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Cytoplasmic expression of a reporter gene by co-delivery of T7 RNA polymerase and T7 promoter sequence with cationic liposomes

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ABSTRACT

Expression of bacteriophage T7 RNA polymerase in mammalian cells can efficiently drive the transcription of a foreign gene controlled by the T7 promoter (Elroy-Stein et al., Proc. Natl. Acad. Sci. USA. 86, 6126–6130, 1989). We have tested the hypothesis that purified T7 RNA polymerase can be co-delivered into mammalian cells together with a reporter gene (chloramphenicol acetyltransferase, CAT) controlled by the T7 promoter (pT7-EMC-CAT) using DC-chol cationic liposomes. Indeed, significant level of CAT activity was observed in human lung adenocarcinoma (A549-1) cells which had been incubated with a complex of T7 RNA polymerase, pT7-EMC-CAT DNA and DC-chol cationic liposomes. The expression was specific in that T3 RNA polymerase could not replace the T7 RNA polymerase, and that co-delivered T7 RNA polymerase did not enhance the expression of a CAT gene controlled by the SV40 early promoter. The system was optimized in terms of enzyme, DNA and liposome concentrations. Time course experiment indicated that the expression of the T7 system was about 8–10 hours sooner than the SV40 system, consistent with the notion that T7 RNA polymerase does not enter into the nucleus and the transcription takes place in the cytoplasm of the transfected cells. The expression of the T7 system was transient; it declined after 30 hours post transfection, probably due to turnover of the phage enzyme in the mammalian cells. The expression system described here should be useful for gene transfer experiments which require a fast but transient expression of a foreign gene. We have also compared our delivery system with a commercial reagent, Lipofectin® , which has been used to deliver T3 or T7 RNA polymerase with a reporter plasmid encoding the T3 or T7 promoter.

INTRODUCTION

Gene therapy is one of the fastest growing areas in biomedical sciences. Both viral and nonviral vectors have been developed for efficient transfer of foreign genes into the target cells (1). Among the nonviral vectors, cationic liposomes have the advantages of low toxicity, high efficiency, non-immunogenicity and easiness to use. Our laboratory has developed several different cationic liposome formulations (2–4). One of these formulations, termed DC-chol liposomes, contains a cationic lipid which is a tertiary amine conjugated to a cholesterol group via a short spacer arm and a carbamoyl bond (2). The structural feature of this lipid conforms with the empirical rules derived by us for the maximal activity and minimal toxicity of a cationic lipid as the result of a structure-activity study (4). The DC-chol liposome formulation, which also shows excellent storage stability, is currently used in a clinical trial for cancer gene therapy (5).

Successful gene transfer is a combination of delivery and expression of the foreign gene in the target cell. Although considerable progress has been made in the development of delivery vehicles for gene therapy, relatively little has been done for the controlled expression of the foreign gene. Viral promoters, tissue or cell-type specific promoter sequences have been employed to achieve specific expression in the target cells. Both types of expression system rely on the endogenous transcriptional machinery, i.e. the RNA polymerase II or III and the associated regulatory factors, for gene expression. Although efficient and sometimes specific expression can be achieved, unexpected down regulation of gene expression is sometimes encountered. For example, expression of the transgene in fibroblasts is turned off after the transduced cells were transplanted into the host (6). It is highly desirable if the foreign gene is co-delivered with an exogenous transcription machinery which is not sensitive to the endogenous regulatory mechanism. A prokaryotic promoter and its corresponding RNA polymerase would satisfy this requirement if a delivery vehicle is versatile enough to deliver the foreign gene, the cis-acting promoter and the exogenous RNA polymerase. The cationic liposome reagent, Lipofectin® , has been used successfully to co-deliver T3 or T7 RNA polymerase with a reporter plasmid encoding the T3 or T7 promoter (Ciccarone et al., manuscript in preparation). We report here that cationic liposomes, DC-chol, can also be used to co-deliver into human cells a reporter gene, CAT, under the control of the bacteriophage T7 promoter, together with purified T7 RNA

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polymerase. This novel gene transfer system has been optimized in terms of the ratio of DNA to protein and the optimal cationic liposome composition. These results should be useful in gene therapy applications.

