RESEARCH COMMUNICATION
Evaluation of the use of the luciferase-reporter-gene system for gene-regulation studies involving cyclic AMP-elevating agents
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The effects of cyclic AMP (cAMP)-elevating agents on the activity of cis-acting gene promoter sequences are frequently studied using the luciferase-reporter-gene system. The aim of the present study was to assess whether cAMP-elevating agents induce any changes in the level of luciferase activity independently of a transcriptional activation of promoter elements. Chloramphenicol acetyltransferase (CAT) and luciferase reporter genes under the control of the same promoter elements were transiently expressed in primary cultures of human vascular smooth-muscle cells. Transfected cells were treated with a cell-permeable and non-hydrolysable cAMP analogue, 2'-O-monobutyryl-8-bromo cAMP, or with the cAMP-elevating agents forskolin and prostaglandin E1 (PGE1). Forskolin and PGE1 induced a significant increase in the level of luciferase activity, but had no effect on CAT activity. Conclusions based solely on the use of the luciferase-reporter-gene system in studies involving promoter activation by cAMP-elevating agents could therefore be misleading.

INTRODUCTION
The regulation of eukaryotic gene transcription is mediated by cis-acting sequence motifs which are typically, although not exclusively, located within the 5'-flanking region of genes [1]. The in vitro study of transcriptional regulation commonly involves the transfection of cultured cells with reporter-gene constructs. These constructs comprise the putative regulatory sequences under investigation linked to a downstream reporter gene [2]. The transcriptional effects of potential inducers or repressors of gene expression are usually assessed by treating the transfected cells with chemical or biological agents prior to preparation of the transfected cell lysates and measurement of reporter protein activity. The most common reporter-gene systems, such as chloramphenicol acetyltransferase (CAT), neomycin phosphotransferase (neo), and firefly luciferase (LUC) fulfil the following requirements: the activity of the encoded protein is easy to assay and is either absent from non-transfected cells or present only at very low levels [3,4].

Firefly LUC offers several advantages over the other reporter proteins: the assay for LUC activity is more sensitive and easier to use than the assay for CAT activity [5,6]. In addition, LUC is a nonomeric protein which does not require post-translational modification and hence its activity can be assessed immediately upon translation [7,8]. The LUC reaction emits light in the visible spectrum (560 nm) through mono-oxygenation of luciferin with O2 and ATP as co-substrates according to the reaction [9]:

\[ \text{LUC} + \text{luciferin} + \text{ATP} + \text{Mg}^{2+} \rightarrow \text{LUC-luciferyl-AMP} + \text{PP}_i \]

\[ \text{LUC-luciferyl-AMP} + \text{O}_2 \rightarrow \text{LUC} + \text{oxyluciferin} + \text{AMP} + \text{CO}_2 + \text{light} \]

In the in vitro LUC reaction assay, saturating concentrations of luciferin, ATP and Mg2+ should be used so that they are not rate-limiting. The emission of light is therefore proportional to the amount of LUC present, which therefore correlates with the level of reporter-gene expression. The luciferin–LUC system is one of the most sensitive assays for the direct measurement of ATP and hence for the indirect measurement of ADP, AMP and cyclic AMP (cAMP) [9]. This luminescence assay of adenosine phosphate molecules is based on their conversion into ATP and the subsequent determination of ATP by its luminescent reaction with firefly luciferin and LUC following the reaction described above.

It is well established that cAMP plays an important role in many cellular processes through its action as second messenger, transmitting the biological effects of various hormones and growth factors [10,11]. The downstream targets of this messenger are cAMP-responsive elements present in the regulatory regions of many genes [12]. The regulatory effects of cAMP-elevating agents on various gene promoters are frequently studied using the LUC-reporter-gene system [13–20]. Bearing in mind the sensitivity of firefly LUC to adenosine phosphate molecules, the measured emission of light may not necessarily reflect the level of LUC generated from the reporter-gene construct. The aim of the present study was to assess whether or not cAMP would interfere with the activity of transiently expressed LUC. It is demonstrated here that treatment of cells with a cAMP-elevating agent could result in a substantial increase in the level of measurable LUC activity. The use of this reporter-gene system in studies involving promoter regulation by cAMP-elevating agents could therefore lead to misleading conclusions.

