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High-Efficiency, Temperature-Sensitive Suppression of Amber Mutations in *Escherichia coli*

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We have constructed a high-copy-number plasmid carrying an allele of the *supD* gene (*supD43,74*). The plasmid conferred temperature-sensitive suppression of amber mutations. Strains carrying the plasmid exhibited 50 to 60% suppression at 30°C but little or no suppression at 42°C. After a temperature shift from 30 to 42°C the efficiency of suppression decreased gradually over a 60- to 90-min period before reaching the 42°C steady-state level of suppression.

Nonsense mutations have been extremely useful for studies of gene organization, regulation of gene expression, and function of gene products. In most cases the selection or exploitation of nonsense mutants depends on the phenotype of the unsuppressed nonsense mutant, i.e., when no functional gene product is produced. Such procedures are obviously not workable if a given gene specifies a function that is essential for survival of the cell. One way to obtain nonsense mutants in essential genes is to use temperature-sensitive suppressors. However, many of the proteins which perform essential functions are presumably also needed in concentrations which are not significantly lower that that found in wild-type cells. This is probably the case for DNA polymerase, RNA polymerase, and ribosomal proteins. To be useful in studies of nonsense mutants of such genes, the temperature-sensitive suppressor must therefore yield very efficient suppression at the permissive temperature. However, all temperature-sensitive suppressors described so far show only moderate or poor suppression (9). The efficiency of suppression can be increased by mutations at several loci (3, 10). Since these loci are unlinked to the suppressor mutations, transfer of the high-efficiency suppressor property to other strains requires transfer of at least one gene in addition to the suppressor gene. It would therefore be desirable to obtain high-efficiency temperature-sensitive suppression by another method which allows the high-efficiency suppression character to be transferred more readily between strains. Hoffman and Wilhelm (4) demonstrated that strains which are diploid for *supD* have an increased suppressor efficiency compared with isogenic haploid strains. It therefore seemed possible that the efficiency of temperature-sensitive suppressors could be increased to a more useful level if the dosage of the suppressor gene were increased. To test this idea, we have transferred a gene for a temperature-sensitive suppressor to a multicopy plasmid by recombinant DNA technology. We chose the *supD43,74* allele (here called *supD74*) described by Oeschger and Woods (12) for our studies because it had been quite well characterized and because suppression by *supD* inserts serine, a relatively small neutral amino acid. In this paper we show that a plasmid derived from pMB9 and carrying *supD74* confers 50 to 60% suppression at 30°C and gives weak or no suppression at 42°C.

MATERIALS AND METHODS

Media. Two media were used: LB (8) and AB minimal (2) supplemented with 0.4% glycerol or 0.2% glucose and the indicated amino acids at 40 μg/ml. Where appropriate, oxytetracycline was added to 20 μg/ml.

Bacterial and phage strains. All bacterial strains used in this study were *Escherichia coli* K-12. The genotypes of bacterial and phage strains are listed in Table 1. Two different alleles of the *supD* gene were used. One is a "regular" suppressor mutation (*supD43*) conferring suppression of amber mutations at all temperatures. The other is a temperature-sensitive allele (*supD74*) derived from *supD43*, which is active at 30°C but not at 42°C (12).

Construction of λ *supD43* and λ *supD74*. Specialized transducing phages carrying the *supD43* and *supD74* genes were constructed by the general method described by Schrenk and Weisberg (14). Bacterial deletion mutants lacking the λ attachment site (*attλ*) were derived from LL88 and LL89, carrying the *supD74* and *supD43* alleles, respectively. The Δattλ mutants were infected with 5 to 10 λ Δ*b* phage per cell, and lysogenic survivors were selected on LB plates by superinfection with a clear-plaque λ mutant (λ *c*I2 or λ *c*I Δ*9h*80). Surviving colonies were scraped off the plates and grown in LB at 30°C. Prophages were then induced at 42°C. LL87, which carries a normal λ attachment site and amber mutations in the *trpA* gene and in one of the *his* genes, was infected with the resulting mixed lysates, and transductants were selected at 30°C on AB glucose plates in the absence of both histidine and tryptophan. Such transductants

