through transcripts applies a stringent selection for integration by homologous recombination. Our strategy can now be tested for isolating other chromosomal regions of interest by targeting the *neo* selectable marker to any closely linked, expressed gene for which sequence information is available. We might expect to see significant variation between different target sequences in the ability to promote homologous recombination. In this regard, the high efficiency of targeting with pTAGNEO may reflect in part the possible recombinogenic function of SV40 T antigen. However, as a large number of other SV40-transformed human and human-mouse hybrid cells are already available, we can use pTAGNEO immediately to access many new regions of DNA. If comparable frequencies of precise homologous integration can be achieved at other loci, then our approach should provide an efficient strategy by which specific gene mutations can be created or repaired.

Note added in proof. Two recent papers describe strategies in which enhancer- or promoter-deficient genes were used to select for homologous recombination events (*19*).

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12. Vectors were constructed in Bluescribe (Vector Cloning Systems) by standard cloning methods. The bacterial *neo* was cloned initially into M13mp8 on a Bcl I–Hind III fragment. To remove a stop codon 5′ to the *neo* methionine, 0.2 pmol of single-stranded DNA was heated for 10 min to 70°C in buffer (50 m*M* NaCl, 10 m*M* $MgCl_2$, and 10 m*M* tris-HCl, *p*H 8.0) containing 200 pmol of antisense oligonucleotide (5′-ACGATCCTCCTCCTGTCTC) containing the required mutation. *Escherichia coli* JM 101 were then transformed with the precipitated DNA, and colonies were screened with the oligonucleotide to identify clones containing the mutation. Positive clones were transferred to a strain of *E. coli* deficient in adenine methylase, and the Bcl I–Hind III *neo* was isolated and spliced onto the 3-kbp Kpn I–Bcl I fragment of SV40 T antigen to create p(pro$^+$)TAGNEO. The deletion of the Avr II–Kpn I DNA fragment that contains the SV40 early promoter, origin of replication, and 72-bp enhancer boxes 5′ to T antigen results in the construction of pTAGNEO. Both vectors contain a unique BstXI site that lies 428 bp downstream from the Avr II site in the SV40 T antigen sequence, and a unique Sma I site in the *neo* gene ~1 kbp 3′ to the Bcl I site. DNA sequencing confirms the SV40 T antigen splice region to be as shown below. This construct results in the deletion of the last 27 amino acids of the SV40 antigen, which now terminates with valine (V) and histidine (H). The methionine (M) indicated is the start of the normal *neo* open reading frame. The asterisked base was altered by the site-directed mutagenesis (described above) so that there would not be a termination codon when translated in-frame from the T antigen sequence. The *neo* gene contains the HSV-tk poly (A) addition signal.

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        ↓ Bcl I
GTT CAT GAT CAA GAG ACA GGA ĠGA GGA TCG TTT CGC ATG ATT
V   H                                       M   I
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15. Probe A is a subclone of SV40 DNA not contained in pTAGNEO. The 1.4-kbp fragment lies between the Kpn I site (position 294) and the Eco RI site (position 1782) numbering clockwise from the Bgl I site in the direction of late genes to early genes [W. Fiers *et al.*, *Nature* **273**, 113 (1978)].
16. The amplifying oligonucleotides were specific for the *neo* mutation (*12*) and the SV40 promoter region (5′-CGCCTCGGCCTCTGAGCTA). Primers for PCR were synthesized on an Applied Biosystems DNA Synthesizer. DNA was prepared from 10^6 cells and PCR was carried out on an Intelligent Heating Block (Hybaid Ltd.) with *Thermus aquaticus* DNA polymerase (Perkin-Elmer Cetus), buffer, and oligonucleotide concentrations according to the manufacturer's instructions and the method of R. K. Saiki *et al.* [*Science* **230**, 1350 (1985)]. The DNA was denatured at 95°C for 7 min, enzyme added, and then 30 cycles of 45 s at 92°C, 1 min at 55°C, and 5 min at 72°C were carried out. Samples were then run on a 1% agarose gel and blotted onto Hybond N (Amersham). Gels were probed at 58°C with oligonucleotide 116, specific for T antigen (5′-ATCCGAGAAGCCTCCAAAG), to distinguish specific from spurious bands.
17. CMGT was performed exactly as described by J. E. N. Morten *et al.* [*Anticancer Res.* **7**, 573 (1987)].
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20. Cl21 and Cl27 cells were grown in Dulbecco's modified Eagle's medium (DMEM) plus 10% fetal calf serum, under 10% CO_2. Cells to be electroporated were harvested with trypsin-versene when rapidly growing, washed in DMEM, counted, and resuspended in phosphate-buffered saline at a concentration of 2×10^6 cells per milliliter. Just before electroporation, the recombination vector was added to a concentration of 20 μg/ml and 0.5 ml of cells, and these were exposed to a single 3000 V/cm pulse, 4-μF capacitance direct discharge through 22-ohm resistance in series (at room temperature on a home-made apparatus), and then allowed to recover on ice for 10 min. The current supplied was 8.8 A and the current density 0.44 A/mm^2. The survival rate was ~40 to 60% for both cell lines. G418 selection was applied after 24 hours at 200 μg/ml, and after 5 days the concentration was increased to 400 μg/ml. Colonies arising were subcloned into G418 at 800 μg/ml. G418-resistant cell lines were propagated and analyzed as described in the text.

