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CONSTRUCTION AND CHARACTERIZATION OF *lck*- AND *fyn*-SPECIFIC tRNA:RIBOZYME CHIMERAS

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Abstract—Two *src*-family protein tyrosine kinases (PTKs), $p56^{lck}$ and $p59^{fyn}$, are thought to play an important role in the antigen-specific T cell receptor (TCR)/CD3-initiated signaling pathway, but their relative contribution to these events is not clearly defined. Here, we have explored the potential of catalytic RNA molecules, or ribozymes, as tools for selectively inhibiting expression of the corresponding target genes in T cells. Several *lck*- or *fyn*-specific hammerhead ribozymes were synthesized, cloned into a bacterial transcription vector, and found to display specific catalytic activity *in vitro*. In order to achieve stable high-level ribozyme expression in intact cells, selected ribozymes were subsequently cloned into a retroviral vector (DC-T5T) immediately downstream of a $tRNA^{met}$ transcription unit. Upon retroviral transduction of a human leukemic T cell line (Jurkat), two out of four chimeric tRNA:ribozymes, fyn-1 and lck-1, were stably expressed at levels of ~10,000 or ~25,000 copies/cell, respectively. Ribozyme expression was associated with a reduction of up to 80% (*lck*) or 61% (*fyn*) in endogenous target mRNA by comparison to the corresponding transcript levels in control clones transfected with vector alone. By contrast, expression of the corresponding target proteins was not reduced, suggesting a post-transcriptional compensatory mechanism that increases translation or stability of the $p56^{lck}$ and/or $p59^{fyn}$ proteins.

Key words: protein tyrosine kinases, $p56^{lck}$, $p59^{fyn}$, T cells, ribozymes, tRNA transcription unit.

INTRODUCTION

Tyrosine phosphorylation is an early obligatory event in the pathway leading to T cell activation (reviewed by Mustelin and Altman, 1991; Klausner and Samelson, 1991; Samelson and Klausner, 1992; Burns and Ashwell, 1993; Mustelin and Burn, 1993), as indicated by kinetic studies of T cell activation (June *et al.*, 1990*a*) and by the use of protein tyrosine kinase (PTK) inhibitors (Mustelin *et al.*, 1990; June *et al.*, 1990*b*). Two members of the *src* family of PTKs, namely $p56^{lck}$ (Rudd *et al.*, 1988; Veillette *et al.*, 1988) and $p59^{fyn}$ (Samelson *et al.*, 1990; Gassmann *et al.*, 1992), are known to be noncovalently associated with the intracellular domains of the CD4/CD8 coreceptors or the TCR/CD3 complex, respectively. Studies using T cell lines overexpressing (Abraham *et al.*, 1991; Davidson *et al.*, 1992; Luo and Sefton, 1992) or lacking (Straus and Weiss, 1992) these kinases, *lck*- or *fyn*-transgenic mice (Abraham *et al.*, 1991; Cooke *et al.*, 1991), and *lck*- or *fyn*-deficient mutant mice (Appleby *et al.*, 1992; Stein *et al.*, 1992; Molina *et al.*, 1992), have suggested that these two PTKs play an essential role in T cell activation. However, the relative contribution of each kinase to TCR/CD3-initiated signaling has not been clearly defined. In order to address this question, we employed catalytic RNA molecules or ribozymes as specific endoribonucleases in an attempt to selectively cleave and inactivate *lck* or *fyn* mRNA transcripts and, consequently, reduce the expression of the corresponding PTKs.

Naturally occurring ribozymes have been described in a number of systems (reviewed by Edgington, 1992). Among these, hammerhead ribozymes, originally identified in plant pathogens (reviewed by Symons, 1992), represent the best characterized class of catalytic RNA molecules, and simple rules have been established for the design of short synthetic ribozymes that can cleave RNA molecules in *trans* in a highly sequence-specific manner (Haseloff and Gerlach, 1988; Herschlag, 1991). In addition to the conserved catalytic domain, these synthetic ribozymes contain flanking antisense sequences that direct them to complementary sense sequences in target mRNA transcripts. Various hammerhead ribozymes were engineered and found to inhibit target gene expression in cultured cells (Cotten and Birnstiel, 1989; Cameron and Jennings, 1989; Sarver *et al.*, 1990; Saxena and Ackerman, 1990; Scanlon *et al.*, 1991; Dropulic *et al.*, 1992; Steinecke *et al.*, 1992; Sioud *et al.*, 1992; Chen *et al.*, 1992; Koizumi *et al.*, 1992; Heidenreich and Eckstein, 1992). The continuous catalytic degradation of the target mRNA by the regenerated ribozyme

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represents a theoretical improvement over the more conventional antisense RNA approach for blocking gene expression (Uhlenbeck, 1987).

Intracellular expression of ribozymes has usually been directed by different RNA polymerase II-driven promoters, such as SV40, viral LTR or β-actin. Cotten and Birnstiel, on the other hand, employed hybrid tRNA:ribozyme molecules in which the ribozyme was placed between the A and B boxes of the internal RNA polymerase III promoter of a *Xenopus*-derived $tRNA^{met}$ gene, leading to a high-rate transcription of functional tRNA:ribozyme molecules in injected oocytes (Cotten and Birnstiel, 1989). These studies relied mostly on transient expression systems, and demonstrated a requirement for high ribozyme:substrate ratios in order to achieve effective inhibition of target gene expression in cultured cells. Here we report the use of a highly efficient RNA polymerase III-based tRNA transcription unit, encoded in the double-copy retroviral vector construct, DC-T5T (Sullenger *et al.*, 1990*a*), to achieve a high-level steady-state expression of ribozymes and efficient long-term reduction of target gene expression at the mRNA level in stably transduced T cell clones.