TABLE 1. *Bacterial and phage strains*

Strain	Description	Source and reference
Bacteria		
MX419	*thi-1 relA1 lacZ2210*(Am) *nalA21 rpsL183 tsx-85 supD74*	B. Bachmann (12)
MX383	F′ *purF his supD32/trp-49*(Am) *his-90 lacZ53*(Am) *nalA20 relA1*	B. Bachmann (12)
KL153	*thi-1 relA1 pro-51 supD43*	B. Bachmann (12)
LL87	*ilv-1 pro-2 trpA9605*(Am) *his-29*(Am)	Our strain collection
LL88	LL87*supD74*, by P1 transduction using MX419 as donor	This work
LL89	LL87*supD43*, by P1 transduction using KL153 as donor	This work
LL161	*trp-49*(Am) *his-90 lacZ53*(Am) *nalA20 relA1*, spontaneous haploid segregant of MX383	This work
LL202	*ilv-1 pro-2 his-29*(Am) *trpA9605*(Am) *aroE recA tsx ara nalA*	Our strain collection
LL217	pLL1/LL202	This work
LL231	LL202(λ *supD74*)(λ$^+$)	This work
LL233	LL202(λ *supD43*)(λ$^+$)	This work
Phages		
λ Δb	λ *c*I857 *S*am7 Δ*b*515 Δ*b*519 *xis*am6	M. Nomura
T4D$^+$	Wild-type T4	J. Celis
T4H36	Amber mutation in gene 43 (head protein)	J. Celis (1)
λ *supD74*	λ *c*I857 *S*am7 *supD74*; specialized transducing phage	This work
λ *supD43*	λ *c*I857 *S*am7 *supD43*; specialized transducing phage	This work

were tested for their ability to produce *supD74* or *supD43* transducing phages by spotting the transductants on an LB plate and incubating the plate at 30°C for 15 to 20 h and then at 42°C for 30 to 60 min followed by 2 h at 37°C. The cells were exposed to chloroform vapor for 10 to 15 min and then replicated onto an LB plate (to check for sterility) and onto AB glucose plates lacking histidine or tryptophan and seeded with LL87(λ$^+$). We tested 140 of the original *supD74* transductants and 160 of the original *supD43* transductants for their ability to produce λ *supD74* and λ *supD43*, respectively. Among these we found three λ *supD74*-producing strains and four λ *supD43*-producing strains.

Construction of a *supD74* plasmid. Conditions for recombinant DNA techniques have been described (J. M. Zengel, D. Mueckl, and L. Lindahl, Cell, in press). The *supD74* gene was transferred from one of the three λ *supD74* phages to the cloning vector pMB9, a multicopy plasmid derived from ColE1 that confers tetracycline resistance (13). DNA from the transducing phage and from pMB9 was digested with *Eco*RI. After heat inactivation of the restriction endonuclease, the pMB9 and λ *supD74* fragments were mixed in a mass ratio of 1:3. The mixture was adjusted to a total DNA concentration of 25 μg/ml and treated with T4 DNA ligase for 3 h at 12°C and then at 0°C overnight. After the ligation, an amount of the resultant mixture of DNA molecules corresponding to about 1 μg of pMB9 DNA was used to transform 5×10^9 cells of the strain NO1188 [*his*(Am) *trp*(Am) *rec*A]. We selected colonies carrying the desired type of plasmid by plating the transformed cells on AB glucose plates containing tetracycline but lacking histidine and tryptophan and incubating at 30°C. Survivors were then tested for their ability to grow at 42°C on minimal plates without histidine and tryptophan. All clones grew at both 30 and 42°C on LB but were temperature sensitive for growth on the minimal medium, indicating that the clones carry a temperature-sensitive suppressor gene. Plasmid DNA was isolated from one of the clones. The plasmid was larger than pMB9 and consisted of two *Eco*RI fragments, one comigrating in agarose gel electrophoresis with linear pMB9 and one comigrating with an *Eco*RI fragment from λ *supD74*. The new plasmid was designated pLL1. Transformation of LL87, LL161, or LL202 with pLL1 and selection at 30°C for suppression of the amber mutations carried by these strains generated transformants with a temperature-sensitive suppressor which were also tetracycline resistant. Conversely, transformation and selection for tetracycline resistance generated transformants which also exhibited the temperature-sensitive suppressor character. These results confirmed that the temperature-sensitive suppressor and the tetracycline resistance character both are carried by pLL1.

