Regulated expression at high copy number allows production of a growth-inhibitory oncogene product in *Drosophila* Schneider cells

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The *Drosophila* metallothionein promoter (Mtn) was used to obtain efficient, regulated expression of foreign gene products inserted in high copy numbers into *Drosophila melanogaster* Schneider 2 cells. An expression unit comprised of a reporter gene [*Escherichia coli* galactokinase (*galK*)] fused to the Mtn promoter was stably introduced into Schneider 2 cells in up to several hundred copies per cell in a single transfection-selection event. This system contrasts dramatically with other eukaryotic systems that permit only a few copies of a gene to be stably inserted in a single transfection-selection event. The transfected *Drosophila* S2 cell lines expressed high levels of both *galK* mRNA and protein in response to metal induction. Most important, and in contrast to mammalian cells, expression remained fully regulated even at high copy number, with low basal expression maintained in the absence of inducer. This regulated system was used to obtain efficient expression in *Drosophila* cells of an otherwise lethal or growth-inhibitory gene product, the human H-*ras* oncogene. The ability to obtain regulated high-level expression of potentially lethal foreign proteins is unique to the *Drosophila* cell system.

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Metallothioneins (MTs) are low-molecular-weight, cysteine-rich proteins that bind heavy metals. Although their complete physiological role is not yet known, they appear to protect cells against toxic metals and may be involved in homeostatic regulation of essential metals such as copper and zinc (Hamer 1986; Kagi and Kojima 1987; Karini et al. 1983; Otto et al. 1986). In mammals, there are multiple MT genes whose transcription is regulated by heavy metals (Brinster et al. 1982; Durnam et al. 1981; Hamer and Walling 1982; Karin et al. 1984). Several of these genes have been cloned and were found to retain their metal-regulated expression following transfer at low gene copy number into heterologous animal cells (Hamer and Walling 1982; Mayo et al. 1982; Searle et al. 1984; Otto et al. 1987). Two MT genes *Mtn* and *Mto* have been isolated from *Drosophila melanogaster*. The complete nucleotide sequence of *Mtn* has been determined, and its regulatory elements have been identified (Lastowsky-Perry et al. 1985; Maroni et al. 1985; Otto et al. 1987). Recently, it was demonstrated that a chimeric *Drosophila* gene driven by the Mtn pro-

motor was transcriptionally regulated when introduced into cultured *Drosophila* cells (Bunch et al. 1988). The *Mtn* gene was also introduced into *Drosophila* flies, as well as into baby hamster kidney cells, and was found to retain metal-regulated expression in both systems. However, the relative activity of the *Drosophila* Mtn promoter in hamster cells was only 5% of the mouse MT-I promoter in the same system.

The mouse metallothionein I (MT-I) promoter region has been used to express a variety of mammalian gene products in cultured mouse cells. For example, genes encoding human growth hormone, hepatitis B surface antigen, and influenza hemagglutinin, have been fused to the regulatory sequences of the MT-I gene on bovine papilloma virus vectors and expressed at high levels (Pavalakis and Hamer 1983; Hsiung et al. 1984; Sambrook et al. 1985). Expression from an exogenously introduced MT-I promoter also has been monitored transiently in R1610 hamster cells and C127 mouse cells and found to give only a 4- to 5-fold increase in expression when fully induced with heavy metals (Pavalakis and Hamer 1983; Johansen et al. 1984), whereas the endogenous single-copy gene reaches induction levels of up to 50-fold. Ap-

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parently, introducing the promoter at higher copy number results in increased basal levels of expression, which, in turn, result in loss of regulation and increased constitutive expression.

In this paper we investigate the efficiency and regulation of the *Drosophila* Mtn promoter in *Drosophila* S2 cells and the use of Mtn to express foreign genes in *Drosophila* cell culture in a regulated manner. We demonstrate that, in contrast to mammalian systems, the *Drosophila* Mtn promoter can be used to obtain regulated, efficient, and stable expression of genes introduced into *Drosophila* cells at high copy number in a single transfection-selection event. This system permits selective and regulated expression of gene products that may exhibit lethal or growth-retarding effects when expressed. We demonstrate the application of this system to the regulated expression of the proto-oncogenic and oncogenic forms of the human *H-ras* gene in *Drosophila* cells.

