

## BRIEF COMMUNICATION

# Polyethylenimine-mediated cellular uptake, nucleus trafficking and expression of cytokine plasmid DNA

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Although polyethylenimine (PEI) has been widely used as a nonviral vector, there is little mechanistic understanding on PEI-mediated delivery. Here, we studied whether the expression of murine interleukin-2 (mIL-2) plasmids could be improved by complexation with PEI at various N/P ratios, and whether the cellular uptake, nuclear translocation, and retention of plasmids could be affected by the N/P ratios. Compared with the naked mIL-2, PEI/mIL-2 complexes showed at least two orders of magnitude higher expression at Raw264 cells in the N/P ratio-dependent manner. PEI-mediated cellular uptake and nuclear trafficking of plasmids, quantitated by competitive polymerase chain reaction, also depended on the N/P ratios showing the highest cell and nuclear levels of plasmids at 10/1. The higher cellular levels

of plasmid DNA after PEI-mediated delivery were also observed in other cell lines. Unlike naked plasmids, PEI/mIL-2 complexes (N/P ratios  $\geq 4/1$ ) showed prolonged cellular and nuclear retention of mIL-2 plasmids. The nuclear translocation and higher cellular level of plasmids given in PEI complexes were similarly observed by fluorescence microscopy. Moreover, PEI/mIL-2 complexes revealed high stability against DNase I, partly explaining the prolonged subcellular retention. These results indicate that the expression of plasmid mIL-2 might be highly enhanced by complexation with PEI and that such increased expression could be attributed by the higher cellular uptake, nuclear translocation and prolonged retention.

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Cytokine gene therapy has been studied for the treatment of cancer and infectious diseases, and for the development of effective molecular adjuvants.<sup>1</sup> Recently, cytokine genes have been administered in nonviral gene delivery systems to increase the stability and the efficiency of cytokine gene expression. Lipid-complexed forms of human interleukin-2 gene are currently in phase I clinical trial for prostate cancer.<sup>2</sup> Polyvinyl pyrrolidone-formulated cytokine genes resulted in higher antitumor immunity against head and neck cancer.<sup>3</sup>

Of nonviral vectors, polyethylenimine (PEI) has been studied as a versatile, inexpensive, and useful transfection system.<sup>4</sup> *In vivo*, PEI was shown to be an efficient transfection vector in several organs, such as the liver,<sup>5</sup> and lung.<sup>6</sup> Recently, *in vivo* fates and the prolonged organ retention of plasmid DNA administered in PEI complexes have been reported.<sup>7</sup> However, in the cellular level there is lack of understanding on the efficiency of PEI-mediated plasmid DNA delivery into the cell and the nucleus compartment. Moreover, despite that various N/P ratios have been used in PEI studies, the underlying

mechanisms by which the N/P ratios could affect the expression of plasmid DNA have not been well analyzed.

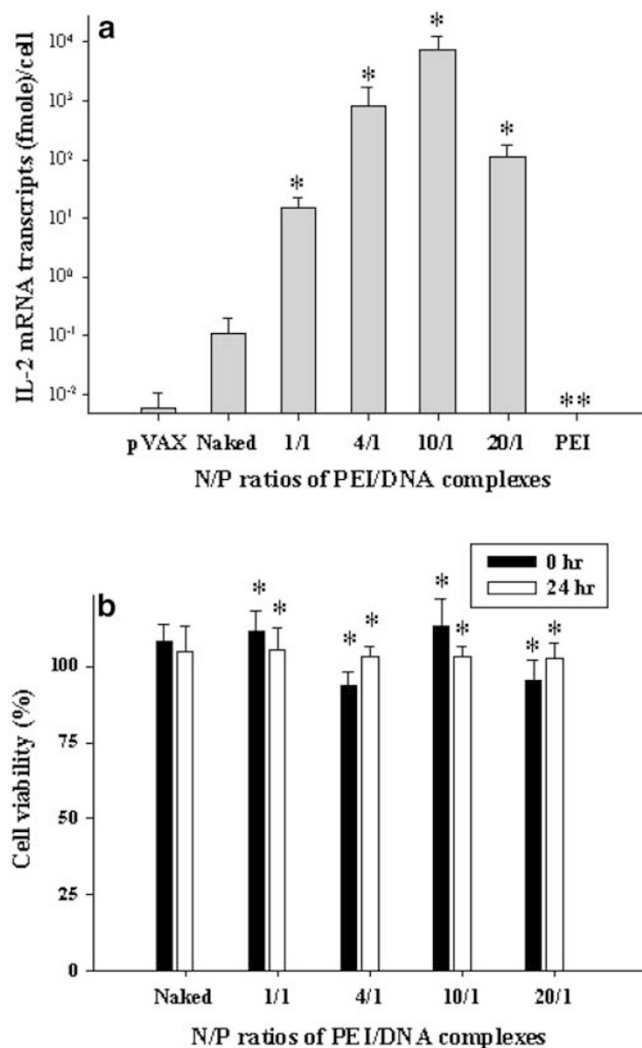
In this study, we aimed to test whether PEI could enhance the expression of murine interleukin-2 (mIL-2) plasmids in macrophage cell lines, and if so, whether the N/P ratios of PEI/mIL-2 complexes could affect the cellular uptake, retention, and nuclear trafficking of plasmid DNA. Here, we report that the cellular expression of cytokine plasmid DNA given in PEI complexes could be highly increased in the N/P ratio-dependent manner, and that the N/P ratios could influence the cellular uptake, nuclear translocation, and subcellular retention of plasmid DNA.

