Multi-Unit Ribozyme-Mediated Cleavage of bcr-abl mRNA in Myeloid Leukemias

By Lance H. Leopold, Scott K. Shore, Todd A. Newkirk, Ramana M.V. Reddy, and E. Premkumar Reddy

Chronic myelogenous leukemia is characterized by the Philadelphia chromosome, which at the molecular level results from the fusion of the bcr gene on chromosome 22 and the abl gene on chromosome 9. The bcr-abl fusion gene encodes a novel tyrosine kinase with transforming activity. In this study, we have synthesized a multi-unit ribozyme that targets bcr-abl mRNA. In vitro ribozyme cleavage reactions show increased cleavage efficiency of this multi-unit ribozyme compared with single or double ribozymes. The multi-unit ribozyme was then transfected into murine myeloblasts transformed with the bcr-abl gene (32D cells). Ribozyme transfection was accomplished either by liposomes or using folic acid-polylysine as a carrier. Multi-unit ribozyme transfection reduced the level of bcr-abl mRNA 3 logs when transfected via folic acid-receptor-mediated uptake into transformed 32D cells. These results suggest that a multi-unit ribozyme could be an effective therapeutic agent for the treatment of Philadelphia chromosome-positive chronic myelogenous leukemia.

© 1995 by The American Society of Hematology.

From the Temple University Hospital and The Fels Institute for Cancer Research and Molecular Biology, Philadelphia, PA.

Submitted August 8, 1994; accepted November 28, 1994.

Supported by the American Society of Clinical Oncology Young Investigator Award and Career Development Award (L.H.L.), the W.W. Smith Charitable Trust (S.K.S.), and National Institutes of Health Grant No. 1 ROI, 5POICA21124-15 (E.P.R.).

Address reprint requests to Lance H. Leopold, MD, Temple Cancer Center, 3232 N Broad St, Philadelphia, PA 19140.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. section 1734 solely to indicate this fact.

© 1995 by The American Society of Hematology.

0006-4971/95/8508-0002$3.00/0

© 1995 by The American Society of Hematology.

0006-4971/95/8508-0002$3.00/0

2162

From www.bloodjournal.org by guest on October 22, 2017. For personal use only.
The conjugation number was determined by measuring the optical density at 363 nm of the conjugate solution, and the conjugation number was transfection mix was added to 4 cells. RIBONEM-MEDIATED THERAPY OF CML 2163

The folate conjugates were introduced into 32D cells by electroporation. Liposomes containing ribozyme RNA were prepared in 100 mM NaCl, 40 mM HEPES, pH 7.3, using RNAse-free water and were gently mixed for 15 minutes. The transfection mix was added to 4 mL of serum-free, folate-free Dulbecco’s modified Eagle’s medium containing 1 × 10⁶ cells. Before transfection, bcr-abl–transformed 32D cells were grown in this medium with 10% FBS for 3 days. FBS and IL-3, to a final concentration of 10%, were added 3 hours after the completion of the transfection protocol.

**BCR-ABL mRNA analysis.** A total of 1 × 10⁶ 32D cells were added to increasing numbers of bcr-abl–transformed 32D cells and transfected with ribozyme molecules using either liposomes or folate-lysine vectors. Inactive ribozyme or transfection medium containing no ribozyme was used as negative control. Plasmid pGEM 3z described above, containing the bcr-abl breakpoint, was used to transcribe RNA used as the positive control for the reverse transcription–polymerase chain reaction (RT-PCR) analysis. The number of bcr-abl–transformed 32D cells added varied from 1 to 1 × 10⁶ cells per 1 million normal 32D cells. Cells were transfected at time zero, and 3 hours after transfection, the medium was supplemented with FBS and IL-3 as described above. Total cellular RNA was isolated by lysing cells in Ultraspec solution (Biotecx, Houston, TX) according to the manufacturer’s protocol. RT-PCR was performed as previously described.21 After RT with an oligo dT primer, PCR amplification was performed as follows: pre-cyclic at 95°C for 2 minutes and 30 cycles at 94°C for 30 seconds, 54°C for 30 seconds, and 72°C for 1 minute. After PCR amplification, the samples were heated to 72°C for 10 minutes to allow completion of extension. The primer sequences were 5'-GGAGCTGCAGATGCTGACCA C and 5'-TCAGACCTCGAGCTTCTTAAATG TC. The β-actin gene transcript was also amplified as an internal control. Twenty microliters of the PCR product was analyzed by electrophoresis on a 1.5% agarose gel and photographed. Southern blotting was performed as described elsewhere26 using a 32P-kilnased oligonucleotide probe spanning the bcr-abl junction. The probe sequence was 5'-GCT- GAAGGGCTTGGGACTC TGCCTTA.

