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Establishment of Mammalian Cell Lines Containing Multiple Nonsense Mutations and Functional Suppressor tRNA Genes

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Summary

We describe the generation of mammalian cell lines carrying amber suppressor genes. Nonsense mutants in the herpes simplex virus thymidine kinase (HSV *tk*) gene, the Escherichia coli xanthine–guanine phosphoribosyl transferase (Eco-*gpt*) gene and the aminoglycoside 3′ phosphotransferase gene of the Tn5 transposon (*NPT-II*) were isolated and characterized. Each gene was engineered with the appropriate control signals to allow expression in both E. coli and mammalian cells. Expression in E. coli made possible the use of well developed bacterial and phage genetic manipulations to isolate and characterize the nonsense mutants. Once characterized, the nonsense mutants were transferred into mammalian cells by microinjection and used, in turn, to select for amber suppressor genes. Xenopus laevis amber suppressor genes, prepared by site-specific mutagenesis of a normal X. laevis tRNA gene, were microinjected into the above cell lines and selected for the expression of one or more of the amber mutant gene products. The resulting cell lines, containing functional amber suppressor genes, are stable and exhibit normal growth rates.

Introduction

A nonsense mutation generates an in-phase polypeptide chain termination codon (UAA, UAG or UGA) in the interior portion of a structural gene. As a consequence of this mutation, an amino-terminal polypeptide fragment, rather than the completed polypeptide chain, is synthesized (Sarabhai et al., 1964). In bacteria and yeast, and more recently in Caenorhabditis elegans, suppressors of nonsense mutations arise from mutations in tRNA genes which permit the mutant tRNA to translate a termination codon as an amino acid codon (Capecchi and Gussin, 1965; Engelhardt et al., 1965; Goodman et al., 1968; Capecchi et al., 1975; Gesteland et al., 1976; Waterston and Brenner, 1978; N. Wills, R. F. Gesteland, J. Karns, L. Barnett, S. Bolton and R. Waterston, unpublished results). In the presence of a suppressor tRNA the completed polypeptide product of a nonsense mutation is synthesized. If the amino acid inserted at the site of the mutation does not markedly alter the protein structure, an active gene product is restored.

Nonsense mutants clearly represent a class of conditional-lethal mutations, since the physiological effect of the mutation can be studied in the presence and absence of the suppressor. This allows, for example, the isolation of viral nonsense mutants on the basis of host range. It should be recalled that the availability of Escherichia coli strains with a variety of defined suppressors was crucial for the rapid development of procaryotic genetics.

A number of cellular and viral nonsense mutants have been identified in mammalian cell systems, including the mouse *hprt* gene (Capecchi et al., 1977), the human β-globin gene (Chang and Kan, 1979), the fusion protein in adenovirus 2–SV40 hybrid virus (Gesteland et al., 1977), the thymidine kinase (*tk*) gene of herpes simplex virus (HSV) (Cremer et al., 1979) and the *A* gene of SV40 (Rawlins and Muzyczka, 1980). As yet no mammalian cell line containing a functional suppressor tRNA gene has been identified. In addition, the known set of cellular and viral nonsense mutants was not suitable for isolating and maintaining such cell lines. With the advent of genetic engineering and an efficient method for transferring genes into cultured mammalian cells, new approaches for isolating such cell lines became practical.

For this purpose, we have isolated nonsense mutants in the HSV *tk* gene, the E. coli xanthine–guanine phosphoribosyl transferase (Eco-*gpt*) gene and the aminoglycoside 3′ phosphotransferase gene coded for by the Tn5 transposon (*NPT-II*). Each gene has been engineered to be transcriptionally and translationally competent in both mammalian cells and E. coli, and when introduced into either cell type each gene confers a selectable phenotype. Eco-*gpt* and *NPT-II* are particularly useful genes for DNA-mediated gene transfer studies, since they confer a dominant selectable phenotype in mammalian cells. Mulligan and Berg (1980) cloned the Eco-*gpt* gene into an SV40 vector that allowed expression of this gene in mammalian cells. Furthermore, since mammalian cells cannot ordinarily use xanthine as a purine source, Mulligan and Berg (1981) were able to develop a selection procedure in which cell survival depends on acquisition of a functional *gpt* gene. Colbere-Garapin et al. (1981) obtained expression of the Tn5 kanamycin resistance gene (*NPT-II*) in mammalian cells by linking it to HSV *tk* controlling sequences. Transfer of this gene into mammalian cells renders the cells resistant to the aminoglycoside G418 (a derivative of gentamycin).

Our strategy was to obtain and characterize nonsense mutations in E. coli and then to transfer them into cultured mammalian cells. These multiply marked somatic cell lines were then available for isolation and maintenance of nonsense suppressor genes. In using this approach we have in effect coupled bacterial and

somatic cell genetics.

Previously, we have described the synthesis of an amber (UAG) suppressor gene by site-specific mutagenesis of an X. laevis tyrosine-tRNA gene (Laski et al., 1982). After it was cloned into SV40 DNA, this gene was shown to function as a suppressor in vivo by coinfection of monkey cells (CV1) with amber mutants in adenovirus 2–SV40 hybrid virus. Here we have microinjected this amber suppressor gene into mouse cells containing HSV *tk*, Eco-*gpt* and *NPT-II* amber mutants and applied one or more selections to isolate permanent cell lines containing functional suppressor tRNA genes.

Results

Generation and Characterization of Nonsense Mutations in HSV *tk*, Eco-*gpt* and *NPT-II* Genes

Plasmids capable of expressing Eco-*gpt* and *NPT-II*, respectively, in both E. coli and mammalian cells were prepared by placing the coding sequence of these genes under the control of the avian sarcoma virus (ASV) long terminal repeat (LTR) promoter (see Figure 1 for the plasmid constructions). Mitsialis et al (1981) have shown that sequences in ASV LTR function as promoters in bacteria and could substitute for the normal bacterial tetracycline and neomycin resistance promoters. The Eco-*gpt* and *NPT-II* plasmid DNAs efficiently transformed appropriate E. coli mutant strains to yield, respectively, Gpt^+ and kanamycin-resistant colonies. The HSV *tk*-coding sequence when placed down stream from the ASV LTR also functions in E. coli but not well enough to permit easy isolation of suppressible nonsense mutations. Thus a two-step procedure was adopted. The HSV *tk*-coding sequence was first placed under the control of the tetracycline resistance gene of pBR322 for expression in E. coli (Garapin et al., 1981; Kit et al., 1981). Following the isolation and characterization of the HSV *tk* nonsense mutations in E. coli, the HSV *tk*-coding sequences were placed under the control of the retroviral ASV LTR promoter for expression in mammalian cells.

An added advantage of the ASV LTR promoter for these experiments is its strength in mammalian cells. Use of the ASV LTR protomer to initiate transcription of the HSV *tk*-coding sequences (Bgl II to Bam HI) consistently yielded ASV LTR–HSV *tk* hybrid mRNAs that were tenfold the number of normal HSV *tk* transcripts yielded by the authentic HSV *tk* promoter (P. Luciw and M. R. Capecchi, unpublished results; Folger et al., 1982).

