Selection of Guide Sequences That Direct Efficient Cleavage of mRNA by Human Ribonuclease P

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Any RNA, when in a complex with another oligoribonucleotide known as an external guide sequence (EGS), can become a substrate for ribonuclease P. Simulation of evolution in vitro was used to select EGSs that bind tightly to a target substrate messenger RNA and that increase the efficiency of cleavage of the target by human ribonuclease P to a level equal to that achieved with natural substrates. The most efficient EGSs form transfer RNA precursor-like structures with the target RNA, in which the analog of the anticodon stem has been disrupted, an indication that selection for the optimal substrate for ribonuclease P yields an RNA structure different from that of present-day transfer RNA precursors.

Ribonuclease P (RNase P) is an enzyme that cleaves tRNA precursors to generate the 5′ termini of mature tRNAs (*1*). Using this enzyme, we have developed a method for specific inactivation of any RNA (*2*, *3*); we designed a small RNA which, when complexed with the target RNA, generated a structure that resembles a tRNA precursor and rendered the target susceptible to cleavage by RNase P. The small RNA is designated an external guide sequence (EGS) (*4*) because it guides RNase P to the cleavage site in the target RNA, which is hydrogen-bonded to the EGS in the complex. This method differs from other methods in which RNA enzymes are used to inactivate mRNAs in that the enzyme, unchanged, resides normally in the cells under study.

We showed that an EGS custom-designed for the mRNA for chloramphenicol transacetylase (CAT) (Fig. 1A) can direct the specific cleavage of the mRNA by human RNase P in vitro or in cells in tissue culture (*3*). However, the cleavage reaction is inefficient compared to cleavage of natural tRNA precursor substrates. Furthermore, the precise details of the relation between the tertiary structure of a tRNA precursor molecule and its ability to be recognized and cleaved efficiently by RNase P are not completely understood, so that there are no comprehensive guidelines for engineering EGSs with improved efficiency. Evolution in vitro (*5*, *6*), however, has been used to identify RNA molecules with desired properties from pools of molecules that contain randomized sequences. We have now successfully used this method for the isolation of efficient EGSs. These new EGSs, when complexed with CAT mRNA, allow cleavage of the target by human RNase P at rates similar to those achieved with natural substrates.

The proposed secondary structure of a complex of CAT mRNA and EGS^{CAT} (Fig. 1A) resembles the tRNA cloverleaf structure, but it includes sequences not normally found in the tRNA from which it was originally derived [tyrosyl tRNA ($tRNA^{Tyr}$) of *Escherichia coli*]. To ensure that appropriate tertiary interactions that facilitate the process of enzyme-substrate recognition would exist in the complex, we attempted to change parts of the EGS that participate in tertiary interactions in the analogous tRNA structures (*7*) in two ways. First, four nucleotides in the equivalent of the T loop and five in the equivalent of the variable loop were randomized by incorporation of equimolar quantities of the deoxynucleotides dA, dG, dC, and T into a DNA template to yield an initial population of 2.6×10^5 sequence variants (*8*). Second, during each round of selective amplification (*9*), random mutations were introduced by performing polymer chain reaction (PCR) at an error rate of approximately 0.1% per nucleotide incorporated (*6*).

To select sequences for the EGS that made it more efficient in guiding RNase P to the target mRNA, we used a chimeric, covalently linked mRNA-EGS substrate (Fig. 2A) and selected for the ability of this substrate to be cleaved by human RNase P (Fig. 2B). The chimeric RNA, with partially randomized sequence, was prepared by transcription in vitro of synthetic DNA templates (*9*). In each round of selection, the pool of RNAs was digested with human RNase P and the cleaved products were isolated by electrophoresis and then amplified to produce progeny RNAs (Fig. 2B). One of the template-creating oligonucleotides (SEC-1A) was also used as the 5′ primer for the PCR in order to allow restoration of the T7 promoter sequence and the leader sequence of the chimeric RNA for the next cycle of selection. The stringency of selection was increased at each cycle by reducing the amount of enzyme and the time allowed for the cleavage reaction, such that only those substrates that were cleaved rapidly by the enzyme were selected.

After eight cycles of selection and amplification, the pool of selected DNA molecules was cloned into the Bluescript vector (*10*). Sixteen individual clones were sequenced (Fig. 3). From the sequence that had been randomized in the T loop, two particular sequences were most frequently selected: UUCGUGC (seven clones) and UUCGCCC (seven clones). The T loop sequences of the two remaining clones contained single transition mutations of the two major sequences (UUCGUCC and UUCACCC). By contrast, no significant sequence-related bias was seen in the sequence of five nucleotides in the variable loop.