## RESULTS

Ribozyme design, synthesis, and packaging. To develop ribozyme-based reactions as a potential therapy for human volumes. All RNA was suspended in 50 mMol/L Tris-HCl, pH 7.5, containing 1 mMol/L EDTA and was heated to 95°C for 5 minutes and immediately chilled on ice. Reactions were initiated by adding 1 μL of 200 mMol/L MgCl₂ and stopped after 2 hours by adding 2 μL of stop solution containing 95% formamide, 20 mMol/L EDTA, and 2% bromophenol blue. Cleavage products were analyzed by electrophoresis on a 6% denaturing gel followed by autoradiography and quantitative densitometry (Fujix, Tokyo, Japan). The kinetics of ribozyme cleavage were determined in identical experiments by removing 1 μL of the reaction mixture at specified times and adding 1 μL of stop solution. Cleavage products were analyzed as above.

### 32D cell transformation.

32D cells, a murine BM myeloblast cell line, was maintained in Iscove’s modified Dulbecco’s medium (IMDM) with 10% fetal bovine serum (FBS), and 10% WEHI-322 saline before transfection. In liposome transfection experiments, the transfection mix was added to 4 cells. RIBONEM-MEDIATED THERAPY OF CML 2163

Transformed cells were selected by growth in L-3-deficient medium, was maintained in Iscove’s modified Dulbecco’s media (IMDM) with 10% fetal bovine serum (FBS), and 10% WEHI-322 saline before transfection. In liposome transfection experiments, the transfection mix was added to 4 cells. RIBONEM-MEDIATED THERAPY OF CML 2163

The bcr-abl-transformed 32D cells were suspended in serum-free, folate-free Dulbecco’s modified Eagle’s medium containing 1 × 10⁶ cells. Before transfection, bcr-abl–transformed 32D cells were grown in this medium with 10% FBS for 3 days. FBS and IL-3, to a final concentration of 10%, were added 3 hours after the completion of the transfection protocol.

**BCR-ABL mRNA analysis.** A total of 1 × 10⁶ 32D cells were added to increasing numbers of bcr-abl–transformed 32D cells and transfected with ribozyme molecules using either liposomes or folate-lysine vectors. Inactive ribozyme or transfection medium containing no ribozyme was used as negative control. Plasmid pGEM 3z described above, containing the bcr-abl breakpoint, was used to transcribe RNA used as the positive control for the reverse transcription–polymerase chain reaction (RT-PCR) analysis. The number of bcr-abl–transformed 32D cells added varied from 1 to 1 × 10⁶ cells per 1 million normal 32D cells. Cells were transfected at time zero, and 3 hours after transfection, the medium was supplemented with FBS and IL-3 as described above. Total cellular RNA was isolated by lysing cells in Ultraspec solution (Biotecx, Houston, TX) according to the manufacturer’s protocol. RT-PCR was performed as previously described.21 After RT with an oligo dT primer, PCR amplification was performed as follows: pre-cyclic at 95°C for 2 minutes and 30 cycles at 94°C for 30 seconds, 54°C for 30 seconds, and 72°C for 1 minute. After PCR amplification, the samples were heated to 72°C for 10 minutes to allow completion of extension. The primer sequences were 5'-GGAGCTGCAGATGCTGACCA C and 5'-TCAGACCTCGAGCTTCTTAAATG TC. The β-actin gene transcript was also amplified as an internal control. Twenty microliters of the PCR product was analyzed by electrophoresis on a 1.5% agarose gel and photographed. Southern blotting was performed as described elsewhere26 using a 32P-kilnased oligonucleotide probe spanning the bcr-abl junction. The probe sequence was 5'-GCT- GAAGGGCTTGGGACTC TGCCTTA.