Isolation of nonsense mutations in HSV *tk*, Eco-*gpt* and *NPT-II* is described in the Experimental Procedures. Plasmid DNAs were mutagenized in vitro with hydroxylamine (Hall and Tessman, 1966) and transfected into the appropriate E. coli strain. Eco-*gpt* mutants were selected directly on 6-thioguanine plates. HSV tk^- and *NPT-II*$^-$ mutants were identified by first isolation of the bacterial transformants on ampicillin plates, followed by screening of the mutants by replica plating to selective and nonselective plates and scoring them for the loss of thymidine kinase or NPT-II activity. Among the collection of null mutants, nonsense mutants were identified by their suppressibility with specialized transducing phages containing amber (UAG) or ochre (UAA) suppressor genes.

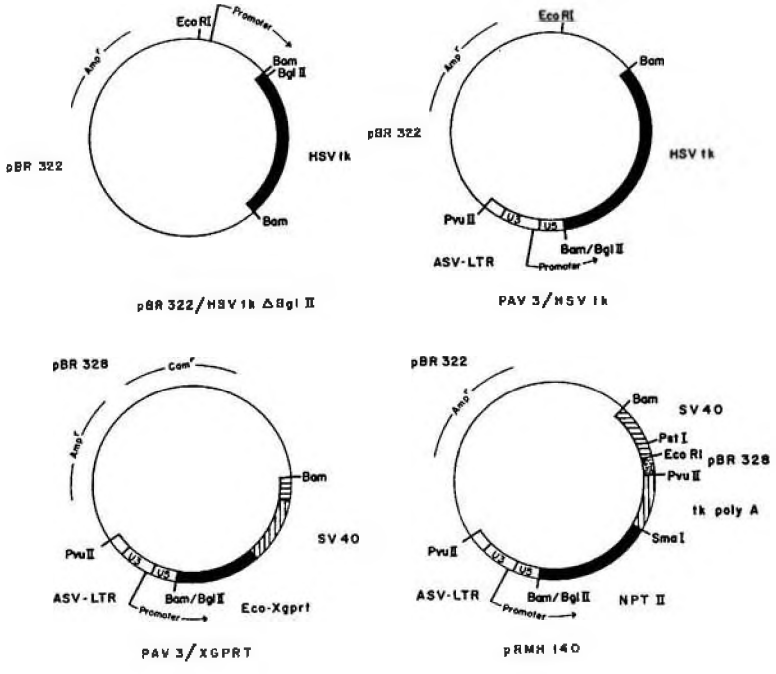

Figure 1. Recombinant Plasmids Used for the Generation of the HSV *tk*, Eco-*gpt* and *NPT-II* Amber Mutations

pBR322/HSV *tk* ΔBgl II was derived from a derivative of pBR322/HSV *tk* that contains two Bgl II restriction sites in the 3.4 kb HSV *tk* Bam HI fragment approximately 10 and 600 bp from the 5' Bam HI site. Cleavage of this plasmid DNA with Bgl II followed by religation removes the HSV *tk* promoter and places the HSV *tk*-coding sequence under the control of the pBR322 tetracycline promoter. In the plasmids PAV3/HSV tk, PAV3/gpt and pRMH-140, the HSV *tk*, Eco-*gpt* and *NPT-II* coding sequences are under the control of sequences in the ASV LTR. The fusion of the virus LTR with the respective coding sequence occurs at the LTR Bst EII site (Swanstrom et al., 1981), which was converted by P. Luciw to a Bam HI site.

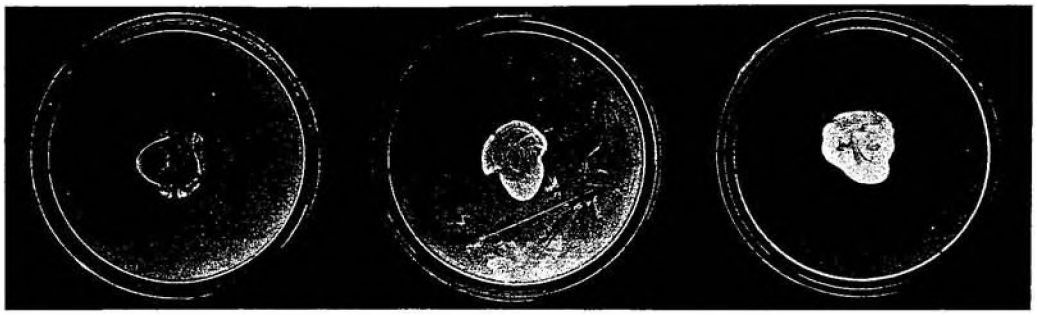

Figure 2. Spot Tests for Amber Mutations in the HSV *tk*, Eco-*gpt* and *NPT-II* Genes

Bacteria bearing a null mutation in the HSV *tk*$^-$(*am1142*), Eco-*gpt*$^-$(*am63*) or *NPT-II*$^-$(*am28*) genes were spread on a plate of the appropriate selection medium (see the Experimental Procedures). A drop of a phage lysate (ϕ80pSuIII, a specialized transducing phage carrying the E. coli SuIII amber suppressor tRNA gene) was placed on each plate. Bacterial growth is observed where the virus drop was added.

Shown in Figure 2 are plates onto which HSV *tk*$^-$, Eco-*gpt*$^-$ or *NPT-II*$^-$ mutants were plated on agar containing the appropriate selective medium. A drop of a phage lysate carrying an amber suppressor gene was placed on each plate. Growth occurs only where the phage carrying the nonsense suppressor gene was added. With this procedure a series of amber and ochre mutants varying in their ability to be suppressed by the different transducing phages was identified. For all subsequent experiments we selected a single amber mutant in each gene on the basis of its ability to be efficiently suppressed by a tyrosine-inserting amber suppressor (SuIII). These mutants are designated *PAV3/HSV-tk*$^-$(*am1142*), *PAV3/Eco-gpt*$^-$ (*am63*) and *PAV3/NPT-II*$^-$(*am28*).

Introduction of the HSV *tk*, Eco-*gpt* and *NPT-II* Amber Mutations into Cultured Mammalian Cells

Microinjection with glass micropipettes was used to introduce the plasmid DNAs carrying the amber mutations into two recipient cell lines, LMtk$^-$/APRT$^-$ and LMtk$^-$/APRT$^-$/HPRT$^-$ (Capecchi, 1980). The second line was generated from the parental LMtk$^-$/APRT$^-$ cell line by mutagenesis to HPRT$^-$ with a frameshift mutagen; the HPRT$^-$ derivative reverts to HPRT$^+$ at a very low frequency (that is, less than 1 cell per 10^7 cells per generation; M. R. Capecchi, unpublished results). Plasmid DNAs bearing the amber mutations were linearized with a restriction endonuclease, and approximately ten copies were injected into each recipient cell. Transformants were selected by coinjecting plasmids containing either a wild-type *aprt* or *NPT-II* gene and selecting for APRT$^+$ or for G418-resistant cell lines, respectively. Each injected plasmid DNA contained either an SV40 or an ASV LTR "enhancer" sequence (Folger et al., 1982). We have shown previously that when the conditions described above for microinjection are used, high transformation frequencies are obtained, the number of gene copies integrated into the host chromosome is roughly proportional to the number of plasmid DNA molecules injected, the donated genes are integrated predominantly in a head-to-tail concatamer at one or a very few sites in the host chromosome and the injected DNA is not extensively rearranged after integration into the host chromosome (Folger et al., 1982). These conditions for gene transfer favor transcriptional activity of the injected DNA.

