

# RNA

## Inhibition of viral gene expression by human ribonuclease P

D. Kawa, J. Wang, Y. Yuan and F. Liu

*RNA* 1998 4: 1397-1406

---

### References

Article cited in:

<http://www.rnajournal.org/cgi/content/abstract/4/11/1397#otherarticles>

### Email alerting service

Receive free email alerts when new articles cite this article - sign up in the box at the top right corner of the article or [click here](#)

---

### Notes

---

To subscribe to *RNA* go to:  
<http://www.rnajournal.org/subscriptions/>

---

# Inhibition of viral gene expression by human ribonuclease P

DIANE KAWA,<sup>1</sup> JUN WANG,<sup>1</sup> YAN YUAN,<sup>2</sup> and FENYONG LIU<sup>1</sup>

<sup>1</sup>Program in Infectious Diseases and Immunity, School of Public Health, University of California, Berkeley, California 94720, USA

<sup>2</sup>Department of Microbiology, School of Dental Medicine, University of Pennsylvania, Philadelphia, Pennsylvania 19104, USA

## ABSTRACT

External guide sequences (EGSs) are small RNA molecules which consist of a sequence complementary to a target mRNA and render the target RNA susceptible to degradation by ribonuclease P (RNase P). EGSs were designed to target the mRNA encoding thymidine kinase (TK) of herpes simplex virus 1 for degradation. These EGSs were shown to be able to direct human RNase P to cleave the TK mRNA sequence efficiently *in vitro*. A reduction of about 80% in the expression level of both TK mRNA and protein was observed in human cells that steadily expressed an EGS, but not in cells that either did not express the EGS or produced a “disabled” EGS which carried a single nucleotide mutation that precluded RNase P recognition. Thus, EGSs may represent novel gene-targeting agents for inhibition of gene expression and antiviral activity.

**Keywords:** gene targeting; herpes simplex virus; RNase P; thymidine kinase

## INTRODUCTION

Antisense technology has been shown to be a promising gene targeting approach for use in basic research and clinical therapeutic applications. The gene-targeting agents used can be a conventional antisense oligonucleotide, an antisense catalytic molecule (ribozyme or DNA enzyme), or an antisense molecule with an additional (guide) sequence that targets the mRNA for degradation by endogenous RNases such as RNase L and RNase P (Stein & Cheng, 1993; Maran et al., 1994; Poeschla & Wong-Staal, 1994; Yuan & Altman, 1994; Rossi, 1995; Santoro & Joyce, 1997; Plehn-Dujowich & Altman, 1998). Antisense molecules with guide sequences have several unique features as gene targeting agents. It has been shown that targeting of mRNAs with these molecules for degradation by RNase P and RNase L results in irreversible cleavage and the cleavage can be in a catalytic fashion (Yuan et al., 1992; Maran et al., 1994; Yuan & Altman, 1994). Moreover, this targeting approach uses the cellular endogenous RNases for degradation of the target mRNA and, therefore, assures the stability and efficiency of the targeting enzymes in the cellular environment.

Ribonuclease P (RNase P) has been found in all organisms examined and is one of the most abundant, stable, and efficient enzymes in cells (Altman et al., 1993; Pace & Brown, 1995). Its enzymatic activity is responsible for the maturation of 5' termini of all tRNAs which account for about 2% of total cellular RNA (Gopalan et al., 1995). This enzyme is a ribonucleoprotein complex and catalyzes a hydrolysis reaction to remove the leader sequence of precursor tRNA (Altman et al., 1993; Pace & Brown, 1995). RNase P from *Escherichia coli* has been extensively studied. It contains a catalytic RNA subunit of 377 nt termed M1 RNA and a single polypeptide of 119 amino acids known as C5 protein (Altman et al., 1993; Pace & Brown, 1995). In the presence of a high concentration of Mg<sup>2+</sup>, M1 RNA itself can hydrolyze tRNA precursors *in vitro* (Guerrier-Takada et al., 1983). However, the addition of C5 protein dramatically increases the rate of cleavage *in vitro* and is required for cleavage *in vivo* (Guerrier-Takada et al., 1983). Human RNase P also contains an RNA subunit, H1 RNA, 344 nt in length (Bartkiewicz et al., 1989). However, in the absence of protein factors, H1 RNA does not exhibit enzymatic activity by itself *in vitro* (Bartkiewicz et al., 1989; Yuan & Altman, 1995).

Extensive studies have been carried out to understand how RNase P recognizes its substrates. It has been shown that any complex of two RNA molecules which resembles a tRNA molecule can be recognized

Reprint requests to: Fenyong Liu, School of Public Health, University of California, 140 Earl Warren Hall, Berkeley, California 94720, USA; e-mail: liu\_fy@uclink4.berkeley.edu.

and cleaved by RNase P and M1 RNA ribozyme (Fig. 1A,B) (Forster & Altman, 1990; Yuan et al., 1992). One of the molecules is called the external guide sequence (EGS) because the EGS guides RNase P to recognize the site of cleavage (Fig. 1B). In principle, any RNA sequence can be targeted for RNase P cleavage by using EGSs to hybridize with the target RNA and direct RNase P to the site of cleavage. Subsequent studies have shown that mRNAs encoding  $\beta$ -galactosidase and chloramphenicol acetyltransferase (CAT) were cleaved in vitro and in both bacterial and human cell culture by *E. coli* and human RNase P with custom-designed EGSs (Li et al., 1992; Yuan et al., 1992). These studies provided the first evidence of targeting cleavage of a specific mRNA by RNase P both in vitro and in cultured cells. The EGSs used to direct human RNase P for targeting cleavage resembled three-quarters of a tRNA molecule and consisted of two sequence elements: a targeting sequence complementary to the mRNA sequence and a guide sequence which is a portion of natural tRNA sequence and is required for RNase P recognition (Fig. 1B) (Yuan et al., 1992; Yuan & Altman, 1994).

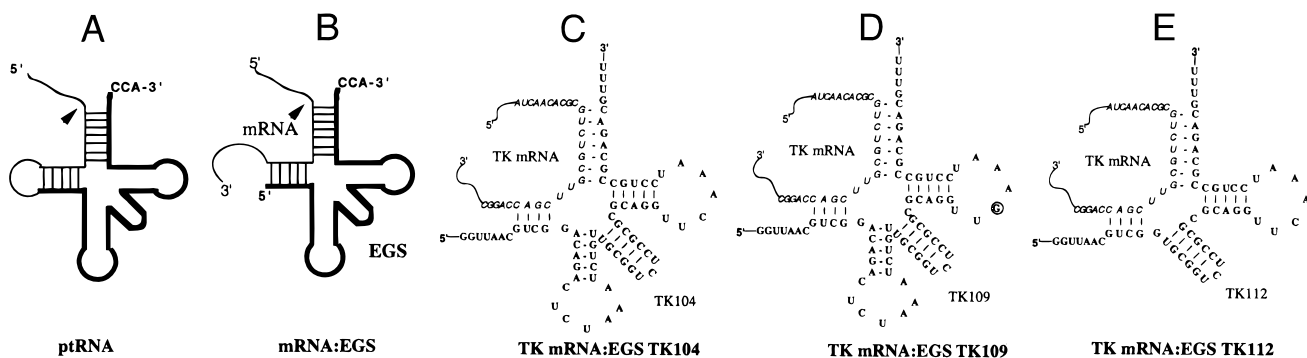
In the studies reported here, we show that targeting cleavage by endogenous RNase P can be used to inhibit expression of a viral gene in human cells by depleting its mRNA. The mRNA that encodes thymidine kinase (TK) of human herpes simplex virus 1 (HSV-1) was used as the target. The TK gene has been extensively studied and its activity can easily be assayed (Roizman & Sears, 1996). EGSs were designed and shown in vitro to target the TK sequence for cleavage by RNase P. Furthermore, when an EGS was expressed in human cells that were infected with HSV-1, a reduction of about 80% in the expression level of TK mRNA and protein was observed. A reduction of less than 10% was detected in cells that either did not express the EGS or expressed one that contained a mu-

tation known to preclude RNase P recognition. These results demonstrate the feasibility of utilizing EGSs as novel gene-targeting agents for specific inhibition of viral gene expression and antiviral therapy.