In addition to the sequences in the T and variable loops that were selected from the totally randomized sequence, a considerable number of mutations were introduced into the EGS in the chimeric substrates as a consequence of the conditions for PCR (*6*). Some of these mutations were beneficial and, therefore, the sequences that included them were selected and accumulated. To our surprise, in almost all the individual selected clones, the integrity of base-pairing in the anticodon stem was disrupted (see Figs. 1B and 3).

The best chimeric substrate we selected (clone 9) was cleaved about five-and-a-half times more efficiently than the parent, nonrandomized, chimeric substrate (mRNA-EGS^{CAT} chimera). Using the sequences of the selected chimeric substrates (*11*), we then prepared nine individual EGS RNAs (Fig. 4) in order to probe the function of these selected EGSs in directing RNase P to the target CAT mRNA. Each of the individual EGS RNAs was mixed with ^{32}P-labeled CAT mRNA, and the mixtures were then exposed to RNase P. Every selected EGS RNA increased the initial rate of the cleavage reaction, as measured during the linear phase of the reaction, over that with EGS^{CAT} and cleavage occurred at the expected site in the target mRNA.

The rates improved substantially with the sequence based on clone 9 (EGS 9), which directed cleavage of the CAT mRNA at an overall rate more than 30 times faster than that observed with EGS^{CAT} in the complex. The three most efficient EGSs tested, derived from clone 6, clone 8, and clone 9, had a common sequence, UUCGUGC, in the T loop (Fig. 3).

The proposed secondary structure of the complex of CAT mRNA and EGS 9 (Fig. 1B) can be compared with the parent CAT mRNA-EGS^{CAT} complex (Fig. 1A). The structures of CAT mRNA-EGS complexes were confirmed in part by partial digestion with RNases T1 and T2 (*12*) under conditions that allowed formation of the mRNA-EGS complex and identification of single-stranded regions in RNA. Double-stranded regions were identified by digestion with

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cobra venom nuclease (*12*). The sites of cleavage by the various nucleases in CAT mRNA-EGS^{CAT} are indicated by arrows in Fig. 1, A and D. The tRNA domain in this structure is similar to that found in a natural tRNA. For example, the results obtained with cobra venom nuclease indicate that the first few nucleotides in the analog of the D loop are involved in a tertiary interaction, presumably with nucleotides in the variable loop. This interaction is either absent or much less extensive in the complex with EGS 9 (Fig. 1E) and, indeed, the same region in the analog of the D loop is susceptible to attack by RNases T1 and T2, an indication that it is in a single-stranded conformation (Fig. 1B). This result, together with the appearance of new sites of susceptibility to attack by cobra venom nuclease in the anticodon loop of EGS 9 confirm that this EGS endows the complex of the EGS and CAT mRNA with new tertiary interactions that enhance the rate of cleavage by RNase P.

The results of cleavages by nucleases also confirmed that the anticodon stem in EGS 9 was disrupted as a result of a single nucleotide deletion. A natural substrate for RNase P with such a disorganized "anticodon stem-loop" structure has been identified in *E. coli* (*13*). Our results suggest that this last mentioned RNA, whatever its function, has a selective advantage as a result of the absence of an intact anticodon stem and loop.

EGSs 9 and 14 are not similar to each other in terms of their efficiency in directing RNase P to a target substrate (Fig. 4). The only difference in their nucleotide sequences is in the variable loop (Fig. 3). This difference alone must account for the relative inefficiency of EGS 14 in terms of targeting ability. Furthermore, digestion with RNase T1 of the complex that contained EGS 9 revealed strong protection of the last nucleotide (G) in the variable loop from attack by RNase T1 (Fig. 1). The role of this G may be similar to that of nucleotide 57 in tertiary interactions in tRNA molecules; namely, the G may form hydrogen bonds with a nucleotide in the CAT mRNA sequence to ensure folding of the tRNA domain.

In an independent study of recognition of precursor tRNA substrates by human RNase P, we found that the anticodon stem and loop form a dispensable structural feature in the recognition of substrates by human RNase P (*14*). These results, together with our observations recorded here, indicate that the anticodon stem, when present in an EGS RNA, acts in a negative fashion on the overall rate of cleavage by RNase P of the target RNA. To test this hypothesis, we made two more EGS RNAs; one was a deletion mutant that lacked the equivalent of the anticodon stem and loop (EGS^{CAT} ΔAC), as compared to the parent EGS^{CAT} (Fig. 1, A and C), and the other (EGS 19) was a derivative of EGS 9 in which the structure of the anticodon stem was restored (*15*). In EGS^{CAT} ΔAC, the