The mRNA expression of mIL-2 in macrophage cell lines could be orders of magnitude augmented by PEI-mediated delivery of mIL-2 plasmids (Figure 1a). Furthermore, the extent of enhancement depended on the N/P ratio. Empty pVAX1 plasmid, used as a control, showed as low as  $0.006 \pm 0.004$  fmole of mRNA/cell. Of various N/P ratios, 1/1 showed the lowest transfection efficiency, whereas 10/1 revealed the highest efficiency with more than four orders of magnitude higher mRNA expression levels relative to naked plasmid.

To determine whether the lower expression of PEI-complexed DNA at the N/P ratio of 20/1 than the 10/1 N/P ratio is due to the cytotoxicity of PEI, we performed the MTT assay for cell viability. Figure 1b shows that the cell viability of PEI-treated groups did not significantly

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**Figure 1** mIL-2 expression and viability of PEI/mIL-2 complex-treated cells. PEI/mIL-2 complexes of various N/P ratios were prepared by adding the appropriate amounts of 0.1 M PEI (25 kDa, Aldrich) to mIL-2 plasmid DNA in 5% glucose. After vortexing, the solution was incubated for 10 min at room temperature. (a) For expression study, Raw264 cells ( $1 \times 10^6$  cells/60-mm dish) were incubated for 2 h with 1  $\mu$ g of mIL-2 plasmid DNA in naked or PEI-complexed forms. As a control, the cells were treated for 2 h with PEI alone (30  $\mu$ M, equivalent to the concentration of PEI at the N/P ratio of 10/1). After 24 h incubation, total cellular RNA was extracted from the cells and the mRNA levels of mIL-2 were determined by quantitative RT-PCR using the synthetic standard mIL-2 RNA kindly provided by Dr Kagnoff (University of California, San Diego, CA, USA) as previously described.<sup>14</sup> The forward primer was 5'-GTCAA-CAGCGCACCACTTCAAGC-3' and the reverse primer was 5'-GCTTGTTGAGATGATGCTTTGACA-3'. The results are expressed as the mean  $\pm$  s.e. ( $n = 4$ ). \*Significantly different from naked DNA-treated group (ANOVA and Duncan's multiple range test,  $P < 0.05$ ). \*\*Under the detection limit ( $1.6 \times 10^{-5}$  fmole/cell). (b) Cell viability was assessed by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay as described previously.<sup>15</sup> In brief, at 0 and 24 h after the cells were treated with naked or PEI-complexed plasmids for 2 h, the cells were added with 1.2 mM of MTT solution. After incubation at 37°C for 2 h, the dark blue crystals were thoroughly dissolved with 100% ethanol. After a few minutes of incubation at room temperature, the absorbance was read at a test wavelength of 570 nm and a reference wavelength of 630 nm. Absorbance readings taken from survival cells were converted to percentage cell viability. The cell viability of untreated cells was regarded as 100%. The results are expressed as the mean  $\pm$  s.e. ( $n = 4$ ). \*Not significantly different from naked DNA-treated group (ANOVA,  $P > 0.05$ ).

