Functional Suppression in Mammalian Cells of Nonsense Mutations in the Herpes Simplex Virus Thymidine Kinase Gene by Suppressor tRNA Genes

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A nonsense mutation (UAG) in the thymidine kinase gene of herpes simplex virus type 1 can be suppressed in vivo to produce active thymidine kinase by prior infection with a defective simian virus 40 stock which acts as a vector to introduce a functional suppressor tRNA gene into mammalian cells in culture. The suppression is specific for UAG, but not UGA or missense, mutants and restores thymidine kinase activity to 20 to 40% of the wild-type level. These results suggest that many cell lines susceptible to simian virus 40 infection may be transiently converted to a suppressor-positive phenotype for use in the genetic study of mammalian viruses.

Translational suppression is a phenomenon well known in procaryotic and lower eucaryotic organisms (7). The availability of cells which carry suppressor mutations makes possible the isolation of conditional lethal mutations in viruses and cells which depend on suppression of nonsense codons for growth. Amber mutants in phage T4 are the classic example of such suppressor-sensitive or conditional lethal virus mutants (3). Mammalian cells expressing genes encoding suppressor tRNAs should allow isolation and propagation of nonsense mutants of animal viruses which are identifiable by their failure to grow on nonpermissive cells lacking the suppressor allele.

Previously we have isolated and characterized nonsense mutations in the thymidine kinase (TK) gene of herpes simplex virus type 1 (HSV-1) (2, 6, 9). These mutant viruses do not make functional TK (a nonessential enzyme in normal culture) when grown on normal cells. Such virus mutants were designed as test virus for functional suppression in mammalian cells.

Construction, by in vitro mutagenesis, of suppressor tRNA genes from eucaryotic organisms has been reported recently (5, 10). One of these suppressor tRNA genes was also shown to suppress, in vivo, nonsense mutations in hybrid adenovirus type 2-ND1 virus (5). In this report we describe the function of one of these suppressor tRNA genes (5) to suppress a known nonsense (amber) mutation in the HSV TK gene to restore functional TK expression. These experiments demonstrate the potential of such a trans-

lation suppressor system for studies of animal virus genetics.

The *su*⁺ tRNA *tyr* gene was cloned in the late region of simian virus 40 (SV40) and propagated with helper virus as a mixed stock of defective SV40 virions, as previously described (5). CV-1 monkey kidney cells were infected with the *su*⁺ SV40 vector, and after a period of time to permit expression of *su*⁺ tRNA, the cells were infected with HSV containing nonsense mutations in the TK gene. TK enzyme activity was measured after HSV infection to detect functional suppression of the HSV TK mutations.

HSV-1, strain CL101, and the TK-negative mutants derived from this strain have been described previously (2, 6, 9). HSV TK4, HSV TK43, and HSV TK37 all fail to make full-length TK polypeptides and are thought to be nonsense mutants. TK4 and TK43 can be suppressed in vitro with UAG and UGA suppressor tRNAs, respectively, from yeast (2). The mutation in TK37 has not been defined yet. HSV TK19 is presumably a missense mutation since it produces a full-length TK protein and induces TK activity to about 1 to 3% of the wild-type level. Although HSV TK43 induces no detectable TK activity during the lytic cycle, HSV TK4 is "leaky" and induces 2 to 4% of the wild-type level. This leakiness may be the result of a low level of synthesis of TK-related polypeptide from an internal AUG, since TK4 maps very near the amino terminus of the protein (6), and it is known from the work of Smiley (personal communication) that sequences encoding at

FIG. 1. Assay of TK activity in cells with and without suppression. The CV-1 monolayers were infected with SV40 at an approximate multiplicity of 10 PFU per cell for wild-type SV40 and with what was estimated to be the equivalent amount of virus for the SV(*su*⁻) or SV(*su*⁺) tRNA viruses. At 40 h after infection with SV40, the monolayers (20 cm²) were infected with HSV-1 at multiplicities of 10 PFU per cell. Wild-type HSV (TK⁺) and two nonsense mutants, TK4 (UAG) and TK43 (UGA), were used. Each infection was done in duplicate, and separate extracts were made at 24 h after HSV infection. The cells were harvested and sonicated, centrifuged extracts were prepared as previously described (8, 9), and the HSV TK was assayed by the [¹²⁵I]deoxycytidine phosphorylation assay (8). The use of iododeoxycytidine as the assay substrate measures HSV TK specifically (8). These assays were linear for at least 2 h (or until substrate depletion). Protein concentrations in the extracts were determined with Bio-Rad dye reagent concentrate (Bio-Rad Laboratories) (1). Duplicate assays are shown where phosphorylation of [¹²⁵I]-deoxycytidine is measured as a function of incubation time at 37°C.

least the 45 N-terminal amino acids can be removed by deletion from the 5' end of the TK-coding sequence without producing a phenotypically TK-negative gene. HSV-1 virus stocks were prepared by low-multiplicity passage on

