Efficient Transfer of Synthetic Ribozymes into Cells Using Hemagglutinating Virus of Japan (HVJ)-Cationic Liposomes

APPLICATION FOR RIBOZYMES THAT TARGET HUMAN T-CELL LEUKEMIA VIRUS TYPE I tax/rex mRNA*

(Received for publication, April 3, 1997, and in revised form, August 13, 1997)

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We investigated the usefulness of ribozymes in inhibiting the expression of human T-cell leukemia virus type I (HTLV-I) gene. Two hammerhead ribozymes that were against HTLV-I rex (RR) and tax (TR) mRNA were synthesized. Both ribozymes were sequence-specific in the in vitro cleavage analysis of run-off transcripts from tax/rex cDNA. Intracellular activities of the ribozymes were studied in HTLV-I tax cDNA-transfected rat embryonic fibroblasts (Rat/Tax cells), which expressed the Tax but not Rex. Ribozymes were delivered into cells using anionic or cationic liposomes fused with hemagglutinating virus of Japan (HVJ). Cellular uptake of ribozymes complexed with HVJ-cationic liposomes was 15–20 times higher than that of naked ribozymes, and 4–5 times higher than that of ribozymes complexed with HVJ-aniocic liposomes. HVJ-cationic liposomes promoted accumulation of ribozymes in cytoplasm and accelerated transport to the nucleus. Tax protein levels were decreased about 95% and were five times lower when the same amount of TR was introduced into the cells using HVJ-cationic, rather than HVJ-aniocic liposomes. Inactive ribozyme and tax antisense oligodeoxynucleotides reduced Tax expression by about 20%, whereas RR and tax sense oligodeoxynucleotides had no effect. These results suggest that the ribozymes’ effect against tax mRNA was sequence-specific, and HVJ-cationic liposomes can be useful for intracellular introduction of ribozymes.

The discovery of RNA molecules with sequence-specific RNA-cleaving properties, called ribozymes, led to investigations of their potential use as specific inhibitors of gene expression (1–3). Several ribozyme configurations have been identified, of which the “hammerhead” (4) and “hairpin” (5) structures are the simplest, and most suitable, for biomedical applications (6–8). Several factors appear to contribute to the intracellular efficency of ribozymes and thus the success of ribozyme gene therapy. Most importantly, the ribozyme must co-localize with its molecular target in the appropriate cellular compartment and must be present in a sufficiently high concentration to promote hybridization.

Previous studies have generally assessed the catalytic activity of ribozymes in cell-free assay systems (6–8). The present study describes an experimental system that allows one to assess the effects of ribozymes in living cells. The in vivo application of ribozymes will depend on the availability of efficient delivery methods. The methods of gene transfer are classified as viral or non-viral. Ribozymes generally have been introduced into cells via a viral infection or the transfection of expression vectors (6, 9, 10). Viral vectors can potentially lead to the incorporation of ribozyme-encoding genes into the cellular chromosomes, thereby allowing their permanent expression, and many studies now are aimed at developing suitable vectors that present a minimal associated risk to the host. Alternatively, ribozymes can be delivered to the cells by non-viral methods, such as lipophilic vesicles (liposomes) and cholesterol (6).

We recently developed a highly efficient method for gene transfer that involves the entrapment of DNA or RNA using hemagglutinating virus of Japan (HVJ,1 Sendai virus) to enhance the fusion of anionic liposomes to cell membranes (11, 12). However, especially in cultured cells, the level of transgene expression achieved with this method is somewhat lower than that obtained with some of the viral vectors. We then improved this gene delivery system using cationic lipids for the liposomes (13). In the present study, we compared the effects of ribozymes that had been transferred into living cells, using anionic or cationic liposomes.

