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Targeted cleavage of mRNA by human RNase P

(external guide sequence/gene inactivation)

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ABSTRACT **Ribonuclease P from *Escherichia coli* can cleave RNAs in simple, hydrogen-bonded complexes of two oligoribonucleotides that resemble the aminoacyl stem and 5′ leader sequence of tRNA precursors. RNase P from human (HeLa) cells cannot catalyze the cleavage *in vitro* of the 5′-proximal oligoribonucleotide that contains the leader sequence in such simple complexes but can do so when the 3′-proximal oligoribonucleotide (external guide sequence) is altered to resemble three-quarters of a tRNA molecule. In such a complex, the efficiency of cleavage of the mRNA for chloramphenicol acetyltransferase, as the 5′-proximal oligoribonucleotide, depends on the structural details of the external guide sequence and on the choice of target site within the mRNA. The presence of the appropriately designed external guide sequence in cells in tissue culture reduces chloramphenicol acetyltransferase activity and the level of the corresponding intact mRNA in the cells. Thus, it appears that the use of such external guide sequences may provide a general technique for gene inactivation.**

Ribonuclease P, an enzyme that consists of protein and RNA subunits, cleaves tRNA precursors to generate the 5′ termini of tRNAs (1, 2). This essential enzymatic activity has been found in all types of cell examined, both prokaryotic and eukaryotic. During studies of the recognition of its substrates by RNase P from *Escherichia coli*, it was found that the enzyme can cleave substrates that lack specific domains (the D and anticodon stems and loops) of the usual tRNA structure (3). A half turn of an RNA helix and a 3′-proximal CCA sequence contain sufficient recognition elements to allow the reaction to proceed. Furthermore, the 5′-proximal sequence of the RNA helix does not have to be covalently linked to the 3′-proximal sequence of the helix (4). The 3′-proximal sequence of the stem can be regarded as an external guide sequence (EGS) because it identifies the site of cleavage in the 5′-proximal sequence by base pairing with the 5′-proximal oligonucleotide (4). In principle, any RNA of known sequence can be targeted for specific cleavage by RNase P by use of a custom-designed EGS RNA. Recently, in fact, EGS-directed cleavage of mRNA has been achieved *in vitro* with RNase P from *E. coli* (5).

Human RNase P is a functional analog of RNase P from *E. coli*. The similarity in secondary structure between M1 and H1 RNAs (the RNA components of RNase P from *E. coli* and human cells, respectively) suggests that they have a common ancestor (6, 7). However, the substrate specificity of human RNase P is much narrower than that of the enzyme from *E. coli*. For example, although RNase P from *E. coli* can cleave the model substrates mentioned above, none of them is a substrate for the human enzyme (unpublished experiments). Therefore, the design of an EGS for use with RNase P from human cells has to be different from that of an EGS for use with RNase P from *E. coli*. Here we describe an EGS which, when in a complex with the mRNA for chloramphenicol acetyltransferase (CAT), can direct specific cleavage of the mRNA by human RNase P both *in vitro* and in cultured cells. Expression of this EGS in cultured human cells caused a decrease in CAT enzymatic activity in the cells. This result suggests a possible method for the inactivation of specific genes.

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MATERIALS AND METHODS

Construction of Plasmids. The *Hin*dIII–*Bam*HI fragment of the CAT gene from the pCAT plasmid (Promega) was subcloned into pGEM-2. The EGS sequence was synthesized by the polymerase chain reaction (PCR), using DNA that encodes $tRNA^{Tyr}$ from *E. coli* as template, with oligonucleotides EC-1A, 5′-GCCAAA**CTG**AGCAGACTC-3′ and EC-1B, 5′-gccgaagcttTAAATGGT**GAGGCATG**AAGGATTC-GAACC-3′, where the bold letters in the oligonucleotide sequences indicate bases chosen to be different from those in the gene for $tRNA^{Tyr}$ in order to create base pairs with CAT mRNA and the lowercase letters define an extra linker sequence. The fragment obtained by PCR was digested with *Hin*dIII and then inserted into pUC19 with a T7 promoter sequence upstream from the insertion. For experiments with cultured cells, EGS^{CAT} DNA was synthesized by PCR with oligonucleotides EC-1A and EC-1C, 5′-GCGCGGTAC-CAAAAATGGTGAGGCATGAAGG-3′. The fragment from PCR was digested with *Kpn* I and inserted into the plasmid pmU6(−315/1) (kindly provided by Ram Reddy, Baylor College of Medicine) (8) at the *Pst* I (blunted)/*Kpn* I site. This plasmid contains the promoter for the gene for U6 small nuclear RNA and a signal for termination of transcription (T cluster) by RNA polymerase III.

