RNAi Silencing of Exogenous and Endogenous Reporter Genes Using a Macrocyclic Octaamine as a "Compact" siRNA Carrier. Studies on the Nonsilenced Residual Activity

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A macrocyclic octaamine **1** having a covalently linked lipid-bundle structure was introduced as a new type of siRNA carrier. Gel electrophoresis, DLS, and SPR results indicate that it strongly binds to a luciferase-targeting 21-mer (42P) siRNA with a ratio of **1**/P ≅ 0.3 (**1**/N ≅ 2.4) to give remarkably compact **1**−siRNA complexes with an average size of ~10 nm. The **1**-mediated siRNA silencing of the exogenous luciferase gene occurs with a 90−95% efficiency. The overall suppression−[siRNA] profile with a 5−10% residual activity in the saturation region is commonly observed irrespective of the cell type (HeLa, HepG2, or HEK293), the order, or timing (stepwise or simultaneous) of supply of the siRNA and that of the luciferase-encoding plasmid, the level of mRNA transcribed, or the type of carriers (**1** vs lipofectamine 2000). The silencing of the endogenous DsRed2 gene stably incorporated in the genome of HeLa cells also has a similar overall profile. These results suggest that (1) the cellular uptake of the plasmid and that of the siRNA are basically independent of each other and (2) the incomplete silencing is not due to insufficient siRNA delivery. Implication of item 2 is briefly discussed.

INTRODUCTION

RNA interference (RNAi) is the posttranscriptional gene silencing process mediated by a 21−23-mer duplex siRNA (si = small or short interfering) which is homologous in sequence with the silenced gene (*1−5*). The siRNA taken up in the cells forms the so-called RISC (RNA-induced silencing complex) containing the antisense strand; consequently the complex then attacks the sense mRNA to result in degradation and hence silencing of the latter (*6*, *7*). The activity of synthetic siRNA was demonstrated for the first time by Tuschl et al., in terms of siRNA-dependent suppression of an exogenous reporter gene provided in the form of plasmid (*8*). This simple analysis was based on the assumption that a substantial portion of the plasmid-transfected cells also contains siRNA. Actually, the suppression, in this pioneering work, of pGL3 luciferase was complete (~100%) in Drosophila cells but less complete (90−95%) in mammalian cells (*8*). Efficient but incomplete silencing has been generally observed in many subsequently studies using mammalian cells (*9−15*).

We are concerned here about two matters. One is the nonsilenced "residual" ≥5% gene-expression activity. Does it simply reflect incomplete siRNA delivery or is it a consequence of essential incompleteness of RNAi? The other is size control of carrier−siRNA complexes, particularly in view of their in vivo performance. As is often a problem in artificial (nonviral) gene delivery, huge particles cannot simply diffuse into vascular periphery (*16*). Small particles, on the other hand, may be selectively taken up in malignant tissues by the so-called EPR (enhanced permeation and retention) effect (*17*). The size we aim at is ≤50 nm, which has not been precedented before for the siRNA complexes to the best of our knowledge.

We introduced a cone-shaped macrocyclic octaamine **1** of an amphiphilic character as siRNA carrier. We report here that it forms an unprecedented compact (~10 nm) complex with siRNA and mediates effective gene silencing but not completely regardless of the cell type, transfection order (siRNA vs plasmid), extent of gene transcription, carrier type (**1** vs lipofectamine 2000), or gene type (exogenous vs endogenous). This led us to suggest that the incomplete silencing should be interpreted in terms of intrinsic incompleteness of RNAi.

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EXPERIMENTAL PROCEDURES

Materials and General Analysis. Octaamine **1** in the form of octa(hydrochloride) was obtained from the corresponding free-base, prepared as described (*18*), upon treatment with HCl. Thus, 2 mL of concentrated hydrochloric acid was added to an ethanol solution (200 mL) of the free-base (200 mg). Most of the ethanol was removed and the white precipitates that separated were collected and dried in vacuo to give 225 mg (94%) of the octa(hydrochloride). Plasmids (pGL3, pGL2, and pGL3-Promoter), siRNAs, and lipofectamine were commercial products of Promega, TAKARA BIO, and Invitrogen, respectively. HeLa (Riken Cell Bank, RCB0007), HepG2 (Dainippon Pharmaceutical Co. Ltd., HB8-65), and HEK293 (Riken Cell Bank, RCB1637) cells were cultivated in DMEM (Dulbecco's modified Eagle's medium) containing 10% FCS (fetal calf serum) and antibiotics (penicillin−streptomycin, 50 μg/mL) with (HepG2) or without (HeLa, HEK293) 1% NEAA (nonessential amino acids) at 37 °C in a humidified atmosphere containing 5% CO_2. HeLa cells stably expressing DsRed2 were provided by Prof. Kazunari Taira and Dr. Yutaka Ikeda (Tokyo University) and were cultivated in a similar manner as above. Gel electrophoresis was performed with 0.7% agarose gel in Tris-acetate buffer and 20% polyacrylamide gel in Tris-borate-EDTA buffer for pGL3 and siRNA, respectively. Size distribution profiles (histograms) in reference to number of particles were obtained by NICOMP analysis of dynamic light scattering (DLS) data for solutions of pGL3 or siRNA (2 μM P) in water in the presence of varying amounts of octaamine **1** at 23 °C using a Particle Sizing Systems NICOMP 380 ZLS zeta potential/particle sizer at 100 mW green laser (532 nm). DLS measure-

