Volume 306, number 2,3, 229–233 FEBS 11314 July 1992

Loss of transcriptional repression contributes to the ectopic expression of the calcitonin/α-CGRP gene in a human lung carcinoma cell line

Aviva J. Symes, Roger K. Craig and Paul M. Brickell

Medical Molecular Biology Unit, University College and Middlesex School of Medicine, Windeyer Building, Cleveland Street, London WC1B 8Y, UK

Received 3 June 1992

The calcitonin/α-CGRP (CT/CGRP) gene is ectopically expressed in a wide variety of neoplasia. We have investigated the molecular mechanisms responsible for this ectopic expression in the human cell line BEN, which is derived from a poorly differentiated squamous cell lung carcinoma. We show that a *trans*-acting factor which represses expression of the CT/CGRP gene in HeLa cells is absent or inactive in BEN cells, and have localised the repressor binding site to a 53 bp fragment 1500 bp upstream of the transcription start site.

Calcitonin; CGRP; Transcription; Repression; Ectopic expression

1. INTRODUCTION

The aberrant or ectopic expression of certain peptide hormones, a common occurrence in many forms of non-endocrine neoplasia [1], often results in adverse clinical syndromes [2]. Several theories have been proposed to explain ectopic expression, yet the molecular mechanisms underlying this aberrant gene expression remain unknown. The CT/CGRP gene is ectopically expressed in a wide variety of neoplasia, including carcinomas of the lung, breast, colon and pancreas, and leukaemias [3,4]. We have examined the regulation of CT/CGRP gene expression in the lung carcinoma cell line BEN, which is derived from a poorly differentiated squamous cell carcinoma which ectopically expresses the CT/CGRP gene [5]. We have compared this with that in the cervical carcinoma cell line, HeLa, which does not express this gene [6]. We report that repression of the gene in HeLa cells is mediated by a *trans*-acting factor that is inactive in BEN cells. We have localised the repressor binding site to a 53 bp fragment 1500 bp upstream of the transcription start site.

2. MATERIALS AND METHODS

2.1. *Plasmids*

A series of plasmids (calgcat 1–6) were constructed, containing regions of the 5′ flanking region of the CT/CGRP gene placed upstream of the bacterial chloramphenicol acetyl transferase (CAT) reporter gene, as shown in Fig. 1. CT/CGRP gene fragments were obtained from the cosmid genomic clone cos CT1 [6], and inserted upstream of the CAT gene in the vectors Gcat-a and Gcat-c [8]. The 3′ end of the CT/CGRP insert was the same in each of the calgcat plasmids and corresponded to the *Sfa N*1 site which lies 9 bp into exon 1. pAJS7 was constructed by ligation of a 946 bp *Kpn*I/*Bgl*II fragment from calgcat 1 to *Kpn*I/*Bam*HI digested pGemBlue 4 (Promega). RCAT 1 was constructed by ligating a 53 bp *Hin*dIII/*Pst*I fragment from calgcat 1 to *Hin*dIII/*Pst*I digested Bleat 2 [9].

Correspondence address: A. Symes, Molecular Neurobiology Laboratory, Massachusetts General Hospital, 149 13th Street, Charlestown, MA 02129, USA. Fax: (1) 617-726-5677.

2.2. *Cell lines*

BEN cells were cultured in α-MEM supplemented with 10% FCS as previously described [10]. HeLa cells were grown in DMEM supplemented with 10% FCS in the same way.

2.3. *Calcium phosphate transfections and CAT assays*

Cells were plated out the day before transfection; BEN cells at 3×10^6 cells/60 mm Petri dish and HeLa cells at 7×10^5 cells/100 mm Petri dish. Cells were transfected according to the procedure of Gorman [11], with 10 μg of plasmid DNA, except where stated otherwise. The precipitate was left on the cells for 14–18 h, the cells glycerol shocked (BEN cells 120 s; HeLa cells 30 s) and fresh medium added. Cells were harvested 36–72 h later, lysed by 3 cycles of freeze/thawing and assayed for protein using the BCA protein assay kit (Pierce; Rockford, IL). Extracts equalised for protein were then assayed for CAT activity [11]. In some experiments, cells were co-transfected with 2.5 μg pRSVluc DNA and the extracts were assayed for luciferase activity [15] directed by pRSVluc, as well as for CAT activity. We found no evidence for any significant differences in transfection efficiency for different CAT constructs.

3. RESULTS

In order to investigate the promoter activity of the CT/CGRP gene in both BEN and HeLa cells, a series of plasmids were constructed as shown in Fig. 1. Initially, calgcat 1, 2, 3 and 4 were transfected into both BEN and HeLa cells. Fig. 2A shows that in BEN cells 1670 bp of 5′ flanking sequence (calgcat 1) induced 30-fold greater levels of CAT expression when transfected into BEN cells than was observed with a fusion gene containing only 150 bp of CT/CGRP promoter (calgcat