Transcription *in Vitro*. EGS^{CAT} RNA was prepared by transcription from plasmid DNA that contained the appropriate sequence (see above) and had been linearized by treatment with *Dra* I. CAT mRNA was prepared from the appropriate plasmid DNA (see above) that had been linearized by treatment with *Eco*RI. The transcription reaction was carried out in 40 mM Tris·HCl, pH 7.9/6 mM $MgCl_2$/10 mM dithiothreitol/2 mM spermidine containing 0.5 mM NTPs in the presence of [α-^{32}P]GTP or 2 mM NTPs in the absence of labeled GTP, with 10–20 units of T7 RNA polymerase at 37°C. The transcripts were purified by electrophoresis in 5–8% polyacrylamide gels that contained 7 M urea.

Assay for Cleavage by RNase P. Human RNase P was partially purified (6) from HeLa cells. Assays were performed in 10 μl of 50 mM Tris·HCl, pH 7.5/10 mM $MgCl_2$/100 mM NH_4Cl containing 1000–2000 cpm ($\approx$0.2 pmol) of substrate RNA and an appropriate amount of EGS RNA, and reaction mixtures were incubated with 1 μl of a solution of partially purified human RNase P at 37°C for 1 hr. The reaction was stopped by the addition of an equal volume of dye solution

Abbreviations: CAT, chloramphenicol acetyltransferase; EGS, external guide sequence; hGH, human growth hormone; nt, nucleotide(s).

that contained 10 mM EDTA, and samples were electrophoresed in 5% polyacrylamide/7 M urea gels.

Primer Extension Analysis. Reverse transcription was performed with the products of cleavage by RNase P, using an end-labeled oligodeoxyribonucleotide primer (5′-GGCCGTAATATCCAGCTGAACGG-3′) complementary to nucleotides 129–107 of CAT mRNA. The reaction mixture contained 100 mM Tris·HCl (pH 8.3), 10 mM KCl, 6 mM $MgCl_2$, 10 mM dithiothreitol, 0.1 M dNTPs, and 2 units of avian myeloblastosis virus reverse transcriptase (Amersham) and was incubated with primer at 46°C for 2 hr. cDNAs were analyzed in an 8% sequencing gel in parallel with reference sequence ladders obtained from the same oligonucleotide primer.

Transfection and Assay for CAT Activity. Human lung cancer cells (A549; American Type Culture Collection) were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. One day prior to transfection, A549 cells were split 1:10 and plated in 100-mm-diameter Petri plates. Cells at 80–90% confluence were transfected with 5 μg of pCAT-control (Promega), 1 μg of $pEGS^{CAT}$, and 1 μg of pXGH5 by a calcium phosphate precipitation method (9). Control cells were transfected with 1 μg of mU6(−315/1) plasmid instead of $pEGS^{CAT}$. Cells were shocked with 15% glycerol after a 12-hr incubation. At several times posttransfection, cells were harvested and cell extracts were assayed for CAT activity. The acetylated forms of chloramphenicol were separated by thin-layer chromatography (10) and quantitated with a Betascope (Betagen, Waltham, MA). The level of human growth hormone (hGH) secreted into the medium was measured with the Allegro hGH immunoradiometric assay system (Nichols Institute Diagnostics, San Juan Capistrano, CA).

