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8-Br-cAMP inhibits the transient expression of firefly luciferase

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The genes for firefly luciferase and chloramphenicol acetyltransferase (CAT) were used as reporter genes to explore the activation of heterologous promoters by 8-Br-cAMP. Cells were transfected with a CAT gene/tyrosine hydroxylase promoter, which contains a cAMP response element. Extracts from cells treated with 8-Br-cAMP had 340% more enzyme activity than untreated cells. In contrast, treated cells transfected with a tyrosine hydroxylase/luciferase construct had 30% less activity than control cells. Simian virus and rous sarcoma virus promoters/luciferase constructs also had lower activities in cells treated with 8-Br-cAMP than untreated cells. The inhibition of luciferase enzyme activity by cAMP appears to be posttranscriptional since both luciferase and CAT RNA levels were similarly increased in cells treated with 8-Br-cAMP or 1-methyl-3-isobutylmethylxanthine. The lower level of luciferase activity was not due to simple allosteric inhibition. We conclude that constructs using the firefly luciferase as a reporter gene are unsuitable for studying the effects of cAMP on the regulation of promoters.

Luciferase; Transfection; Bromo cyclic AMP, 8-; Reporter gene; Methyl-3-isobutylmethylxanthine, 1-

1. INTRODUCTION

The firely luciferase gene is being increasingly used as a reporter gene to explore the regulation of heterologous promoters (e.g. [1,2]). As such it offers a number of advantages over other reporter genes in that the luciferase enzyme assay is easy, sensitive, does not require radioactive material, and is amenable to automation [3]. However, the luciferase reporter gene has not been tested in as many situations as the most commonly used reporter gene, chloramphenicol acetyltransferase (CAT). We have tested the effect of a cAMP derivative on heterologous promoters fused to the luciferase reporter gene.

A number of genes have been identified as being regulated at the transcriptional level by cAMP [4]. Two such genes are tyrosine hydroxylase (TH) and somatostatin. In this report we show that treatment of transiently transfected cells by 8-Br-cAMP reduces the activity of various promoter/luciferase fusion genes.

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Abbreviations: CAT, chloramphenicol acetyltransferase; IBMX, 1-methyl-3-isobutylxanthine; TH, tyrosine hydroxylase

2. MATERIALS AND METHODS

2.1. *Plasmids*

The somatostatin promoter linked to the CAT reporter gene was provided (pD(−71)CAT) by Dr M. Montminy (UCSD); pRSVCAT (rous sarcoma virus promoter/CAT) was provided by Dr B. Howard (NIH); pRSVL (rous sarcoma virus promoter/luciferase reporter gene), pSV232AL (an enhancerless simian virus promoter/luciferase) and pSV0AL (luciferase reporter gene) were gifts from Dr J. deWet (UCSD). To facilitate cloning we constructed a plasmid that contained an *Eco*RV site flanked by *Hin*dIII sites. An *Alu*I fragment (27 to −276) from the rat TH promoter was inserted into the *Eco*RV site [5]. This fragment was excised using *Hin*dIII and inserted in the proper orientation at the *Hin*dIII site of pSV0AL [3] creating prTHluc and into pSV0CAT, creating prTHCAT.

2.2. *Transfection*

Rat fibroblast cells (NIH 3T3) were grown in Dulbecco's modified Eagle's media supplemented with 10% fetal calf serum. Rat pheochromocytoma cells (PCG2) were grown in the same media supplemented with 10% fetal calf and 5% horse serum. Cells (5×10^5) were transferred to 10-cm plates and transfected the next day using calcium phosphate-mediated transfection [6]. Cells were treated either with a mixture of test

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plasmids (10 μg) or with the test plasmid (20 μg) plus pRSVCAT or pRSVL (2 μg) as an internal control. 6 h later the cells were treated with 25% glycerol in media without serum for 1 min, rinsed once and fresh complete media added. Isobutylmethylxanthine (IBMX; Sigma) and freshly prepared 8-Br-cAMP (Sigma) were added to final concentrations of 1 mM. The cells were harvested 24 h later and assayed for luciferase [3] and CAT [7] enzyme activity or for RNA levels [8]. Luciferase activity was computed by measuring the height of the peak immediately after injection of ATP.