Results and discussion

Stable integration of a pMtn-directed transcription unit at varying copy number

To characterize the function of the *Drosophila* Mtn promoter in *Drosophila* cells, we fused it to a reporter gene, *Escherichia coli* galactokinase (*galK*), and introduced it stably into *Drosophila* cells at varying copy number (Fig. 1). *galK* serves as a reporter gene in this cell type because no endogenous *galK* protein or activity is found in S2 cells (Kamerow et al. 1981). The vector carrying the pMtn-*galK* chimeric transcription unit was introduced into S2 cells by cotransfection with a second vector, which carries a selectable marker *E. coli dhfr* gene (pHGCO), which confers resistance to methotrexate selection or carries the *E. coli* hygromycin B phosphotransferase gene (*pcodhygro*), which, in turn, confers resistance to hygromycin B. Either vector can be used to achieve stably integrated copies of transfected DNAs (Bourouis and Bruno 1983; Van der Straten et al. 1989). To achieve integration of high copy number of the *galK* gene, we used a ratio of 1 : 1 of the pMtK to pHGCO vector or a ratio of 10 : 1 of the pMtK to *pcodhygro* vector. For the following experiments, we used cotransfection with the pHGCO vector to obtain methotrexate-resistant cell lines that contained different copy numbers of the pMtK vector DNA. Copy number was controlled by varying the ratio of pMtK to pHGCO DNA used in the cotransfection. Independent transfections were performed using pMtK to pHGCO ratios of A 1 : 100, B 1 : 10, and C 1 : 1. In each case, methotrexate-resistant cell lines were generated, chromosomal DNA was isolated, and integrated sequences examined by genomic Southern blot analysis, using both *galK*- and *dhfr*-specific probes. The results show that cell lines selected at all three vector ratios contain both vector DNAs. Moreover the *galK* and *dhfr* copy numbers indeed varied in the three cell lines in proportion to the ratios of input DNA (Fig. 2, lanes 1–3). The three cell

lines were estimated to contain ~1–2, 50, and ~500 copies of the pMtn-*galK* transcription unit, respectively (Fig. 2, lanes 3, 2, 1). All three cell lines were passaged both with and without selection for several months in culture and reexamined by genomic Southern blot analysis. No change in copy number was observed, indicating that the sequences had been stably integrated into the host cell. We emphasize that this system allows for rapid selection of stable cell lines with varying copy number of exogenously introduced sequences and that copy numbers up to ~500 are readily obtained in a single transfection-selection experiment.

Regulated gene expression by pMtn at varying copy number

We examined both expression levels and metal regulation in the cell lines described above by measuring the product of the reporter gene *galK*. Cells were harvested before and after metal induction, and total cellular protein was analyzed by SDS-PAGE, followed by Western blot analysis using *galK*-specific antisera (Fig. 3). Alternatively, the cell extract was subjected directly to *galK* enzymatic assay. The recombinant *galK* product produced in these cells was found to be of the expected size, 41 kD, and to be fully active, indicating that *Drosophila* cells correctly express the bacterial gene. Moreover, *galK* expression now was subject to metal regulation. Addition of heavy metals resulted in a dramatic increase (~50-fold) of *galK* expression, as measured by radiograph scanning (Fig. 3) and dot blot analysis (data not shown). Most remarkably, all of the cells, including cell line C containing the very high copy number (~500 copies) of the *galK* transcription unit, retained full regulation and expressed only very low or undetectable basal levels of *galK* in the uninduced state (Fig. 3).

To establish optimum induction conditions, the high-copy-number cell line C was induced using various concentrations of cadmium, copper, or zinc for different lengths of time. Maximum induction with cadmium occurred using 10 μ M CdCl₂ (Fig. 3), with product accumulation beginning about 4 hr after the start of cadmium treatment and reaching a maximal level after 20 hr of induction. This high level of *galK* synthesis could be maintained for 14 days of continuous induction. Copper was also an effective inducer but required a 20-fold higher concentration of metal (i.e., 200 μ M CuSO₄) to reach maximum expression (Fig. 3). ZnSO₄ also was tested for its inducing capability and was found to be a relatively poor inducer. We conclude that among the metals tested, cadmium was the most effective inducing agent and that the same high level of expression could be reached using a 20-fold higher concentration of CuSO₄. Further analysis indicated that cells could be maintained for up to 4 months in the presence of 10 μ M CdCl₂ or 200 μ M CuSO₄ without any apparent detrimental effect to growth and could maintain their fully induced levels of expression.