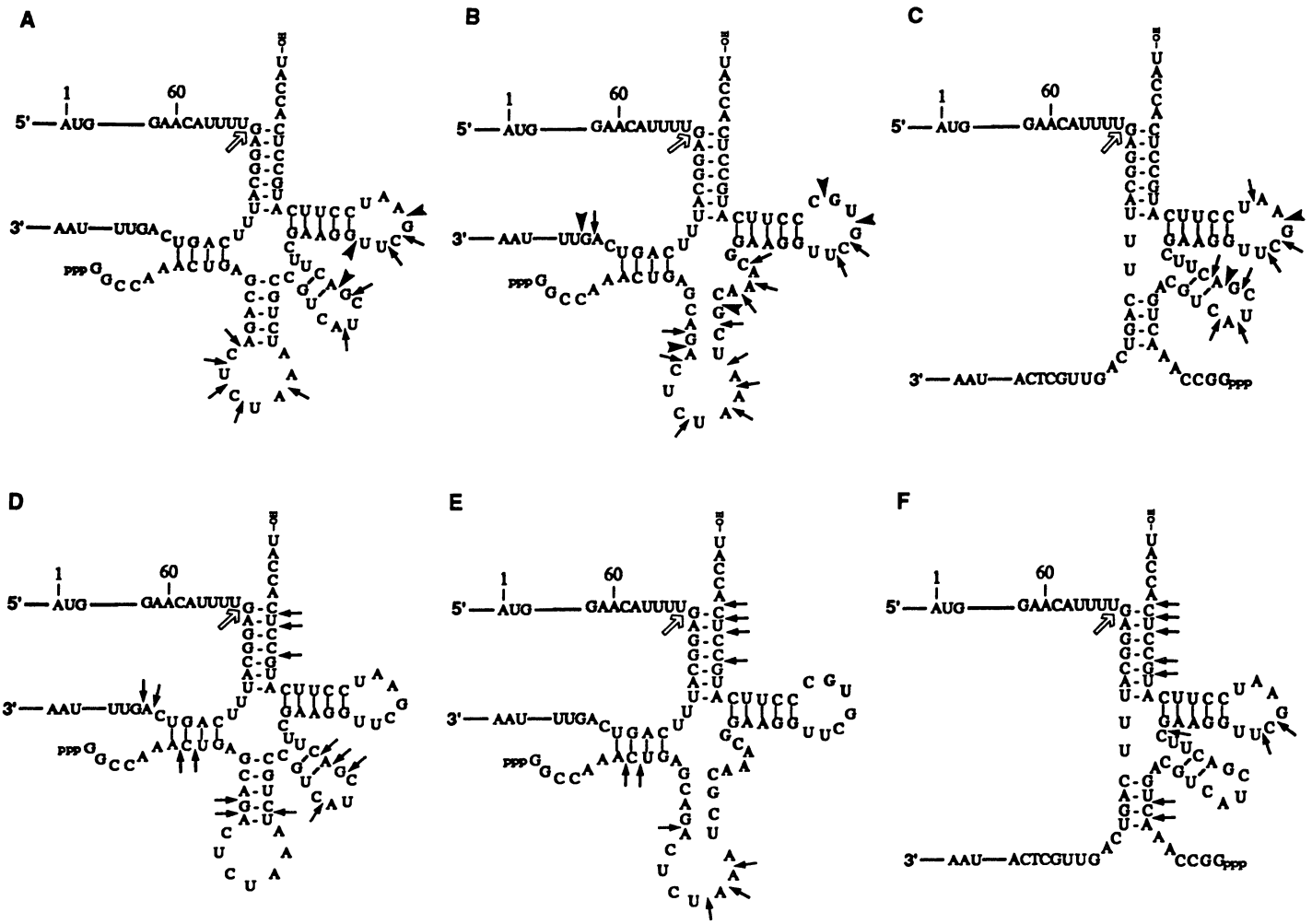

Fig. 1. Proposed secondary structures of complexes of CAT mRNA and various EGSs. (**A**) Complex of CAT mRNA and EGS^{CAT} RNA. (**B**) Complex of CAT mRNA and EGS 9. (**C**) Complex of CAT mRNA and EGS^{CAT} ΔAC. Sites of cleavage by RNase T1 are indicated by arrowheads. Arrows indicate sites of cleavage by RNase T2. Hollow arrows denote sites of cleavage by human RNase P. (**D** to **F**) The same structures as those in (A) to (C), respectively, but the arrows indicate sites of cleavage by cobra venom nuclease.

length of EGS^{CAT} was reduced by 25%; the shorter deletion mutant directed cleavage of target RNA about six times more efficiently than the parent EGS (Fig. 4). Restoration of the anticodon stem structure, as in EGS 19, reduced the rate of cleavage of the target RNA with EGS 19 to four times lower than that with EGS 9. These results, together with the measurement of the rates of reaction with EGSs selected in vitro, indicate a significant inverse correlation between the efficiency of an EGS in the cleavage reaction and the existence of an anticodon stem in EGS RNA.