MATERIALS AND METHODS

Design and cloning of ribozymes into transcription vectors

Ribozymes were synthesized on a Gene Assembler Plus™ DNA Synthesizer (LKB, Uppsala, Sweden) as single-stranded oligonucleotides encompassing the conserved catalytic motif of a hammerhead ribozyme (Haseloff and Gerlach, 1988), flanking antisense sequences complementary to selected sequences in the human *lck* or *fyn* transcripts, and convenient restriction sites for directional cloning (Table 1). The flanking *lck* or *fyn* sequences were selected based on the presence of a GUX triplet (where X is C, U or A), which serves as a consensus cleavage site for hammerhead ribozymes (Haseloff and Gerlach, 1988), within the targeted *lck* or *fyn* sequences. Oligonucleotides were kinased and cloned into the *Eco*RI and *Pst*I sites of the transcription vector, pGEM-3Z (Promega, Madison, WI), or into the *Sac*II-*Bam*HI sites or the DC-T5T vector (Sullenger *et al.*, 1990*a*). Second strand was filled-in with the Klenow fragment of DNA polymerase I before bacterial transformation. DNA sequences were confirmed by the chain termination method, using a Sequenase 2.0 sequencing kit (USB, Cleveland, OH) according to the manufacturer's protocol.

Cloning of lck *and* fyn *substrate RNAs*

lck or *fyn* cDNA fragments corresponding to nucleotide residues 117–373 or 607–824 of human cDNA clones YT16 (Koga *et al.*, 1986) or c-syn (Semba *et al.*, 1986), respectively, were amplified from oligo(dT)-primed human peripheral blood-derived lymphocyte cDNA in PCRs using gene-specific 5′ and 3′ primers, respectively (Table 1). These cDNA fragments were cloned into the *Sma*I site of pGEM-3Z downstream of the T7 bacteriophage RNA promoter.

Table 1. Oligonucleotides and primers used in this study[a]

pGEM-3Z ribosymes
(1) lck-1: 5′-AATTCTCCAGTGG**CTGATGAGTCCGTGAGGACGAAAC**TATGGGCTGCA-3′
(2) lck-2: 5′-AATTCTCGTAGGT**CTGATGAGTCCGTGAGGACGAAAC**CAGTGCTGCA-3′
(3) lck-3: 5′AATTCGAGCGAT**CTGATGAGTCCGTGAGGACGAAAC**CAGGTTCTGCA-3′
(4) lck-4 (lck-1 and lck-2): 5′AATTCTCGTAGGT**CTGATGAGTCCGTGAGGACGAAAC**CAGT GAGATCTTCCAGTGG**CTGATGAGTCCGTGAGGACGAAAC**TATGGGCTGCA-3′
(5) fyn-1: 5′-AATTCATAGCGG**CTGATGAGTCCGTGAGGACGAAAC**CCAGACTGCA-3′
(6) fyn-2: 5′-AATTCCCTCCAAA**CTGATGAGTCCGTGAGGACGAAAC**GGTGAGCTGCA-3′
(7) fyn-3 (fyn rz4 and rz2): 5′-AATTCCGTATGA**CTGATGAGTCCGTGAGGACGAAAC**GAA GAGAGATCTCCTCCAAA**CTGATGAGTCCGTGAGGACGAAAC**GGTGAGCTGCA-3′
DC-T5T ribozymes
(8) lck-1: 5′-GATCCCATA**GTTTCGTCCTCACGGACTCATCAG**CCACTGGAGC-3′
(9) lck-3: 5′-GATCAACCTG**GTTTCGTCCTCACGGACTCATCAG**ATCGCTCCGC-3′
(10) fyn-1: 5′-GATCCTCTGG**GTTTCGTCCTCACGGACTCATCAG**CCGCTATCCGC-3′
(11) fyn-2: 5′-GATCCTCACC**GTTTCGTCCTCACGGACTCATCAG**TTTGGAGGC-3′
Recombinant PCR primers
(12) 5′-tRNA-T7-hybrid primer: 5′-GCGGTACCTAATACGACTCACTAT AGGGAAGCAGAGTGGCGCAGCGG-3′
(13) 3′-termination site primer: 5′-CGCCGGCGACGCGTCCAAAAACG-3′
RT-PCR primers
(14) lck-5′-primer: 5′-GAA GAT GAC TGG ATG GAA AA-3′
(15) lck-3′-primer: 5′-CCG CTC TGC TCC AGG AT-3′
(16) fyn-5′-primer: 5′-GAA GCA ACA AAA CTG ACG G-3′
(17) fyn-3′-primer: 5′-TTC CTC CTC TCG TAC GCA-3′
(18) 5′-tRNA:rz primer: 5′-AGC AGA GTG GCG CAG C-3′
(19) 3′-tRNA:rz primer: 5′-CCT CAC GGA CTC ATC AG-3′

[a]Restriction enzyme sites used for cloning are underlined, and the hammerhead core sequence is outlined in bold.