Northern Blot Analysis and RNase Protection Assay. Total RNA was prepared from monolayer cells (grown in 100-mm-diameter Petri plates) by the SDS/phenol method (for the Northern blot of EGS RNA) or the guanidinium thiocyanate method (for the RNase protection assay of CAT mRNA) (11). Ten micrograms of RNA was loaded onto an 8% polyacrylamide/7 M urea gel. After electrophoresis, the RNA was transferred to a Zeta-Probe nylon membrane (Bio-Rad) and probed with oligonucleotide EC-1C that had been labeled at its 5′ terminus with ^{32}P by polynucleotide kinase.

The RNase protection assay was carried out by the method of Zinn *et al.* (12). The probe was antisense CAT RNA [complementary to nucleotides (nt) −36 to 219; the first nucleotide of the codon for initiation of translation is taken as no. 1] transcribed *in vitro* by SP6 polymerase in the presence of [α-^{32}P]GTP. A probe for the human mRNA for γ-actin, similarly labeled, was included as an internal control (13). The relative levels of the CAT and γ-actin mRNAs were quantitated by particle counting with a Betascope and levels of CAT mRNA were normalized by reference to the amount of γ-actin mRNA in each sample.

RESULTS

An EGS Can Target a Small RNA for Cleavage by Human RNase P. A small RNA fragment, pAva (Fig. 1*A*), that contains the 5′ precursor sequence and the first 14 nt from the 5′ terminus of $tRNA^{Tyr}$ from *E. coli* can be cleaved correctly by human RNase P when another piece of RNA that contains

