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Genetic regulation of galactokinase in *Tetrahymena* by cyclic AMP, glucose, and epinephrine

(transcription/dibutyryl adenosine 3′:5′-cyclic monophosphate)

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ABSTRACT **We have found evidence that transcription of the galactokinase (ATP:D-galactose 1-phosphotransferase; EC 2.7.1.6) gene is inhibited, in the animal-like protozoan *Tetrahymena*, by dibutyryl adenosine 3′:5′-cyclic monophosphate, glucose, and epinephrine. The specific activities of galactokinase in *Tetrahymena* cells grown in defined media with galactose or glycerol as the principal carbon source are equivalent; the specific activity in glucose minimal medium is 1/7 the value. Thus, while there seems to be no specific induction of the enzyme by the substrate, galactose, there is a strong "repression" by glucose. This repression by glucose is *mimicked*, in glycerol-grown cells, by the addition of millimolar amounts of dibutyryl adenosine 3′:5′-cyclic monophosphate or phosphodiesterase inhibitors such as caffeine and theophylline. When glucose-grown cells are washed and resuspended in carbohydrate-free medium, the galactokinase specific activity increases by as much as 10-fold within 12 hr. This increase is blocked by dibutyryl adenosine 3′:5′-cyclic monophosphate and by epinephrine (synthesized by *Tetrahymena*, and previously shown to activate a membrane-bound adenylate cyclase in extracts of this organism), as well as by inhibitors of mRNA synthesis, maturation, and translation. Our results suggest that glucose and epinephrine can regulate transcription of the galactokinase gene by modulation of cyclic nucleotide levels. The observation that the nonmetabolized sugars 2-deoxyglucose, 2-deoxygalactose, and α-methylglucoside are as effective as glucose suggests that the sugar itself, or an immediate metabolite such as the 1-phosphate derivative, may be the effector.**

While many aspects of gene regulation in prokaryotic cells have been elucidated at the molecular level, success in the analysis of genetic control mechanisms in higher eukaryotes has been limited. Progress has been hampered, in part, by the lack of eukaryotic model systems amenable to both biochemical and genetic manipulation, which were largely responsible for the progress made with *Escherichia coli* in the study of prokaryotic gene expression.

Tetrahymena thermophila [formerly *Tetrahymena pyriformis*, syngen 1 (1)] is a unicellular eukaryote which, by virtue of its extensive biochemical (2) and genetic (3) characterization and tractability, appears to be especially suitable as a model system for the investigation of gene regulation in animal cells. Although nutritionally similar to mammalian cells, *Tetrahymena* can be grown rapidly in a defined minimal medium, in which growth is strongly stimulated by various carbohydrates (unpublished observations); we have taken advantage of this system to study the regulation of galactose metabolism in this animal-like eukaryote. Since the regulation of galactose metabolism has been extensively investigated in bacteria (4) and yeast (5, 6), it could be expected that results derived from this investigation, in addition to their relevance to the control of gene expression in animal cells, may provide some insights into the evolution of genetic regulatory mechanisms.

In this paper, we describe experiments which suggest that the expression of the galactokinase (ATP:D-galactose 1-phosphotransferase; EC 2.7.1.6) gene in *Tetrahymena* can be regulated at the transcriptional level by glucose and the hormone epinephrine, acting through modulation of adenosine 3′:5′-cyclic monophosphate (cAMP) levels; this cyclic nucleotide mediates *inhibition* of transcription of the galactokinase gene.

METHODS AND MATERIALS

Cells. All experiments reported in this paper were performed with wild-type strain BIII of *Tetrahymena thermophila* [formerly *Tetrahymena pyriformis*, syngen 1 (1)].

Growth Conditions. For enzyme assays, 15-ml cultures were grown in 25 × 200 mm screw-cap test tubes on a roller at 30°. Larger cultures (50–100 ml) were grown at 30° in 125- or 250-ml erlenmeyer flasks on a rotary shaker set at 110 rpm. Cultures were inoculated at an initial density of 2–5 × 10^3 cells per ml.

