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Kinetic analysis of protein production after DNA transfection

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Abstract

The production of an exogenous protein by the transfection of a plasmid DNA encoding the protein was kinetically analyzed, to determine the efficiency of the transfection. Cultured NIH3T3 or HeLa cells, and the luciferase protein were used as a model system in this experiment. The findings indicate that at least a 8×10^4- and 4×10^3-fold molar amounts of luciferase protein was produced from one copy of the plasmid DNA molecule in NIH3T3 and HeLa cells, respectively. The rate of elimination of luciferase activity upon DNA transfection was smaller than that for the luciferase protein itself (k_{el} for DNA transfection $< k_{el}$ for the luciferase protein), suggesting that a decrease in intranuclear active DNA was the main determinant of the elimination rate in this case. A preliminary pharmacokinetic model is proposed, based on the results obtained.

Keywords: DNA transfection; AUC; Intranuclear disposition

1. Introduction

Gene delivery is a promising approach and considerable efforts have been made to improve the efficiency of protein production (Mahato et al., 1997; Rolland, 1998; Kamiya et al., 2001; Niidome and Huang, 2002). To achieve successful protein production, an efficient and targeted gene delivery system in which intracellular trafficking is also considered is necessary. In addition, protein production from exogenous genes is a transient phenomenon in most cases of non-viral vectors. We previously proposed that the 'controlled intranuclear disposition' of delivered genes would also be highly important for achieving practical gene therapy (Kamiya et al., 2003). Thus, it would be both interesting and important to evaluate protein production as the result of DNA transfection in a quantitative manner.

In this study, we report on the analysis of protein production in cultured mammalian cells by DNA transfection with cationic lipids, in an attempt to better understand the kinetic features that are involved. The luciferase protein was chosen as a model protein because its activity decreases relatively rapidly (Nguyen et al., 1989; Thompson et al., 1991) and

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this characteristic would be suitable for the analysis. Based on our kinetic analysis, it was estimated that huge amount of luciferase protein could be produced from one copy of the plasmid DNA molecule in mouse NIH3T3 cells. The rate of elimination of luciferase activity when DNA transfection was used was much smaller than that for the protein itself, suggesting that a decrease in intranuclear active DNA was a major factor in the rate of elimination of luciferase activity. Similar tendencies were obtained with HeLa cells. A preliminary pharmacokinetic model is proposed, based on the results obtained in this study.

2. Experimental

2.1. Materials

The firefly (*Photinus pyralis*) luciferase protein (molecular weight: 1.2×10^5) was obtained from Sigma–Aldrich (St. Louis, MO, USA). The pcDNA 3.1 (+)-luc 2 plasmid (7037 bp) was constructed by inserting the firefly luciferase gene (*Hin*dIII–*Xba*I fragment) of the pGL3-Control plasmid (Promega, Madison, WI, USA) into the pcDNA 3.1 (+) plasmid (Invitrogen, Groningen, the Netherlands) pretreated with the same restriction enzymes. The luciferase gene in the pcDNA 3.1 (+)-luc 2 plasmid is expressed under the control of the cytomegalovirus promoter. The pTriEx-3 Neo plasmid was obtained from Novagen (Madison, WI, USA). These plasmid DNAs were purified with a Qiagen (Hilden, Germany) Plasmid Mini Kit.

2.2. Protein and DNA deliveries

NIH3T3 cells (4×10^4 cells/well) were incubated in DMEM medium supplemented with 10% fetal calf serum under an atmosphere of 5% CO_2/air at 37 °C for 24 h. The luciferase protein (1 and 3 μg (8.3 and 25 pmol)) was mixed with 0.4 and 1.2 μg, respectively, of Chariot reagent (Active Motif, Carlsbad, CA, USA) and incubated at room temperature for 30 min. The cells were washed with phosphate-buffered saline (PBS), and the protein–Chariot mixture (total 100 μl) and DMEM medium without serum (100 μl) were added to the cells. After a 1 h incubation under 5% CO_2 at 37 °C, 1 ml of DMEM medium supplemented with 10% serum was added, and the cells were incubated at 37 °C. The cells were washed with PBS before the luciferase assay.