The presence of functional HSV *tk* and Eco-*gpt* amber mutant genes in the APRT$^+$ or G418-resistant transformants was demonstrated by injection into the cytoplasm of partially purified amber suppressor tRNA isolated from yeast suppressor strains (Capecchi et al., 1975, 1977). As shown in Figure 3, microinjection of amber suppressor tRNA, but not ochre or wild-type tRNA, restores thymidine kinse and xanthine–guanine phosphoribosyl transferase (Gpt) enzymatic activity to these cells. The presence of thymidine kinase and Gpt enzymatic activity in the cells receiving an injection of tRNA was determined 24 hr after injection by following the incorporation of ^{3}H-thymidine, ^{3}H-guanine or ^{14}C-xanthine into nucleic acid by autoradiography. Since mammalian cells cannot utilize xanthine as a purine source, incorporation of ^{14}C-xanthine allows detection of Gpt enzymatic activity in the presence of cellular HPRT activity. Guanine and xanthine are incorporated into both DNA and RNA, which explains the cytoplasmic and nuclear labeling pattern.

In a typical experiment, LMtk$^-$/APRT$^-$ and LMtk$^-$/APRT$^-$/HPRT$^-$ cells were coinjected with approximately ten copies per cell of HSV *tk* (amber), Eco-*gpt* (amber) and *NPT-II*$^+$ plasmid DNAs. The transformation frequency to G418 resistance (250 μg/ml) for both cell lines was approximately 15%. Of six independent G418-resistant transformants tested, five were transiently converted to a TK$^+$ phenotype by microinjection of the yeast amber suppressor tRNA and thus were shown to contain transcriptionally competent HSV *tk* (amber) genes. All six cell lines could be transiently converted to Gpt$^+$ phenotype by microinjection of yeast amber suppressor tRNA and thus would contain active Eco-*gpt*$^-$ (amber) genes. The

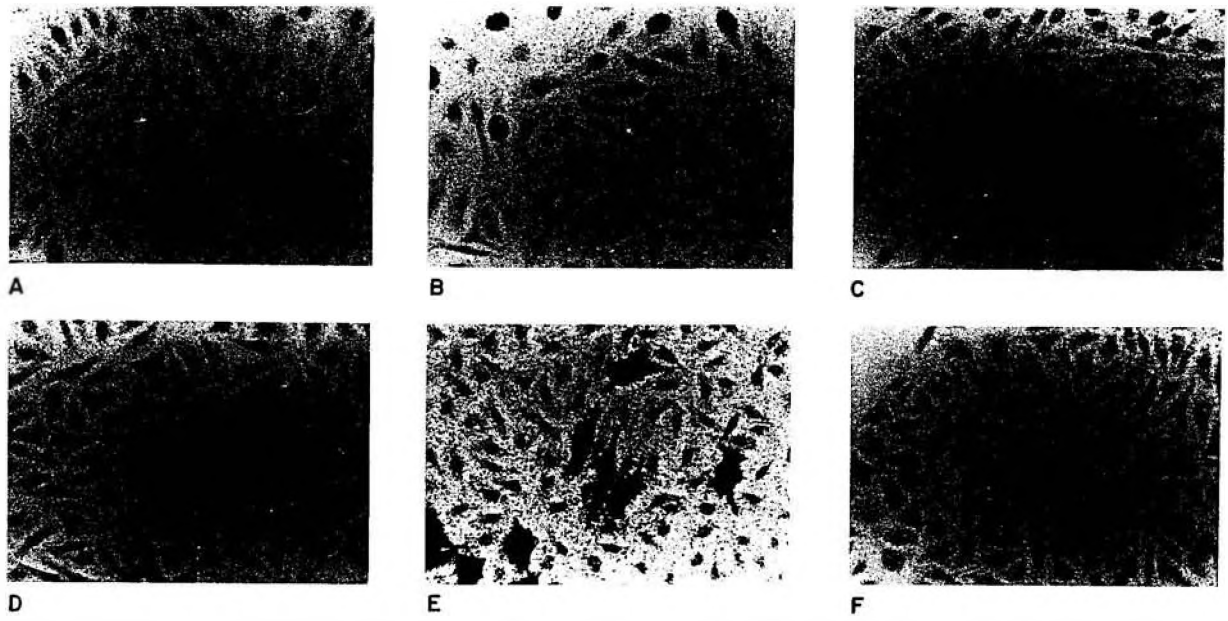

Figure 3. Restoration of Thymidine Kinase and Gpt Activity in Cells Containing HSV *tk* and Eco-*gpt* Amber Mutations by Microinjection of Yeast Amber Suppressor tRNA

Plasmids bearing HSV *tk* and Eco-*gpt* amber mutations were introduced into a LMtk$^-$/APRT$^-$/HPRT$^-$ cell line by coinjection with *NPT-II*$^+$ genes and selection for G418-resistant cell lines. tRNA was isolated from three closely related strains of yeast: SL110-4D, D599-4B and D604-7D, containing no suppressor, an amber suppressor and an ochre suppressor, respectively. After partial purification of the tRNA as previously described (Capecchi et al., 1975), the tRNAs were injected into cells of one of the above G418-resistant cell lines (20). Cells shown in (A) and (D) received injections of SL110-4D tRNA, cells shown in (B) and (E) received injections of D599-4B tRNA and cells shown in (C) and (F) received injections of D604-7D tRNA. The thymidine kinase activity and guanine phosphoribosyltransferase activity present in each cell were determined by measuring the capacity of the cells to incorporate ^{3}H-thymidine into DNA and ^{3}H-guanine into RNA and DNA, respectively. Following injection of the tRNA, the cells were incubated for 24 hr at 37°C in minimal essential medium (MEM) with 10% fetal calf serum to allow translation of the mutant message. The cells were then transferred into MEM with 10% fetal calf serum containing either 25 μCi/ml of ^{3}H-thymidine (A, B and C) or ^{3}H-guanine (D, E and F) and incubated at 37°C for an additional 16 hr. The cells were then washed four times with phosphate-buffered saline and fixed with 2% glutaraldehyde. After fixing, the cells were washed two times with distilled water and two times with 30% ethanol. The coverslips containing the fixed cells were then mounted on microscope slides and dipped in NTB-2 autoradiographic emulsion. Following exposure for 72–84 hr, the slides were developed. Thymidine kinase and Gpt enzymatic activity were observed only in cells receiving injections of amber suppressor tRNA. These experiments demonstrate that the plasmids containing the HSV *tk* and Eco-*gpt* amber mutations were successfully cotransferred into the cell line in a transcriptionally competent state. Particularly noteworthy is the low background incorporation of ^{3}H-thymidine and ^{3}H-guanine in uninjected cells. The low background attests to the stringency of these amber mutations in mammalian cells.

cotransfer index for both unselected genes was nearly one.