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ments were also carried out for solutions of **1** with or without siRNA in the transfection medium Opti-MEM. However, the formation of big (≥50 nm) particles as a result of siRNA-independent aggregation processes prohibited detailed analysis of the **1**−siRNA complexes. Surface plasmon resonance (SPR) response curves were recorded on a BIAcore X system docked with a hydrophobized (HPA) sensor chip by repeated injection of an aqueous solution of octaamine **1** (100 μM, 25 μL) or siRNA (100 μM P, 25 μL) under the flow of degassed water (10 μL/min). Microscopic images were taken with an Olympus IX70 microscope.

Transfection and Subsequent Analysis. Cells (Hela, HepG2, or HEK293) were seeded at a density of 1.0×10^4 cells/well in a 96-well plate and cultivated. After 24 h, the cells became ~60% confluent and were washed with 100 μL of PBS(−) (magnesium/calcium-free phosphate buffer). To the cells in each well was added under cotransfection conditions a transfection medium (100 μL of Opti-MEM (GIBCO)) containing 200 ng of pGL3 (625 μM P) and 0, 2, 10, 42, or 126 ng of siRNA (0, 6, 30, 125, or 372 μM P) in the absence or presence of a carrier, octaamine **1** (1088 ng; **1**/P = 1.0 (N/P = 8.0) with respect to pGL3) or lipofectamine (500 ng, according to the manufacturer's protocol). After incubation for 6 h at 37 °C, the medium was replaced by 100 μL of fresh DMEM containing 10% FCS, and the cells were further incubated for 48 h. Under pretransfection conditions, the cells were first treated with an Opti-MEM solution (100 μL) containing siRNA (0, 2, 10, 42, or 126 ng) and octaamine **1** (1088 ng) or lipofectamine 2000 (500 ng) for 6 h, washed with 100 μL of PBS(−), and then transfected with pGL3 (200 ng) and lipofectamine (500 ng) in 100 μL of Opti-MEM. After 6 h, the medium was replaced by DMEM and incubation was continued for 48 h as above.

Luciferase assay was performed by the conventional chemiluminescence method using a Lumat LB5907 luminometer (Berthold Detection System) and the luciferase assay kit (Promega). Luciferase relative light units (RLU) integrated over 10 s are normalized for the total protein content of the cell lysate, obtained by the Bradford assay (Bio-Rad Laboratories) using bovine serum albumin as a standard. For MTT assay of lively cells, 20 μL of a solution of the MTT reagent (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide) in PBS(−) (5 mg/mL) was added to each transfection mixture in a well. After incubation for 3 h, the medium was removed, a 10% SDS solution (200 μL) was added, and the absorbance at 570 nm of the resulting homogeneous solution was measured.

Gene silencing in HeLa cells stably expressing DsRed2 was carried out in essentially the same manner as above. After washing with PBS(−), the cells were dissolved in 25 μL of PLB (passive lysis buffer, Promega). The resulting solution was divided into two parts; one (2.5 μL) was analyzed for the total proteins and the other (20 μL) for the fluorescence intensity at 580 nm (excitation at 563 nm) after dilution with 380 μL of PBS(−).

RESULTS AND DISCUSSION

Complexation Behavior. The new carrier introduced here is a cone-shaped macrocyclic octaamine **1** (Figure 1), having four long alkyl (undecyl) chains and eight amino groups on the opposite sides of the calix[4]resorcarene macrocycle. It is obtained as octaammonium octachloride. Because of its amphiphilic structure, it is what may be called a covalently linked lipid bundle. The free-base compound served as precursor of glycocluster compounds (*18*, *19*) capable of stoichiometric gene coating to give transfectious "glycoviruses" (*16*, *20*). Cationic lipid aggregates (cationic liposomes) and polymers have so far been extensively used as nonviral gene carriers (*21*−*23*). While being a small molecule (mw, 1740), compound **1** unimolecularly presents a characteristic amine/ammonium cluster motif with a well-defined geometry.

Figure 1. Structures of octaamine **1**, siRNA, and siRNA-relevant sequences (only sense strands are shown) of the luciferase genes in plasmids pGL3 and pGL2.