## RESULTS

Ribozyme design, synthesis, and packaging. To develop ribozyme-based reactions as a potential therapy for human
leukemias, we have designed hammerhead motifs that can cleave specifically \textit{bcr-ab1} mRNA, thereby preventing the synthesis of the transforming fusion protein. Single-, double-, and triple-unit ribozymes were designed and constructed targeting GUX sequences near the \textit{bcr-ab1} fusion site (Fig 1). Single-unit ribozymes were synthesized as cDNA oligomers, whereas double- and triple-unit ribozyme expression vectors were produced by ligation of single-unit ribozyme cDNAs. Nonfunctional ribozymes (antisense controls), with a G substituted for the A in the conserved GAG sequence (marked with an asterisk in Fig 1) in the catalytic unit eliminating catalytic function, were similarly cloned and transcribed. Sense ribozymes were synthesized by transcription from the opposite strand in pGEM3z vector using T7 RNA polymerase.

\textit{In vitro ribozyme cleavage reactions}. To test the efficiency of single-, double-, and triple-unit ribozymes, we performed in vitro cleavage reactions. Four single-unit ribozymes, two double-unit ribozymes, and one triple-unit ribozyme, whose structures are shown in Fig 1, were used in these studies. A constant amount of \textsuperscript{32}P-labeled substrate \textit{bcr-ab1} RNA was incubated with equal molar amounts of various ribozymes for 2 hours, and the cleavage products were analyzed on a denaturing polyacrylamide gel as described in Materials and Methods. As shown in Fig 2A, single-, double-, and triple-unit ribozymes specifically cleave \textit{bcr-ab1} mRNA. After 2 hours, single ribozymes cleaved target \textit{bcr-ab1} mRNA into 2 fragments with variable efficiency. Ribozymes A, B, C, and D cleaved 45%, 79%, 5%, and 7% of target mRNA, respectively. Comparison among these ribozymes shows that ribozymes targeting the same site but with shorter annealing arms are more efficient (A v C) and GUC cleavage sites (ribozyme B) appear to be more efficiently cleaved. The variability in cleavage of substrate RNA by ribozymes B, C, and D is not surprising and reflects the expected cleavage efficiency at different GUX cleavage sites. Double-unit ribozymes E and F cleaved 72% and 13% of target mRNA, respectively. There was no significant improvement in cleavage over the best single ribozyme of the pair. However, ribozyme F, the triple-unit ribozyme, cleaved 95% of target mRNA in 2 hours. Figure 2B shows the cleavage obtained when all 3 single unit ribozymes are added together in one reaction. This resulted in little additional cleavage compared with that of the best single ribo-

![Image](image-url)

\textbf{Fig 2.} Autoradiogram of ribozyme-mediated cleavage of \textit{bcr-ab1} mRNA. \textsuperscript{32}P-labeled \textit{bcr-ab1} mRNA was synthesized from a plasmid vector containing a 499-bp segment of the \textit{bcr-ab1} chimeric gene. Substrate and ribozyme reactions were performed in 10 \textmu L volumes. All RNA concentrations were 100 nmol/L. Transcribed RNAs were resuspended in 50 mmol/L Tris-HCl, pH 7.5, containing 1 mmol/L EDTA and heated to 95°C for 5 minutes and immediately chilled on ice. Reactions were initiated by adding 1 \textmu L of 200 mmol/L MgCl\textsubscript{2} and stopped after 2 hours by adding 2 \textmu L of stop solution containing 95% formamide, 20 mmol/L EDTA, and 2% bromophenol blue. Cleavage products were separated by electrophoresis on a 6% denaturing gel. In (A) Lane S, a control and contains substrate without ribozymes; lane A, cleavage products from a single ribozyme targeting site no. 2; lane B, cleavage products from a single ribozyme targeting site no. 1; lane C, cleavage products from a single ribozyme targeting site no. 2; lane D, cleavage products from a single ribozyme targeting site no. 1; lane E, cleavage products from a double ribozyme targeting sites no. 1 and 2; lane F, cleavage products from a double ribozyme targeting site no. 3; lane G, cleavage products from a triple ribozyme targeting sites no. 1, 2, and 3; Ribozyme A and C differ in the length of their annealing arms. Ribozyme A has shorter annealing arms of 9 bp each and was described earlier. Cleavage with double- and triple-unit ribozymes release small fragments that run at the bottom of the gel and are not shown. P1 through P6 are 314-, 286-, 263-, 235-, 212-, and 184-base fragments respectively. (B) Single-unit ribozymes B, C, and D were incubated with substrate RNA as described above.
zyme (ribozyme B). There was no cleavage of bcr-abl mRNA using sense-oriented ribozymes and cleavage-inactive antisense ribozymes (data not shown).