3. RESULTS

Extracts of PCG2 cells transfected with prTHCAT and pD(−71)CAT (somatostatin promoter) had 3.4–5.5-fold higher levels of CAT activity on treatment of cells with 1 mM 8-Br-cAMP (fig.1). In contrast, extracts of cell transfected with the same TH promoter fragment placed in front of the luciferase reporter gene (prTHluc) treated with 8-Br-cAMP had lower activities than untreated cells. To determine if the reduction in enzyme activity was intrinsic to the TH promoter, we also examined two viral promoters, simian virus (pSV232AL delta 5′) and rous sarcoma virus (pRSVL), attached to luciferase gene. Treatment with 8-Br-cAMP of cells transfected with both of these plasmids also resulted in lower enzyme activities than untreated cells. Similar results were obtained when we used NIH 3T3 cells (not shown).

In order to confirm and extend these results, PCG2 cells were cotransfected with prTHluc and prTHCAT and treated with the phosphodiesterase inhibitor, IBMX, and/or 8-Br-cAMP (fig.2). Treatment with 8-Br-cAMP or with IBMX resulted in 6- and 8-fold enhancement of CAT activity over that of control. Addition of IBMX and 8-Br-cAMP resulted in higher levels than when either component was added separately. In contrast, luciferase activity was not stimulated but was either reduced or remained the same when either IBMX or 8-Br-cAMP was added.

To determine if the inhibition of enzyme activity was transcriptional or posttranscriptional, we assayed RNA from control and treated cells. In contrast to enzyme activities, the levels of luciferase and CAT RNAs were increased to a similar degree by treatment with either IBMX or 8-Br-cAMP (fig.2).

To test whether the diminished activity was the result of inhibition of luciferase enzyme activity by either 8-Br-cAMP or 8-Br-AMP, we assayed commercial luciferase (Sigma) in the presence of several different concentrations of these reagents. Firefly luciferase has two active catalytic sites [9]; one site, I_0, responds to addition of ATP by a rapid pulse of activity and a rapid decay (within 30–60 s). The second site, I_1, is active at about 10–20% of I_0 at saturating levels of ATP and persists over several minutes. Both AMP and cAMP are competitive inhibitors of ATP at I_0 [10] with K_i values of 0.25 and 0.9 mM, respectively. As shown in fig.3, 8-Br-AMP when present at 0.25 mM in-

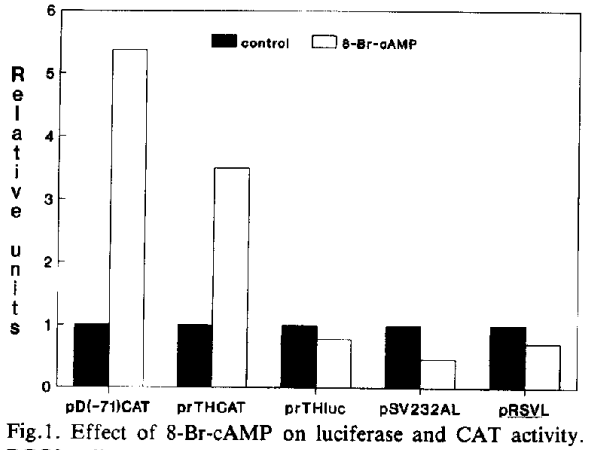

Fig.1. Effect of 8-Br-cAMP on luciferase and CAT activity. PCG2 cells were transfected with a mixture of test plasmid (20 μg) and the control plasmids, pRSVcat or pRSVL. After 23 h incubation with 1 mM 8-Br-cAMP, cells were extracted and enzyme activities determined. Activities were normalized to respective control activities. Values are averages of 3 replicates (SE < 10%).