DNA transfection was carried out with the Lipofectamine Plus Reagent (Invitrogen) essentially according to the supplier's instructions. NIH3T3 cells (4×10^4 cells/well) were incubated in DMEM medium with 10% fetal calf serum under 5% CO_2/air at 37 °C for 24 h. 0.3 and 1 ng (0.08 and 0.23 fmol) of the pcDNA 3.1 (+)-luc 2 plasmids containing the luciferase gene were mixed with 'carrier DNA', the pTriEx-3 Neo plasmid, to give a total amount of 400 ng. The DNA was mixed with lipids and transfected into the cells. After a 1 h incubation under 5% CO_2 at 37 °C, 1 ml of DMEM medium containing 10% serum was added, and the cells were incubated at 37 °C. After a further 23 h, the lipid–DNA complex was removed and the cells were incubated in DMEM medium supplemented with 10% serum. The medium was changed at 24 h intervals. The cells were washed with PBS before the luciferase assay.

Introduction of the luciferase protein and DNA into HeLa was carried out using the same procedures.

Luciferase activity was measured with a Luciferase Assay System with a Reporter Lysis Buffer Kit (Promega). The activity is expressed as fmol/well based on known amounts of the luciferase protein as standards.

2.3. Quantitative evaluation of protein production after DNA transfection

We applied the simple one-compartment model to the amount (activity) of luciferase per well. The luciferase protein was assumed to be cleared by first-order kinetics. The rate constants for elimination and half-lives were calculated using data for the elimination phase. AUC (area under the luciferase amount–time curve) values, which are equal to $\int_0^t (\text{luciferase amount})\,dt$, were used to evaluate the amount of luciferase protein, considering the time factor. For a linear system, the AUC value is proportional to amount of protein (Eq. (1)).

$$\text{luciferase uptake} = k \cdot \text{AUC} \quad (1)$$

where k is a constant. Thus, the value of AUC/uptake is constant (and equal to $1/k$). This constant value was used to estimate the amount of luciferase protein produced after DNA transfection.

Table 1
Pharmacokinetic parameters of direct protein delivery

Cell line	Dose	k_{el}[a] (h^{-1})	$t_{1/2}$[b] (h)	Luciferase uptake[c] (fmol/well)	AUC[d] (h fmol/well)	AUC/luciferase (h)
NIH3T3	1 μg (8.3 pmol)	0.245	2.8	3.8 (2 h)	14.5	3.8
	3 μg (25 pmol)	0.243	2.9	9.5 (2 h)	31.6	3.3
HeLa	1 μg (8.3 pmol)	0.263	2.6	2.0 (6 h)	8.5	4.3
	3 μg (25 pmol)	0.211	3.3	14.5 (6 h)	59.5	4.1

[a] Elimination constant of the luciferase activity.
[b] Half-life of the luciferase activity.
[c] Amount of the luciferase protein per well at the time point shown in parenthesis.
[d] Area under the luciferase amount–time curve. The AUC values from 2 h to ∞ and from 6 h to ∞, for NIH3T3 and HeLa cells, respectively, were obtained according to the methods described in the text.

These AUC/uptake values were calculated based on the data for the direct protein delivery. When delivery of the protein terminated (elimination phase), the AUC values are proportional to the amount of protein. The luciferase activity decreased exponentially after 2 and 6 h post-introduction in NIH3T3 and HeLa cells, respectively, and the AUC values were calculated from 2 or 6 h to ∞. The AUC values from 2 h to ∞ for NIH3T3 cells were obtained using the actual areas of the luciferase amount–time curves from 2 to 12 h, and using AUC values from 12 h to ∞, which were calculated by integration of the fitted curves. Likewise, the AUC values from 6 h to ∞ for HeLa cells were obtained from the actual areas of the luciferase amount–time curves from 6 to 24 h, and from AUC values from 24 h to ∞, which were calculated by integration of the fitted curves. The AUC values obtained using the actual areas of the luciferase amount–time curves were more than 90% of the total AUC values from 2 or 6 h to ∞. The average AUC/uptake values were 3.55 and 4.2 h for NIH3T3 and HeLa cells, respectively (Table 1), and were used for the calculation of the total amount of the protein upon DNA transfection.

