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Optimization of Gene Transfer into Neonatal Rat Cardiomyocytes and Unmasking of Cytomegalovirus Promoter Silencing

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ABSTRACT

Cardiomyocytes are notoriously difficult to transfect using standard techniques unless viral vectors such as recombinant adenoviruses are used. Generation of recombinant adenoviruses is, however, a complex and time-consuming procedure and not possible for every DNA construct. We therefore optimized DNA/polylysine/adenovirus complexing for efficient gene transfer in neonatal rat cardiomyocytes determining the critical parameters for this method. Importantly, not only the concentration of the various components but also the method used for plasmid purification is critical for this transfection technique. Cesium–chloride-purified DNA is inferior to anion-exchange methods for this purpose possibly because of altered ionic properties. In the second part of this study, we could demonstrate silent gene transfer into cardiomyocytes applying this optimized technique to plasmids encoding luciferase or β-galactosidase cDNAs under the control of the cytomegalovirus immediate-early promoter. Phorbol myristate acetate and/or forskolin increased the amount of β-galactosidase positive cells up to fivefold. Luciferase activity could even be increased as much as ninefold. These results demonstrate that the cytomegalovirus promoter is not maximally active in neonatal rat cardiomyocytes under basal conditions. In fact, a large proportion of cells is silently transfected and seems to express (an) inhibitor(s) of transcription from the CMV promoter that can be overcome by stimulation of cAMP- or protein kinase C-dependent pathways.

INTRODUCTION

CARDIOMYOCYTES ARE GENERALLY considered to be one of the most difficult cell types to transfect both *in vitro* and *in vivo*. Recent advances in the construction of genetically engineered adenovirus vectors have facilitated gene transfer into both adult (Stratford-Perricaudet *et al*., 1992; Kirshenbaum *et al*., 1993) and neonatal rat cardiomyocytes (nCMCs) (Kass-Eisler *et al*., 1993) *in vitro* as well as into neonatal (Zhang *et al*., 1999) and adult myocardium (Barr *et al*., 1994) *in vivo*. Construction of adenoviral vectors, however, is a time-consuming technique and not possible for all potential transgenes. Another major limitation of these vectors is the size constraint for the introduction of a transgene including the promoter, especially for large cDNAs such as dystrophin or the ryanodine receptor. For tissue-specific expression, longer promoters (in the range of several kb) are usually used adding to the problem of limited packaging capacity. Further problems include potential toxicity to the parent cell line used to generate and amplify the virus thereby inhibiting production of recombinant viruses. So far, no convincing technique has been established to overcome this problem. An important application of plasmid DNA transfer is the use of numerous constructs for the identification of regulatory gene elements. It would be very cumbersome to generate recombinant adenoviruses for the numerous constructs required.

We therefore evaluated a system that takes advantage of the efficient gene transfer capabilities of adenoviruses but obviates the construction of recombinant adenoviruses by a simple noncovalent complex of adenovirus and plasmid DNA with polylysine (Kohout *et al*., 1996). This technique has initially been introduced by experiments by E. Wagner's and D.T. Curiel's

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groups (for review, see Michael and Curiel, 1994). It is feasible for large constructs such as cosmid DNA (Cotton *et al*., 1992), and might even be extended to YAC transfection (Marschall *et al*., 1999).

The most commonly used promoters in gene transfer experiments are of viral origin, since they have evolved for optimal gene expression in their host cells. One of the most commonly used is the cytomegalovirus (CMV) immediate-early promoter, which is considered to be among the strongest constitutive promoters in most cells. Recent publications, however, have suggested that there is regulation of this promoter in various cell types (Clesham *et al*., 1996) including primary cardiac cells (Maass *et al*., 2003).

In addition to the adaptation of the method of adenovirus/polylysine/DNA complexing for primary rat cardiomyocytes, this study confirms a surprising extent of regulation of the CMV promoter in a plasmid background that is important to consider when using this promoter in gene transfer experiments.

