all, the design provides a more controlled system with much greater reliability. In addition, because the medium bathing the cells is only in contact with the biologically inert Delrin, toxic effects on the cells are minimized.

The ability to dynamically monitor signals in living cells provides unprecedented spatial and temporal resolution in the study of the distribution of chemical and electrical activity. This flow-cell design provides a convenient means of displacing the medium bathing the cells in a controlled manner, so that various kinds of stimuli can be delivered to cells during imaging.

REFERENCES


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Co-Transfected SV40 Origin of Replication Activates Expression from SV40 Promoterless Constructs

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ABSTRACT

Co-transfection with expression plasmids is widely used to control DNA uptake efficiency in transient transfection experiments. However, a number of problems have been associated with their use. Here, we describe the activation of expression of constructs not containing the simian virus 40 (SV40) origin of replication (ori) by co-transfection in COS-7 cells with plasmids containing the SV40 ori. This effect has consequences for the use of such plasmids to control transfection efficiency.

INTRODUCTION

Where cells are transfected in separate culture dishes or wells and it is required to compare expression between cells transfected in separate batches, it is necessary to correct results for efficiency of the transfection process (1). This is usually achieved by co-transfection with a control-expression plasmid, often one expressing a conveniently measured product such as β-galactosidase (β-gal) or luciferase. Although this procedure is used widely, there are problems associated with it. For instance, there may be inhibitory effects of one plasmid on another (2), possibly as a result of competition for transcription factors or unexplained, synergistic effects between co-transfected plasmids (3). In comparing control plasmids for use with COS-7 cells, we have observed activation by plasmids containing the simian virus 40 (SV40) origin of replication (ori) of those that do not contain this sequence. This can lead to a high background of reporter gene expression. Since many commonly used control plasmids contain the SV40 ori, this is a frequently encountered but easily avoided problem.

MATERIALS AND METHODS

pCAT® Basic and pCAT Promoter Vectors were from Promega (Southampton, England, UK). pCMV5hER (11), expressing the human estrogen receptor was the gift of Prof. B.S. Katzenellenbogen (University of Illinois, Urbana-Champaign, IL, USA). pBL2CATERE, expressing chloramphenicol acetyltransferase (CAT) under the control of the vitellogenin A2 estrogen-response element and herpes simplex virus thymidine-kinase promoter, and pM2mER, expressing the mouse estrogen receptor, were derived from the constructs described by Luckow and Schütz (10) and Kaufman et al. (9)

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and were kindly provided by Dr. M.G. Parker (Imperial Cancer Research Fund, London, England). pCMVβ was from CLONTECH Laboratories (Palo Alto, CA, USA) and pCH110 was from Amersham Pharmacia Biotech (St. Albans, England, UK). The SV40 ori, 27-bp palindromic region and the large T-antigen binding site I (positions 7030–7110 relative to the SV40 ori at 7023–7098) were removed from pCH110 with BplI (Fermentas, Vilnius, Lithuania) followed by S1 nuclease, and the plasmid religated with DNA ligase; the removed region was confirmed by sequencing. pCMV5β-gal was constructed by subcloning the HindIII-EcoRI fragment (lacZ gene) from pCH110 into pCMV5 (pCMV5hER following removal of the hER cDNA). pCATbOTR was prepared by subcloning a 1315-bp, upstream-region PstI fragment from the bovine oxytocin receptor gene (2) into pCAT basic in the appropriate orientation.