The AUC values from time 0 to ∞ for transfection into NIH3T3 cells were obtained from the actual areas of the luciferase amount–time curves from time 0 to 144 h, and from AUC values from 144 h to ∞, which were calculated by integration of the curves fitted to data from 36 to 144 h. The AUC values from time 0 to ∞ for transfection into HeLa cells were obtained from the actual areas of the luciferase amount–time curves from time 0 to 48 h, and from AUC values from 48 h to ∞, which were calculated by integration of the curves fitted to data from 24 to 48 h. The AUC values obtained using the actual areas of the luciferase amount–time curves were ∼99 and ∼50% of the total AUC values from time 0 to ∞ for NIH3T3 and HeLa cells, respectively. Division of these calculated AUC values from time 0 to ∞ by the average AUC/uptake values obtained by the direct protein delivery yielded the total amounts of luciferase protein produced by the cultured cells.

3. Results and discussion

3.1. Kinetics of direct protein delivery in NIH3T3 cells

To obtain kinetic parameters for the firefly luciferase protein in mouse NIH3T3 cells, we first delivered the luciferase protein (1 and 3 μg (8.3 and 25 pmol)) to NIH3T3 cells with Chariot, a peptide-based protein introduction reagent (Morris et al., 2001; Deshayes et al., 2004). This reagent was reported to introduce other proteins in an endocytosis-independent manner, and thus probably introduce them into the cytosol directly (Morris et al., 2001). NIH3T3 cells were treated with the protein–Chariot complex, and cytosolic luciferase activity was measured at 2–12 h after the initiation of treatment. As shown in Fig. 1A, the luciferase activity decreased exponentially starting at 2 h post-introduction. This exponential decrease indicates that degradation and/or excretion of the protein predominantly occurred at this stage, because no protein would enter the cells or the process would be highly impaired by the addition of serum after 1 h post-introduction. The luciferase protein was assumed to be cleared by

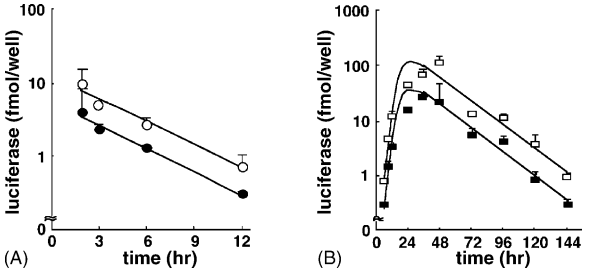

Fig. 1. Time course for luciferase activity (A) upon direct protein delivery and (B) upon DNA transfection in NIH3T3 cells. (A) The firefly luciferase protein was mixed with Chariot and incubated at room temperature for 30 min. The protein–Chariot mixture and DMEM medium, without serum, were added to NIH3T3 cells. After a 1 h incubation at 37 °C, DMEM medium supplemented with 10% serum was added and the cells were incubated at 37 °C for the indicated times. The cells were washed before the luciferase assay. Open circles: 3 μg (25 pmol); closed circles: 1 μg (8.3 pmol). (B) The pcDNA 3.1 (+)-luc 2 plasmid containing the luciferase gene was mixed with 'carrier DNA', the pTriEx-3 Neo plasmid, to a total amount of 400 ng. DNA transfection was carried out using the Lipofectamine Plus Reagent. The DNA–lipid complex and DMEM medium without serum were added to NIH3T3 cells. After a 1 h incubation at 37 °C, DMEM medium supplemented with 10% serum was added and the cells were incubated at 37 °C for the times indicated. At 24 h after the initiation of transfection, the lipid–DNA complex was removed and the cells were incubated in DMEM medium supplemented with 10% serum. At 48, 72, 96 and 120 h after the initiation of transfection, the medium was exchanged. The cells were washed prior to the luciferase assay. Open squares: 1 ng (0.23 fmol); closed squares: 0.3 ng (0.08 fmol). Data are expressed as means + standard deviation.

first-order kinetics in the elimination phase. The calculated elimination rate constant and half-life were ∼0.24 h^{-1} and ∼3 h, respectively, based on the 2–12 h data (Table 1). This half-life (∼3 h) is in agreement with that for luciferase protein in cells, obtained by different methods (Nguyen et al., 1989; Thompson et al., 1991).