TABLE 1. Summary of TK activities induced by HSV-1 wild-type and mutant virus upon infection of CV-1 monolayers which had been infected 40 h previously with SV(*su*⁺), SV(*su*⁻), or SV40 virus stocks^a

Induction by:	HSV TK activity (cpm μg of protein ⁻¹ h ⁻¹)			
	<i>su</i> ⁺	<i>su</i> ⁻	SV40	Mock
HSV TK ⁺	230	290	230	160
HSV TK4 (UAG)	53	11	7.0	8.4
Mock	1.4	2.2	1.3	2.0
HSV TK43 (UGA)	1.1	1.2	0.88	ND ^b
HSV TK19 (missense)	1.8	1.5	1.4	0.96
HSV TK37 (?nonsense)	1.1	1.2	1.4	ND

^a HSV TK37 makes a shortened TK peptide and is presumed to be a nonsense mutant. TK activities were calculated from the initial linear slopes of incorporation data such as shown in Fig. 1. Duplicate samples were used for each measurement.

^b ND, Not determined.

Vero cells and were assayed for virus titer on these cells. SV40 wild-type stocks and the SV40 stocks carrying the wild-type or suppressor allele were prepared as recently described (5). CV-1 cells were infected with a mixture of the helper SV-rINS-7, which has a deletion/substitution in the early region, and the tRNA-carrying SV-tT-2(*su*⁻) or SV-tT-2(*su*⁺) virus, which has the tRNA gene inserted in the late region. About 1 week after infection, cytopathic degeneration was observed, and virus stocks were harvested. These were designated SV(*su*⁻) and SV(*su*⁺), respectively. In some experiments a second- or third-passage stock of the mixed virus stock was used, prepared by infecting CV-1 cells with progeny of the original mixed infection or the second passage, respectively.

The experiment shown in Fig. 1 and Table 1 demonstrates that infection with wild-type SV40, SV40 with the normal *su*⁻ tyrosine tRNA gene, SV40 with the *su*⁺ allele of the tyrosine tRNA gene, and mock infection have no significant effect on TK induction by wild-type HSV. Also, mutants TK43, TK19, and TK37 all fail to respond to the suppressor allele. However, for the amber (UAG) mutant TK4 (which is known to be leaky [6]) TK activity is restored to a level of about 20% of the wild-type activity.

This experiment allows three important conclusions. First, the suppressor gene functions to restore TK-inducing activity to a TK⁻ virus. Second, the suppression is allele specific; i.e., only the UAG mutation is suppressed (Table 1). Third, the specific mutation in HSV TK4 when suppressed with tyrosine tRNA gives a functional product. The actual insertion of tyrosine at the nonsense codon, however, has yet to be demonstrated. In the first half of the wild-type sequence tyrosine occurs at positions 4, 53, 80, 87, 101, and 152 (11). It is important to note, however, that the functional suppression by tyrosine insertion does not imply that tyrosine is present in the wild-type protein at the site of the mutation, but only that tyrosine insertion is compatible with enzymatic activity. In fact, mutant TK4 was characterized originally as an amber mutant by restoration of TK activity by serine-inserting amber suppressor tRNA (2).

To characterize this system further and show its potential utility for HSV genetic studies, we examined the time course of the development and maintenance of the suppressor state in SV(*su*⁺)-infected cells. In Fig. 2 is shown an experiment in which the interval between SV(*su*⁺) infection and HSV-1 infection varied from 0 to 60 h. The samples were harvested at 24 h after HSV infection. Efficient levels of suppression developed during the 24- to 48-h interval after SV(*su*⁺) infection and persisted until at least 60 h. Since the last set of samples was

FIG. 2. Suppression of HSVTK4 (UAG) as a function of time between SV(*su*⁺) infection and HSV infection. The experimental procedures were the same as for Fig. 1. The TK activity induced by HSV TK⁺ at each time interval was taken as 100%, and the activities induced by the mutant HSVs are expressed as a percentage of the wild-type control. All HSV infections proceeded for 24 h, at which time extracts were prepared and the TK activity was measured as described in the text.

FIG. 3. Capacity of cells infected with SV(*su*⁺) virus to support plaquing and growth of HSV. Near-confluent cultures of CV-1 cells were infected with different volumes of SV(*su*⁺) stock virus preparation. After 40 h the cells were infected with HSV: wildtype WT; (×) or mutant TK4 (O). Cells were infected with HSV at a multiplicity of 0.01. Two days later the cultures (cells and medium) were twice frozen and thawed and sonicated, and the virus was titrated on Vero cells to determine virus yield. HSV yield in the controls with no SV40 (1.0) was approximately 2×10^4 times the inoculum. (A) Cells were infected with HSV at a multiplicity of 0.01. Two days later the cultures (cells and medium) were twice frozen and thawed and sonicated, and the virus was titrated on Vero cells to determine virus yield. HSV yield in the controls with no SV40 (1.0) was approximately 2×10^4 times the inoculum. (B) Cells were infected with 100 PFU of HSV per 2 cm² of culture. Efficiency of plating (E.O.P.) was determined by counting plaques after 2 days. The number of plaques in controls (no SV40) was approximately 40% of the number obtained on comparable cultures of Vero cells, the cells routinely used for growth and titrating of the virus. (C) Cells were infected with HSV at a multiplicity of 10. Twenty-four hours later TK activity was measured as in the legend to Fig. 1. Activities are expressed as the fraction of activity of CV-1 cells not infected with SV40 and infected with wild-type HSV. This SV(*su*⁺) virus stock suppressed the amber mutant TK virus to produce 40% of wild-type TK activity.

