Proc. Natl. Acad. Sci. USA
Vol. 84, pp. 8031–8034, November 1987
Genetics

Physiological levels of normal $tRNA^{Gln}_{CAG}$ can effect partial suppression of amber mutations in the yeast *Saccharomyces cerevisiae*

(genetic code/wobble/ciliated protozoa/evolution)

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Communicated by John R. Preer, Jr., July 27, 1987

ABSTRACT **A number of ciliated protozoa are known to read the stop codons UAA and UAG as sense codons that specify glutamine during protein synthesis. In considering evolutionary mechanisms for this curious divergence from the standard genetic code, we propose the existence of progenitor tRNAs for glutamine that can weakly suppress UAA and UAG codons. It has been previously shown that multicopy plasmids that overexpress normal $tRNA^{Gln}_{CAA}$ and $tRNA^{Gln}_{CAG}$ genes from the yeast *Saccharomyces cerevisiae* can partially suppress a number of yeast ochre and amber mutations, respectively. In the present study we show that the $tRNA^{Gln}_{CAG}$ gene can also function as a weak amber suppressor when expressed in cells at physiological levels. This observation is consistent with a role of $tRNA^{Gln}_{CAG}$ as an evolutionary progenitor of tRNAs that strongly decode UAG codons.**

In previous studies (ref. 1; I.E. and M.R.C., unpublished data) we isolated a normal $tRNA^{Gln}_{CAG}$ gene from the yeast *Saccharomyces cerevisiae* and demonstrated that its overexpression from a multicopy plasmid results in partial suppression of a number of different amber mutations in the yeast genome. In related studies it was shown that a multicopy plasmid containing the normal gene for $tRNA^{Gln}_{CAA}$ can partially suppress ochre mutations exclusively (2). In both situations anticodon–codon interactions require G·U mispairing at the first codon position, thus raising the interesting possibility that during protein synthesis in eukaryotes wobble may not be restricted to the third codon position, as is traditionally assumed (3).

The observation of nonsense codon suppression by two different *normal* tRNAs, both of which decode glutamine, may have implications for the evolution of divergence from the universal genetic code recently documented for several ciliated protozoa. In these organisms amber and ochre codons have been demonstrated in the coding region of nuclear genes and are decoded with high efficiency by tRNAs normally charged with glutamine (4–8). In one of these protozoa (*Tetrahymena thermophila*), two glutamine tRNA species with the anticodon CUA or UUA have been identified (9, 10) that can decode UAG and UAA codons, respectively, by normal base pairing.

It is not obvious how such divergence from the normal genetic code evolved. It has been suggested that in the progenitors of these ciliated protozoa the stop codons UAG and UAA served as weak translational stop signals and were only rarely used (10). We have previously suggested (11) that such circumstances might have set the stage for the selection of mutational alterations in glutamine tRNAs that normally decode CAG and CAA, such that they could also weakly decode the erstwhile stop codons UAG and UAA. Weak nonsense suppression could allow the random genetic drift of amber and ochre codons into the coding region of essential nuclear genes and their subsequent loss as functional stop codons. The observation of partial suppression of amber and ochre codons by normal glutamine tRNAs overexpressed in yeast (ref. 1; I.E. and M.R.C., unpublished data) may thus have relevance for the evolution of glutamine tRNAs in ciliates that decode these codons with high efficiency.

The existence of natural suppressor tRNA genes in eukaryotes has been documented in many organisms; however, the molecular basis for this phenomenon is poorly understood. One case in which the mechanism of natural suppression has been characterized is that of a UAG suppressor isolated from *Drosophila*, tobacco, and wheat (12–14). This suppressing species has been identified as a normal $tRNA^{Tyr}$ in which the QψA anticodon is undermodified and exists instead as GψA. Only the undermodified $tRNA^{Tyr}$ species can suppress amber codons (12–14). Although undermodified $tRNA^{Tyr}$ exists normally in *Drosophila* and plant cells, such undermodification could potentially result from the overexpression of normal tRNA genes. Undermodification of an overexpressed tRNA species could lead to suppression events that would not be observed physiologically. Thus, biologically significant nonsense suppression must be examined in the context of physiological levels of that nonsense suppressor.