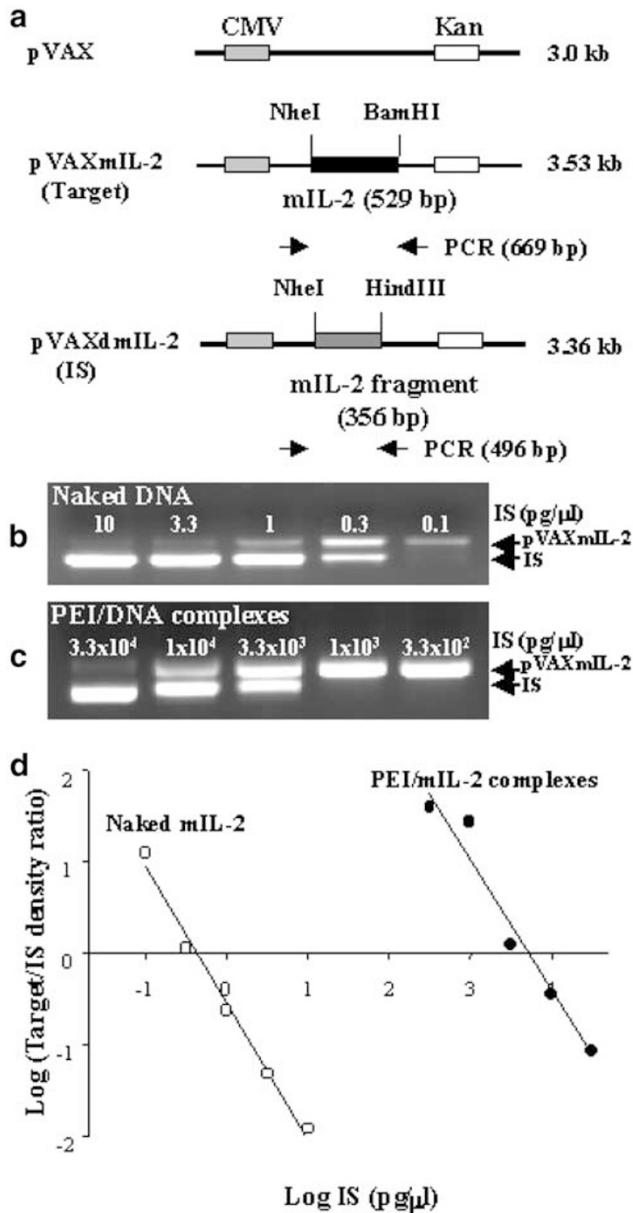
differ from that of naked plasmid DNA-treated group (ANOVA,  $P > 0.05$ ). This result indicates that the less effective mRNA expression of PEI-complexed plasmid DNA at the highest N/P ratio may not be attributed by the cytotoxicity of PEI.

To explore the contribution of cellular uptake process to the enhanced expression of mIL-2 genes delivered in PEI complexes, the amounts of plasmid DNA taken up by the cells were determined using a competitive and quantitative polymerase chain reaction (PCR). Quantitative PCR is based on co-amplification of the sample template together with various amounts of internal standard competitor molecule (IS) sharing with the target the primer recognition sites, but differing in size. For quantitative PCR, a 173 bp-deleted mutant IS plasmid was constructed to share the same primers used for target amplification (Figure 2a). As an example, the gel bands and internal standard curves of competitive PCR are shown for nuclear DNA extracts obtained at 24 h after treatment of naked DNA or PEI/DNA complexes (N/P ratio, 10/1). The resulted PCR product was 669-bp and 496-bp for the target and the IS, respectively. The band densities of the target gene PCR product diminished as the amounts of IS increased (Figure 2b and c). The quantitation of gel band density generated internal standard curves of high linearity (Figure 2d). The amounts of plasmid DNA in the samples were calculated from each internal standard curve based on the x-axis intercept, where the log of the ratio between the PCR product originating from the sample and the corresponding IS becomes zero. In this study, thanks to the competitive PCR technique sensitive enough to detect the fg/ $\mu$ l level of plasmids in the samples, we could quantitate the cellular and nuclear levels of plasmids after delivery of plasmid DNA in various forms.

