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

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CHRONIC myelogenous leukemia (CML) accounts for 20% of all cases of leukemia and carries a death rate of 1.5 per 100,000 population. In 1960, Nowell and Hungerford discovered that the Philadelphia chromosome (Ph') was consistently associated with CML. Molecular studies have shown that during the formation of the Ph chromosome, a portion of the c-abl gene is translocated from chromosome 9q34 to chromosome 22q11 resulting in the formation of a chimeric gene consisting of sequences derived from the *bcr* and *abl* loci. This translocation is detectable in over 95% of patients with CML. The transcripts of this gene either includes (b3a2) or excludes (b2a2) exon 3 of the *bcr* gene. The fusion gene, named *bcr-abl*, transcribes a chimeric mRNA of 8.5 kb that is translated into a p210 fusion protein with altered tyrosine kinase activity. The p210*abl* protein has been shown to transform myeloid precursor cells in vitro, and infection of primary murine bone marrow (BM) stem cells with a retrovirus encoding the *bcr-abl* protein predictably produced a disorder in mice similar to human CML.

In the past decade, the use of antisense nucleic acid sequences to block the translation of mRNA has been developed as a strategy to inhibit viral and malignant diseases. Recently, it has been shown that specific RNA sequences, termed ribozymes, have the ability to cleave other RNA molecules in a catalytic manner. Several different catalytic sequences have been described that share a common need for divalent cations and result in the nonhydrolytic transesterification of specific RNA target regions. These catalytic sequences have been adapted to create targeted antisense molecules that bind to and cleave target RNA molecules.

The "hammerhead" ribozyme has been developed into a targeted ribozyme, employing a catalytic "hammerhead" domain and flanking oligonucleotides that specifically bind to target sequences. Cleavage occurs 3' to a GUX triplet where X can be C, U, or A. Any GXU sequence can be targeted by the appropriate design of the flanking oligonucleotide sequence. Recently, a few studies have used antisense molecules or ribozymes to specifically target the *bcr-abl* gene. Examination of the b3a2 *bcr-abl* junction sequence shows the presence of three closely spaced GUX sequences near the breakpoint. In this communication, we have designed a multi-unit ribozyme that targets these three cleavage sites and have compared its efficacy with single-unit ribozymes that target these GUX sequences individually. Our results show that multi-unit ribozymes have a greater efficacy of cleavage compared with that of single-unit ribozymes. To test the feasibility of using multi-unit ribozymes as a purging strategy for autologous BM transplantation (BMT) in CML, we studied the in vitro activity of this ribozyme and the effects of ribozyme transfection on *bcr-abl* mRNA levels in *bcr-abl*-transformed 32D cells. Our results show that folic acid-polylysine is an effective carrier molecule for ribozymes and that *bcr-abl*-transformed 32D cells transfected with an anti-*bcr-abl* triple-unit ribozyme via the folic acid-polylysine method reduced the level of *bcr-abl* mRNA 3 logs.

MATERIALS AND METHODS

Synthesis of the multi-unit ribozyme. cDNAs encoding single-unit ribozymes were synthesized as complimentary oligonucleotide molecules. Double- and triple-unit ribozyme expression vectors were produced by ligation of single-unit ribozyme cDNAs. Single-, double-, and triple-unit ribozyme cDNAs were cloned into pGEM3z vector (Promega, Madison, WI). Ribozyme RNA was transcribed using SP6 RNA polymerase in vitro as per manufacturer's protocol (Promega). Cleavage-inactive control ribozymes, with a G substituted for the A in the conserved GAG sequence 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.

Target mRNA synthesis. cDNA encoding the *bcr-abl* breakpoint region, a portion of the *bcr* gene, and a portion of the *abl* gene were cloned into pGEM3, pGEM5, pGEM3z vectors (Promega), respectively. RNA was transcribed using T7-UTP and T7, T3, and SP6 RNA polymerases (Promega), respectively, according to the manufacturer's protocol.

Ribozyme cleavage reactions. Substrate and ribozyme reactions were performed using RNA concentrations of 100 nmol/L in 10-μL.

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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 million normal 32D cells. Cells were transfected at time zero, and 3 hours after transfection, the medium was supplemented with 10% 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 × 10^9 32D cells were added to increasing numbers of bcr-abl-transformed 32D cells and transfected with ribozyme molecules using either liposomes or folate-polylysine 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 × 10^8 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. After RT with an oligo dT primer, PCR amplification was performed as follows: precoat 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’-GGAGGCTGACAGATGCTGACCA C and 5’-TCAAGATGCTTCCAAGGCTC. 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 elsewhere using a 32P-kinaed oligonucleotide probe spanning the bcr-abl junction. The probe sequence was 5’-GGAGGCTGACAGATGCTGACCA C and 5’-TCAAGATGCTTCCAAGGCTC.