EGSs selected in vitro may have mediated enhanced rates of cleavage by RNase P in part by increasing the stability of the EGS-mRNA complex. Using each EGS we examined the stability of such complexes by measuring both the binding constants between the mRNA and each EGS and the dependence on Mg^{2+} ions of the cleavage reaction (we presumed that relatively high concentrations of Mg^{2+} ions were needed to stabilize relatively unstable complexes). The dissociation constants (K_d) of mRNA-EGS complexes were measured directly by a gel mobility shift assay in polyacrylamide gels that contained 10 mM Mg^{2+} (*16*) (Table 1). The K_d values of selected EGSs were 4 to 40 times lower than that of the parent EGS. Thus, the selected EGSs had higher affinity for the target RNA than did EGS^{CAT}. The chimeric substrate derived from clone 9 was cleaved by RNase P at a rate only about 1.5 times faster than the rate for the target in the mRNA–EGS 9 complex, an indication that the ability of the EGS to bind tightly to the target RNA in solution can be a critical determinant in the efficiency of the substrate complexes.

The differences in K_d values between complexes with EGS^{CAT} and the selected EGSs correspond to the contribution of −1 to −2.4 kcal/mol to the free energy of binding (ΔG^O) with selected EGSs (ΔG^O was −8.5 kcal/mol for the complex with EGS^{CAT}, −10.1 for the complex with EGS 9, and −10.9 for the complex with EGS^{CAT} ΔAC), thus revealing interactions in the selected EGS-mRNA complexes.

Deletion of the anticodon stem from EGS^{CAT} resulted in a K_d (for EGS^{CAT} ΔAC) that was 44 times lower, and resto-

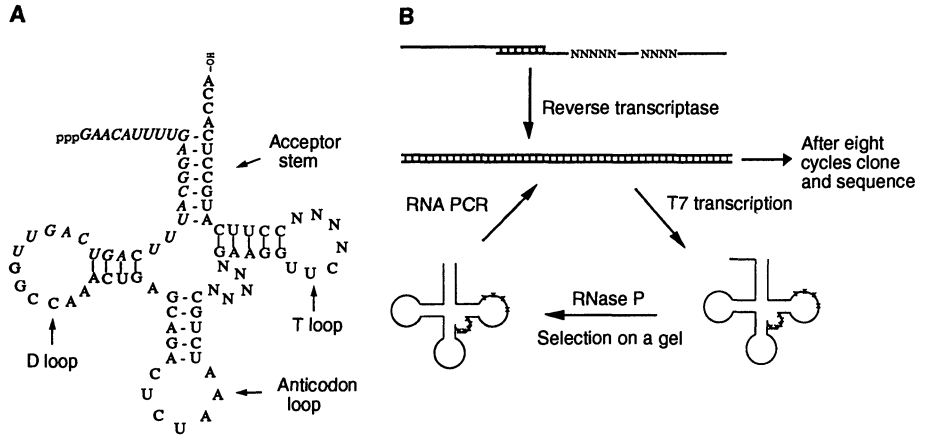

Fig. 2. Components of the selection scheme. (**A**) Proposed secondary structure of the chimeric substrate used in the selection experiment. The italicized sequence is from CAT mRNA and the remaining sequence, aside from changes made to assure hydrogen bonding to CAT mRNA, is based on the sequence of *E. coli* tRNA^{Tyr}. The nine nucleotides that were randomized are each indicated by N. The analogs of the various parts of a tRNA (for example, T loop) are indicated in the figure. (**B**) Scheme for in vitro selection and amplification. The method is described in (*8–10*). The DNA polymerase capability of reverse transcriptase was used to create the double-stranded template DNA from the overlapping DNA oligonucleotides. RNA PCR refers to reverse transcription coupled to PCR.

Fig. 3. Partial sequences of the EGS segment of some individual chimeric RNAs obtained as a result of the selection experiment. Nucleotides that differ from the parent EGS^{CAT} sequence (listed as P) are given in bold letters and are underlined. Deletions are indicated by hyphens. The remaining part of the sequence of each EGS is shown in Fig. 2A. The numbering of clones is not uniformly consecutive, since some clones did not have appropriate inserts, and only 16 sequences are given.