21. We thank H. J. Evans for his support and encouragement, V. van Heyningen and N. Hastie for their helpful discussion; E. Emslie for technical assistance, and M. Wilkinson for carrying out SV40 T antigen assays. Thanks also to N. Davidson, S. Bruce, and D. Stuart for photographic preparation, and to S. Farrow for calculating the parameters of the electroporation. J.D. is supported by the Cystic Fibrosis Research Trust (U.K.).

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Phylogenetic Stains: Ribosomal RNA–Based Probes for the Identification of Single Cells

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Rapid phylogenetic identification of single microbial cells was achieved with a new staining method. Formaldehyde-fixed, intact cells were hybridized with fluorescently labeled oligodeoxynucleotides complementary to 16*S* ribosomal RNA (rRNA) and viewed by fluorescence microscopy. Because of the abundance of rRNA in cells, the binding of the fluorescent probes to individual cells is readily visualized. Phylogenetic identification is achieved by the use of oligonucleotides (length 17 to 34 nucleotides) that are complementary to phylogenetic group-specific 16*S* rRNA sequences. Appropriate probes can be composed of oligonucleotide sequences that distinguish between the primary kingdoms (eukaryotes, eubacteria, archaebacteria) and between closely related organisms. The simultaneous use of multiple probes, labeled with different fluorescent dyes, allows the identification of different cell types in the same microscopic field. Quantitative microfluorimetry shows that the amount of an rRNA-specific probe that binds to *Escherichia coli* varies with the ribosome content and therefore reflects growth rate.

CLASSICAL CHEMICAL STAINS FOR VISUALIZING microorganisms seldom provide much information beyond the morphology of the organisms detected. Reagents such as fluorescent antibodies can be used for the identification of particular organisms, but their high specificity limits their utility to well-studied organisms. We have developed a staining method that provides phylogenetic information on single microbial cells and requires no previous knowledge of the organisms detected. The approach is based on oligodeoxynucleotide hybridization probes complementary to ribosomal RNA (rRNA) sequences that are diagnostic for selected phylogenetic groups. When these probes are labeled with fluorescent dyes, they can be used for the detection and phylogenetic characterization of orga-

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nisms with a microscope.

In situ nucleic acid hybridization with isotopically or fluorescently labeled probes is widely used for the intracellular localization and quantitation of RNAs and genes (*1*). Isotopically labeled oligodeoxynucleotides bind to the rRNAs of intact, fixed cells and, together with autoradiography, can be used for the phylogenetic identification of organisms (*2*). However, microautoradiography of such in situ hybridization probes requires long exposure to photographic emulsions. Moreover, the useful resolution is no less than about 1 μm because of the scatter of radioactive disintegrations. The abundance of cellular ribosomes, 10^4 to 10^5 per cell in rapidly growing bacteria, suggested that the binding of phylogenetic group-specific probes for the rRNAs might be viewed directly in the fluorescence microscope.