MATERIALS AND METHODS

Isolation and culture of neonatal rat CMCs

Hearts of 1–3-day-old rats were excised and cardiomyocytes were isolated by trypsin digestion as previously described (Maass *et al*., 2003). Cells were cultured in serum-free MEM supplemented with transferrin, insulin, and BSA for 24–48 h before experiments were carried out.

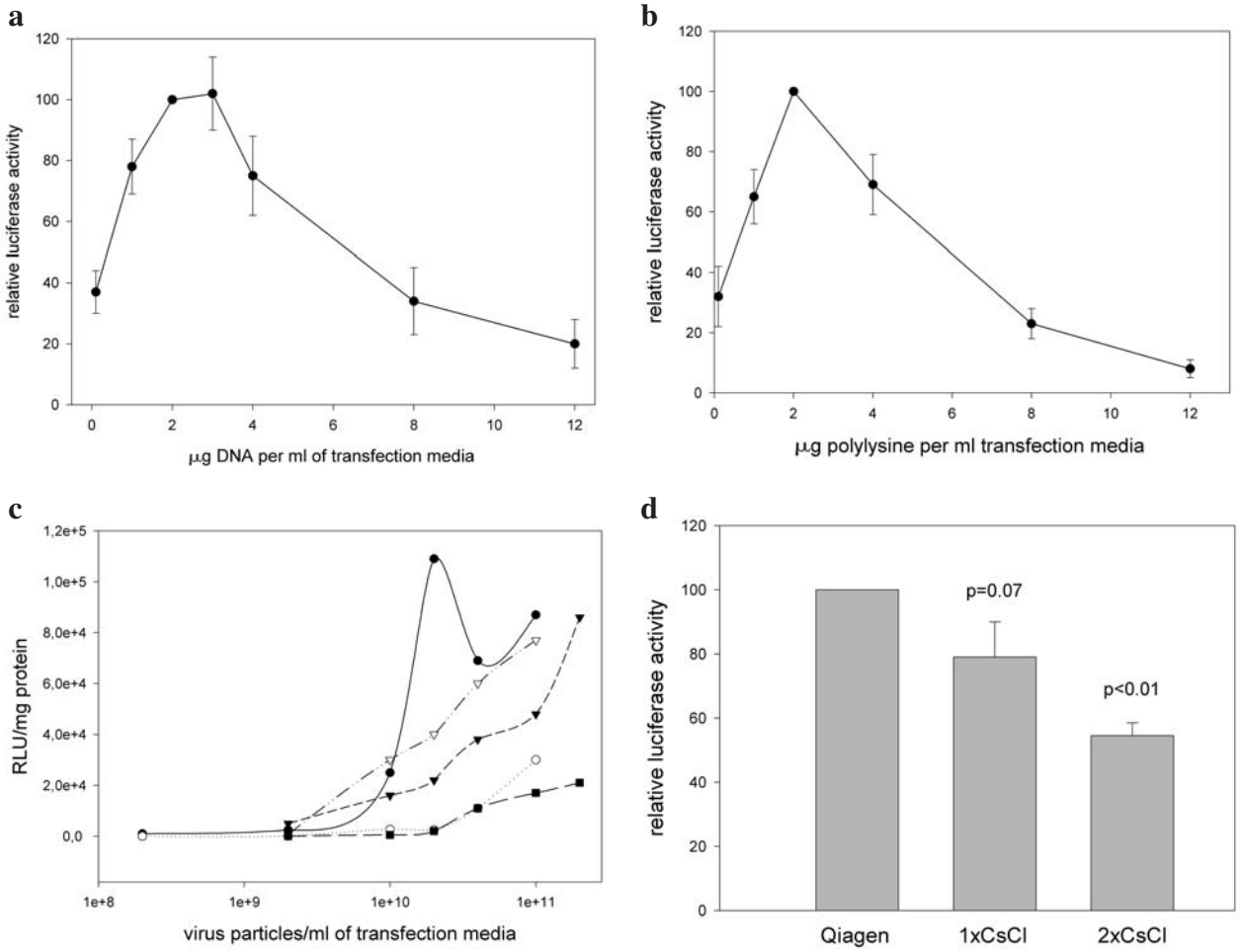


FIG. 1. Optimization of gene transfer by adenovirus/polylysine/DNA complexing in neonatal rat cardiomyocytes assessed by luciferase activity. **(a)** Complexing of variable amounts of plasmid DNA with polylysine and adenovirus. Relative luciferase activity normalized to cells transfected with a concentration of 2 μg DNA per ml of culture media. Polylysine constant at 2 μg/ml, adenovirus constant at 5×10^{10} particles/ml. $n = 5$ for all concentrations. **(b)** Complexing of variable amounts of polylysine with 2 μg plasmid DNA and adenovirus. Luciferase activity normalized to cells transfected with 2 μg polylysine per ml of culture media. DNA constant at 2 μg/ml, adenovirus constant at 5×10^{10} particles/ml. $n = 5$ for all concentrations. **(c)** Luciferase activity normalized to protein content in the sample after complexing with increasing amounts of adenovirus particles using five different adenovirus preparations (labeled by different symbols ○, ●, ▼, ■, □). **(d)** Luciferase activity using plasmid DNA prepared by different isolation techniques. Qiagen: plasmid DNA prepared by a commercial kit, 1 × CsCl: plasmid DNA prepared after alkaline lysis and single banding on a CsCl gradient, 2 × CsCl: plasmid DNA banded twice on a CsCl gradient. $n = 4$ for each DNA preparation. Significances calculated versus Qiagen DNA.

Luciferase and β-galactosidase assays