Previous studies from our laboratories examined suppression of UAG and UAA codons in yeast cells in which the $tRNA^{Gln}_{CAG}$ and $tRNA^{Gln}_{CAA}$ genes were overexpressed from multicopy plasmids (refs. 1 and 2; I.E. and M.R.C., unpublished data). Unlike most tRNA genes, the former is present in the yeast genome in only a single copy (ref. 1; I.E. and M.R.C., unpublished data) and hence provides the opportunity for investigating whether suppression of amber mutations by normal $tRNA^{Gln}_{CAG}$ can also occur under physiologically more relevant conditions. Experimental examination of this issue is not trivial because the $tRNA^{Gln}_{CAG}$ gene is essential and haploid strains lacking a functional copy of the gene are inviable (ref. 1; I.E. and M.R.C., unpublished data). We therefore devised a modified approach to study this question. It has recently been demonstrated (I.E. and M.R.C., unpublished data) that transformation with a multicopy plasmid that encodes the major glutamine isoacceptor species $tRNA^{Gln}_{CAA}$ [which weakly suppresses ochre but not amber mutations (1)] rescues the lethality of cells in which the $tRNA^{Gln}_{CAG}$ gene is disrupted. Such rescue presumably reflects the ability of $tRNA^{Gln}_{CAA}$ to decode the CAG glutamine codon by conventional G·U wobble in the third codon position. We show here that the introduction of the $tRNA^{Gln}_{CAG}$ gene into such rescued cells by transformation with a centromeric plasmid [an autonomously replicating yeast plasmid typically present in

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only one or two copies per cell (15)] results in partial suppression of *rad10* and *ade2* amber mutations in yeast.

MATERIALS AND METHODS

Yeast Strains and Plasmids. Construction of the yeast strains YH50 (*RAD10*, $\Delta tDNA^{Gln}_{CAG}$, *ade2*-101_a) and YH60 (*rad10*-1_a, $\Delta tDNA^{Gln}_{CAG}$, *ade2*-101_a) are as described below. The diploid yeast strain YD10 (*RAD10/rad10*-1_a, $tDNA^{Gln}_{CAG}/\Delta tDNA^{Gln}_{CAG}$) and the haploid strains YH10 (*Δrad10*), LP2817-2D (*rad10*-1_a), and AB1841 (*RAD10*, *ade2*-101_a) have been described (1). Plasmids pJDB207, pSUP207, YCp19ΔURA3, and pNF4015 are also as described (1). Plasmid YCp19HIS was constructed by cloning a *Bam*HI–*Xho* I fragment containing the yeast *HIS3* gene into the *Bam*HI and *Sal* I sites of YCp19ΔURA3. The centromeric plasmids $pC689_{UAG}$, $pC689_{UAA}$, and $pC689_{UGA}$, carrying the amber, ochre, and opal alleles, respectively, of *sup4*, were from Maynard Olson (16).

Construction of Yeast Strains for Amber Suppression Studies. A *RAD10/rad10*-1_a diploid strain deleted in one of the two chromosomal $tRNA^{Gln}_{CAG}$ genes (YD10) was transformed with pSUP19HIS, a centromeric plasmid containing a single copy of the cloned $tRNA^{Gln}_{CAG}$ gene (1). The diploid was sporulated and a haploid *rad10*-1_a segregant was isolated that carries the tDNA deletion and plasmid pSUP19HIS. This haploid was mated to strain AB1841 (*RAD10*, *ade2*-101_a) to generate the *RAD10/rad10*-1_a, *ADE2/ade2*-101_a, $tDNA^{Gln}_{CAG}/\Delta tDNA^{Gln}_{CAG}$ diploid shown at the top of Fig. 1. The diploid was sporulated and *ade2*-101_a, $\Delta tDNA^{Gln}_{CAG}$ haploids (carrying pSUP19HIS) were isolated that were additionally *RAD10* or *rad10*-1. Both haploids were transformed with the multicopy plasmid pNF4015 carrying the cloned $tRNA^{Gln}_{CAA}$ gene. The resulting transformants [YH50 (*RAD10*, $\Delta tDNA^{Gln}_{CAG}$, *ade2*-101_a) and YH60 (*rad10*-1_a, $\Delta tDNA^{Gln}_{CAG}$, *ade2*-101_a)] were cured of plasmid pSUP19HIS by mitotic selection for *his3* auxotrophs (Fig. 1). Cured strains were transformed with the centromeric vector without the tRNA gene (YCp19HIS) to generate the control strains YH51 and YH61 (Fig. 1).