In vitro *transcription and ribozyme cleavage reactions*

Substrate or ribozyme RNAs were transcribed from *Hind*III-linearized DNA templates in the presence of [α-^{32}P]UTP (3000 Ci/mmol) or [α-^{35}S]UTP (1000 Ci/mmol), respectively, using T7 RNA polymerase (Promega) according to the manufacturer-supplied protocol. After incubation for 1 hr at 37°C, DNA templates were removed by a 15 min RQ1-DNase digestion, RNA was extracted with an equal volume of water-saturated phenol:chloroform (1:1), precipitated with ethanol at −70°C and dissolved in diethyl pyrocarbonate-treated water. Aliquots of TCA-precipitated transcription reactions were counted, and the radiolabeled RNA was quantitated based on the specific activity of the incorporated isotopes (Goodchild and Kohli, 1991). Following denaturation at 95°C, ribozyme RNAs (2 pmol) were resuspended in ice-cold Tris–HCl (50 mM, pH 8.0) and combined with 0.2 or 2 pmol substrate RNA to produce ribozyme:substrate ratios of 10:1 or 1:1, respectively. An undigested aliquot was retained on ice as a control, and the remainder was brought to 20 mM $MgCl_2$ at 37°C or 50°C in a final volume of 100 μl. Reactions were terminated at different times by transferring 10 μl aliquots to dry ice. Samples were analyzed by electrophoresis on a 6% polyacrylamide gel containing 7 M urea and autoradiography.

Retroviral transduction of T cell lines

Vector DNA was converted to corresponding recombinant virus using established procedures (Aldovini and Walker, 1990). Briefly, the amphotropic packaging cell line PA317 was transfected with 20 μg circular ribozyme-encoding or control vector DNA by calcium phosphate precipitation techniques. Two days later, Jurkat cells, a human leukemic T cell line, were infected with recombinant retroviruses by cocultivation. The cultures were supplemented with G418 (1000 μg/ml) and drug-resistant cells were expanded, cloned by limiting dilution in the presence of G418, cryopreserved, and used for further analysis.

RT-PCR

A semi-quantitative RT-PCR was used to determine expression levels of ribozyme RNA or target mRNAs in stable, G418-resistant Jurkat transfectants. Total RNA was isolated from 3×10^6 cells by lysis in 100 μl RNazol B solution (Cinna/Biotecx, Friendswood, TX), phenol/chloroform extraction and isopropanol precipitation as described by the manufacturer, and used for reverse transcription of cDNA templates. Each tube contained 2 μg total RNA, 500 units Mu-MLV reverse transcriptase (GIBCO-BRL, Gaithersburg, MD) and a mixture of 3′ primers consisting of 10 ng each gene-specific (*lck*, *fyn* or ribozyme) primer (Table 1) and, as a control (see below), 10 ng of a glyceraldehyde 3-phosphate dehydrogenase (GAPDH)-specific primer (Baier *et al.*, 1993*a*, *b*). Reverse transcription was performed at 42°C for 60 min, followed by 30 min at 56°C and 3 min at 94°C. The templates were then amplified in a PCR using ribozyme- or target mRNA- plus GAPDH-specific sense (5′) and antisense (3′) primer pairs. The sense primers were kinased prior to their use with [γ-^{32}P]ATP and T4 polynucleotide kinase in order to radiolabel the PCR products synthesized in each round of amplification. PCRs were conducted on a TwinBlock™ thermal cycler (Ericomp, Inc., San Diego, CA) for 1 min at 94°C, 2 min at 50°C and 0.5 min at 72°C for 20 (ribozymes, *lck* or *fyn* mRNAs) or 15 (GAPDH) cycles. The PCR mixture consisted of 30 ng ^{32}P-labeled sense primers, 100 ng unlabeled antisense primers, all four dNTPs (200 μM each), 1.5 mM $MgCl_2$ and 2.5 units *Taq* polymerase (Promega) in a final volume of 50 μl. Under these standardized conditions, which were selected based on titration experiments, the PCR was found to proceed in a linear fashion. One fifth of the PCR products were resolved by 2.4% agarose gel electrophoresis, dried, visualized by autoradiography and quantified by laser densitometry using an Ultroscan XL enhanced laser densitometer (LKB, Bromma, Sweden). For each sample, the signal was normalized to the corresponding GAPDH signal in order to allow a comparison of ribozyme or target mRNA expression levels among different Jurkat clones as described (Baier *et al.*, 1993*b*).

Immunoblots

Membranes were prepared by sonicating cells in TSEM buffer (25 mM Tris–HCl pH 7.5, 25 mM sucrose, 0.1 mM EDTA and 5 mM $MgCl_2$) and centrifugation at 150,000*g* and 4°C for 30 min. The resulting membrane pellet was resuspended in TSEM buffer by sonication and protein concentration was assayed using the Bradford Protein Assay (Bio-Rad, Melville, NY). Equal amounts of membrane-derived protein preparations (30 μg) from different clones were subjected to 10% SDS–PAGE and immunoblotting with optimal dilutions of $p56^{lck}$- or $p59^{fyn}$-specific rabbit antibodies, respectively.