Injection of X. laevis Amber Suppressor tRNA Genes into Cell Lines Containing Multiple Amber Mutations

We previously synthesized an amber suppressor tRNA gene by site-specific mutagenesis of a wild-type X. laevis tyrosine-tRNA gene (Laski et al., 1982). As outlined in Figure 4, this was accomplished by cloning the tRNA gene into the vector M13mp5 so that the positive strand was packaged into phage. An oligonucleotide containing a single base pair mismatch in the anticodon region of the tRNA gene (GTA → CTA) was used to prime the in vitro synthesis of the negative strand. After purification, the closed duplex DNA was used to transfect E. coli. The desired site-specific mutation generated a new Hinf site within the tRNA gene which was used to screen phage plaques for the suppressor tRNA gene. DNA sequencing confirmed that two clones containing the new Hinf site possessed the desired mutation.

Having shown that both the HSV *tk*$^-$ and Eco-*gpt*$^-$ ambers were suppressible by yeast suppressor tRNA, we tested whether the X. laevis tyrosine amber suppressor gene described above would also suppress these mutations. Figure 5 shows the results of such an experiment. Murine cell lines containing the HSV *tk*$^-$ and Eco-*gpt*$^-$ amber mutations exhibit thymidine kinase and Gpt enzymatic activity following injection of X. laevis amber suppressor tRNA genes. No activity was detected in cells receiving injections of wild-type X. laevis tyrosine-tRNA genes.

Establishment of Permanent Cell Lines Containing Functional Suppressor tRNA Genes

A cell line containing HSV *tk*$^-$ (amber), Eco-*gpt*$^-$ (amber) and *NPT-II*$^-$ (amber) genes was injected with approximately ten copies per cell of X. laevis amber suppressor tRNA genes. Transformants expressing

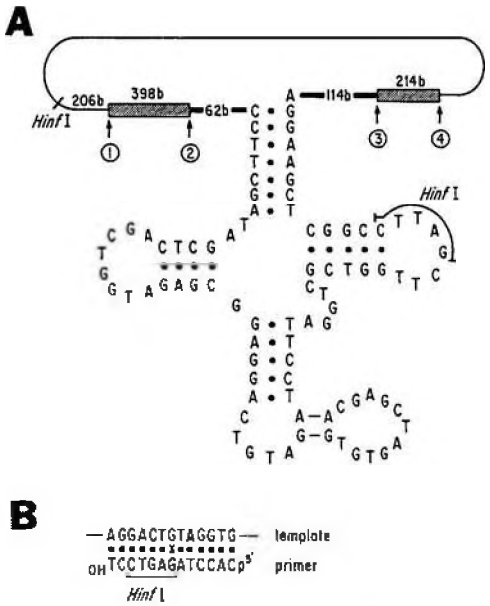

Figure 4. Sequence of X. laevis Tyrosine-tRNA Gene and Oligonucleotide Used for Site-Specific Mutagenesis

(A) A 263 bp segment of X. laevis DNA containing a tyrosine-tRNA gene was first cloned into the late region of SV40 DNA (hatched bar) by ligation at sites 2 and 3. Sites in SV40 DNA (sites 1 and 4 above) were cleaved, and the segment containing the tRNA gene was subcloned into a M13mp5 vector (this line) so that the positive strand was packaged in phage. (B) For site-specific mutagenesis, single-stranded phage DNA was used as template for priming with the oligonucleotide shown. The template DNA and primer were mismatched at a single position (denoted by ×), and conversion of the gene sequence to that of the oligonucleotide mutated the tyrosine-tRNA gene to an amber tRNA suppressor sequence. The conversion also conveniently created a novel Hinf I site in the tRNA gene, and cleavage by this restriction endonuclease was used in screening during site-specific mutagenesis. The sequence of the generated X. laevis tyrosine amber gene was determined (for details see Laski et al., 1982).

thymidine kinase activity were selected on HAT medium and those expressing *NPT-II* activity on G418 medium. As shown in Table 1, both cell types were obtained at a transformation frequency of approximately 10%. No transformants were obtained after injection of 5, 10 or 25 copies per cell of wild-type X. laevis tyrosine-tRNA genes.

A useful property of nonsense suppressors is that they can phenotypically correct separate nonsense mutants in the same cell line. In Figure 6 we show that G418-resistant cell lines, obtained by injecting X. laevis amber suppressor tRNA genes into the multiply marked cell lines, also express thymidine kinase and Gpt activity. Similarly, cell lines selected for growth in HAT medium (Table 1) also expressed Gpt and *NPT-II* enzymatic activity (data not shown). We also obtained transformants by injecting X. laevis suppressor tRNA genes into the multiply marked cell line and coselecting for TK^+ and Gpt^+ phenotype (that is, growth in medium containing thymidine, xanthine, mycophenolic acid and methotrexate; Mulligan and Berg, 1981).

In Table 2 we compare the growth rate of the parental LMtk$^-$/APRT$^-$/HSV-*tk*$^-$(amber)/Eco-*gpt*$^-$(amber)/*NPT-II*$^+$ cell line with three independent HAT-resistant transformants obtained by injecting X. laevis amber suppressor tRNA genes into the above cell line and selecting for growth in HAT medium. The growth rates of all four cell lines were very similar. Thus the presence of functional suppressor tRNA genes in these mammalian cell lines does not markedly alter their growth rates. The three HAT-resistant transformants selected for the TK^+ phenotype (that is, growth in HAT medium) also exhibited Gpt enzymatic activity (data not shown). Each of the above cell lines (Table 2) has been maintained in its respective selection medium (G418 or HAT) for over 70 generations without observable phenotypic change. Thus they can be considered stable cell lines when grown in their respective selection media. For example, the parental cell line LMtk$^-$/APRT$^-$/HSV-*tk*$^-$(*am1142*)/Eco-*gpt*$^-$(*am63*)/*NPT-II*$^+$ has been periodically checked for the presence of transcriptionally competent HSV *tk* and Eco-*gpt* amber mutations by injections of yeast amber suppressor tRNA. Furthermore, the derivative cell lines, 35, 37 and 39, containing functional X. laevis suppressor tRNA genes have been periodically checked for Gpt activity. The latter cell lines are grown in HAT medium, which does not require Gpt activity for survival. Reversion of the *tk* amber mutation followed by loss of the suppressor tRNA genes would have been detected as a loss of Gpt activity.