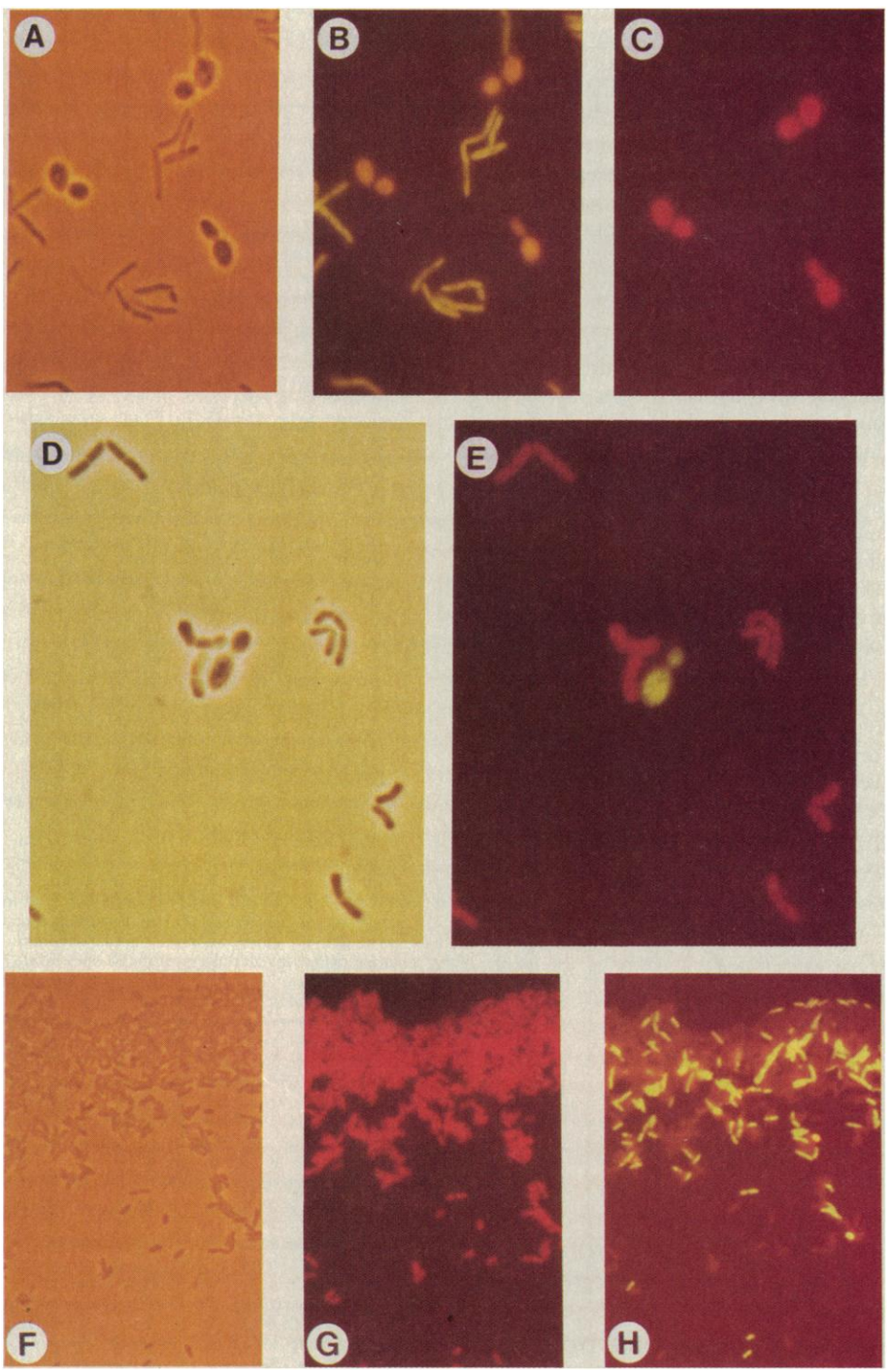

Fig. 1. In situ hybridization of formaldehyde-fixed cells with fluorescently labeled oligodeoxynucleotides complementary to 16*S* RNA sequences (*7, 8*). (A to E) *Saccharomyces cerevisiae* and *Bacillus megaterium*. (F to H) *Proteus vulgaris* and son-killer bacterium. (**A**, **D**, and **F**) Phase-contrast microscopy. Epifluorescence microscopy shows the binding of the fluorescein-labeled universal probe (**B**), the X-rhodamine–labeled eukaryote probe (**C**), the fluorescein-labeled eukaryote probe (**E**), the X-rhodamine–labeled eubacteria probe (**G**) (also in E), and the fluorescein-labeled son-killer probe (**H**).