MATERIALS AND METHODS

Chemicals

T7 RNA polymerase (New England BioLabs, Baverly, MA) was used without further purification. T3 RNA polymerase was from Promega (Madison, WI). 1,2-Dioleoyl-sn-glycero-3-phosphatidylethanolamine (DOPE) and 1,2-dioleoyl-sn-glycero-3-phosphatidylcholine (DOPC) were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL). Acetyl coenzyme A, chloramphenicol, Triton X-100 were from Sigma (St Louis, MO). [^{3}H]acetyl coenzyme A and Beta-Max™ scintillation cocktails were from ICN Biomedicals (Costa Mesa, CA). Cationic liposomes composed of DC-cholesterol (60 mol%) and DOPE or DOPC (40 mol%) were prepared by a sonication method as described previously (2). Lipofectin® reagent was obtained from Life Technologies, Inc. (Gaithersburg, MD).

Plasmids and cells

pT7-EMC-CAT, an expression plasmid of CAT gene under the control of bacterial phage T7 promoter (7) was a kind of gift of Dr B.Moss. pUCSV2CAT, an expression plasmid of CAT gene under the control of SV40 early promoter, was provided by Dr M.Magnuson. Both plasmids were amplified in *E.coli* and purified by CsCl gradient method (8). All the culture media were supplied by Gibco BRL (Gaithersburg, MD). Human lung adenocarcinoma A549 cells were from American Type Culture Collection, (Rockville, MD), and were propagated with Ham's F12 medium containing 10% fetal bovine serum. The A549-1 cells were a variant derived from the original A549 cells and contained some less differentiated cells.

Transfection and CAT assay

A549-1 cells were transfected according to the published method with minor modification (2). Briefly, plasmid DNA at concentration of 100 μg/ml in serum free medium CHO-S-SFM (Gibco BRL) was incubated with varying amounts of T7 RNA polymerase for 10 min at room temperature, then the mixture was diluted with CHO-S-SFM to 2μgDNA/ml. Cationic liposomes (2 mM total lipids in 20 mM HEPES buffer, pH7.6) were directly added to the DNA/polymerase complex and were mixed immediately. Half ml of the complex were added to each well of the A549-1 cells (about 60% confluency). Cells had been plated for 32 h in 24 well plates and were washed once with CHO-S-SFM before use. Transfection incubation was done at 37°C for 4 h. The cells were washed and further cultured with complete medium. For the dual transfection experiments, A549-1 cells were first transfected for 4 h, then cells were allowed to recover for 1 h in F12 medium containing 15% fetal bovine serum before second transfection which was done identically as the first transfection. All transfection experiments were done in triplicate.

At indicated time post transfection, cells were washed once with saline and lysed with 100 μl of lysis buffer which consisted of 0.05% Triton, 2mM EDTA, 2mM dithiothritol in 200 mM Tris−HCl (pH 7.8) at 4°C for 10 min. Cell lysates were prepared by centrifuging at 15000 rpm for 5 min and the protein concentration of the supernatant was estimated using Coomassie Plus Protein Assay Reagent (Pierce, Rockford, IL). CAT assay was performed according to the method of Sanharan (9) with a few modifications. We used 1mM chloramphenicol, 0.1mM acetyl CoA, and 0.1 μCi [^{3}H]acetyl CoA for the assay mixture. The reaction was performed at 37°C for 20 min, followed by extraction with 1ml toluene at 4°C for 10 sec. One half of the organic phase was mixed with 3 ml Beta-Max™ and counted for the radioactivity. One unit of CAT activity was defined as the amount of enzyme converting 1 nanomole of acetyl groups to chloramphenicol per min at above reaction condition.

RESULTS

Bacteriophage T7 RNA polymerase produced in the transfected mammalian cells localizes only in the cytoplasm (10). Thus, transcription of the reporter gene by the exogenously supplied T7 RNA polymerase is expected to take place in the cytoplasm, and the transcripts would not be capped properly. An internal ribosome entry site (IRES) of the myocardiotitis virus was inserted into the 5′ untranslated region such that the uncapped message can be translated efficiently in the cytoplasm (7).