COS-7 cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM; Life Technologies, Paisley, England, UK), supplemented with 10% fetal calf serum (Life Technologies), penicillin (100 iu/mL) and streptomycin (100 µg/mL). Transfections were carried out either with diethylaminoethyl (DEAE) dextran or by electroporation. For transfection using DEAE dextran, cells were transferred to 24-well plates 24 h before use in numbers resulting in 50%–60% confluency on the day of transfection. All cultures were carried out in quadruplicate. Medium was removed; cells were rinsed with phosphate-buffered saline (PBS) and incubated with DEAE dextran (500 µg/mL) and chloroquine (100 nmol/mL; Sigma, Poole, England, UK) in PBS (0.2 mL) containing plasmid DNA. Unless otherwise stated, plasmid DNAs were used at the following concentrations: pCMV5hER, 0.1 µg/mL; pMT2mER, 0.05 µg/mL; all CAT constructs, 0.25 µg/mL; pCMV5β-gal and pCH110, 0.15 µg/mL. After 3 h at 37°C, transfection mixtures were thoroughly removed. Cells were rinsed with PBS, and 1 mL of medium was added. For electroporation, cells growing exponentially were trypsinized, washed twice in serum-free medium (DMEM) and once in Optimix A (Flowgen, Shenstone, England, UK). They were suspended in 0.4-mL Optimix B (Flowgen) with DNA constructs (0.5 µg each construct) and subjected to a single pulse at 280 V, 1500 µF (Easyject® Plus; EquiBio, Kent, England, UK). Cells were then immediately transferred to DMEM containing 10% calf serum and antibiotics (penicillin, 100 U/mL; streptomycin, 100 µg/mL) and grown in 24-well plates. Following transfection by either method, medium was changed after 24 h, and cells were incubated for a further 48 h. Levels of CAT activity in different experiments varied, and absolute values cannot be compared between experiments.

CAT was measured in precleared cell lysates with acetylcoenzyme A (0.80 µmol/mL; Sigma), 2.31 KBq d-
threo-[dichloroacetyl-1,14C]chloramphenicol (2.04 GBq/mmol; Amersham Pharmacia Biotech) in a total volume of 125 µL 0.25 mol/L Tris-HCl, pH 8.0. Ethyl acetate extracts were separated by thin-layer chromatography and mono-acetylated chloramphenicol counted after autoradiography. For β-gal assay lysates were incubated in 96-well plates with MgCl₂ (1 mmol/L), 2-mercaptoethanol (50 mmol/L), chlorophenol red-β-D-galactopyranoside (0.5 mg/mL; Boehringer Mannheim, Lewes, England, UK) in 0.1 mol/L sodium phosphate, pH 7.3 and OD₅₇₄ measured against reaction blanks containing reporter-lysis buffer.

RESULTS

CAT expression by 3 vectors not containing the SV40 ori (pCAT basic, pBLCATERE and pCATbOTR) was increased up to 300-fold by co-transfection with each of 4 constructs containing the SV40 ori (pCH110, pCMV5hER, pCMV5β-gal and pMT2mER; Figure 1). It was not affected by co-transfection with a construct not containing the SV40 ori (pCMVβ). CAT expression by pCAT promoter alone (which contains the SV40 ori; 50357

![Figure 1. Effects of co-transfection with SV40 origin-containing plasmids on CAT expression. Basal expression of SV40 origin-negative CAT constructs in absence of co-transfected construct was: for pCAT basic, 8200 ± 2433; pBLCATERE, 2205 ± 160; pCATbOTR, 1107 ± 107 cpm/mg cell protein; these values were obtained in different experiments and cannot be compared. N = 4 wells/experiment in each case (means ± SEM; in some cases, multiple experiments are pooled). All cultures were carried out in the absence of estrogen.](image1)

![Figure 2. Activation of CAT expression from pCATbOTR by pCMVhER was concentration-dependent. Various concentrations of pCMVhER as indicated were co-transfected with a constant concentration (250 ng/mL) of pCATbOTR. Basal CAT activity in the absence of pCMVhER was 1990 ± 312 cpm/mg protein.](image2)

![Figure 3. Co-transfection of pBLCATERE with pCMV5β-gal or pCMVβ. Cells were electroporated with 0.5 µg pBLCATERE with or without 0.2 µg pCMV5β-gal or 10 µg pCMVβ. Levels of β-gal expression (arbitrary U/mg protein) were: pCMV5β-gal, 2.20 ± 0.01; pCMVβ, 1.56 ± 0.04.](image3)
counts per minute [cpm]/well) was not affected by co-transfection with pMT2mER (39592 cpm/well). Activation of pCATβOTR expression by pCMVhER was dose-dependent (Figure 2). To confirm that absence of CAT activation with pCMVβ was not due to low amplification of this construct, quantities of pCMVβ and pCMV5β-gal transfected were adjusted to give similar levels of β-gal expression. This was achieved using a 20- to 50-fold excess of pCMVβ over pCMV5β-gal; under these conditions, CAT expression was unaffected by pCMVβ, but increased up to 11-fold on co-transfection with pCMV5β-gal (Figure 3). Removal of the SV40 ori, 27-bp palindrome and large T-antigen binding site I from pCH110 prevented activation of CAT expression (Figure 4).