One set of fluorescent probes that we developed for single-cell analysis consists of oligodeoxynucleotides that distinguish the three primary lines of evolutionary descent: the eubacteria, the eukaryotes, and the archaebacteria (*3*). The probes, 17 to 34 nucleotides in length, are the complements of sequences that are characteristic of known 16*S* rRNA sequences of each of the respective lineages (*2, 4*). The oligodeoxynucleotides were synthesized (Applied Biosystems DNA synthesizer), and in the last stage an aminoethyl phosphate linker (*5*) was attached to the 5′ end. The 5′-aminoethyl oligodeoxynucleotides were then coupled to fluors and purified (*6*).

Cells fixed on microscope slides were hybridized in situ as described (*2*) with some modifications (*7*). Phase-contrast and epifluorescence microscopy (*8*) were used to show (Fig. 1, A to E) the specificities of the fluorescent probes that identify the primary kingdoms. A mixture of *Bacillus megaterium* (a eubacterium) and *Saccharomyces cerevisiae* (a eukaryote) was hybridized with a fluorescein-labeled "universal" probe (that binds to the rRNA of all organisms that we have examined) and, simultaneously, with an X-rhodamine–labeled probe specific for eukaryotes. Phase contrast microscopy (Fig. 1A) reveals both cell types, as does epifluorescence microscopy with a fluorescein filter set (Fig. 1B), because the universal probe binds to the rRNA of all cell types present. In the same field, the eukaryotes are exclusively identified with epifluorescence filters specific for the excitation and emission wavelengths of rhodamine (Fig. 1C). In a similar experiment, but with rhodamine-labeled eubacteria and fluorescein-labeled eukaryote probes, we used double exposure to display both fluorescent dyes simultaneously (Fig. 1, D and E). In other experiments (not shown) the specified archaebacteria probe was similarly efficient in distinguishing an archaebacterium (*Methanosarcina acetivorans*) from eubacteria (*B. megaterium* and *Proteus vulgaris*) and from a eukaryote (*S. cerevisiae*).

Oligodeoxynucleotide probes also can distinguish closely related phylogenetic groups. For example, a probe was developed to identify a bacterium dubbed "son-killer" that is found in association with the parasitoid wasp *Nasonia vitripennis* (*9*). A mixture of son-killer and its closest known relative, *Proteus vulgaris* (*10*), was hybridized simultaneously with a rhodamine-labeled eubacteria probe and a fluorescein-labeled probe specific for son-killer (*10*), then viewed by phase

contrast (Fig. 1F) or by fluorescence at wavelengths that discriminate between the fluors (Fig. 1, G and H). These two closely related organisms (92% similarity in 16*S* rRNA sequences), differing morphologically only slightly in size and shape, are easily distinguished with the son-killer probe even where densely packed on the slide.

The sequential application of a panel of fluorescent probes, with incrementally finer specificities, could provide phylogenetic identification without resort to direct sequence analysis of the rRNAs or their genes. The binding of a universal probe (Fig. 1B) is a positive control for the presence of detectable target sequences; an oligonucleotide that is not complementary to the rRNA serves as a control for non-specific binding (below). This staining approach to phylogenetic analysis should be particularly useful in the study of natural microbial populations, where unknown, often uncultivatable organisms are routinely encountered. A phylogenetic characterization of an organism can provide perspective on its biochemical nature because the organism is expected to share properties common to its phylogenetic group.

The rRNA content of microorganisms is proportional to growth rate over a wide range (*11*). Therefore, the amount of a fluorescent, rRNA-targeted probe that binds to cells should reflect growth rates and metabolic activities. We quantified by means of microfluorimetry the binding of the fluorescent, universal probe to *Escherichia coli* grown in media that support different growth rates (*12*). Background fluorescence resulting from nonspecific oligonucleotide binding or autofluorescence of cells was assessed with the same cultures by hybridizing cells with a fluorescently labeled complement of the universal probe (*2*). This latter probe should not bind specifically because it is not complementary to the rRNA of *E. coli*.