Figure 2. Electrophoretic gel shifts for pGL3 (a) and siRNA (b) in the absence (lane 1) and presence (lanes 2−6) of increasing amounts of octaamine **1** using 0.7% agarose gel in Tris-acetate buffer (a) or 20% polyacrylamide gel in Tris-borate-EDTA buffer (b); N and P stand for an ammonium group of octamine **1** and a phosphate moiety of the plasmid, respectively.

RNAi experiments were carried out using the same set of plasmid and siRNA (Figure 1) as used before (*8*). The plasmid is pGL3 (5256 base pairs) encoding a firefly protein luciferase under control of an SV40 promoter and an SV40 enhancer. The siRNA is a 21-mer duplex having two-nucleotide overhangs at the 3′-ends, the sense strand copying the 440−458 nucleotide sequence (shown in bold letters) of that of the luciferase gene in pGL3.

The complexation of octaamine **1** with pGL3 (a) and with siRNA (b) was monitored by gel (agarose and polyacrylamide, respectively) electrophoresis (Figure 2). The huge plasmid DNA duplex (pGL3) and the short linear RNA duplex (siRNA) are rendered completely immobile at a ratio of **1**/P in the range 0.1−0.2 (N/P 0.8−1.6) or 0.2−0.3 (N/P 1.6−2.4), respectively (N and P stand for an ammonium group of **1** and a phosphate moiety of DNA/RNA, respectively). Dynamic light scattering (DLS) also confirmed the complexation. When alone, octaamine

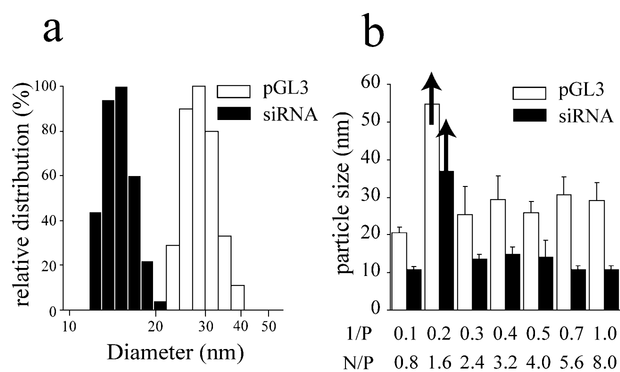

Figure 3. Typical size-distribution profiles (histograms) for the **1**−pGL3 and **1**−siRNA complexes at **1**/P = 0.4 (2 μM P) (a) and variation in the mean sizes thereof as a function of **1**/P or N/P (b) in reference to number of particles as evaluated by NICOMP analysis of the DLS data taken after 1 h from mixing of components. N and P stand for an ammonium group of octaamine **1** and a phosphate moiety of the plasmid, respectively.

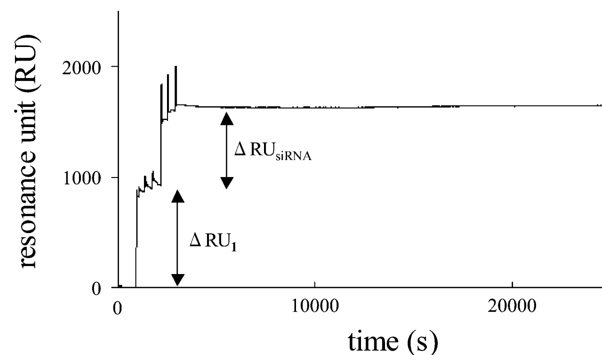

Figure 4. SPR response curve for the immobilization of octaamine **1** on a hydrophobized sensor chip and further adsorption of siRNA on the resulting monolayer of **1**. An aqueous solution of **1** (100 μM, 25 μL) or siRNA (100 μM P, 25 μL) was injected successively three times for immobilization of **1** or adsorption of siRNA, respectively, in the flow of water at a flow rate of 10 μL/min.

1 shows no tendency of forming aggregates of a size of ≥10 nm. However, in the presence of pGL3 or siRNA (2 μM P), the formation of particles (**1**-pGL3 or **1**−siRNA complexes) in that size range with a well-behaved size−distribution profile can be readily detected. In Figure 3a are shown typical histograms for the pGL3 and siRNA complexes at **1**/P = 0.4 (N/P = 3.2), both having a polydispersity index of 1.02. The mean sizes are plotted in Figure 3b as a function of **1**/P (N/P). The particle sizes are changeable and time-dependently grow at **1**/P = 0.2 (N/P = 1.6) probably as a result of cross-linking but are rendered time-stable and leveled off upon further increase in the amount of **1**. Saturation occurs at **1**/P ≅ 0.3 (N/P ≅ 2.4), and the saturation sizes for the **1**-pGL3 and **1**−siRNA complexes are d_{DLS} ≅ 30 and 10 nm, respectively, the latter being remarkably smaller than the size of cationic liposome−siRNA complexes (>100 nm) (*15*). The saturation ratio of **1**/P ≅ 0.3 or N/P ≅ 2.4 suggests that the complexation is primarily electrostatic in origin and that each siRNA molecule (42P) binds ∼12 molecules of **1**, having a width of 1.5−2.0 nm and a height of ∼2 nm (Figure 1). Amphiphile **1** in the cone shape may form small micelle-like nanoparticles but not huge vesicular/liposomal aggregates (*18*, *19*), and this may be at least partly why the present **1**−siRNA complex can be kept exceptionally compact (∼10 nm).

