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Magnesium transport in *Salmonella typhimurium*: biphasic magnesium and time dependence of the transcription of the *mgtA* and *mgtCB* loci

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Salmonella typhimurium has three distinct Mg^{2+} transport systems, the constitutive high-capacity CorA transporter and two P-type ATPases, MgtA and MgtB, whose transcription is repressed by normal concentrations of Mg^{2+} in the growth medium. The latter Mg^{2+}-transporting ATPase is part of a two-gene operon, _mgtCB_, with _mgtC_ encoding a 23 kDa protein of unknown function. Transcriptional regulation using fusions of the promoter regions of _mgtA_ and _mgtCB_ to _luxAB_ showed a biphasic time and Mg^{2+} concentration dependence. Between 1 and 6 h after transfer to nitrogen minimal medium containing defined concentrations of Mg^{2+}, transcription increased about 200-fold for _mgtCB_ and up to 400-fold for _mgtA_, each with a half-maximal dependence on Mg^{2+} of 0·5 mM. Continued incubation revealed a second phase of increased transcription, up to 2000-fold for _mgtCB_ and up to 10000-fold for _mgtA_. This secondary increase occurred between 6 and 9 h after transfer to defined medium for _mgtCB_ but between 12 and 24 h for _mgtA_ and had a distinct half-maximal dependence for Mg^{2+} of 0·01 mM. A concomitant increase of at least 1000-fold in uptake of cation was seen between 8 and 24 h incubation with either system, showing that the transcriptional increase was followed by functional incorporation of large amounts of the newly synthesized transporter into the membrane. Regulation of transcription by Mg^{2+} was not dependent on a functional stationary-phase sigma factor encoded by _rpoS_, but it was dependent on the presence of a functional _phoPQ_ two-component regulatory system. Whereas _mgtCB_ was completely dependent on regulation via _phoPQ_, the secondary late Mg^{2+}-dependent phase of _mgtA_ transcription was still evident in strains carrying a mutation in either _phoP_ or _phoQ_, albeit substantially diminished. Several divalent cations blocked the early phase of the increase in transcription elicited by the decrease in Mg^{2+} concentration, including cations that inhibit Mg^{2+} uptake (Co^{2+}, Ni^{2+} and Mn^{2+}) and those which do not (Ca^{2+} and Zn^{2+}). In contrast, the second later phase of the transcriptional increase was not well blocked by any cation except those which inhibit uptake. Overall, the data suggest that at least two distinct mechanisms for transcriptional regulation of the _mgtA_ and _mgtCB_ loci exist.

Keywords: magnesium transport, magnesium regulation, *phoPQ* two-component system, *Salmonella typhimurium*

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INTRODUCTION

Magnesium is a vital divalent cation in living organisms, functioning as both a cofactor and a regulator of

numerous proteins and as a stabilizing factor for membranes, ribosomes and other cellular structures (Altura, 1992; Grubbs & Maguire, 1987; Maguire, 1990; Romani *et al.*, 1993). Studies in mammalian and other systems have shown that Mg^{2+} transport is far more active and the intracellular content of Mg^{2+}, both free and total, is far greater than previously appreciated (Clausen *et al.*, 1991; Grubbs *et al.*, 1985; Maguire, 1990; Romani *et al.*, 1993). In *Salmonella typhimurium*, three Mg^{2+} transport systems have been identified and designated as CorA, MgtA and MgtB, each encoded by the respective locus (Hmiel *et al.*, 1989; Snavely *et al.*, 1989). Transport via the CorA system is constitutive and of high capacity. This locus encodes a single protein of about 40 kDa lacking homology to any known protein and capable by itself of mediating Mg^{2+} influx (Smith *et al.*, 1993a). In contrast, the *mgtA* and *mgtCB* loci encode P-type ATPases (Snavely *et al.*, 1991a), and are thus members of a large family of homologous proteins responsible for membrane transport of cations (Pedersen & Carafoli, 1987). Interestingly, the MgtA and MgtB proteins have relatively little similarity to other prokaryotic P-type ATPases but much greater similarity to eukaryotic P-type ATPases, especially the muscle sarcoplasmic reticulum Ca^{2+}-ATPases (Snavely *et al.*, 1991a; Smith *et al.*, 1993b). The *mgtA* locus consists of a single gene encoding MgtA whereas the *mgtCB* locus is a two-gene operon, where *mgtC* encodes a 23 kDa protein of unknown function and *mgtB* encodes a Mg^{2+}-transporting P-type ATPase. Both loci are tightly repressed under normal laboratory growth conditions. This repression, however, is relieved when Mg^{2+} concentration in the medium is lowered (Snavely *et al.*, 1991b) and upon phagocytosis into mammalian epithelial cells (Garcia-del Portillo *et al.*, 1992). Recent evidence has indicated that this repression is mediated at least in part by a two-component regulatory system, *phoPQ*, with the PhoQ protein being a membrane sensor-kinase for Mg^{2+} (Garcia-Vescovi *et al.*, 1996; Soncini *et al.*, 1996).

Since the *phoPQ* system is an important virulence factor in *Salmonella* and other species, the ability of Mg^{2+} to control a two-component regulatory system that in turn regulates Mg^{2+} transport is of interest. Here we report that the derepression of the promoters for both the *mgtA* and *mgtCB* loci has a biphasic time and Mg^{2+} concentration dependence, that such derepression results in extremely large increases in both gene transcription and translation of functional transport protein even in the absence of cell growth, and that this response appears to involve a second regulatory pathway, in addition to the *phoPQ* system.

METHODS

Plasmids and strains. These are shown in Table 1.

Buffers. Luria–Bertani (LB) broth was used for routine cultures with antibiotics supplemented as required (Hmiel *et al.*, 1989; Snavely *et al.* 1989). For luciferase assays as well as $^{63}Ni^{2+}$ transport assays, nitrogen minimal medium supplemented with 1 mM leucine, 0·1% Casamino acids, 0·4% glucose, 1 µg $FeSO_4$ ml^{-1} and the indicated concentration of Mg^{2+} was used as culture broth (Hmiel *et al.*, 1989). Luciferase assay buffer is 50 mM sodium phosphate buffer, pH 7·5, containing 0·01% (v/v) dodecyl aldehyde.