We have first demonstrated that the expression of CAT gene was dependent on the co-delivery of T7 RNA polymerase. As shown in Fig. 1, CAT activity was only observed in cells which had been incubated with a complex of DNA and T7 RNA polymerase. No activity was observed if T7 RNA polymerase was absent in the incubation mixture. Furthermore, optimal CAT expression was detected when 0.75−2 μg DNA was used.

The action of T7 RNA polymerase on the T7 promoter sequence should be very specific (11,12). We have tested the hypothesis by adding T7 RNA polymerase to a plasmid construct of CAT gene controlled by the SV40 early promoter (pUCSV2CAT). As can be seen from the data in Fig. 1, T7 RNA polymerase had little effect on the expression of CAT gene driven by the SV40 promoter. Data in Fig. 1 also indicate that the optimal DNA concentration (0.75−2 μg) for the delivery of pUCSV2CAT DNA was the same as the optimal concentration for the delivery of pT7-EMC-CAT plasmid DNA. Thus, the

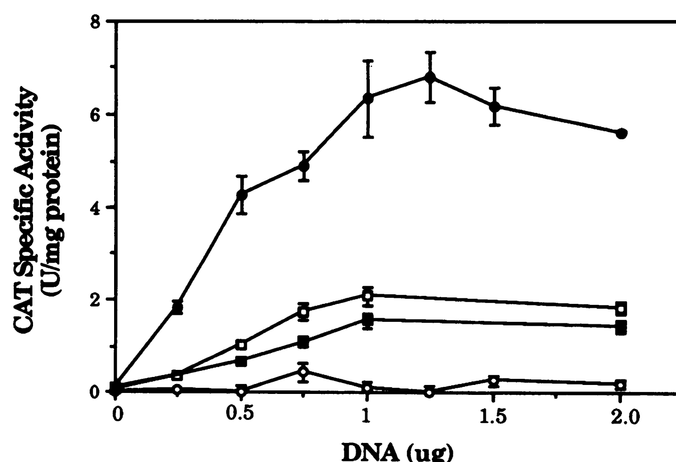

Figure 1. Expression of CAT reporter gene as a function of DNA concentration. Plasmid pT7-EMC-CAT (circles) or pUCSV2CAT (squares) were used at indicated concentrations, together with (filled symbols) or without (open symbols) 100 units of T7 RNA polymerase. DC-chol liposomes (15 nmoles) were used to deliver the complex to A549-1 cells. CAT activity was determined at 20 h after transfection.

presence of a DNA binding protein, T7 RNA polymerase in this case, did not alter the optimal conditions for the delivery of DNA by cationic liposomes.

The expression system was also optimized with respect to the concentration of T7 polymerase used in the experiment. Data shown in Fig. 2 indicate that maximal activity was obtained if 100 or more units of T7 RNA polymerase were used in the experiment. Also shown in the figure is the fact that 50 and 100 units of bacteriophage T3 RNA polymerase had no effect in enhancing the expression of CAT gene controlled by the T7 promoter. This result again indicates the specificity of the polymerase binding to its own promoter sequence.

Delivery of plasmid DNA with cationic liposomes requires the use of optimal liposome concentration (2,13). We had tested if the delivery of DNA/protein complex also show an optimal liposome concentration. Fig. 3 shows that 15–20 nmoles of DC-chol liposomes were optimal for the delivery of the complex; higher amounts of liposomes showed less activity and more toxicity to the treated cells (data not shown). Also shown as a comparison is the effect of a commercially available cationic liposome reagent, Lipofectin® (13). The optimal dose appeared to be 20–25 nmoles, but the treated cells showed some cytotoxicity at these doses.