## RESULTS

### Design of EGSs and in vitro studies of their targeting activity

Since most mRNA species inside cells are usually associated with proteins and are present in a highly organized and folded conformation, it is critical to choose a targeting region that is accessible to binding of EGSs in order to achieve efficient targeting. In vivo mapping with dimethyl sulphate (DMS) has been extensively used to determine the accessibility of mRNA and structure of RNAs in cells (Climie & Friesen, 1988; Ares & Igel, 1990; Liu & Altman, 1995; Zaug & Cech, 1995). Using this method, we mapped the region around the translation initiation site of TK mRNA because this region is supposed to be accessible to ribosome binding (Liu & Altman, 1995). A position 29 nt downstream from the TK translational initiation codon was chosen as the cleavage site for human RNase P. This site appeared to be one of the regions most accessible to DMS modification (data not shown, Liu & Altman, 1995). Moreover, its flanking sequence exhibited several sequence features that need to be present in order to interact with an EGS and RNase P to achieve efficient cleavage. These necessities are that (1) the nucleotides 3' and 5' adjacent to the site of cleavage are a guanosine and a pyrimidine, respectively; and (2) a uracyl is 8 nt downstream from this cleavage site (Yuan & Altman, 1994; Yuan et al., 1992). The interactions of these sequence elements with the EGS facilitate the formation of the mRNA-EGS complex into a tRNA-like structure while

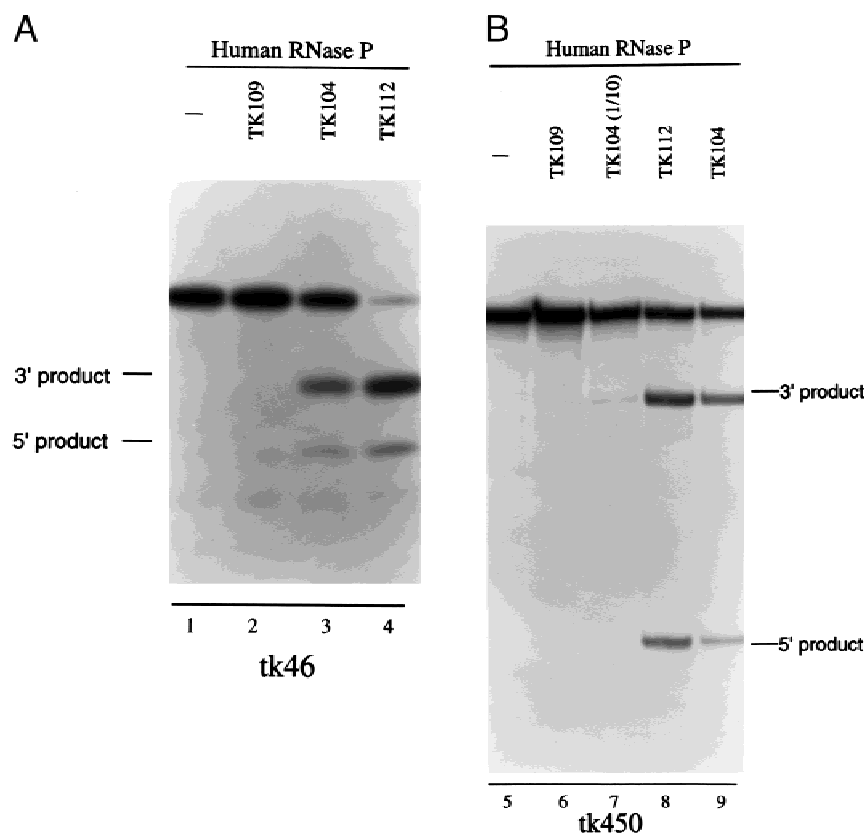


**FIGURE 1.** Schematic representation of substrates for RNase P. **A:** Natural substrate (ptRNA). **B:** A hybridized complex of a target RNA (e.g. mRNA) and an EGS that resembles the structure of a tRNA. **C, D, E:** Complexes between TK mRNA sequence and EGS TK104, TK109 and TK112, respectively. The sequence of these EGSs equivalent to the tRNA sequence (i.e. T-stem and loop, anticodon stem and loop, and variable region) was derived from tRNA<sup>Ser</sup>. Only the exact sequence of the TK mRNA around the targeting site was shown and italicized. The EGS sequence was shown in bold type. The design and construction of these EGSs can be found in Materials and Methods.

those with RNase P are critical for recognition and cleavage by the enzyme (Yuan & Altman, 1994).

Three EGSs were designed based on the sequence of tRNA<sup>ser</sup> and constructed (Fig. 1C–E) as described previously (Yuan & Altman, 1994; Yuan et al., 1992). EGS TK104 (Fig. 1C) resembles a three-quarters tRNA<sup>ser</sup> structure (Fig. 1B) (Yuan et al., 1992); EGS TK109 (Fig. 1D) was constructed from TK104 by introducing a point mutation (from C to G) at a highly conserved position in the T-loop. This cytosine was found in the sequences of all known natural tRNAs (Sprinzl et al., 1991) and in substrates selected to be efficiently cleaved by human RNase P in simulated evolution *in vitro* experiments (Yuan & Altman, 1994). These observations suggested that this nucleotide may be important for the interaction between the tRNA domains and human RNase P. Finally, EGS TK112 (Fig. 1E) was constructed from TK104 by deleting the anticodon domain. Previous studies have shown that deletion of the anticodon stem and loop improved EGS activity to target CAT mRNA for cleavage by human RNase P (Yuan & Altman, 1994). The DNA sequences coding for the EGSs were generated by polymerase chain reaction (PCR) using tRNA<sup>ser</sup> as the template and primers that

contained the sequences complementary to the targeting region of TK mRNA. These DNA sequences were inserted in a plasmid vector and under the control of the promoter for T7 RNA polymerase. EGS RNAs were synthesized *in vitro* from these EGS DNA sequences by T7 RNA polymerase and subsequently incubated with TK mRNA substrates and human RNase P. Two RNA substrates were used: tk46 and tk450 which contain a 46- and 450-nt-long TK mRNA sequence, respectively. Cleavage of these two substrates by RNase P yielded cleavage products of 21 and 25 nt, and 150 and 300 nt, respectively (Fig. 2). In the absence of EGS RNAs (Fig. 2, lanes 1 and 5), no cleavage of TK mRNA sequence was observed. Among the three EGSs tested, cleavage of the TK mRNA substrates by human RNase P was most efficient in the presence of TK112 (Fig. 2, lanes 4 and 8). In contrast, cleavage of the same substrates was hardly detected in the presence of TK109 (Fig. 2, lanes 2 and 6). The cleavage efficiency with TK112 is at least twofold higher than that with TK104 and at least 20 times higher than that with TK109 (Fig. 2, lanes 5–9; data not shown). These results are consistent with the previous observations that deletion of the anticodon domain significantly increases the