We also characterized the RNA induction levels and ratios of the three different cell lines A, B, and C car-

Figure 1. Construction of the pMtK vector. The pSV40K vector containing the *E. coli galk* gene fused to the SV40 promoter was digested with *Sma*I and *Hind*III, which excised the SV40 early promoter from the vector. The vector was subsequently end-filled and ligated to an end-filled 430-bp *Eco*RI-*Stu*I fragment containing the *D. melanogaster* Mtn regulatory sequences. The Mtn promoter then was fused to the *galk*-coding sequences in such a fashion that the untranslated leader sequences consist of 60 bp derived from the Mtn 5'-noncoding leader region fused to 70 bp of the 5' leader segment from the *E. coli galk* operon.

rying different copy numbers of the pMtn-*galk* transcription unit. Total cellular RNA was isolated as described in Methods from each cell line, both prior to and after cadmium induction. The RNA was analyzed by agarose-formaldehyde gel electrophoresis followed by Northern blotting and hybridization to a *galk*-specific probe. In each case, a transcript of the expected size, 1.5 kb, was visualized. Figure 4 shows that cadmium induces a large accumulation of this *galk* RNA in the high-copy-number cell line C (lane 1), compared with

relatively low, almost undetectable amounts of this RNA found in uninduced cells (lane 2). The RNA induction ratio was found to be ~50-fold using dot blot analysis of total RNA (data not shown). This induction ratio is consistent with that observed at the protein level. Cell lines A and B, containing low and medium copy numbers, respectively, showed similar results, except that lower amounts of total *galk* RNA and protein were found to accumulate after induction in approximate proportion to their relative copy number.

Figure 2. Determination of copy number. Southern analysis of genomic DNA from the methotrexate-resistant cell lines. One microgram of total DNA extracted from each cell line was digested with *HindIII* and *XhoI*, which excises the *galK* gene on a 1.4-kb fragment. The samples were electrophoresed on a 1% agarose gel, and the DNA was transferred to nitrocellulose paper prior to hybridization with a 1.2-kb ^{32}P -labeled *galK*-specific probe. (Lane 1) Cell line C; (lane 2) cell line B; (lane 3) cell line A; (lane 4) untransformed control cells. The standard lanes (1–4) contain the *galK* gene on a 1.2-kb fragment loaded in exact amounts corresponding to 1, 10, 100, and 1000 *galK* gene copies, respectively. Three independent analyses confirmed the copy numbers.

Constitutive expression of the human *H-ras* oncogene in *Drosophila* cells is lethal

Phylogenetically, the *ras* genes are among the most conserved oncogenes, and close homology is found in organisms ranging from yeast to man (Varmus 1984; Barbacid 1987). These genes are implicated in mammalian cell growth regulation, and point mutations resulting in single amino acid changes in the *ras* proteins are observed in a variety of tumor cells and tumor cell lines. Introduction of these mutant *ras* genes into cell lines that exhibit contact growth inhibition (e.g., NIH-3T3

Figure 3. Induction of the Mtn promoter with copper and cadmium. Cells of cell line C (5×10^6) were harvested after 20 hr of exposure to various concentrations of CdCl_2 or CuSO_4 , as indicated. The proteins were electrophoresed on 10% SDS-PAGE and analyzed by Western blotting techniques. The *galK* protein was visualized using *galK*-specific antiserum followed by incubation with ^{125}I -labeled protein A. Arrows indicate the position of the *galK* protein. Relative amounts of *galK* protein were determined by radiograph scanning. Similar results were obtained in repeated experiments.

Figure 4. RNA analysis. Total RNA extracted from cell line C and control cells was subjected to Northern analysis. RNA extracted from 10^6 cells was loaded in each lane. (Lane 1) Cell line C RNA (cells had been incubated with $10 \mu\text{M}$ CdCl_2 for 24 hr prior to harvesting; (lane 2) cell line C RNA; (lane 3) control cell line RNA (untransfected cells). The RNA was denatured with formaldehyde and run on a 1% denaturing agarose gel. The RNAs were transferred to nitrocellulose paper and hybridized to a 1.2-kb ^{32}P -labeled *galK*-specific probe. Repeated experiments were performed.

