7. The complementing plasmid (*rad25*) was integrated and four independent strains were crossed to *rad*$^+$ *ura*$^-$ cells. For each strain, approximately 20% of *rad*$^-$ *ura*$^-$ segregants were generated, which suggests that integration had occurred at a locus distinct from *rad24.T1*. As was consistent with this possibility, crosses between the *rad25* null mutant and the *rad24.T1* mutant yielded progeny that were approximately 30% *rad*$^+$.
8. Overexpression of *rad25*, even from a high-level expression vector, resulted in only a slight increase in cell length at division. Similar observations have been made with *rad24*.
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11. Four degenerate PCR primers (Fig. 2) were used in amplification reactions on genomic *S. pombe* DNA. Three different pairs of the primers were used to generate distinct DNA fragments. Representative DNAs from each of these fragments were cloned and sequenced. Analysis of 24 independent clones suggested that *S. pombe* contained two 14-3-3 homologs. One of these was *rad25*. The entire coding region of the second 14-3-3 homolog was isolated as follows: Genomic DNA was digested with Hind III and recircularized. Polymerase chain reaction was done with primers pointing away from each other, thus amplifying (to the nearest Hind III site) the unknown sequences that flank the original fragment. The amplified regions contained the 3′ end of the coding region but not the entire 5′ end. To obtain the 5′ coding region, we performed anchored PCR on a complementary DNA (cDNA) library template with one primer that was unique to the known coding region and one primer that was designed to hybridize to the vector region flanking the 5′ end of the insert. Finally, we amplified the entire region, including the intron, by PCR from genomic DNA and sequenced two independent clones with synthetic primers.
12. When the *rad24* null mutant was crossed with the *rad24.T1* mutant, no wild-type recombinants were observed among 5×10^3 progeny.
13. To test the viability of the double mutant, we dissected 24 tetrads from a cross between the *rad24* and *rad25* null mutants to obtain four spores. Seven tetrads segregated four colonies (two *rad24::ura*$^+$ and two *rad25::ura*$^+$), eleven tetrads segregated three colonies (one *rad*$^+$ *ura*$^-$, one *rad24::ura*$^+$, and one *rad25::ura*$^+$), and six tetrads segregated two colonies (two *rad*$^+$ *ura*$^-$).
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17. Wild-type cells containing the *chk1* cDNA in the REP1 vector (*16*) were shifted to a medium without thiamine in order to induce expression of *chk1*. The cells elongated and mitosis was reduced to a rate at which colonies did not form. Cell mass accumulation continued. After 24 hours, the average cell length was 42 μm (cells containing only the empty vector divided at 13.9 μm). Under identical circumstances, *rad24* null mutant cells continued to divide (but at a reduced rate), and colonies formed in approximately twice the time required for control cells containing the empty vector. This indicated that an appreciable rate of cell division had occurred. After 24 hours, cells were only partially elongated, with an average cell length of 22.3 μm at division (cells containing only the empty vector divided at 9.3 μm). The *rad25* null mutants behaved like wild-type cells when *chk1* was overexpressed (cells were 42 μm in length after 24 hours). When *chk1* overexpression was reduced by use of the REP41 vector (*16*), both *rad24* and *rad25* cells could form large colonies, whereas the growth of wild-type cells was still impeded. Although we cannot eliminate the possibility that *rad24* and *rad25* activate *chk1*, these results are consistent with the idea that the 14-3-3 function acts downstream of *chk1*.
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Association of Polyomavirus Middle Tumor Antigen with 14-3-3 Proteins

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To carry out its transformation function, the middle tumor antigen (MT) of murine polyomavirus associates with a number of cellular proteins involved in regulation of cell proliferation, including pp60$^{c\text{-}Src}$, phosphatidylinositol 3-kinase, protein phosphatase 2A, Src homologous and collagen protein and growth factor receptor–binding protein 2. Here, two additional MT-associated proteins were identified as members of the 14-3-3 family of proteins. Yeast homologs of 14-3-3 proteins have recently been shown to play a role in the timing of mitosis. Thus, regulation of 14-3-3 protein function by MT may contribute to the development of neoplasia.