Nonsense Suppression Assays. UV survival of various strains was as described (2). Suppression of the *ade2*$_a$ mutants was measured by growing these strains in minimal medium containing limiting amounts of adenine (3 μg/ml) to $OD_{600} = 1.3$. Samples from each culture were removed and plated to quantitate cell number. Cells were harvested by centrifugation and the color of the cell pellets was examined to evaluate amber suppression.

Quantitation of Plasmid Copy Number. DNA from strains YH50, YH51, YH60, and YH61 was prepared as described (17), restricted with *Eco*RI, and electrophoresed in a 0.7% agarose gel. This gel was transferred to Genetran 45 paper (Plasco, Woburn, MA) and probed with an *Eco*RI–*Pvu* II fragment from CEN4 (18). *Eco*RI linearizes plasmids YCp19HIS and pSUP19HIS, and these plasmid bands migrate ≈1 kilobase larger than an *Eco*RI fragment containing the chromosomal copy of CEN4. The ratio of band intensities for plasmid and chromosomal copies of CEN4 was determined by densitometry.

RESULTS AND DISCUSSION

The *RAD10* gene of *S. cerevisiae* is required for the excision repair of DNA damage produced by exposure of cells to UV radiation (19). It was previously shown that the UV sensitivity of a *rad10* mutant strain (*rad10*-1) is partially suppressed by a multicopy plasmid containing the cloned $tRNA^{Gln}_{CAG}$ gene (1, 17). This gene suppresses a number of well-characterized yeast amber mutations (ref. 1; I.E. and

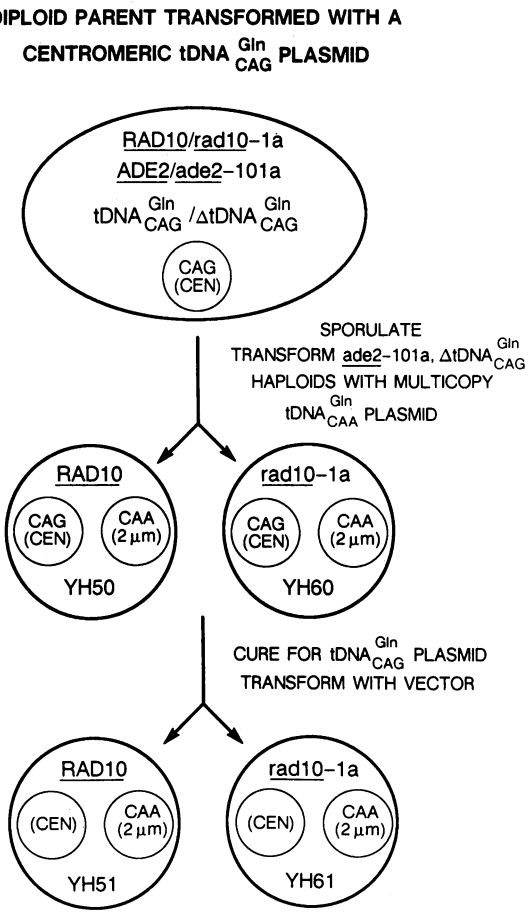

FIG. 1. Construction of isogenic strains carrying one or no copies of the $tRNA^{Gln}_{CAG}$ gene. Strains YH50 and YH51 represent isogenic *RAD10* strains that both carry a multicopy (2-μm based) plasmid with the $tRNA^{Gln}_{CAA}$ gene. In addition, YH50 carries a centromeric (CEN) plasmid with the $tRNA^{Gln}_{CAG}$ gene, whereas YH51 carries the same plasmid without the tRNA gene. The same plasmids are present, respectively, in the *rad10*-1_a strains YH60 and YH61.

M.R.C., unpublished data), thus suggesting that the *rad10*-1 allele also harbors an amber mutation (1). In the present studies we have confirmed this result by showing that the UV sensitivity of *rad10*-1 is suppressed by a plasmid carrying the tyrosine-inserting amber-specific suppressor *sup4* (Fig. 2, closed circles). Plasmids containing the isogenic *sup4* ochre (Fig. 2, closed squares) and opal (data not shown) suppressors have no effect on the UV sensitivity of *rad10*-1. In keeping with conventional terminology, we designate the amber mutation in *rad10*-1 as *rad10*-1_a.

