similarity, as would be expected if both were controlled by the same regulatory gene. The fact that there is no significant increase in the Na^+,K^+-ATPase pump activity, measured in the presence of monensin when cells were derepressed, even though there was an increase in mRNA for the α_1 subunit for this enzyme, suggests that amino acid starvation somehow interferes with the synthesis of an active Na^+,K^+-ATPase. We have no explanation for this phenomenon.

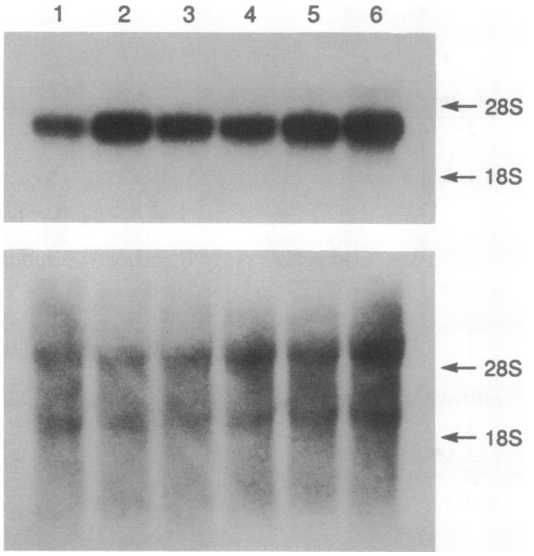

FIG. 2. RNA blot hybridization (Northern analysis) of WT for the mRNA of the α_1 subunit of the Na^+,K^+-ATPase under conditions that regulate expression of the A system of amino acid transport. WT RNA was extracted and processed from cells grown in MEMCHO-4 and then incubated under various conditions. Lane 1, MEMCHO-4 (repressed); lanes 2–6, amino acid starved for 24 hr in the absence or presence of the following 5 mM amino acids: lane 2, no amino acids; lane 3, MeAIB; lane 4, β-alanine; lane 5, phenylalanine; lane 6, lysine. Upper autoradiogram, probed with α_1-subunit Na^+,K^+-ATPase cDNA. Lower autoradiogram, blot was stripped and reprobed with ^{32}P-labeled oligo(dT) as an internal control.

Table 1. Effect of repression and derepression on A system activity and on the activity of the Na^+,K^+-ATPase and abundance of the mRNA for the α_1 subunit

Condition	A system activity, ng per mg of protein per min	Increase over WT repressed value	Na^+,K^+-ATPase		Ratio derepressed/ repressed‡	A system/ mRNA
			Activity*	α_1-subunit mRNA†		
Repressed	0.16 ± 0.03	1	0.25 ± 0.06 0.86 ± 0.20 (m)	1.04	1	1
Derepressed	0.42 ± 0.01	2.6	0 0.96 ± 0.10 (m)	3.0	2.9	0.9
+ MeAIB	0.23 ± 0.01	1.4	ND	1.9	1.8	0.78
+ β-Ala	0.19 ± 0.00	1.2	ND	1.7	1.6	0.75
+ Phe	0.35 ± 0.04	2.2	ND	2.7	2.6	0.85
+ Lys	0.38 ± 0.01	2.4	ND	3.6	3.5	0.69

ND, Na^+,K^+-ATPase activity was not determined since derepressed cells had no activity in the absence of monensin; (m), activity determined in the presence of monensin. Repressed cells were grown in MEMCHO-4; derepressed cells were starved for amino acids.

*Ouabain-inhibitable Rb^+ uptake in μmol of Rb^+ per mg of protein per hr.

†Densitometer readings of x-ray film of RNA blot hybridization (see Fig. 2) normalized to the respective oligo(dT) lane.

‡Ratio of normalized densitometer reading of x-ray film of RNA blot hybridization from derepressed over repressed cells (Fig. 2).

Table 2. Comparison of the Na^+ and K^+ content of repressed and derepressed CHO-K1 cells

Condition	nmol per 10^6 cells		mmol per liter per cell	
	Na^+	K^+	Na^+	K^+
Repressed	24.2 ± 0.6	293.6 ± 7.2	18.7 ± 0.5	227.6 ± 5.6
Derepressed (starved)	17.6 ± 2.5	299.0 ± 6.9	13.6 ± 2.0	231.8 ± 5.3

Experiments were performed in quintuplicate. The results are averaged and standard deviations are given.

Na^+ and K^+ Levels in Derepressed CHO-K1 Cells. Na^+ and K^+ levels in CHO-K1 cells cultured in minimal essential medium or after a 24-hr starvation period are shown in Table 2. Na^+ levels decreased after maintaining CHO-K1 cells in an amino acid-free medium, which makes sense on the basis of the decreased activity of Na^+ cotransporters. K^+ levels were not significantly altered after cells were starved. These observations do not support the concept suggested by others (27, 31) that induction of Na^+,K^+-ATPase gene expression might be triggered by increases in intracellular Na^+ levels. Our experiments show that there was a significant increase in abundance of the mRNA for the Na^+,K^+-ATPase α_1-subunit gene when cells were starved, even in the presence of low intracellular Na^+ levels. Furthermore, the fact that MeAIB and phenylalanine, amino acids that are substrates for the A system and therefore cotransport Na^+, have different effects on α_1-subunit mRNA levels also negates a role for Na^+ intracellular levels in mediating the level of mRNA for the Na^+,K^+-ATPase α_1 subunit. Since we could not detect any Na^+,K^+-ATPase activity in derepressed cells, which had lowered Na^+ content, and we found that this activity was restored in the presence of monensin, it is reasonable to conclude that the loss of activity is the result of the lowered intracellular Na^+ concentration.