The primary transforming protein of murine polyomavirus is MT, a membrane protein with no known enzymatic activity (*1*). MT is thought to transform cells and induce tumors in animals by virtue of its association with cellular proteins involved in the control of cell proliferation. These proteins include pp60$^{c\text{-}Src}$ and other members of the Src family of protein-tyrosine kinases (*1*), phosphatidylinositol 3–kinase (*2*), two subunits of protein phosphatase 2A (*3*), Src homologous and collagen protein (SHC) (*4*), and growth factor receptor–binding protein 2 (GRB2) (*4*).

Among the MT-associated proteins that remain to be characterized are two with molecular masses of 27 and 29 kD (*5*). As is the case for the protein phosphatase 2A subunits, the 27- and 29-kD proteins associate efficiently with baculovirus-expressed polyomavirus MT in insect cells (Fig. 1, A and B) (*6*). To identify these proteins, we purified them from human 293 cells by preparative two-dimensional isoelectric focusing/SDS-polyacrylamide (2D) gels and subjected them to microsequence analysis (*7*). The sequences of multiple tryptic peptides (Table 1) indicated that both proteins belong to the 14-3-3 family of proteins; the 29-kD protein corresponds to the epsilon subspecies of 14-3-3 proteins, whereas the 27-kD protein appears to be a mixture of several 14-3-3 subspecies. This family of proteins was originally thought to be brain-specific and to function as a coregulator of tryptophan and tyrosine hydroxylases (*8*). Subsequently, these proteins have been found in a wide variety of mammalian tissues and in other eukaryotic organisms including plants and yeast and have had a number of activities ascribed to them (*8*).

The identity of these proteins was confirmed by immunoblot analysis of MT immunoprecipitates with an antibody raised to a peptide sequence conserved within several 14-3-3 subspecies (*9*). The antibody detected a 27-kD protein in MT immunoprecipitates prepared from mouse NIH 3T3 cells stably expressing MT (Fig. 1, C and D) (*10*). This same antibody recognized the 27-kD but not the 29-kD protein from cell lysates (*11*). This result is consistent with the fact that the peptide used as immunogen is not well conserved in the epsilon subspecies of 14-3-3 proteins.

The 14-3-3 proteins are an obligatory

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cofactor in vitro for an adenosine 5′-diphosphate (ADP)–ribosyltransferase enzyme (exoenzyme S) from *Pseudomonas aeruginosa* that has a substrate preference for $p21^{ras}$ and several other small guanosine 5′-triphosphate–binding proteins (G proteins) (*12*). MT immunoprecipitates prepared from MT-expressing insect or NIH 3T3 cells were also found to activate ADP-ribosylation by exoenzyme S (Fig. 1E) (*13*). In contrast, when the same antiserum was used to immunoprecipitate polyomavirus small t antigen (ST), which shares its first 191 amino acids with MT, substantial amounts of this activity were not found. This result is consistent with previous data showing that ST does not associate with the 27- and 29-kD proteins (*14*). Immunoprecipitates prepared from v-*src*–transformed cells with the same antibody also contained little activity, demonstrating that the activity from MT-expressing NIH 3T3 cells is not simply the result of transformation.