We constructed two strains in which the chromosomal $tRNA^{Gln}_{CAG}$ gene was inactivated by partial deletion and which additionally carry the *ade2*-101_a allele and either the *RAD10* or *rad10*-1_a allele. Both strains remain viable because they also carry the $tRNA^{Gln}_{CAG}$ gene on a centromeric plasmid (1). These two haploid strains were independently transformed with a multicopy plasmid carrying the cloned $tRNA^{Gln}_{CAA}$ gene. When the resulting transformants [YH60 (*rad10*-1_a, $\Delta tDNA^{Gln}_{CAG}$, *ade2*-101_a) and YH50 (*RAD10*, $\Delta tDNA^{Gln}_{CAG}$, *ade2*-101_a)] were cured of the centromeric plasmid, viability was maintained by overexpression of $tRNA^{Gln}_{CAA}$ from the multicopy plasmid (Fig. 1). The cured strains were then transformed with a centromeric vector to generate a pair of control strains (YH61 and YH51) without a functional $tRNA^{Gln}_{CAG}$ gene (Fig. 1).

We carried out quantitative measurements on the UV sensitivity of the four strains described above to determine whether expression of $tRNA^{Gln}_{CAG}$ from the small number of

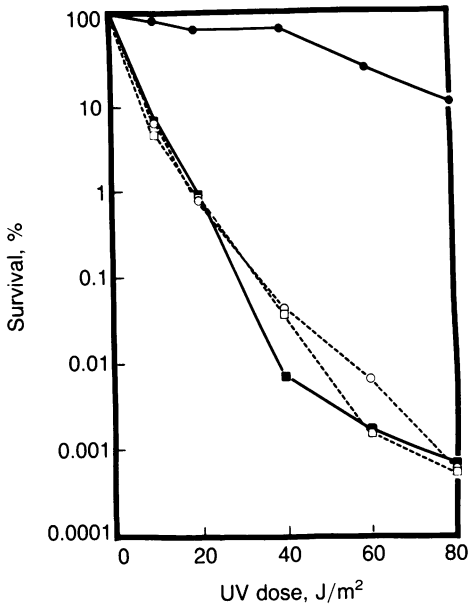

FIG. 2. A plasmid carrying the $sup4_a$ gene suppresses the $radl0\text{-}1_a$ mutation. Strain LP2817-2D ($radl0\text{-}1_a$) was transformed with the multicopy plasmid vector pJDB207 and with either the $sup4_a$ plasmid $pC689_{UAG}$ (●), $pC689_{UAA}$ (■), or the vector YCp19ΔURA3 (□). The survival of strain YH60 is shown for comparison (○). This strain also carries the $radl0\text{-}1_a$ mutation but is deleted in the chromosomal $tRNA^{Gln}_{CAG}$ gene. However, this gene is present on the centromeric plasmid pSUP19HIS. The strain is additionally transformed with the multicopy plasmid pNF4015 carrying the $tRNA^{Gln}_{CAA}$ gene.