Surface plasmon resonance (SPR) shed more light on the **1**−siRNA interaction. Octaamine **1** (molecular weight, 1456) having four long alkyl chains can be readily immobilized on a hydrophobized (alkanethiol-coated) sensor chip (HPA) of SPR with an increase in resonance unit of ΔRU_1 = 960 (Figure 4). The latter, in light of the known relationship that ΔRU = 1000 corresponds to 1 ng/mm^2 of adsorbate, indicates that compound **1** is adsorbed with a packing density of 0.40 molecules/nm^2 or an occupation area of 2.5 nm^2/molecule, which is very close to the expected area of the octaammonium macrocycle with a diameter of 1.5−2.0 nm (Figure 1). Compound **1** must thus form a closely packed monolayer on the surface with its alkyl chains embedded in the hydrophobic forest of the sensor chip and the arrayed ammonium groups exposed to bulk water, as has been confirmed for the **1**-derived glycocluster compounds (*24*).

Treatment of the resulting **1**-coated sensor chip with siRNA gave a further increase in RU (Figure 4) as a result of adsorption of the siRNA (molecular weight per P or nucleotide, 320) via interaction with the ammonium groups on the surface. The interaction is strong or practically irreversible, since no notable dissociation of siRNA adsorbed was observed even after 24 h (ΔRU ≤ 20). The increase in RU of ΔRU_{siRNA} = 700 corresponds to a packing density of 1.3 P/nm^2 or an occupation area of 0.76 nm^2/P. These values, taken in conjunction with the corresponding values for octaamine**1** shown above, reveal the stoichiometry of **1**/P = 0.40/1.3 ≅ 0.3, which is in excellent agreement with that (**1**/P ≅ 0.3) evaluated from the DLS results. Neighboring phosphate groups in each strand of B-type duplex polynucleotides are separated by ∼0.7 nm from each other (*25*). The open conformation of the octaammonium motif of **1** has a diameter of ∼2 nm (Figure 1). Each molecule of **1** could thus cover three (2/0.7) P or nucleotide moieties with a stoichiometry of P/**1** ≅ 3. This is indeed what was observed (**1**/P ≅ 0.3). While the actual structure of complex **1**−siRNA is still not ready to be imaged, the present results and analysis suggest that (1) the anionic surface of siRNA is effectively coated with the cyclic array of the ammonium groups of rigid macrocycle **1** which is possibly micellized in small nanoparticles, (2) the coating stoichiometry is governed by steric factors, and (3) the resulting **1**−siRNA complex with insulated negative charges is free from further electrostatic aggregation.

Cotransfection. Cotransfection of cell culture was carried out by incubating HeLa (a human uterine cell line), HepG2 (a human hepatic cell line), or HEK293 (a human embryo kidney cell line) cells for 6 h in a transfection medium (Opti-MEM, 100 μL) containing a fixed amount (200 ng; 0.6 nM or 6.2 μM P) of pGL3 and a varying amount of siRNA in the presence of octaamine **1** as a carrier of both pGL3 and siRNA, followed by further incubation of the cells for 48 h in a fresh medium of DMEM (100 μL). The amount of carrier **1** (1088 ng per well) was set at **1**/P = 1.0 (N/P = 8) with respect to pGL3 to ensure complete complexation thereof. Each transfection mixture was analyzed for luciferase expression efficiency and for cytotoxicity. The cytotoxicity in reference to cell morphology and cell viability (Supporting Information) turned out to be slightly more pronounced than that of widely used lipofectamine 2000. In Figure 5 are shown the yields of luciferase, in terms of chemiluminescence relative light units (RLU) per mg total protein (top) or relative percentages (bottom), as a function of the amounts of siRNA (0, 2, 10, 42, and 126 ng; 0, 1.8, 7.0, 29, and 88 nM or 0, 0.08, 0.3, 1.2, or 3.7 μM P) for HeLa (a), HepG2 (b), or Hek293 (c) cells. The saturation-type yield-[siRNA] profile indicates that octaamine **1** acts as carrier for both pGL3 and siRNA. The **1**-mediated expression of luciferase becomes sharply suppressed with increasing amounts of siRNA but not perfectly. Thus, the suppression is evident at 2 ng of siRNA, reaches a plateau at ≥ 10 ng with 90−95% silencing, and remains in that level upon further increase in the amount of siRNA up to 126 ng. The suppression profiles with a 5−10% residual activity are remarkably similar or even superimposable for the three independent cell lines.