The fluorescence intensity of single cells due to hybridization with the universal probe varies linearly with growth rate in parallel to measured and known values (*11*) for RNA per cell and for ratios of RNA to DNA content (Fig. 2). Only low nonspecific fluorescence is observed in cells grown at each of the different rates and exposed to the probe that is not complementary to the rRNA. Thus, from a calibration of the extent of probe binding as a function of growth rate for a particular organism, the growth rate of that organism can be estimated in natural populations.

Potential problems with the use of fluorescently labeled probes include high autofluorescent background and low staining intensity of target cells. Potential solutions to autofluorescence include the bleaching of fixed cells before hybridization or the use of fluors with emission wavelengths that do not coincide with the autofluorescence. Weak fluorescence intensity could result from a poor permeability of fixed cells to the oligonucleotide probes or to low cellular ribosome contents, a significant concern in slowly growing natural populations. All cell types that we have examined, including thick-walled Gram-positive bacteria and yeast spores, and a variety of Gram-negative bacteria and eukaryotic tissues, seem freely permeable to these short oligonucleotides after fixation. However, probes that are a few hundred nucleotides in length do not efficiently enter some types of fixed cells unless the cells are mildly digested with lysozyme or proteinase K (*13*). Weak fluorescence in organisms containing relatively few ribosomes should be enhanced by the use of multiple probes or oligodeoxynucleotides labeled with multiple fluors.

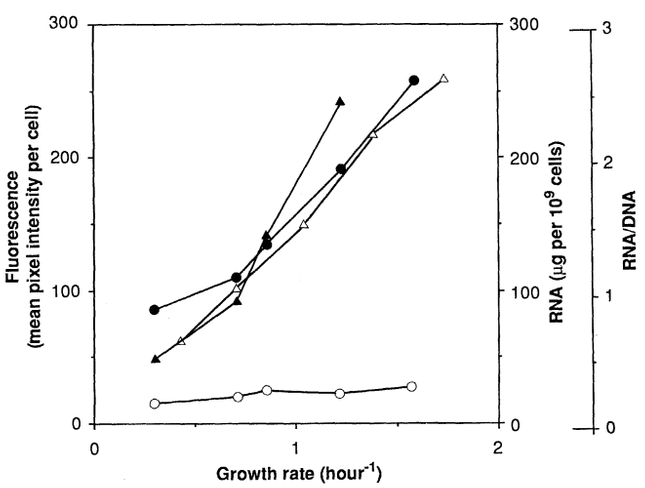

Fig. 2. Fluorescence intensity per cell as a function of growth rate. Fluorescence intensity per cell resulting from the binding of the universal rRNA probe (●) or the nonspecific control probe (○), measured RNA per cell (▲), and RNA to DNA ratios (△) are plotted as a function of growth rate (*12*). The ratios of RNA to DNA for *E. coli* strain B/r were obtained from (*11*). Fluorescence intensity per cell was measured with an MRC-Lasersharp fluorescence scanning confocal microscope (MRC500, Bio-Rad), in conjunction with an Optiphot biological microscope (Nikon). An argon laser operating at 455 nm was used as the excitation source. The raster size of the video image was 768 by 512 pixels. Mean pixel intensity per cell at each growth rate was determined from measurements on 25 individual cells in five randomly chosen microscopic fields. Values were reproducible from cell to cell and experiment to experiment. Standard deviations for fluorescence per cell due to binding of the universal rRNA probe at each growth rate were, respectively: 10.6, 0.29 hour^{-1}; 16.6, 0.7 hour^{-1}; 15.9, 0.85 hour^{-1}; 11.8, 1.22 hour^{-1}; 13.7, 1.58 hour^{-1}. The standard MRC500 image analysis software was used. Concentrations of RNA were determined by the orcinol method (*15*) and concentrations of cells by Petroff-Hausser counter.