MATERIALS AND METHODS
Materials

Anti-(human smooth-muscle cell α-actin) monoclonal antibody, 3-isobutyryl-1-methylnanthine (IBMX), forskolin, prostaglandin E1 (PGE1), 2'-O-monobutyryl-8-bromo cyclic AMP (Bu-8-Br-cAMP), dibutyryl cAMP, sodium salt AMP, ADP, ATP and cAMP were purchased from Sigma. The cAMP 125I-radioimmunoassay kit was purchased from Amersham Inter-

Abbreviations used: HVSMC, human vascular smooth-muscle cells; FCS, foetal-calf serum; DMEM, Dulbecco's modified Eagle's medium; IBMX, 3-isobutyryl-1-methylnanthine; PGE1, prostaglandin E1; cAMP, cyclic AMP; Bu-8-Br-cAMP, 2'-O-monobutyryl-8-bromo cyclic AMP; CAT, chloramphenicol acetyltransferase; LUC, luciferase.

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national. Transfectam reagent (liposomes: cationic lipopoly-
amine molecule), reporter lysis buffer, LUC-reporter-gene vectors
(pGL2-Control DNA), CAT reporter gene vectors (pCAT-
control DNA), pSV-β-galactosidase-reporter-gene vectors, LUC
and β-galactosidase enzyme assay were from Promega
(Southampton, U.K.). Pure firefly LUC was obtained from
Boehringer Mannheim.

Cell culture
Human vascular smooth-muscle cells (HVSMC) were isolated
from sections of normal abdominal aorta obtained from kidney
transplant donors (10-45 years of age) as described previously
[21]. Cells were subcultured at a split ratio of 1:4 and used
between passages 2 and 8. HVSMC were identified as such by
their characteristic ‘hills-and-valleys’ pattern of growth and by
their positive immunostaining with an anti-(human smooth-
muscle cell α-actin) antibody [22].

Transfections, CAT and LUC assays
Transient expression of CAT and LUC constructs in HVSMC
was achieved after transfection by liposomes [23,24]. At 1 day
prior to transfections, HVSMC were plated at a density of
1.5 × 10^4/cm^2 in Dulbecco’s modified Eagle’s medium
(DMEM)/10% FCS. On the day of transfection, cells were
incubated in serum-free medium for 12 h with liposomes/DNA
containing 1 μg of pSV-β-galactosidase control plasmid in addi-
tion to 1 μg of LUC-positive control (pGL2-control DNA) or
CAT-positive (pCAT-control DNA). At 48 h post-transfection,
cells were treated for 1 h with 10 μM forskolin, PGE_1, Bu-8-Br-cAMP or
diluent. When the cells were treated with IBMX
(10 μM), this was added 5 min before the start of the treatment.
At the end of the assay period, cell extracts were prepared. CAT
activity was determined by liquid-scintillation counting [25], and
LUC activity was determined by measuring its activity in 20 μl of
cell extract with 100 μl of LUC assay reagent on a model 20e
Turner Designs (Sunnyvale, CA, U.S.A.) luminometer. The
values for CAT and LUC activity were corrected for transfection
efficiency using β-galactosidase activity [26].

cAMP measurement
Parallel cultures of HVSMC pre-treated for 5 min with IBMX
(10 μM) were incubated for 1 h in the presence of forskolin
(10 μM), PGE_1 (10 μM) or diluent. The cells were harvested by
scraping in PBS, collected by centrifugation and treated with
95% ethanol for 30 min on ice. Samples were then centrifuged,
and the supernatant collected, dried under vacuum and used for
the measurement of cAMP. The pellets were lysed in 0.1 M
NaOH and used for the measurement of protein concentration
using the Pierce bicinchoninic acid (BCA) kit. cAMP levels were
measured using an 111In-radioimmunoassay kit according to the
manufacturer’s (Amersham International) instructions.