Production of β-galactosidase. Cultures were grown at the indicated temperatures in AB glycerol medium supplemented with histidine, arginine, and tryptophan. Cultures were allowed to grow exponentially for at least two doublings before β-galactosidase synthesis was induced. The doubling times were 120 to 130 min at 30°C and 70 to 85 min at 42°C. At a cell density of approximately 2×10^8 cells/ml, the synthesis of β-galactosidase was induced by addition of isopropylthiogalactoside (IPTG) to 2 mM, and at intervals thereafter 0.2-ml samples of the culture were withdrawn, shaken with about 20 μl of toluene, and kept at 0°C until the enzyme activity was measured. The toluene was evaporated from the samples by placing them in an evacuated desiccator at room temperature for about 0.5 h. The enzyme activity was determined at 28°C essentially as described by Miller (8), except that AB medium was used for the enzyme reaction instead of Z buffer. One unit of enzyme activity corresponds to hydrolysis of 1 nmol of *o*-nitrophenylgalactoside per min at 28°C.

Synthesis of T4 head protein. Cells growing exponentially at 30 or 40°C in AB glucose medium supplemented with histidine, arginine, and tryptophan were harvested by centrifugation at a density of 2×10^8 cells/ml. The cells were suspended in one-fifth volume of AB containing 40 μg of tryptophan per ml. Each sample of cells was then infected with the indicated T4 phage strain at a multiplicity of about five phage per cell. The phage were allowed to absorb for 5 min at 0°C. The infected cells were diluted fivefold with AB medium supplemented with glucose, histidine, arginine, and tryptophan and shaken at the temperature used for growth of the original culture. Six minutes after the dilution the cells were superinfected with about five phage per cell. After 45 min (42°C) or 60 min (30°C), infected cultures were labeled

with 2 μCi of [^{35}S]methionine (specific activity, 800 Ci/mmol) for 0.5 min (42°C) or 1 min (30°C). The labeling was terminated by addition of a 10^4-fold excess of nonradioactive methionine, and the cultures were incubated longer, as indicated. Finally, the samples were harvested on ice and lysed by boiling with sample buffer. Total proteins were fractionated by electrophoresis in 12% sodium dodecyl sulfate polyacrylamide gels (6). The gels were then stained and dried. The appropriate bands were identified by autoradiography and cut from the gels. Each gel piece was then placed in a vial with 5 ml of scintillation liquid, and the radioactivity was determined by liquid scintillation spectroscopy. We assumed that the recovery of the protein and the efficiency of counting were constant for a given sample. In fact, results calculated from duplicate gel runs of the same samples never differed by more than 20%.

RESULTS

Effects of multiple copies of the temperature-sensitive gene on suppression efficiency. To obtain a high gene dosage of the *supD74* allele, we cloned the gene on a multicopy plasmid vector, pMB9. This was accomplished by first constructing a λ *supD74* specialized transducing phage by using "natural" means of recombination. For comparison, a λ *supD43* phage was also constructed. DNA from λ *supD74* was then cleaved with restriction endonuclease *Eco*RI, and the resulting fragments were inserted into pMB9. By selection for suppression of amber mutations, pLL1 was obtained (see Materials and Methods for details). This plasmid has two *Eco*RI fragments, one corresponding to the vector pMB9 and one corresponding to an *Eco*RI fragment from the λ *supD74* DNA carrying the gene for the temperature-sensitive suppressor tRNA. The transducing phages λ *supD74* and λ *supD43* and the plasmid pLL1 were then introduced into LL161 (see Table 1). To obtain single lysogens of the transducing phages, we used a low multiplicity of infection (0.1). The probability for infection of a single cell with more than one phage is 0.01, and we therefore assume that our transductants were singly lysogenic for the transducing phages. Wild-type λ was used as a helper phage to permit growth of the lysogenic strains at 42°C. We confirmed by Southern hybridization experiments and fingerprint analysis that the *Eco*RI fragment transferred from λ *supD74* to pLL1 carries a tRNA gene (data not shown).