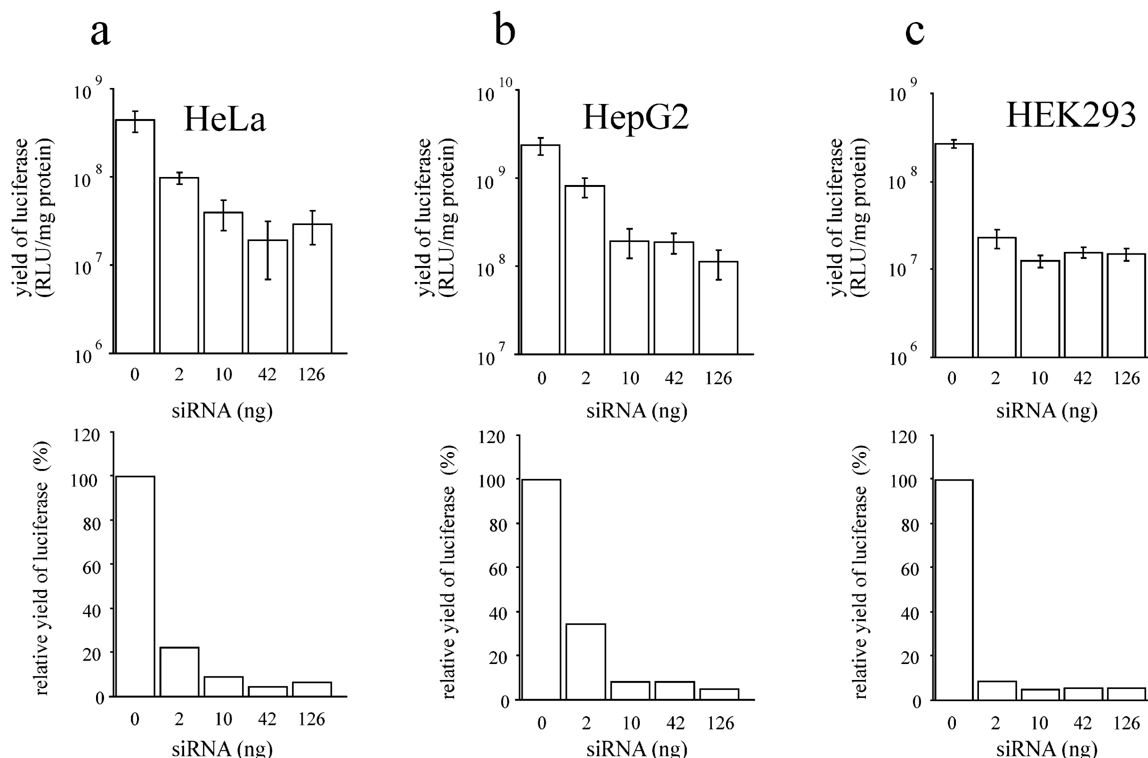

Figure 5. Transfection of HeLa (a), HepG2 (b), and HEK293 (c) cells under cotransfection conditions with siRNA and pGL3 (200 ng) as mediated by octaamine **1** (1088 ng). Yields of luciferase in terms of chemiluminescence relative light units (RLU) per mg total protein (top) or percentages relative to siRNA-free runs (bottom) are plotted as a function of the amounts of siRNA. Data are averages of about five experiments and error bars represent standard deviations.

Pretransfection. There are many conceivable explanations for the incomplete silencing. A simple one would assume that a small fraction (5−10%) of the cells cotreated with plasmid and siRNA actually incorporates only the plasmid and no siRNA for a statistical reason or as a result of competition between plasmid and siRNA. There is no way of efficient suppression in such plasmid(+)siRNA(− or Δ) cells. The question in a more general term is if or not the residual 5−10% activity arises anyhow from insufficient delivery of siRNA. If this is the case, the performance of the silencer siRNA might be improved when it is introduced in advance to enhance its population in the cells and to *wait* for its target, i.e., the mRNA coming from the plasmid taken afterward. If this pretransfection method is to work, the siRNA should be amply stable in the cells and should not seriously inhibit subsequent cellular uptake of the plasmid.