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6. Aminoethyl oligodeoxynucleotides were purified by electrophoresis on nondenaturing 20% polyacrylamide gels; 5′-amino groups were then coupled to either fluorescein isothiocyanate (Molecular Probes) or X-rhodamine isothiocyanate (Research Organics) in 250-μl reaction mixtures containing 200 m*M* $NaHCO_3$ buffer, *p*H 9, 100 μg of 5′-aminoethyloligodeoxynucleotide, and 400 μg of isothiocyanate dye. Reactions were carried out in the dark at room temperature for 16 hours. Oligodeoxynucleotides were separated from unreacted dyes by passage through a Sephadex G-50 column equilibrated with 10 m*M* tris-HCl, *p*H 7.5. The materials eluting in the void volume were lyophilized and resuspended in 50 μl of 80% formamide, then labeled oligonucleotides were separated from unlabeled ones by electrophoresis on 20% nondenaturing polyacrylamide gels. The intensely fluorescent bands, well resolved from unlabeled oligomers, were recovered by passive elution in 10 m*M* tris-HCl, *p*H 7.8. The eluate was filtered to remove polyacrylamide and used in hybridizations without further purification.
7. Cells were harvested and resuspended in phosphate-buffered saline (145 m*M* NaCl, 100 m*M* sodium phosphate, *p*H 7.5), fixed by the addition of one-tenth volume of 37% formaldehyde, and stored at 4°C. In each experiment, 30 μl of fixed cells (1×10^7 cells per milliliter) were smeared onto slides coated with gelatin (*2*). The smears were dried in air and again fixed in 3.7% formaldehyde and 90% methanol for 10 minutes at room temperature. After a brief rinse in water, slides were immersed in 100 m*M* tris-HCl, *p*H 8.0, 50 m*M* sodium borohydride, and held for 30 minutes in the dark with rapid stirring. Slides were then rinsed briefly in water and dried in air. Fluor-labeled oligodeoxynucleotides were lyophilized and resuspended (1.7 ng/μl) in a hybridization mixture that contained five times SET (750 m*M* NaCl, 100 m*M* tris-HCl *p*H 7.8 and 5 m*M* EDTA), 0.2% bovine serum albumin (Sigma; fraction V), 10% dextran sulfate (Sigma; average molecular weight 5000), and 0.01% polyadenylic acid (Sigma). This mixture (30 μl) was applied to

each smear and covered with a cover slip. Slides were placed in airtight chambers containing a tissue saturated with 2 ml of the hybridization mixture and incubated at 37°C for 5 to 16 hours. Cover slips were removed by immersion in five times SET, and the slides were immediately washed three times in 0.2 times SET at 37°C for 10 minutes each time. Slides were then dried in air in the dark and viewed immediately, or stored in the dark at 4°C until they were evaluated.
8. Samples were mounted in Citifluor (Citifluor, Ltd., London) and viewed under oil immersion with a Neofluor 100 times objective on a Zeiss Photomicroscope III fitted with an epifluorescence condenser, a mercury lamp, and Zeiss filter sets #48-77-09 and #48-77-15. Photomicrographs were taken with Kodachrome or Fujichrome 400 ASA color film.
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Nucleotides in Yeast $tRNA^{Phe}$ Required for the Specific Recognition by Its Cognate Synthetase

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An analysis of the aminoacylation kinetics of unmodified yeast $tRNA^{Phe}$ mutants revealed that five single-stranded nucleotides are important for its recognition by yeast phenylalanyl-tRNA synthetase, provided they were positioned correctly in a properly folded tRNA structure. When four other tRNAs were changed to have these five nucleotides, they became near-normal substrates for the enzyme.