Media. Cells were grown in a defined, minimal medium containing amino acids, vitamins, guanosine, uracil, trace metals, and salts (unpublished data). Penicillin and streptomycin (250 μg/ml) were present to prevent bacterial growth. Significant growth in this medium is dependent upon the presence of added carbohydrate. When present, carbohydrates were added at a concentration of 1% (wt/vol); growth rates in the carbohydrate-supplemented media were approximately one doubling per 8–12 hr. Resuspension buffer contains 2 mM Na citrate/1 mM Na_2HPO_4/1 mM NaH_2PO_4/1.5 mM $CaCl_2$ (7).

Preparation of Cell Extracts. Exponential-phase cultures (15 ml; 1–2 × 10^4 cells per ml) were harvested in 15-ml conical centrifuge tubes by centrifugation at room temperature for 2 min at 700 × *g* in an International clinical centrifuge. The cell pellets were then resuspended in 5 ml of buffer warmed to 30°, mixed with a Vortex mixer, and recentrifuged for 1.5 min. This step was repeated and the final pellets were resuspended in 0.5 ml of 50 mM Tris·HCl, pH 7.5/2 mM dithiothreitol, at 0°. These suspensions were transferred to 10.9 × 77 mm plastic centrifuge tubes, sonicated (50 W, 30 sec) with a Branson sonifier, and centrifuged for 20 min at 40,000 × *g* in a Sorval RC-5 refrigerated centrifuge at 4°. The supernatant, which contained 0.5–1.5 mg of soluble protein per ml, was removed with a fine-tipped pasteur pipette and used for enzyme assays. Protein was determined by the procedure of Lowry *et al.* (8).

Galactokinase Assay Procedure. Reaction mixtures con-

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Abbreviations: cAMP, adenosine 3′:5′-cyclic monophosphate; Bt_2cAMP, N^6,$O^{2'}$-dibutyryl adenosine 3′:5′-cyclic monophosphate.

Table 1. Reaction requirements for galactokinase assay

Reaction mixture	Relative activity, %
Complete	100
Omit extract	1.9
Boiled extract (3 min, 90°)	2.3
Omit EDTA	87
Omit Mg^{2+}	2.4
Omit ATP	2.4

Conditions for the assay (60 min, 37°) were as described in *Methods and Materials*; the complete system contained 50 μl of an extract prepared from cells grown in glycerol-supplemented medium.

tained 50 mM Tris·HCl (pH 7.5), 2 mM dithiothreitol, 5 mM ATP, 10 mM $MgCl_2$, 5 mM EDTA, 1 mM D-galactose, 0.5 μCi of D-[*1*-^{3}H]galactose (specific activity 14.2 Ci/mmol), and 0–50 μl of cell extract in a total volume of 100 μl. Reaction mixtures were incubated at 37° in 10 × 75 mm glass tubes for 1 hr; the reactions were then terminated and enzyme was denatured by incubation at 90° for 3 min. Reaction mixtures containing 50 μl of denatured cell extract were incubated as blanks. Duplicate 25-μl aliquots of each reaction mixture were spotted on Whatman DE-81 filter discs (23 mm diameter) and air-dried. To separate free galactose from the galactose-1-phosphate bound to the DEAE-paper, the prenumbered filters were washed in bulk in a 1-liter erlenmeyer flask with three successive 500-ml aliquots of 5 mM D-galactose, followed by one wash with 95% ethanol. The filters were then dried under an infrared lamp for 10 min, and radioactivity was measured by conventional liquid scintillation techniques. Overall counting efficiency was determined for each experiment by spotting 25 μl of a solution containing a known amount of D-[*1*-^{3}H]galactose on filters that had been washed as described above, and then drying and counting. Enzyme units are expressed as nmol of galactose 1-phosphate produced per hr at 37°. The errors shown in the tables and figures represent one standard deviation.

Reagents. Chemicals were obtained from the following sources: glucose from Mallinckrodt; galactose, lactose, maltose, raffinose, melibiose, 2-deoxyglucose, and 2-deoxygalactose from Pfansteihl; actinomycin D, puromycin, norepinephrine, and caffeine from Calbiochem; cellobiose from Matheson, Coleman and Bell; stachyose, α-methylglucoside, *p*-fluorophenylalanine, cordycepin, bacterial alkaline phosphatase, epinephrine, serotonin, Tris, EDTA, dithiothreitol, N^6,$O^{2'}$-dibutyryl adenosine 3′:5′-cyclic monophosphate (Bt_2cAMP), and cAMP from Sigma; theophylline from Schwarz/Mann; glycerol from Baker; and D-[*1*-^{3}H]galactose (specific activity 14.2 Ci/mmol) from New England Nuclear.

cAMP, Bt_2cAMP, and cordycepin were dissolved in buffer A of Makman and Sutherland (9) prior to addition to cell cultures or reaction mixtures.