Luciferase assay. Previous work (Maguire *et al.*, 1992; Smith *et al.*, 1993b, Snavely *et al.*, 1991b, Tao *et al.*, 1995) has shown that a decrease in the Mg^{2+} concentration in minimal medium greatly increases transcriptional activity at both the *mgtA* and *mgtCB* loci. Previous studies were performed using *mgtB*::*lacZ* and *mgtA*::*lacZ* fusions, measuring β-galactosidase activity at a single 6 h time point. The use of the luciferase reporter constructs allows easier continuous monitoring of much smaller volumes of individual cultures over any incubation period. Further, since at 37 °C the luciferase protein is unstable, a time course of luciferase activity can give some measure of the temporality of transcriptional activity, unlike β-galactosidase measurements which give only a sum of activity over time without necessarily indicating when the activity occurred. The constructs were tested over a range of supplemental Mg^{2+} concentrations from 0·3 µM to 100 mM Mg^{2+}. We have previously determined that the contaminant Mg^{2+} concentration inherent in nitrogen minimal medium as measured by atomic absorption spectrometry is approximately 15 µM (Hmiel *et al.*, 1986, 1989). However, this amount appears to be bound completely since we can detect effects of added Mg^{2+} concentrations as low as 1–2 µM (Snavely *et al.*, 1991b).

The luciferase assay has been previously described (Tao *et al.*, 1995). Briefly, an aliquot of cells (5–50 µl) is mixed with 500 µl luciferase assay buffer at room temperature in a 0·7 ml clear plastic microfuge tube. The capped tube is mixed for 10 s, placed in a carrier scintillation vial, and luciferase activity determined immediately by light counting in a Beckman LS7000 liquid scintillation counter for 0·5 min using a full channel setting. To correct for coincidence detection in the scintillation counter, net c.p.m. activity is calculated as the square root of the c.p.m. detected in the counter. All data are normalized for cell number as represented by simultaneous measurement of turbidity as OD_{600}. Luciferase activity was linear over cell densities in the scintillation counter (as OD_{600}) from 0·001 to at least 1·0 as long as the total c.p.m. was less than 1×10^6. Activities greater than this amount were measured by dilution so that the total c.p.m. per cell aliquot used was less than 1×10^6.

It is important to note that the luciferase enzyme is temperature sensitive, being most stable at 28–30 °C. Transcriptional data in this report were obtained with cells grown both at 30 °C and at 37 °C. Control experiments indicated that qualitatively similar results were obtained with cells grown at either temperature although the maximal response obtained at 37 °C was somewhat less and slightly more variable, presumably because of ongoing denaturation and proteolysis of the luciferase. The growth temperature also has some effect on the temporal dependence of the transcriptional response, with lower temperatures resulting in a lengthening in the lag period before increases in transcription were observed. Finally, estimation of the *fold* increase in transcription (and transport itself) is inherently variable because of the extremely low, possibly zero, levels of activity seen in the presence of high extracellular Mg^{2+} concentrations, thus making the denominator in the calculation a small, variable number. However, both the baseline and maximal responses varied slightly with inoculum density and from experiment to experiment. Overall, the most consistent presentation of the data is by fold increase. Representative absolute values for responses are given in the figure legends; $K_{0.5}$ values are

Table 1. *S. typhimurium* strains and plasmids used in this work

Strain	Genotype/plasmid	Reference/source
MM281	*DEL485(leuBCD) mgtB10*::MudJ *corA45*::MudJ *mgtA21*::MudJ *zjh1628*::Tn*10*(Cam^r)	Hmiel *et al.* (1989)
MM387	*DEL485(leuBCD) corA185*::Tn*10*Δ16Δ17(Tet^r)	Smith *et al.* (1993a)
MM1101	MM387/pTT5SE	This study
MM1102	MM387/pTT6SE	This study
MM1103	MM387/pTT-CALux	This study
MM1104	MM387/pTT-ALux	This study
MM1106	MM387/pTT-CLux	This study
MM1267	*phoP5170*::MudJ (14028s)	E. Groisman*
MM1270	*phoQ5172*::MudJ (14028s)	E. Groisman
MM1269	*S. typhimurium* 14028s (wild-type)	ATCC
MM1512	MM1269/pTT-ALux	This study
MM1511	*phoP5170*::MudJ/pTT-ALux	This study
MM1551	*phoQ5172*::MudJ/pTT-ALux	This study
MM1520	MM1269/pTT-Clux	This study
MM1521	*phoP5170*::MudJ/pTT-CLux	This study
MM1552	*phoQ5172*::MudJ/pTT-CLux	This study

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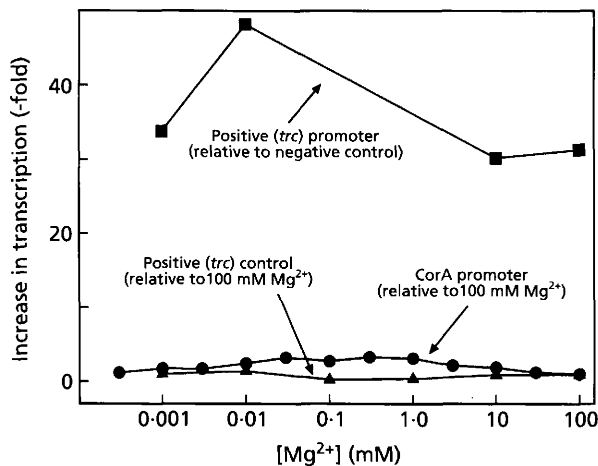

Fig. 1. Effect of Mg^{2+} on transcription from the *trc* promoter and from pTT-CALux. Transcription was measured as described in Methods for the *trc* promoter in the positive orientation in pTT6SE and the *corA* promoter in pTT-CALux. All data were normalized for cell number. Response of the *corA* promoter (●) is plotted relative to the response at 100 mM Mg^{2+}. Per 10^8 cells, the absolute value of the response in a typical experiment was 18200 c.p.m. with no added Mg^{2+}, 15600 c.p.m. at 1 mM Mg^{2+} and 19100 c.p.m. at 100 mM Mg^{2+}. The positive control is plotted either relative to the response at 100 mM Mg^{2+} (▲) or relative to the response of the negative (pTT5SE) control measured at the same Mg^{2+} concentration (■). Negative control responses averaged about 30 c.p.m. per 10^8 cells, while the positive promoter control averaged about 5000 c.p.m. per 10^8 cells. The ability of IPTG to induce transcription from the *trc* promoter is also unaffected by changes in extracellular Mg^{2+} (Tao *et al.*, 1995).

estimates. The small number of points on the steep part of the dose response curve made curve-fitting error prone. The estimated $K_{0.5}$ values varied no more than threefold between experiments.