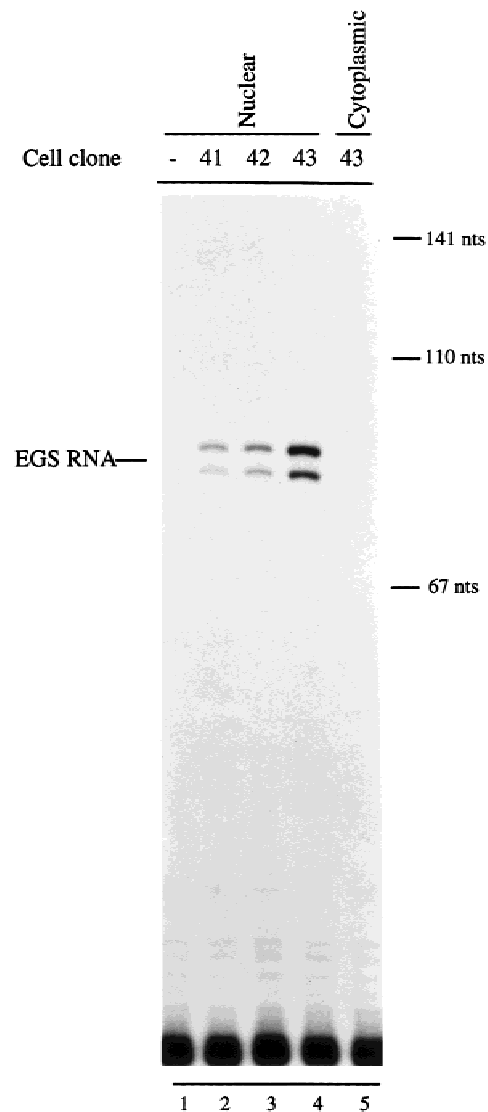
**FIGURE 2.** Cleavage of TK mRNA sequence of 46 (A) and 450 nt (B) by human RNase P in the presence of different EGSs. No RNase P was added to the reaction mixture in lanes 1 and 5. In (B), 20 nM (lanes 6, 8, and 9) and 2 nM of the EGS (lane 7) were incubated with [<sup>32</sup>P]-labeled TK RNA substrate (20 nM) and human RNase P (2 units) at 37 °C in a volume of 10  $\mu$ l for 45 min in buffer A (50 mM Tris, pH 7.4, 100 mM NH<sub>4</sub>Cl, and 10 mM MgCl<sub>2</sub>) (Yuan et al., 1992). Experimental details can be found in Materials and Methods.

cleavage efficiency by RNase P (Yuan & Altman, 1994). In addition, the highly conserved cytosine in the T-loop is important for recognition by RNase P and mutation of this nucleotide will probably disrupt the interaction (Kahle et al., 1990; Nolan et al., 1993; Liu & Altman, 1994; Yuan & Altman, 1994).

Experiments were carried out to further determine whether the differential cleavage efficiencies observed with TK104 and TK109 are possibly due to their different binding affinities to the TK mRNA sequence. The binding between the EGS and substrate tk46 was assayed in the absence of human RNase P and the EGS-tk46 complexes were separated in polyacrylamide gels under nondenaturing conditions. Similar amounts of complexes formed by these EGSs and TK mRNA sequence were observed when the same amounts of EGSs were used (data not shown). Further detailed assays using different concentrations of the EGSs and TK sequences indicated that the binding affinity of TK109 to substrate tk46, measured as the dissociation constant ( $K_d$ ), is similar to that of TK104 (data not shown). Meanwhile, very small amounts of cleavage products were observed in the presence of TK109 even under high concentrations of RNase P and prolonged incubation period (Fig. 2, lanes 2 and 6; data not shown). These observations indicated that the T-loop mutation does not significantly affect the binding affinity between TK109 and TK mRNA sequence but disrupts the recognition of EGS-TK mRNA complex by RNase P. Thus, TK109 may be used as a control for the antisense effect in our experiments in cultured cells (see below).

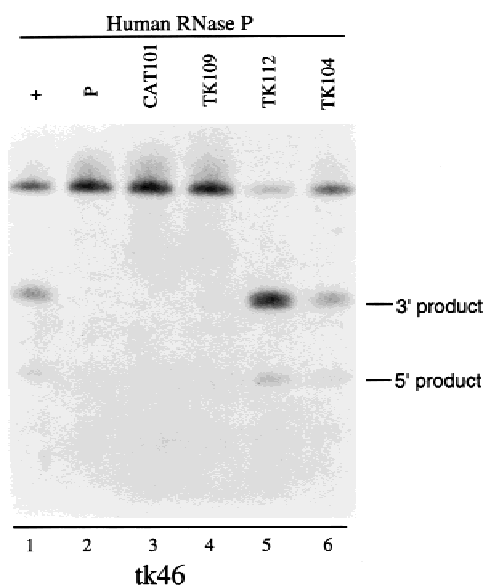
### Expression of EGS in human cell culture

The DNA sequences coding for TK104, TK109, and TK112 were subcloned and placed under the control of the promoter for small nuclear U6 RNA. This promoter is transcribed by RNA polymerase III and its transcripts are highly expressed and primarily present in the nucleus, where RNase P is localized (Das et al., 1988; Yuan et al., 1992; Liu & Altman, 1995; Bertrand et al., 1997; Good et al., 1997). To construct cell lines that express EGSs, human 143tk<sup>-</sup> cells were cotransfected with each of these three EGS DNA constructs and a plasmid containing a neomycin resistance gene. These cells were then selected in culture medium that contained neomycin and cells that exhibited neomycin resistance were cloned. The presence of EGS genes in these constructed cell lines was confirmed by detecting the EGS RNA transcript (Fig. 3). An additional cell line was also constructed which expressed an EGS, CAT101, that targeted the CAT mRNA (Yuan et al., 1992). No cleavage of tk46 substrate by RNase P was observed in vitro in the presence of CAT101 (data not shown; see Fig. 4, lane 3). This cell line was used to determine whether EGS RNA with an incorrect targeting sequence can



**FIGURE 3.** Detection of the expression of EGS TK104 from human cultured cells by RNase protection analyses. RNase protection analyses were carried out using RNA isolated from the nuclei of parental human 143tk<sup>-</sup> cells (lane 1) and three cloned cell lines that expressed TK104 (lanes 2–4) and from the cytoplasm of one cloned cell line (lane 5). 2  $\mu$ g of either nuclei or cytoplasmic RNA was used in each lane. 5' end-[<sup>32</sup>P]-labeled fragments obtained from digestion of pUC19 DNA with MspI were also separated on the same gel and the positions of the three fragments of 141, 110, and 67 nt were shown at the right as size markers. The RNA probe contained a sequence complementary to TK104. Products of the RNase protection assay were separated in 8% polyacrylamide gels that contained 8M urea and the gels were then subjected to autoradiography.

direct human RNase P to cleave TK mRNA in tissue culture (see below). The level of EGS RNA expression in each individual cell clone was determined with an RNase protection assay. The same amount (2  $\mu$ g) of RNA fractions was used and Fig. 3 shows the result from TK104-expressing cells with an RNA probe complementary to the 71-nt-long TK104 sequence (Fig. 1C). This EGS was expressed exclusively in the nuclei as it was only detected from the nuclear but