	Anticodon stem-loop	V loop	T stem-loop
	30	47	52
P	GCAGACUCUAAAUCUGC	NNNNN	GAAGGUUCNNNNCCUUC
1	GCAGACUCUAAAUC**G**GC	**CCUUC**	GAAGGUUC**GCCC**CCUUC
2	GCAGACUCUAAAUCUGC	**ACGAGA**	GAAGGUUC**GUGC**CCUUC
4	GCAGACUCUAAA**CUG**GC	**CUAAC**	GAAGGUUC**GCCC**CCUUC
5	GCAGACUCUAAAU - UGC	**CCAAC**	GAAGGUUC**ACCC**CCUUC
6	GCAGACUC**C**AAAUC - - C	**ACCAA**	GAAGGUUC**GUGC**CCUUC
8	GCAGACUCUAAA - CU**C**C	**UCCCA**	GAAGGUUC**GUGC**CCUUC
9	GCAGACUCUAAAUC - GC	**AAACG**	GAAGGUUC**GUGC**CCUUC
10	GCAGACUCUAAAUC**G**GC	**CUACG**	GAAGGUUC**GCCC**CCUUC
11	GCAGAC**G**CUAAAUCU**A**C	**CCCGU**	GAAGGUUC**GUCC**CCUUC
12	GCAGACUCUAAAU**U**UGC	**CACCA**	GAAGGUUC**GCCC**CCUUC
13	GCAGACUC-AAAUCU**G**GC	**CAUUC**	GAAGGUUC**GCCC**CCUUC
14	GCAGACUCUAAAUC - GC	**AGUGU**	GAAGGUUC**GUGC**CCUUC
15	GCAGACUCUAAAUC**A**GC	**GCGUG**	GAAGGUUC**GUGC**CCUUC
16	GCAGACUCUAAAUC**G**GC	**CGCAC**	GAAGGUUC**GCCC**CCUUC
17	GCAGAC**A**CUAAAU**U**UGC	**ACGAG**	GAAGGUUC**GCCC**CCUUC
18	GCAGAC**C**CUAAAUCUGC	**CCCCG**	GAAGGUUC**GUGC**CCUUC

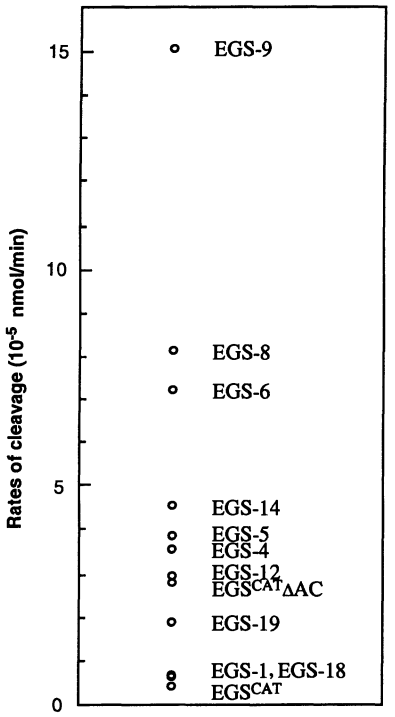

Fig. 4. Rates of cleavage directed by 12 individual EGS RNAs. Nine individual EGS RNAs, numbered as shown, prepared from selected and sequenced clones, as well as two derivatives prepared by mutagenesis in vitro, namely EGS 19 and EGS^{CAT} ΔAC, and the original EGS^{CAT} RNA, were assayed for their ability to direct cleavage of CAT mRNA by human RNase P. Conditions were as described (*11*), and the results are presented as initial rates (nmol/min) of cleavage of substrate by RNase P during the linear phase of each reaction. The units on the ordinate are consistent with those in Table 1.

ration of the stem of EGS 9 (EGS 19) resulted in a K_d that was 10 times higher than that for EGS 9 and was close to that of EGS^{CAT} (Table 1). Thus, the intact anticodon stem stabilized a conformation of the EGS that could not bind as strongly to the target RNA compared to an EGS with no organized anticodon stem. Accordingly, the enhancement of the ability of the selected EGSs for targeting RNA can be assigned, in part, to the increase in the strength of their binding to target RNA.

Cleavage of mRNA directed by the original EGS^{CAT} requires Mg^{2+} ions with an optimum concentration of 25 mM. By contrast, the reaction with certain selected EGSs (6 and 9) proceeded optimally in 2 to 10 mM Mg^{2+} ions (*14*). This 2 to 10 mM concentration is close to the optimal concentration of Mg^{2+} ions for processing of tRNA precursors by human RNase P (*17*). Since high concentrations of Mg^{2+} ions are especially effective in the neutralization of repulsion between adjacent regions of the phosphate backbone and the stabilization of RNA folding, our results indicate that the selected EGSs can achieve the appropriate folded structures in the complex with target RNAs without high concentrations of Mg^{2+} ions.