After the indicated incubation period, cells were washed twice with phosphate buffered saline (PBS) and lysed in 1× passive lysis buffer (Promega, Madison, WI). Protein concentrations were measured by the modified Bradford method (Bio-Rad, Hercules, CA) and luciferase activity determined after mixing with 100 μl assay reagent (Promega) in a standard luminometer (Turner Designs, Sunnyvale, CA). Relative light units (RLU) were normalized to microgram protein content in the sample. Cells transfected with β-galactosidase containing constructs were washed twice with PBS, fixed for 10 min in 2% formaldehyde, and stained in potassium ferricyanide/potassium ferrocyanide and X-gal overnight. β-Galactosidase positive cells were counted by phase-contrast microscopy.

Adenovirus/polylysine complexing

Replication-deficient adenovirus (Addl312) was grown by standard methods as previously described (Maass *et al*., 2003). Briefly, HEK293 cells (ATCC, Rockville, MD) were infected, and virus was harvested after 24 h with freeze/thaw cycles. Virus was purified over a cesium chloride gradient, dialyzed, and stored at −20°C in storage buffer containing glycerol. Particle numbers were determined photometrically.

Plasmid DNA was isolated either by a commercially available kit (Qiagen, Hilden, Germany) or by standard cesium chloride banding (one or two rounds of purification) followed by dialysis and precipitation. Poly-L-lysine with an average molecular weight of 35 kDa (Sigma, St. Louis, MO) was used as a stock solution of 33 μg/ml in MEM. Two-thirds of the total poly-L-lysine (optimal dose 1.33 μg) was mixed with adenovirus (10^{11} particles) in 200 μl MEM and incubated for 30 min. Plasmid DNA (2 μg) was added, and after a further 30-min incubation the remaining third of polylysine (0.6 μg) was added. The complex was incubated for 10 min before it was added to the cells in serum-free MEM.