**FIGURE 4.** Cleavage of TK mRNA sequence of 46 nt by human RNase P in the presence of TK104 RNA synthesized in vitro (+, lane 1), and RNA fractions isolated from the parental 143tk<sup>-</sup> cells (P, lane 2) and cells that expressed EGS CAT101, TK109, TK112, and TK104, respectively (lanes 3–6). Cleavage products in lanes 5–6 comigrated with those in lane 1. 20 nM TK104 or 1  $\mu$ g of cellular RNA and [<sup>32</sup>P]-labeled TK RNA substrate (20 nM) were incubated with human RNase P (2 units) at 37 °C in a volume of 10  $\mu$ l for 45 min in buffer A (50 mM Tris, pH 7.4, 100 mM NH<sub>4</sub>Cl, and 10 mM MgCl<sub>2</sub>) (Yuan et al., 1992). Experimental details can be found in Materials and Methods.

not cytoplasmic RNA fractions (Fig. 3, lanes 4 and 5). Similar results were observed with cell clones which expressed TK109 and TK112 (data not shown). The different level of TK104 RNA expression in each individual clone (e.g. clones 41, 42, and 43) (Fig. 3, lanes 2–4) is presumably due to random incorporation of the EGS gene into the host chromosome, and its expression is influenced by the flanking sequence in the insertion site (Sambrook et al., 1989). The nature of the doubled bands of TK104 RNA and of TK109 and TK112 (data not shown) is unknown. Primer extension experiments with reverse transcriptase to map the 5' terminus of TK104 showed that the extension terminated prematurely before it reached the 5' end (data not shown). This observation suggested that some unknown modifications (e.g. modified nucleotides) at this region of the EGS blocks the reverse transcription. Alternatively, the nature of the doubled bands may possibly be attributed to the heterogeneity of the 3' termini of these transcripts or yet other unknown modification of EGS RNAs in the cultured cells.

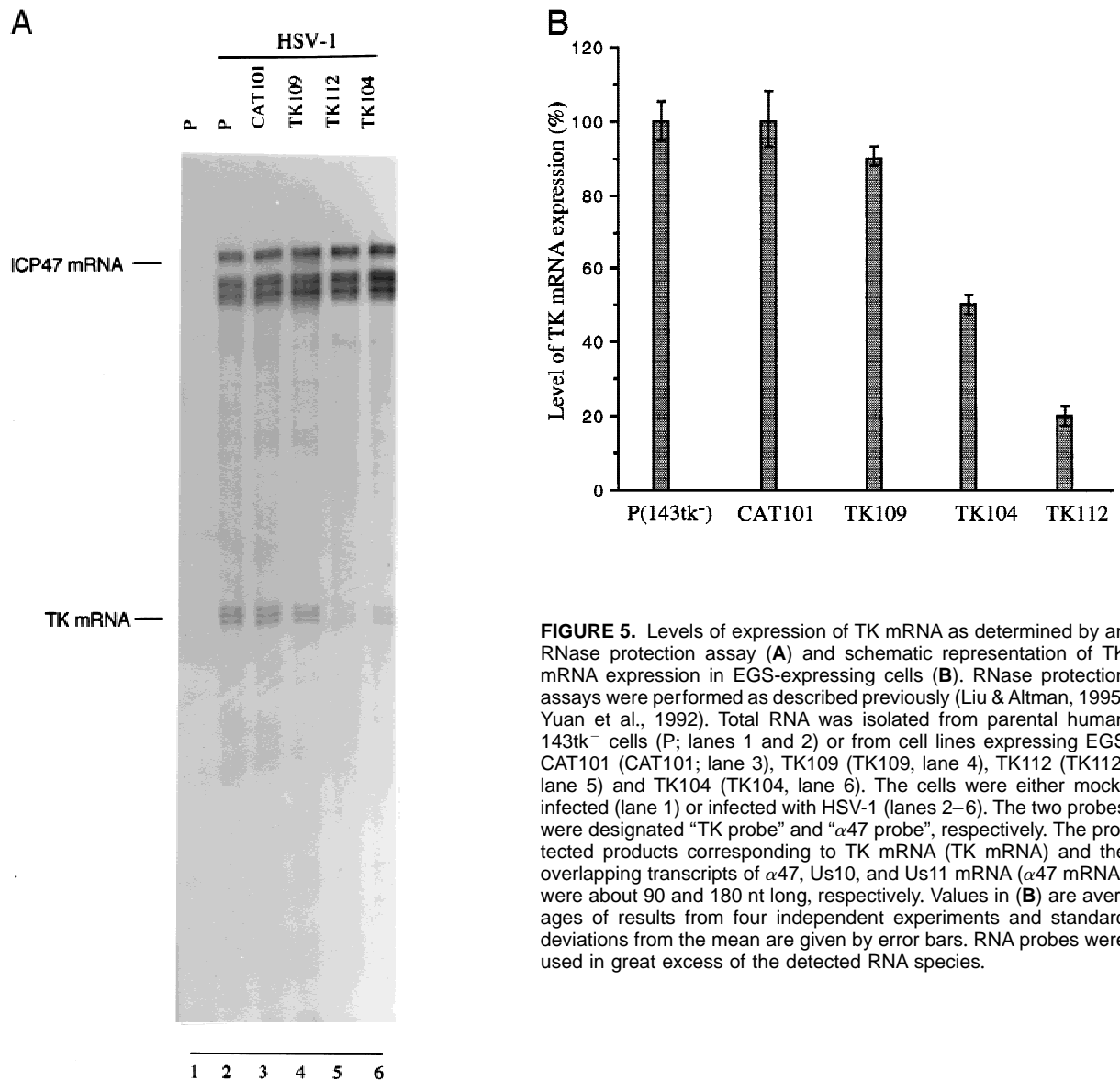
To determine whether the EGSs expressed in cultured cells were still active to direct RNase P to cleave TK mRNA, total RNAs were isolated from the EGS-expressing cells and incubated with substrate tk46 and human RNase P in vitro. Cleavage of tk46 was ob-

served in RNAs from cells that expressed TK112 and TK104 (Fig. 4, lanes 5 and 6) but not from those that did not express an EGS or expressed CAT101 or TK109 (Fig. 4, lanes 2–4). Cleavage products generated in the presence of these cellular RNA fractions comigrated with those generated in the presence of TK104 synthesized in vitro (Fig. 4, lane 1), suggesting that the cleavage occurred at the same position. Among cells that expressed TK104, TK109 and TK112, RNA isolated from TK112-expressing cells exhibited the highest targeting efficiency while that from TK109-expressing cells exhibited the lowest. These data were in agreement with those when EGSs synthesized in vitro were used (Fig. 2).