System A Activity and Na^+,K^+-ATPase α_1-Subunit Gene Expression in CHO-K1–ala^{r}4 Hybrids. As mentioned above, ala^{r}4 is the result of a mutation in the regulatory gene R1 that has led to constitutive expression of A system. Evidence for this mutation affecting regulatory gene R1 that functions by negative control is provided by the fact that CHO-K1–ala^{r}4 hybrids show the WT phenotype. As an experimental approach to further test the hypothesis that gene R1 regulates both A system and Na^+,K^+-ATPase genes, we studied A system activity and levels of Na^+,K^+-ATPase α_1-subunit mRNA in CHO-K1, ala^{r}4, and CHO-K1–ala^{r}4 hybrids. Two independently derived hybrid clones were studied (7). Results on A system activity and Na^+,K^+-ATPase α_1-subunit gene expression are shown in Table 3 and Fig. 3. In this experiment, ala^{r}4 showed a 3.9 times increase in A system activity and a 2.2 times enhancement in mRNA levels for Na^+,K^+-ATPase α_1 subunit as compared to the WT. Both hybrid clones showed a phenotype similar to the WT and, as expected if coordinate regulation occurs, both A system and Na^+,K^+-ATPase α_1-subunit mRNA levels decreased to a similar extent in both hybrid clones. The possibility that the phenotype of the hybrids might be due to the absence of the pertinent chromosome seems unlikely since two independently isolated hybrids were analyzed and the chances that the same chromosome was lost in each case would appear to be remote. In addition, the chromosome number of each hybrid is what is expected from such a cross (Table 3).

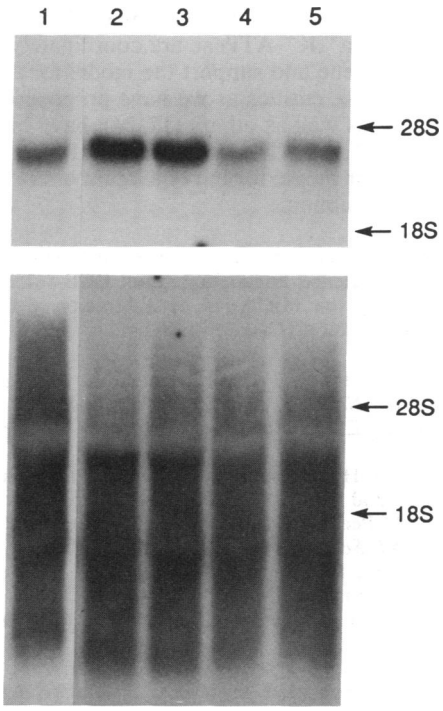

FIG. 3. RNA blot hybridization (Northern analysis) of WT, ala^{r}4, and WT–ala^{r}4 hybrids. Lane 1, WT in MEMCHO-4 (repressed); lane 2, WT starved for amino acids for 24 hr (derepressed); lane 3, ala^{r}4 in MEMCHO-4; lane 4, WT–ala^{r}4, hybrid 3 in MEMCHO-4; lane 5, WT–ala^{r}4, hybrid 4 in MEMCHO-4. Upper autoradiogram, blot probed with α_1-subunit Na^+,K^+-ATPase cDNA. Lower autoradiogram, blot above was stripped and reprobed with ^{32}P-labeled oligo(dT) as an internal control.

The increase in abundance and activity of A system and that of the Na^+,K^+-ATPase and the mRNA for the α_1 subunit of the latter in a mutant constitutive for the A system (1), the increase in the A system activity accompanied by an increase in abundance of the mRNA for the α_1 subunit of the Na^+,K^+-ATPase upon subjecting CHO-K1 to conditions that derepress the A system, and the demonstration that the constitutive levels of the A system and the increased abundance of the mRNA for the α_1 subunit of the Na^+,K^+-ATPase are

Table 3. Constitutive A system activity and increased abundance of mRNA for the α_1 subunit of Na^+,K^+-ATPase are recessive

Cells	A system activity, nmol per min per mg of protein	Increase over WT activity	α_1-subunit mRNA*	Increase over WT level	Chromosome†
CHO-K1 (WT)	0.19 ± 0.01	1	0.12	1	20 (18)
ala^{r}4	0.74 ± 0.15	3.89	0.26	2.2	20 (20)
WT–ala^{r}4(H3)‡	0.13 ± 0.01	0.68	0.08	0.7	40 (37)
WT–ala^{r}4(H4)‡	0.24 ± 0.02	1.26	0.11	0.9	40 (40)

System A activity was determined in triplicate. Averages and standard deviations are given.
*Densitometer readings of x-ray film of RNA blot hybridization normalized to the respective oligo(dT) spot (Fig. 3).
†Values were determined from at least 25 spreads prepared by the usual procedure and represent the modal number with the mean in parentheses.
‡Independent hybrid clones.

recessive to that of the WT offer strong evidence that the A system and the Na^+,K^+-ATPase are coordinately regulated by the same R1 gene and support the model for the coregulation of both these entities as we have proposed.

Since we (1), as well as others (33, 34), have been unable to detect the Na^+,K^+-ATPase β-subunit mRNA, we do not know whether our model for the regulation of the α_1 subunit applies to the β subunit.

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