RESULTS

Initial experiments using adenovirus/polylysine/plasmid complexes in neonatal rat cardiomyocytes showed efficiencies

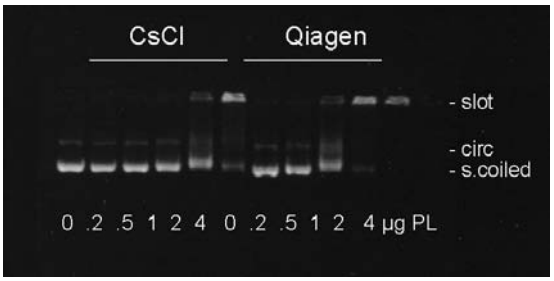


FIG. 2. Gel retardation assay of plasmid DNA complexed with increasing amounts of polylysine. Left half: plasmid DNA purified by CsCl gradients. Right half: plasmid DNA purified by anion exchange (Qiagen). Slots: slots of agarose gel where DNA/polylysine complex was loaded. Circ, circular form of the plasmid; S.coiled, supercoiled form of the plasmid; PL, polylysine.

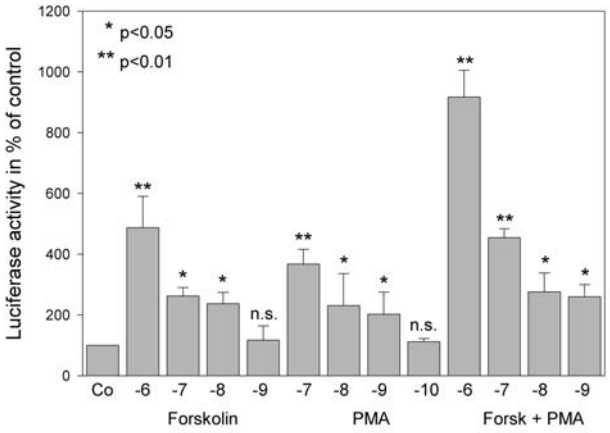


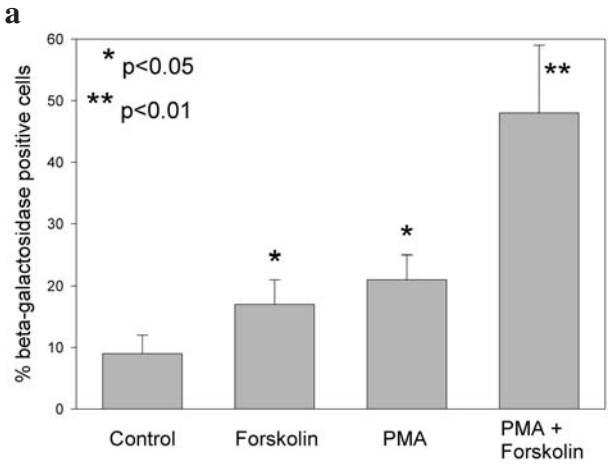
FIG. 3. Stimulation of luciferase activity with forskolin and phorbol-myristate-acetate (PMA) for 48 h at different concentrations 18 h after transfection. Logarithmic scale with numbers representing the log concentration (e.g., −6: 10^{-6} M). Co, control, no stimulation. Significances versus control. n.s., no significance.

below 5% and we therefore wanted to determine critical parameters that had not been published previously (Kohout *et al*., 1996). The first variable we tested was DNA concentration at a constant DNA/polylysine ratio and a constant amount of adenovirus. There was a sharp maximum at a DNA dose of 2–3 μg/ml media with luciferase activity decreasing rapidly at higher and lower DNA concentrations (Fig. 1a). The second variable was the polylysine to DNA ratio. Keeping the DNA concentration constant at 2 μg/ml, we tested polylysine concentrations from 0.2 to 12 μg/ml media. There was a sharp optimum at a polylysine concentration of 2 μg/ml, with luciferase activity again falling rapidly at higher or lower concentrations (Fig. 1b). The third variable tested was the amount of viral particles in the complexes. The results differed dramatically for different preparations of the virus (Fig. 1c). There was a steady increase for most of the different virus preparations without reaching a maximum even at 2×10^{11} virus particles per milliliter of media. There was no correlation between infectious particles as assessed by plaque assays and the efficiency of transfection (data not shown), and in all further experiments we used the photometrically determined amount of virus particles to calculate the appropriate amount for each transfection. The maximum amounts of adenovirus used in this study corresponded to a multiplicity of infection of 5×10^4 plaque forming units per cell, a concentration that is generally considered toxic to cells. We could, however, not detect any toxic effects on our cells as assessed by conversion of the formazan dye MTT (data not shown). This could be explained by the use of a recombinant virus not encoding for any transgene or promoter elements that might be toxic at higher doses.