Fig. 1 shows control data for the promoter constructs pTT5SE and pTT6SE and for the promoter plasmid containing the *corA* promoter sequence, pTT-CALux. These plasmids were derived from pTrc99A (Pharmacia) and carry the *trc* promoter under *lacI*^{q} control. There is no significant alteration in *corA* transcription by the concentration of extracellular Mg^{2+}. Likewise, transcription from the *trc* promoter of the parental construct was not affected by Mg^{2+} whether expressed relative to the negative control plasmid or to its activity at 100 mM extracellular Mg^{2+}.

Control experiments for cation toxicity. Cell exposure to high concentrations of many divalent cations is toxic; therefore, in experiments to determine cation specificity of transcriptional response, we used three parameters to gauge toxicity. First, cells carrying the various plasmids were tested by a disk inhibition assay in which a filter paper disk was placed in the centre of a lawn of cells. Cells that are not inhibited by the test ion grow up to the disk while those that are sensitive form a ring some distance from the disk with no visible growth within the ring (Hmiel *et al.*, 1986, 1989). Second, in liquid cultures, we have used the cell density as a guide. In nitrogen minimal medium containing no added Mg^{2+}, cell density (OD_{600}) usually increases by a factor of about 3–5 with a starting inoculum of OD_{600} 0·1 over the first several hours of incubation and remains constant for the remainder of the 24 h incubation period. (Toxicity results were similar at initial densities of OD_{600} 0·02–0·2.) Cations were considered as non-toxic if, at the tested concentration, they did not significantly affect final cell density in liquid culture or cause formation of a centre ring devoid of growth on plates. Based on these considerations (data not shown), Co^{2+}, Ni^{2+}, Mn^{2+} and Zn^{2+} were toxic at approximately the same or slightly higher concentrations than those at which they diminished transcription of the *luxAB* fusions. Ba^{2+} and Sr^{2+} showed no significant toxicity. The effect of Ca^{2+} depends on the system being tested. In cells dependent on the MgtB Mg^{2+} transport system for Mg^{2+} uptake (e.g. *mgtA corA* double mutants), Ca^{2+} is highly toxic (Hmiel *et al.*, 1986, 1989). In contrast, in cells dependent on the MgtA system (*mgtCB corA* double mutants), even very

high concentrations of extracellular Ca^{2+} have no effect on growth.

As a third test of possible cation toxicity, we measured the ability of the various cations to alter transcription of the *treR* promoter carried on pTT-DLux. The *treR* gene is adjacent to and transcribed in the opposite direction from *mgtA* (Tao *et al.*, 1995), with their promoters carried on the same segment of DNA between the two genes. This is probably the most informative assay because it measures the same parameter (transcription) and utilizes the same segment of DNA as the Mg^{2+}-sensitive promoter carried on pTT-ALux (see above). Using transcription of *treR* as a guide, we concluded (data not shown) that regardless of any effect of divalent cations to inhibit growth, no cation is severely toxic based on inhibition of *treR* transcription. Only Ni^{2+} represses transcription from pTT-DLux more than 50% after 24 h incubation.

$^{63}Ni^{2+}$ transport. MM281 was transformed by electroporation with plasmids carrying inserts to be tested. The resulting strains were grown overnight in LB broth supplemented with appropriate antibiotics. Subcultures (20 ml) were started by 1:50 inoculation of nitrogen minimal medium containing 1 mM Mg^{2+}. After 8 h growth, the cells from the subculture were collected by centrifugation at 1000 *g* for 5 min and washed twice in the same volume of nitrogen minimal medium containing no added Mg^{2+}. New subcultures were then started from the washed cells by resuspension in nitrogen minimal medium containing the indicated Mg^{2+} concentration to a final OD_{600} of 0·1. After incubation for the indicated time, cells were collected and washed three times in nitrogen minimal medium without added Mg^{2+} before suspension in the same medium at a final OD_{600} of 1·0 for use in the transport assay (Snavely *et al.*, 1989; Grubbs *et al.* 1989).

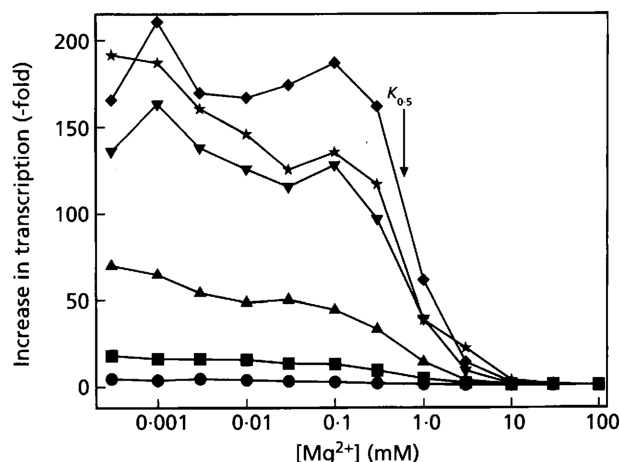

Fig. 2. Effect of Mg^{2+} on transcription from the *mgtCB* promoter from 1 to 6 h incubation. Transcription was measured as described in Methods for the *mgtCB* promoter in cells carrying pTT-CLux at the indicated Mg^{2+} concentrations and times. At each time the data are normalized to the apparent transcription at 100 mM Mg^{2+} and for cell number. The $K_{0.5}$ for Mg^{2+} of 0·5–1·0 mM is estimated from the data for the 4, 5 and 6 h plots. A single experiment is shown representative of three similar experiments. Values are the mean of three independent replicates at each concentration and time. The variation for any single value was about 10%, but for clarity error bars are not plotted. Absolute values of the responses were 50–100 c.p.m. per 10^8 cells at 100 mM Mg^{2+} and 18500 c.p.m. per 10^8 cells after 6 h at 0·01 mM Mg^{2+}. ●, 1 h; ■, 2 h; ▲, 3 h; ▼, 4 h; ◆, 5 h; ★, 6 h.