RESULTS

Catalytic activity of ribozymes in vitro

Since target mRNA accessibility to ribozyme action may depend, to a large degree, on local secondary RNA structures (Lee *et al.*, 1992), several distinct ribozymes, targeted to different sites in the target *lck* or *fyn* transcripts, were constructed in order to increase the probability of identifying a suitable target sequence that will be efficiently cleaved by ribozymes in the context of the native mRNA. The target sites for three *lck*-specific ribozymes within a 257-nucleotide *lck* substrate RNA are shown in Fig. 1A. An additional ribozyme, lck-4, consisted of ribozymes 1 and 2 covalently linked in tandem. The ribozymes were initially cloned into pGEM-3Z, an *in vitro* transcription vector, and T7 RNA polymerase-transcribed ribozymes were tested for their ability to degrade catalytically the target *lck* substrate RNA *in vitro*. Bands having sizes consistent with both the full-length primary transcript and the expected cleavage products, respectively, were seen (Fig. 1B), thereby

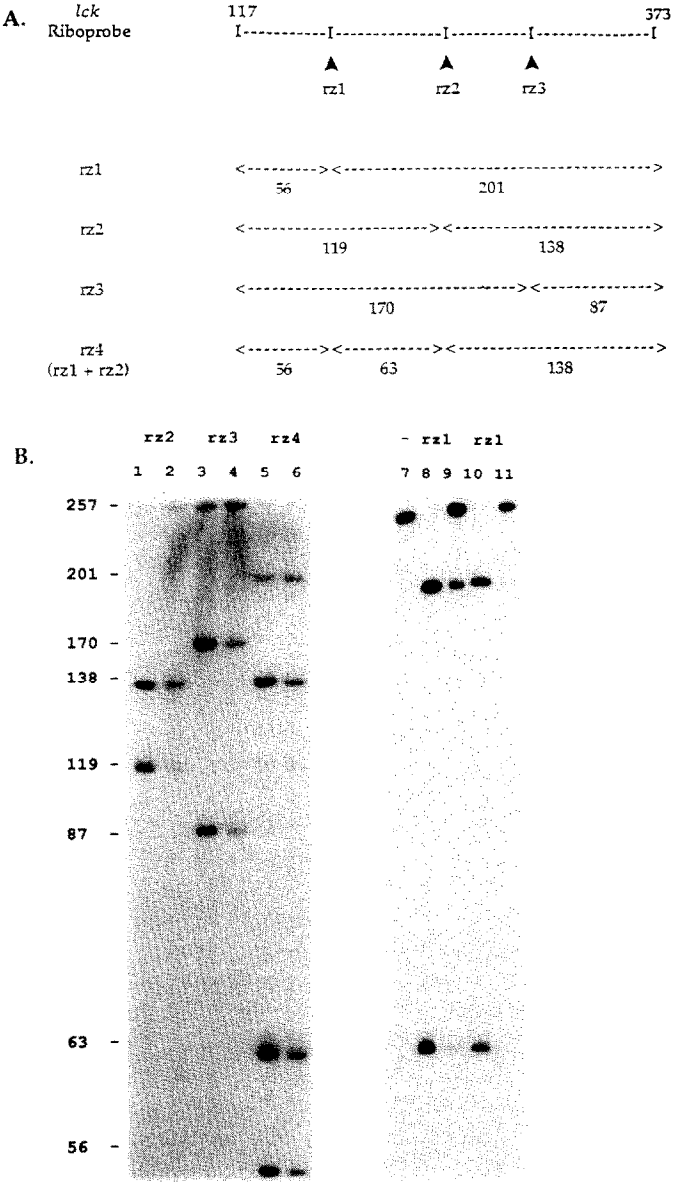

Fig. 1. *In vitro* cleavage of substrate RNA by *lck*-specific ribozymes. (A) Schematic representation of the 257-nucleotide *lck* substrate RNA indicating the selected cleavage sites and the predicted fragments generated by each ribozyme. (B) Ribozymes were incubated for 60 min with an [α-^{32}P]UTP-labeled *lck* substrate RNA at 50°C (lanes 1, 3, 5, 8 and 10) or 37°C (lanes 2, 4, 6, 9 and 11) at ribozyme:target ratios of 10:1 (lanes 1–9) or 1:1 (lanes 10 and 11). Cleavage products were analyzed by gel electrophoresis and autoradiography. The sizes of the substrate and the cleavage products are given at the left. Fragments having the expected size can be observed.

confirming the specificity and effectiveness of these catalysts at 50°C and 37°C. Incubation of these *lck*-specific ribozymes with an unrelated *fyn* substrate RNA did not result in any detectable cleavage products. Similarly, several *fyn*-specific ribozymes cleaved a target 217-nucleotide *fyn* substrate RNA but not the unrelated *lck* substrate RNA *in vitro* (not shown).