RESULTS

Characteristics of Galactokinase Assay. Under the conditions used, the conversion of galactose to a derivative that binds to DEAE-paper (presumably galactose 1-phosphate) is dependent upon the presence of an undenatured *Tetrahymena* cell extract, ATP, Mg^{2+}, and, to a lesser extent, EDTA (Table 1). The yield of the reaction is linearly proportional to the time of incubation for 90 min and (in the range of concentrations assayed) is essentially proportional to the amount of cell extract added. To verify that the product measured by this procedure is, in fact, galactose 1-phosphate (rather than some other product or conjugate of the [^{3}H]galactose that similarly might be adsorbed by DEAE-paper), we determined the sensitivity of the final product to bacterial alkaline phosphatase (EC 3.1.3.1). Aliquots of a final galactokinase reaction mixture (after reaction and heat denaturation) were treated with H_2O, a buffer containing 0.5 mM NH_4HCO_3/0.1 mM $ZnSO_4$/25 mM $MgSO_4$, or this buffer containing bacterial alkaline phosphatase (5 units/ml), prior to application and analysis of samples on DEAE-paper filters. Results of this experiment indicate that ≥97% of the DEAE-bound reaction product is, in fact, galactose phosphate.

Table 2. Effect of carbohydrate on galactokinase specific activity

Carbohydrate	Galactokinase: Specific activity, units/mg	Galactokinase: Relative specific activity, %
Glycerol	650 ± 55	100
Galactose	543 ± 100	83
Lactose	634 ± 84	97
Stachyose	432 ± 65	66
Maltose	296 ± 21	46
Glucose	99 ± 7	15

Exponential-phase cells grown in glycerol were washed twice in buffer and used to inoculate media containing the indicated carbohydrates present at 1% (wt/vol). These cultures were grown at 30° for 24 hr; cells were harvested and extracts prepared for assay as described in *Methods and Materials*.

Galactokinase Levels in Presence of Various Carbohydrates. To determine if intracellular levels of galactokinase are regulated by the presence of galactose or other carbohydrates, the steady-state specific activity of galactokinase was determined in cells grown for several generations in the presence of various sugars. There is no increase in galactokinase levels in the presence of galactose or galactose-containing oligosaccharides such as lactose or stachyose (Table 2). Other sugars such as melibiose, raffinose, and cellobiose give results similar to galactose (data not shown). The most striking result was the extremely low galactokinase activity observed in the presence of glucose. Mixing experiments performed with extracts from cells grown in glucose and either galactose or glycerol (which consistently gave the highest galactokinase specific activity) failed to show the presence of any inhibitors or activators affecting the activity of preformed enzyme in the cell extracts.

Effect of Bt_2cAMP on Galactokinase Levels. The results shown in Table 2 suggest that repression of enzyme activity by glucose is a major feature of the physiological regulation of galactokinase levels in *Tetrahymena*. Since the repression of galactokinase activity by glucose in *E. coli* (10–12) and yeast (13–15) appears to reflect a *decrease* of intracellular cAMP, and can be prevented by supplementing glucose-grown cultures of these organisms with exogenous cAMP, the effect of Bt_2cAMP on galactokinase levels in glucose- and glycerol-grown cells was investigated (Table 3). The effect of glucose on galactokinase levels can be mimicked in glycerol-grown cells by millimolar concentrations of exogenous Bt_2cAMP or by cyclic nucleotide phosphodiesterase inhibitors such as caffeine and theophylline, rather than reversed in glucose-grown cells, as in *E. coli*. Caffeine and theophylline are effective in inhibiting the cyclic nucleotide phosphodiesterase (16, 17) and elevating internal cAMP levels (18) in *Tetrahymena*. The repression of galactokinase by glucose and Bt_2cAMP together is less than strictly additive (Table 3), suggesting that glucose and Bt_2cAMP may effect repression through a common mechanism; evidence for such a glucose-mediated increase in internal cAMP levels has been obtained in *Tetrahymena* (19), and may account for the data in Table 3.