It has been speculated that supercoiled plasmid DNA is required for efficient gene transfer. To ensure high quality of our plasmid DNA, we generally purified the DNA by double cesium chloride (CsCl) banding followed by dialysis to remove remaining CsCl. We compared this method with single CsCl banding and a commercially available kit for plasmid DNA isolation utilizing anion exchange (Qiagen). Surprisingly, there

was a significant decrease in luciferase activity when we used CsCl purified DNA (Fig. 1d). A possible explanation might lie in structural differences in plasmid DNA prepared by different methods. The precise structural requirements for DNA used in different transfection techniques have not been elucidated. In some cases such as direct injection *in vivo*, supercoiled DNA seems to be more efficient (Buvoli and Leinwand, 2002). Both methods that were employed yielded almost exclusively supercoiled DNA (Fig. 2). The major difference between the anion-exchange method and CsCl purification is the amount of endotoxin in the preparation, which is much lower for CsCl (Wicks *et al*., 1995). We therefore compared an endotoxin lowering kit (Endo-free Qiagen kit) with no difference to the regular kit (data not shown). To further elucidate the mechanism of better performance of DNA produced by the ion-exchange method, we complexed equal amounts of each DNA with increasing amounts of polylysine and separated them by agarose gel electrophoresis. Surprisingly, for CsCl-purified DNA more polylysine was needed to retard migration into the gel. This result suggests a lower binding affinity of CsCl-purified DNA for polylysine (Fig. 2).

When assaying transfection efficiency at our optimized conditions with β-galactosidase encoding plasmids, the amount of visibly transfected cells (by positive β-galactosidase staining) was approximately 10%. This is in stark contrast to the results from Kohout *et al*. (1996) reporting transfection efficiencies of