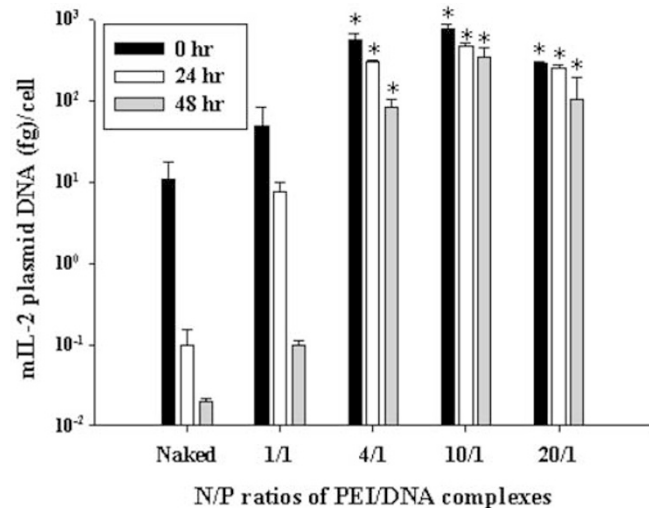
Although PEI/mIL-2 complexes of various N/P ratios resulted in much higher cellular uptake than the naked plasmid treatment, the N/P ratio of the complexes significantly affected the extent of the uptake (Figure 3). Immediately after the cells were treated with naked DNA for 2 h, the cellular level of plasmid DNA was  $11 \pm 7$  fg/cell. As compared with the naked plasmid DNA, PEI-complexed DNA showed 52- and 69-fold higher cellular mIL-2 plasmid levels at the N/P ratios of 4/1 and 10/1, respectively. However, when the N/P ratio was 20/1, the cellular uptake of plasmid DNA was only 26-fold higher relative to naked DNA.

PEI complexation prolonged the cellular retention of plasmid DNA (Figure 3). In case of naked pVAXmIL-2, the cellular levels of plasmid DNA rapidly decreased with time, showing as low as  $0.02 \pm 0.002$  fg plasmid DNA/cell at 48 h. Similarly, substantial reduction of plasmid mIL-2 levels was observed in the PEI/DNA complex with the N/P ratio of 1/1. Unlike naked DNA, the PEI/DNA complexes with the N/P ratios  $\geq 4/1$  displayed more prolonged retention of plasmid DNA in the cells (ANOVA and Duncan's multiple range test,  $P < 0.05$ ). At 48 h, the cellular level of plasmid DNA was 17 400-fold higher in the cells treated with PEI/DNA complexes of the N/P ratio of 10/1 than the cells incubated with naked DNA. Moreover, at each time-point the N/P ratio of 10/1 showed significantly higher levels of cellular DNA as compared with other N/P ratios (ANOVA and Duncan's multiple range test,  $P < 0.05$ ).

The higher cellular uptake and prolonged retention of



**Figure 2** Representative gel and internal standard curves for quantitative PCR of pVAXmIL-2. (a) pVAXmIL-2 was constructed by inserting the 529-bp mIL-2 gene from pCIneoIL-2 into the pVAX1 vector (Invitrogen, Carlsbad, CA, USA) using NheI and BamHI restriction enzymes. The competitor internal standard (IS), a 173-bp deleted mutant of pVAXmIL-2, was constructed by inserting the 356-bp partial fragment of mIL-2 gene from pCIneoIL-2 into pVAX1 vector using NheI and HindIII restriction enzymes. (b, c) Total nuclear DNA was extracted from the cells at 24 h after treatment of naked plasmid (b) or PEI/DNA complexes of N/P ratio, 10/1 (c). Competitive PCR was performed by adding various amounts of IS to the reaction mixtures containing the unknown amounts of sample DNA. The amounts of IS varied from 0.1 to 10 pg (b) and 330 to 33000 pg (c). The primers used for competitive PCR were 5'-ACGACTACTA-TAGGGAGACCC-3' (sense) and 5'-CAACTAGAAGGCACAGTC GAGG-3' (antisense). The PCR product of IS plasmid was 496-bp as compared with 669-bp of the wild-type. PCR was performed in a 0.2 ml reaction tube containing 50 μl of PCR mixture composed of 1 μl sample DNA, 1 μl the competitor plasmid, 4 μl 2.5 mM dNTPs, 5 μl 10× reaction buffer (50 mM Tris pH 8.0, 100 mM NaCl, 0.1 mM EDTA, 1 mM dithiothreitol, 50% glycerol, and 1% Triton X-100), 10 pmole each primer, and 1 U Taq polymerase. The mixture was cycled in a Perkin-Elmer/2400 thermocycler at 95°C for 40 s, 56°C for 30 s, and 72°C for 30 s for 33 cycles. (d) Calibration curves were used to determine the quantity of the target DNA in the samples. The density of each band was measured using a gel-doc image analyzer (Vilber Lourmat, France). In each competitive PCR run, an internal standard curve was obtained by plotting the log values of target/competitor density ratios against the log amounts of IS initially added to the reaction.