The effect of gene dosage on the efficiency of suppression by the *supD74* allele was determined for two different amber mutations: *lacZ53* of the β-galactosidase gene and H36 of the T4 phage coat protein gene. To measure the suppression of the *lac* amber mutation, cultures of LL161 and the *supD* derivatives described above were grown at 30 and 42°C, and the synthesis of β-galactosidase was induced by IPTG. A constant rate of accumulation of β-galactosidase activity was reached after about 10 min at 30°C and about 5 min at 42°C. These rates of accumulation of enzyme activity were used to calculate the relative efficiency of suppression at 30 or 42°C (Table 2). At the permissive temperature, suppression in the strain carrying multiple copies of the *supD74* gene (pLL1/LL161) was seven- to eightfold as high as in the strain harboring a single copy of the *supD74* gene [LL161(λ *supD74*) (λ$^+$)]. In fact, the multiple *supD74* genes generated essentially the same level of suppression as was found in the strain with a single copy of the non-temperature-sensitive *supD43* suppressor gene. At 42°C the efficiency of suppression was strongly reduced in both the single and the multiple gene strains carrying the *supD74* allele.

To determine the level of suppression of the H36 mutation of the T4 phage head protein gene, steady-state cultures growing in glucose minimal medium at 30 or 42°C were infected with T4H36 (1); late in infection, when only late proteins were being synthesized, radioactive methionine was administered to the cultures for a short time. An excess of nonradioactive methionine was added in order to chase all radioactivity into complete polypeptide chains. After the chase, the cells were harvested on ice and the proteins were fractionated on sodium dodecyl sulfate-polyacrylamide gels (see Materials and Methods for details). As expected, after a short (approximately 1 min) chase, the majority of the radioactivity was found in the precursor of the T4 head protein (called P23) or in the amber fragment (called P23A) of this protein, or in both (Fig. 1). To check the relative stability of P23 and P23A, we also prepared cells which had

TABLE 2. *Suppression of the lacZ53 amber mutation*[a]

Strain	Suppressor status	Rate of accumulation of β-galactosidase at:	
		30°C	42°C
LL161	*sup*^{0}	<0.01 (<1)	0.007 (0.4)
LL231	λ *supD74*	0.11 (11)	0.01 (1)
LL233	λ *supD43*	0.99 (100)	1.77 (100)
LL217	pLL1*supD74*	0.89 (90)	0.13 (7)

[a] Rate of accumulation is given as units of β-galactosidase accumulated per minute per absorbancy unit at 450 nm (about 3×10^8 cells/ml), determined after enzyme accumulation had reached a constant rate. Rates were usually measured between 5 and 30 min after induction. The numbers in parentheses are percentages indicating accumulation of enzyme activity normalized to LL233 grown at the same temperature.