RESULTS

Regulation of *mgtCB*

Regulation of the *mgtCB* promoter (pTT-CLux) over the first several hours of incubation at 37 °C is shown in Fig. 2. An increase in transcription can be seen as early as 1 h after resuspending cells in low Mg^{2+} concentrations. A marked increase occurs beginning after about 2 h incubation, reaching about 200-fold for *mgtCB* by 6 h. The apparent half-maximal (extracellular) Mg^{2+} concentration ($K_{0.5}$) at which this increase in transcription occurs is 0·5–1 mM. Continued incubation for a total of 24 h provided an additional increase in transcription of *mgtCB* (Fig. 3). Beginning at approximately 7 h incubation, a second phase of transcriptional activity ensues, reaching a maximum of 1500–2000-fold in most experiments. The $K_{0.5}$ for Mg^{2+} of this second phase is about 10 μM, significantly lower than that of the initial phase. The time course of the increase in activity is clearly at least biphasic, as shown in the inset to Fig. 3.

Regulation of *mgtA*

Identical experiments with the *mgtA* promoter construct (pTT-ALux) in cells grown at 37 °C gave results qualitatively comparable to those with the *mgtCB*

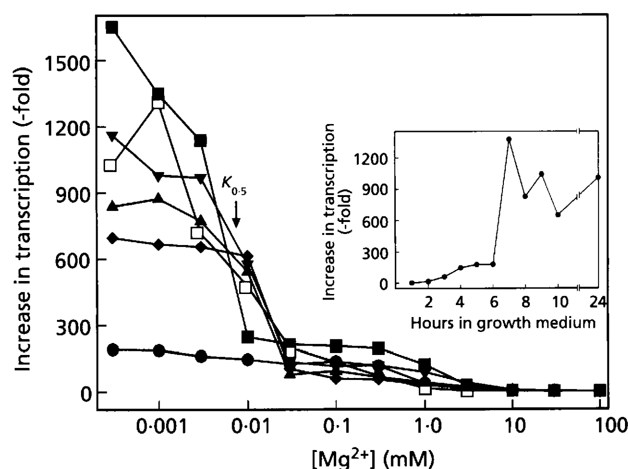

Fig. 3. Effect of Mg^{2+} on transcription from the *mgtCB* promoter from 6 to 24 h incubation. Transcription was measured as described in Methods and the legend to Fig. 2 for the *mgtCB* promoter in cells carrying pTT-CLux at the indicated Mg^{2+} concentrations and times. At each time the data are normalized to the apparent transcription at 100 mM Mg^{2+} and for cell number. The $K_{0.5}$ value of 10 μM is estimated using the curves at 8–24 h. The inset shows a time course of the data from Figs 2 and 3 averaging the responses at the three lowest added Mg^{2+} concentrations. Absolute values of the responses were 50–100 c.p.m. per 10^8 cells at 100 mM Mg^{2+} and 88000 c.p.m. per 10^8 cells after 12 h at 0·01 mM Mg^{2+}. ●, 6 h; ■, 7 h; ▲, 8 h; ▼, 9 h; ◆, 10 h; □, 24 h.

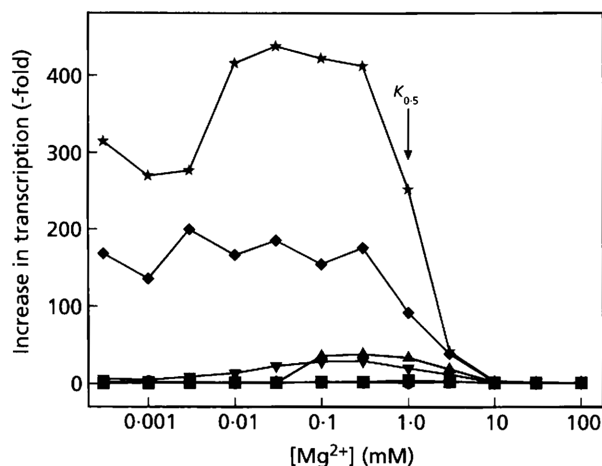

Fig. 4. Effect of Mg^{2+} on transcription from the *mgtA* promoter from 1 to 6 h incubation. Transcription was measured as described in Methods for the *mgtA* promoter in cells carrying pTT-ALux at the indicated Mg^{2+} concentrations and times. At each time the data are normalized to the apparent transcription at 100 mM Mg^{2+} and for cell number. The $K_{0.5}$ value of 10 μM is estimated using the curves at 5 and 6 h. Absolute values of activity at 100 mM Mg^{2+} were similar to the activity of the pTT-CLux construct (see legends to Figs 2 and 3) while maximal values at low Mg^{2+} concentrations were usually 3–4 times higher than with pTT-CLux. ●, 1 h; ■, 2 h; ▲, 3 h; ▼, 4 h; ◆, 5 h; ★, 6 h.

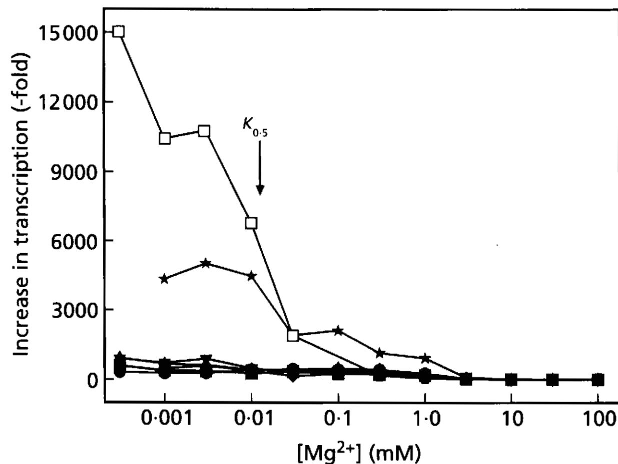

Fig. 5. Effect of Mg^{2+} on transcription from the *mgtA* promoter from 6 to 24 h incubation. Transcription was measured as described in Methods for the *mgtA* promoter in cells carrying pTT-ALux at the indicated Mg^{2+} concentrations and times. At each time the data are normalized to the apparent transcription at 100 mM Mg^{2+} and for cell number. The $K_{0.5}$ value of 10 μM is estimated using the curves at 12 and 24 h. ●, 6 h; ■, 7 h; ▲, 8 h; ▼, 9 h; ◆, 10 h; ★, 12 h; □, 24 h.