**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 \textit{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,\textsuperscript{12} 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 vs C) and \textit{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 \textit{GUX} cleavage sites.\textsuperscript{15} 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-

![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 \(\mu\)L volumes. All RNA concentrations were 100 mmol/L. Transcribed RNAs were resuspended in 50 mmol/L Tris-Cl, 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 \(\mu\)L of 200 mmol/L MgCl\textsubscript{2} and stopped after 2 hours by adding 2 \(\mu\)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. 3; lane E, cleavage products from a double ribozyme targeting sites no. 1 and 2; lane F, cleavage products from a double ribozyme targeting sites no. 2 and 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.\textsuperscript{15} Cleavage with double- and triple-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.](Image)
zyme (ribozyme B). There was no cleavage of *bcr-ab1* 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-ab1* 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-ab1* junction. Ribozymes B and D target normal *bcr* and *abl* sequences and would not be specific for *bcr-ab1* 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-ab1* 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-ab1* mRNA. In Fig 4B, ribozyme B cleaved 83% and ribozyme G cleaved 45% of target *bcr* mRNA. Thus, the more efficient triple-unit *bcr-ab1* ribozyme does cleave *c-ab1* and *bcr* mRNA, although less efficiently as compared with *bcr-ab1* sequences.

**Ribozyme transfection of transformed 32D cells.** Having shown that the triple-unit ribozyme efficiently cleaved *bcr-ab1* mRNA, we next compared two transfection techniques to standardize a transfection protocol for use in subsequent experiments. For comparison, we used either lipofectin 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.
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 × 10⁶ cells were transfected with 1 μg of ³²P-labeled triple-unit ribozyme via 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 was 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 μg of lipofectin was diluted in 50 μL of water and mixed with 1 μg of RNA suspended in 50 μL of water or 1 μL of folic acid-polylysine was diluted in 50 μL HEPES-buffered saline and mixed with 1 μg of RNA suspended in 50 μL 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 ³²P-labeled ribozymes over a 48-hour period. Three identical folic acid-polylysine transfection experiments were performed at time zero with either ³²P-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 ³²P-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 ³²P-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 × 10⁶ 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
RIBOZYME-MEDIATED THERAPY OF CML

Fig 6. Ribozyme uptake standardization curves for liposome- and folate receptor-mediated transfection in 32D cells. 32D cells (1 x 10^6) 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 9.6 This results in the generation of an 8.5-kb chimeric mRNA and an abnormal p210^bcr-abl tyrosine kinase.6 This appears to be the transforming signal that leads to the development of CML.9

There are no conventional therapies that have resulted in cures in CML, including chemotherapy, 32P, and splenic irradiation.14 In 5% to 15% of patients, α-interferon therapy may suppress the expression of the Ph-positive clone in CML.15-17 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.17-19 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.16 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 -

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Fig 7. Autoradiogram from serial 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 with unlabeled and \( ^{32}\)P-labeled ribozymes over 48 hours. Total cellular RNA was isolated 3 hours after each transfection experiment. 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.

neic HLA-matched donor. Current use of matched unrelated donors has resulted in high mortality because of GVHD and infections.\(^{35}\) Thus, the development of an autologous BM transplant program using Ph-negative stem cells would provide an alternative for patients without other curative options. An autologous BMT would avoid GVHD and should be curative in up to 50% of patients, provided the BM is pure of CML stem cells. Thus far, autologous BMT has not been successful in CML because Ph-positive stem cells are invariably reinfused into the patient.\(^{36}\) In an attempt to provide Ph-negative stem cells for autologous BMT, chemotherapy purging,\(^{37}\) interferon purging,\(^{38}\) long-term BM cultures,\(^{39}\) and CD34 antigen-based stem cell selection\(^{40}\) result only in transient Ph-negative hematopoiesis.

Szczylík et al.\(^{41}\) reported the inhibition of proliferation of isolated blasts from CML patients after the addition of antisense oligonucleotides directed against the \( bcr-abl \) gene in vitro. More recently, we and others\(^{42,43}\) have reported the use of single-unit ribozyme molecules for cleavage of \( bcr-abl \) mRNA that, in turn, appeared to reduce or eliminate the synthesis of \( bcr-abl \) protein. Reduction in \( bcr-abl \) RNA with single-unit ribozymes appeared variable and, to a large extent, was dependent on ribozyme activity and intracellular levels. Therefore, it was a logical next step to develop ribozymes with increased catalytic activity. Analysis of the sequence of the \( bcr-abl \) gene adjacent to the fusion site showed 3 GUX sequences in close proximity. Therefore, we attempted to generate ribozymes that can bind to \( bcr-abl \) mRNA and cleave all the three sites that lie in close proximity to the junction point. Results presented in this report show that a triple-unit ribozyme has substantially increased catalytic potential when compared with single, double, or mixtures of individual ribozymes. Furthermore, the kinetics of cleavage show that the triple-unit ribozyme functions quickly. This may provide an additional advantage in the intracellular environment where nucleases would be present.

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,\(^{43}\) 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 pathway.

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-polyllysine vectors. From 1 to 1 \( \times \) 10\(^3\) \( bcr-abl \)-transformed 32D cells were added to 1 \( \times \) 10\(^9\) 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.
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 p210bcr-abl protein levels, and reversing the transformed phenotype in living cells. Our results show a reduction in bcr-abl mRNA levels in transfected 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 similar model may show greater efficacy.

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

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