Functional Suppressor tRNA Is Present in Cell Lines Transformed with the Suppressor Gene

tRNA was isolated from a number of cell lines which had been reverted as described above by injection of X. laevis amber suppressor tRNA genes. After purification, this tRNA was injected into cells containing the HSV *tk* and Eco-*gpt* amber mutations. As a control, tRNA was also isolated from the parental LMtk$^-$/APRT$^-$/HPRT$^-$ cell line. As expected, cells that received injection of tRNA from Su^+ cells, but not cells that received injections of control tRNA, exhibited thymidine kinase and Gpt enzymatic activity (see Figure 7).

Discussion

We isolated nonsense mutations in the HSV *tk* gene, the Eco-*gpt* gene and *NPT-II* gene. Each gene was engineered with the appropriate control signals to allow expression in both E. coli and mammalian cells. Expression in E. coli made it possible to apply bacterial and phage genetic manipulations to generate and characterize nonsense mutants. From the pattern of suppression with a series of specialized transducing phages, the codon specificity (that is, amber, UAG; or

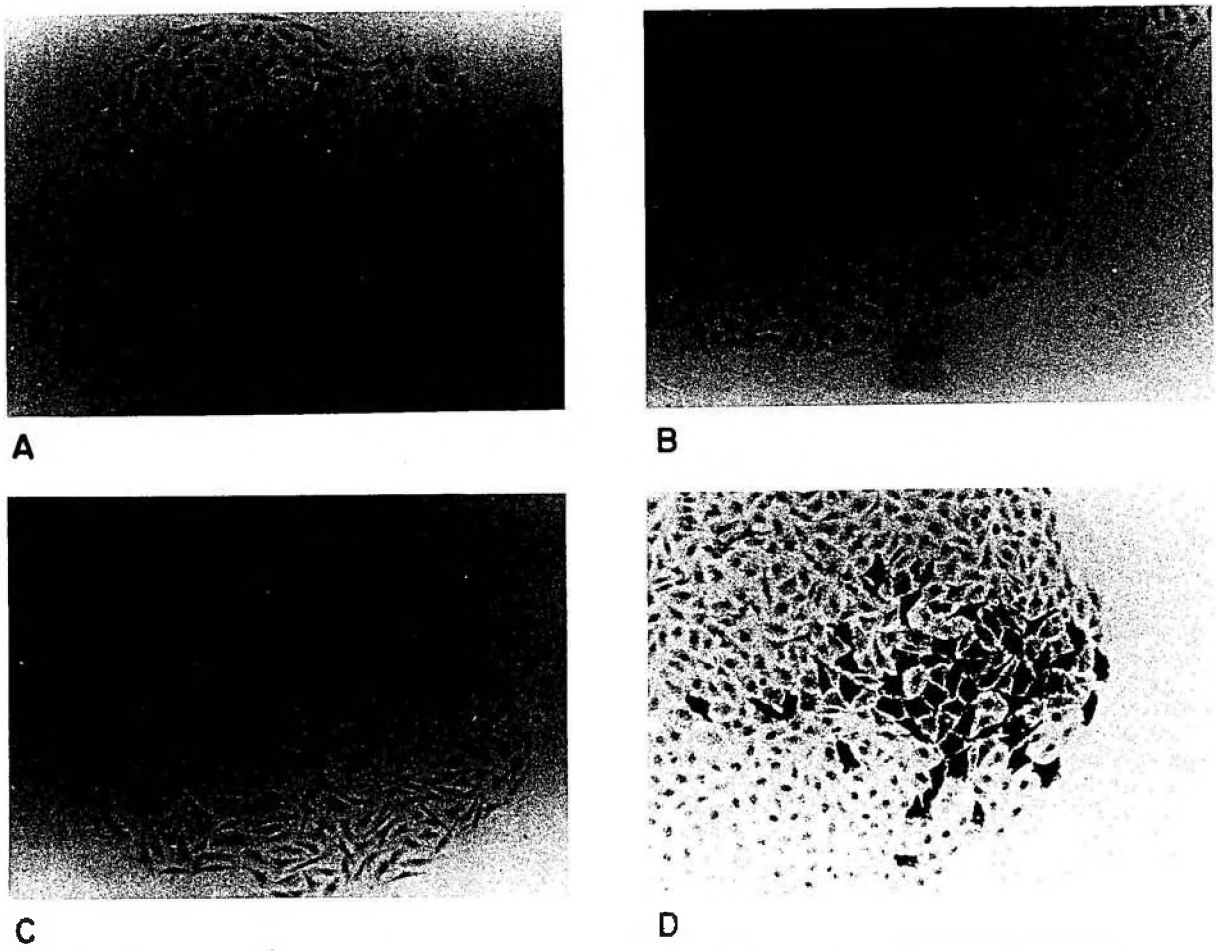

Figure 5. Detection of Thymidine Kinase and Gpt Activity in Cells Containing HSV *tk* and Eco-*gpt* Amber Mutations following Injection of X. laevis Amber Suppressor tRNA Genes

X. laevis wild-type tyrosine-tRNA genes (A and C) and amber suppressor tRNA genes (B and D) were injected into LMtk$^-$/APRT$^-$/HPRT$^-$/HSV-*tk*$^-$(amber)/Eco-*gpt*$^-$(amber)/*NPT-II*$^+$ cells (cell line 20) and assayed for thymidine kinase and Gpt enzymatic activity as described in the legend to Figure 3. After injection, the cells on coverslips (A) and (B) were incubated in MEM with ^{3}H-thymidine, and the cells on coverslips (C) and (D) were incubated in MEM with ^{3}H-guanine. Thymidine kinase and Gpt enzymatic activity were observed only in cells that received injections of the X. laevis amber suppressor tRNA genes and not in cells that received injections of wild-type X. laevis tyrosine-tRNA genes. Comparable numbers of cells received injections of wild-type or amber suppressor tRNA genes.

ochre, UAA) and efficiency of suppression with a tyrosine-inserting suppressor was determined for each mutant.

A subset of the amber mutations was transferred into cultured mammalian cells by microinjection with glass micropipettes (Capecchi, 1980). The conditions of gene transfer were selected to favor transcriptional competence of the injected genes (Folger et al., 1982). As many as four genes, one selectable and three nonselectable, could be cotransferred with an index approaching one. The presence of transcriptionally competent HSV *tk* and Eco-*gpt* amber mutant genes in the transformed cell lines was established by assaying for thymidine kinase and Gpt enzymatic activity in cells which received injections of suppressor tRNA isolated from yeast.