The composition of the cationic liposomes is an important factor for efficient gene transfer (4). We have prepared several liposome preparations with various contents of the cationic lipid, DC-chol. As can be seen in Fig. 4, high efficiency delivery of the protein/DNA complex requires that the liposomes contain a high

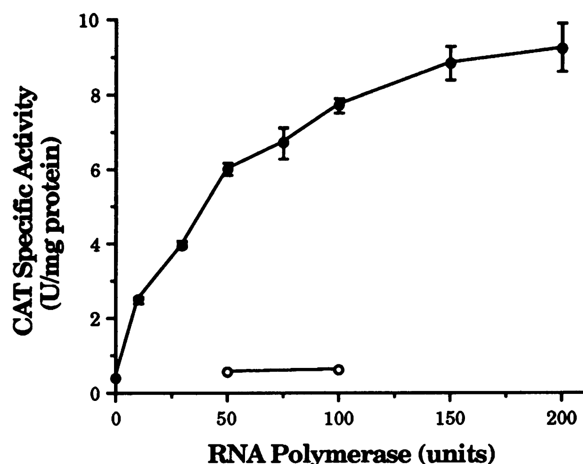

Figure 2. Expression of CAT reporter gene as a function of RNA polymerase concentration. A549-1 cells were transfected with 0.75 μg DNA of pT7-EMC-CAT, 15 nmoles DC-chol liposomes and various amounts of T7 RNA polymerase (●) or T3 RNA polymerase (○). CAT activity was determined at 24 h after transfection.

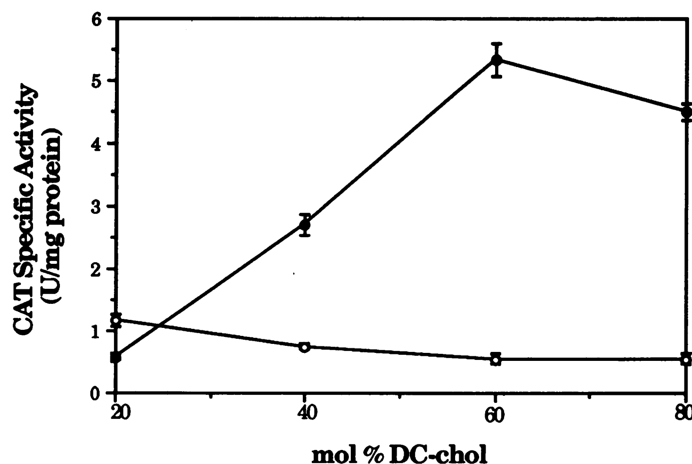

Figure 4. Effect of lipid composition of the cationic liposomes on the CAT reporter gene expression. A549-1 cells were transfected with 0.75 μg DNA of pT7-EMC-CAT, 100 units of T7 RNA polymerase complexed with 15 nmoles liposomes composed of various amounts of DC-chol and DOPE (●) or DOPC (○). CAT activity was determined at 20 h after transfection.

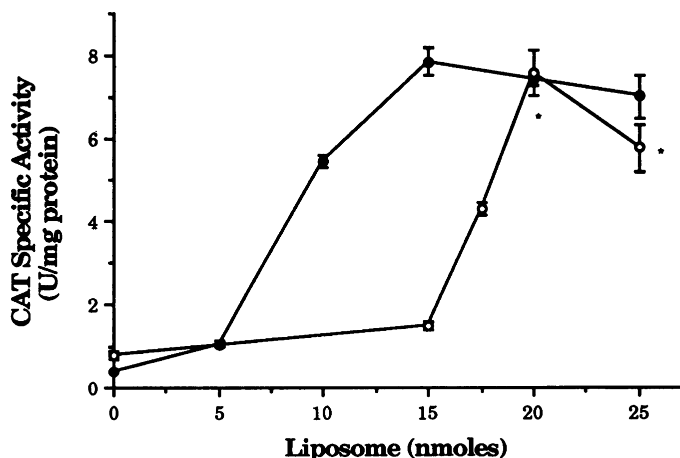

Figure 3. Expression of CAT reporter gene as a function of liposome concentration. A549-1 cells were transfected with 0.75 μg DNA of pT7-EMC-CAT, 100 units of T7 RNA polymerase, complexed with DC-chol liposomes (●) or Lipofectin™ (○). Data points marked with * indicate that cells showed cytotoxicity with reduced total protein recovery. CAT activity were assayed at 20 h after transfection.