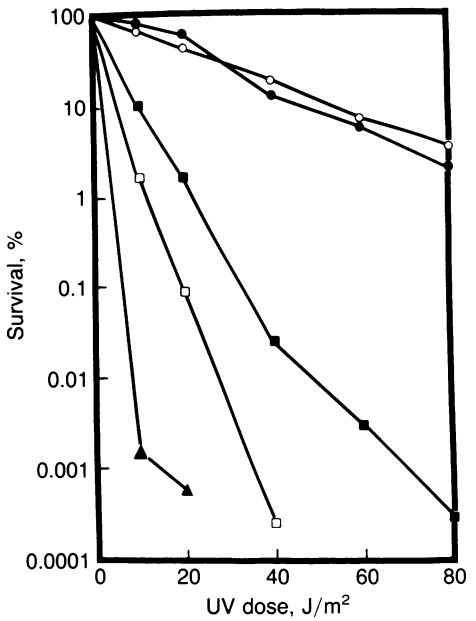

FIG. 3. A centromeric plasmid carrying the $tRNA^{Gln}_{CAG}$ gene can effect partial suppression of the $radl0\text{-}1_a$ mutation. Strains described in the text and in the legend to Fig. 1 were UV irradiated and survival was measured. ●, Strain YH50 transformed with the multicopy plasmid pNF4015 carrying the $tRNA^{Gln}_{CAA}$ gene and the centromeric plasmid pSUP19HIS carrying the $tRNA^{Gln}_{CAG}$ gene; ○, strain YH51 carrying the multicopy plasmid pNF4015 and the centromeric vector YCp19HIS; ■, strain YH60 carrying plasmids pNF4015 and pSUP19HIS; □, strain YH61 carrying plasmids pNF4015 and YCp19HIS; ▲, strain YH10 (Δ*rad10*) carrying the multicopy plasmid vector pJDB207 and YCp19HIS.

copies typically present in cells transformed with centromeric plasmids results in suppression of the $radl0\text{-}1_a$ mutation. The *RAD10* strains (YH50 and YH51) show equivalent UV resistance at levels expected of Rad$^+$ yeast cells (Fig. 3). Thus, it is evident that the absence of a functional $tRNA^{Gln}_{CAG}$ gene in YH51 does not affect UV sensitivity nonspecifically. Strain YH61 carrying the $radl0\text{-}1_a$ allele (and which is deleted of the $tRNA^{Gln}_{CAG}$ gene) is distinctly less UV resistant than the Rad$^+$ strains (Fig. 3). However, in the presence of a centromeric plasmid carrying the $tRNA^{Gln}_{CAG}$ gene the level of UV resistance of strain YH60 is increased (Fig. 3) to that of a $radl0\text{-}1_a$ mutant without a disruption of the gene (Fig. 2). Thus, there is significant enhancement of UV resistance in $radl0\text{-}1_a$ mutant cells carrying the wild-type $tRNA^{Gln}_{CAG}$ gene on a centromeric plasmid, as compared with isogenic strains lacking a wild-type copy of this gene.

The $tRNA^{Gln}_{CAG}$ gene on plasmid pSUP19HIS is clearly expressed, since, as indicated above, it supports the viability of cells deleted in the chromosomal gene (1). The level of $tRNA^{Gln}_{CAG}$ expressed in YH60 could not be directly measured by hybridization because $tRNA^{Gln}_{CAG}$ is 97% homologous with $tRNA^{Gln}_{CAA}$ (1). Nonetheless, the observation that the UV sensitivity of this strain is essentially the same as that of the parent $radl0\text{-}1_a$ strain carrying a single chromosomal copy of $tDNA^{Gln}_{CAG}$ (Fig. 2) suggests that the two strains express comparable levels of $tRNA^{Gln}_{CAG}$. These experiments therefore demonstrate that $tRNA^{Gln}_{CAG}$ decodes the UAG amber codon when expressed at levels adequate to maintain the viability of cells carrying an otherwise lethal disruption of the tRNA gene (ref. 1; I.E. and M.R.C., unpublished data).

To directly quantitate plasmid copy number in these strains, chromosomal and plasmid-borne CEN4 sequences were examined by Southern hybridization (20) using a CEN4-specific probe. Total cellular DNA was extracted from strains YH50, YH51, YH60, and YH61 and examined as described in *Materials and Methods* (data not shown). This analysis demonstrated YH50, YH51, YH60, and YH61 to have centromeric plasmids in copy numbers of 1.3, 1.6, 2.4, and 1.7, respectively, values consistent with those determined by others for centromeric plasmid copy number (15). These data thus demonstrate that the $tRNA^{Gln}_{CAG}$ gene on plasmid pSUP19HIS is present at one or two copies per cell.

Strain YH61 is not as UV sensitive as a strain carrying a deletion in the *RAD10* gene (YH10, Fig. 3). Thus, the difference in UV sensitivity of the *rad10* deletion mutant and the $radl0\text{-}1_a$ mutant is only partially explained by suppression of the amber mutation by $tRNA^{Gln}_{CAG}$. The slightly enhanced UV resistance of strain YH61 relative to that of the deletion mutant may reflect the expression of a truncated Rad10 protein with residual excision repair activity. Alternatively or additionally, the $radl0\text{-}1_a$ mutation may be subject to other forms of partial suppression in yeast cells.