We made a kinetic analysis to determine the Michaelis constant (K_m) and the maximum velocity (V_{max}) of the enzymatic reactions (*18*). The effective concentrations of substrate used were calculated as the concentration of the complex of target mRNA with EGS as determined from the K_d values shown in Table 1. The K_m for the precursor to $tRNA^{Tyr}$ is 10 nM with human RNase P, whereas the K_m value of the complex of mRNA-EGS^{CAT} is 12 times higher (Table 1). The K_m for all the selected EGSs tested was the same as that of EGS^{CAT}. However, the maximum velocities of the reactions with selected EGSs were up to 10 times higher than that with the original EGS. Thus, the value of V_{max}/K_m for selected EGSs was increased. The value of V_{max}/K_m of EGS 9 was, for example, 10 times higher than that of EGS^{CAT} and was very close to that of the $tRNA^{Tyr}$ precursor. When the anticodon stem of EGS 9 was restored, however, as it was in EGS 19, V_{max} fell about twofold, suggesting that the rate of release of the product was specifically reduced by physical interactions of the enzyme with an intact anticodon stem. These data show that the enhanced abilities for targeting of the selected EGSs, as measured in the overall rate of the cleavage reaction, were due to both enhanced affinity of binding to substrate RNAs and to increases in the velocity of the enzymatic reaction.

The tRNAs and their precursors interact with many different enzymes and proteins, as well as with ribosomes, in vivo. These interactions, combined with the processes of natural selection, produced as they evolved the present-day structure of tRNAs. We selected for only one trait in a tRNA precursor-like structure—the ability to be cleaved efficiently by human RNase P. This selection process led to isolation of tRNA domains that lacked a recognizable anticodon stem and loop (a part of the tRNA structure that we know participates in a translational function unrelated to RNase P activity in vivo) and that had sequences in the T and variable loops that usually do not occur naturally. Earlier work with model substrates for RNase P (*19*) and aminoacyl synthetases (*20*) from *E. coli* also illustrate simplified structural requirements in interactions with tRNAs or tRNA precursors when these interactions are restricted to single enzyme substrate systems rather than when they involve the full panoply of interactions encountered by tRNAs in vivo. However, mutations in parts of tRNAs that are dispensable in vitro, such as the anticodon stem in our experiments, can still affect the function of RNase P in vivo (*21*).

Our specific goal was to optimize the design of an EGS RNA such that, when complexed with a target RNA, the EGS would render the mRNA especially susceptible to cleavage by human RNase P. After the selection procedure, two characteristics of the EGSs, the binding affinity of EGS to the target RNA, represented by K_d with a concomitant decrease in the free energy of binding, and the V_{max} of the enzymatic reaction improved. The changes in K_d and V_{max} that we observed with the most efficient EGS (EGS 9) were reversed when the anticodon stem, a structure lost in EGS 9, was restored.

Removal of the anticodon stem and loop from EGSs directed against mRNAs other than CAT mRNA also improves their targeting efficiency (*22*). Given that an EGS must first bind to its target and then form a structure that is recognized efficiently by RNase P, we consider that our simple selection process in vitro was remarkably successful. It is possible to repeat the selection process with the EGSs that we have isolated and to select for a decrease in K_m rather than an increase in k_{cat}, as we have already done. When EGSs are selected in this way the substrate complex may compete too well with natural substrates for binding to the enzyme in vivo. Finally, the relevance of these studies to the use of the new EGSs as tools in gene inactivation experiments must now be determined by experiments with cells in tissue culture.

Table 1. Kinetic parameters of EGS-directed cleavage of CAT mRNA in vitro by RNase P from HeLa cells. K_d refers to measurements of the dissociation constant for binding of EGS to CAT mRNA. The other parameters were determined in standard assays of enzyme kinetics. V_{max} is the value obtained with 0.5 ml (0.6 unit) of human RNase P. pTyr refers to the precursor to $tRNA^{Tyr}$ from *E. coli*.

Substrates	K_d (nM)	K_m (nM)	V_{max} (nmol/min)	V_{max}/K_m
pTyr		10	2.9×10^{-5}	2.90×10^{-6}
CAT mRNA				
+ EGS^{CAT}	880	120	2.9×10^{-5}	0.24×10^{-6}
+ $EGS^{CAT}\Delta AC$	20	150	11.4×10^{-5}	0.76×10^{-6}
+ EGS-5	210	125	16.3×10^{-5}	1.30×10^{-6}
+ EGS-8	25	125	21.3×10^{-5}	1.70×10^{-6}
+ EGS-9	78	125	30.0×10^{-5}	2.40×10^{-6}
+ EGS-19	710	130	15.6×10^{-5}	1.20×10^{-6}