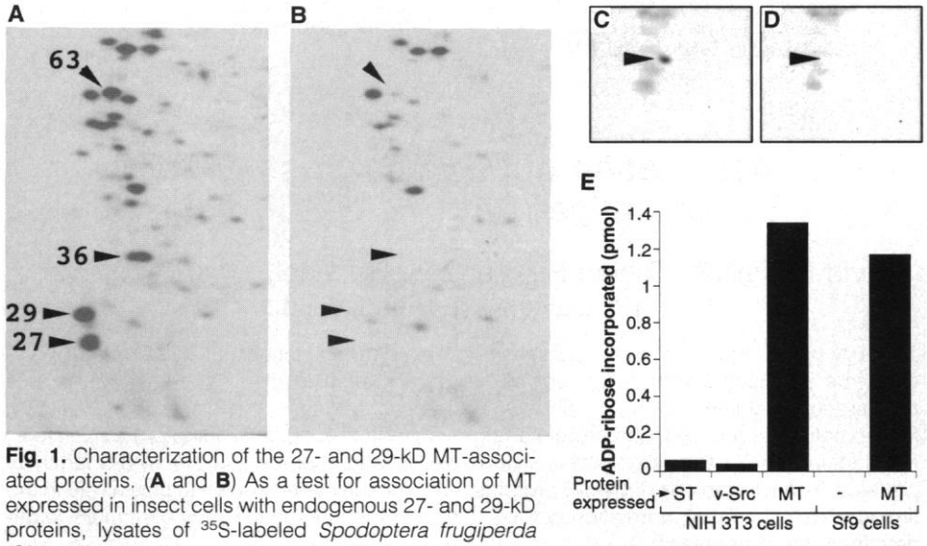

Fig. 1. Characterization of the 27- and 29-kD MT-associated proteins. (**A** and **B**) As a test for association of MT expressed in insect cells with endogenous 27- and 29-kD proteins, lysates of ^{35}S-labeled *Spodoptera frugiperda* (Sf9) cells were immunoprecipitated 48 hours after infection with MT-expressing baculovirus with (A) rabbit antitumor antigen serum recognizing MT (*18*) or (B) control serum, and the immunoprecipitates analyzed on 2D gels (*14*). The position and size (in kilodaltons) of the major MT-associated proteins are indicated. The 36- and 63-kD proteins are subunits of protein phosphatase 2A. (**C** and **D**) MT immunoprecipitates from mouse NIH 3T3 cells expressing MT were probed for the presence of 14-3-3 proteins. Immunoprecipitates were prepared with antitumor antigen serum (C) or with preimmune serum (D), analyzed on 2D gels, and immunoblotted with rabbit polyclonal antiserum specific for a peptide found in several subspecies of the 14-3-3 proteins (*9*). Arrowhead in (C) indicates an immunoreactive spot corresponding to a protein of 27 kD. In another experiment performed with metabolically labeled cells, this spot was found to comigrate on 2D gels with ^{35}S-labeled 27-kD MT-associated protein (*11*). (**E**) MT immunocomplexes were tested for their ability to activate the ADP-ribosylating activity of exoenzyme S from *P. aeruginosa*. Immunoprecipitates were prepared from NIH 3T3 cells stably expressing polyomavirus ST, MT, or $pp60^{v\text{-}Src}$ (v-Src) and from Sf9 insect cells infected with wild-type (−) or MT-expressing baculovirus with antitumor antigen serum. Washed immunoprecipitates were assayed for activation of exoenzyme S activity.

Table 1. Sequence comparison of tryptic peptides from the 27- and 29-kD MT-associated proteins with 14-3-3 proteins (*19*).

Peptide*	Sequence†	Sequence in 14-3-3 protein (isoform)‡
27-t15	EMQPTHPIR	Identical (ζ; most others)
27-t25	VISSIEQK	Identical (most; not ζ)
27-t28	TVTEQGAEL-NEER	SVTEQGAELSNEER (ζ)
27-t41	VFYLK	Identical (ζ; most others)
27-t47	DNLTLWTSDTQGD	Identical (ζ)
27-t48	NLLSVAYK	Identical (all)
27-t52	GIVDQSQQAYQEAFEI	Identical (ζ)
27-t66	TAFDEAIAELDTLSEESY	Identical (ζ)
29-t26	IISSIEQK	Identical (ε)
29-t33	YDEMVE-MK	Identical (ε)
29-t34a	EAAENSLVAYK	Identical (ε)
29-t45	YLAEFATGNDR	Identical (ε)
29-t55	NLLSVAYK	Identical (all)
29-t59	-NLTLWTSDMQGDGEEQNK	Identical (ε)
29-t93	AAFDDAIAELDTLSEESY	Identical (ε)

*The first number of the peptide name indicates its origin from the 27- or 29-kD protein. The second number indicates the peak number from the high-pressure liquid chromatography separation of tryptic (t) peptides from each protein. †Internal amino acid sequences were obtained as in (*21*). Dashes indicate an amino acid whose identity could not be determined. ‡Only mammalian isoforms (β, γ, ε, ζ, η sequences) are considered in this comparison.