construct. The onset of the increase in transcriptional activity appears somewhat delayed with *mgtA* (Fig. 4) compared to *mgtCB* (Fig. 2), with the initial increase in transcription not occurring until after about 4 h incubation. The marked increase seen with *mgtCB* at 3–4 h incubation is delayed with *mgtA* until 5–6 h incubation. While the overall activity of *mgtA* during this initial phase is significantly higher than that of *mgtCB*, the $K_{0.5}$ for Mg^{2+} is similar, about 0·5–1 mM. As with the initial response, the second phase of the transcriptional response was delayed for *mgtA* compared to *mgtCB*, occurring after at least 10 h incubation, and was greater for *mgtA* (Fig. 5) than for *mgtCB* (Fig. 3). Increases of up to 10000-fold have been routinely measured. As with *mgtCB*, the $K_{0.5}$ of Mg^{2+} for this second phase is significantly lower than for the first phase. The variability in the assay and their lengthy time course make determination of the second $K_{0.5}$ for *mgtA* more difficult, but the $K_{0.5}$ values for both *mgtA* and *mgtCB* appear similar at approximately 10 μM Mg^{2+}. Previous data on *mgtA* using the *mgtA*::*lacZ* fusion showed only the initial phase of the Mg^{2+}-dependent increase in transcription (Snavely *et al.*, 1991b). However, the second phase was probably not seen since β-galactosidase activity was measured only at a single 6 h time point, well before the second phase of the increase in transcription occurs with *mgtA*.

Regulation of Mg^{2+} transport

The question of whether these large increases in transcription result in a similarly large translation into functional protein was addressed using $^{63}Ni^{2+}$ uptake as surrogate for the unavailable $^{28}Mg^{2+}$ (Snavely *et al.*, 1989, 1991b). We have previously shown that activation of the (single-copy) chromosomally encoded *mgtCB* locus results in significant increases in $^{28}Mg^{2+}$ and $^{63}Ni^{2+}$ uptake when the Mg^{2+} concentration of the medium is decreased (Snavely *et al.*, 1991b). Since the luciferase reporter plasmids assayed above are pBR322-based and therefore have a relatively high copy number, $^{63}Ni^{2+}$ uptake was assayed under conditions as similar as possible to the promoter plasmids using pDS107 (Smith *et al.*, 1993b), a pBR322-based plasmid expressing the intact *mgtCB* operon, carried in MM281. Since the chromosomal Mg^{2+} transport genes have been inactivated in MM281 (Hmiel *et al.*, 1989, Snavely *et al.*, 1989), the strain is dependent on MgtB protein encoded by pDS107 for Mg^{2+} uptake and Mg^{2+}-independent growth. When extracellular Mg^{2+} is lowered, Mg^{2+} uptake measured as $^{63}Ni^{2+}$ accumulation markedly increases, with a time course similar to that of the transcriptional increase (Fig. 6). Similar increases in transport via the MgtA system are also seen under these incubation conditions (data not shown). The degree of increase is a function of the extracellular Mg^{2+} concentration (Maguire *et al.*, 1992; data not shown). Estimation of the exact fold increase is difficult however because of the low initial level of uptake.

Effect of *phoPQ* on transcription

The finding by Groisman and colleagues (Garcia-Vescovi *et al.*, 1996; Soncini *et al.*, 1996) that the *phoPQ* two-component regulatory system, important for virulence in *Salmonella* and other enteric bacteria (Miller *et*

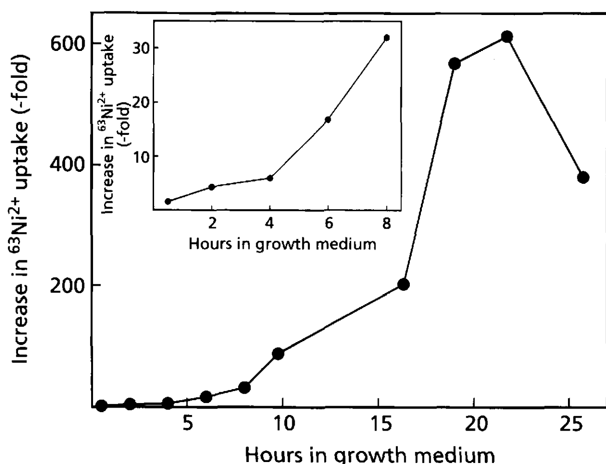

Fig. 6. Time course of induction of Mg^{2+} uptake by the MgtB transport system. Strain MM281 carrying pDS107 (Smith *et al.*, 1993b) was grown overnight in nitrogen minimal medium containing 1 mM Mg^{2+}. The cells were washed twice in the same medium without added Mg^{2+}, resuspended in the same medium at OD_{600} 0.1, transferred into flasks containing medium at 37 °C either without added Mg^{2+} or containing 1 mM Mg^{2+} and incubated at 37 °C. At the indicated times, triplicate 1 ml aliquots were withdrawn from each flask, centrifuged for 10 s in a microfuge, resuspended in nitrogen minimal medium containing 100 µM $^{63}Ni^{2+}$ and no added Mg^{2+}. Uptake was measured as previously described for 20 min at 37 °C (Snavely *et al.*, 1989; Grubbs *et al.*, 1989). The basal level of uptake in cells grown in 1 mM Mg^{2+} was 100–200 net c.p.m. $^{63}Ni^{2+}$ per 10^8 cells over the 20 min incubation period over a scintillation counter background of 100–200 c.p.m. The fold increase at each time point was calculated by dividing the uptake in cells grown without added Mg^{2+} by uptake in the cells grown with added Mg^{2+} after normalizing for cell number. The uptake in cells grown in 1 mM Mg^{2+} varied less than twofold over the time course of the experiment. The variation in uptake at each time point was ≤5% for cells grown without added Mg^{2+} and ≤20% for cells grown in 1 mM Mg^{2+}. The inset shows the same data at the early time points on an expanded scale.