The HSV *tk* and Eco-*gpt* amber mutants are stringent. In cell lines containing these mutations, neither thymidine kinase nor Gpt enzymatic activity was detected by autoradiography under labeling conditions that would have detected 0.1% of the enzymatic activity present in cell lines containing normal HSV *tk* or Eco-*gpt* genes. The observed stringency of nonsense mutants in cultured mammalian cells is important, since application of the common conditional-lethal system (temperature-sensitive mutations) to mammalian cell genetics has met with some difficulty. The versatility of temperature-sensitive mutants has been limited because mammalian cells cannot be grown over as wide a temperature as bacteria or yeast. As a consequence, many temperature-sensitive mutants isolated in mammalian cells are "leaky"; that is, they exhibit measurable activity at the nonpermissive temperature, which has also been observed in viral mu-

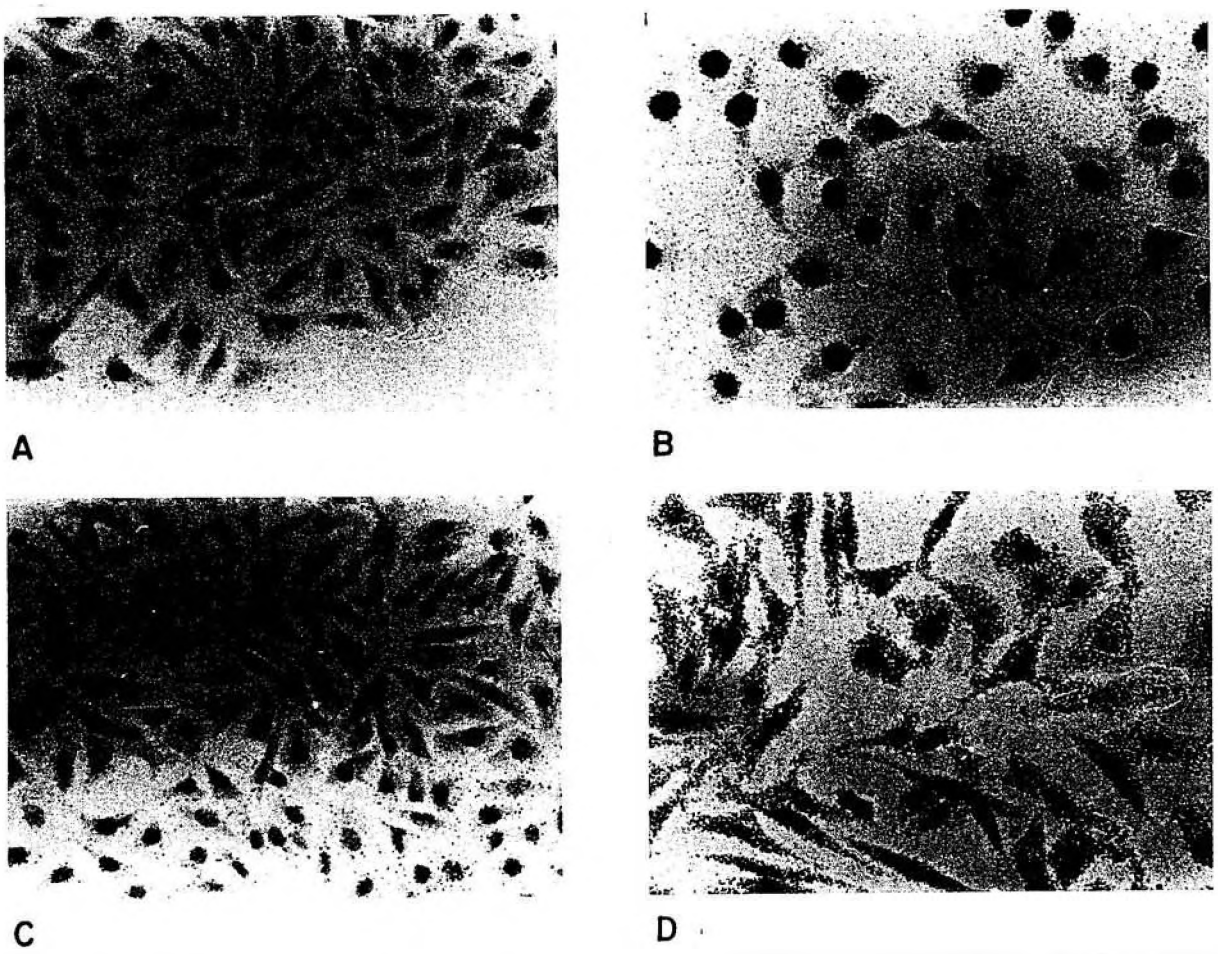

Figure 6. G418-Resistant Cells Obtained by Injection of X. laevis Amber Suppressor tRNA Genes into LMtk$^-$/APRT$^-$/HSV-*tk*$^-$(amber)/Eco-*gpt*$^-$(amber)/*NPT-II*$^-$(amber) Cells Also Express Thymidine Kinase and Gpt Activity

Cells containing amber mutations in the HSV *tk*, Eco-*gpt* and *NPT-II* genes (cell line 16) were injected with approximately ten copies per cell of X. laevis amber suppressor tRNA genes. Twenty-four hours after injection the cells were transferred into MEM with 10% fetal calf serum and G418 (250 μg/ml). Approximately one in ten cells that received an injection of amber suppressor tRNA genes gave rise to a G418-resistant colony. Following cloning, one of these cell lines (7) was incubated in MEM with 10% fetal calf serum and either ^{3}H-thymidine (25 μCi/ml; B) or ^{14}C-xanthine (3 μCi/ml; D). As a control, the parental cell line (that is, containing the amber mutations in the HSV *tk*, Eco-*gpt* and *NPT-II* genes) was also incubated in medium containing ^{3}H-thymidine (A) or ^{14}C-xanthine (C). After 16 hr of incubation in radioactively labeled medium, the cells were processed for autoradiography as described in the legend to Figure 3. As expected, cells selected for expression of *NPT-II* gene product also express thymidine kinase and Gpt activity.

tants. The use of tight nonsense mutants will allow more definitive complementation and recombination analysis of viral and host cell mutants.

The cell lines with defined amber mutations in the HSV *tk*, Eco-*gpt* and *NPT-II* genes were used to establish permanent cell lines containing functional amber suppressor genes. Previously, we synthesized an amber suppressor tRNA gene by site-specific mutagenesis of an X. laevis tyrosine-tRNA gene (Laski et al., 1982). Infection of monkey cells with an SV40 recombinant containing this amber suppressor produced a transient state which was permissive for amber mutants. Coinfection of these cells with adenovirus 2–SV40 hybrid viruses containing amber mutations in the 30 kilodaltons (kd) fusion protein yielded wild-type levels of this protein. We injected this X. laevis suppressor tRNA gene into murine cell lines containing one or more of the HSV *tk*, Eco-*gpt* or *NPT-II* amber mutations. Suppressor-positive transformants were selected on the basis of growth that required wild-type activity for one or more of the nonsense mutant gene products. The following observations indicate that the resulting transformants arose by acquiring a functional suppressor tRNA gene. First, the observed transformation frequency (~10%) is approximately 10^6-fold higher than the expected intergenic reversion frequency of an amber mutation. Second, no transformants were obtained after injection of wild-type X. laevis tyrosine-tRNA genes. Third, following injection of suppressor tRNA genes into a cell line