The kinetics of in vitro cleavage are shown in Fig 3. As described in Materials and Methods, equal molar amounts of 32P-labeled bcr-abl substrate was incubated with single- or triple-unit ribozymes for various times, and aliquots were analyzed over time for percentage of cleavage. Ribozyme C was used as the single-unit ribozyme for comparison because its sequence was contained within the triple-unit ribozyme and targets the bcr-abl junction. Ribozymes B and D target normal bcr and abl sequences and would not be specific for bcr-abl sequences. These ribozymes would have little clinical utility. By 30 minutes, cleavage of target mRNA by the triple-unit ribozyme is nearly complete, whereas the single ribozyme has had little effect on the same target mRNA.

To test the specificity of the triple-unit ribozyme, c-abl and c-bcr mRNA containing the breakpoint region was transcribed with 32P-CTP and used as a target for single- and triple-unit ribozymes in similar 2-hour cleavage reactions. Figure 4 shows specific cleavage products from these reactions. In Fig 4A, ribozyme D cleaved 3% and ribozyme G cleaved 87% of c-abl mRNA. In Fig 4B, ribozyme B cleaved 83% and ribozyme G cleaved 45% of target bcr mRNA. Thus, the more efficient triple-unit bcr-abl ribozyme does cleave c-abl and bcr mRNA, although less efficiently as compared with bcr-abl sequences.

Ribozyme transfection of transformed 32D cells. Having shown that the triple-unit ribozyme efficiently cleaved bcr-abl mRNA, we next compared two transfection techniques to standardize a transfection protocol for use in subsequent experiments. For comparison, we used either lipofectin2' or folic acid-polylysine because these two reagents have been reported to yield high levels of transfection. The transfection protocols were standardized by varying both the concentration of transfection vehicle and RNA over 4 logs and the incubation time. After 3 hours of ribozyme exposure, total cellular RNA was isolated from bcr-abl-transformed 32D cells and was analyzed by electrophoresis on a 6% sequencing gel followed by autoradiography and quantitative densitometry. Figure 5A shows the improvement in 32P-labeled ribozyme uptake in 32D cells that results by incorporating the ribozyme in a liposome. Naked multi-unit ribozymes are poorly taken up by cells (Fig 5A, lane 1), whereas liposomes augmented delivery 100-fold (Fig 5A, lane 2). In addition to isolating total cellular RNA and comparing ribozyme delivery, the supernatant from these transfection experiments was monitored for ribozyme stability. Naked ribozymes were degraded over several hours, whereas ribozymes packaged in liposomes remained stable in serum-free medium for 24 hours (data not shown). Analysis of cellular RNA after 3 hours of ribozyme exposure insured that uptake comparisons were made during the period of time in which ribozymes were present under all transfection conditions.

Figure 5B compares liposome-mediated and folate receptor-mediated transfection techniques in 32D cells. Using 1 µg of 32P-labeled ribozyme, cells were transfected with different concentrations of lipofectin or folic acid-polylysine.

![Fig 3. Comparison of triple-unit and single-unit ribozyme cleavage kinetics. Equal molar concentrations of 32P-labeled substrate bcr-abl mRNA and ribozyme G or C were mixed at time zero. Aliquots of the reaction mixture were removed and placed in stop solution at the specified time points. Cleavage products were separated on a 6% denaturing gel and percentage of cleavage was determined by quantitative densitometry.](image)

![Fig 4. Autoradiogram of ribozyme-mediated cleavage of abl and bcr mRNA. 32P-labeled abl and bcr mRNA were synthesized from plasmid vectors containing a 447-bp segment of the abl gene and a 359-bp segment of the bcr gene, respectively, which included the bcr and abl regions present in the bcr-abl breakpoint region. Specific cleavage products are shown. (A) The cleavage of control (SI) abl mRNA with ribozymes D and G is shown. (B) The cleavage of control (SI) bcr mRNA with ribozymes B and G is shown. Ribozyme names correspond to those in Fig 2. (A) P1 and P2 are 363 and 84 bases respectively; (B) P1 and P2 are 220 and 139 bases, respectively.](image)
that varied over 4 logs. Ribozyme uptake was more efficient (sevenfold) by folate receptor-mediated transfection. Similar to liposome vectors, folic acid-polylysine vectors protected ribozymes from being degraded in serum-free medium (data not shown).