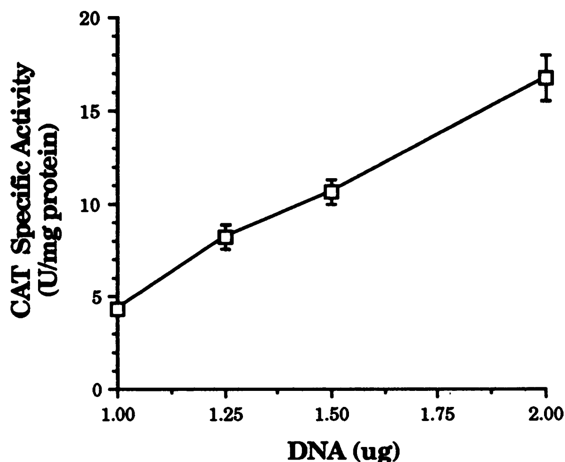

Figure 5. Effect of total dose of polymerase/DNA liposome complex on the CAT reporter gene expression. A549-1 cells were transfected with various amounts of a complex containing the following ingredients: DNA/T7 RNA polymerase/DC-chol liposome = 1μg/100 units/10 nmoles. pT7-EMC-CAT was used in the experiment and its concentration is used to indicate the total dose of the complex. CAT activity was determined at 20 h after transfection.

concentration of DC-chol, with the optimal concentration being 60%. Furthermore, the identity of the helper phospholipid in the liposomes is also important. Activity was only seen if DOPE was in the liposomes; DOPC had no activity at any of the DC-chol concentrations used.

The optimal ratio for the co-delivery of T7 RNA polymerase and pT7 EMC CAT plasmid DNA obtained thus far are as follows: 0.75−2 μg DNA, 100 or more units of T7 RNA polymerase and 15−20 nmoles DC-chol liposomes composed of 60 mol% DC-chol and 40 mol% DOPE. Using these optimal ratios, the transfection activity increased almost linearly with the total amount of complex in the incubation medium with cells (Fig. 5). It should be noted that the maximal level of the CAT gene expression at the highest complex concentration used represents nearly total consumption of the substrate during the standard incubation time for the CAT assay. This was an excellent level of gene expression which is not commonly seen with standard transfection protocols. Further increase of the complex concentration resulted in cytotoxicity of the treated cells (data not shown).

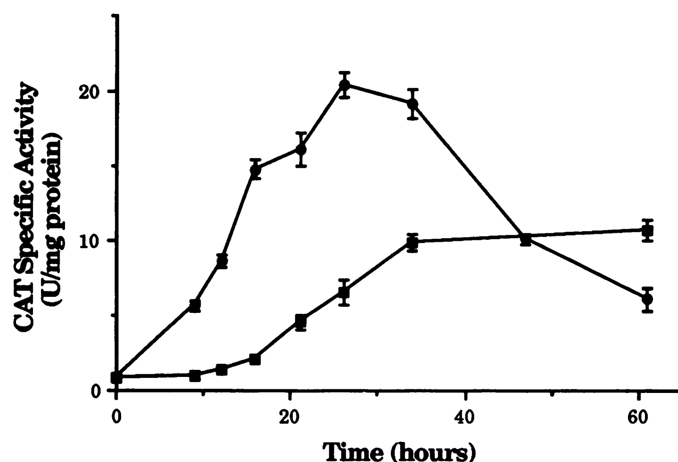

Figure 6. Time course of CAT reporter gene expression. A549-1 cells were transfected with 0.75 μg DNA of either pT7-EMC-CAT (●) or pUCSV2CAT (■), 100 units of T7 RNA polymerase and 15 nmoles DC-chol liposomes. Cells were harvested at indicated time post transfection.

Using these optimal conditions the time course of the gene expression was studied with cells incubated with the DNA/protein complex for 4 h. Data in Fig. 6 show that expression of CAT activity could be detected as early as 9 h after the incubation and reached the maximum level by about 30 h post transfection. The activity then steadily declined afterwards. Little activity could be detected in the treated cells 61 h after the incubation. Thus, the expression of CAT activity was relatively rapid but did not sustain for long periods of time. This is in contrast to the expression kinetics of pUCSV2CAT, also shown in Fig. 6, which took place more slowly but the activity had lasted much longer (up to 5−6 days).