RESULTS AND DISCUSSION
LUC and CAT reporter-gene constructs under the control of the
same regulatory elements (simian-virus-40 promoter and
enhancer elements) were transiently expressed in primary cultures
of HVSMC. At 48 h post-transfection, cells were treated for 1 h
with 10 μM of cAMP-elevating agents forskolin or PGE_1, or
10 μM Bu-8-Br-cAMP, which is a cell-permeable and non-
hydrolysable cAMP analogue [10,11]. Bu-8-Br-cAMP mimics
some of the biological effects of cAMP which do not involve the
hydrolysis of cAMP. The above treatments were performed in
the presence or absence of 10 μM IBMX, a potent inhibitor of

<table>
<thead>
<tr>
<th>Treatment</th>
<th>10^-3 x Activity (c.p.m.)</th>
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<tbody>
<tr>
<td></td>
<td>CAT</td>
</tr>
<tr>
<td></td>
<td>-IBMX</td>
</tr>
<tr>
<td>Diluent control</td>
<td>147.6 ± 19.6</td>
</tr>
<tr>
<td>Forskolin</td>
<td>133.6 ± 7.9</td>
</tr>
<tr>
<td>PGE_1</td>
<td>151.3 ± 28.7</td>
</tr>
<tr>
<td>Bu-8-Br-cAMP</td>
<td>136.6 ± 6.7</td>
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HVSMMC were transfected with liposomes/DNA of pSV β-galactosidase control plasmid in addition to either luciferase-positive-control or CAT-positive-control plasmids. At 48 h post-
transfection, cells were treated for 1 h with 10 μM of either forskolin, PGE_1, Bu-8-Br-cAMP or
diluent in the presence or absence of 10 μM IBMX. Cell extracts were prepared, and β-
galactosidase, LUC and CAT activities were determined. Values for CAT and LUC activities were
both corrected for transfection efficiency using β-galactosidase activity. Values are
+ S.E.M. of three independent determinations.

Table 1 Effect of forskolin, PGE, and Bu-8-Br-cAMP on LUC and CAT activity

phosphodiesterases, enzymes which hydrolyse cAMP to AMP
[10,11]. Measurement of cAMP levels in cultured HVSMMC
confirmed that forskolin and PGE_1 used at 10 μM for 1 h
induced 5.2- and 4.8-fold increases respectively in the cellular
levels of cAMP. It should be noted here that, in the present
study, dibutylryl-cAMP, another cell-permeable cAMP analogue,
was used. Since dibutylryl-cAMP and Bu-8-Br-cAMP gave
rigorously similar results, only the data obtained with Bu-8-Br-
cAMP are presented.

Table 1 depicts the changes in the levels of CAT and
LUC activity in transfected HVSMC treated with the various
agents described above. In both the presence or absence of
IBMX, neither agent induced any significant change in the level
of CAT activity as compared with cells treated with diluent.
The level of LUC activity, however, showed a marked change as
a result of treatment with forskolin and PGE_1. In the absence
of IBMX, treatment of the cells with forskolin or PGE_1, resulted
in 4.7- and 3.5-fold increases in LUC activity respectively over
that of diluent-treated cells. The LUC activity of cells treated with
Bu-8-Br-cAMP was similar to that of diluent-treated cells. Pre-
 incubation of cells with IBMX reduced the level of LUC activity
in both diluent-treated cells and cells treated with forskolin,
PGE_1 or Bu-8-Br-cAMP. However, forskolin and PGE_1, induced
a 3.2- and 3.0-fold increase in the level of LUC activity as
compared with diluent-treated cells. The LUC activity of Bu-8-
Br-cAMP-treated cells was similar to that of diluent-treated cells.
Using a range of concentrations of pure CAT, LUC and β-
galactosidase enzymes, it was verified that the assays for these
three reporter-gene activities were performed within a linear
range. In the experiments described above, both LUC and CAT
reporter genes were placed under the control of the same
promoter elements. Since IBMX, forskolin and PGE_1 induced a
significant change in the level of LUC activity but had no
detectable effect on the level of CAT activity, we may infer that
these agents did not affect the extent rate of LUC gene tran-
scription, but rather interfered with the expressed LUC protein
activity.