Ribozymes may become useful molecular therapies for several diseases of humans, including human T-cell leukemia virus type I (HTLV-I) infection, which is etiologically associated with adult T-cell leukemia (14, 15). The HTLV-I Tax protein has oncogenic properties that may play a key role in tumorigenesis (16, 17). We previously evaluated gene therapy for the treatment of HTLV-I-related diseases using tax antisense oligodeoxynucleotides (ODNs) (18–20). In the present study, we describe an approach involving ribozyme-mediated cleavage of HTLV-I tax/rex mRNA. We investigated whether ribozymes

1 The abbreviations used are: HVJ, hemagglutinating virus of Japan; FITC, fluorescein isothiocyanate; HTLV-I, human T-cell leukemia virus type I; mAb, monoclonal antibody; ODN, oligodeoxynucleotide; PBS, phosphate-buffered saline; PI, propidium iodide; Tia/Tax cell, tax-expressing rat embryonic fibroblast; RR, ribosome against HTLV-I rex mRNA, RC, inactive control ribosome derived from RR; TR, ribosome against HTLV-I tax mRNA; TC, inactive control ribosome derived from TR; TRITC, tetramethylrhodamine isothiocyanate; bp, base pair(s).

* This work was funded by Grant 09470172 from the Ministry of Education, Science, Sports and Culture of Japan and grants from Funds for Comprehensive Research on Long Term Chronic Disease. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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can cleave their target RNAs in the cells and whether HVJ-cationic lipidosome-mediated gene transfer allows efficient introduction of ribozymes into living cells.

**EXPERIMENTAL PROCEDURES**

**Synthesis of Ribozymes Targeted against HTLV-I tax/rex mRNA**—Ribozymes were chemically synthesized on a 1-μmol scale using a DNA synthesizer (model 8909 Expedit System, PerSeptive Biosystems, Framingham, MA). We generated two hammerhead ribozymes that targeted the tax/rex mRNA based on previously published sequence information (21). The ODNs were designed to bracket position 7308 of the HTLV-I genome, had the sequence 5′-G₃G₆C₃C₃UCCU₅₉-C₅U₃G₉-3′. It cleaves the mRNA at position 5139 of the HTLV-I genome. The ribozyme targeting the rex mRNA (TR), which cleaves the mRNA at position 7308 of the HTLV-I genome, had the sequence 5′-A₅C₇C₇C₉G₉C₉G₉-U₅-3′. It cleaves the mRNA at position 5139 of the HTLV-I genome.

**Ribozyme Transfer into Cells Using HVJ-Cationic Liposomes**—Ribozymes were introduced into Rat-1 cells by the HVJ-cationic liposome method (22). Briefly, ribozymes were mixed with cationic liposomes (HVJ-cationic liposomes) or anionic liposomes (HVJ-anionic liposomes). Liposomes, or 1 mM TR complexed with HVJ-cationic liposomes, 1 mM TR complexed with HVJ-cationic liposomes, 1 mM TC complexed with HVJ-cationic liposomes, 1 mM tax antisense ODNs complexed with HVJ-cationic liposomes, or 1 mM tax sense ODNs complexed with HVJ-cationic liposomes. Furthermore, cells were treated with 1 μM TR complexed with HVJ-cationic or -anionic liposomes diluted by a factor of 1 × 10⁻⁷ to 1 × 10⁻³. The cells were lysed with radiomunnu precipitation buffer (25 mM Tris-HCl, pH 7.5, 50 mM NaCl, 0.5% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 1% aprotinin, and 1 mM phenylmethylsulfonyl fluoride) at 98 °C. The proteins were fractionated on an 8% SDS-polyacrylamide gel and transferred to an Immobilon-S membrane (Millipore Corp., Bedford, MA). The membrane was blocked with 3% fat-free milk in PBS for 1 h at 4 °C and incubated for 1 h with anti-HTLV-I Tax monoclonal antibody (mAb) (gift of Prof. M. Hatanaka, Kyoto University) (diluted 1:1000). The membrane then was washed four times with PBS containing 0.1% Tween, followed by incubation with peroxidase-conjugated goat anti-mouse IgG antibody (Cappel Research Products, Durham, NC) (diluted 1:10,000) for 1 h at room temperature. Immunoreactive proteins were visualized by enhanced chemiluminescence using the ECL system (Amersham, Buckinghamshire, UK).