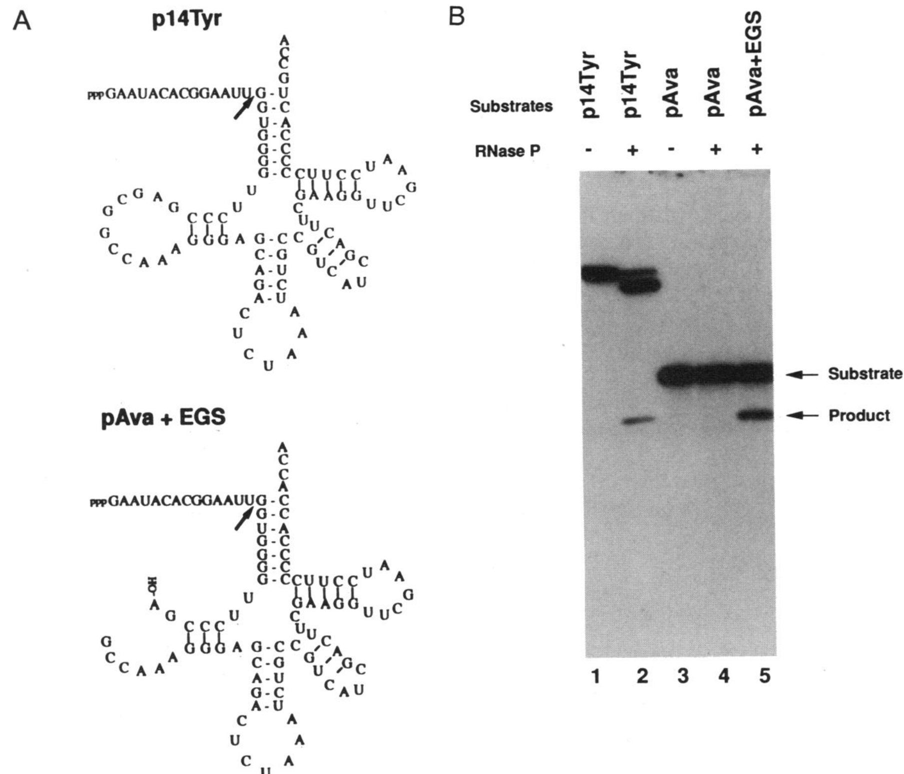

FIG. 1. EGS RNA-directed cleavage of substrates by human RNase P. (*A*) Secondary structures of complexes of substrates and EGS RNAs. p14Tyr, the precursor to $tRNA^{Tyr}$ from *E. coli* with 14 nt in the leader sequence; pAva, substrate RNA with 5′ leader sequence and the first 14 nt of mature $tRNA^{Tyr}$. The RNA was prepared by transcription with T7 polymerase of the DNA for $tRNA^{Tyr}$ from *E. coli* truncated with *Ava* I; EGS^{Tyr} RNA was transcribed from a derivative of the gene for $tRNA^{Tyr}$ from *E. coli* from which the leader sequence and first 18 nt of the 5′ end of the mature tRNA had been removed. Arrows, sites of cleavage by human RNase P. (*B*) Cleavage of substrates by human RNase P in the presence or absence of EGS RNAs. [α-^{32}P]GTP-labeled pAva RNA (28 nt) was mixed with unlabeled EGS^{Tyr} RNA (lane 5) and the reaction mixture (see *Materials and Methods*) was incubated with human RNase P for 30 min. pAva RNA was also incubated alone with (lane 4) or without (lane 3) enzyme, as was p14Tyr (lanes 2 and 1, respectively).

the remaining 3′-proximal sequence of the $tRNA^{Tyr}$ sequence is hybridized to it (Fig. 1*B*). This substrate closely resembles a tRNA precursor (p14Tyr; Fig. 1*A*) with the exception that it consists of two noncovalently linked oligoribonucleotides. The 3′-proximal oligoribonucleotide is the EGS (4). Because the lengths of the precursor-specific segments, their sequences, and the sequences of the mature domain are not conserved among precursors to different tRNAs, the main determinants for cleavage by human RNase P must be in some portion of the conserved, higher-order structural features of the various tRNAs. This hypothesis is supported by the observation that several other EGSs that did not mimic exactly the structure of a tRNA (i.e., the number of base pairs in the D or aminoacyl stems and the nucleotides at positions 8 and 9 were changed as compared to those found in mature tRNA sequences) did not target complementary RNAs. However, neither the anticodon nor the variable stem and loops are absolutely required in EGSs that can be used with human RNase P (unpublished experiments).

Our results suggest that if an mRNA, rather than part of a precursor tRNA sequence, is incorporated into the double-stranded region of the acceptor and D stems of a putative target complex, and if the resulting hybrid contains the structural features required of a substrate for human RNase P, that mRNA should be cleaved by human RNase P.

An EGS Can Target CAT mRNA for Specific Cleavage by Human RNase P. Because the gene coding for CAT can be easily manipulated on plasmids and the gene product is readily expressed in cells in tissue culture, we chose CAT mRNA as a target substrate for RNase P. A complex in which an EGS can base pair with nt 67–79 of CAT mRNA and which could theoretically direct human RNase P to cleave that mRNA at nt 67 is shown in Fig. 2. The EGS^{CAT} construct was derived from the gene for $tRNA^{Tyr}$ from *E. coli*. The first 18 nt at the 5′ terminus of the tRNA sequence have been deleted and sequences in the D and aminoacyl acceptor stems have been changed to allow base pairing with CAT mRNA.

Labeled CAT mRNA was mixed without prior denaturing or annealing with various amounts of EGS^{CAT} RNA and assayed for susceptibility to cleavage by human RNase P *in vitro*. In the presence of EGS^{CAT}, this mRNA was cleaved efficiently, giving two products of expected sizes (Fig. 3, lanes 3–7). Control experiments in which other RNAs, not predicted to function as EGSs for this mRNA, were included in the reaction mixtures failed to result in cleavage of the target RNA (data not shown). The precise site of cleavage of CAT mRNA by human RNase P was determined by primer extension analysis with reverse transcriptase and a primer

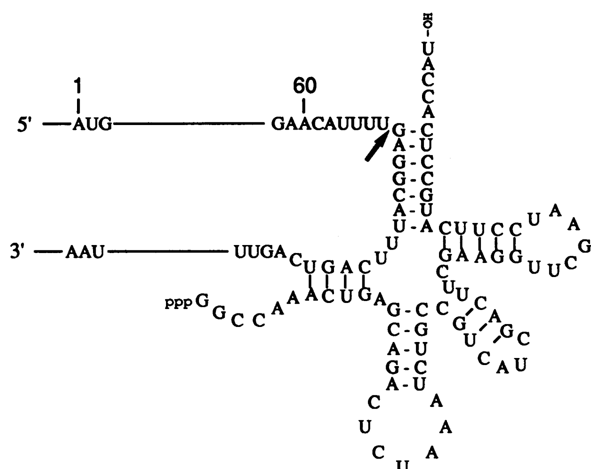

FIG. 2. Schematic representation of a complex of CAT mRNA and EGS^{CAT} RNA. Arrow, expected site of cleavage in CAT mRNA.

