Effect of Cationic Liposomes in an in Vitro Transcription and Translation System

Rieko TACHIBANA, a)Hideyoshi HARASHIMA, b) Tatsuhiro ISHIDA, a) Yasuo SHINOHARA, a) Hiroshi TERADA, a) Yoshinobu BAB, a)c and Hiroshi KIWADA a)*

Faculty of Pharmaceutical Sciences, The University of Tokushima,a) Shomachi-1, Tokushima 770–8505, Japan, Hokkaido University Graduate School of Pharmaceutical Sciences, b) Kita 12 Nishi 6, Sapporo, Hokkaido 060–0812, Japan, and CREST, Japan Science and Technology Corporation, c) Kawaguchi, Saitama 332–0012, Japan.

Received August 6, 2001; accepted January 11, 2002

The effects of cationic liposomes complexed with plasmid DNA on the process of transcription was examined using a recently developed rapid cell free translation system. The findings indicate that the liposome itself inhibited the process when the ratio of DNA/liposome typically used in transfection studies was used.

Key words cationic liposome; transcription; rapid translation system (RTS500); gene delivery

Cationic liposomes, an attractive nonviral vector, have several advantages, which include low immunogenicity, ease of handling, and large-scale preparation, but the efficiency of transfection is lower than that of a typical viral vector. To develop more effective vectors, an understanding of the effects of cationic liposomes on individual processes such as cellular uptake and subcellular delivery are very important. In the case of subcellular delivery, the transport of plasmid DNA into the nucleus is a particularly important process. Furthermore, an understanding of the nature of the plasmid DNA delivered to the nucleus and the effects of the chemical modification of plasmid DNA and/or cationic liposomes delivered with the plasmid DNA on such processes after the nuclear transport of the plasmid DNA is important. However, these issues have not been intensively investigated.

The issue of the form of the plasmid DNA transfection with cationic liposomes that are delivered to the nucleus is also important. Xu and Szoka proposed a model in which plasmid DNA is released from the complex after its escape from endosomes. However, the issue of precisely where the cationic lipids and plasmid DNA dissociate is presently unclear. Electrostatic interaction between cationic lipids and plasmid DNA is strong, and the components of the complexes are bound together tightly. It is particularly noteworthy that when the nuclear uptake of exogenous DNA occurs in cells where the nuclear envelope disappears during mitosis, the possibility that undissociated complexes are transported into the nucleus cannot be excluded.

Thus the mechanism of the transfer of plasmid DNA complexed with cationic liposomes to the nucleus remains highly speculative. If such DNA/liposome complexes are to be utilized in gene transfer, it will be necessary to understand the effect of liposomes on transcription in the nucleus. In mammalian cells in particular, however, transcription is a very complex process. For accurate measurements of activities, a simple system would be desirable.

In this study, we report on a study of the effects of cationic liposomes on the process of transcription using a recently developed bacterial cell free transcription and translation system.

MATERIALS AND METHODS

Materials A Rapid Translation System (RTS500), its in vitro protein synthesis kit, and maleimide-activated hemocyanin (code 1-376-438) were purchased from Roche Diagnostics K.K. (Tokyo, Japan). An enhanced chemiluminescence (ECL) immunodetection kit was purchased from Amersham Pharmacia Biotech (Uppsala, Sweden). The cationic liposome (O, O′-ditetradecanoyl-N-(α-trimethylammonioacetyl) diethanolamine chloride/dioleoylphosphatidylethanolamine/cholesterol=1/0.75/0.75 in molar ratio) was a generous gift from Daiichi Pharmaceutical Company, Ltd. (Tokyo, Japan).

Plasmins and Preparation of Plasmid DNA/Cationic Liposome Complex A bacterial expression vector of the C-terminal half of rat-type II hexokinase (HKIIc) was prepared as described previously. In this paper, this expression vector is referred to as pET/HKIIc.

Appropriate amounts of a cationic liposome solution and plasmids, respectively, were diluted in 25 μl of phosphate-buffered saline. The liposomal solution was then gently added to the plasmid solution, and the resulting solution was incubated for 15 min.

Protein Synthesis by RTS500 Fifty microliters of a plasmid solution or a solution of the plasmid/cationic liposome complex was added to 1 ml of a solution containing an Escherichia coli lysate and carefully mixed by rolling. The reaction solution was loaded into the 1-ml reaction compartment. After closing the lids, the reaction device was turned upside down and the 10-ml feeding compartment was filled with feeding solution. After closing the lids and turning the reaction device again, the device was inserted into the RTS500 instrument. Protein synthesis was carried out in the RTS500 instrument with stirring at 30 °C. During the coupled translation and transcription reaction, amino acids, energy substrates, and nucleotides were supplied from the feeding compartments via a semipermeable membrane. After a specified period of incubation, the incubation mixture was rapidly frozen in dry ice and stored at −25 °C until use.

Preparation of Antibody An antibody against HKIIc was prepared using a synthetic peptide with an amino acid sequence of GEELFDHVQCIADFLEYMGKGV (amino acids 571—594 of rat-type II hexokinase) as the antigen. The specific recognition of HKIIc by this antibody has been
conditions. As shown in Fig. 1, when 15 g of pET/HKIIc as the expression vector at 30°C. After the indicated periods of incubation, aliquots of the reaction mixture were separated and immediately frozen on dry ice. They were then subjected to SDS-polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane. Synthesized HKIIc was detected with an anti-HKIIc antibody. The faster migrating of the two immunoreactive protein bands represents HKIIc.