How might the role of the 14-3-3 proteins in normal cell growth relate to MT-mediated transformation? Their extreme conservation between species and their interaction with MT are consistent with a potential role in regulation of cell proliferation. Interestingly, one member of the 14-3-3 protein family that is preferentially expressed in epithelial cells was found to be down-regulated in several human mammary carcinoma cell lines examined (*15*). MT presumably modulates 14-3-3 proteins, but their function is unclear. Several activities have been ascribed to the 14-3-3 proteins, including inhibition of protein kinase C in vitro and activation of exocytosis (*8*). We have measured ADP-ribosylation cofactor activity; however, it remains to be seen whether 14-3-3 functions in a similar reaction in vivo. It is striking that the ADP-ribosylation cofactor activity is conserved in yeast 14-3-3 proteins (*16*). The data here place 14-3-3 proteins in a complex with MT, which is also known to bind SHC, GRB2, and perhaps SOS (son of sevenless, a guanine nucleotide exchange factor known to complex with GRB2 and to activate ras). Thus, 14-3-3 proteins may exist in proximity to ras, and it is conceivable that these proteins might play a physiological role in the function of ras or other small G proteins. The most direct evidence to date that these proteins participate in cell cycle control is the finding that they play a role in the timing of mitosis in fission yeast (*17*). Taken together, these results suggest that MT may contribute to neoplasia by regulating the function of yet another protein involved in the control of cell proliferation.

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6. Sf9 insect cells (2×10^6) were infected by incubation for 1 hour with baculovirus expressing MT. The infection medium was then removed and the cells labeled with ^{35}S-methionine [1 mCi per 5 ml of Grace's methionine-minus labeling medium (Gibco/BRL) supplemented with 0.5% fetal bovine serum]. Five hours later, 0.25 ml of fetal bovine serum was added to the medium. At 48 hours after infection, cell lysates were prepared as described in (*2*)

except that lysis buffer lacked $CaCl_2$ or $MgCl_2$. Rabbit polyclonal antiserum, prepared against polyomavirus ST but recognizing all three tumor antigens because of their shared NH_2-terminal sequence identity (*18*), was used to immunoprecipitate MT.

7. Microsequencing was performed on proteins purified from lysates that were shown previously by tryptic peptide analysis to be identical to the 27- and 29-kD MT-associated proteins (*5*).
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10. Subconfluent NIH 3T3 cells (5×10^6) stably expressing wild-type MT from an integrated retroviral vector (*20*) were washed and lysed as in (*6*). Polyclonal rabbit antiserum, and preimmune serum from the same animal were used to immunoprecipitate MT (*18*), and the immunoprecipitates were analyzed by 2D gel electrophoresis (*14*). Proteins were transferred electrophoretically and immunoblotted (*21*) with the antibody described in (*9*).
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13. Subconfluent NIH 3T3 cells (2×10^6) stably expressing polyomavirus ST, MT, or $pp60^{v\text{-}Src}$ from an integrated retroviral vector and Sf9 insect cells (2×10^6) infected with wild-type or MT-expressing baculovirus were lysed as described in (*6*). Immunoprecipitates were prepared from cell lysates with polyclonal rabbit antitumor antigen serum (*18*) and with control serum and tested for their ability to activate the ADP-ribosyltransferase activity of exoenzyme S of *P. aeruginosa* as in (*12*), using 3 μl of immune complexes and the artificial substrate soybean trypsin inhibitor (100 μg/ml; Sigma) in a final reaction volume of 10 μl. The assay was performed at 23°C for 30 min. [^{32}P]ADP-ribose incorporation into substrate was verified by analysis on gels. In the absence of the immune complexes, exoenzyme S had no detectable ADP-ribosyltransferase activity. Background values obtained with preimmune sera were subtracted from each sample.
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Sensing Starvation: A Homoserine Lactone–Dependent Signaling Pathway in *Escherichia coli*

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When nutrients become limiting, many bacteria differentiate and become resistant to environmental stresses. For *Escherichia coli*, this process is mediated by the σ^s subunit of RNA polymerase. Expression of σ^s was induced by homoserine lactone, a metabolite synthesized from intermediates in threonine biosynthesis. Homoserine lactone–dependent synthesis of σ^s was prevented by overexpression of a newly identified protein, RspA. The function of homoserine lactone derivatives in many cell density–dependent phenomena and the similarity of RspA to a *Streptomyces ambofaciens* protein suggest that synthesis of homoserine lactone may be a general signal of starvation.