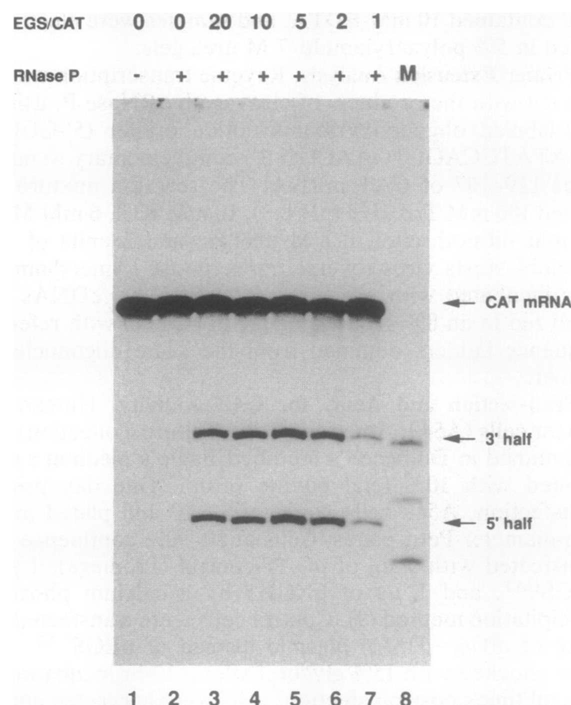

FIG. 3. EGS^{CAT}-directed cleavage of CAT mRNA. A mixture of unlabeled and [α-^{32}P]GTP-labeled fragments of CAT mRNA (0.2 pmol) (265 nt in length) was mixed with 4 pmol (lane 3), 2 pmol (lane 4), 1 pmol (lane 5), 0.4 pmol (lane 6), or 0.2 pmol (lane 7) of EGS^{CAT} RNA, and reactions were carried out and analyzed as described. CAT mRNA was incubated alone without (lane 1) and with (lane 2) RNase P. Lane M, molecular size markers.

that was complementary to nt 107–129 of CAT mRNA. Cleavage occurred between nt 66 and 67, as predicted (Fig. 4). The products of cleavage by RNase P were digested completely with RNase T1; further analysis of these latter products showed that the end groups of the initial products of the reaction catalyzed by RNase P were 5′ phosphoryl and 3′ hydroxyl, as expected (data not shown).

The cleavage reaction proceeded in a linear fashion for >3 hr at 37°C. Denaturation and reannealing of the oligonucleotides in the target complex did not improve the efficiency of cleavage. The reaction had an absolute requirement for Mg^{2+} ions, with an optimal concentration of 25 mM. By contrast, the reaction with the precursor to $tRNA^{Tyr}$ from *E. coli* proceeds optimally with Mg^{2+} in the range of 2–10 mM. The efficiency of cleavage of CAT mRNA was proportional to the amount of EGS^{CAT} in the reaction mixture up to a 5-fold molar excess of added EGS^{CAT} RNA relative to CAT mRNA. However, a >10-fold excess of EGS^{CAT} RNA caused a decrease in the efficiency of cleavage (Fig. 3), presumably because of nonspecific inhibition of the enzymatic activity by excess EGS^{CAT} RNA. Under our conditions, no detectable cleavage of the target mRNA occurred when the EGS was made of DNA (data not shown).