**Quantitative Analysis of Tax Expression by Flow Cytometry**—Ribozymes were introduced into Rat-Tax cells (2 × 10⁶) were incubated for 4 days without ribozymes, with 1 mM TR complexed with HVJ-cationic liposomes diluted by a factor of 1 × 10⁻⁷, or 1 mM TR complexed with HTLV-I Tax monoclonal antibody (mAb) (diluted 1:1000). The membrane then was washed four times with PBS containing 0.1% Tween, followed by incubation with peroxidase-conjugated goat anti-mouse IgG antibody (Cappel Research Products) as a second antibody. Control samples were treated with anti-FITC-labeled mouse IgG alone. The expression of Tax protein was quantified by flow cytometry (Coulter Electronics Inc., Hialeah, FL). The cut-off value for positive staining by PI was set at 10 on the y axis, and the cut-off value for positive Tax protein expression was set at 10 on the y axis in the histogram.

**RESULTS**

In Vitro Cleavage of HTLV-I tax/rex mRNA by the Ribozymes—We synthesized two hammerhead ribozymes that were sequence-specific for a site downstream of the AUG start...
codons of the HTLV-I tax mRNA (TR) or rex mRNA (RR) (Fig. 1A). These sites had previously been targeted successfully by antisense ODNs (18, 20) and thus were expected to be accessible for ribozyme-mediated cleavage. Incubation of the 220-base substrate HTLV-I tax/rex mRNA with RR resulted in the predicted 97-base and 123-base cleavage products, whereas 148-base and 72-base cleavage products were observed following incubation with TR (Fig. 1B). Both cleavage reactions were dose-dependent and were also dependent on the Mg²⁺ concentrations (Fig. 1C). No cleavage products were observed when the mRNA was incubated with the inactive control ribozymes, RC and TC (Fig. 1B).

Ribozyme Transfer into Cells Using HVJ-Cationic Liposomes—We previously established a novel and highly efficient method for gene transfer using HVJ-liposomes (11, 12). Gene delivery was even most efficient if the liposomes consisted of cationic rather than non-cationic lipids (13). In the present study, we have compared the gene transfer efficiency of ribozymes in the absence of HVJ-liposomes (naked ribozymes) and in the presence of HVJ-cationic or -anionic liposomes. To evaluate the intracellular distribution of the ribozymes, FITC-labeled ribozymes were visualized by confocal laser scanning microscopy. Cells of control samples containing FITC-conjugated rabbit IgG exhibited no staining (Fig. 2A).

Incubation of Rat/Tax cells with naked ribozymes for 24 h resulted in weak staining and spotty distribution of ribozymes in 1–3% of the cells (Fig. 2B). Examination of the cells at a higher magnification demonstrated that the naked ribozymes were distributed diffusely throughout the cytoplasm. TR complexed with HVJ-anionic liposomes showed a significantly improved cellular uptake compared with the naked ribozymes (Fig. 2C). Most of these ribozymes were retained in the cytoplasm and existed as vesicles in the endosomes or lysosomes (data not shown). Approximately 20% of cells also exhibited staining in their nuclei. In contrast, more than 90% of the Rat/Tax cells incubated with TR in the presence of HVJ-cationic liposomes exhibited cytoplasmic and nuclear fluorescence, which was more intense than in cells treated with anionic liposomes (Fig. 2D).

To quantitate the cellular uptake of TR complexed with
HVJ-cationic liposomes, cells containing FITC-labeled ribozymes were counted by flow cytometry. Positive staining was indicated by a signal shift to the right on the x axis, compared with the signal obtained after non-specific staining with FITC-labeled rabbit IgG (closed histogram). Right panels, nuclear staining of 1 × 10⁶ cells was performed using PI. The cut-off value for positive staining was 10 on the y axis (regions a and b). For cells containing FITC-labeled TR, the cut-off value was 10 on the x axis (regions b and d). The characteristics of the cells in the different regions are as follows: a, PI/FITC = +/+; b, PI/FITC = +/−; c, PI/FITC = −/−; d, PI/FITC = −/+.