### Inhibition of TK expression in EGS-expressing cells

To investigate whether EGSs expressed in the cell lines can inhibit viral TK expression, the cells were infected with HSV-1 at MOI (multiplicity of infection) of 0.1–0.5, which resembles that in natural infection. Total RNA and protein fractions were isolated from cells that were harvested at 6 and 9 h postinfection, respectively. At these time points, TK mRNA and protein expression reached optimal levels (Roizman & Sears, 1996). The expression level of TK mRNA was determined by an RNase protection assay with an RNA probe (TK probe) which contained a 87-nt sequence complementary to the 5' TK mRNA sequence. An RNA probe ( $\alpha$ 47 probe) which contained a 181-nt sequence complementary to the overlapping region of  $\alpha$ 47, Us10, and Us11 mRNAs was used to determine the levels of these mRNAs. The expression levels of the latter RNAs were used as the internal control for quantitation of expression of TK mRNA. Figure 5A shows the result of an RNase protection experiment with both TK and  $\alpha$ 47 probes and Figure 5B summarizes the results obtained from four different experiments. Cells that expressed TK112 and TK104 exhibited a significant reduction in the level of TK mRNA expression ( $80 \pm 7\%$  and  $50 \pm 7\%$ , respectively) although cells that expressed TK109 only exhibited a reduction of  $8 \pm 5\%$ . No reduction in the expression level of TK mRNA was observed in cells that expressed CAT101 (Fig. 5A, lane 3; 5B). These results suggested that the significant reduction of TK mRNA expression in cells that expressed TK112 and TK104 was due to the targeting cleavage by RNase P. The low level of inhibition observed in TK109-expressing cells was presumably due to the antisense effects of the EGS. No products of the cleavage of the TK mRNA were detected in our RNase protection assays presumably because these RNAs, which lack either a cap structure or a poly(A) sequence, are rapidly degraded by intracellular RNases (Sambrook et al., 1989).

It is expected that the level of TK protein should decrease in EGS-expressing cells because of the de-



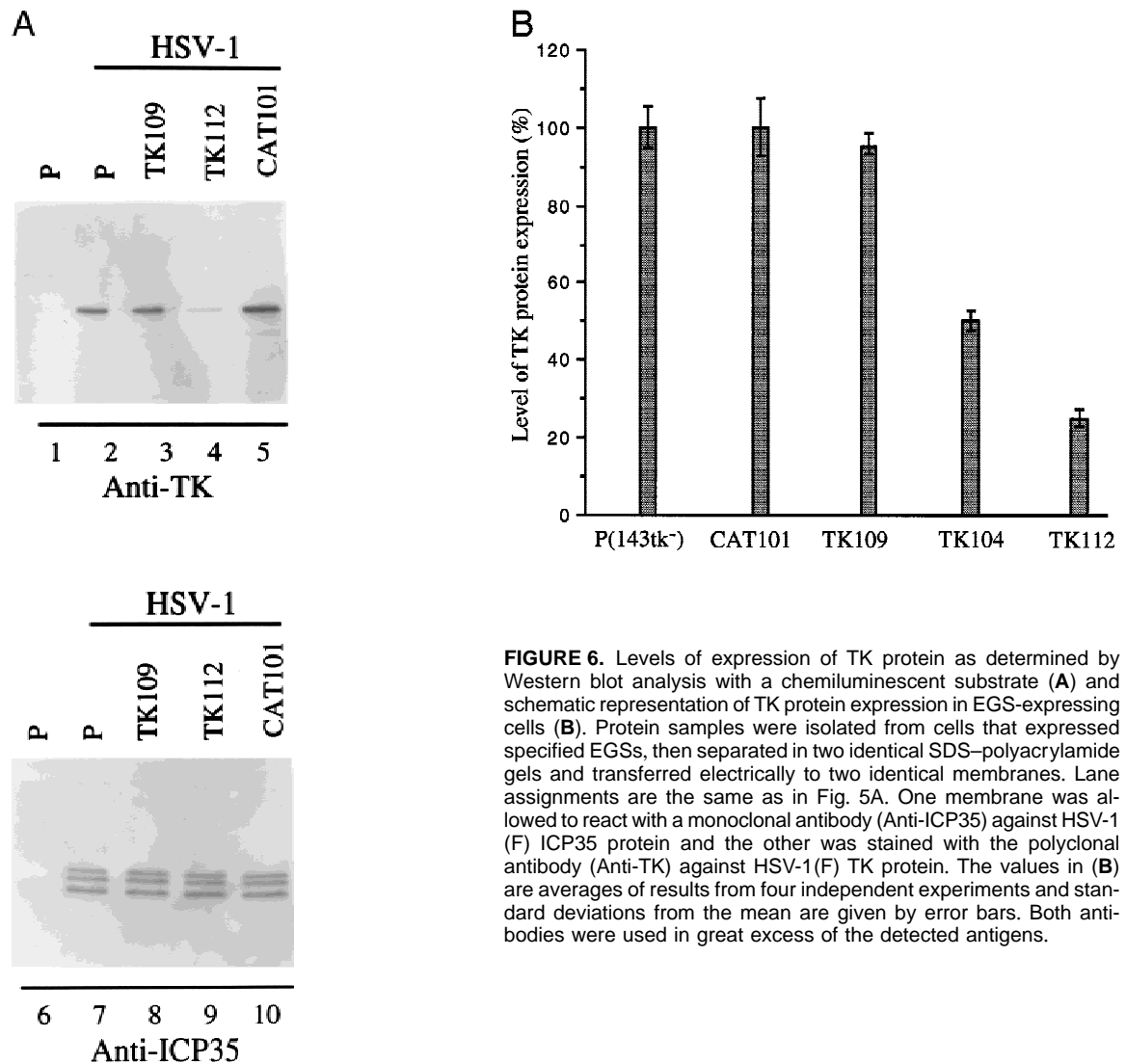
**FIGURE 5.** Levels of expression of TK mRNA as determined by an RNase protection assay (**A**) and schematic representation of TK mRNA expression in EGS-expressing cells (**B**). RNase protection assays were performed as described previously (Liu & Altman, 1995; Yuan et al., 1992). Total RNA was isolated from parental human 143tk<sup>-</sup> cells (P; lanes 1 and 2) or from cell lines expressing EGS CAT101 (CAT101; lane 3), TK109 (TK109, lane 4), TK112 (TK112, lane 5) and TK104 (TK104, lane 6). The cells were either mock-infected (lane 1) or infected with HSV-1 (lanes 2–6). The two probes were designated “TK probe” and “ $\alpha$ 47 probe”, respectively. The protected products corresponding to TK mRNA (TK mRNA) and the overlapping transcripts of  $\alpha$ 47, Us10, and Us11 mRNA ( $\alpha$ 47 mRNA) were about 90 and 180 nt long, respectively. Values in (**B**) are averages of results from four independent experiments and standard deviations from the mean are given by error bars. RNA probes were used in great excess of the detected RNA species.

creased level of TK mRNA. Protein extracts were isolated from cells either mock infected or infected with HSV-1. Viral proteins were separated electrophoretically in SDS–polyacrylamide gels and electrically transferred to two identical membranes. One of these membranes was stained with an anti-TK antibody (anti-TK) (Liu & Summers, 1988) and the other was stained with a monoclonal antibody against the HSV-1 capsid protein ICP35 (anti-ICP35) (Liu & Roizman, 1993) (Fig. 6A). The latter is used to detect the expression of ICP35, which serves as an internal control for the quantitation of TK protein expression. By using a chemiluminescent substrate for the antibody staining, the expression of TK and ICP35 proteins was quantitated (Fig. 6A). The results are summarized in Figure 6B. A reduction of  $75 \pm 7\%$  and  $50 \pm 5\%$  in the expression level of TK protein was observed in cells that ex-

pressed TK112 and TK104, respectively. In contrast, a reduction of only  $5 \pm 3\%$  was found in TK109-expressing cells. No reduction in the level of TK protein expression was observed in cells that expressed CAT101. The low level of reduction in the expression level of TK protein observed in cells that expressed TK109 was plausibly attributed to the antisense effect of the EGS.