The A549-1 cells were not alone in expressing the reporter gene when it was co-delivered together with T7 RNA polymerase. Four other cell lines, i.e. the Baby Hamster Kidney BHK21 cells, the human epidermal carcinoma A431 cells, the human small cell carcinoma SCC25-CP cells and the human bronchial epithelial cell line IB3-1 cells, were also receptive to this novel expression system (data not shown). However, the level of CAT expression varied somewhat, with the SCC25-CP cells showing the highest activity and A431 cells the lowest.

All the above described experiments were done by mixing T7 RNA polymerase with DNA and liposomes, the complex was then added to cells. We tested if T7 RNA polymerase could be separately delivered to cells as a complex with an irrelevant piece of DNA. A dual transfection experiment was designed in which T7 RNA polymerase was co-delivered with pCMVLacZ plasmid (the *E.coli* LacZ gene controlled by the cytomegalovirus immediate-early promoter, a kind gift from Dr W.F. Goins) either before or after the delivery of pT7-EMC-CAT DNA. The two separate incubations were both 4 h in length. A one-hour interim incubation of cells with F12 medium containing 15% FBS was included to improve the cell viability. Cells treated with both incubations were washed and returned to the growth medium. The CAT activity expressed by the cells was assayed after 20 h. The results of this experiment shown in Table 1 indicate that T7 RNA polymerase did not have to be delivered together with a piece of DNA containing the T7 promoter sequence; approximately 50% of CAT gene expression was found in cells

Table 1. CAT activity of dually transfected A549-1 cells

Sample	First transfection	Second transfection	Relative CAT Activity
1	T7 RNAP/pT7EMCCAT/DC-Chol	none	100.0±2.1
2	T7 RNAP	pT7 EMCCAT/DC-Chol	3.0±5.0
3	T7 RNAP/DC-Chol	pT7 EMCCAT/DC-Chol	8.7±4.8
4	T7 RNAP/pCMVLacZ/DC-Chol	pT7 EMCCAT/DC-Chol	45.2±3.6
5	T7 RNAP/pT7EMCCAT/DC-Chol	pT7 EMCCAT/DC-Chol	100.7±4.9
6	T7 RNAP/pT7EMCCAT/DC-Chol	pCMVLacZ/DC-Chol	93.3±9.7
7	pT7 EMCCAT/DC-Chol	T7 RNAP	5.9±2.2
8	pT7 EMCCAT/DC-Chol	T7 RNAP/DC-Chol	17.3±13.9
9	pT7 EMCCAT/DC-Chol	T7 RNAP/pCMVLacZ/DC-Chol	53.7±5.5
10	pT7 EMCCAT/DC-Chol	T7 RNAP/pT7EMCCAT/DC-Chol	108.7±4.3
11	pCMVLacZ/DC-Chol	T7 RNAP/pT7EMCCAT/DC-Chol	75.2±7.1

A549-1 cells were treated for 4 h with 100 units of T7 RNA polymerase (T7 RNAP) alone (2), complexed with 15 nmoles of DC-Chol liposomes (3), complex with 0.75μg of either nonspecific DNA (pCMVLacZ) (4) or a plasmid carrying T7 promoter (pT7 EMCCAT) (1), (5), (6) and 15 nmoles of DC-Chol liposomes. Some cells were treated with pT7 EMCCAT or pCMVLacZ complexed with DC-Chol. After the treatment, cells were allowed to recover in F12 medium containing 15% of FBS for one h before treated for another 4 h with 0.75μg of pT7 EMCCAT (2), (3), (4), (5) or 0.75 μg of pCMVLacZ (6) complexed with 15 nmoles of DC-Chol. Some cells were treated with 100 units of T7 RNA polymerase alone (7), T7 RNA polymerase complexed with 15 nmoles of DC-Chol liposomes (8) or complexed with 0.75μg of pT7 EMCCAT (10), (11) or same amount of pCMVLacZ (9) and 15 nmoles of DC-Chol liposomes. Cells were cultured for 20 h after the dual transfection.

which received the enzyme as a complex with an irrelevant piece of DNA, i.e. pCMVLacZ (samples 4 and 9). Free enzyme, with or without liposomes, probably did not enter the cells as little CAT activity was observed (samples 2,3,7 and 8). Other positive controls (samples 5,10 and 11) indicated that additional incubation did not interfere with the transfection of cells with pT7-EMC-CAT/T7 RNA polymerase complex as delivered by the DC-chol liposomes. Thus, T7 RNA polymerase can be delivered into cells with cationic liposomes together with a piece of DNA which contains either the T7 promoter sequence or not.