We designed a second EGS^{CAT}, in addition to the one that targeted nt 67 in CAT mRNA, to target nt 417. Although CAT mRNA was cleaved by RNase P nt 417 as a result of targeting with the second EGS, the efficiency of cleavage was very low, indicating that the sequence around nt 417 was not very accessible (data not shown). It is likely that this region of the mRNA participates in a higher-order structure that prevents the mRNA from binding to the EGS.

Since transcripts of genes for eukaryotic tRNA do not contain 3′-terminal CCA sequence, we tested an EGS^{CAT} that lacked this sequence. While the rate of cleavage of the target mRNA by human RNase P decreased by about 50% with the

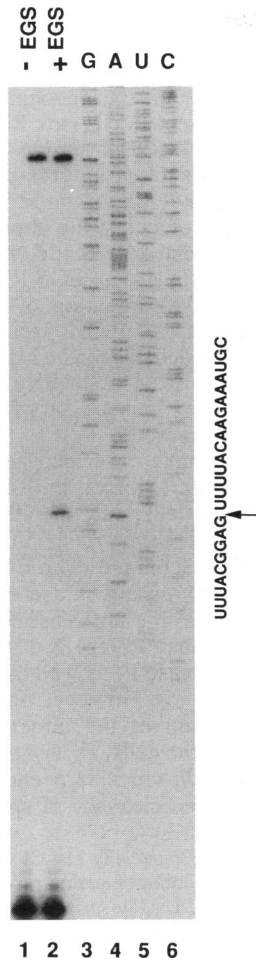

FIG. 4. Primer extension analysis of the products of EGS^{CAT}-directed cleavage of CAT mRNA by human RNase P. Reverse transcription was performed with the uncleaved (lane 1) and cleaved (lane 2) CAT mRNA. Lanes 3–6, labeled G, A, U, and C are reference analyses of DNA sequences that correspond to the CAT mRNA template. The band representing the cleaved product and the site of cleavage in the nucleotide sequence of CAT mRNA are marked by an arrow.

EGS that lacked the 3′-terminal CCA, the presence of this sequence was clearly not essential for the reaction to proceed.

Inhibition of Expression of CAT Activity in Human Cells in Tissue Culture by EGS^{CAT}. To examine whether EGS^{CAT} can function "*in vivo*," we monitored the CAT activity in cells in tissue culture that expressed both EGS^{CAT} RNA and CAT mRNA. The EGS^{CAT} sequence was inserted downstream from the promoter of a mouse gene for U6 snRNA (8) and was followed by a signal for termination of transcription, T_5. Thus, the sequence of the EGS^{CAT} could be transcribed by RNA polymerase III and, in fact, transcription terminated accurately in S100 extracts. Further, when the plasmid that contained the synthetic gene for EGS^{CAT} was used to transfect human lung cancer cells (A549) by calcium phosphate precipitation, expression of EGS^{CAT} RNA of 69 nt was readily detected by Northern analysis (Fig. 5*A*).

We then cotransfected A549 cells with pCAT and pEGS^{CAT} DNA. At various times posttransfection, cells were harvested and CAT activity was assayed. Cotransfected cells exhibited a decrease in their ability to convert chloramphenicol to its acetylated forms as compared with cells cotransfected with pCAT and pmU6(−315/1), which lacked the EGS sequence. The maximum inhibition measured was about 60%