Cloning and characterization of chimeric tRNA:ribozymes in vitro

The double-copy retroviral vector DC-T5T (Sullenger *et al.*, 1990*a*), that carries an SV40 early promoter-driven neomycin resistance gene, was previously used as an efficient transcription unit for expressing noncatalytic antisense and decoy RNAs in murine fibroblasts and in a human leukemic T cell line or *BCRABL*-specific ribozymes in K562 cells (Sullenger *et al.*, 1990*a*, *b*, 1991; Shore *et al.*, 1993). Four selected ribozymes (lck-1, lck-3, fyn-1 and fyn-2) were cloned immediately downstream of the $tRNA^{met_i}$ transcription unit (Fig. 2A) contained within the 3′-LTR of DC-T5T. In order to test these hybrid tRNA:ribozymes transcripts for their catalytic activity *in vitro*, a bacteriophage T7 promoter sequence was introduced upstream of the hybrid gene. A recombinant polymerase chain reaction (PCR) amplification was performed in order to amplify the chimeric tRNA:ribozymes using the ribozyme-encoding DC-T5T plasmids as templates. The PCR primers consisted of a sense hybrid primer spanning the 5′ sequence of the tRNA gene together with an upstream bacteriophage T7

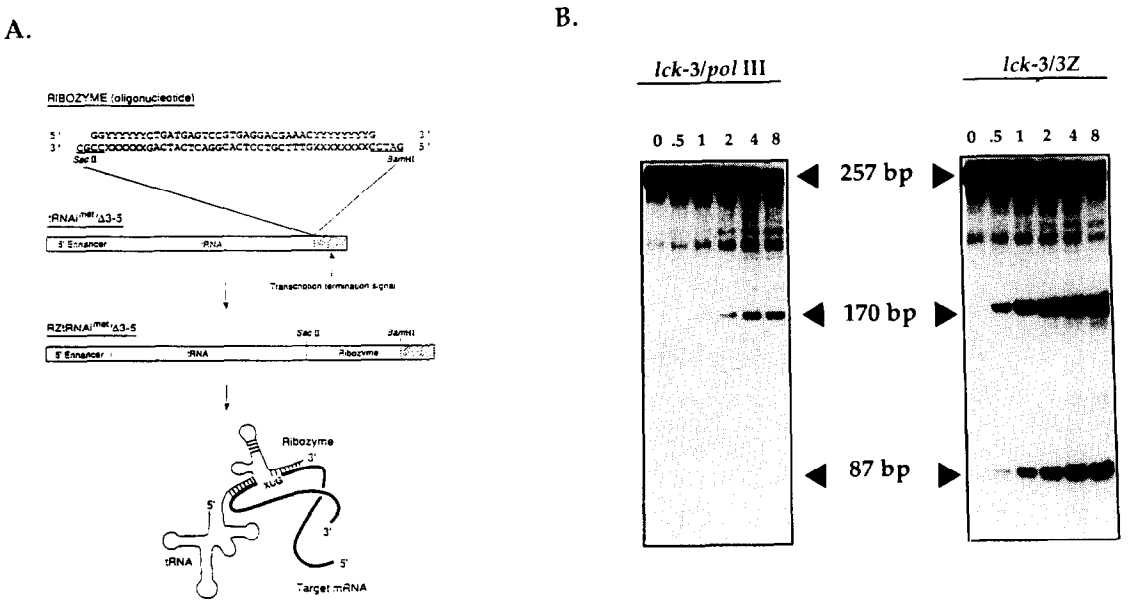

Fig. 2. (A) Cloning and schematic structure of tRNA:ribozymes. Oligonucleotides representing the catalytic ribozyme core, flanking target-specific antisense sequences (represented by X) and restriction sites for directional cloning (underlined) were cloned downstream of a tRNA transcription unit. This unit, a derivative of a human $tRNA^{met_i}$ gene lacking 18 bp at its 3′ end (Δ3–5 mutant; Adeniyi-Jones *et* al., 1984), is encoded in the 3′-LTR of the DC-T5T vector (Sullenger *et al.*, 1990*a*). Correct read-through transcription is expected to generate the hybrid tRNA:ribozyme transcript represented schematically at the bottom. (B) Catalytic activity of different forms of an *lck*-specific ribozyme. The *in vitro*-transcribed tRNA:ribozyme (lck-3/pol III) or pGEM-3Z-ribozyme (lck-3/3Z) were incubated at 37°C with an *lck* substrate RNA at a 10:1 ratio for the indicated times (in hours). Reactions were terminated by freezing on dry ice, and analyzed by gel electrophoresis and autoradiography.

promoter site, and an antisense primer spanning the transcription termination site within the DC-T5T flanking sequence (Table 1). These PCRs gave rise to fragments having the expected size (~165 nucleotide-long) which were used directly as templates for *in vitro* T7 RNA polymerase-mediated transcription. The resulting ~125-nucleotide tRNA:ribozyme hybrid (e.g. lck-3/polIII, Fig. 2B), and the corresponding pGEM-3Z-transcribed ~50-nucleotide ribozyme RNA (lck-3/3Z), were compared for their catalytic activity at 37°C. Although the catalytic activity of tRNA:ribozyme was ~7-fold lower compared to the pGEM-3Z-transcribed ribozyme, the former retained a specific, time-dependent catalytic activity and, thus, appears to possess sufficient structural flexibility that may be exploited for the inhibition of target gene expression *in vivo*.