Thus, under the pretransfection conditions, the cells (HeLa, HepG2, or HEK293) were treated first with a mixture of siRNA (0−126 ng) and octaamine **1** (1088 ng) in Opti-MEM for 6 h, medium was removed, and the cells were washed for 1 h and then treated with a mixture of pGL3 (200 ng) and lipofectamine 2000 (500 ng) in Opti-MEM for 6 h, followed by further incubation for 48 h in a fresh medium of DMEM. It was in order to make the siRNA-delivery and plasmid-delivery steps distinguishable that we used different carriers for the plasmid and siRNA. We also used a reference gene contained in plasmid pGL2 (6047 base pairs) (Figure 1) (*8*). This also encodes luciferase but the key siRNA-relevant sequence (shown in bold letters) is different from that in pGL3 at three points underlined. Thus, the mRNA transcribed from pGL2 cannot be a target of the present siRNA. Each transfection mixture was analyzed for luciferase expression efficiency (Figure 6) and for cytotoxicity (Supporting Information), as above. An inspection of Figures 5 and 6 immediately shows that the overall feature remains unchanged as regards the general saturation profile and particularly the persisting 5−10% activity even at 126 ng of siRNA for the three different types of the cells. The siRNA introduced in the cells prior to the plasmid may competitively inhibit the uptake of the latter in a [siRNA]-dependent manner. This could explain the decreasing yields of luciferase with increasing [siRNA], but this cannot be the case. This is because the uptake of, and hence the expression of luciferase from, the RNAi-irrelevant pGL2 is not inhibited at all by the siRNA even at the highest 126 ng level (Figure 6, black bars). In this context, the [siRNA]-dependent suppression of pGL3 under the present pretransfection conditions should be ascribed to specific RNAi as in the case of cotransfection.

The similar suppression profiles under the cotransfection and pretransfection conditions can be interpreted as suggesting that cellular uptake of the plasmid and that of the siRNA are basically independent of (orthogonal with) each other. There is no evidence that the preintroduced siRNA notably inhibits subsequent uptake of the plasmid. There is no evidence either that the siRNA can be taken in the cells much more efficiently when alone under the pretransfection conditions than when in combination with the plasmid under the cotransfection conditions. Thus, the siRNA and the plasmid are not competitors with each other.

Control Runs. A couple of control runs were carried out. In one set of experiments, we used the pGL3-Promoter vector in place of pGL3; the former lacks the SV40 enhancer sequence which is present in the latter. With pGL3-Promoter, the nonsuppressed yields of luciferase at siRNA = 0 ng were one- or two-orders of magnitude lowered than those with the enhancer-containing pGL3 vector. Nevertheless, the general siRNA-suppression profile with a 5−10% residual activity under both cotransfection and pretransfection conditions (Supporting Information) remained remarkably the same as that shown in the bottom panels of Figures 5 and 6. In the other control, we carried out the same set of cotransfection and pretransfection experiments using lipofectamine 2000 in place of octaamine **1** under the otherwise identical conditions. All the results (shown in Supporting Information) not only reproduce the ~90%