Table 1. Transformation Frequency Obtained by Injecting X. laevis Amber Suppressor or Wild-Type Tyrosine-tRNA Genes

Recipient Cell Line	Genes Injected	Selected on G418	Selected on HAT
HSV *tk*$^-$ (amber) Eco-*gpt*$^-$ (amber) *NPT-II*$^-$ (amber)	X. laevis amber suppressor tRNA gene	122/1000	97/1000
	X. laevis wild-type tyrosine-tRNA gene	0/1000	0/1000

Approximately ten copies per cell of either X. laevis amber suppressor or wild-type tyrosine-tRNA genes were injected into LMtk$^-$/APRT$^-$ cells containing HSV *tk*, Eco-*gpt* and *NPT-II* amber mutations. After injections, the cells were incubated for 24 hr in nonselective medium and then transferred to either G418 medium (250 μg/ml) or HAT medium (that is, selected for either NPT-II$^+$ or TK$^+$ phenotype). After 3 weeks in selective medium, the plates were scored for large healthy colonies. The LMtk$^-$/APRT$^-$/HSV-*tk*$^-$(amber)/Eco-*gpt*$^-$(amber)/*NPT-II*$^-$(amber) recipient cell line (designated 16) was obtained by coinjecting LMtk$^-$/APRT$^-$ cells with plasmid DNA containing HSV *tk*$^-$(*am1142*), Eco-*gpt*$^-$(*am63*), *NPT-II*$^-$(*am28*) and wild-type APRT and selecting for APRT$^+$ transformants in adenine–azaserine medium (Lowy et al., 1980).

Table 2. Comparison of Growth Rates of a Parental Cell Line with Three Derivative Cell Lines Containing Functional X. laevis Amber Suppressor tRNA Genes

Cell Line	Hours per Cell Division
LMtk$^-$/APRT$^-$/HSV-*tk*$^-$(*am1142*)/Eco-*gpt*$^-$(*am63*)/*NPT-II*$^+$ Cell Line 37 phenotype TK$^-$, Gpt$^-$	26
LMtk$^-$/APRT$^-$/HSV-*tk*$^-$(*am1142*)/Eco-*gpt*$^-$(*am 63*)/*NPT-II*$^+$/X. laevis amber suppressor tRNA gene Cell Line 35 phenotype TK$^+$, Gpt$^+$	30
LMtk$^-$/APRT$^-$/HSV-*tk*$^-$(*am1142*)/Eco-*gpt*$^-$(*am63*)/*NPT-II*$^+$/X. laevis amber suppressor tRNA gene Cell Line 37 phenotype TK$^+$, Gpt$^+$	24
LMtk/APRT$^-$/HSV-*tk*$^-$(*am1142*)/Eco-*gpt*$^-$(*am63*)/*NPT-II*$^+$/X. laevis amber suppressor tRNA gene Cell Line 39 phenotype TK$^+$, Gpt$^+$	27

The growth rates of one parental cell line and three derivative cell lines containing functional X. laevis suppressor tRNA genes were compared (see text). Eighteen 100 mm plates were seeded with an equal aliquot of each cell line and incubated at 37°C in 5% CO_2. Every 24 hr two plates of each cell line were treated with trypsin and the cell density was determined by multiple counts in a hemacytometer. The growth rate for each cell line was derived from the best-fit curve of the logarithm of cell density versus time.

containing amber mutations in the HSV *tk*, Eco-*gpt* and *NPT-II* genes and selection for *NPT-II* gene product (G418 resistance), the selected cell lines were also found to express wild-type thymidine kinase and Gpt activity as would be expected if the HSV *tk* and Eco-*gpt* amber mutations were also being suppressed. Finally, injection of tRNA isolated from the transformed cell lines described above into cell lines containing HSV *tk* and Eco-*gpt* amber mutations resulted in the transient expression of the HSV *tk* and Eco-*gpt* genes.

The procedure described above for establishing murine cell lines which contain functional suppressor tRNA genes should be applicable to the isolation of similar lines in a wide spectrum of cultured mammalian cells. Particularly useful for this purpose are the nonsense mutations in the Eco-*gpt* and *NPT-II* genes, which, when suppressed, confer a dominant selectable phenotype. Coreversion of multiple nonsense mutations may allow selection of endogenous suppressor genes, an alternative approach to the introduction of exogenously engineered suppressor genes. Both approaches should allow the generation of cell lines with suppressor genes of other codon specificity (for example, ochre) or ones that suppress by insertion of other amino acids. Furthermore, by modulating the number of copies of the amber mutants and amber suppressor genes in a given transformant, it may be possible to create cell lines with different levels of suppressor activity. We are currently determining how many suppressor tRNA genes are present in each transformant, and whether this number correlates with the amount of suppressor tRNA being synthesized and with the level of suppression exhibited in each cell line. We are also examining the stability of the transformants in nonselective medium. This parameter should provide insight on the existence or nonexistence of selection pressure to eliminate a suppressor tRNA gene from the cell.

Many tRNA genes are susceptible to conversion by site-specific mutagenesis to a suppressor tRNA gene. Temple et al. (1982) have synthesized an amber suppressor from a human lysine-tRNA gene and have shown that this modified gene functions as an amber suppressor in injected Xenopus oocytes. It is not unreasonable to expect that a battery of suppressor tRNA genes capable of inserting a spectrum of amino acids at amber and ochre mutations will soon become available. The availability of cell lines containing such suppressor genes will make it possible to isolate many new viral mutants by simple host-range screening procedures. The affected mutant gene product can often be identified, since it is possible to distinguish the mutant polypeptide fragment from the suppressed completed polypeptide chain. The stringency of nonsense mutations should facilitate complementation analysis. With these new genetic tools it should be possible to dissect the complex virus–host interactions leading to many virus-induced diseases.

Experimental Procedures

The methods used for culturing cells, microinjection, plasmid DNA preparation and autoradiography have been described elsewhere (Capecchi et al., 1977; Capecchi, 1980; Folger et al., 1982).