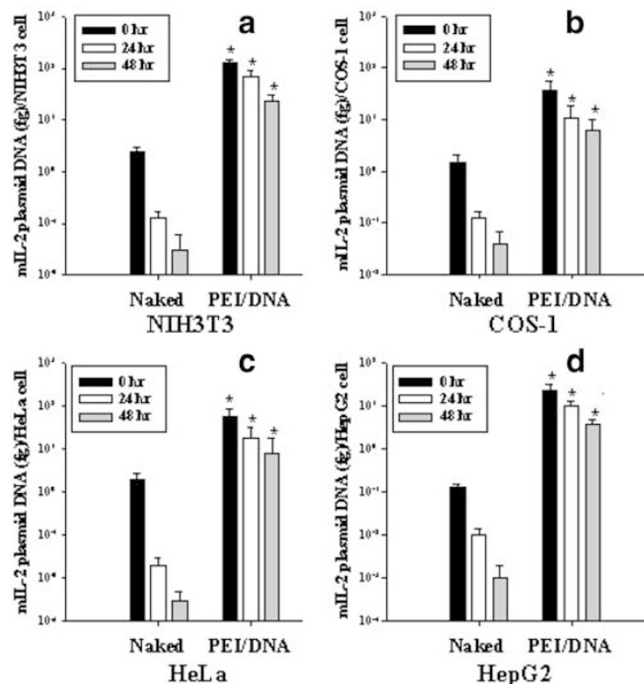
**Figure 3** Uptake and retention of mIL-2 plasmid DNA in Raw264 cells. The macrophage cell lines, Raw264 cells were maintained in RPMI-1640 medium supplemented with 10% heat-inactivated fetal calf serum. Cells ( $1 \times 10^6$  cells/dish) were incubated for 2 h with 1 μg of plasmid DNA in naked or PEI-complexed forms. The cells were then washed, and replaced with culture medium. At various time-points, the pellets of cell harvests were resuspended in 300 μl of TE buffer (pH 8.0), added with 300 μl of the lysis buffer (50 mM Tris-HCl, 10 mM EDTA, 5% β-mercaptoethanol, 0.4% sodium dodecyl sulfate), proteinase K (1 mg/ml), RNase (0.1 mg/ml), and incubated for 1 h. Subsequently, phenol/chloroform solution was added to the cell lysates, and DNA was obtained by the ethanol-precipitation method. The cellular levels of pVAXmIL-2 were measured by quantitative PCR. The results are expressed as the mean ± s.e. (n = 4). \*Significantly different from naked DNA-treated group (ANOVA and Duncan's multiple range test,  $P < 0.05$ ).

PEI-complexed plasmid DNA was also observed in other cell lines (Figure 4). The higher cellular uptake of PEI-mediated plasmid delivery was shown in various cell lines such as NIH3T3 fibroblast, COS-1, HeLa and HepG2 cell lines. Although the initial uptake levels of plasmid DNA after 2 h of incubation differed among the cell lines, the higher uptake and prolonged retention patterns of PEI-complexed plasmid DNA were consistently observed in all the cell lines tested in this study. At 48 h after treatment, the cellular DNA levels of PEI-complexed group were 570-, 168-, 1326-, 3707-fold higher in PEI-complexed group as compared with the corresponding naked DNA-treated groups for NIH3T3, COS-1, HeLa and HepG2 cell lines, respectively. This result indicates that the higher uptake and retention patterns of plasmid DNA after PEI-mediated delivery might not be limited to the macrophage-like cells, rather applicable to other cell lines.

To further understand the mechanisms by which PEI-complexation increased the cellular expression of mIL-2

plasmids, we studied the nuclear translocation and retention of pVAXmIL-2 at Raw264 cells. The nuclear trafficking of plasmids given in PEI complexes significantly differed from that of naked plasmids (Figure 5). The intracellular distribution of plasmid DNA to nucleus was