mouse cells) results in growth transformation (Pulciani et al. 1985). These transformed cells often show a reduced requirement for added growth factors and added serum supplements (Pulciani et al. 1985). The *Drosophila* S2 cell line is an immortalized cell line that requires relatively high serum levels (i.e., 10% fetal calf serum) for growth. In an attempt to examine the effect of *ras* on the growth properties of insect cells, we introduced and expressed both the wild-type *H-ras* and transforming VAL¹² mutant human *ras* oncogene (T24 *ras*) into *Drosophila* S2 cells. Initially we placed both the normal gene and the T24 mutant gene under the control of the *copia* 5'LTR (pcodraswt and pcodT24) to achieve constitutive expression of these products in insect cells. pcodraswt and pcodT24 were introduced separately into S2 cells by cotransfection, with the pcodhygro vector containing the selectable marker for hygromycin B resistance. A vector DNA ratio of 10 (pcodraswt or pcodT24) to 1 (pcodhygro) was chosen to obtain high copy number of the *ras* expression unit. Hygromycin-B-resistant transformants were selected and examined for *ras* gene expression. Only early on in the selection could *ras* expression be detected transiently in the polyclonal population. The vast majority of the cells did not recover from the selection and died. Plating efficiency experiments demonstrated a marked reduction in stable transformants compared to the control cell line (2; Table 1). Moreover, neither of the transformants expressed detectable levels of the *ras* protein. Southern blot analysis of genomic DNA showed that the cells did carry copies of either the normal or the T24 *ras* transcription unit integrated into the host genome (data not shown). However, both cell lines had lost the ability to express the recombinant product (Fig. 6B, lanes 4 and 5).

Table 1. *Plating efficiency*

Cells transfected with	Number of clones – CdCl ₂	Number of clones + CdCl ₂
pMT24	45	4
pcodT24	2	
pcodhygro	35	30

The *ras*-transfected cells and control cells transfected with *pcodhygro* were plated in 0.3% soft agar containing M3 media + 300 μ g/ml hygromycin B $\pm$ 10 μ M CdCl₂. Three plates were seeded with 10⁶ cells for each transfection 7 days post-transfection, and colonies were counted 3 weeks later.

To prove that the *copia-ras* transcription unit constructed into the vector was indeed functional, we examined transient expression of the *ras* product from the same vectors. Cells were again transfected with the *ras* gene DNA constructs, but rather than being placed in selection for stable expression, they were analyzed 48–96 hr after transient transfection for *ras* expression. We found that both the normal and oncogenic *ras* forms were expressed and readily detected by this method (Fig. 6C). Thus, the majority of the cells died only after prolonged selection under conditions of constitutive expression (i.e., several weeks), and those few cells that were obtained containing the ‘competent’ vector DNA did not express the gene product.

Regulated expression of a lethal human oncogene

In an attempt to circumvent this apparent toxicity or growth inhibition resulting from constitutive *ras* gene expression, we placed the normal and the T24 *ras* genes separately under the control of the Mtn promoter to create the vectors pMtraswt and pMT24, respectively, to achieve regulated expression of the gene products in S2 cells. Each vector construct was cotransfected with the *pcodhygro* vector, as described earlier in a ratio of 10 : 1, and resistant cell lines were selected. Southern blot analysis of the genomic DNA showed that the cells contained 20–25 copies of either the wild-type *ras* or T24 *ras* oncogene (Fig. 5, lanes 4 and 2). Then cells were examined for metal-inducible *ras* gene expression by protein immunoblot analysis. Efficient expression of both normal and oncogenic forms of *ras* could now be obtained after induction with 10 μ M CdCl₂ (Fig. 6). We estimate by dot blot analysis that after 20 hr of induction, *ras* expression could account for ~0.2–0.5% of the total cellular protein. Moreover, these cells could be carried in culture under selection in the uninduced state for >10 months and they remained fully inducible for *ras* expression (Fig. 6B, lane 1). In contrast, if these cultures were maintained under permanent Cd induction and under selection for several weeks, a dramatic loss in *ras* expression was observed (Fig. 6A). This confirms the growth-inhibitory effect of constitutive expression of *ras* in this cell type. Southern blot analysis of the cells before and after permanent Cd induction showed that, at

least in part, the loss of *ras* expression resulted from reduction of the number of integrated *ras* DNA sequences in the induced cell population (Fig. 5, lanes 3 and 5). Our results clearly demonstrate that expression of both the normal and the mutant *ras* gene is toxic or at least growth static in *Drosophila* S2 cells and that cell lines containing high copy numbers of a competent transcription unit for this gene can be maintained only by using a regulatable promoter system.