cAMP regulates the activity of a variety of proteins through

Table 3. Effect of Bt_2cAMP on galactokinase specific activity in glycerol and glucose

Additions to medium	Galactokinase specific activity
Glucose	173
+ Bt_2cAMP (1 mM)	101
+ Bt_2cAMP (5 mM)	45
Glycerol	910
+ Bt_2cAMP (1 mM)	267
+ Bt_2cAMP (2.5 mM)	144
+ Bt_2cAMP (5 mM)	135
+ Caffeine (2 mM)	40
+ Theophylline (2 mM)	88

Cultures were prepared and assayed as described in Table 2, with additions to the final growth media as indicated above.

the activation of target-specific protein kinases (20). To investigate the possibility that the effect of Bt_2cAMP on galactokinase levels might be due to the action of a protein kinase on preformed galactokinase, we added samples of a crude extract derived from cells grown in glycerol to galactokinase reaction mixtures without galactose and preincubated them for various periods of time in the presence and absence of Bt_2cAMP prior to initiation of the galactokinase assay by addition of substrate. Since the reaction mixtures contained ATP and Mg^{2+}, any cAMP-dependent, galactokinase-specific protein kinase present in the crude extract would be active during the preincubation. The results of this experiment, however, show no significant effect of Bt_2cAMP on galactokinase activity in crude extracts; thus, the effect of Bt_2cAMP on galactokinase activity does not appear to be mediated through inactivation of the preformed enzyme by a protein kinase.

Derepression of Galactokinase in Cells Pregrown in Glucose. The experiments described above were all performed with cells pregrown in glycerol media (in which galactokinase specific activity is high) which were then transferred to other media for evaluation of subsequent effects on galactokinase levels 24 hr later. Since the results of previous experiments suggest that glucose and Bt_2cAMP were not regulating the activity of preformed galactokinase, control of enzyme synthesis was measured directly. Cells pregrown in glucose were transferred to various media with or without carbohydrates, nutrients, or Bt_2cAMP to ascertain the effect of these conditions on the *appearance* of galactokinase activity (Table 4). We find a significant increase in galactokinase activity in cells transferred from glucose to glycerol; this increase is blocked by the additional presence of Bt_2cAMP (Table 4, Exp. A; compare Table 3). Transfer of cells to medium that is otherwise complete, but contains no carbohydrate, actually results in a greater increase in enzyme level after 16 hr than that seen in the culture with added glycerol; these data may reflect repression resulting from gluconeogenesis occurring in the culture with glycerol. That the increase in galactokinase seen in the carbohydrate-free medium is not simply a result of the decreased cell division is suggested by the observation that cells transferred to buffer (in which cell division is also arrested) show very little increase in galactokinase levels after removal of glucose. This latter result also suggests that the increased enzyme levels seen after glucose removal may be the result of *de novo* enzyme synthesis, since the carbohydrate-free medium, in which a large increase is observed, contains compounds required for RNA and protein synthesis which are absent from the buffer. The kinetics of derepression of galactokinase in the absence of significant cell growth are shown in Fig. 1.

Table 4. Effect of various additions to growth media on derepression of galactokinase

Additions to medium	Galactokinase: Specific activity, units/mg	Galactokinase: Relative specific activity, %
Experiment A		
Glucose	44	8
Glycerol	327	62
+ Bt_2cAMP (1 mM)	119	23
+ Bt_2cAMP (2.5 mM)	65	12
No carbohydrate	524	100
Resuspension buffer	131	25
Experiment B		
Glucose	90	17
+ Bt_2cAMP (1 mM)	44	8
+ Bt_2cAMP (2 mM)	34	6
No carbohydrate	544	100
+ Bt_2cAMP (1 mM)	192	35
+ Bt_2cAMP (2 mM)	172	32
Experiment C		
Glucose	130	18
No carbohydrate	728	100
+ Caffeine (1 mM)	402	55
+ Theophylline (1 mM)	621	85
+ Caffeine (1 mM) + cAMP (10 mM)	352	48
+ Theophylline (1 mM) + cAMP (10 mM)	492	68
Experiment D		
Glucose	61	14
No carbohydrate	440	100
+ Norepinephrine (0.5 mM)	284	65
+ Norepinephrine (1 mM)	46	10
+ Epinephrine (0.5 mM)	235	53
+ Epinephrine (1 mM)	156	35
+ Serotonin (1 mM)	436	99
Experiment E		
Glucose	106	30
No carbohydrate	362	100
+ 2-Deoxyglucose (250 μg/ml)	54	15
+ 2-Deoxygalactose (250 μg/ml)	74	20
+ α-Methylglucoside (1 mg/ml)	154	43
Experiment F		
Glucose	61	14
No carbohydrate	440	100
+ Actinomycin D (1 μg/ml)	220	50
+ Actinomycin D (2 μg/ml)	113	26
+ Cordycepin (50 μg/ml)	117	27
+ Cordycepin (200 μg/ml)	32	7
+ p-Fluorophenylalanine (100 μg/ml)	38	8
+ Puromycin (0.5 mM)	35	8