A, cells treated with 1 μM TR in the absence of HVJ-liposomes. The proportion of cells in the various regions was as follows: a = 92.2%, b = 5.0%, c = 2.1%, d = 0%. B, cells treated with 1 μM TR complexed with HVJ-anionic liposomes (a = 76.2%, b = 22.8%, c = 1.0%, d = 0%). C, cells treated with 1 μM TR complexed with HVJ-cationic liposomes (a = 3.2%, b = 91.6%, c = 0.7%, d = 0%).

Fig. 3. Flow cytometric analysis of cellular uptake of ribozymes. Left panels, the cellular uptake of 1 μM FITC-labeled TR was analyzed using flow cytometry. Positive staining of the TR samples (open histogram) was indicated by a shift to the right of the histogram compared with the signal obtained after non-specific staining with FITC-labeled rabbit IgG (closed histogram). Right panels, nuclear staining of 1 × 10⁶ cells was performed using PI. The cut-off value for positive staining was 10 on the y axis (regions a and b). For cells containing FITC-labeled TR, the cut-off value was 10 on the x axis (regions b and d). The characteristics of the cells in the different regions are as follows: a, PI/FITC = +/+; b, PI/FITC = +/−; c, PI/FITC = −/−; d, PI/FITC = −/+.

A, cells treated with 1 μM TR in the absence of HVJ-liposomes. The proportion of cells in the various regions was as follows: a = 92.2%, b = 5.0%, c = 2.1%, d = 0%. B, cells treated with 1 μM TR complexed with HVJ-anionic liposomes (a = 76.2%, b = 22.8%, c = 1.0%, d = 0%). C, cells treated with 1 μM TR complexed with HVJ-cationic liposomes (a = 3.2%, b = 91.6%, c = 0.7%, d = 0%).

Sequence-specific Ribozyme-mediated Cleavage of tax mRNA in Rat/Tax Cells—Rat/Tax cells express high levels of tax mRNA, but no rex mRNA, because the tax cDNA used transfected these cells contained naked 5’-rex sequences (17) (Fig. 4A). Thus, the tax mRNA from Rat/Tax cells could be cleaved by TR but not by RR. We used Western blot analysis to investigate whether the ribozymes introduced into the cells by HVJ-cationic liposome-mediated gene transfer possessed sequence-specific catalytic activity and therefore down-regulated Tax expression. The Tax protein signals were quantified by densitometric scanning. The 40-kDa Tax protein was highly detectable in Rat/Tax cells treated with 1 μM naked TR (Fig. 4B, lane 1). Treatment with 1 μM TR complexed with HVJ-cationic liposomes significantly reduced Tax expression by approximately 95%, compared with cells treated with 1 μM naked TR (Fig. 4B, lane 2). In contrast, Tax protein expression was not affected by treatment with 1 μM RR complexed with HVJ-cationic liposomes (Fig. 4B, lane 3). A reduction of nearly 20% in Tax protein expression was observed in cells treated with 1 μM TC complexed with HVJ-cationic liposomes (Fig. 4B, lane 4). However, this effect of TC may be due to an antisense effect of the flanking TR sequences. Consistent with this hypothesis,
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**Fig. 4.** Tax expression in Rat/Tax cells and sequence specific intracellular ribozyme activity. A, schematic diagram of the HTLV-I tax cDNA incorporated in Rat/Tax cells. The tax cDNA (pH2R40M) contains the full-length tax mRNA (from position 5128 to position 8339 of the HTLV-I genome), including the tax initiation codon (ATG). The tax mRNA was cleaved by TR but not by cleaved by RR. The position of the splice donors (sd) and splice acceptors (sa) involved in the generation of tax/rix mRNA are indicated. B, immunoblot analysis for Tax protein expression in Rat/Tax cells treated with TR, RR, and tax antisense and sense ODNs. The positions of the molecular size markers are shown at the left (in kDa). The position of the 40-kDa Tax protein (p40 Tax) is indicated at the right. Lane 1, Rat/Tax cells treated with 1 μM TR in the absence of HVJ-liposomes (NK). Lane 2, cells treated with 1 μM TR complexed with HVJ-cationic liposomes (TR). Lane 3, cells treated with 1 μM RR complexed with HVJ-cationic liposomes (RR). Lane 4, cells treated with 1 μM TC complexed with HVJ-cationic liposomes (TC). Lane 5, cells treated with 1 μM tax antisense ODNs complexed with HVJ-cationic liposomes (AS). Lane 6, cells treated with 1 μM tax sense ODNs complexed with HVJ-cationic liposomes (SS).