To demonstrate suppression of an amber mutation in a different gene we studied the $ade2\text{-}101_a$ mutation (D. Garza and M. V. Olson, personal communication). Like $radl0\text{-}1_a$, this mutation can be weakly suppressed in cells transformed with a multicopy plasmid carrying the $tRNA^{Gln}_{CAG}$ gene (1). Suppression can be qualitatively monitored with considerable sensitivity, since Ade$^+$ colonies are white whereas mutant colonies are red (21). Cells in which the amber mutation is partially suppressed are uniformly pink or light red, depending on the extent of suppression, whereas cells in which the amber mutation is efficiently suppressed are white. As shown in Fig. 4, cells harboring the centromeric plasmid with the $tRNA^{Gln}_{CAG}$ gene are lighter pink than an equivalent number of cells carrying the vector control.

The present studies show that when expressed at physiologically relevant levels—i.e., from a plasmid present in one or two copies per cell—the $tRNA^{Gln}_{CAG}$ gene can weakly decode UAG codons in the yeast *S. cerevisiae*. These results confirm our earlier contention (1, 2) that G·U wobble at the

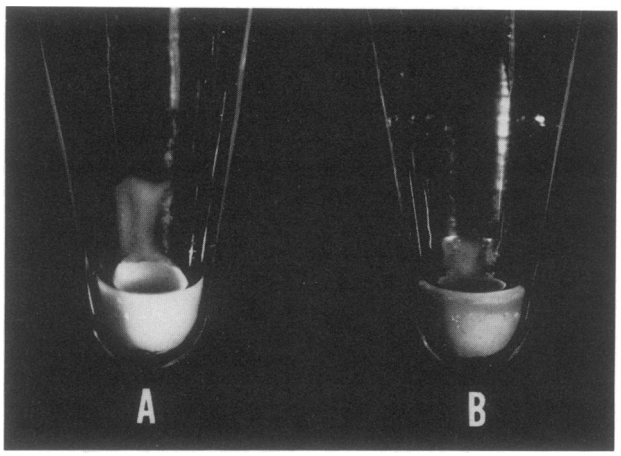

FIG. 4. A single-copy plasmid carrying the $tRNA^{Gln}_{CAG}$ gene can effect partial suppression of the *ade2*-101_a mutation. Strains YH50 and YH51 are identical except for the presence of the plasmid-borne $tRNA^{Gln}_{CAG}$ gene in the former (Fig. 1). The two strains were grown in medium containing limiting amounts of adenine and cells were harvested by centrifugation to examine the cell pellet color. The YH50 cell pellet (*A*) is lighter pink than the YH51 cell pellet (*B*), indicating partial suppression of the *ade2*-101_a mutation. The numbers of cells in the cultures are approximately equal (YH60 = 6.1×10^6 per ml and YH51 = 4.5×10^6 per ml). An identical result was obtained using strains YH60 and YH61 (data not shown).

first codon position may occur during anticodon–codon pairing associated with protein synthesis in eukaryotes. In addition, these experiments suggest that weak *in vivo* suppression of amber codons by $tRNA^{Gln}_{CAG}$ may *normally* occur in yeast cells. Such low-level decoding of a nonsense codon might reflect the vestiges of a mechanism that operated before the yeasts and ciliates split from the main eukaryotic evolutionary line. This weak suppression was apparently retained in *S. cerevisiae*, which uses $tRNA^{Gln}_{CAG}$ and $tRNA^{Gln}_{CAA}$ as the only two glutamine isoacceptor species. We suggest that in at least some ciliated protozoa further selection led to gene duplication and to changes at the anticodon sequences corresponding to the first codon position. These organisms thus evolved new genes that encode tRNAs that read the codons UAG and UAA as sense codons for glutamine (4–7).

We are grateful to Dan Garza and Maynard Olson for the *ade2*-101_a mutant and for the *sup4* plasmids. We also thank Ching Kung, Louie Naumovski, and Gordon Robinson for helpful discussions and numerous colleagues in our laboratories for their critical review of this manuscript. These studies were supported by U.S. Public Health Service Research Grants CA-12428 (to E.C.F.) and GM-26217 (to M.R.C.). W.A.W. and I.E. are supported by U.S. Public Health Service Training Grants CA-09302 and GM-07133 to the Stanford Program in Cancer Biology and the Department of Genetics, University of Wisconsin, respectively.

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