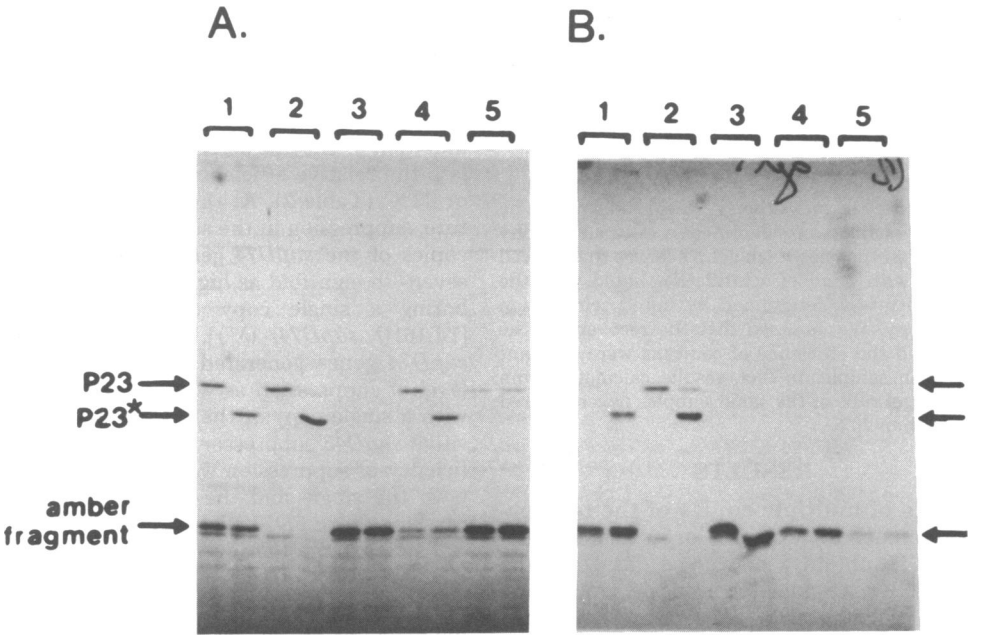

FIG. 1. *Suppression of the H36 amber mutation in the T4 head protein gene (gene 23). Cells grown at 30 or 42°C were infected with the indicated T4 strains. After 45 min (42°C) or 60 min (30°C), the infected cultures were pulse-labeled with [^{35}S]methionine followed by a chase with nonradioactive methionine for the indicated time. Extracts of the labeled cells were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Autoradiograms of the dried gels are shown. (A) Cells grown at 30°C. Each pair of lanes shows two samples of a given infected culture. The left sample was chased for 1.1 min; the right sample was chased for 20 min. (B) Cells grown at 42°C. Chase periods were 0.8 min (left) and 15 min (right). (1) LL233 infected with T4H36; (2) LL161 infected with T4$^+$; (3) LL161 infected with T4H36; (4) LL217 infected with T4H36; (5) LL231 infected with T4H36.*

been exposed to nonradioactive methionine for a relatively long time after the pulse-labeling. We found no evidence for instability of either of the proteins, with the exception of the expected cleavage of P23 to form the mature head protein (P23*) (Fig. 1).

To determine the efficiency of suppression of the H36 mutation, we measured the radioactivity in the P23, P23*, and P23A bands. If it is assumed that methionine residues are distributed evenly along the length of P23, the absolute suppressor efficiency can be calculated as:

$$\frac{\text{(cpm in P23/mol wt P23)} + \text{(cpm in P23*/mol wt P23*)}}{\text{(cpm in P23/mol wt P23)} + \text{(cpm in P23*/mol wt P23*)} + \text{(cpm in P23A/mol wt P23A)}}$$

assuming the molecular weights (mol wt) of P23, P23*, and P23A are 60,000, 50,000, and 35,000, respectively (1). Suppression at 30°C in the strain carrying multiple *supD74* genes was 50 to 60% (Table 3), slightly higher than in the strain carrying a single copy of the temperature-stable *supD* allele. A single copy of *supD74* resulted in only 8% suppression at 30°C. At 42°C, suppression was almost or completely absent in both *supD74* strains, whereas suppression in the *supD43* strain was only slightly reduced. Thus, the relative effects of gene dosage and temperature on suppression of the amber mutation in the T4 head protein by the various *supD* strains are consistent with the suppression levels observed for the *lac* amber strains.

Suppressor efficiency after a temperature shift. The original description of the *supD74* allele (12) indicated that the efficiency of suppression changes rapidly after an increase in temperature. To test that this property had been preserved after the gene had been transferred to the plasmid, we tried to measure the level of suppression of the *lacZ53*(Am) mutation after a temperature shift. Strains carrying pLL1 or λ *supD43* in conjunction with *lacZ53* were grown exponentially at 30°C and then shifted to 42°C simultaneously with addition of IPTG (Fig. 2). Unfortunately, the interpretation of this experiment was obscured by a reduction in the rate of β-galactosidase synthesis during the first

TABLE 3. *Suppression of the H36 amber mutation in the T4 head protein*[a]

Temp (°C)	Strain	Suppressor	Radioactivity (cpm) in:			Absolute efficiency of suppression (%)
			P23	P23*	P23A	
30	LL231	λ *supD74*	1,087	315	5,934	8
	LL233	λ *supD43*	2,065	1,262	2,436	43
	LL217	pLL1*supD74*	1,939	349	1,325	56
42	LL231	λ *supD74*	65	67	791	0
	LL233	λ *supD43*	2,311	257	2,574	36
	LL217	pLL1*supD74*	168	101	3,038	0.3

[a] Bands of T4 head precursor protein (P23), T4 mature head protein (P23*), and the H36 amber fragment (P23A) were cut out from the gels shown in Fig. 1, using the "short-chase" samples. The efficiency of suppression was calculated as explained in the text, correcting for the background radioactivity found in the appropriate positions of the lanes loaded with radioactive extracts from *sup*^{0} cells infected with T4$^+$ and T4H36.

20 min after the temperature shift, even in the strain carrying the non-temperature-sensitive allele *supD43* (Fig. 2B). We assume that this temporary inhibition of the β-galactosidase synthesis, which was observed consistently in our experiments, was due to a temperature effect on the transcription or translation of the *lac* operon. A similar effect was not reported by Oeschger and Woods (12), but temporary temperature-induced perturbations of gene expression have been described for a number of other *E. coli* proteins (7). In spite of this complication, the results shown in Fig. 2 suggest that suppression by the *supD74* gene on pLL1 of the *lacZ53* mutation declines slowly: the level of β-galactosidase in LL217 cells shifted to 42°C at the time of induction was considerably higher than the level in cells which had been grown for several generations at 42°C before induction. Similar results were obtained for the λ *supD74* lysogen (data not shown).