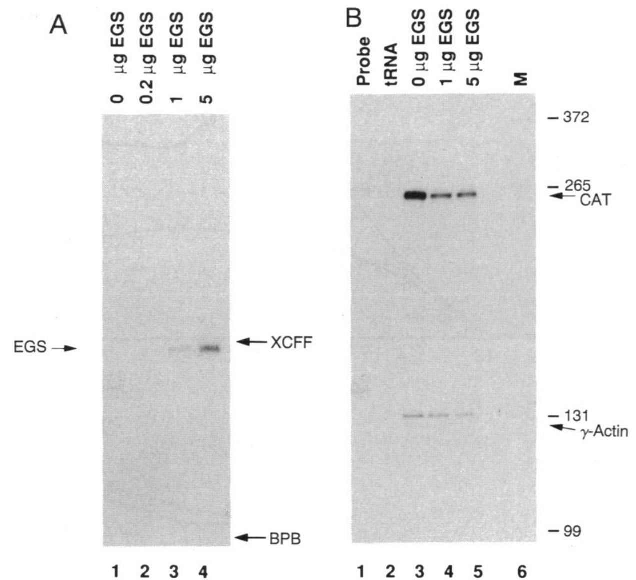

FIG. 5. (*A*) Northern blot analysis of EGS^{CAT} RNA in human lung cancer cells (A549). Samples (10 μg) of total RNA, prepared from cells transfected with 0 μg (lane 1), 0.2 μg (lane 2), 1 μg (lane 3), or 5 μg (lane 4) of EGS^{CAT} plasmid at 48 hr after transfection, were fractionated in an 8% polyacrylamide/7 M urea gel, transferred to a Zeta-Probe membrane, and probed with the end-labeled antisense oligonucleotide that corresponded to EGS^{CAT}. The band that corresponded in size to the EGS is marked by the arrow at left. Arrows at right indicate positions of xylene cyanol FF (XCFF) and bromophenol blue (BPB) tracking dyes. (*B*) RNase protection assay of CAT mRNA in A549 cells. Samples (40 μg) of total RNA from cells cotransfected with 5 μg of pCAT and various amounts of pEGS^{CAT} (lanes 3–5) and a sample (40 μg) of tRNA (lane 2) were hybridized with 10^6 cpm of probe complementary to CAT mRNA and 500 cpm of probe complementary to γ-actin mRNA. RNA was extracted from the cells 48 hr after cotransfection. Positions of the protected fragments of CAT and γ-actin mRNA, as well as the size (in nucleotides) of molecular size markers, are indicated.

at 48 hr posttransfection when 1 μg of pEGS^{CAT} was used (Fig. 6*A*). After 48 hr the kinetics were no longer reproducible, perhaps because the transfected cells began to lose the plasmids. Similar results were obtained in several experiments with African green monkey cells (CV-1; data not shown).

To assure that the EGS^{CAT}-dependent inhibition was specific to CAT, we simultaneously cotransfected the A549 cells with a third plasmid, pXGH5, which contained the gene for hGH, and we monitored the secretion of hGH into the culture medium. There was no significant difference in hGH expression between cells that had or had not been cotransfected with pEGS^{CAT} (Fig. 6*B*). Therefore, the EGS^{CAT} RNA specifically inhibited function of the CAT gene.

The mechanism of inhibition of CAT activity in the cells is unclear. To determine whether cleavage of the CAT mRNA by RNase P was responsible for the inhibitory effect, we carried out an RNase protection experiment with an RNA probe that was complementary to CAT mRNA and that covered the expected site of cleavage in CAT mRNA. Cells cotransfected with either 1 μg or 5 μg of pEGS^{CAT} contained less CAT mRNA than the cells with no EGS^{CAT} plasmid (Fig. 5*B*, lanes 3–5). The reduction in intact CAT mRNA, normalized to the amount of control mRNA for γ-actin in each sample, was 41% and 33%, respectively, for 1 μg and 5 μg of pEGS^{CAT}. These numbers are in good agreement with the extent of inhibition of CAT enzymatic activity measured in various experiments (note that inhibition was less efficient at the highest level of pEGS^{CAT} DNA used). However, we failed to detect the fragments of CAT mRNA that should have