It was somewhat surprising that expression of the human *ras* oncogene in this serum-dependent embryonic cell line was growth inhibitory rather than growth stimulating, as it appears to be in many mammalian and avian cell types. However, for at least one mammalian cell line, *ras* expression has been shown to arrest growth (Hirakawa and Ruley 1988). Our results in *Drosophila* mimic these results, as well as those observed both in wild-type yeast cells expressing a chimeric mammalian/yeast *ras* oncogene (Kataoka et al. 1985) and in *Dictyostelium* transformed with an oncogenic form of the *ras* gene. In both cases, efficient *ras* expression was shown to be a lethal or growth-arresting event (Reymond et al. 1986).

Methods

Vector construction

pSV40K was constructed by ligating a 1780-bp *SalI* cassette containing the *galK* transcription unit into a *SalI*-cut pML2 plasmid. [pML2 is a pBR322 derivative that has deleted the poison-minus sequence and a portion of the tetracycline-resistant gene (Mellon et al. 1981)]. The *SalI* cassette contained the SV40 early promoter, the *E. coli galK* gene, and the SV40 early polyadenylation site. A *HindIII* site positioned downstream of

Figure 5. DNA analysis of *ras* transfected cells. High-molecular-weight DNA was extracted from cells before or after 2.5 months of cadmium induction. DNA (250 ng) was digested with *PvuII*, which excises the *ras* gene from the vector, electrophoresed on a 0.8% agarose gel, and transferred onto a nitrocellulose filter. The presence of integrated genes in the host DNA was detected with a ³²P-nick-translated *ras* probe. (Lane 1) Control lane; (lanes 2 and 4) pMT24- and pMtraswt-transfected cells; (lanes 3 and 5) same cultures after 2.5 months under cadmium induction.

Figure 6. Loss of T24 *ras* expression under permanent induction of the Mtn promoter. (A) pMT24-transfected cells were seeded in complete medium containing 10 μ M CdCl₂. Protein extracts (30 μ g) from the cells before (lane 5) and after 1 week (lane 2), 3 weeks (lane 3), 6 weeks (lane 4), and 12 weeks (lane 1) of induction were analyzed on 15% SDS-PAGE and transferred onto a nitrocellulose filter. The filter was subsequently treated with anti-*ras* antiserum followed by incubation with ¹²⁵I-labeled protein A. (B) pMT24-transfected cells were maintained in culture for 10 months and were induced for 48 hr with 10 μ M CdCl₂ (lane 1) or uninduced (lane 3) and analyzed as in A. Survivors from pcodT24-transfected cultures were analyzed for constitutive expression (lanes 4 and 5). (C) Cells were transfected transiently. The precipitate was left on the cells for 96 hr before the cells were harvested and analyzed, as in B. (Lane 1) 30 μ g protein extract from pcodT24-transfected cells.

the SV40 promoter was destroyed, leaving a unique *Hind*III site between the SV40 promoter and the *galK* gene (Fig. 1).

pMtK was derived from pSV40K by removing the SV40 promoter with a *Sma*I plus *Hind*III digestion and end-filling and replacing it with an *Eco*RI–*Stu*I end-filled 430-bp fragment containing the *Drosophila* Mtn promoter. The promoter sequence spans from 370 bp upstream to 50 bp downstream of the initiation site of transcription. The fragment contains all the regulatory sequences needed for metal-regulated expression of the Mtn gene in *Drosophila* flies (Fig. 1).

pMT24 is a derivative of a puc18 vector that carries a transcription unit composed of the thymidine kinase promoter, the mutant T24 *ras* gene, and the SV40 early polyadenylation site inserted between the *Pst*I site and the *Bam*HI site. The thymidine kinase promoter was excised by digestion with *Bgl*II followed by a fill-in reaction and a subsequent digestion with *Aat*II and was replaced by a 430-bp *Eco*RI–*Stu*I *Drosophila* Mtn promoter fragment ligated to an *Aat*II–*Eco*RI linker.

pMtraswt is also a derivative of a pBR322 vector carrying a transcription unit composed of the thymidine kinase promoter, the wild-type *ras* gene, and the SV40 early polyadenylation site. The thymidine kinase promoter was replaced by the Mtn promoter in similar fashion as described above.

pcodT24 was constructed as follows: The T24 *ras* gene then was moved into a puc18 derivative, pucopiadATG, carrying the *copia* 5'LTR inserted at the *Bam*HI site. The T24 *ras* gene and SV40 early poly(A) site were isolated from pTKT24 *ras* on a *Bgl*II–*Eco*RI fragment and were inserted immediately downstream of the *copia* 5'LTR into a *Bgl*II–*Eco*RI-digested vector.

pcodraswt was constructed from pcodT24 by excising the T24 *ras* gene using *Hind*III–*Bam*HI digestion and replacing it with the wild-type *ras* wild-type gene on a *Hind*III–*Bam*HI fragment.