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a similar reduction in Tax expression of about 20% was also found in cells treated with 1 μM tax antisense ODNs (18, 20) complexed with HVJ-cationic liposomes (Fig. 4B, lane 5). In contrast, Tax expression was not reduced after treatment with 1 μM tax sense ODNs, complexed with HVJ-cationic liposomes (Fig. 4B, lane 6).

**Inhibition of Tax Protein Synthesis in TR-treated Cells**—We compared the degree of down-regulation of Tax expression obtained when cationic or anionic liposomes that carried equal amounts of TR were used. Under these conditions, TR complexed with HVJ-cationic or HVJ-anionic liposomes produced a similar level of Tax down-regulation (data not shown). To evaluate the ribozyme achieved with each type of HVJ-liposome more precisely, we also compared Tax suppression after ribozyme transfer using diluted HVJ-liposomes. When the Rat/Tax cells treated with 1 μM TR complexed with HVJ-cationic liposomes diluted by a factor of 1 × 10⁻², a reduction of Tax expression occurred (Fig. 5, lanes 2–4). In contrast, 1 μM TR complexed with HVJ-anionic liposomes diluted by a factor of 1 × 10⁻³ showed a decrease in Tax protein expression (Fig. 5, lanes 5–7). Densitometric scanning analysis of a Tax immunoblot demonstrated that Tax signals were approximately 5 times lower in cells incubated with 1 μM TR complexed with 1 × 10⁻² diluted HVJ-cationic liposomes (Fig. 5, lane 3), compared with equally diluted HVJ-anionic liposomes (Fig. 5, lane 6).

We quantified the inhibitory effects of TR on Tax expression by flow cytometry using an anti-Tax mAb as reported previously (20). Rat/Tax cells expressed Tax protein at a high level (Fig. 6A). In cells treated with 1 μM naked TR, the level of Tax expression was almost unaffected (Fig. 6B). Incubation of the cells with 1 μM TR complexed with HVJ-anionic liposomes diluted by a factor of 1 × 10⁻² (Fig. 6C), however, considerably down-regulated Tax expression, although to a lesser extent than did 1 μM TR complexed with an equal volume of HVJ-cationic liposomes (Fig. 6D).

**Tax Protein Degradation Induced by TR Accumulation in Rat/Tax Cells**—The intracellular distribution and kinetics of ribozyme activity in the Rat/Tax cells was visualized using FITC-labeled ribozymes. The relationship between the ribozymes and Tax protein was investigated by a two-color confocal laser-fluorescence-microscopy scanning system using FITC-labeled ribozyme, which produces a green signal, and TRITC-labeled Tax protein, visible as an orange signal. A yellow signal indicated the co-localization of ribozyme and Tax protein. All untreated Rat/Tax cells expressed Tax protein both in the cytoplasm and in the nucleus, and the expression level did not differ among individual cells (data not shown). When the cells were incubated with TR complexed with HVJ-cationic liposomes for 4 days, Tax protein expression decreased markedly, resulting in the degradation of Tax protein, as indicated by the loss of orange signals (Fig. 7A). Approximately 5% of the cells did not incorporate TR and still expressed Tax protein (Fig. 7A, arrow). In contrast, following the introduction of RR mediated by HVJ-cationic liposomes, yellow signals were clearly evident in the cytoplasm and nuclei of the Rat/Tax cells (Fig. 7B). This observation suggests that Tax protein was still highly expressed following the nuclear deposition of RR.