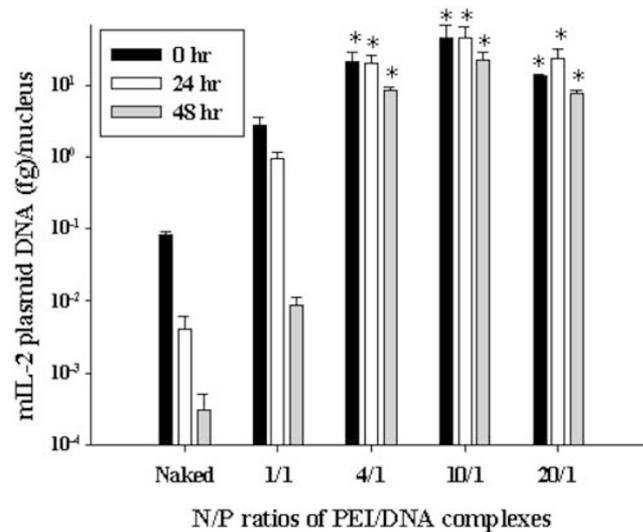




**Figure 4** Uptake and retention of mIL-2 plasmid DNA in various cell lines. Various cell lines were maintained in DMEM (NIH3T3, HepG2) or RPMI-1640 (COS-1, HeLa) medium supplemented with 10% heat-inactivated fetal calf serum. Cells ( $1 \times 10^6$  cells/dish) were incubated for 2 h with 1  $\mu$ g of plasmid DNA in naked or PEI-complexed forms (N/P ratio, 10/1). The cells were then washed, and replaced with culture medium. At each time-point, the pellets of cell harvests were resuspended in 300  $\mu$ l of TE buffer (pH 8.0), added with 300  $\mu$ l of the lysis buffer (50 mM Tris-HCl, 10 mM EDTA, 5%  $\beta$ -mercaptoethanol, 0.4% sodium dodecyl sulfate), proteinase K (1 mg/ml), RNase (0.1 mg/ml), and incubated for 1 h. Subsequently, phenol/chloroform solution was added to the cell lysates, and DNA was obtained by ethanol-precipitation method. The cellular levels of pVAXmIL-2 were measured by quantitative PCR. The results are expressed as the mean  $\pm$  s.e. ( $n = 3$ ). \*Significantly different from naked DNA-treated group (Student's *t* test,  $P < 0.05$ ).

studied at 0, 24 and 48 h after treatment. Immediately after 2 h treatment of the cells with PEI/DNA complexes of the various N/P ratios, the nuclear translocation of naked plasmids was the highest at the N/P ratio of 10/1 followed by 4/1, 20/1, and 1/1. In addition to the enhanced trafficking to nucleus, PEI/DNA complexes showed prolonged retention in the nucleus. At 48 h after treatment of the cells with naked DNA, the nucleus levels of plasmid DNA represented as much as a 267-fold decrease from the 0 h level. In contrast, PEI/DNA complexes with the N/P ratios  $\geq 4/1$  revealed much slower decline in the nucleus levels of plasmid DNA (ANOVA and Duncan's multiple range test,  $P < 0.05$ ). At 48 h after treatment with PEI/DNA complexes with the N/P ratios (4/1, 10/1), the nucleus levels of plasmid DNA were lowered to only less than three-fold. These results indicate that the nucleus retention of plasmid DNA could be prolonged by delivery of plasmids in PEI complexes of the proper N/P ratios.

The fluorescence microscopy data support the higher cellular level and nuclear translocation of plasmids given in PEI complexes. After treatment with fluorescent dye-labeled naked plasmids, no visible fluorescence was observed in the nucleus (Figure 6a and c). In contrast, fluorescent dye-labeled plasmids given in PEI complexes



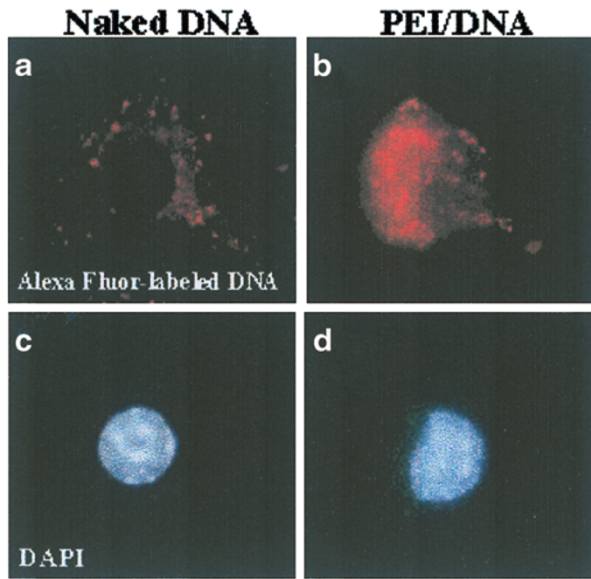
**Figure 5** Nuclear translocation and retention of mIL-2 plasmid DNA. At various time-points after 2 h treatment with plasmid DNA of various forms, nuclear DNA was isolated from Raw264 cells according to the method of Clesham et al.<sup>16</sup> In brief, at each time-point the cells were washed with PBS, then resuspended in 400  $\mu$ l of Nonidet P-40 lysis buffer at 4°C. The cells were maintained on ice for 3 min and centrifuged at 8000 r.p.m. for 5 min. The supernatant was removed, and the nuclear pellet was resuspended in 50  $\mu$ l of proteinase K solution (50  $\mu$ g/ml proteinase K, 10 mM Tris, pH 7.8, 5 mM EDTA, and 0.5% SDS), and incubated for 6 h at 37°C. The nuclear DNA was extracted using phenol/chloroform, precipitated with ethanol, and resuspended in 20  $\mu$ l of Tris EDTA buffer. The levels of pVAXmIL-2 in the nuclear extracts were measured by quantitative PCR. The results are expressed as the mean  $\pm$  s.e. ( $n = 4$ ). \*Significantly different from naked DNA-treated group (ANOVA and Duncan's multiple range test,  $P < 0.05$ ).