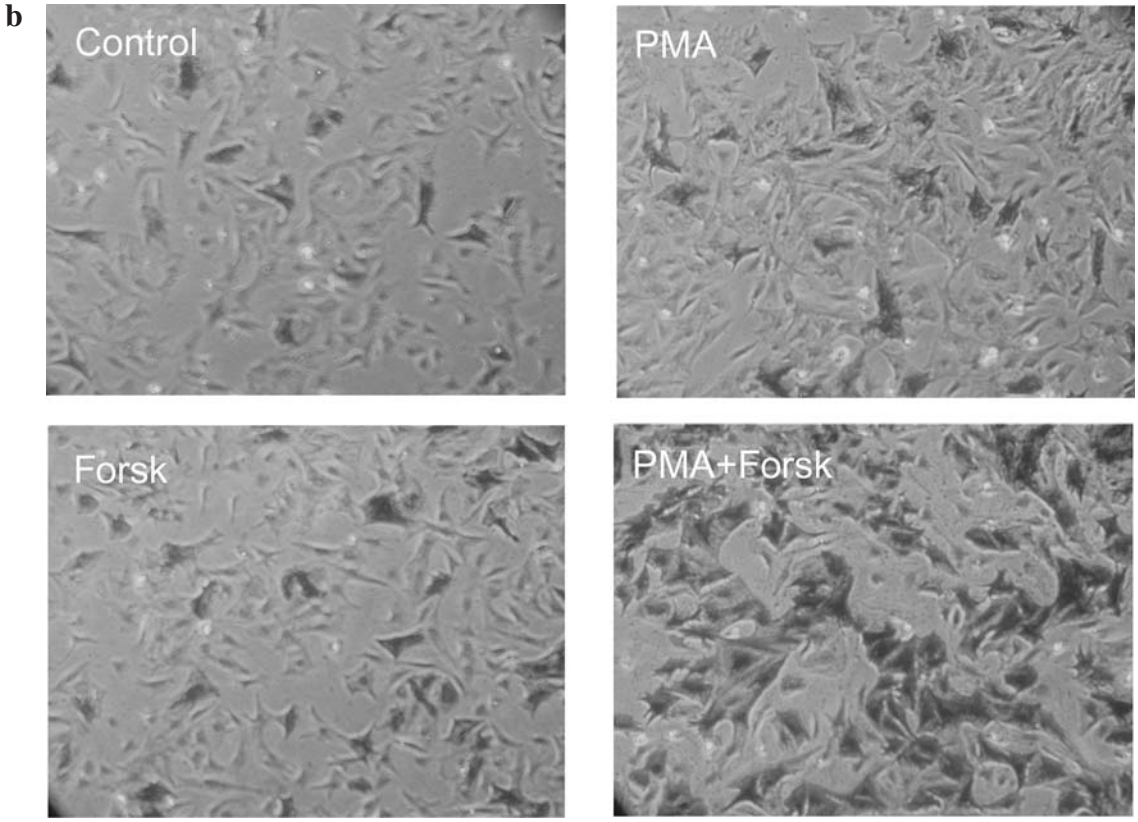


FIG. 4. Increase in percentage of β-galactosidase-positive neonatal rat cardiomyocytes after transfection with plasmid/polylysine/adenovirus complexes. **(a)** Percentage of beta-galactosidase positive neonatal rat cardiomyocytes after transfection for 18 h and subsequent treatment with Forskolin (10^{-6} M) and/or phorbol-myristate-acetate (PMA, 10^{-6} M) for 48 h. Co, control, no stimulation. $n = 5$ independent experiments. Significances calculated versus control. **(b)** Representative photographs of neonatal rat cardiomyocytes after transfection for 18 h (Co) and treatment with PMA (10^{-6} M), or both PMA and Forskolin (10^{-6} M) for 48 h.

up to 50%. A possible explanation for this discrepancy might lie in the different culture conditions used. In contrast to other investigators (Kohout *et al*., 1996), we cultured our cells in conditions that do not promote autonomous hypertrophy, that is, serum-free conditions (Simpson *et al*., 1982). Serum contains a suite of growth-inducing factors, some of which might also act as transcriptional activators of the CMV early promoter (Ghazal *et al*., 1987). We sought to identify possible signal transduction pathways that can activate the CMV early promoter in neonatal rat CMCs. Four repeat elements have been described that occur four to six times each in the CMV immediate-early promoter (Ghazal *et al*., 1997): A 17-bp element with an NF1 half site, an 18-bp element binding NFκB (Niller and Hennighausen, 1991), a 19bp element containing a cAMP response element (Stamminger *et al*., 1990), and a 21 bp element binding YY1 (Liu *et al*., 1994). An up to 23-fold activation of the CMV promoter by forskolin and phorbol ester has been discovered in human vascular smooth muscle cells (Clesham *et al*., 1996), accompanied with an identification of silent gene transfer in these cells.

We used two defined inducers of different signal transduction pathways to assay activation of the CMV promoter in neonatal rat CMCs after transfection with DNA/polylysine/adenovirus complexes. PMA was used to stimulate protein kinase C and its downstream effectors. Forskolin was used to activate cAMP and its downstream effectors protein kinase A and CREBP.