Stable expression and quantitation of tRNA:ribozymes in transduced cells

We next wished to express stably the *lck*- and *fyn*-specific ribozymes in the human leukemic T cell line, Jurkat. An amphotropic packaging cell line, PA317, was transfected with ribozyme-encoding or control vectors, and the resulting recombinant retroviruses were used to infect Jurkat cells. G418-resistant clones were selected, and ribozyme expression was verified and quantitated by a reverse transcriptase PCR (RT-PCR). PCRs yielded cDNA fragments corresponding to the predicted size of the hybrid tRNA:ribozyme transcripts (Fig. 3). As

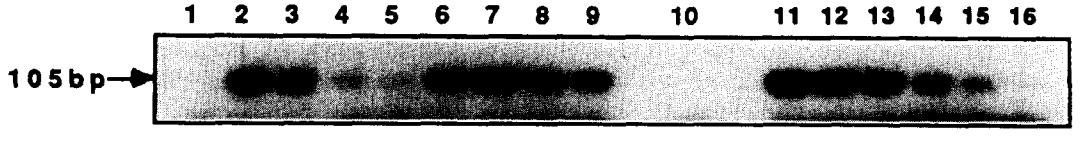

Fig. 3. RT-PCR analysis of tRNA:ribozymes expression in Jurkat clones. Total cellular RNA was used for cDNA synthesis primed by a ribozyme-specific antisense primer spanning the catalytic domain of the hammerhead sequence. The resulting cDNAs were subsequently employed as amplification templates in PCRs using ribozyme (3′)- and $tRNA^{met_i}$ (5′)-specific primers, respectively. Representative clones transduced with a vector control (1), lck-1 (2, 3), lck-3 (4, 5), fyn-1 (6, 7) or fyn-2 (8, 9) were analyzed. Lane 10 represents an RT-PCR of the lck-1 clone shown in lane 2 in the absence of reverse transcriptase. For quantitation purposes, parallel PCRs were conducted using 400, 80, 40, 8, 4 and 0.8 pg (lanes 11–16, respectively) of plasmid-derived tRNA:ribozyme cDNA templates.

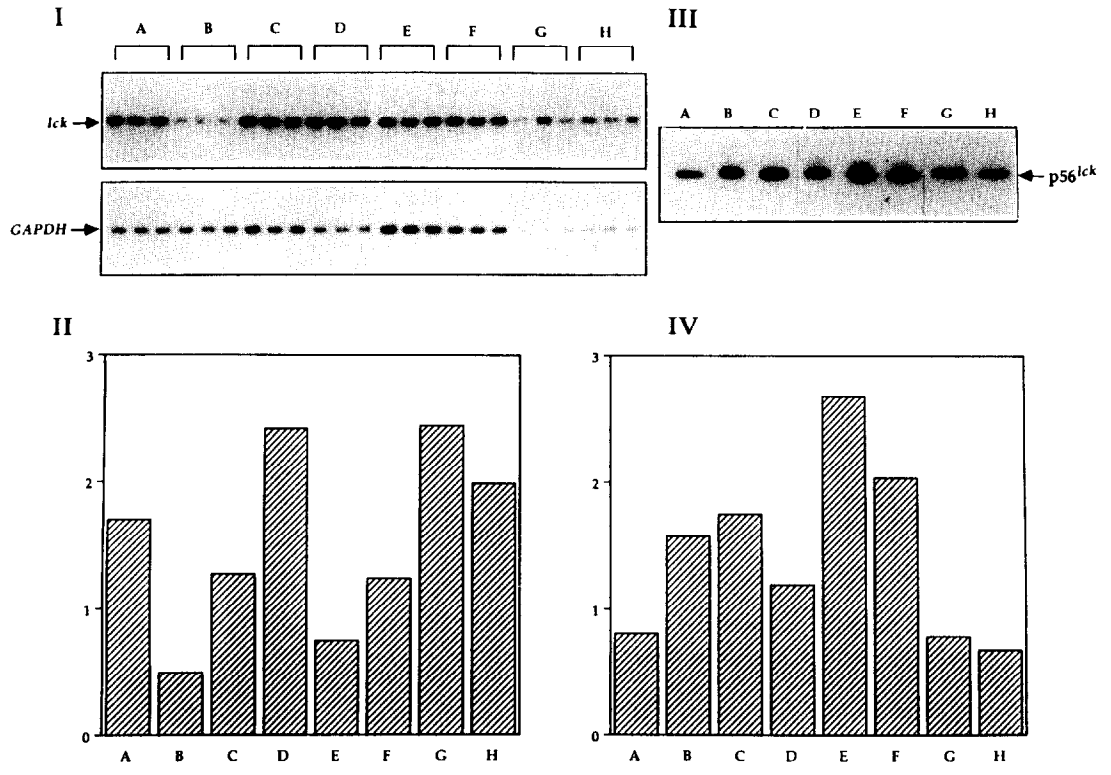

Fig. 4. (I) Target *lck* mRNA levels in stably transduced ribozyme-expressing Jurkat clones. Transcripts were amplified in PCR reactions and analyzed as described in Materials and Methods. The PCR product from each clone was analyzed in triplicate to minimize sampling errors. Sizes of the PCR products shown were as expected, namely, 257 or 299 bp for *lck* or GAPDH, respectively. (II) Relative levels of *lck* transcripts normalized to the control GAPDH gene. (III) Immunoblot analysis of $p56^{lck}$ protein expression in ribozyme-expressing Jurkat clones. Membrane-derived protein preparations (30 μg) from different clones were subjected to SDS–PAGE and immunoblotting with $p56^{lck}$-specific rabbit antibodies as described (Mustelin *et al.*, 1990). (IV) Relative intensity of the $p56^{lck}$ band as determined by scanning laser densitometry. The different clones represented are: A (lck-1.1), B (lck-1.2), C (lck-1.3), D (lck-3.1), E (lck-1.4), F (lck-1.5), G (fyn-2.1) and H (vector control). Protein or mRNA expression levels in other control clones were similar to those shown in groups G, H ($\leq$25% variability; not shown).