THE ACCURATE INCORPORATION OF amino acids into proteins depends on the correct aminoacylation of each tRNA by its cognate aminoacyl-tRNA synthetase. How each synthetase recognizes its set of iso-acceptor tRNAs among all of the tRNAs in the cell remains unknown. We have used a biochemical approach to identify nucleotides in yeast $tRNA^{Phe}$ (Fig. 1A) that are required for its specific recognition and subsequent aminoacylation by yeast Phe-tRNA synthetase (FRS). Anticodon loop replacement experiments established that substitution of any one of the anticodon nucleotides G_{34}, A_{35}, or A_{36} resulted in a 3- to 12-fold reduction of the rate of aminoacylation with purified FRS (*1*). When yeast $tRNA^{Tyr}$ was modified to have a Phe anticodon by changing $\psi_{35} \rightarrow A_{35}$, it became a much better substrate for misacylation by FRS, yet still aminoacylated poorly when compared to $tRNA^{Phe}$ (*2*). These data suggested that although FRS, like many other synthetases (*3*), requires the anticodon for the specific recognition of $tRNA^{Phe}$, other features in $tRNA^{Phe}$ must contribute as well. A method that allows substitution of nucleotides elsewhere in the $tRNA^{Phe}$ sequence involves in vitro transcription by T7 RNA polymerase (*4*). Although the wild-type $tRNA^{Phe}$ transcript lacked all of the modified nucleotides normally found in yeast $tRNA^{Phe}$, it was a good substrate for FRS, thus allowing extensive structure-func-

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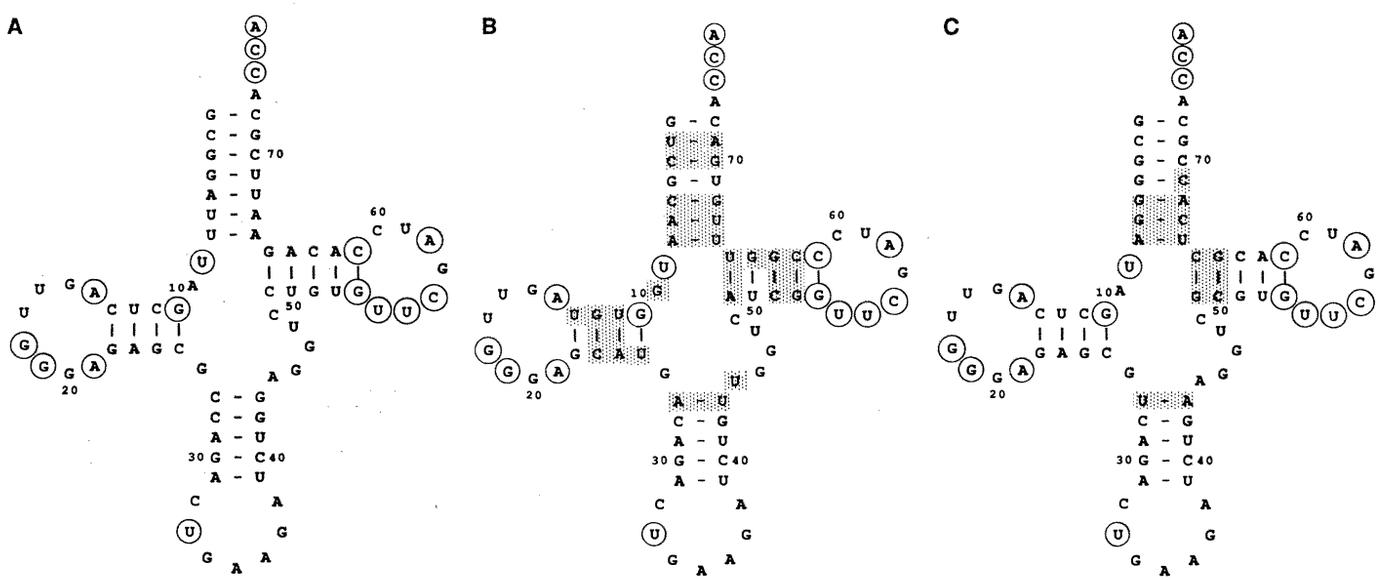

Fig. 1. Three tRNAs [(**A**) yeast $tRNA^{Phe}$, (**B**) *S. pombe* $tRNA^{Phe}$, and (**C**) wheat germ $tRNA^{Phe}$] that are active substrates for yeast FRS. The circled nucleotides are conserved in all cytoplasmic yeast tRNAs and the nucleotides in *S. pombe* $tRNA^{Phe}$ and wheat germ $tRNA^{Phe}$ that differ from yeast $tRNA^{Phe}$ are shaded. *S. pombe* $tRNA^{Phe}$ aminoacylates with the same k_{cat} and K_m as yeast $tRNA^{Phe}$ (*7*), whereas wheat germ $tRNA^{Phe}$ has the same k_{cat} and 1.3-fold lower K_m (*6*).