(Figure 6b and d) showed intense spots in the nucleus and the higher cellular levels of DNA at 6 h after treatment.

Given the cellular and nuclear retention results, we next examined the stability of PEI/DNA complexes against DNase I. From 5 min after treatment of naked plasmid DNA with 0.1 units of DNase I, the intact form of plasmid DNA disappeared and only the smaller fragments of nucleotides were observed (Figure 7a). However, PEI/DNA complexes (Figure 7b) did not show any sign of degradation even after 8 h of incubation with DNase I (1 unit), indicating the higher stability of PEI/DNA complexes against DNase I.

In this study, we demonstrated that the expression of mIL-2 plasmids could be enhanced by complexation with PEI in proper N/P ratio, and that such higher transfection could be contributed by orders of magnitude higher PEI-mediated cellular and nuclear delivery of mIL-2 plasmid DNA. Moreover, we report that the cellular and nuclear retention of mIL-2 plasmid DNA could be significantly prolonged by PEI complexation.

Our observation that the transfection efficiencies of PEI/mIL-2 complexes depended on the N/P ratios agrees with the previous report which showed the highest transfection efficiency after delivery of luciferase-encoding plasmids in PEI complexes at the N/P ratio of 10/1.<sup>8</sup> In this study, we further analyzed the mechanisms by which the N/P ratio could affect the transfection efficiency. The N/P ratio-dependent cellular and nucleus levels of plasmid DNA given in PEI complexes (Figures 3 and 5) indi-



**Figure 6** Cellular distribution of plasmid DNA given in naked or PEI-complexed forms. pVAXmIL-2 was labeled using an ARES Alexa Fluor 546 DNA labeling kit (Molecular Probes, Eugene, OR, USA) according to the manufacturer's instructions. Raw264 cells ( $3 \times 10^5$  cells/12-mm coverslip) were incubated for 2 h with 1  $\mu$ g of Alexa Fluor 546-labeled plasmid DNA in naked (a, c) or PEI-complexed forms (b, d). The cells were then washed, and replaced with culture medium. At 6 h, the cells were fixed for 0.5 h in periodate-lysine-paraformaldehyde as described previously,<sup>17</sup> and the nucleus was stained with diamino-2-phehyindole (DAPI). The cells were observed under a Nikon TE-300 epifluorescence microscope ( $\times 1000$ ). Each image is representative of about 250 cells observed in this study.

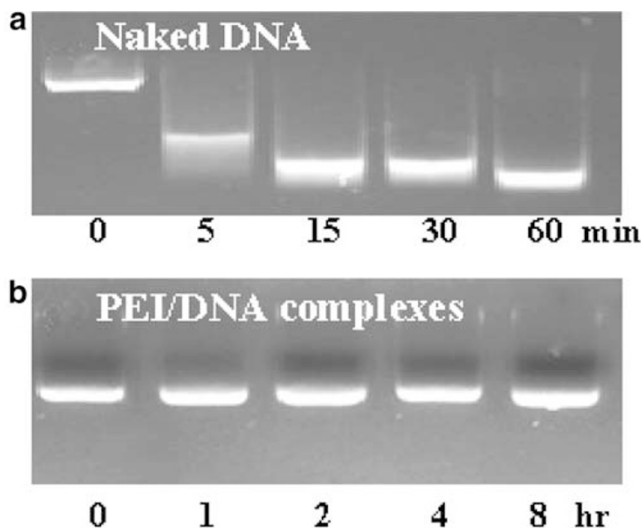
cate that the highest transfection efficiency of plasmid DNA offered by the N/P ratio, 10/1, might be attributed to the higher cellular uptake and nucleus trafficking of plasmid DNA.