We calculated the AUC values from 2 h to ∞ (Table 1) and these values were divided by the amounts of luciferase protein present at 2 h. For example, amount of intracellular luciferase protein was calculated to be 3.8 fmol/well at 2 h after treatment with 1 μg (8.3 pmol) of the protein. The AUC value from 2 to 12 h (13.3 h fmol/well) was obtained using the actual areas of the luciferase amount–time curves from 2 to 12 h. The AUC value from 12 h to ∞ (1.2 h fmol/well) was calculated by integration of the fitted curves (luciferase = $5.52 \times e^{-0.245t}$). The AUC value from 2 h to ∞ (14.5 h fmol/well) was obtained with the observed (2–12 h) and calculated (12 h–∞) values. The AUC value from 2 to 12 h is more than 90% of that from 2 h to ∞. Assuming a simple one-compartment model, the AUC/uptake values would be constant in the case of linear conditions, as described above. As shown in Table 1, the calculated AUC/uptake values were constant (3.3 and 3.8 h), and the average value (3.55 h) was used in the quantitative evaluation of DNA transfection (see below).

3.2. Kinetics of luciferase activity on DNA transfection in NIH3T3 cells

We next transfected plasmid DNA (0.3 and 1 ng (0.08 and 0.23 fmol)) encoding the firefly luciferase protein into NIH3T3 cells with the aid of cationic lipids. NIH3T3 cells were treated with the DNA–lipid complex, and the complex was removed after 24 h. Cytosolic luciferase activity was determined at 6–144 h after the initiation of treatment. As shown in Fig. 1B, maximum luciferase activity was observed at 36–48 h. After these time points, the luciferase activity decreased exponentially with time and the calculated elimination rate constant and half-life, based on the 36–144 h data, were determined to be ∼0.04 h^{-1} and 16 h, respectively (Table 2). The rate of elimination of the luciferase protein was 6 times lower upon transfection than by direct protein delivery. The actual AUC values from 0 to 144 h, and the calculated AUC values (0 h–∞) indicate that ∼99% of the luciferase proteins in the cells were produced within 144 h, respectively (Table 2).

Using 0.3 ng (0.08 fmol) of DNA, the maximum amount of luciferase (28 fmol of luciferase protein/well) was observed at 36 h. This value was more than a 350-fold molar excess over the amount of DNA transfected (0.08 fmol). This indicates that DNA transfection serves as an efficient protein delivery system in NIH3T3 cells. The calculated AUC value (0 h–∞), 1100 h fmol/well, corresponds to 310 fmol/well luciferase protein, based on the average AUC/uptake value (3.55 h) obtained by direct protein delivery (Tables 1 and 2). This calculation indicates that ∼4000-fold amount of luciferase protein was produced from the DNA used for transfection.

Using 1 ng (0.23 fmol) of DNA, the maximum luciferase activity (119 fmol of luciferase protein/well) was observed at 48 h. This value was more than a 520-fold molar excess over the amount of DNA

Table 2
Pharmacokinetic parameters of DNA transfection

Cell line	Dose	k_{el}[a] (h^{-1})	$t_{1/2}$[b] (h)	Maximum luciferase dose[c] (fmol/well)	AUC[d] (h fmol/well)	Total luciferase produced[e] (fmol/well)
NIH3T3	0.3 ng (0.08 fmol)	0.0424	16.3	28.1 (36 h)	1100	310
	1 ng (0.23 fmol)	0.0421	16.5	119 (48 h)	3360	950
HeLa	0.3 ng (0.08 fmol)	0.0151	45.9	1.0 (24 h)	61	15
	1 ng (0.23 fmol)	0.0144	48.1	3.7 (24 h)	262	62

[a] Elimination constant of the luciferase activity.
[b] Half-life of the luciferase activity.
[c] Amount of the luciferase protein per well at the time of the maximum luciferase activity. The time point is shown in parenthesis.
[d] Area under the luciferase amount–time curve. The AUC values from 0 h to ∞ were obtained according to the methods described in the text.
[e] The AUC values (0 h–∞) were divided by average AUC/uptake values obtained with the direct protein delivery.

transfected (0.23 fmol). It was calculated that a total of 950 fmol/well of luciferase protein (∼4100-fold molar excess) was produced from the total DNA transfected (0.23 fmol), calculated based on the AUC value (0 h–∞) (3360 h fmol/well, Table 2).