**DISCUSSION**

The present study evaluated the efficiency of a method of gene transfer using anionic or cationic liposomes complexed with HVJ to introduce hammerhead ribozymes targeted against HTLV-I tax/rix mRNA into living cells. We also investigated the sequence specificity and activity of these ribozymes.
in the cells. We found that transfer efficiency was higher for ribozymes complexed with HVJ-cationic liposomes rather than with HVJ-anionic liposomes. The ribozymes specifically cleaved the HTLV-I \textit{tax/rex} target mRNA, and TR suppressed Tax protein expression in the cells.

The ultimate goal of gene therapy for HTLV-I infections is the inactivation of viral genes in the infected cells. Tax protein has been suggested to play a role in tumorigenesis by modulating the expression of cellular genes (26). We previously reported that Rat/Tax cells were transformed and exhibited a marked increase in a cell refracton cell density (17, 27). Treatment of Rat/Tax cells and \textit{tax}-expressing murine fibroma cells derived from an HTLV-I \textit{tax} transgenic mouse with TR that was complexed with HVJ-cationic liposomes reduced the density to the cells and inhibited the transformation, suggesting that TR can induce the ablation of tumorigenesis of HTLV-I \textit{tax}-expressing cells. 2 TR that was complexed with HVJ-cationic liposomes successfully cleaved \textit{tax} mRNA in the HTLV-I infected synoviocytes obtained from patients with chronic arthritis and induced apoptosis in these \textit{tax}-expressing synoviocytes (28).

These are several reasons for choosing ribozyme-mediated, rather than antisense-based, strategies for gene therapy in the treatment of disorders such as HTLV-I infection. We reported previously that antisense ODNs against \textit{tax} successfully reduced Tax protein expression by up to 80% in HTLV-I-\textit{tax} transformed cells \textit{in vitro} and \textit{in vivo} (18). However, this approach required large doses (10–20 \textmu M) of \textit{tax} antisense ODNs to obtain a significant inhibition of Tax expression. Attempts to inhibit gene expression using antisense ODNs have often been complicated by non-antisense effects, such as direct interactions with proteins (hybridization-independent effects) or hy-

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**FIG. 6. Quantitative analysis of Tax protein synthesis.** Rat/Tax cells treated for 4 days with or without TR were incubated with an anti-Tax monoclonal antibody. The inhibitory effect of TR on Tax expression was analyzed quantitatively using flow cytometry. Specific staining of anti-Tax monoclonal antibody is represented by the open histograms (indicated as \textit{Tax}), while closed histograms represent nonspecific staining by the secondary antibody alone (indicated as \textit{Control}). A, untreated Rat/Tax cells (\textit{no treatment}). B, cells treated with 1 \mu M TR in the absence of HVJ-liposomes (\textit{naked}). C, cells treated with 1 \mu M TR complexed with 1 \times 10^{-2} diluted HVJ-anionic liposomes (\textit{anionic}). D, cells treated with 1 \mu M TR complexed with 1 \times 10^{-2} diluted HVJ-cationic liposomes (\textit{cationic}).

**FIG. 7. Immunostaining analysis of Tax protein degradation in Rat/Tax cells exhibiting TR accumulation.** A, Tax protein expression (\textit{orange}) was markedly decreased in Rat/Tax cells treated for 4 days with 1 \mu M FITC-labeled TR complexed with HVJ-cationic liposomes (\textit{green}). The Tax protein signal disappeared when TR accumulated in the cytoplasm and was translocated into the nucleus. A few cells that contained no TR still expressed Tax protein. Arrow, Tax-expressing cell; magnification, \times 1000. B, high Tax protein expression was evident in cells treated for 4 days with 1 \mu M FITC-labeled RR complexed with HVJ-cationic liposomes (\textit{green}, FITC-labeled ribozyme; \textit{orange}, TRITC-labeled anti-\textit{tax} monoclonal antibody; \textit{yellow}, co-localization of ribozyme and Tax protein; magnification, \times 1000).
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There was no indication of significant cytotoxicity, even when viability in Rat/Tax cells and mouse fibroblast, Balb/3T3 cells. The present study used a ribozyme designed to be transfection efficiency. The present study used a ribozyme designed to be transfection efficiency.