## DISCUSSION

Ribozymes and conventional antisense molecules have been shown to be promising antiviral agents for inhibition of viral gene expression and replication (Stein & Cheng, 1993; Rossi, 1995; Poeschla & Wong-Staal, 1994). Clinical trials are currently being carried out to use antisense molecules and ribozymes for



**FIGURE 6.** Levels of expression of TK protein as determined by Western blot analysis with a chemiluminescent substrate (**A**) and schematic representation of TK protein expression in EGS-expressing cells (**B**). Protein samples were isolated from cells that expressed specified EGSs, then separated in two identical SDS-polyacrylamide gels and transferred electrically to two identical membranes. Lane assignments are the same as in Fig. 5A. One membrane was allowed to react with a monoclonal antibody (Anti-ICP35) against HSV-1 (F) ICP35 protein and the other was stained with the polyclonal antibody (Anti-TK) against HSV-1(F) TK protein. The values in (**B**) are averages of results from four independent experiments and standard deviations from the mean are given by error bars. Both antibodies were used in great excess of the detected antigens.

therapy for HIV and human cytomegalovirus (HCMV) infections. Ribozyme technology represents an attractive approach for gene inactivation because it exhibits most of the properties of the conventional antisense targeting method and, in addition, catalytic and irreversible cleavage of the target RNA. Much is known about how to construct ribozymes that exhibit highly functional activity *in vitro*. However, the intracellular efficacy of these gene-targeting agents is primarily dictated by their stability, catalytic activity, localization, and delivery in the cellular environment and, therefore, does not always correlate with their *in vitro* catalytic efficiency. EGS-directed cleavage by RNase P also leads to catalytic and irreversible cleavage of the target mRNA (Yuan et al., 1992; Yuan & Altman, 1994). The EGS approach is unique in using endogenous RNase P as the targeting enzyme and therefore obviates the concerns for its stability, activity, localization, and delivery in cells.

Several criteria must be satisfied if successful targeting with the EGS technology is to be achieved. Among these are high efficiency of cleavage, sequence specificity of the EGS, and efficient delivery of the reagents. We have constructed EGSs that target HSV-1 TK mRNA and have shown that these EGSs directed human RNase P to cleave TK mRNA sequence efficiently *in vitro*. Moreover, we have shown that these EGSs were expressed stably in human cell culture and a reduction of about 80% in the TK expression level was achieved with a designed EGS. A reduction of less than 10% in the TK expression level was observed in cells that expressed TK109. EGS TK109 bound efficiently to TK mRNA but contained a single point mutation that disrupted RNase P recognition. No reduction was observed in cells that expressed EGS CAT101 that targeted another mRNA. These results suggest that the overall observed inhibition with TK112 and TK104 was primar-



ily due to targeted cleavage by RNase P as opposed to the antisense effect or other nonspecific effects of the EGSs. The extent of inhibition of TK gene expression observed in cells containing TK112 is similar to that seen when the M1GS ribozyme was introduced into mammalian cells via a retroviral vector and used to cleave TK mRNA (Liu & Altman, 1995). These studies demonstrate that the efficiency of target cleavage directed by TK112 and human RNase P is comparable to that achieved with the M1GS ribozyme. Another recent study has also shown that the EGS-directed targeting approach is extremely effective in inhibiting replication and gene expression of influenza virus (Plehn-Dujowich & Altman, 1998).

Our results also show that the designed EGS is highly sequence-specific. Efficient cleavage of TK mRNA by human RNase P was observed *in vitro* with the EGSs (i.e. TK104 and TK112) that target TK mRNA but not with CAT101. Moreover, a significant reduction of the TK expression was observed in cells that expressed TK112 and TK104 but not CAT101. Two types of interaction between the EGS and target mRNA govern the specificity of the EGS targeting approach (Yuan et al., 1992; Yuan & Altman, 1994). One is the Watson–Crick base pairing interaction between the antisense domain of the EGS and the target mRNA. The other type of interaction is between the RNase P recognition domain (e.g. T-loop and stem) and the mRNA. This interaction facilitates the folding of the EGS–mRNA complex into a tRNA-like molecule and stabilizes the mRNA–EGS complex. Manipulation of this latter interaction would make the EGS technology potentially more sequence specific than the conventional antisense approach.

EGSs introduced into human cells were stably expressed and localized primarily in the nuclei along with RNase P. The expression cassette we used to produce these EGSs is the promoter for small nuclear U6 RNA (Das et al., 1988; Yuan et al., 1992). This promoter has been used extensively to express functional RNAs and ribozymes for gene targeting applications and the transcript from this promoter is quite stable and primarily localized in the nuclei (Yuan et al., 1992; Liu & Altman, 1995; Bertrand et al., 1997; Good et al., 1997). In addition, the efficient delivery and proper localization of the EGS may be mediated by cellular tRNA-binding proteins. These proteins could interact with the tRNA-like domains of the EGS and target the EGS to the nuclear compartment that contains RNase P.

A recent study has shown that the EGS-directed targeting approach can effectively abolish replication and production of influenza virus in tissue culture (Plehn-Dujowich & Altman, 1998). Although the activity of TK has been shown to be important for HSV replication and reactivation *in vivo* in sensory neurons, this enzyme is dispensable for viral replication in tissue culture (Roizman & Sears, 1996). Therefore, the level of HSV replication in the EGS-expressing cells in our studies is not affected.

To assess the efficacy of the EGS technology for inhibition of HSV replication in tissue culture, EGSs can be constructed to target viral mRNAs that encode proteins essential for viral replication, such as ICP4, the viral major transcriptional activator (Kawa, D & Liu, F., unpubl.). These studies will further facilitate the development of EGSs as novel gene-targeting agents for treatment and studies of human herpesviruses.

## MATERIALS AND METHODS

### Viruses, cells and antibodies

HSV-1 (F), a prototype of human herpes simplex virus 1, was a gift from Dr. Bernard Roizman of the University of Chicago (Ejercito et al., 1968). The human 143tk<sup>-</sup> cells and Vero (African green monkey kidney) cells (gifts from Dr. Bernard Roizman and Dr. Bill Summers of Yale Medical School) were maintained and propagated in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum. The anti-rabbit polyclonal antibody against HSV-1 (F) thymidine kinase was a gift from Dr. Bill Summers (Liu & Summers, 1988) and the anti-mouse monoclonal antibody MCA406 against HSV-1 ICP35 protein (Liu & Roizman, 1993) was purchased from Bioproduct for Sciences Inc. (Indianapolis, Indiana). The anti-rabbit or anti-mouse IgG conjugated with horseradish peroxidase was purchased from Vector Laboratories, Inc. and Bio-Rad, Inc., respectively.