Under similar experimental conditions, ∼5% of the DNA transfected entered the nuclei of NIH3T3 cells (Moriguchi et al., unpublished results). Thus, it was calculated that one copy of the luciferase gene produced, at least, $\sim 8 \times 10^4$ luciferase protein molecules (Fig. 2). This calculation appears to underestimate the transcription/translation numbers from the gene, because the amount of plasmid DNA would decrease with time (Tachibana et al., 2004).

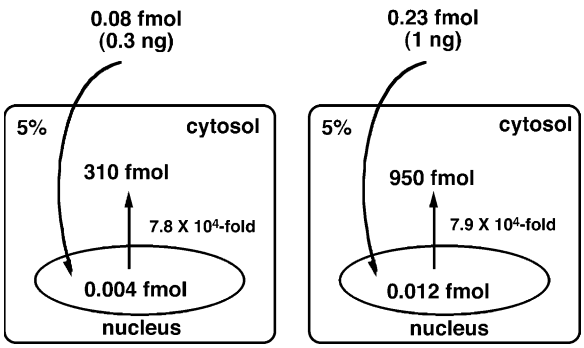

Fig. 2. Production of luciferase protein by the transfection of luciferase-coding DNA in NIH3T3 cells. According to the AUC (0 h–∞) values shown in Table 2, 310 and 950 fmol of the protein are produced by the transfection of 0.08 and 0.23 fmol of DNA, respectively. Based on the experimental results that 5% of the transfected DNA entered the nuclei under similar conditions, at least 8×10^4 molecules of luciferase protein were calculated to be produced from one DNA molecule.

3.3. Kinetics of luciferase activity on DNA transfection in HeLa cells

Similar experiments were carried out using human HeLa cells. As shown in Fig. 3 and Table 2, the expression of luciferase was less efficient in HeLa cells than in NIH3T3 cells. A nearly 30-fold lower amount of luciferase protein was produced in HeLa cells than in NIH3T3 cells, as the result of the transfection of the same amount of DNA, at the time point where the maximum luciferase activity was observed (Table 2). When 0.08 fmol (0.3 ng) and 0.23 fmol (1 ng) of DNA were used, 15 and 62 fmol of luciferase protein, respectively, were produced, as calculated using the AUC

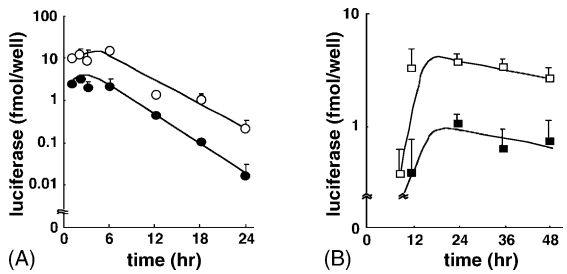

Fig. 3. Time course for luciferase activity (A) upon direct protein delivery and (B) upon DNA transfection in HeLa cells. (A) Delivery of the firefly luciferase protein with Chariot was carried out as described in the legend to Fig. 1. Open circles: 3 μg (25 pmol); closed circles: 1 μg (8.3 pmol). (B) DNA transfection was carried out with the Lipofectamine Plus Reagent as described in Fig. 1 (legend). At 24 h after transfection initiation, the lipid–DNA complex was removed and the cells were incubated in DMEM medium supplemented with 10% serum. Open squares: 1 ng (0.23 fmol); closed squares: 0.3 ng (0.08 fmol). Data are expressed as means + standard deviation.

(0 h–∞) values. These values correspond to ∼190- and ∼270-fold amounts of DNA transfected. Under similar experimental conditions, ∼5% of the DNA transfected entered the nuclei of HeLa cells (Iwasa et al., unpublished results). Thus, it was calculated that one copy of the luciferase gene produced, at least, as described above, a 4–5 × 10^3-fold molar amount of luciferase protein. The half-life was ∼50 h in HeLa cells, longer than in NIH3T3 cells, in the case of DNA delivery, although the half-lives for the direct introduction were similar in both cell lines (∼3 h, Table 1). These results can be attributed to difference in the half-lives of the intranuclear active DNAs, as described below.

