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.

[Key Words: Gene regulation; high copy number; oncogene; insect cell]

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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; Scarle 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 promoter 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-
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 pMtK–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 µM 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 µM 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 µM CdCl₂ or 200 µM 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.
Figure 1. Construction of the pMtK vector. The pSV40K vector containing the \textit{E. coli galK} gene fused to the SV40 promoter was digested with \textit{SmaI} and \textit{HindIII}, which excised the SV40 early promoter from the vector. The vector was subsequently end-filled and ligated to an end-filled 430-bp \textit{EcoRI}–\textit{StuI} fragment containing the \textit{D. melanogaster} \textit{Mtn} regulatory sequences. The Mtn promoter then was fused to the \textit{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 \textit{E. coli galK} operon.

rying different copy numbers of the pMtn–\textit{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 \textit{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 \textit{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 \textit{galK} RNA and protein were found to accumulate after induction in approximate proportion to their relative copy number.
Regulated expression at high copy number in S2 cells

**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 32P-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 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).

**Figure 3.** Induction of the Mtn promoter with copper and cadmium. Cells of cell line C (5 x 10^6) were harvested after 20 hr of exposure to various concentrations of CdCl2 or CuSO4 as indicated. The proteins were electrophoresed on 10% SDS-PAGE and analyzed by Western blotting techniques. The galK protein was visualized using galK-specific antisera followed by incubation with 125I-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 µM CdCl2 for 24 hr prior to harvesting; (lane 2) cell line C RNA; (lane 3) control cell line RNA (untransformed 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 32P-labeled galK-specific probe. Repeated experiments were performed.
ras vector, as described earlier in a ratio of 10 : 1, 
amined for metal-inducible pMT24 45 4 
pcoDHygro 35 30 
protein immunoblot analysis. Efficient expression of both 
pression {i.e., several weeks), and those few cells that 
continued selection under conditions of constitutive ex-

The ras-transfected cells and control cells transfected with 
pcoDHygro were plated in 0.3% soft agar containing M3 media 
+ 300 μg/ml hygromycin B ± 10 μM CdCl₂. Three plates were 
seeded with 10⁶ cells for each transfection 7 days post-transfec-
tion, and colonies were counted 3 weeks later.

To prove that the copia-ras transcription unit con-
structed into the vector was indeed functional, we ex-
tained 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 pro-
longed selection under conditions of constitutive expres-
sion [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 con-
tained 20–25 copies of either the wild-type ras or T24 
ras oncogene [Fig. 5, lanes 4 and 2]. Then cells were ex-
amined for metal-inducible ras gene expression by pro-
tein immunoblot analysis. Efficient expression of both 
normal and oncogenic forms of ras could now be ob-
tained after induction with 10 μM CdCl₂ [Fig. 6]. We esti-
mate 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 be-
fore and after permanent Cd induction showed that, at 
least in part, the loss of ras expression resulted from re-
duction 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 embry-
onic 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 
[Hiarakawa 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 Dicyostel-
telium 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-resis-
tant 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-molec-
ular-weight DNA was extracted from cells before or after 2.5 
months of cadmium induction. DNA (250 ng) was digested 
with Pvull, which excises the ras gene from the vector, electro-
phoresed on a 0.8% agarose gel, and transferred onto a nito-
 cellulose filter. The presence of integrated genes in the host DNA 
was detected with a [32P]-nick-translated ras probe. [Lane 1] Control 
track; [lanes 2 and 4] pMT24- and pMtraswt-transfected 
cells; [lanes 3 and 5] same cultures after 2.5 months under cad-
mium induction.
Regulated expression at high copy number in S2 cells

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

pMTK was derived from pSV40K by removing the SV40 promoter with a Smal plus HindIII digestion and end-filling and replacing it with an EcoRI–Stul 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 PstI site and the BamHI site. The thymidine kinase promoter was excised by digestion with BglII followed by a fill-in reaction and a subsequent digestion with AatII and was replaced by a 430-bp EcoRI–Stul Drosophila Mtn promoter fragment ligated to an AatII–EcoRI 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 pc181 vector derivative, pucopiADATG, carrying the copia 5′LTR inserted at the BamHI site. The T24 ras gene and SV40 early poly(A) site were isolated from pTKT24 ras on a BglII–EcoRI fragment and were inserted immediately downstream of the copia 5′LTR into a BglII–EcoRI-digested vector.

pcodraswt was constructed from pcodT24 by excising the T24 ras gene using HindIII–BamHI digestion and replacing it with the wild-type ras wild-type gene on a HindIII–BamHI 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 × 10^6 cells seeded in 4 ml of medium the previous day were cotransfected with 20 μg 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 1983] 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 μg/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 selective 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 μM CdCl_2 or 200 μM CuSO_4 to the culture medium for 20 hr.

Northern analysis of mRNA levels

Total cellular RNA was isolated as described previously [McCarré 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 NaCl, 0.01 M Tris (pH 8.0), and 1 mM EDTA (final pH 7.5), at a concentration of 10^6 cells per milliliter. The solution was brought to a final concentration of 20 mM EDTA and 0.2% SDS. RNase was added at 100 μg/ml, and the reaction was in-

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 μM CdCl_2. Protein extracts (30 μg) 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 antisemur 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 μM CdCl_2 (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 transfected cells were maintained in culture for 10 months and were induced for 48 hr with 10 μM CdCl_2 (lane 1) or uninduced (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 antisemur 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 μM CdCl_2 (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 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 μg protein extract from pcodT24-transfected cells.
Johansen et al.

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 HindIII and XhoI, 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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H Johansen, A van der Straten, R Sweet, et al.

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