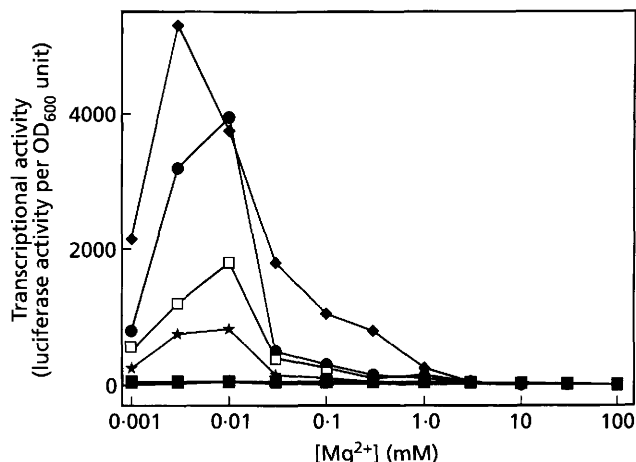

Fig. 7. Effect of Mg^{2+} and *phoP*/*phoQ* on transcription from the *mgtA* and *mgtCB* promoters measured after 6 h incubation. The parental strain for these studies was *S. typhimurium* 14028s which carries a wild-type *rpoS* allele. Transcription was measured as described in Methods and the legend to Fig. 2 after incubation with the indicated Mg^{2+} concentrations. At each time the data were normalized to cell density (OD_{600}) and to the apparent transcription at 100 mM Mg^{2+}. Absolute values of activity were similar in this experiment to those noted in the legends to Figs 2 and 3. ●, pTT-CLux; ■, pTT-CLux/*phoP*⁻; ▲, pTT-CLux/*phoQ*⁻; ◆, pTT-ALux; ★, pTT-ALux/*phoP*⁻; □, pTT-ALux/*phoQ*⁻.

al., 1989; Soncini & Groisman, 1996), regulates a variety of genes via its ability to sense Mg^{2+} indicates that Mg^{2+} transport and its regulation are of potential importance in pathogenesis. We had previously shown that *mgtCB* transcription is increased upon *S. typhimurium* invasion of MDCK epithelial cells (Garcia-del Portillo *et al.*, 1992). We therefore determined the time and Mg^{2+} dependence of *mgtA* and *mgtCB* transcription in the presence of mutations in *phoP* or *phoQ*. In the presence of either a *phoP* or a *phoQ* mutation, *mgtCB* transcription fails to be induced upon lowering of extracellular Mg^{2+}. This lack of increase in transcription occurs whether *mgtCB* transcription is measured after 6 h Mg^{2+} deprivation (Fig. 7) or after 12–24 h Mg^{2+} deprivation (data not shown). In sharp contrast, *mgtA* transcription does not appear to be completely under *phoPQ* control. Decreased extracellular Mg^{2+} elicits a substantial induction of *mgtA* transcription in the presence of either a *phoP* or a *phoQ* mutation (Fig. 7). The dose–response curve for *mgtA* induction also suggests that it is the second, later phase of *mgtA* transcription, induced by Mg^{2+} concentrations in the low micromolar range, that remains in the presence of a *phoP* or *phoQ* mutation. This dose dependence is also consistent with the concentration dependence of the interaction of Mg^{2+} with the PhoQ membrane sensor protein, which is in the range of 0·5 mM (Garcia-Vescovi *et al.*, 1996; Vescovi *et al.*, 1997). Together, these data suggest that a second level of transcriptional control exists for *mgtA* but that a similar second level of control may not exist for *mgtCB*.

Relationship of growth and transcriptional response

The transcriptional results presented above are somewhat complicated by the fact that at very low Mg^{2+} concentration in the growth medium, Mg^{2+} becomes growth limiting. We have examined this by measuring the medium Mg^{2+} concentration during growth (Fig. 8). In Mg^{2+} concentrations above about 0·2 mM in the supplemented nitrogen minimal medium described in Methods, Mg^{2+} is not growth limiting. With cell growth, an initial Mg^{2+} concentration in the medium of 1 mM falls rapidly to about 150–200 µM within 2 h and remains constant throughout the remainder of the incubation period even though the cells continue to increase in density for at least 6–8 h (data not shown). Cell viability remains constant between 2 and 24 h. In Mg^{2+} concentrations of 0·1 mM or below, Mg^{2+} concentration in the medium falls to about 5–10 µM over at least 6 h. With such low Mg^{2+} concentrations, OD_{600} doubles within 2–3 h and cell growth ceases even before

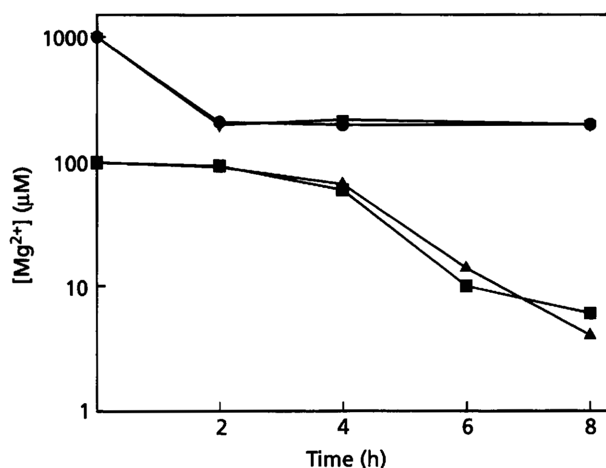

Fig. 8. Mg^{2+} content of growth medium during growth of cells. MM1269 (wild-type) and MM387 (*corA*) were inoculated at OD_{600} 0·1 into a 25 ml flask with the supplemented nitrogen minimal medium as described in Methods and grown at 37 °C with either 1 mM or 0·1 mM Mg^{2+} added to the medium. At each time point, a 1 ml aliquot was withdrawn, the cells removed by spinning in a microfuge for 1 min, and 0·5 ml of the supernatant withdrawn for analysis by atomic absorption using a Ca^{2+}/Mg^{2+} lamp and standards made in the same medium. Values are corrected for the approximately 15 μM Mg^{2+} inherent to the nitrogen minimal medium (Hmiel *at al.*, 1989). ▼, Wild-type (1 mM Mg^{2+}); ●, *corA* (1 mM Mg^{2+}); ▲, wild-type (100 mM Mg^{2+}); ■, *corA* (100 mM Mg^{2+})