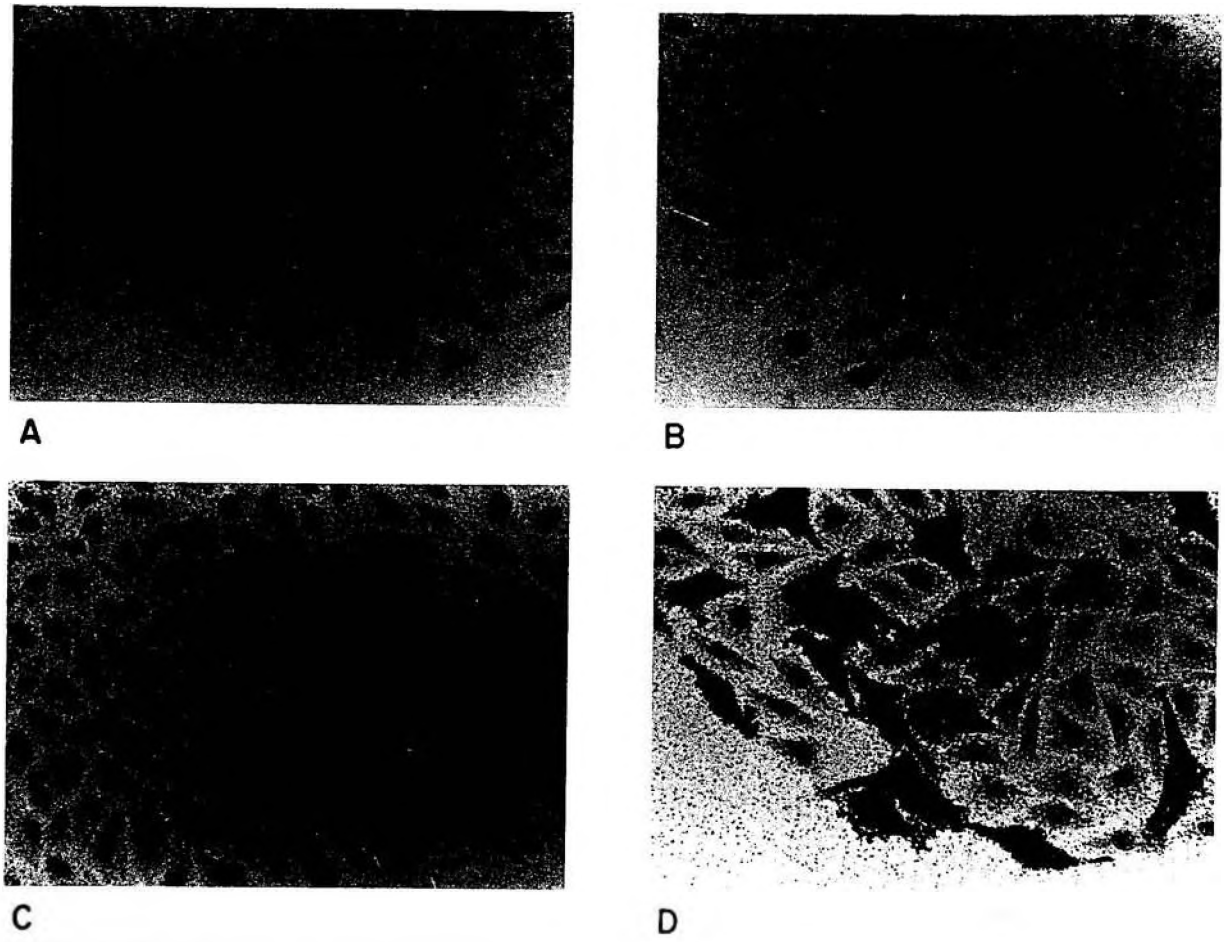

Figure 7. Functional Suppressor tRNA Is Present in Cell Lines Transformed with the Amber Suppressor Gene

tRNA was isolated from a cell line which was transformed by injection of X. laevis amber suppressor tRNA genes (cell line 39). Following purification, this tRNA (2 mg/ml) was injected into cells (cell line 20) containing the HSV *tk* and Eco-*gpt* amber mutations (B and D). As a control, tRNA isolated from the parental LMtk$^-$/APRT$^-$/HPRT$^-$ cell line was also injected into the cell line containing HSV *tk* and Eco-*gpt* amber mutations (A and C). Following injections of the tRNAs, the cells were incubated in medium containing ^{3}H-thymidine (A and B) or ^{3}H-guanine (C and D) and processed for autoradiography as described in the legend to Figure 3, except that the specific activity of the ^{3}H-thymidine (5 Ci/mmole) and ^{3}H-guanine (8.5 Ci/mmole) was threefold higher and the exposure time of the coverslips following dipping in NTB-2 autoradiographic emulsion was twofold longer.

Mutagenesis

Mutations were induced in the HSV *tk*, Eco-*gpt* and *NPT-II* genes by a procedure adapted from Hall and Tessman (1966) for in vitro chemical mutagenesis of bacteriophage T4 DNA. Several micrograms of plasmid DNA in 50 μl were made 0.4 M hydroxylamine and 1 mM EDTA (pH 6.0) and incubated at 37°C for 24–48 hr. The mutagen was removed by drop dialysis (Marusyk and Sargeant, 1980) against 4 mM Tris and 1 mM EDTA for 1 hr, and the DNA was transfected into the appropriate E. coli K12 strain. The frequency of mutants was 1/200–1/50.

Isolation and Characterization of Nonsense Mutations in the HSV *tk*, Eco-*gpt* and *NPT-II* Genes

For *gpt*$^-$ mutants, the transfected E. coli were plated into selective medium. To isolate HSV *tk*$^-$ and *NPT-II*$^-$ mutants the cells were first plated on L-ampicillin plates. Colonies were then picked with sterile toothpicks, stabbed to selective and nonselective replica plates and scored for the loss of function at 24–48 hr.

The host bacterial strain for isolating the *gpt* mutants was the *pur*H, J, Δ*gpt*$^-$, *hprt*$^-$ mutant S$_\phi$609 of Jochimsen et al. (1975). This strain was inhibited by adenine, thus all of the experiments were done with an adenine-resistant derivative. The scoring medium for the Gpt$^+$ characteristic was glucose, basal salts (Clark and Maaløe, 1967), 0.2% casamino acids, 20 μg/ml guanine and 100 μg/ml ampicillin. The selection medium for the Gpt$^-$ phenotype contained glucose, basal salts, 0.2% casamino acids, 15 μg/ml adenine, 5.5 μg/ml 6-thioguanine and 100 μg/ml ampicillin (Holden et al., 1976).

The host strain for the HSV *tk*$^-$ mutants was the KY895 strain of Igarashi et al. (1967), which is deficient for the endogenous E. coli thymidine kinase. Plates to differentiate between TK$^+$ and TK$^-$ bacteria contained glucose, basal salts, 0.2% casamino acids, 25 μg/ml 5-fluorouracil, 25 μg/ml uridine, 50 μg/ml thymidine and 100 μg/ml ampicillin. The 5-fluorouracil blocks the de novo pathway of thymidylate synthesis, making the bacteria dependent on exogenous thymidine.

The host strain for the *NPT-II*$^-$ mutants was DHI. Transfectants were scored for resistance to 10–70 μg/ml of kanamycin on L plates supplemented with 100 μg/ml of ampicillin. The collection of HSV *tk*, Eco-*gpt* and *NPT-II* mutants were then tested for suppressibility with specialized φ80p transducing phages carrying E. coli amber or ochre suppressor tRNA genes.

Candidate clones were grown in liquid L broth and 100 μl was plated onto selective plates and spread. A drop of phage lysate was then placed on the plate and incubated at 37°C for 24–48 hr. Nonsense suppressibility was indicated by growth only where the virus drop was placed.

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