DISCUSSION

T7 RNA polymerase has been used to express transgenes in mammalian cells (7,14,15,16). It is a highly efficient RNA polymerase with an activity about 8-fold higher than the bacterial RNA polymerase (17). Previous uses of this enzyme in transgene expression require that the gene coding for the T7 RNA polymerase be transfected into the host cells, often with a lytic viral vector such as the vaccinia virus (7,14,15). We describe here that the enzyme itself can be delivered into mammalian cells with the help of DC-chol cationic liposome/DNA complex.

Previous studies have shown that T7 and T3 RNA polymerase can be co-delivered with plasmid DNA using Lipofectin® reagent (Ciccarone et al., manuscript in preparation). We have optimized the conditions for the co-delivery of T7 RNA polymerase and pT7-EMC-CAT plasmid DNA (Fig. 1−5) using DC-chol liposomes. The optimal conditions for the delivery are similar to those for the delivery plasmid DNA alone (2), even when substantial amount of T7 polymerase is used in the experiment (approximately 0.3 μg T7 polymerase for 0.75−1 μg DNA). It can be viewed that T7 RNA polymerase is piggy-backed into the cells by the delivery of the liposome/DNA complex. This hypothesis is confirmed by the data of Table 1. Apparently, T7 RNA polymerase form a weak complex with a piece of irrelevant DNA and once entered the cells, it can dissociate and bind with the sequence containing T7 promoter. The binding affinity of T7 RNA polymerase with T7 promoter is quite high with an estimated dissociation constant of 10^{-8} M (18,19). Thus, the enzyme could be piggy-backed into the cell by an irrelevant piece of DNA and then find the correct specific promoter sequence inside the cells. Free enzyme in the absence of DNA cannot be delivered by liposomes.

Since T7 polymerase does not enter the nucleus of the mammalian cell (10), the observed gene expression must be cytoplasmic. It is not surprising that expression was relatively rapid (Fig. 6) because there is no need for the DNA and enzyme to enter the nucleus for transcription. Such cytoplasmic expression system is potentially advantageous for somatic gene therapy because most cells in an adult organism are not dividing. Translocation of DNA from cytoplasm into the nucleus appears to be an inefficient process, since the expression of DNA microinjected into the cytoplasm is much less efficient than those injected directly into the nucleus (20). However, the maximum level of expression of the T7 system in the A549-1 cells was only approximately two-fold higher than that of the SV40 system (Fig. 6) which is expressed in the nucleus. This is probably due to the turnover of T7 RNA polymerase in the cytoplasm. The net level of gene expression should be determined by, among other factors, the amount of enzyme entering into the cytoplasm and the amount removed by degradation. After a short incubation, the level of CAT gene expression peaked at about 30 h and declined steadily afterwards (Fig. 6). These data are consistent with the enzyme turnover hypothesis. Different cell types may show different turnover rate of the enzyme, resulting in different net expression of the CAT gene. In any case, the T7 expression system described here should be useful for any gene transfer experiments which require fast but transient expression of a foreign gene.

Data presented here are consistent with the notion that a cytoplasmic gene expression system may be useful in somatic gene therapy, provided that the life time of T7 polymerase in the cytoplasm can be prolonged. Ideally and more practically, a cytoplasmic regeneration system for the T7 RNA polymerase should be co-delivered together with the T7 system described here. For example, a T7 RNA polymerase gene driven by the T7 promoter, similar to the 'autogene' described by Dubendorf and Studier (21), would allow continuous synthesis of T7 RNA polymerase in the cytoplasm and driving a theoretically long lasting expression of a therapy gene controlled by the T7 promoter. These experiments are currently in progress.

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ABBREVIATIONS

CAT, Chloramphenicol acetyltransferase
DC-chol, 3β[N-(N',N'-dimethylaminoethane)-carbamoyl] cholesterol
DOPE, 1,2-dioleoyl-sn-glycero-3-phosphatidylethanolamine
DOPC, 1,2-dioleoyl-sn-glycero-3-phosphatidylcholine

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