RESULTS AND DISCUSSION

The RTS 500 Rapid Translation System (Roche Diagnostics) was used to evaluate the effects of cationic liposomes used for transfection on the process of transcription. This apparatus enables the cell free synthesis of a particular protein coded by an expression vector having a T7 promoter. Using this system, the plasmid DNA is first transcribed into mRNA by the T7 RNA polymerase and is then translated to form protein by ribosomes, which are then included in the E. coli lysate. Generally, this system is utilized to prepare a particular protein for use in functional characterization studies. However, in this study the system was used as a model system for mammalian transcription, and the effect of cationic liposomes on the process was evaluated.

We used an expression vector of the HKIIc, of which the effectiveness in the expression of HKIIc in bacterial cells has been well established, as a model gene for the transfection studies. Prior to examining the possible effect of cationic liposomes on the processes of transcription and translation, we examined protein synthesis by RTS500 under a variety of conditions. As shown in Fig. 1, when 15 μg (recommended by the supplier as the amount to be used in the standard procedure) of pET/HKIIc was used as the expression vector and 1 μl of the total reaction mixture was directly subjected to SDS-PAGE followed by immunodetection, two major immunoreactive protein bands were observed. Of these two bands, the upper one with an apparent molecular size of about 60 kDa represents an unassigned protein involved in protein synthesis. This protein shows cross-immunoreactivity with the anti-HKIIc antibody, because it was also observed even in the absence of an expression vector (data not shown). The lower band, with an apparent molecular size of about 50 kDa, was not detected in the absence of the expression vector and represents HKIIc. Furthermore, differing from the time course of the control vector, the maximum synthesis of HKIIc had already been achieved after 4-h incubation.

As a result of these findings, we next examined protein synthesis during a shorter incubation period. As shown in Fig. 2, when 15 μg of pET/HKIIc was used, the amount of HKIIc produced increased in a linear manner as a function of incubation time and reached a plateau after 2 h. On the contrary, when the amount of pET/HKIIc was decreased, the rate of synthesis of HKIIc was slower and the amount of synthesized HKIIc decreased in a nearly linear manner with incubation time up to 4 h. The time dependence of protein synthesis and the maximum amount produced may be related to the characteristics of the expression vector and the expressed protein. Thus the profile of protein synthesis observed above is characteristic of the pET/HKIIc expression vector used.

Based on the above results, we selected experimental conditions in which 5 μg of expression vector was used, since the conditions appear to be highly susceptible to the contents of the incubation mixture used in protein synthesis and are suitable for the detection of possible effects of cationic liposome on the process of transcription. Furthermore, since cationic liposomes were used in previous studies, we examined the effects of the complex of pET/HKIIc with this liposome on the cell free synthesis of HKIIc. Compared to the results observed in the absence of liposome, the addition of 12.5 nmol of cationic lipid (5 nmol cationic lipid) slightly suppressed the synthesis of HKIIc (Fig. 3). When the concentration of cationic liposomes was increased, the synthesis of HKIIc was significantly inhibited. However, no inhibitory effect was observed when plasmids and cationic liposomes containing 15 nmol of cationic lipid were separately added to the reaction device (Fig. 3, open triangles). This indicates that cationic liposomes themselves do not affect transcription and translation. The findings clearly show that the cationic liposomes complexed with plasmids have an in-
hibitory effect on the process of transcription in a dose-dependent manner. It should be noted that Zabner et al. examined the effect of cationic lipids on the efficiency of transcription and translation by injecting a DNA/lipid complex into the nucleus of Xenopus oocyte cells. Results similar to ours were obtained, but the procedure described in this study is more convenient and suitable for detailed studies.

For ordinary transfection studies, 10 μg of plasmid DNA was mixed with 20 to 200 nmol of cationic lipid. Thus from the standpoint of DNA/liposome ratio, the experimental conditions employed in this study (5 μg of plasmid plus 5 to 50 nmol lipid) did not differ greatly from that of actual transfection, and we conclude that even under ordinary experimental conditions, the cationic liposome itself had an inhibitory effect on transcription and translation.

It is known that the ratio of DNA/cationic liposomes is one of the crucial parameters determining overall transfection efficiency. However, since a cationic liposome itself was found to have an inhibitory effect on the transcription process, the ratio of DNA/cationic liposomes would be important for the processes before the transport of plasmid DNA into the nucleus, e.g., cellular uptake, endosomal escape, or the nuclear transport of plasmid DNA.

In the future, a system will be needed to examine the effects of various chemical modifications of plasmid DNA using a nuclear localization signal, etc. The system described here will be useful in detailed studies of the transcription of plasmids under a variety of conditions.

Acknowledgement This work was supported in part by research fellowships of the Japan Society for the Promotion of Science for Young Scientists (to R.T.), by a grant from the New Energy and Industrial Technology Development Organization (NEDO) of the Ministry of Economy Trade and Industry, Japan (to Y.B.), and by a grant from the Ministry of Education, Culture, Sports, Science and Technology (No. 11557194 to H.K.).

REFERENCES