**Transfection standardization.** To further optimize the transfection conditions, a series of experiments were performed to determine the dose-response relationship between ribozyme and transfection vehicle concentration in 32D cells. First, $1 \times 10^6$ cells were transfected with $1 \mu g$ of $^{32}P$-labeled triple-unit ribozyme via increasing concentrations of liposomes or folic acid-polylysine. Next, based on these dose-response curves, the quantity of transfection vehicle was held constant and the quantity of ribozyme varied. Then, using optimum ratios of ribozyme and transfection vehicle, the time course for ribozyme uptake and survival was determined. Figure 6 graphically shows the results from these experiments in 32D cells. Although both transfection techniques augmented ribozyme uptake, folate receptor-mediated transfection improved uptake up to 10-fold. Furthermore, multi-unit ribozyme RNA remained at significant intracellular levels in 32D cells after 24 hours. This occurred despite the addition of FBS to the culture medium after 3 hours, which quickly degraded ribozyme RNA in the medium (data not shown). In subsequent transfection experiments, either 5 $\mu g$ of lipofectin was diluted in 50 $\mu L$ of water and mixed with 1 $\mu g$ of RNA suspended in 50 $\mu L$ of water or 1 $\mu L$ of folic acid-polylysine was diluted in 50 $\mu L$ HEPES-buffered saline and mixed with 1 $\mu g$ of RNA suspended in 50 $\mu L$ HEPES-buffered saline was used.

**Serial transfections.** To test the availability of the folate receptor for serial transfections, 32D cells were serially transfected with unlabeled and $^{32}P$-labeled ribozymes over a 48-hour period. Three identical folic acid-polylysine transfection experiments were performed at time zero with either $^{32}P$-labeled (one sample) or unlabeled ribozymes (two samples). After 3 hours, the cells transfected with labeled ribozymes were lysed and RNA was isolated, whereas cells transfected with unlabeled ribozymes were supplemented with FBS and IL-3 as described above. After 24 hours, one of the two remaining samples was then washed in purified buffered saline and transfected with $^{32}P$-labeled ribozyme. After 3 additional hours, these cells were washed and lysed, and RNA was isolated. A second transfection with unlabeled ribozymes was performed on the remaining cells (second dose), and these cells were supplemented as described. After 48 hours, these cells were transfected with labeled ribozymes (third dose), and, after 3 hours, RNA was isolated as described above. The RNA samples were analyzed by electrophoresis on a 6% denaturing polyacrylamide gel electrophoresis followed by autoradiography and quantitative densitometry. Figure 7 shows the autoradiogram that demonstrates that serial folic acid-polylysine transfection results in a decrease in ribozyme uptake. Compared with the quantity of $^{32}P$-labeled ribozyme recovered from cells at 3 hours, 50% was recovered after the second transfection, and 60% was recovered after the third transfection. These experiments show that less ribozyme is delivered during sequential folate receptor-targeted transfections in 32D cells, probably because of receptor occupancy or downregulation of receptor density on the cell surface.

**RNA analysis.** To test the ability of the multi-unit ribozyme to cleave bcr-abl mRNA in cells, untransformed 32D cells were added to various numbers of bcr-abl-transformed 32D cells and were transfected 1 or 3 times over 24 or 48 hours respectively, with triple-unit ribozyme via liposomes or folic acid-polylysine vectors. Total cellular RNA was isolated after 24 and 48 hours and was reverse-transcribed into cDNA. The cDNA was amplified by PCR using primers that amplify the bcr-abl breakpoint. Purified cellular RNA was DNA-free, and there was no amplification of DNA in the absence of RT, suggesting only reverse-transcribed RNA is being amplified (data not shown). Figure 8 shows the results of a Southern blot performed on the PCR products using an oligonucleotide probe that spans the bcr-abl breakpoint. Control cells transfected with folic acid-polylysine vectors alone show that this technique has a sensitivity for detecting 1 transformed cell in 1 x 10^6 normal cells. A single transfection of the triple-unit ribozyme via folic acid-polylysine vectors into bcr-abl-transformed 32D cells resulted in a 3-log