More conclusive evidence for the slow loss of suppressor activity after an increase in temperature was obtained by measuring the suppression of the T4H36 amber mutation in cells shifted from 30 to 42°C. The results (Fig. 3) show a small reduction in suppressor efficiency immediately after the temperature shift, but the remaining ability to suppress the H36 amber mutation decayed slowly and reached the 42°C steady-state level only after 60 to 90 min.

DISCUSSION

We have constructed *E. coli* strains carrying multiple copies of a gene for a temperature-sensitive allele of *supD* (sul). These strains were constructed by insertion of the *supD*(Ts) gene into a multicopy plasmid (pMB9) by recombinant DNA techniques. Measurements of protein synthesis from genes harboring amber mutations show that the suppression at the permissive temperature is seven to eightfold higher in the multicopy strain than in the strain with a single *supD*(Ts) gene. The absolute suppressor efficiency in the plasmid strain is 50 to 60% at 30°C, but very low (~1%) at 42°C. The new plasmid described in this report therefore allows a con-

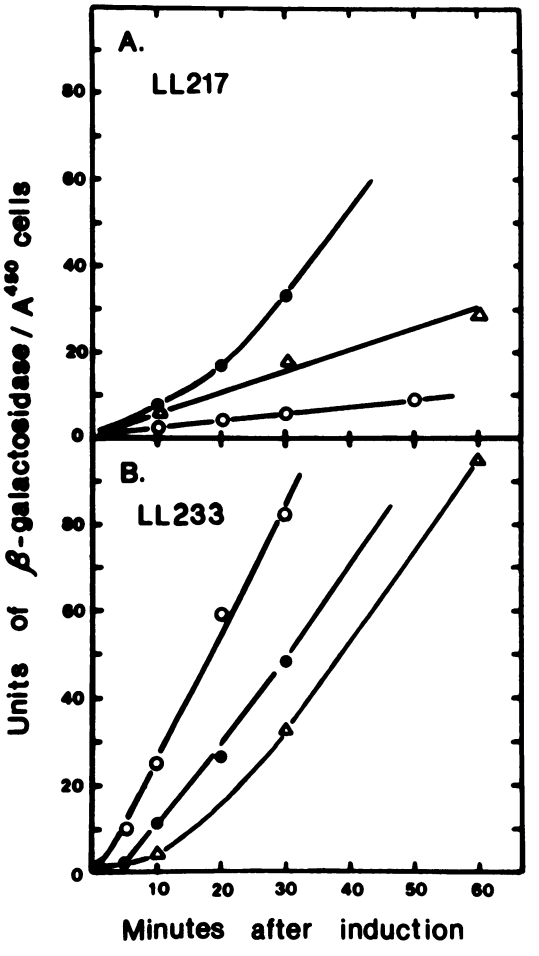

FIG. 2. *Suppression of the lacZ53 amber mutation after temperature shift. Cultures of LL217 and LL233 growing at 30°C were induced with IPTG at time zero. Simultaneously, a portion of each induced culture was switched to 42°C. At the indicated times after induction, portions of the cultures were withdrawn and assayed for β-galactosidase activity. A control culture of each strain was grown at 42°C for 2.5 h and then induced. Symbols: △, temperature shift from 30 to 42°C at the time of induction; ●, control at 30°C; ○, control at 42°C.*