Previously, Godbey *et al*<sup>9</sup> suggested that PEI alone may have some biological effects after localization to the nucleus. To determine whether PEI itself can induce the expression of mIL-2 in the cells, the expression of mIL-2 was tested after PEI single treatment. The treatment of PEI 30  $\mu$ M (equivalent to the PEI concentration of PEI/DNA complex at the N/P ratio of 10/1) did not induce the detectable amount (detection limit in this study:  $1.6 \times 10^{-5}$  fmole/cell) of mIL-2 by the quantitative RT-PCR. Given the PEI alone control data (Figure 1a), it is unlikely that the enhanced expression of mIL-2 in PEI-DNA-treated groups could be due to the induction of endogenous IL-2 by PEI *per se*.

Although plasmid DNA given in various PEI complexes showed at least more than one order of magnitude higher cellular uptake than did naked plasmids, the N/P ratio of 10/1 showed the highest cellular uptake. The mechanism by which plasmids given in PEI complex of the specific N/P ratio resulted in the highest gene transfer into the cells remains to be further elucidated. Considering that PEI-complexed plasmids could be taken up into the cells by adsorptive endocytosis,<sup>10</sup> we cannot exclude the possibility that the positive zeta potential<sup>8</sup> of PEI/DNA complexes at the N/P ratio of 10/1 enhanced the adsorption of plasmids on the cell surface, resulting in more efficient endocytosis. However, the lower cellular uptake of plasmid DNA delivered in the N/P ratio of 20/1 with the highest zeta potential<sup>11</sup> implies that there might be additional factors other than zeta potential, contributing to the higher uptake of PEI-complexed plasmids at the N/P ratio of 10/1.

The prolonged cellular and nuclear retention of plasmid DNA after delivery in PEI complexes might be attributed to the enhanced protection and stability of PEI-complexed plasmids to nucleases (Figure 7) and other degradative elements in the nucleus. It has been proposed that the degradation of plasmid DNA by cytosolic nucleases might be a potential barrier of nuclear gene delivery.<sup>12</sup> The stability of PEI/DNA complexes against nucleases indicates that the PEI/mIL-2 complexes might exist in a more stable form in the cytosol and get a higher chance of entering the nucleus. Our observation about PEI-mediated nuclear delivery is consistent with the previous finding of Pollard *et al*<sup>13</sup> who reported the nuclear localization of plasmid DNA given in PEI-complexed forms. In this study, we found that the extent of nuclear delivery could be affected by the N/P ratios and that the nuclear retention time might be prolonged by PEI complexation. The nuclear translocation of PEI previously reported by Godbey *et al*<sup>9</sup> and the prolonged nuclear retention of plasmids given in PEI complexes (Figure 5) imply that plasmid DNA might exist in PEI-complexed forms in the nucleus. Moreover, the nucleus trafficking pattern of plasmid DNA delivered in PEI-complexed forms (Figure 5) showed similarity to the cellular uptake pattern (Figure 3), although the magnitude of plasmid levels differed between the cell and the nucleus. Such similarity indicates the possibility that PEI-complexed plasmid DNA might enter the nucleus by diffusion, rather than some active transport mechanisms.

In conclusion, we report that the enhanced expression



**Figure 7** Stability of PEI/mIL-2 complexes against DNase I. (a) Naked pVAXmIL-2 (1  $\mu$ g) was incubated with 0.1 unit of DNase I (Gibco BRL, Grand Island, NY, USA) in buffer (20 mM Tris-HCl, pH 8.4, 2 mM  $MgCl_2$ , 50 mM KCl). (b) One  $\mu$ g of PEI/mIL-2 complex (N/P ratio, 10/1) was incubated with 1 unit of DNase I. At each time-point, the samples were mixed with 0.3% sodium dodecyl sulfate, electrophoresed on a 0.8% agarose gel, and stained with ethidium bromide.

of mIL-2 plasmid delivered in PEI complexes of suitable N/P ratios might be attributed by the orders of magnitude higher cellular uptake, nuclear trafficking, and prolonged subcellular retention of plasmid DNA. Moreover, our results suggest that PEI complexation might be a useful strategy to increase the expression and the delivery of cytokine genes to immune cells, such as macrophages and other cell lines.

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