Both TR and RR successfully cleaved a target tax/rex mRNA in a cell-free system. The specificity of the TR or RR-mediated cleavage was supported by our findings that inactive ribozymes that contained mutations in their catalytic domains (TC and RC) showed no cleavage activity. However, only TR, but not RR, effectively cleaved tax mRNA in Rat/Tax cells. These results confirmed the sequence-specific activities of our synthetic ribozymes in vitro and in cultured cells. In the cultured cells, however, we also observed a nearly 20% reduction in Tax expression after treatment with TC. It is likely that this effect is due to antisense effects of the flanking sequences of TR, because a similar down-regulation of Tax expression occurred in the presence of tax antisense ODNs. Scherr et al. (32) recently reported a noticeable antisense effect of ribozymes, because the incubation of HeLa cells with inactive variants of hammerhead ribozymes targeted against N-ras also resulted in a 20% reduction of N-ras levels. Thus, whereas the effects of ribozymes in cultured cells mainly result from the cleavage activities of the ribozymes, other factors (e.g., antisense effects) also may contribute to the gene inhibition. Although these effects may only be weak, they should not be ignored.

Only a few studies have previously investigated the activity of ribozymes in the living cell, which is difficult for ribozymes to penetrate effectively (7, 9, 10). Several factors appear to contribute to the efficiency of ribozyme uptake and activity in living cells. For example, unmodified RNA is subject to rapid degradation by nucleases upon its delivery to the cells. The half-life of a hammerhead ribozyme in serum is less than 0.1 min (33). However, a combination of modifications, including the introduction of several phosphorothioate linkages at the 5'-ends of the ribozyme, can substantially increase ribozyme stability (34, 35). We therefore used hammerhead ribozymes containing extensive phosphorothioate modifications at the 5'- and 3'-ends, which have been shown to confer higher stability in serum than the 5'-end modification alone, without reducing the catalytic efficiency of the ribozyme (36). The efficacy of ribozymes in inhibiting gene expression also depends on their cellular uptake. Ribozymes generally have been introduced into cells by viral infection or by the transfection of expression vectors (6, 9, 10). Viral vectors can potentially lead to the incorporation of ribozyme-encoding genes into cellular chromosomes, thereby allowing their permanent expression. Consequently, many ongoing studies are aimed at developing suitable vectors that present a minimal risk to the host. However, in addition to the possibility that viral expression vectors may exert their own biological effects, they generally require long periods of incubation and usually have a low transfection efficiency. The present study used a ribozyme delivery system that employs cationic liposomes fused to the viral coat of HVJ. Ribozymes complexed with these HVJ-cationic liposomes showed a 15–20 times higher cellular uptake than naked ribozymes, and a 4–5 times higher uptake than ribozymes complexed with HVJ-anionic liposomes. We also assessed the effect of the HVJ-cationic liposomes on cellular viability in Rat/Tax cells and mouse fibroblast, Balb/3T3 cells. There was no indication of significant cytotoxicity, even when excess amounts of HVJ-liposome-complexed ribozymes were used (data not shown).