Forskolin dose dependently increased luciferase activity up to fivefold after transfection of the complex (Fig. 3). PMA also induced luciferase activity dose dependently up to fourfold (Fig. 3). Both substances acted additively, inducing luciferase activity up to ninefold (Fig. 3). Interestingly, when we assayed the number of cells visibly expressing β-galactosidase, this number increased after the use of forskolin and PMA from 10% in control cells to 50% when using both substances (Fig. 4), suggesting that the number of cells being transfected in the unstimulated state was underestimated because of transgene expression below the detectable limit.

To assess if the observed promoter induction was restricted to the CMV promoter, we tested both a different viral (the Rous Sarcoma Virus promoter) and a muscle-specific promoter (MEF2-responsive promoter) (Maass *et al*., 2003). These exhibited a slightly higher basal activity but were only minimally inducible by PMA and/or forskolin (Fig. 5). When staining with a luciferase-specific antibody, we could detect robust expression only in about 10% of the cells, similar to the uninduced CMV promoter (data not shown).

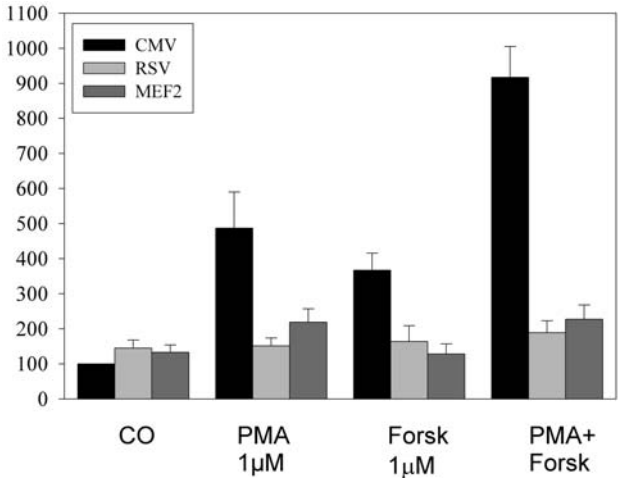


FIG. 5. Comparison of two viral and a tissue-specific promoter and stimulation with forskolin and phorbol-myristate-acetate (PMA) for 48 h, 18 h after transfection with plasmid/polylysine/adenovirus complexes.

DISCUSSION

Gene transfer to the myocardium is of obvious scientific and potentially therapeutic interest, but has proved notoriously difficult. This study therefore aimed at more precisely defining parameters that are critical for using a technically simple method, noncovalent adenovirus/polylysine/plasmid DNA complexing, for gene transfer into cardiomyocytes. This technique has been summarily described (Kohout *et al*., 1996) but has not been used widely because of rather variable results. Defining the parameters governing this variability may therefore contribute to a wider application, potentially even in (animal) experiments of therapeutic gene transfer methods.

Critical parameters defined in this study are both DNA and polylysine concentrations that showed a very sharp peak at 2 μg/ml media. Some virus preparations proved more efficient than others independent from infectivity, suggesting the necessity of testing several preparations to find the optimal batch. CsCl purified DNA proved inferior to plasmid made by a commercially available kit. We showed that this surprising result is not due to different molecular forms, as both methods yield mainly supercoiled plasmid DNA. This finding also cannot be explained by contaminating endotoxin, since that should lead to the opposite effect of better performance by CsCl-purified DNA that contains low levels of endotoxin. Our hypothesis is that the methods for plasmid purification lead to DNA with different ionic properties. This hypothesis is strengthened by the gel retardation assay that shows that the equal amount polylysine binds less CsCl-purified DNA than DNA purified by anion exchange (Fig. 2). The different ionic properties can be explained by the method employed by currently available kits to purify plasmid DNA. The anion exchange technique favors DNA with the maximal number of available negative charges. This property is obviously critical for binding the positively charged polylysine.