Exponential-phase cells grown in glucose were washed twice in carbohydrate-free medium (or resuspension buffer for the last culture in Exp. A) and used to inoculate media (or buffer) supplemented as described above. Cells were harvested and assayed after incubation at 30° for 16 hr (Exp. A) or 12 hr (Exps. B–F); results of galactokinase assays are shown normalized to the value obtained in the absence of carbohydrate in each experiment.

As seen in Table 4 (Exp. B), Bt_2cAMP inhibits the derepression of galactokinase in carbohydrate-free medium after removal of glucose. Caffeine and, to a lesser extent, theophylline

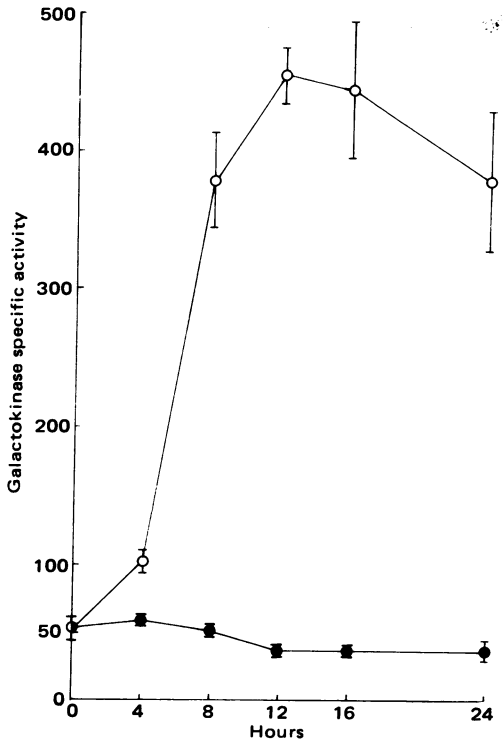

FIG. 1. Kinetics of derepression of galactokinase after removal of glucose. A logarithmic phase culture of cells grown in glucose was harvested by centrifugation and washed twice in carbohydrate-free medium. These cells were used to inoculate 15-ml cultures in media containing no carbohydrate (○) or 1% (wt/vol) glucose (●). These cultures were incubated at 30° prior to harvesting and assaying for galactokinase.

are also inhibitory; their effects are increased by the presence of 10 mM cAMP itself (Table 4, Exp. C). We find, however, that this concentration of cAMP alone has no effect on the increase in galactokinase activity after removal of glucose (data not shown), thus suggesting that the effect of caffeine and theophylline is to protect exogenous or endogenous cAMP from degradation.

Tetrahymena contains both epinephrine (21) and serotonin (22), the hormonal effects of which are often mediated through cAMP in higher systems. These compounds can elevate intracellular cAMP concentrations in *Tetrahymena in vivo* (23, 24); activation of a *Tetrahymena* membrane-bound adenylate cyclase has also been demonstrated *in vitro* (25). Since glucose may regulate galactokinase synthesis through increased cAMP levels, we investigated the effect of these hormones on the derepression of galactokinase (Table 4, Exp. D). Clearly, millimolar concentrations of epinephrine or its immediate precursor, norepinephrine, can completely inhibit the derepression of galactokinase in carbohydrate-free medium. At an equivalent concentration, however, serotonin has no effect on galactokinase derepression; this may reflect the weaker activation of adenylate cyclase by serotonin observed *in vitro* (25). We cannot yet conclude, however, that glucose controls the *in vivo* expression of the galactokinase gene through epinephrine-stimulated cAMP synthesis.