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8. Double-stranded DNA templates for the transcription in vitro of the chimeric, mRNA-EGS RNA substrate were made by annealing and subsequent extension of two overlapping, synthetic oligonucleotides SEC-1A (5′-TAATACGACTCACTATAGAACATTTTGAGGCATTTCAGTCAGTTGGCCAAACTGAGCAGAC-3′) and SEC-1B (5′-TGGTGAGGCATGAAGGNNNNGAACCTTCNNNNNGCAGATTTAGAGTCTGCTCAGTTTGGCC-3′). The complementary sequences that form the double-stranded region in the annealed oligonucleotides are underlined, and the randomized nucleotides (N) were introduced during machine synthesis by inclusion of equimolar amounts of the four nucleotides. A promoter for T7 bacteriophage RNA polymerase is included in SEC-1A. The extension was done with avian myoblastosis virus reverse transcriptase (Boehringer Mannheim) at 46°C for 2 hours.
9. RNA substrate populations were prepared by transcription of template DNA (*8*) with T7 RNA polymerase (Promega) in 40 mM tris-HCl (pH 7.9), 6 mM $MgCl_2$, 10 mM dithiothreitol, 2 mM spermidine, and 1 mM NTPs containing 20 μCi of [α-^{32}P]GTP (Amersham) at 37°C. In the first three rounds of selection, RNA substrates were digested with 3.6 units of human RNase P [purified through the glycerol gradient step (*3*) and M.

Bartkiewicz, H. A. Gold, S. Altman, *Genes Dev.* **3**, 488 (1989)] in 50 mM tris-HCl (pH 7.5), 10 mM $MgCl_2$, and 100 mM NH_4Cl at 37°C for 2 hours. (One unit of human RNase P is defined as that amount of enzyme that cleaves 1 pmol of precursor to $tRNA^{Tyr}$ from *E. coli* in 30 min at 37°C.) For assays in subsequent rounds of selection, the amount of enzyme was reduced, and the incubation time was shortened so that less than 20% of the substrate was cleaved. Cleavage products were separated from uncleaved substrates by electrophoresis on an 8% polyacrylamide, 7 M urea gel. RNA was extracted from the gels by the crush and soak method. The purified cleavage product RNAs were reverse transcribed and amplified by PCR with SEC-1A (*8*) and SEC-1C (5′-TGGTGAGGCATGAAGG-3′) as primers with a Perkin-Elmer RNA PCR kit. The double-stranded DNA generated by PCR regained the T7 promoter sequence and the leader sequence from the sequence in the primer SEC-1A, and it was then used as a template for transcription of RNA for the next round of selection.

10. After eight cycles of selection and amplification, the resulting double-stranded DNAs were cloned into the Bluescript vector (Promega). Sixteen plasmid DNAs were sequenced by means of Sequenase 2.0 (U.S. Biochemical).
11. As a test for how well EGSs derived from the individual chimeric substrate clones can target mRNA, the EGS sequences were amplified by PCR with primers SCE-1C and SCE-1T (5′-TAATACGACTCACTATAGGCCAAACTGAGCAGAC-3′), which contains a promoter sequence for T7 RNA polymerase. RNAs were then transcribed with T7 RNA polymerase and used as substrates for human RNase P. Rates of EGS-directed cleavage of mRNA were determined in 10 μl of 50 mM tris-HCl (pH 7.5), 10 mM $MgCl_2$, and 100 mM NH_4Cl that contained 1 pmol (1000 cpm) of target RNA and 2.5 pmol of EGS RNA. Reaction mixtures were incubated at 37°C for various times with 0.5 μl (0.6 unit) of RNase P from HeLa cells and were then analyzed by electrophoresis in 5% polyacrylamide–7 M urea gels.
12. Partial digestion of CAT mRNA-EGS complexes with RNases T1 and T2 (Pharmacia) were performed in RNase P assay buffer (*9*). The reaction mixture contained substrate RNA labeled with ^{32}P at its 5′ terminus (2000 cpm), rat 5S RNA as carrier (at 0.2 mg/ml), and RNase T1 or RNase T2 (at three different concentrations, 2×10^{-4}, 1×10^{-3} and 5×10^{-3} units/ml). Reactions were incubated at room temperature for 5 min. The samples were analyzed by polyacrylamide gel electrophoresis in 12.5% sequencing gels. Conditions for digestion with cobra venom nuclease (Pharmacia) were as described above except that incubation was at 37°C.
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15. DNA coding for EGS^{CAT} ΔAC was synthesized by PCR with $pEGS^{CAT}$ DNA (*3*) as template; and oligonucleotide EC-1ΔAC (5′-GCCAAACTGACGTCATCGACTTCG-3′) and M13 reverse primer (5′-AACAGCTATGACCATG-3′) were used as primers. The DNA generated by PCR was digested with Hind III and then inserted into pUC19 downstream from a T7 RNA polymerase promoter sequence. EGS^{CAT} ΔAC RNA was prepared by transcription in vitro after the new plasmid DNA had been linearized with Dra I. DNA that coded for EGS 19 was synthesized by PCR procedure in a manner similar to that used for the synthesis of DNA for EGS 9, that is, with oligonucleotides SCE-1C (*9*) and SCE-1I (5′-GTAATACGACTCACTATAGGCCAAACTGAGCAGACTCTAAATCTGCAAACGGAAGGTTC-3′): the newly inserted T residue in SEC-1I is underlined The DNA was transcribed in vitro with T7 RNA polymerase to give EGS 19 RNA. The EGS 19 RNA differs from EGS 9 RNA only in the additional U that restores the structure of the anticodon stem.
16. The dissociation constants of the CAT mRNA-EGS RNA complexes were determined [A. M. Pyle, J. A. McSwiggen, T. R. Cech, *Proc. Natl. Acad. Sci. U S.A.* **87**, 8187 (1990)]. A fragment of CAT mRNA, 160 nucleotides in length, was prepared by transcription with T7 RNA polymerase in the presence of [α-^{32}P]GTP. EGS RNA (10 μl) in 2× binding buffer was heated at 80°C for 4 min before it was mixed with an equal volume of 2 nM CAT mRNA fragment in water (1× binding buffer contains 50 mM tris-HCl (pH 7.5), 10 mM $MgCl_2$, 100 mM NH_4Cl, 3% glycerol, 0.05% xylene cyanol). The mixtures were incubated at 37°C for 20 min and immediately separated on 5% polyacrylamide gels (9 watts). The electrophoresis buffer consisted of 36 mM tris base, 64 mM Hepes, 0.1 mM EDTA, 10 mM $MgCl_2$ (pH 7.5 without any adjustment). Quantitation of free target RNA and of the complex was performed with a Betascope (Betagen, Waltham, MA). The free energies of binding were determined from the equation $\Delta G^{\circ} = -RT\ln(1/K_d)$, where R = 0.00198 kcal/mol and T = 310.15 K.
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Heat-Inducible Degron: A Method for Constructing Temperature-Sensitive Mutants