### Construction of plasmids, EGS RNAs and RNA substrates for studies *in vitro*

pTK101 was constructed in which the HSV-1 (F) TK encoding sequence (*Bam*H I–*Sma* I fragment) was inserted into vector pBluescript (Stratagene, Inc.) and driven by the promoter of bacteriophage T7 RNA polymerase. DNA template coding for substrate tk46 was constructed by annealing oligonucleotide OliT7 (5'-TAATACGACTCACTATAG-3') with oligonucleotide OliTK46 (5'-ACCGCGCAGCCTGGTTCGAACGCAGACGCGTGTTGATGGCAGGGGTCTATAGTGAGTCGTATTA-3'). The DNA sequences coding for the EGSs were synthesized by PCR, using DNA that encoded yeast tRNA<sup>Asp</sup> as a template (Drainas et al., 1989), and were cloned under the control of the T7 RNA polymerase promoter. All oligonucleotides used as PCR primers were synthesized in a DNA synthesizer. To construct pTK104, the 5' and 3' primers were oligo1 (5'-GT TAACGTCGGACAGACTC-3') and oligo2 (5'-AAGCTTTAA ACGTCTGCGGCAGGATTTG-3'), respectively. pTK109 and pTK112 were constructed by PCR with pTK104 as the template. To construct pTK109, the 5' and 3' primers were oligoT7 (5'-TAATACGACTCACTATAG-3') and oligo3 (5'-CTTT AAACGTCTGCGGCAGGATTTCAACCTGCGCG-3'), respectively. To construct pTK112, the 5' and 3' primers were oligo4 (5'-GTTAACGTCGGTGGTCTCC-3') and oligo2, respectively. pCAT that encoded EGS CAT101 was constructed as described previously (Yuan et al., 1992). Plasmids pTK115, pTK126, and pTK127 were constructed by inserting the sequence coding for TK104, TK109, and TK112, respectively, into plasmid pmU6 (–315/1). pmU6 (–315/1) contained the promoter for small nuclear U6 RNA and a signal for termination of transcription (T cluster) by RNA polymerase III (Das

et al., 1988; Yuan et al., 1992). pTK116 was derived from pGEM7Z (Promega, Inc.) in which a neomycin resistance gene driven by the SV40 promoter was inserted. Plasmids pTK129 and pTK141 were constructed by placing the 87-nt-long *Bgl*I–*Mlu*I fragment from pTK101 and the 181-nt-long *Bam*H I–*Acc*I fragment from pRB4059, respectively, under the control of bacteriophage T3 RNA polymerase promoter. These two fragments correspond to the sequences encoding the 5' sequence of TK mRNA and the overlapping transcripts for HSV-1  $\alpha$ 47, Us10, and Us11 genes, respectively (McGeoch, 1989).

### RNase P assay and in vitro studies

Human RNase P was prepared from HeLa cellular extracts as described previously (Bartkiewicz et al., 1989; Yuan et al., 1992). EGS RNAs and the TK mRNA substrates (i.e. tk46 and tk450) were synthesized in vitro by either T7 or T3 RNA polymerase (Promega, Inc., Madison, Wisconsin) following manufacturer's recommendations and further purified on 8% urea/polyacrylamide gels. Subsequently, the EGS RNAs (20 nM from those synthesized in vitro or 1  $\mu$ g from those isolated from cells) and [<sup>32</sup>P]-labeled TK RNA substrate (20 nM) were incubated with human RNase P (2 units). The cleavage reactions were carried out at 37 °C in a volume of 10  $\mu$ l for 45 min in buffer A (50 mM Tris, pH 7.4, 100 mM NH<sub>4</sub>Cl, and 10 mM MgCl<sub>2</sub>) (Yuan et al., 1992). Cleavage products were separated in denaturing gel and subjected to either autoradiography with a Kodak film or quantitated with a STORM phosphorimager (Molecular Dynamics, Sunnyvale, California).

Assaying for the binding between EGSs and TK mRNA sequence in vitro was performed as described previously (Yuan & Altman, 1994). Briefly, 0.5 nM of TK substrate and 0.5 nM of EGSs were preincubated separately in buffer A at 37 °C for 15 min, mixed and incubated at the same temperature for another 15 min, and subjected to separation in 5% polyacrylamide gels under non-denaturing conditions (Pyle et al., 1990). The gels were subjected to autoradiography with a Kodak film and the amount of the bound complex was quantitated with a STORM phosphorimager.

### Construction of cell lines

Human 143tk<sup>-</sup> cells were cotransfected with pTK116 and the EGS plasmid as specified in Results, with the aid of a mammalian transfection kit purchased from Stratagene Inc. (La Jolla, California). At 48 h posttransfection, neomycin (Gibco-BRL) was added to the culture medium in a final concentration of 400  $\mu$ g/ml. Cells were subsequently selected under neomycin for two weeks. Those cells that exhibited resistance to neomycin were cloned and expanded in media that contain neomycin (Sambrook et al., 1989). Finally, aliquots of these cells were either frozen for long-term storage in liquid nitrogen or used for further studies in tissue culture.

### Viral infection and isolation of RNA and protein extracts

A T-25 flask of cells (approximately 10<sup>6</sup> cells) were either mock-infected or infected with HSV-1 in an inoculum of 1.5 ml Medium 199 (M199) (GIBCO) supplemented with 1% fetal

calf serum. The MOI was as stated in Results. After 2-h exposure of cells to the virus at 37 °C, the inoculum was replaced with DMEM supplemented with 5% fetal bovine serum. The infected cells were incubated for a certain period time (as stated in Results) before isolating viral mRNA or protein. Total cellular RNA was isolated from cells either mock-infected or infected with HSV-1 as described previously (Jenkins & Howett, 1984; Liu & Altman, 1995). To prepare protein extracts, cells were harvested, washed twice with phosphate-buffered saline (PBS), and lysed in the disruption buffer (0.05M Tris, pH 7.0, 8.5% [v/v] sucrose, 5% [v/v]  $\beta$ -mercaptoethanol, 2% [v/v] sodium dodecyl sulphate). The protein samples were boiled for 1 min before electrophoretic separation in SDS-polyacrylamide denaturing gels.

### RNase protection assay for EGS and viral mRNA expression

Experiments to isolate both nuclear and cytoplasmic RNA fractions from EGS-expression cells were carried out as described previously (Jenkins & Howett, 1984; Liu & Roizman, 1993). The RNA probes used to detect EGS TK104, TK109, TK112, and CAT101 were synthesized in vitro by T3 RNA polymerase from *Eco*R I-linearized plasmids pTK115, pTK125, pTK126, and pCAT101, respectively. The RNA probes used to detect TK mRNA and the transcripts of  $\alpha$ 47, Us10, and Us11 genes were synthesized in vitro by T3 RNA polymerase from *Eag* I-linearized pTK129 and pTK141, respectively. RNase protection was performed as described previously (Liu & Altman, 1995; Yuan et al., 1992). The protected RNA products were separated in 8% urea/polyacrylamide denaturing gels, and quantitated with a Betascope (Betagen) scanner and a STORM phosphorimager.

### Electrophoretic separation and staining of infected cellular polypeptides with antibodies

The denatured, solubilized polypeptides from cell lysates were separated on 9% (v/v) SDS-polyacrylamide gels crosslinked with *N,N'*-methylenebisacrylamide. The separated polypeptides were transferred electrically to nitrocellulose membranes and reacted in an enzyme-linked immunoassay with either anti-mouse or anti-rabbit IgG conjugated with horseradish peroxidase in addition to the antibodies against HSV-1 TK or ICP35. The membranes were subsequently either stained with a color substrate from the peroxidase substrate kit purchased from Vector Laboratories Inc. or reacted with a chemiluminescent substrate with the aid of the LumiGLO chemiluminescent substrate kit (Kirkegaard & Perry Laboratories Inc.). The membranes stained with the chemiluminescent substrate were subsequently exposed to Kodak films. Finally, the amount of TK and ICP35 protein in the membrane was quantitated by scanning the Kodak films in a densitometer (Bio-Rad, Inc.).

### ACKNOWLEDGMENTS

We are grateful to Dr. Sidney Altman for his guidance and support during the initial stage of this work, and his advice

and reviewing of the manuscript. The authors are also indebted to Dr. Bernard Roizman of the University of Chicago, Dr. William Summers and Dr. Daniel DiMaio of Yale Medical School for stocks of HSV-1 (F) and cells, retroviral vectors and packaging cell lines, and polyclonal antibody against HSV-1(F) TK protein. D.K. is partially supported by a University of California Graduate Opportunity Fellowship. F.L. is a recipient of the Basil O'Connor Starter Scholar Award of the March of Dimes Birth Defects Foundation. This research was, in part, supported by a University of California Junior Faculty Research Award (F.L.) and a grant from the University of California Cancer Research Coordinating Committee (F.L.).