Fig 5. Autoradiogram of liposome- and folate-receptor-mediated ribozyme uptake in 32D cells. 32D cells ($1 \times 10^6$) were transfected with $1 \mu g$ of $^{32}P$-labeled triple-unit ribozyme in 4 mL of serum-free medium as described. After 3 hours, total cellular RNA was isolated and electrophoresed on a 6% denaturing gel. (A) 32D cell transfection is compared with naked ribozymes (lane 1) and liposome vectors (lane 2). (B) 32D cell transfection using a constant 1 $\mu g$ of RNA is compared with increasing quantities of lipofectin or folic acid-polylysine.
RIBOZYME-MEDIATED THERAPY OF CML

![Graph A](image)

**Fig 6.** Ribozyme uptake standardization curves for liposome- and folate receptor-mediated transfection in 32D cells. 32D cells (1 x 10⁶) were transfected with ³²P-labeled ribozyme via liposome or folic acid-polylysine vectors at various concentrations. RNA was isolated at 3 hours and run on a 6% sequencing gel followed by autoradiography and quantitative densitometry. Time course experiments were terminated at 1, 3, and 24 hours as noted. (A) Transfection vehicle concentration was varied and 1 µg of RNA was used. (B) RNA concentration was varied, and transfection vehicle quantity was 5 µg of lipofectin or 1 µL of folic acid-polylysine. (C) RNA uptake over time using 1 µg of RNA and 1 µL of folic acid-polylysine or 5 µg of lipofectin is shown.

decrease in detectable bcr-abl mRNA signal 24 hours after transfection. There was no improvement in reducing the bcr-abl mRNA with multiple transfections over 48 hours compared with single transfections (data not shown). A 1-log reduction in bcr-abl RNA content was observed by transfection of ribozymes via liposomes, despite multiple treatments over 48 hours (24 hour data shown in Fig 8). Cleavage-inactive ribozymes had no effect on elimination of the bcr-abl mRNA signal (data not shown). RT-PCR using primers that amplified the β-actin gene confirmed that similar quantities of RNA were used in RT-PCR amplification for the bcr-abl mRNA signal (data not shown). These results show that delivery of a triple-unit ribozyme using folic acid-polylysine reduces the levels of bcr-abl RNA by 3 logs, whereas only a 1-log reduction is achieved with liposomes. These results are consistent with the levels of ribozyme present in cells after transfection using these two reagents.

**DISCUSSION**

CML is characterized by the presence of Ph chromosome that, at the molecular level, results from the fusion of the bcr gene on chromosome 22 with the abl gene on chromosome 9. This results in the generation of an 8.5-kb chimeric mRNA and an abnormal p210bcr-abl tyrosine kinase. This appears to be the transforming signal that leads to the development of CML.

There are no conventional therapies that have resulted in cures in CML, including chemotherapy, ³²P, and splenic irradiation. In 5% to 15% of patients, α-interferon therapy may suppress the expression of the Ph-positive clone in CML. However, the ability of interferon to cure CML has not been shown. Allogeneic BMT, using HLA-identical siblings, after myeloablative chemoradiotherapy is curative in up to 85% of carefully selected patients in chronic phase CML. In CML patients transplanted with BM grafts from an identical twin after high dose myeloablative therapy, the cure rate is 50%. This difference is because of a graft-versus-leukemia (GVL) effect and is enhanced by the presence of chronic graft-versus-host disease. T-cell depletion of donor BM reduces both the GVL effect and the incidence of graft-versus-host disease, as well as the cure rate. Collectively, these data show that high-dose therapy administered early in the course of CML is potentially curative in 50% of patients. The additional curative effects of allogeneic BMT are because of a GVL effect.

Less than 30% of CML patients will have a normal allogene-
Ribozyme –

| Hours | 3 | 24 | 48 |

Similar results have been described for a nonaribozyme targeting HIV-1 env RNA.\(^{42}\)

An important consequence of targeting normal sequences of the \(bcr\) and \(abl\) genes in the proximity of the fusion site is that normal mRNA may be affected by the ribozyme. Although we observe cleavage of normal \(bcr\) and \(abl\) mRNA, this appears to be less efficient in vitro cell-free cleavage experiments than \(bcr-abl\) cleavage. This could be caused by extensive nonhomology of portions of the ribozyme to normal \(abl\) and \(bcr\) RNAs that, in turn, could reduce the affinity of the ribozyme to the substrate.