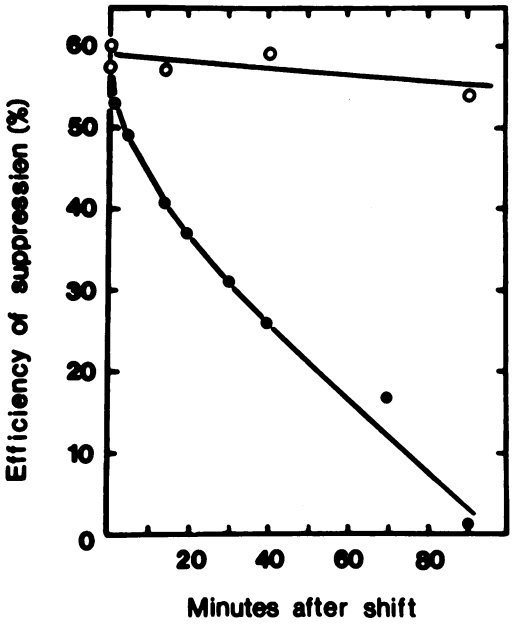

FIG. 3. *Suppression of the T4H36 amber mutation after a temperature shift. A culture of LL217 growing at 30°C was infected with T4H36. Sixty minutes after infection, a portion of the culture was shifted to 42°C; the remainder of the culture was kept at 30°C. At the indicated times, samples from both portions were pulse-labeled with [^{35}S]methionine, followed by a 1-min chase with nonradioactive material. Extracts were analyzed by gel electrophoresis, and the suppressor efficiency was calculated from the amounts of radioactivity in the P23, P23*, and P23A bands. See Fig. 1, Table 3, and text for more details. Symbols: ●, cells shifted from 30 to 42°C; ○, cells maintained at 30°C.*

ditional suppression of amber mutations at an efficiency which is equal to that of the suppression in strains with a temperature-stable suppressor allele. Such a plasmid should be useful for isolating amber mutations in essential genes.

The clear gene dosage effect observed here for the *supD* gene confirms the results of Hoffman and Wilhelm (4). These authors found that the efficiency of suppression in a strain diploid for *supD* is approximately twice that observed in a haploid strain. Even though we have not investigated the rate of synthesis of suppressor RNA in these strains, it seems likely that the synthesis is increased in the plasmid strain compared with strains with single gene copies. This would be in agreement with the elevated synthesis of rRNA and "spacer tRNA" observed by Ikemura and Nomura (5) in strains carrying plasmids derived from ColE1 by insertion of rRNA transcription units.

Other investigators have recently reported mutations mapping outside the suppressor loci, which in an unknown way interact with the suppression process and increase the efficiency of suppression without changing the complement of suppressor genes (3, 10). When certain of these modifying mutations are combined with temperature-sensitive suppressor mutations, the efficiency of suppression at 30°C is reported to be comparable to the level found in the pLL1-carrying strain. The use of these modifying mutations may therefore also be useful for selecting amber mutants in essential genes. However, the plasmid should make strain construction simpler, since new strains can be constructed by a single transformation, instead of two independent transfers of the suppressor gene itself and the modifying mutation.

The suppressor efficiency in pLL1-carrying strains declines gradually after a temperature shift from 30 to 42°C; the level of suppression characteristic of cultures grown in steady state at 42°C is not reached until 60 to 90 min after the shift. This slow decrease in the suppressor activity is in contrast to the behavior reported for the *supD74* allele by Oeschger and his coworkers (11, 12). They reported inactivation of about 80% of the suppressor activity within a few minutes after a temperature shift, even when the *supD74* allele was combined with the suppressor-enhancing mutations. We do not understand the reason for the difference in kinetics of suppressor efficiency after temperature shifts. Three possible explanations can be offered. One is that we might have inadvertently selected a secondary mutation in the *supD74* allele during the construction of λ *supD74* or pLL1 which decreases the rate of inactivation of the suppressor after a temperature shift-up. A second possibility is that the differences in the rate of decay of active suppressor tRNA result from differences in the genetic backgrounds of the strains used by Oeschger et al. and by us. The latter explanation is consistent with the observation that extracts from *supD74* strains maintain their ability to suppress amber mutations in vitro even at 42°C (12). Finally, the estimates of the suppressor efficiency reported by Oeschger and Wiprud (11) for the period immediately after a temperature shift-up may not be accurate. Their estimates were based on the rates of synthesis of RNA polymerase subunit β relative to the β′ subunit in a strain with a nonpolar amber mutation in the β gene. However, Lemaux et al. (7) found that the synthesis of the β subunit is temporarily reduced threefold about 7 min after a shift of wild-type *E. coli* from 28 to 42°C. Since similar studies have not been reported for the effect of temperature shift on the β′ subunit, it is possible that the syntheses of β and β′ respond

noncoordinately after a temperature shift. Therefore, without measurements of the synthesis of the amber fragment of β, it is difficult to interpret the experimental results reported by Oeschger and Wiprud (11).

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