**DISCUSSION**

The factor responsible for the activation of CAT expression is presumably a sequence common to pCH110, pCMV 5β-gal, pCMV5hER and pMT2mER, but absent in pCMVβ. One candidate for such a sequence is the SV40 early promoter/ori. Consistent with this possibility, the SV40 promoter/ori-containing vector pCAT promoter showed a high rate of expression and was not induced further by pMT2mER, suggesting that the sequence responsible was present in the SV40 promoter/ori sequence. The SV40 fragment common to all these constructs is an approximately 75–80-bp sequence, including the 27-bp palindromic ori and the sequences binding large T-antigen tetramers. COS-1 and COS-7 cells express the large T antigen, which triggers high levels of replication of SV40 ori-containing constructs in these cells. We postulate that a recombination event occurring after internalization of transfected DNA causes a sequence containing the SV40 ori to be transferred from plasmids containing it to those that do not. This would be expected to result in increased rates of replication in the CAT constructs involved, with a consequent increase in CAT gene expression. Removal of the 27-bp sequence and one T-antigen binding site from pCH110 blocked the activatory effect, confirming that one of these components of the SV40 ori is involved. It is uncertain whether the recombination event suggested here is a characteristic of COS cells. However, it may be facilitated by the tendency of the large T antigen in these cells to
form DNA loops through polymerization while bound to DNA (12). CV-1 cells, the progenitors of COS cells, have a high rate of recombination efficiency (8). The effect reported here would also be expected to occur in cells other than COS cells, when susceptible reporter plasmids are co-transfected together with an SV40 T antigen-expressing plasmid, as in the SV40 large T-antigen boost procedure of de Chasseval and de Villartay (3).

An alternative explanation is that the highly amplified SV40 origin-containing plasmid DNA could either sequester factors normally suppressing CAT expression or swamp degradative systems responsible for removing the CAT-expression vector. We are unable to distinguish between these possibilities directly.

An effect similar to that observed here was reported by Simoni and Gromoll (14), when growth hormone (GH) production from a metallothionein promoter construct was activated on co-transfection into COS-7 cells with constructs containing the SV40 ori. This effect, an approximately 20-fold induction, reduced the value of the GH/metallothionein promoter construct when used to correct for transfection efficiencies. In general, a high background of reporter gene activity generated by the effect observed here may obscure small changes in activity resulting from other treatments. Furthermore, anomalous results may be obtained if treatments affecting the process (presumably recombination) leading to reporter gene activation by the SV40 ori are applied to cells shortly after transfection. In view of the sensitivity of reporter gene expression to low concentrations of SV40 ori-containing plasmid (Figure 2), this effect may not be easily overcome by transfecting with low concentrations of control plasmid compared with experimental plasmids (1).

Recombination events between endogenous and exogenous SV40 sequences have been reported (7,13,15), with a focus on the repair of incomplete SV40 viral DNA that bears a double-strand break, through the incorporation of SV40 sequences from the COS cell chromosome (8). However, such events are unlikely to be involved in the process reported here. The origin of replication contained in SV40 sequences stably incorporated in the COS cell genome is nonfunctional as a result of a 6-bp deletion (6) and therefore would not be expected to result in increased copies of exogenous SV40 ori-negative constructs, even if recombination from endogenous to exogenous sequences did occur. That no such effect was involved was suggested by the relatively low CAT expression observed in the absence of SV40 ori-positive co-transfectants. On the other hand, the explanation proposed here is consistent with the homologous recombination between co-injected exogenous plasmids in mammalian cells reported by Folger et al. (5).

The data reported here suggest that where it is desirable to reduce background levels of CAT expression following transfection in COS cells, it is advisable to use a control plasmid that does not contain the SV40 ori.

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