Received June 5, 1998; returned for revision July 16, 1998; revised manuscript received July 28, 1998

## REFERENCES

- Altman S, Kirsebom L, Talbot SJ. (1993). Recent studies of RNase P. *FASEB J* 7:7–15.
- Ares M, Igel AH. (1990). Lethal and temperature-sensitive mutations and their suppressors identify an essential structural element in U2 small nuclear RNA. *Genes & Dev* 4:2132–2145.
- Bartkiewicz M, Gold H, Altman S. (1989). Identification and characterization of an RNA molecule that copurifies with RNase P activity from HeLa cells. *Genes & Dev* 3:488–499.
- Bertrand E, Castanotto D, Zhou C, Carbonnelle C, Lee NS, Good P, Chatterjee S, Grange T, Pictet R, Kohn D, Engelke D, Rossi JJ. (1997). The expression cassette determines the functional activity of ribozymes in mammalian cells by controlling their intracellular localization. *RNA* 3:75–88.
- Climie SC, Friesen J. (1988). In vivo and in vitro structural analysis of the *rplJ* mRNA leader of *Escherichia coli*. *J Biol Chem* 263:15166–15175.
- Das G, Henning D, Wright D, Reddy R. (1988). Upstream regulatory elements are necessary and sufficient for transcription of a U6 RNA gene by RNA polymerase III. *EMBO J* 7:503–512.
- Drainas D, Zimmerly S, Willis I, Soll D. (1989). Substrate structural requirements of *Schizosaccharomyces pombe* RNase P. *FEBS Lett* 251:84–88.
- Ejercito PM, Kieff ED, Roizman B. (1968). Characterization of herpes simplex virus strains differing in their effect on social behavior of infected cells. *J Gen Virol* 2: 357–364.
- Forster AC, Altman S. (1990). External guide sequences for an RNA enzyme. *Science* 249: 783–786.
- Good PD, Krikos AJ, Li SX, Bertrand E, Lee NS, Giver L, Ellington A, Zaia JA, Rossi JJ, Engelke, DR. (1997). Expression of small, therapeutic RNAs in human cell nuclei. *Gene Ther* 4:45–54.
- Gopalan V, Talbot SJ, Altman S. (1995). RNA–protein interactions in RNase P. In: Nagai K, Mattaj IW, eds. *RNA–protein interactions*. Oxford: Oxford University Press. pp 103–126.
- Guerrier-Takada C, Gardiner K, Marsh T, Pace N, Altman S. (1983). The RNA moiety of ribonuclease P is the catalytic subunit of the enzyme. *Cell* 35:849–857.
- Jenkins FJH, Howett MK. (1984). Characterization of the mRNAs mapping in the *BgIII* N fragment of the herpes simplex virus type 2 genome. *J Virol* 52:99–107.
- Kahle D, Wehmeyer U, Krupp G. (1990). Substrate recognition by RNase P and by the catalytic M1 RNA: Identification of possible contact points in pre-tRNAs. *EMBO J* 9:1929–1937.
- Li Y, Guerrier-Takada C, Altman S. (1992). Targeted cleavage of mRNA in vitro by RNase P from *Escherichia coli*. *Proc Natl Acad Sci USA* 89:3185–3189.
- Liu F, Altman S. (1994). The differential evolution of substrates for an RNA enzyme in the presence and absence of its protein cofactor. *Cell* 77:1083–1100.
- Liu F, Altman S. (1995). Inhibition of viral gene expression by the catalytic RNA subunit of RNase P from *Escherichia coli*. *Genes & Dev* 9:471–480.
- Liu F, Roizman B. (1993). Characterization of the protease and other products of amino-terminus-proximal cleavage of the herpes simplex virus 1 UL26 protein. *J Virol* 67:1300–1309.
- Liu Q-Y, Summers WC. (1988). Site-directed mutagenesis of a nucleotide-binding domain in HSV-1 thymidine kinase: Effects on catalytic activity. *Virology* 163:638–642.
- Maran A, Maitra RK, Kumar A, Dong B, Xiao W, Li G, Williams BR, Torrence PF, Silverman RH. (1994). Blockage of NF-kappa B signaling by selective ablation of an mRNA target by 2–5A antisense chimeras. *Science* 265:789–792.
- McGeoch DJ. (1989). The genomes of the human herpesviruses: Contents, relationships, and evolution. *Ann Rev Microbio* 43:235–265.
- Nolan JM, Burke DH, Pace NR. (1993). Circularly permuted tRNAs as specific photoaffinity probes of ribonuclease P RNA structure. *Science* 261:762–765.
- Pace NR, Brown JW. (1995). Evolutionary perspective on the structure and function of ribonuclease P, a ribozyme. *J Bact* 177:1919–1928.
- Plehn-Dujowich D, Altman S. (1998). Effective inhibition of influenza virus production in cultured cells by external guide sequences and ribonuclease P. *Proc Natl Acad Sci USA* 95:7327–7332.
- Poeschla E, Wong-Staal F. (1994). Antiviral and anticancer ribozymes. *Curr Op Onc* 6:601–606.
- Pyle AM, McSwiggen JA, Cech TR. (1990). Direct measurement of oligonucleotide substrate binding to wild-type and mutant ribozymes from *Tetrahymena*. *Proc Natl Acad Sci* 87:8187–8191.
- Roizman B, Sears AE. (1996). Herpes simplex viruses and their replication. In: Field BN, Knipe DM, Howley PM, Chanock RM, Melnick JL, Monath TP, Roizman B, Strauss SE, eds. *Field's Virology*, 3rd ed., New York: Raven Press. pp 2231–2296.
- Rossi JJ. (1995). Controlled, targeted, intracellular expression of ribozymes: Progress and problems. *Trends Biotechnol* 13:301–306.
- Sambrook J, Fritsch EF, Maniatis T. (1989). *Molecular cloning: A laboratory manual*. Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press.
- Santoro SW, Joyce GF. (1997). A general purpose RNA-cleaving DNA enzyme. *Proc Natl Acad Sci USA* 94:4262–4266.
- Sprinzi M, Dank N, Nock S, Schon A. (1991). Compilation of tRNA sequences and sequences of tRNA genes. *Nucleic Acids Res* 19 (Suppl):2127–2171.
- Stein CA, Cheng YC. (1993). Antisense oligonucleotides as therapeutic agents—is the bullet really magical? *Science* 261: 1004–1012.
- Yuan Y, Altman S. (1994). Selection of guide sequences that direct efficient cleavage of mRNA by human ribonuclease P. *Science* 263:1269–1273.
- Yuan Y, Altman S. (1995). Substrate recognition by human RNase P: Identification of small, model substrates for the enzyme. *EMBO J* 14:159–168.
- Yuan Y, Hwang E, Altman S. (1992). Targeted cleavage of mRNA by human RNase P. *Proc Natl Acad Sci USA* 89:8006–8010.
- Zaug AJ, Cech TR. (1995). Analysis of the structure of *Tetrahymena* nuclear RNAs in vivo: Telomerase RNA, the self-splicing rRNA intron, and U2 snRNA. *RNA* 1:363–374.