Bacteria lead a "feast or famine" existence, interchanging short periods of rapid growth with prolonged periods of starvation. To survive starvation, bacteria develop stress resistances before nutrients are exhausted (*1*). These developmental changes require the induction of specific genes at the onset of starvation, a process that in *E. coli* is in part regulated by the induction of a stationary phase–specific sigma factor of RNA polymerase, termed σ^s or σ^{38}. The mechanism by which bacteria sense starvation and how this signal is transduced to induce σ^s synthesis is only poorly understood.

The σ^s protein is encoded by the *rpoS* gene and regulates expression of at least 30 genes, some of which influence osmoprotection (*osmB*, *osmE*, *otsAB*, *treA*), cell morphology (*bolA*), or general stress resistance (*katE*, *xth*, *dps*, *appA*, *mccC*) (*1*). Transcription of *rpoS* increases at the onset of stationary phase (*2*), and the σ^s protein also appears at that time (*3*, *4*). To understand further the mechanism by which bacteria sense starvation and respond by inducing σ^s activity, we tested whether any *E. coli* genes, when present in high copy number, could alter the expression of a σ^s-dependent *bolA::lacZ* gene fusion (*5*). We found one chromosomal locus that in high copy completely repressed expression of this fusion (Fig. 1A) (*6*).

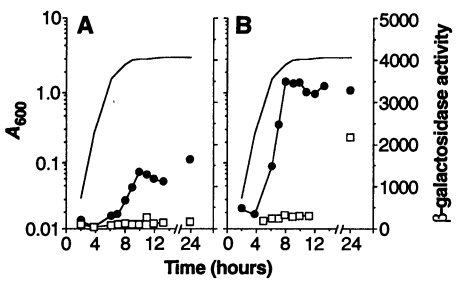

Fig. 1. Repression of σ^s-dependent gene expression by pSPER1. *Escherichia coli* VIP36 (*bolA::lacZ*) (**A**) and *E. coli* HS143 (*rpoS::lacZ*) (**B**) were transformed with pSPER1 (open squares) and pUC19 (closed circles), and β-galactosidase activities (Miller units) were determined during growth and stationary phase (line with no symbols). Cultures were grown in LB medium at 30°C.

Strains harboring this clone (pSPER1) also failed to induce *rpoS* expression at the onset of stationary phase (Fig. 1B). The chromosomal insert present in pSPER1 was located at minute 35.6 of the *E. coli* chromosome (*7*). The nucleotide sequence of the region identified two adjacent genes, designated *rspA* and *rspB*, whose products were overproduced as determined by SDS–polyacrylamide gel electrophoresis (PAGE) (*8*). Repression of *rpoS* expression was correlated with the overproduction of RspA (Fig. 2A).

The amino acid sequence of RspA is similar (*9*) to that of Spa2, a protein from *Streptomyces ambofaciens* (Fig. 2B). Strains in which the locus encoding Spa2 is amplified are deficient in starvation-induced pigmentation (*10*). RspA is also similar to two catabolic enzymes from *Pseudomonas putida*—mandelate racemase and chloromuconate cycloisomerase (*11*)—that have nearly identical three-dimensional structures and share active site residues that are conserved in RspA (Fig. 2B) (*11*).

The similarity of the RspA sequence to that of catabolic enzymes led us to hypothesize that the effect of RspA on *rpoS* transcription could result from degradation of a metabolite that signals starvation (*12*). The similarity of RspA with a lactonizing enzyme (chloromuconate cycloisomerase) suggested that such a metabolite might be a lactone. Homoserine lactone (HSL) derivatives act as inducers in several cell density–dependent systems including bioluminescence, Ti plasmid transfer, and synthesis of exoenzymes and antibiotics (*13*).

Although HSL derivatives are known to act as inducers of diverse phenomena, the biosynthetic pathway of HSL has not been described. We reasoned that possible precursors for the formation of HSL might be homoserine (HS) and homoserine phosphate (HSP)—intermediates in the threonine biosynthetic pathway (Fig. 3). If these

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