3.4. Pharmacokinetics of luciferase activity on DNA transfection

Fig. 4 shows a preliminary pharmacokinetic model for the proteins upon DNA transfection. The amount of intracellular protein can be affected by a variety of factors, including the amount of intranuclear DNA, the rate constant for the elimination of DNA, the transcriptional (and translational) efficiency, and the rate constant for the elimination of the protein. When the nuclear entry of DNA is terminated, the rate of elimination of exogenous DNA and that for the encoded protein determine the amount of protein (its enzymatic activity). In the case of luciferase, the model protein used in this study, the elimination rate constant of the protein itself was larger (6- and 16-fold) in comparison with the elimination rate constant of the protein upon DNA transfection. In this situation, the amount of protein could be assumed to be determined by the DNA in the nucleus.

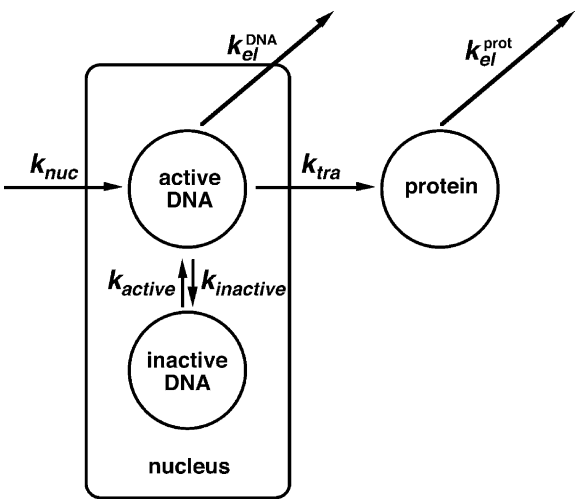

Fig. 4. Proposed pharmacokinetic model for proteins upon DNA transfection. The amount of protein encoded by a transgene is affected by a number of factors, such as the amount of intranuclear active DNA, the elimination rate constant for DNA, the transcriptional (and translational) efficiency, and the elimination rate constant for the protein. k_{nuc}, rate constant for nuclear entry; k_{tra}, rate constant for transcription and translation; k_{el}, elimination rate constant; $k_{inactive}$, constant for transcription suppression; k_{active}, constant for transcription activation.

In addition, the elimination rate constant of the protein upon DNA transfection may reflect the suppression of transcription in the nucleus as the result of the presence of inactive DNA (compartment) (Fig. 4) (Kamiya et al., 2003). In an earlier study, we observed that the same molar amount of plasmid DNA and linearized DNAs capped with loops expressed a transgene with different efficiencies (Tanimoto et al., 2003). Thus, the ratio of inactive DNA, from which the expression of its encoded protein is suppressed, would depend on the DNA structures. Likewise, this ratio might be cell line-dependent, and the difference in the half-lives might reflect the conversion rate from active to inactive DNAs (the rate of transcription suppression) in the two cell lines. Thus, the rate of conversion from active to inactive DNAs might be slower in HeLa cells than in NIH3T3 cells. In the case where the half-life for the protein itself is longer than that for the luciferase protein (such as the LacZ protein), a more complex situation would be expected. However, in any case, DNA transfection with a non-viral vector would result in transient expression (Kamiya et al., 2001). As we suggested previously (Kamiya et al., 2003), the controlled intranuclear disposition of exogenous DNA would become an important factor in the adequate regulation of a protein. Hence, a pharmacokinetic analysis of DNA transfection provides key information related to controlled protein expression.

For the first time, the total amount of luciferase protein produced from an exogenous gene on a transfected plasmid was calculated. This calculation was carried out by division of the AUC (0 h–∞) values upon transfection by the AUC/uptake values obtained with the direct protein delivery. These AUC/uptake values are dose-based and are equal to $1/k_{el}$, and correspond to the

reciprocal of the clearance in the usual pharmacokinetic analysis, in which the AUC is concentration-based. This method of calculation would be applicable to other proteins, in vivo as well as in cultured cells.

The calculations show that one copy of the luciferase gene is responsible for the production of at least $\sim 8 \times 10^4$ and $(4–5) \times 10^3$ luciferase protein molecules in NIH3T3 and HeLa cells, respectively. This 'amplification' could occur at the transcriptional and translational levels. Although these amplification values would dependent on the structure of the DNA cassette (a promoter/enhancer, gene, and poly(A) signal and introns), DNA transfection has the potential to deliver therapeutic proteins very efficiently.

Important features of DNA transfection are described, and a preliminary pharmacokinetic model for proteins upon DNA transfection is proposed. According to this model, room exists for improving non-viral DNA delivery systems and studies concerning this are currently in progress.

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