In an attempt to determine the chemical nature of the compound responsible for repression of galactokinase by glucose, the ability of nonmetabolized analogues of glucose to mimic the effect of glucose was evaluated (Table 4, Exp. E). Millimolar concentrations of 2-deoxyglucose, 2-deoxygalactose, and α-methylglucoside were as effective as glucose itself in repressing the expression of the galactokinase gene. α-Methylglucoside (26) and 2-deoxyglucose (27) are phosphorylated but not further metabolized, while 2-deoxygalactose is converted by the enzymes of the Leloir pathway to UDP-2-deoxyglucose and possibly to 2-deoxyglucose-1-phosphate (28). Since the only feature common to the metabolism of these compounds is the synthesis of a glucose-phosphate analogue, it may be likely that the latter compound, or its synthesis, is responsible for cAMP-mediated repression of galactokinase in *Tetrahymena*.

The presence of inhibitors of the synthesis (actinomycin D), maturation (cordycepin, which is 3-deoxyadenosine), or translation (*p*-fluorophenylalanine and puromycin) of mRNA all prevent the increase in galactokinase levels otherwise seen upon transfer from glucose-containing to carbohydrate-free media (Table 4, Exp. F).

DISCUSSION

The results of this study lead us to propose that high concentrations of glucose result in the inhibition of transcription of the galactokinase gene of *Tetrahymena* through elevation of the intracellular concentration of cAMP. Since the same effect on galactokinase synthesis is obtained with epinephrine, glucose may actually modulate cAMP levels indirectly through effects on intracellular epinephrine levels rather than by a direct effect on adenylate cyclase or cyclic nucleotide phosphodiesterase activities.

The mechanism by which cAMP inhibits the transcription of the galactokinase gene in *Tetrahymena* may involve the activation of a cAMP-dependent protein kinase (29) specific for nonhistone chromosomal proteins (30) or some component of an RNA polymerase (31). Although most regulatory effects of cAMP thus far identified in *E. coli* (32), yeast (15), and *Neurospora* (33) involve the *stimulation* of enzyme synthesis, cAMP has been reported to decrease the activity of the enzymes of glutamate and glutamine interconversion in *E. coli* (34). In mammalian systems, cAMP has been shown to increase the activity of a number of enzymes, but apart from the induction of serine dehydratase (35) and mitochrondial glutamic dehydrogenase (36), these stimulatory effects of cAMP appear to be at a post-transcriptional level (37–39). The induction of several liver enzymes by various treatments has been reported to be repressed by cAMP (40–42). These findings suggest that *inhibition* of gene expression by cAMP in prokaryotes and eukaryotes may be more widespread than previously believed. The study reported here documents inhibition of specific gene transcription by this nucleotide.

The present studies also show that the regulation of galactokinase gene expression in *Tetrahymena* differs markedly from that found in *E. coli* (4) and yeast (5). In these systems, galactokinase synthesis is specifically induced by galactose and this induction is prevented by a glucose-mediated drop in intracellular cAMP levels [effected through the enzymatic phosphorylating components of the glucose membrane transport system (43)]. In *Tetrahymena*, on the other hand, there is no specific induction of galactokinase synthesis by galactose; there is repression by glucose, which in this case apparently is mediated by an *increase* in intracellular cAMP. In a forthcoming communication (J. L. Botsford, C. T. Roberts, Jr., and D. E. Morse, unpublished data) we show that the intracellular levels of cAMP in cells grown in glucose under the conditions described here are indeed significantly higher than those in cells from carbohydrate-free or glycerol-supplemented media. These findings, made with a simple eukaryote with animal-like hormones and metabolism, may provide some guidelines for the

eventual elucidation of the complex and often contradictory data obtained in studies of the regulation of the enzymes of galactose metabolism in human cells (44–47). We have recently isolated and characterized mutants of *Tetrahymena* deficient in galactokinase, which will facilitate further genetic and biochemical analyses of the regulatory mechanisms we have described here. Exploitation of this experimental system should provide a unique opportunity for continued characterization of important genetic regulatory mechanisms in animal cells.

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