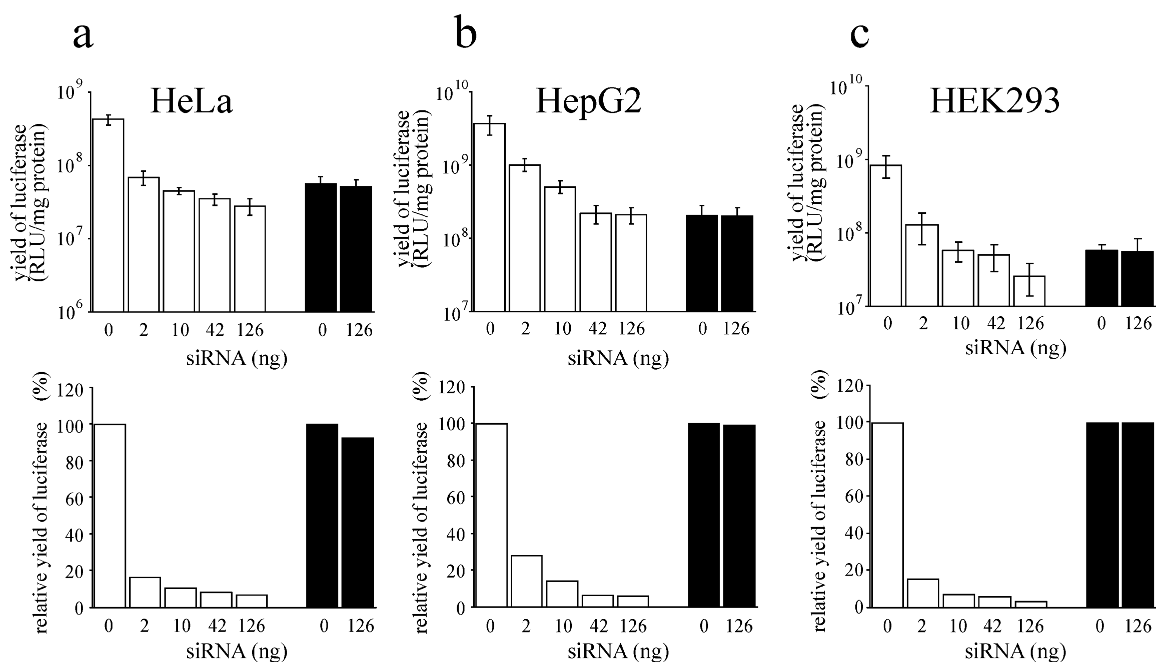

Figure 6. Transfection of Hela (a), HepG2 (b), and HEK293 (c) cells under pretransfection conditions with successive introduction of siRNA by the aid of octaamine **1** (1088 ng) followed by that of pGL3 (white bars) or pGL2 (black bars) (200 ng) by the aid of lipofectamine (500 ng). Yields of luciferase in terms of chemiluminescence relative light units (RLU) per mg total protein (top) or percentages relative to siRNA-free runs (bottom) are plotted as a function of the amounts of siRNA. Data are averages of about five experiments, and error bars represent standard deviations.

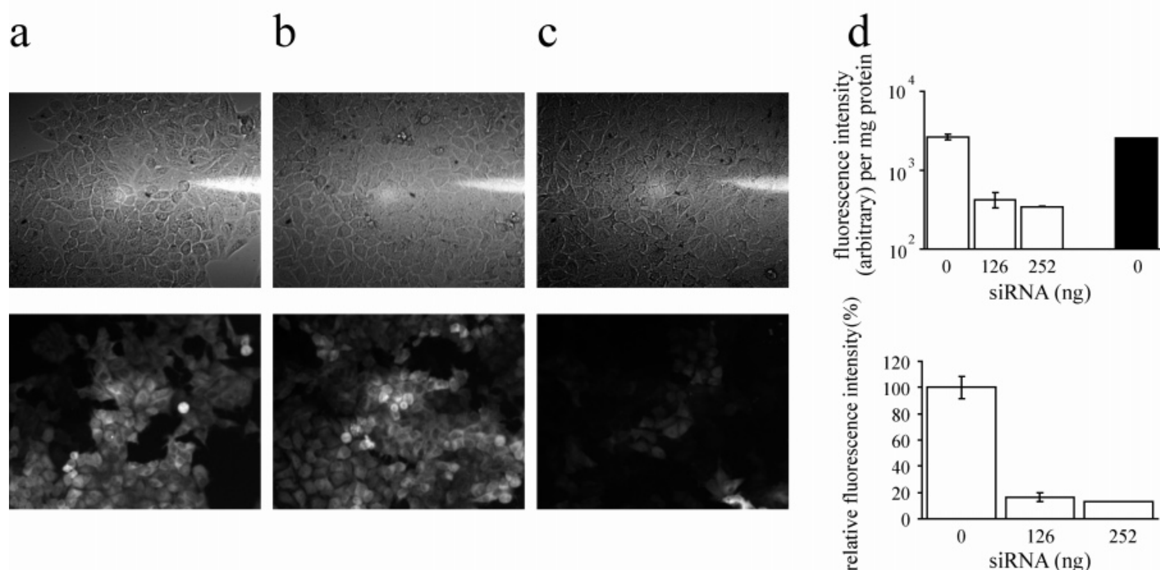

Figure 7. Optical micrographs (top) and their fluorescence images (bottom) of the DsRed2-expressing HeLa cells treated in Opti-MEM (100 μL) for 6 h. (a) In the presence of neither octaamine **1** nor siRNA, (b) in the presence of **1** (500 ng) without siRNA, and (c) in the presence of **1** (500 ng) and siRNA (126 ng). The fluorescence intensities (in arbitrary unit) normalized by the total protein content of the cell lysate (top) or as relative percentages (bottom) are plotted as a function of the amounts of siRNA in panel d. The black bar shows the reference intensity in the presence of neither **1** nor siRNA.

suppression previously reported (*8*) but also reveal that the overall feature with a saturation profile (*8*, *12*) is essentially the same as that for octaamine **1** (Figures 5 and 6). Thus, the surviving 5−10% activity in the saturation range is neither cell-sensitive nor carrier-dependent, in accord with a previous report (*15*).