expected, similar RNA species were not detected in the parental cell line or in clones infected with control, nonrecombinant retrovirus. In order to quantitate the tRNA:ribozyme transcripts expressed by Jurkat clones, transcripts amplified from known amounts of plasmid-derived tRNA:ribozyme cDNA templates were used as a reference. Variable expression levels, ranging between approximately 4 and 400 pg tRNA:ribozyme per μg of total cellular RNA, were found in different clones expressing distinct ribozymes. The calculated number of ribozyme copies per cell was ~25,000 or ~250 for lck-1 or lck-3, and ~10,000 or ~5,000 for fyn-1 or fyn-2, respectively, based on an estimate of 1 pg total RNA per Jurkat cell (Marth *et al.*, 1987). For ribozymes lck-1 and lck-3, these levels represent, on a molar basis, a 650- and 6.5-fold, respectively, of ribozyme excess over endogenous *lck* transcripts, estimated at ~40 copies per Jurkat cell (Marth *et al.*, 1987).

Downregulation of target gene expression in ribozyme-expressing cells

In order to determine whether the tRNA:ribozymes can effectively reduce expression of their target mRNAs in cultured cells, *lck* and *fyn* mRNA expression levels were determined in transduced Jurkat clones by a semi-quantitative RT-PCR. RT-PCR conditions were selected based on titration experiments, and after exact standardization the RT-PCR was found to proceed in a quantitative range (data not shown). Expression of lck-1 (Fig. 4, panels I and II) or fyn-1 (Fig. 5, panels I and II) was associated with a variable but significant reduction in the amount of the respective target mRNAs in different clones, by comparison with clones expressing the vector controls or the opposite ribozyme. The most pronounced downregulation of *lck* or *fyn* mRNA expression, namely, 80% or 61% reduction, was seen in clones lck-1.2 (Fig. 4) and fyn-1.2 (Fig. 5), respectively. In contrast, expression of two other ribozymes, lck-3 and fyn-2, or vector controls, was not associated with a significant reduction in target mRNA levels (Figs 4 and 5). Clonal variations in the expression levels of *fyn* or *lck* mRNA in additional control clones were $\leq$25% (not shown), indicating that the differences in RNA expression levels between clones expressing the specific ribozyme and control clones do not simply reflect clonal variation. These results were also reproducible when the same clones were retested a few weeks later (not

shown). We were unable to determine any deleterious effect of the ribozyme expression in these cells indicating the specificity of the ribozymes for its target sequences.

Ribozyme-expressing clones that demonstrated reduced levels of target mRNAs were then assayed for expression of the corresponding PTKs by immunoblotting using $p56^{lck}$- and $p59^{fyn}$-specific antibodies. Despite the marked reduction in the endogenous mRNA levels, expression levels of either the $p56^{lck}$ (Fig. 4, panels III and IV) or $p59^{fyn}$ (Fig. 5, panels III and IV) proteins were not decreased. On the contrary, upregulation of target PTK protein levels were observed in these clones by comparison with clones expressing inactive ribozymes (lck-3 or fyn-2) or vector controls. These results were found to be reproducible when the same clones were retested a few weeks later (not shown). Furthermore, no defects in TCR/CD3-mediated signaling could be observed in appropriate lck-1 or fyn-1 ribozyme expressing clones, by comparison with clones expressing the vector controls, e.g. no reduction of Ca^{2+}-influx or total tyrosine phosphorylation of cellular substrates after TCR/CD3-ligation was found.

DISCUSSION

A tRNA-based transcription system and retroviral gene transfer were used to express stably *lck*- or *fyn*-specific ribozymes in a human T cell line. The potential advantages of using this RNA polymerase III transcription unit have been discussed previously (Cameron and Jennings, 1989; Sullenger *et al.*, 1990*a*; Cotten, 1990). Recently, such RNA polymerase III transcribed tRNA:ribozymes were demonstrated to be superior to ribozymes transcribed by the human β-actin and the herpes simplex virus thymidine kinase promoters, respectively (Shore *et al.*, 1993). Expression levels of distinct tRNA:ribozyme constructs, as detected by an RT-PCR, varied considerably from one ribozyme to another. These apparently ribozyme sequence-dependent differences in expression levels may represent different steady-state transcription rates and/or intrinsic stabilities of the ribozyme transcripts. Most hybrid tRNA:ribozymes, namely, lck-1, fyn-1 and fyn-2, were expressed at high ribozyme:substrate ratios, and two of these (lck-1 and fyn-1) were active against their target RNA in intact cells, as indicated by the associated marked reduction in the expression level of the corre-