To develop a protocol for ribozyme-mediated treatment of human CML, both efficiency of delivery and ease of application need to be taken into consideration. To accomplish rapid and efficient delivery of ribozymes into myeloid cells, we used three different methods. The first method tried was the receptor-mediated uptake of naked oligonucleotides,\(^{37}\) which proved to be extremely inefficient, possibly because of the delivery of the nucleotide molecules via the endosomal pathway, which exposes the ribozyme molecules to various nucleases of the cell. We next studied nontargeted uptake via cationic lipids and receptor-mediated uptake via the folate receptor. Both augmented ribozyme delivery to 32D cells, although folate-receptor-mediated uptake was more efficient. However, only folate acid-polylysine–mediated delivery of the ribozymes seemed to affect \(bcr-abl\) mRNA levels significantly. Although we cannot rule out a quantitative effect because of improved delivery of ribozymes via folate receptor uptake, the intracellular compartment accessible to ribozymes may be more favorable by this technique.\(^{44}\) As reviewed by Gottschalk et al.,\(^{45}\) folate enters cells through several different receptor systems, including a glycosyl-phosphatidyl-anchored protein that is excluded from clathrin-coated pits and cycles in and out of cells by caveolae.\(^{46}\) RNA transfected via liposomes may be quickly degraded,\(^{47}\) because this uptake is mediated via an endosomal

![Fig 7. Autoradiogram from sequential transfections of ribozymes via folate receptor-mediated uptake in 32D cells. To test the availability of the folate receptor for serial transfections, 32D cells were serially transfected at time zero with unlabeled and \(^{32}P\)-labeled ribozymes over 48 hours. Total cellular RNA was isolated 3 hours after each transfection experiments. Lane 1 contains RNA from cells transfected at time zero with \(^{32}P\)-labeled ribozyme. Lane 2 contains RNA from cells transfected at time zero with unlabeled ribozyme followed by \(^{32}P\)-labeled ribozyme at 24 hours. Lane 3 contains RNA from cells transfected at time zero and 24 hours with unlabeled ribozyme followed by \(^{32}P\)-labeled ribozyme at 48 hours.](image)

![Fig 8. Autoradiogram of a Southern blot of RT-PCR–amplified \(bcr-abl\) mRNA from transformed 32D cells transfected with ribozymes via liposomes or folate acid-polylysine vectors. From 1 to 1 \(\times\) 10\(^6\) \(bcr-abl\)–transformed 32D cells were added to 1 \(\times\) 10\(^6\) untransformed 32D cells. Cells were transfected with ribozymes or vectors containing no ribozymes (controls), and, after 24 hours, total cellular RNA was extracted. RT-PCR was performed with primers that amplified the \(bcr-abl\) chimeric gene and the \(\beta\)-actin gene (an internal control). Southern blotting was performed with a \(^{32}P\)-kinased probe that detects the \(bcr-abl\) breakpoint. + control, RNA transcribed from a plasmid containing the \(bcr-abl\) breakpoint region (see Materials Methods); – control, no template addition.](image)
RIBOZYME-MEDIATED THERAPY OF CML

pathway, which exposes the ribozymes to cellular nucleases. On the other hand, receptor-mediated uptake may protect the ribozymes from intracellular degradation caused by exposure to cellular nucleases. Folate-mediated uptake may suffer from a first-dose effect if receptors are downregulated after ligand binding. In such an event, the problem could be circumvented by targeting different receptors sequentially. Our results show that, although transfection efficiency decreases with serial transfections, ribozymes continue to be delivered into cells when transfections are separated over a 24-hour time period.

The ultimate measure of ribozyme effects on bcr-abl mRNA are decreasing the bcr-abl mRNA signal, reducing p210ab1 protein levels, and reversing the transformed phenotype in living cells. Our results show a reduction in bcr-abl mRNA levels in transformed 32D cells when transfected with ribozymes delivered by folate receptor-mediated uptake. Unfortunately, multiple sequential transfections did not improve on the results obtained by a single transfection over 24 hours. It is possible that constitutive expression of ribozymes transfected by retroviruses in a similiar model may show greater efficacy.

ACKNOWLEDGMENT

This paper is dedicated to the memory of T.A. Newkirk. He was a dedicated scientist and good friend.

REFERENCES

44. Spector D, Purves F, Roizman B: Mutational analysis of the promoter region of the alpha 27 gene of herpes simplex virus 1 within the context of the viral genome. Proc Natl Acad Sci USA 87:5268, 1990
Multi-unit ribozyme-mediated cleavage of bcr-abl mRNA in myeloid leukemias

LH Leopold, SK Shore, TA Newkirk, RM Reddy and EP Reddy