Incubation of transfected cells with the cell-permeable and
non-hydrolysable cAMP analogue Bu-8-Br-cAMP did not affect
the level of LUC activity. IBMX, which inhibits cellular phospho-
diestrases (and hence the conversion of cAMP into AMP, ADP
and ATP), reduced both the steady-state level of LUC activity
and the increase in LUC activity induced by the cAMP-elevating
agents forskolin and PGE$_1$. Taken together, these data indicate that the observed changes in the level of LUC activity subsequent to forskolin or PGE$_1$ treatment are not due to cAMP itself.

In order to determine if cAMP derivative molecules mediate the observed increases in LUC activity, increasing amounts of cAMP, Bu-8-Br-cAMP, AMP, ADP or ATP were added to either pure LUC or extracts prepared from HVSMC transfected with the LUC reporter gene and which had not received any further treatment. Whereas AMP and ADP induced a dramatic decrease in the LUC activity of such extracts, neither cAMP, Bu-8-Br-cAMP nor ATP had any significant effect (Figure 1a).

Apart from its insensitivity to changes in the concentration of cAMP and Bu-8-Br-cAMP, pure LUC behaved differently from the LUC present in cell extracts with regard to changes in the concentration of ATP, ADP, and AMP (Figure 1b). ATP induced a drastic decrease in the activity of pure LUC, whereas AMP and ADP had exerted a rather biphasic effect which was more pronounced for AMP. These data are of particular interest, as they underline the extreme sensitivity of the luminescence reaction to variations in the levels of the cAMP derivative molecules AMP, ADP and ATP. cAMP and Bu-8-Br-cAMP are cyclic nucleotides and do not appear to interfere directly with the LUC reaction. The discrepancies observed between the sensitivity of pure LUC and LUC present in the cell extract to changes in the concentrations of ATP, ADP and AMP strongly suggest that these molecules modulate the activity of the LUC protein and that other as-yet-unidentified compounds, present in cell extracts, interfere with their action. The aim of the experiments described above was to determine whether the observed changes in the LUC activity subsequent to treatment of the cells with cAMP-elevating agents could be attributed to a specific molecule. If that were the case, the conclusion of the present study would have been that the LUC-reporter-gene system could still be used in studies involving treatment of cells with cAMP-elevating agents provided that the cell extracts were normalized with regard to the concentration of the interfering agent. The data presented here demonstrate clearly that the observed increases in LUC activity subsequent to treatment of cells with cAMP-elevating agents do not appear to be mediated solely by variations in the cellular levels of specific compounds, but are rather likely to be a result of complex changes in the levels of multiple compounds. Therefore the normalization of cell extracts with regard to ATP, ADP and AMP cannot be an appropriate solution.

The present study was conducted with HVSMC as an example of primary cell culture. Similar if not identical results were obtained with an established human hepatocyte cell line (HepG 2). Additionally, using forskolin as a cAMP-elevating agent, similar results were obtained with mouse fibroblasts (Swiss 3T3 cells).

The present study demonstrates that treatment of cells with cAMP-elevating agents can lead to a substantial change in the level of the LUC activity independently of a transcriptional activation of promoter elements. The results presented here suggest that variations in LUC activity in studies involving cAMP-elevating agents should be interpreted with caution. Hence we propose that, if this reporter-gene system is to be used in such studies, additional controls, including another reporter-gene system under the control of the same promoter elements, should be included. In light of the data presented here and using a reporter gene other than the LUC system, it would be important to re-examine the conclusions of many reports describing the transcriptional activation of various promoter elements by cAMP-elevating agents.

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REFERENCES


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Figure 1  Effect of cAMP, AMP, ADP, ATP on LUC activity

Increasing amounts of either cAMP (A), AMP (B), ADP (C) or ATP (D) were added to either (a) extracts prepared from untreated cells transfected with the LUC reporter gene or 50 μg/ml of pure LUC (b), and LUC activity was determined. Values are means for three independent determinations. The S.E.M. values were less than 5% and are therefore not shown.

For Bu-8-Br-cAMP the curves obtained were identical with that of cAMP and are also not shown.