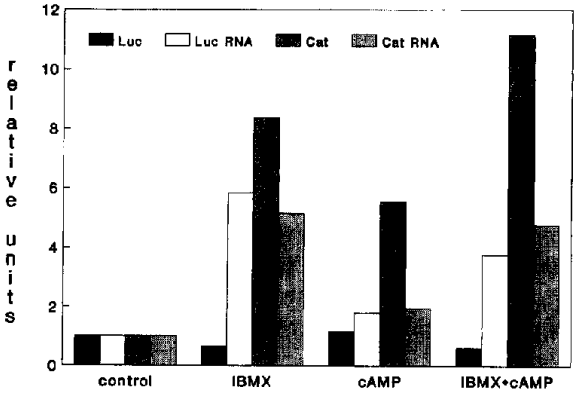

Fig.2. Effect of IBMX and 8-Br-cAMP on luciferase and CAT activity and RNA levels. PCG2 cells were transfected with a mixture of prTHCAT and prTHluc (10 μg each). After 23 h incubation with 1 mM 8-Br-cAMP and/or 1 mM IBMX, cells were extracted and enzyme activities and RNA levels determined. Values are averages of 3 replicates (SE < 10%).

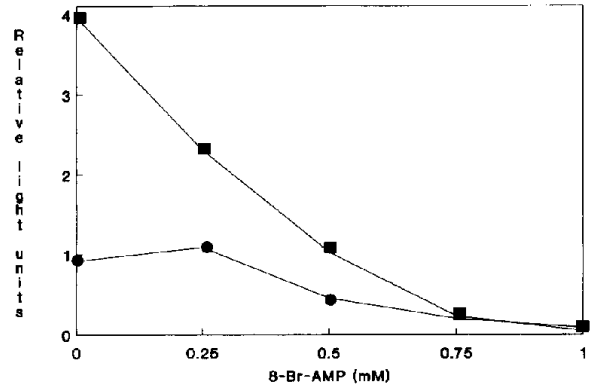

Fig.3. Effect of AMP derivatives on luciferase activity. Luciferase (30 μU) was assayed in the presence of various concentrations of 8-Br-AMP. ATP (final concentration 4.0 mM) initiated the reaction. Squares, I_0 activity; circles, I_1 activity.

hibited I_0 activity by 42% and had no effect on I_1 activity. In contrast, 8-Br-cAMP at a final concentration of 1 mM did not inhibit either site (not shown).

To determine if cell extracts contained a compound that inhibited luciferase activity, commercial luciferase was preincubated for 5 min with extracts of cells that had been treated or untreated with 8-Br-cAMP. There was no effect on exogenous luciferase activity demonstrating the absence of any inhibitory compounds present in cell extracts. In contrast, we did find that certain preparations of ATP contained inhibitory compounds, presumably AMP. To minimize degradation, stock solutions of ATP were rapidly adjusted to pH 7.0 and quick frozen.

4. DISCUSSION

We and others have shown that cAMP derivatives increase the transcription of TH and somatostatin/CAT reporter genes in transient expression assays [11,12]. This increase in transcriptional activity is mediated by the presence of a cAMP response element 5′ to the TATA box in both of these genes [4]. Surprisingly, when an identical TH promoter fragment is fused to luciferase and CAT reporter genes very different responses are elicited by the presence of 8-Br-cAMP (fig.1). In the same experimental paradigm, CAT activity was increased in extracts from treated cells whereas luciferase activity was decreased in comparison to control values (fig.2). Therefore, the reduction of luciferase activity upon addition of 8-Br-AMP does not appear to be due to the nature of the attached promoter or the presence of a cAMP response element as neither pSV232AL delta 5′ nor pRSVL contain this sequence (fig.1). Although luciferase can be inhibited by AMP derivatives (fig.3; [10]) our mixing experiments ruled out simple inhibition of enzyme activity by an allosteric inhibitor such as 8-Br-AMP or AMP.

Because RNA for both CAT and luciferase were induced to a similar degree upon the addition of either 8-Br-cAMP or IBMX (fig.3), the observed decrease in luciferase activity presumably involves its translational efficiency and/or protein stability in the presence of cAMP. As firefly luciferase is intimately involved in ATP metabolism, it is not unreasonable to suggest that cAMP may regulate luciferase protein levels via these mechanisms. Whatever the means of inhibition, our data demonstrate that luciferase should not be used in examining the response of heterologous promoters to cAMP.

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