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A temperature-sensitive (*ts*) mutant retains the function of a gene at a low (permissive) temperature but not at a high (nonpermissive) temperature. Arg-DHFR, a dihydrofolate reductase bearing an amino-terminal (N-terminal) arginine, is long-lived in the yeast *Saccharomyces cerevisiae*, even though arginine is a destabilizing residue in the N-end rule of protein degradation. A *ts* derivative of Arg-DHFR was identified that is long-lived at 23°C but rapidly degraded by the N-end rule pathway at 37°C. Fusions of *ts* Arg-DHFR to either Ura3 or Cdc28 of *S. cerevisiae* confer *ts* phenotypes specific for these gene products. Thus, $Arg\text{-}DHFR^{ts}$ is a heat-inducible degradation signal that can be used to produce *ts* mutants without a search for *ts* mutations.

Conditional mutants make possible the analysis of physiological changes caused by inactivation of a gene or a gene product and can be used to address the function of any gene. Several types of conditional mutants and methods for producing them have been developed (*1, 2*) since Horowitz's demonstration of the utility of *ts* mutants (*3*), but the *ts* phenotype is still the one most frequently used (*4, 5*). One limitation of the *ts* approach is the uncertainty as to whether a given gene can be mutated to yield a *ts* product. For example, only six loci were identified after repeated searches for *ts* lethal mutations mapping to the *S. cerevisiae* chromosome I, which contains many essential genes (*5*). Another problem with conventional *ts* mutations is that they are often too leaky to be useful (*4*). We now describe a strategy for producing *ts* mutants that does not require a search for a *ts* mutation in a gene of interest. This strategy is based on a portable, heat-inducible N-degron (Fig. 1).

The N-degron is an intracellular degradation signal whose essential determinant is a "destabilizing" N-terminal residue of a protein. A set of N-degrons containing different destabilizing residues is manifested as the N-end rule, which relates the in vivo half-life of a protein to the identity of its N-terminal residue (*6*). In eukaryotes, the N-degron consists of at least two determinants: a destabilizing N-terminal residue and a specific internal Lys residue (or residues) of a substrate (*6, 7*). The Lys residue is the site of attachment of a multiubiquitin chain (*6–8*). Ubiquitin is a protein whose covalent conjugation to other proteins plays a role in a number of cellular processes, primarily through routes that involve protein degradation (*6, 8*).

We constructed a thermolabile protein that becomes a substrate of the N-end rule pathway only at a temperature high enough to result in at least partial unfolding of the protein. This unfolding activates a previously cryptic N-degron in the protein by

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