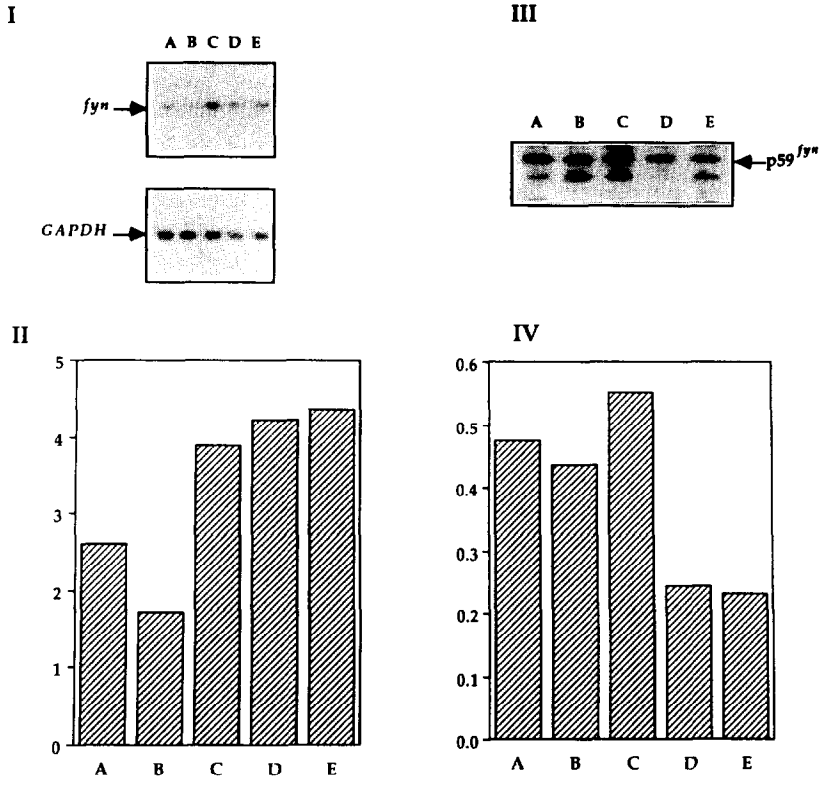

Fig. 5. (I) Target *fyn* mRNA levels in stably transduced ribozyme-expressing Jurkat clones. Transcripts were amplified in PCRs and analyzed as described in Materials and Methods. Sizes of the PCR products shown were as expected, namely, 217 and 299 bp for *fyn* and GAPDH, respectively. (II) Relative levels of *fyn* transcripts normalized to the control GAPDH gene. (III) Immunoblot analysis of $p59^{fyn}$ protein expression in ribozyme-expressing Jurkat clones. Membrane-derived protein preparations (30 μg) from different clones were subjected to SDS–PAGE and immunoblotting with $p59^{fyn}$-specific rabbit antibodies as described (Appleby *et al.*, 1992). (IV) Relative intensity of the $p59^{fyn}$ band as determined by scanning laser densitometry. The different clones represented are: A (fyn-1.1), B (fyn-1.2), C (fyn-1.3), D (fyn-2.1) and E (vector control). Protein or mRNA expression levels in other control clones were similar to those shown for the control clone $\leq$25% variability; not shown).

sponding mRNA targets. The inability of the highly expressed fyn-2 ribozyme to reduce *fyn* mRNA expression may reflect inaccessibility of the relevant target site to the ribozyme, perhaps due to secondary RNA structures.

Despite the significant ribozyme-mediated downregulation of *lck* or *fyn* mRNA transcripts in the stably transduced clones, expression of the corresponding proteins, $p56^{lck}$ and $p59^{fyn}$, was not reduced. Although the data presented here do not provide insights into the mechanisms involved, reports on the translational mechanism controlling $p56^{lck}$ expression in T lymphoma cells appear to be relevant (Voronova and Sefton, 1986; Marth *et al.*, 1988; Perlmutter *et al.*, 1988). The *lck* mRNA in these cells is comprised of the 5′-untranslated region (5′-UTR) of the Moloney murine leukemia virus (Mo-MuLV) and the complete *lck*-coding sequence, thereby representing a viral-*lck* fusion transcript. This hybrid mRNA was found to be translated ~7-fold more efficiently than normal *lck* mRNA (Marth *et al.*, 1988). The elevated translational efficiency is caused by the removal of three short upstream open reading frames (uOFR's), contained in the 5′-UTR of normal *lck* transcripts, which are implicated in translational repression.

Translational regulation through differential utilization of upstream AUG initiation codons has been well characterized for *GCN4*, a transcriptional activator of a large number of unlinked genes encoding amino acids biosynthetic enzymes in *Saccharomyces cerevisiae* (for review see Altmann and Trachsel, 1993). The translational efficiency of the *GCN4* mRNA was found to be modulated by *trans*-acting factors in response to amino-acid availability (Mueller *et al.*, 1987), and its uORFs were shown to confer translational control even upon a heterologous yeast transcript (Mueller and Hinnebusch, 1986). Yeast cells starved of any of a number of amino acids respond thereby by increasing *GCN4* synthesis, which in turn is entirely based on enhanced *GCN4* mRNA translation (Hinnebusch, 1990). Therefore, beside increased protein stability, elevated translational efficiency could represent one putative compensatory mechanism by which levels of $p56^{lck}$ are adjusted upwards in the lck-1 ribozyme-expressing Jurkat clones despite the significant downregulation of the corresponding mRNA. Since upstream AUG codons are also found in the 5′-UTR of the *fyn* transcript, a similar regulatory mechanism may operate for $p59^{fyn}$ in the fyn-1 ribozyme-expressing Jurkat clones. Similar AUG codons are also found within the 5′-UTR of *fgr* and *hck*, two other members of the *src* family, and may constitute an important mechanism regulating protein levels of *src*-family PTKs or other protooncogene products (Kozak, 1987; Perlmutter *et al.*, 1988).

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