harvested at 84 h, the SV(*su*⁺)-treated monolayers were shown to be functional for at least 3.5 days. Morphological observation of the monolayers showed that they were still intact at 5 and 6 days, with only minimal evidence of SV40 cytopathology. It is worth noting that this experiment was done with SV40 stocks made by a second passage of the mixed defective SV40

stock on CV-1 cells, rather than with the original stock made by DNA transfection (5).

Figure 3 shows the effect of SV(*su*⁺) infection on the efficiency of plating and yield of HSV-1 and the level of suppression. Various amounts of third-passage SV(*su*⁺) stock were added to 2-cm² monolayers of CV-1 cells, and then HSV-1 TK4 virus was added at a low multiplicity of infection (1,000 PFU/2 cm²) to determine virus yield, very low multiplicity (about 100 PFU/2 cm²) to determine plaquing efficiency, and high multiplicity (10⁶ PFU/2 cm²) to detect functional suppression by TK enzyme assay. These data show that it is possible to find conditions which give efficient suppression (up to 40%) but interfere with HSV growth only to a limited extent.

Since HSV grows rapidly and makes observable plaques on CV-1 cells in 2 to 3 days, it should be possible to carry out virus genetic studies on SV(*su*⁺)-infected monolayers as a permissive host for nonsense mutants of HSV. This potential ability to make many cell lines into transient suppressor-carrying cells may be useful for genetic studies of other viruses and may complement the alternate approach of construction of cell lines which carry and express the tRNA alleles in a stable form (4).

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LITERATURE CITED

1. Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 72:248-254.
2. Cremer, K. J., M. Bodemer, W. P. Summers, W. C. Summers, and R. F. Gesteland. 1979. In vitro suppression of UAG and UGA mutants in the thymidine kinase gene of herpes simplex virus. *Proc. Natl. Acad. Sci. U.S.A.* 76:430-434.
3. Epstein, R. H., A. Bolle, C. M. Steinberg, E. Kellenberger, E. Boy de la Tour, R. Chevalley, R. S. Edgar, M. Susman, G. H. Denhardt, and A. Lielausis. 1963. Physiological studies of conditional lethal mutations of bacteriophage T4D. *Cold Spring Harbor Symp. Quant. Biol.* 28:375-392.
4. Hudziak, R. M., F. A. Laski, U. L. RajBhandary, P. A. Sharp, and M. R. Capecchi. 1982. Establishment of mammalian cell lines containing multiple nonsense mutations and functional suppressor tRNA genes. *Cell* 31:137-146.
5. Laski, F. A., R. Belagaje, U. L. RajBhandary, and P. A. Sharp. 1982. An amber suppressor tRNA gene derived by site-specific mutagenesis: cloning and function in mammalian cells. *Proc. Natl. Acad. Sci. U.S.A.* 79:5813-5817.
6. Smiley, J. R., M. J. Wagner, W. P. Summers, and W. C. Summers. 1980. Genetic and physical evidence for the polarity of transcription of the thymidine kinase gene of herpes simplex virus. *Virology* 102:83-93.
7. Steege, D. A., and D. Söll. 1979. Suppression, p. 433-485. In R. H. Goldberg (ed.), *Biological regulation and development*, vol. 1. Plenum Press, New York.
8. Summers, W. C., and W. P. Summers. 1977. [¹²⁵I]deoxycytidine used in a rapid, sensitive, and specific assay for herpes simplex virus type 1 thymidine kinase. *J. Virol.* 24:314-318.
9. Summers, W. P., M. Wagner, and W. C. Summers. 1975. Possible peptide chain termination mutants in thymidine kinase gene of a mammalian virus, herpes simplex virus. *Proc. Natl. Acad. Sci. U.S.A.* 72:4081-4084.
10. Temple, G. F., A. M. Dozy, K. L. Roy, and Y. W. Kan. 1982. Construction of a functional human suppressor tRNA gene: an approach to gene therapy for β -thalassaemia. *Nature (London)* 296:537-540.
11. Wagner, M. J., J. A. Sharp, and W. C. Summers. 1981. Nucleotide sequence of the thymidine kinase gene of herpes simplex virus type 1. *Proc. Natl. Acad. Sci. U.S.A.* 78:1441-1445.