We recently evaluated in vivo immunogenic reactions by the repeated injection of HVJ-liposomes into lung of rats. Although anti-HVJ antibody titers were markedly elevated after the second administration of the HVJ-liposomes, the reporter gene was efficiently expressed, and only minimal inflammatory changes were detected after repeated administration. HVJ-liposomes adhere to and fuse with their target cells rapidly (i.e., within 1–2 min) (37), likely before they can be neutralized by antibody. No cytotoxic T-lymphocytes against HVJ were generated even after the repeated injection of HVJ-liposomes into the portal vein of rats. Thus, we conclude that HVJ-liposome-mediated method of gene transfer is safe, only weakly immunogenic, and highly efficient approach in living cells.

The efficiency of ribozymes also depends on the kinetics and location of their accumulation in the cells. The HVJ-cationic liposome-based gene transfer resulted in an accelerated transport of the ribozyme to the nucleus, with the nuclear localization of the ribozyme occurring in approximately 93% of the cells within 24 h. In contrast, HVJ-anionic liposomes were retained in the lysosomes, and ribozyme translocation into the nucleus had occurred in only 23% of the cells after 24 h of treatment. Finally, the naked ribozymes appeared to be localized in endosome vesicles and/or lysosomes for up to 24 h of treatment. This is consistent with the reported distribution of naked ODNs in cells (38). Naked ODNs and ODNs that are complexed with cationic lipids exhibit different intracellular behaviors as a result of their size difference (39). Thus, naked ODNs are taken up by pinocytosis, whereas ODNs associated with cationic lipids are taken up by phagocytosis and exhibit a delayed transfer to the lysosomes. Cationic liposomes can fuse with the cell membranes, allowing their content to be directly transferred into the cytoplasm, thus, avoiding their uptake by lysosomes (40). As a result, ribozymes complexed with treated with HVJ-cationic liposomes can escape lysosomal degradation by passing through the cell membrane, thereby leading to the accumulation of catalytically active ribozymes in the cytoplasm and nucleus.

Several factors may help to explain the difference in transfer efficiency and activity between ribozymes complexed with HVJ-cationic liposomes and HVJ-anionic liposomes. First, the trapping efficiency of ribozymes into the liposomes was about 6 times higher for HVJ-cationic liposomes than for HVJ-anionic liposomes. We previously analyzed the efficiency with which DNAs were trapped into liposomes (12, 41). We found that, whereas the trapping efficiency of HVJ-cationic liposomes was about 60%, the efficiency of HVJ-anionic liposomes was only about 10%. Thus, the use of cationic lipids facilitates the entrapment of large amounts of negatively charged molecules such as plasmids, proteins, ODNs, and ribozymes in the liposomes. Second, the fusion efficiency of cationic liposomes with HVJ appears to be about 5 times higher than that of anionic liposomes. Third, the strong negative net charge of anionic liposomes seems to reduce the possibility that these liposomes associate and fuse with the plasma membranes of cultured cells. In contrast, HVJ-cationic liposomes, which have a neutral net charge, fuse efficiently with the cells (15).

To evaluate the cellular effects of the ribozymes in more detail, we compared the effects of ribozymes complexed with diluted HVJ-cationic or HVJ-anionic liposomes. The densitometric analysis of Tax Immunoblot demonstrated that Tax levels were approximately 5 times lower in cells incubated with...

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3 M. Yoshida and Y. Kaneda, submitted for publication.
4 M. Hirano and Y. Kaneda, submitted for publication.
TR complexed with HVJ-cationic liposomes diluted by a factor of $1 \times 10^{-2}$, compared with TR complexed with equally diluted HVJ-anionic liposomes. Our findings suggest that HVJ-cationic liposomes are more effective in introducing ribozymes into cultured cells than HVJ-anionic liposomes, because the HVJ-cationic liposome-mediated delivery resulted in a more efficient ribozyme entrapment, a more effective cellular delivery, and an accelerated transport to the nucleus.

In summary, our results show that HVJ-cationic liposomes can be useful in delivering macromolecules such as ODNs and ribozymes into living cells. We believe that HVJ-cationic-liposome-mediated gene transfer method can be useful in molecular therapies for various diseases, including HTLV-I infection.

Acknowledgments—We thank Nobue Uto and Satoru Nonaka for their technical assistance.

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