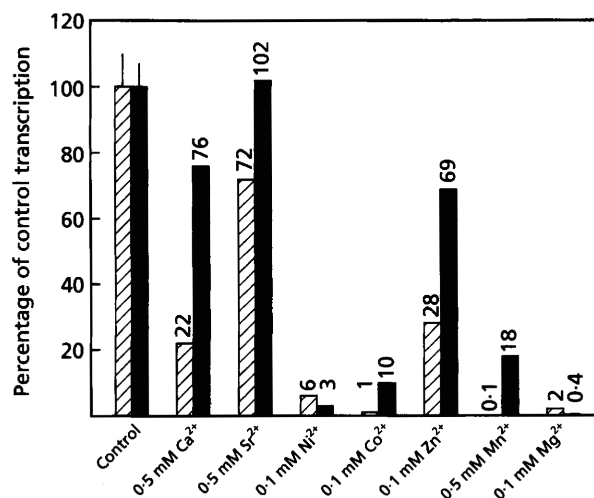

Fig. 9. Effect of various cations on transcription from the *mgtCB* promoter in cells carrying pTT-CLux. Transcription was measured as described in Methods. Cell aliquots were resuspended in the indicated concentrations of cation, transcription measured after incubation for 6 h (▨) or 24 h (■) and the values normalized to transcription at the respective time in the absence of Mg^{2+}. Activity of *luxAB* was measured in triplicate aliquots as described in Methods. The single experiment shown is representative of two such experiments. Triplicate aliquots measured for each individual culture varied ±10% of the absolute value and were similar under all conditions tested. Three independent cell cultures with no added Mg^{2+} were assayed in this experiment and shown as the control with the bars indicating SEM. The percentage transcription relative to the no added Mg^{2+} control is shown by the number above each bar. Absolute values of transcriptional activity were similar to those noted in the legends to Figs 2 and 3.

Mg^{2+} has fallen to its lowest level. Again, both Mg^{2+} concentration and cell viability remain constant for at least 24 h (data not shown). The initial cell density had little effect on these time courses, affecting only the final cell density. Interestingly, there was no difference in medium Mg^{2+} level or the time course of its depletion when tested in wild-type versus *corA* strains. This would imply that the source of Mg^{2+} (CorA versus the Mgt systems) has no direct influence on the cell's Mg^{2+} requirement.

Regulation by other cations

Both MgtA and MgtB mediate Mg^{2+} and, non-physiologically, Ni^{2+} influx, but each transporter has a distinct cation inhibition profile. Previous work has also shown that the initial phase of transcription of both *mgtA* and *mgtCB* induced by decreasing Mg^{2+} concentration could be blocked by the addition of 1 mM extracellular Ca^{2+} (Snavely *et al.*, 1991b), as measured using *mgtB*::*lacZ* fusions. Thus it was of interest to determine how other cations affected transcription of these loci. We therefore tested the Group IIa divalent cations Ba^{2+}, Sr^{2+} and Ca^{2+} and the transition metal cations Ni^{2+}, Co^{2+}, Mn^{2+} and Zn^{2+} for their ability to alter transcription at the *mgtA* and *mgtCB* loci. First, no cation could, of itself, elicit an *increase* in transcription when added in the presence of either low or high Mg^{2+} concentrations (data not shown). Second, a cation's ability to inhibit the increase in transcriptional activity elicited by the absence of extracellular Mg^{2+} depended on the specific cation tested. Of the Group IIa cations, Ca^{2+} but not Sr^{2+} (Fig. 9) blocks the *initial* increase in *mgtA* or *mgtCB* transcription. Ba^{2+} also was unable to block this initial increase (data not shown). About 1 mM Ca^{2+} is required for complete inhibition of transcription at *mgtA* and *mgtCB* (data not shown; Snavely *et al.*, 1991b). Since Ca^{2+} is not toxic in strains dependent on *mgtA* for Mg^{2+} uptake at concentrations that block the initial increase in transcription, the ability to repress the initial phase of transcription at both the *mgtA* and *mgtCB* loci is likely a specific effect of the cation, most probably mediated by binding to PhoQ (see below) which recognizes both Mg^{2+} and Ca^{2+}. In contrast to its ability to inhibit the initial phase of increased transcription, Ca^{2+} is largely ineffectual in blocking the second later phase of transcriptional activity completely (Fig. 9).

Among the transition metal cations, Co^{2+} blocks the increase in transcription at either locus (Fig. 9 and data not shown). Significant inhibition can be seen at <10 μM Co^{2+}, almost complete inhibition is seen at 100 μM Co^{2+} (data not shown), and both the early and late phases of the transcriptional response are inhibited. Test strain MM387, carrying a *corA* mutation, is

resistant to Co^{2+} concentrations up to 350 μM in the growth medium, although growth slows somewhat above 150 μM Co^{2+}. Thus the inhibition of transcription by Co^{2+} could be a combination of a direct effect on transcription of these specific genes and an indirect effect via general cell toxicity, but any toxic effect of Co^{2+} is probably minimal since Co^{2+} had little effect on *treR* transcription (see Methods). By the same argument, only a portion of the ability of Ni^{2+}, Mn^{2+} and Zn^{2+} to diminish transcription (Fig. 9) can be attributed to toxicity. Except possibly for Ca^{2+}, transcriptional inhibition by divalent cations other than Mg^{2+} is rather unlikely to be physiologically relevant since the concentrations required are much greater than the organism is likely to encounter routinely in its various environments. Even for Ca^{2+}, the levels required for repression are relatively high.

Since some reports have suggested that sulfate rather than or in addition to Mg^{2+} may play a role in gene expression (Gross & Rappuoli, 1989; Kertesz *et al.*, 1993; Scarlato & Rappuoli, 1991), we tested the effect of anions on *mgtA* and *mgtCB* transcription as a control. The ability of Mg^{2+} to repress transcription was independent of chloride or sulfate as the counter-ion; further, sulfate alone had no effect as seen by the inability of 10 mM Na_2SO_4 to alter transcription (data not shown).

DISCUSSION

The data presented in this report confirm and extend our previous work on the ability of Mg^{2+} to regulate transcription of its transport genes. The regulation is obviously complex. Distinct transcriptional effects can be seen at different times and different Mg^{2+} concentrations. Divalent cations other than Mg^{2+}, but not anions, also alter transcription. Despite some degree of cell toxicity from other cations, a major proportion of this transcriptional inhibition is probably a direct regulatory effect at the *mgtA* and *mgtCB* loci. Nonetheless, transcriptional inhibition by cations other than Mg^{2+} is unlikely to be of major physiological significance since relatively high concentrations of cation are required.