Stable and transient transformation

The *D. melanogaster* Schneider 2 cell line (Schneider 1972) was maintained in M3 medium (Lindquist et al. 1982), supplemented with 10% fetal bovine serum that was heat-inactivated at 65°C for 30 min. Cells were grown in Corning flasks at 25°C. For DNA transfection, 3 $\times$ 10⁶ cells seeded in 4 ml of medium the previous day were cotransfected with 20 μ g of plasmid DNA using calcium phosphate precipitation, as described by

Wigler et al. (1979). The cotransfection cocktail contained a mixture of the vector that contained the gene of interest and a vector containing a selectable marker, being either pHGCO containing the *E. coli dhfr* gene (Bourouis and Bruno 1983; Moss 1985) or pcodhygro containing the *E. coli* hygromycin B phosphotransferase gene (Van der Straten et al. 1987; 1989). At 15–18 hr post-transfection the cells were spun down, washed twice with M3 medium, and resuspended in 5 ml of fresh M3 medium. After 2 days, the cells were spun down and resuspended in selective medium containing either methotrexate (100 ng/ml) or hygromycin B (300 μ g/ml), depending on the cotransfected plasmids. Selective medium was replaced every 5 days, and stably transformed polyclonal cell populations were isolated after 3 or 6 weeks of selection with hygromycin B or methotrexate, respectively. Hygromycin B was maintained routinely in the media at all times after selection.

For transient expression, cells were transfected as above, except no vector containing a selectable marker gene was included in the transfection mixture. The precipitate was left on the cells for up to 5 days until harvest.

Inductions

We have analyzed and determined the optimum induction procedures for the Mtn promoter. Both transiently and stably transformed cells were induced routinely by the addition of 10 μ M CdCl₂ or 200 μ M CuSO₄ to the culture medium for 20 hr.

Northern analysis of mRNA levels

Total cellular RNA was isolated as described previously (McGarry and Lindquist 1985). Samples were denatured with formaldehyde and separated on 1% agarose–formaldehyde gel, transferred to nitrocellulose, and hybridized to a *galK*-specific nick-translated probe according to Maniatis et al. (1982).

Southern analysis of integrated DNA

Total DNA was prepared by direct lysis of *Drosophila* cells in 0.1 M NaCl₂, 0.01 M Tris (pH 8.0), and 1 mM EDTA (final pH 7.5), at a concentration of 10⁸ cells per milliliter. The solution was brought to a final concentration of 20 mM EDTA and 0.2% SDS. RNase was added at 100 μ g/ml, and the reaction was in-

cubated at 37°C for 2 hr. Proteinase K was added at 100 µg/ml, and the reaction was incubated further for 2 hr at 37°C. The solution was extracted with phenol chloroform until clean interface, and the high-molecular-weight DNA was collected with a sterile tip after ethanol precipitation. The DNA was resuspended into a Tris-EDTA buffer and quantitated by measuring absorbance at 260 nm. After restriction with *Hind*III and *Xho*I, which excises the *galK* transcription unit on a 1.4-kb fragment, the DNA was subject to electrophoresis on 1% agarose gel in standard Tris-borate buffer. Transfer onto nitrocellulose filter and hybridization to a *galK*-specific nick-translated probe were according to Maniatis et al. (1982).

Protein analysis

Total cellular proteins were separated on 10% SDS-polyacrylamide slab gels, and the *galK* and *ras* proteins were detected by Western blotting analysis (Gross et al. 1985) using *galK*- and *ras*-specific rabbit antiserum followed by incubation with ¹²⁵I-labeled protein A (National Cancer Institute).

DNA restriction enzymes were purchased from New England Biolabs or Boehringer-Mannheim, and reactions were carried out according to manufacturers' instructions.

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