Silencing of Endogenous Gene. Silencing of endogenous gene is practically more significant and allows more direct evaluation of the performance of siRNA. The target gene here is that for fluorescent protein DsRed2 stably incorporated in the genome of HeLa cells. The latter constantly fluoresces at 580 nm when excited at 563 nm (Figure 7a). In the presence of the DsRed2-targeting siRNA (126 ng) conjugated with octaamine **1**, the otherwise fluorescent $HeLa_{DsRed2}$ cells were rendered remarkably nonfluorescent (Figure 7c) as a result of siRNA-silencing of the endogenous DsRed2 gene. The suppression is 84% or 87% in the saturation range of siRNA = 126 ng or 252 ng, as evaluated by referring to relative fluorescence intensities (Figure 7d). The residual activity of 13−16% of the present endogenous gene is slightly higher than that of 5−10% observed above in case of exogenous luciferase but the overall features (Figures 5 and 6 vs 7d) are quite similar to each other.

Residual Gene-Expression Activity. We have so far investigated the siRNA silencing of the exogenous luciferase gene using three cell lines, two expression vectors with or without

an enhancer, and two carriers under cotransfection and pretransfection conditions. Different cells have different transfectabilities, giving rise to different yields of luciferase at siRNA = 0 ng. The amounts of mRNA transcribed can be more directly changed by changing the expression vectors for a given cell type. The two carriers have different size properties. Octaamine **1** forms small particles which coat the plasmid or siRNA into a compact size (~30 or ~10 nm, respectively), while lipofectamine forms huge liposomes to which the plasmid or siRNA is attached (*26*). Nevertheless, the effects of increasing amounts of siRNA turned out to be essentially identical, leaving a 5−10% nonsuppressed gene-expression activity. Similar profiles are found in the literature (*9*−*15*). One report shows that the delivery efficacy of siRNA is not affected by the particle size (*15*). As far as residual activity is concerned, there is no essential difference between the exogenous luciferase and the endogenous DsRed2 genes to be silenced. The common profile should be interpreted as reflecting something essential rather than accidental.

It is beyond the scope of this work to go into the mechanistic aspect of RNAi. Obviously, however, the incomplete silencing should not be ascribed to insufficient (relative to mRNA in concern) siRNA delivery. Therefore, it is not a fault of the siRNA carrier. Formally, the mRNA transcribed would be captured either by the ribosome to get decoded into protein or by the RISC (RNA-induced silencing complex containing the antisense strand of siRNA) to give the mRNA−RISC adduct for cleavage. In terms of ribosome−RISC competition, the present results should be interpreted as suggesting that the competition is 90−95% in favor of the RISC pathway and 5−10% of the ribosome route irrespective of the cell type. Another possibility is that the mRNA is well saturated with the RISC, and it is the mRNA−RISC adduct itself that is responsible for the incomplete silencing. There has been considerable recent discussion on the formulation and functions of the RISCs (*26*). On the basis of the type of constituent proteins, these complexes can be divided into two general types, a cleaving RISC and a noncleaving RISC, which direct the complementary target mRNA either for cleavage or for translational repression, respectively. Even the cleaving RISC has such dual functions, depending on the base-pairing features between the small RNA and the target mRNA (*27*, *28*). It is tempting, although by no means convincing, to attribute the present 90−95/5−10 selectivity to the selectivity of the cleaving/noncleaving pathways.

CONCLUDING REMARKS

This work may be summarized as follows. (1) Cone-shaped quadruple-chain/octaammonium amphiphile **1** serves as a nonliposomal lipid capable of complexing plasmid DNA and siRNA in compact sizes of ~30 and ~10 nm, respectively. Its performance in vivo will be a subject of future work. (2) The carrier-mediated siRNA-silencing of reporter genes in the present type of cells is generally 90−95% at best. The residual ≥5% nonsilenced activity is most likely not a fault of the carrier but a fault of the RNAi process itself, where the choice of ~20-nucleotide target sequences of a given mRNA would be of a primary importance (*10*, *11*, *29*−*32*). The abilities of siRNA carriers should be judged in terms of such factors as minimal effective quantity (*8*), speed of action, maximal possible toxicity (*15*), versatility/selectivity (*33*), and particularly in vivo performance. (3) The plasmid and the siRNA are independent in their cellular uptake. The stepwise or pretransfection method works equally as the simultaneous or cotransfection procedure. Such a plasmid/siRNA orthogonality poses a new question. Is that because they are remarkably different in size, because one is DNA and the other RNA, or because the cellular uptake of nucleic acids is reversible to such an extent as to make the timing of supply less important? These remain to be better answered. From a practical point of view, controlled timing of introduction of siRNAs may find its unique application in the sequential knockdown of multiple genes, although possible interference between similarly sized plasmids and/or siRNAs should be kept in mind.

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Supporting Information Available: Suppression profiles for lipofectamine 2000 as a carrier (Figures S1 and S2) and pGL3-Promoter as a control expression-vector (Figure S3), cell morphology (Figure S4), and cytotoxicity (Figure S5). This material is available free of charge via the Internet at http://pubs.acs.org/BC.

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