The mechanism of these effects on transcription is only partially known. The *phoPQ* two-component regulatory system (Garcia-Vescovi *et al.*, 1996; Soncini *et al.*, 1996) would appear to mediate the initial earlier transcriptional response based on the Mg^{2+} concentration required for half-maximal induction and the effect of *phoP* and *phoQ* mutations. These data are consistent with previous data (Miller *et al.*, 1989; Johnston *et al.*, 1996; Groisman *et al.*, 1989, Gunn & Miller, 1996; Guo *et al.*, 1997; Soncini *et al.*, 1996; Soncini & Groisman, 1996, Garcia-Vescovi *et al.*, 1996) showing that the *phoPQ* system is involved in both induction and repression of a relatively large set of genes important for virulence. However, the *phoPQ* system cannot be the only regulatory pathway involved. The most direct evidence for this is the ability of very low extracellular Mg^{2+} concentrations to induce *mgtA* transcription in the presence of a *phoP* or *phoQ* mutation. While the presence of a *phoP/Q* mutation markedly diminishes the transcriptional response at *mgtA*, it is clearly not abolished and still has a dose dependence on Mg^{2+} (Fig. 7). Additional evidence for a second regulatory pathway might be deduced from the fact that the second, later phase of *mgtA* and *mgtCB* transcription has a distinct half-maximal Mg^{2+} concentration dependence, well below the demonstrated affinity for Mg^{2+} interaction with *phoQ*. This interpretation is complicated however by the results in Fig. 7 showing complete dependence of *mgtCB* response on the presence of a functional PhoPQ system. The cation sensitivity data (Fig. 8) suggest that the phase of regulation with a half-maximal Mg^{2+} dependence of about 10 μM appears more selective for Mg^{2+}, while that operative at 1 mM Mg^{2+} may be sensitive to several divalent cations. *mgtA* and *mgtCB* are clearly not the only loci regulated by changes in extracellular Mg^{2+} concentrations and it would be of interest to determine if other *phoPQ*-regulated loci respond with a biphasic pattern. Other examples of apparent Mg^{2+} regulation of gene expression have also been reported (Scarlato & Rappuoli, 1991; Kiyota *et al.*, 1989; Guzzo *et al.*, 1991; Phinney & Hoober, 1992; O'Halloran, 1993).

The time courses of the responses of *mgtA* and *mgtCB* suggest that the initial phase of Mg^{2+}-dependent transcriptional response occurs during or near the end of active growth, but that the second phase, seen after several hours' incubation and at low extracellular Mg^{2+} concentrations, occurs only after cell growth has ceased (and while extracellular Mg^{2+} concentration remains constant). The question therefore arises whether the second phase of response seen with both *mgtA* and *mgtCB* is a stationary-phase response in some part. We do not believe it is. First, cells grown in moderate levels of Mg^{2+} to stationary phase do not show induction of *mgtA* or *mgtCB* regardless of how long they are left at high density. Second, more directly, the stationary-phase sigma factor encoded by *rpoS* has no effect of transcription of *mgtA* or *mgtCB*. The experiments shown in Figs 2–5 were performed in a strain background using *S. typhimurium* LT2 as parent. This nominal wild-type strain carries an *rpoS* mutation (Wilmes-Riesenberg *et al.*, 1997). (We have confirmed by catalase assay and introduction of a wild-type allele of *rpoS* that our strain of *S. typhimurium* LT2 is *rpoS*.) In contrast, the data of Fig. 7 were obtained in a strain background using *S. typhimurium* 14028s as parent, which is a virulent strain with a wild-type *rpoS* allele. From these data, there is little if any difference in the transcriptional response of *mgtA* or *mgtCB* in wild-type and *rpoS* strains. Thus we conclude that neither phase of the Mg^{2+}-associated transcription response at *mgtA* and *mgtCB* is necessarily tied to growth state.

These data also suggest that MgtA and MgtB are not (solely) scavenger systems for Mg^{2+}. Classically, multiple transport systems for nutrients consist of a relatively poor-affinity transporter(s) operative during

growth in media rich in that nutrient while a high-affinity transporter is expressed in media with a very low concentration of that substance. Classic examples are the K^+ transporters of *Escherichia coli*. The TRK system has an affinity of 1–2 mM for K^+ uptake, while in low extracellular K^+ the Kdp P-type ATPase is induced and has an affinity for K^+ of 1–3 μM (Altendorf & Epstein, 1994; Epstein *et al.*, 1993; Dosch *et al.*, 1991). The MgtA and MgtB systems do not fit this model. First, all three Mg^{2+} transporters of *S. typhimurium* and *E. coli* have affinities for Mg^{2+} of between 5 and 30 μM. Second, as noted above, an increase in *mgtA* and *mgtCB* transcription is seen at extracellular Mg^{2+} concentrations at which the constitutive CorA system is still capable of supplying sufficient Mg^{2+}. Third, unlike scavenger systems, both MgtA and MgtB transport the physiologically relevant cation down its electrochemical gradient. Even at very low extracellular Mg^{2+}, the electrochemical gradient for Mg^{2+} should still be directed inward. The purpose of using ATP is therefore unclear. Fourth, it is unclear why the cell would possess two Mg^{2+} uptake carriers. Although they have similar properties and regulation, the nucleotide sequences of *mgtA* and *mgtCB*, and the amino acid sequences of their products, are not exceptionally similar. This suggests that the alleles must have been maintained separately within *S. typhimurium* for a considerable time in evolutionary terms or that one of the ATPases has been acquired by *Salmonella* by horizontal transfer. Presumably the cell would not maintain two such functionally similar alleles unless one or both had some specific, useful property. Thus, while the extraordinarily large transcriptional responses of these loci indicate that they play a role in providing Mg^{2+} at very low extracellular concentrations, this may not be their only role. Indeed, it is possible that Mg^{2+} transport is a secondary function, with their primary role yet to be discovered.

Finally, it must be noted that the function of the *mgtC* gene is currently unknown. The MgtC protein sequence yields no clues from current database entries. It is entirely possible the Mg^{2+} regulation described herein is directed primarily at expression of the MgtC protein rather than at MgtA or MgtB. The complex regulation of the two loci suggests that these genes are expressed under some specific growth or metabolic condition. Regardless of the mechanism of regulation and the growth conditions under which regulation of these loci occur, these data demonstrate that extracellular Mg^{2+} can have a profound influence on gene expression, indicating that Mg^{2+} may play an important role in cellular metabolic homeostasis and gene expression.

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