A very important finding is the up to ninefold induction of the CMV promoter, but not the RSV or MEF2-responsive promoters in cardiomyocytes by PMA and forskolin. This demonstrates that, other than previously thought, even a strong viral promoter might not be maximally active in these cells under basal conditions. This phenomenon is not restricted to the cells used in this study, but has also been described in other cells such as smooth muscle cells (Clesham *et al*., 1996) or primary cardiac fibroblasts (Maass *et al*., 2003). A regulation might be caused by various stimuli, such as α-interferon (Acsadi *et al*., 1998; downregulation), PMA/forskolin (Clesham *et al*., 1996; induction) in various cell types and the CMV promoter has also been shown to be inactivated after *in vivo* gene transfer into

mouse liver (Löser *et al*., 1997). In the present study, we show dose-dependent induction of this promoter in a plasmid background by PMA and forskolin in cardiomyocytes. The mechanism of this induction, however, remains unclear. An increase in the actual uptake of the complexes or adenoviruses and thereby increase in gene transfer efficiency appears unlikely because the substances were added 18 h after the transfection/infection and after the cells had been washed with fresh media. One might argue that the uptake of complexes still attached to the cell membrane after washing was facilitated. It has been shown, however, that adenovirus uptake into cells is very rapid (Greber *et al*., 1993), and is usually completed after a few hours. The prolongation of the previously described short half-life of luciferase in mammalian cells of about 3 h (Thompson *et al*., 1991) by these substances is also not likely, since the effect was also seen with β-galactosidase, a protein with much longer half-life. The most likely explanation is the induction or activation of specific transcription factors stimulating transcription from the CMV early promoter. Binding sites for multiple transcription factors have been identified in this promoter, some like cAMP response element binding protein (CREBP) (Stamminger *et al*., 1990) and serum response factor (Chan *et al*., 1996) being implicated in stimulation, while YY1 is thought to be a negative regulator (Liu *et al*., 1994). Phorbol esters are inducers of the signal transduction molecule protein kinase C that induces multiple cellular pathways including the transcription factor NFκB. Forskolin leads to increased cellular cAMP levels that induce protein kinase A and the transcription factor CREBP.

An interesting and surprising finding of this study is that a high percentage of neonatal rat CMCs is silently transfected. This inactivation can be overcome by treatment with PMA and/or forskolin. Inactivation of the CMV promoter has been described *in vivo* after adenoviral infection of the mouse liver (Löser *et al*., 1997). This could be overcome by reinfection with an "empty" replication-deficient adenovirus. The likely molecular mechanism was the induction of the transcription factor NFκB. This transcription factor is ubiquitously expressed in resting CMCs but at a very low level, and is increased in (patho)physiological conditions such as ischemia and aging (Helenius *et al*., 1996). The abundance of other transcription factors with demonstrated binding to and activation of the CMV promoter are also low in normal CMCs, whereas the transcription factor YY1 is of high abundance in neonatal cardiomyocytes (Patten *et al*., 2000). This expression pattern of transcription factors in neonatal cardiomyocytes possibly tips the balance between positive and negative transcriptional regulators towards the latter. Preliminary experiments with transcription factor decoy using the repeat elements point to an involvement of YY-1 and NFκB (data not shown).

In conclusion, we determined the critical parameters for the use of adenovirus/polylysine/DNA complexing for gene transfer to cardiac myocytes. Furthermore, the induction of the CMV promoter demonstrated here is important to take into consideration when using this promoter in gene transfer experiments. Caution should be used when the CMV promoter is employed as normalization for studies involving analyses of regulatory elements. Another direct consequence of our results is that gene expression can be enhanced dramatically in cardiomyocytes by well-defined substances with low toxicity. If uninducible, but lower expression is required, the RSV promoter should be considered. Further experiments are aimed at validation of CMV promoter regulation *in vivo* and preliminary results show an induction of the CMV promoter by hypertrophic stimuli after direct plasmid injection *in vivo*.

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