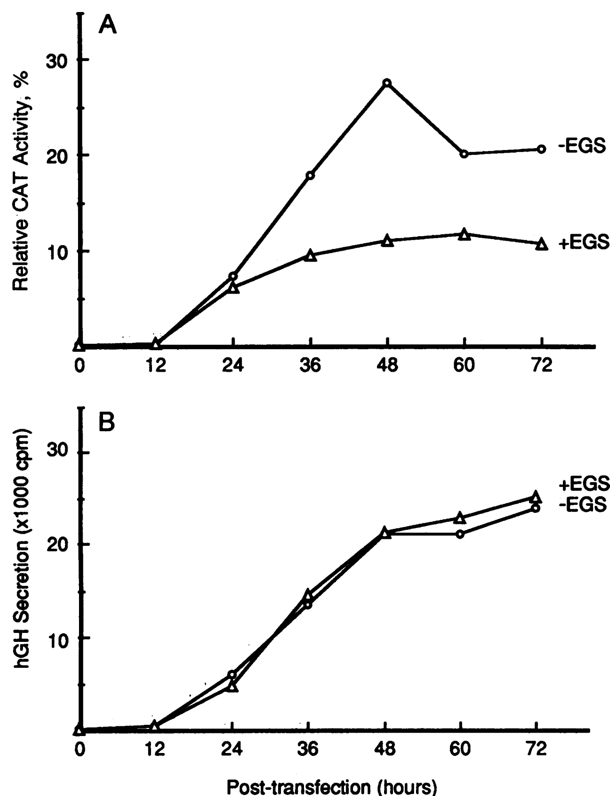

FIG. 6. Inhibition of expression of CAT activity by EGS^{CAT} in cells in tissue culture. Human lung cancer cells (A549) were transfected with 5 μg of pCAT, 1 μg of $pEGS^{CAT}$, and 1 μg of pXGH5 (△) or with 5 μg of pCAT, 1 μg of pmU6(−315/1), and 1 μg of pXGH5 (○). Cells were harvested 12, 24, 36, 48, 60, and 72 hr after transfection. Cell extracts were assayed for CAT activity (*A*), and the amount of hGH in 100 μl of culture medium was determined by radioimmunoassay (*B*).

resulted from cleavage by RNase P and that were predicted from our experiments *in vitro*. These cleavage products may be degraded too quickly to allow detection. We cannot rule out the possibility that some unidentified RNase may degrade the mRNA that is in the complex with the EGS.

DISCUSSION

We have described a method for site-specific cleavage of mRNA by human RNase P. Although mRNAs are not natural substrates of RNase P, complexes of mRNA and EGS RNA share common structural features with tRNA that allow the mRNA to be cleaved by RNase P. The design of EGS^{CAT} was based on our current, rudimentary understanding of the recognition by human RNase P of its substrate. Hence, it is expected that further details of the mechanism of such recognition and identification of minimal requirements for cleavage by human RNase P will lead to the design of more efficient EGSs. Clearly, an EGS for human RNase P is more complex than one for RNase P from *E. coli*. The nature of human cells demands that these EGSs be complex; otherwise RNase P would not be sufficiently specific and would cleave many RNAs that contain appropriate, simple, hairpin structures. An EGS for use in human cells has two short, sequence-specific recognition elements that are oriented in space with respect to each other in a well-defined fashion. This complex recognition element provides the necessary specificity for RNase P. A single sequence-specific element (allowing formation of a double-stranded RNA) of length ≥11 nt would not provide adequate specificity in human cells. Perhaps the complexity of the recognition elements for RNase P explains why human cells lack RNase III activity—i.e., an activity that cleaves "simple" double-stranded RNAs—since too many such structures exist *in vivo*.

Given the extensive secondary and tertiary structure associated with all RNA molecules, the choice of target site in mRNA may play a crucial role in determining whether a target substrate is accessible to an EGS RNA. Some general methods, such as the determination of sensitivity to nucleases, use of chemical or nucleic acid probes that are specific for single-stranded regions (14), and analysis of sequences to identify regions accessible to components of the translation apparatus, may be necessary for identification of suitable target sites in mRNA.

The method that we have described here may provide a powerful tool with which to block the function of specific mRNAs in mammalian cells. mRNAs that code for gene products that act in a stoichiometric fashion are good targets for inactivation by this technique. The EGS-directed cleavage of certain RNAs has an advantage over techniques of gene inactivation that involve antisense RNA or other, exogenous ribozymes because RNase P destroys the targeted RNA irreversibly and RNase P, as well as the EGS, can act in a catalytic fashion (5, 15). However, further work on the mechanism of degradation of the target mRNA, and the location of this action in the cells, as well as on the molecular biology of the reaction *in vitro*, is needed to improve the efficiency of EGS-directed cleavage of specific mRNAs.

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