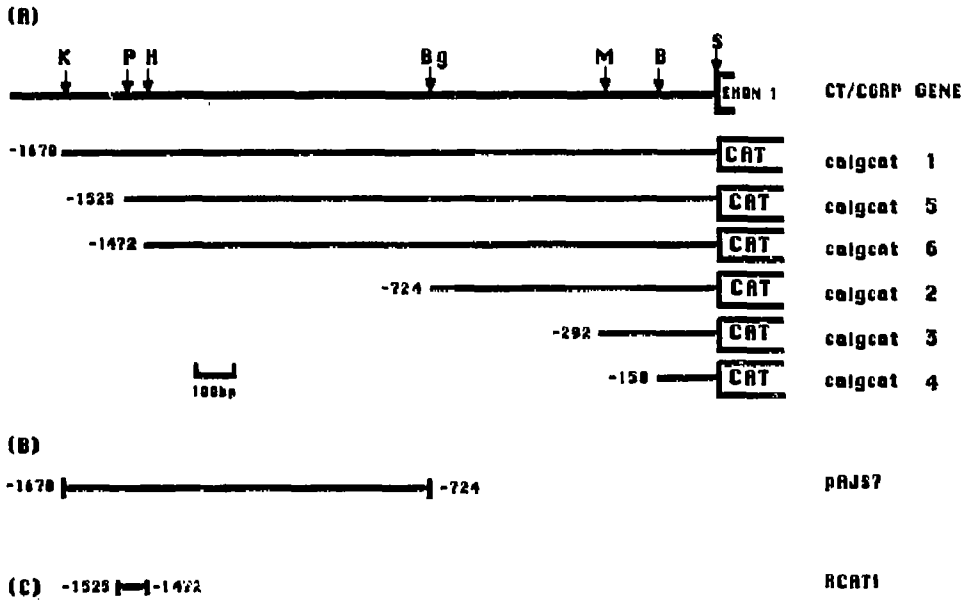

Fig. 1. Representation of CT/CGRP promoter constructs. The 5′ flanking region of the CT/CGRP gene is shown. Restriction enzyme sites used in the construction of the promoter plasmids are indicated: (K=*Kpn*I; P=*Pst*I; H=*Hin*dIII; Bg=*Bgl*II; M=*Msp*I; B=*Bam*HI; S=*Sfa*NI). (A) Construction of the series of CT/CGRP promoter–CAT fusion plasmids. The 3′ end of the CT/CGRP promoter, and its fusion to the CAT coding sequences was identical in all plasmids (see Methods). (B) 946 bp *Kpn*I/*Bgl*II fragment of the CT/CGRP promoter was ligated to pGemBlue 4 to form pAJS7. (C) 53 bp *Hin*dIII/*Pst*I fragment of the CT/CGRP promoter was ligated into BLcat 2 to form Rcat 1.

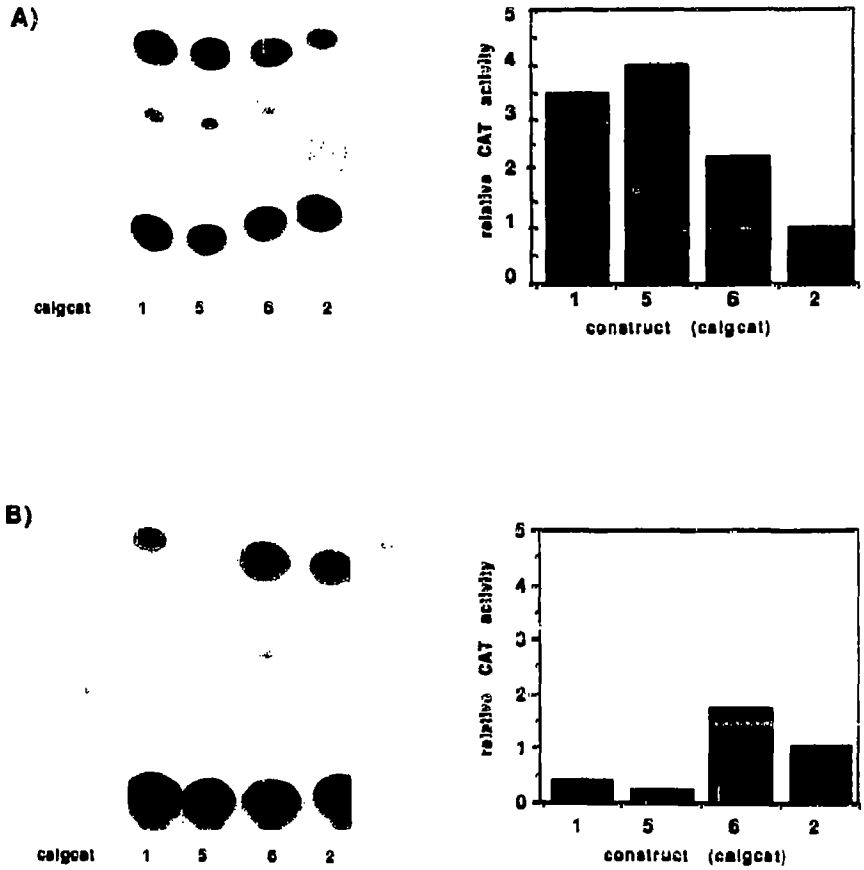

Fig. 2. Transient expression of calgcat 1–4 in (A) BEN cells and (B) HeLa cells. Cells were transfected by calcium phosphate precipitation, with 10 μg plasmid DNA, and harvested 36 h after the precipitate was removed. Resultant CAT activity was determined in extracts equalised for protein. CAT activity was quantitated by liquid scintillation counting of the thin-layer chromatography plates, and calculated as the percentage conversion of chloramphenicol to its acetylated products, relative to that generated by calgcat 4 for each cell line. Values shown are the mean of triplicate determinations of duplicate experiments ± SEM.

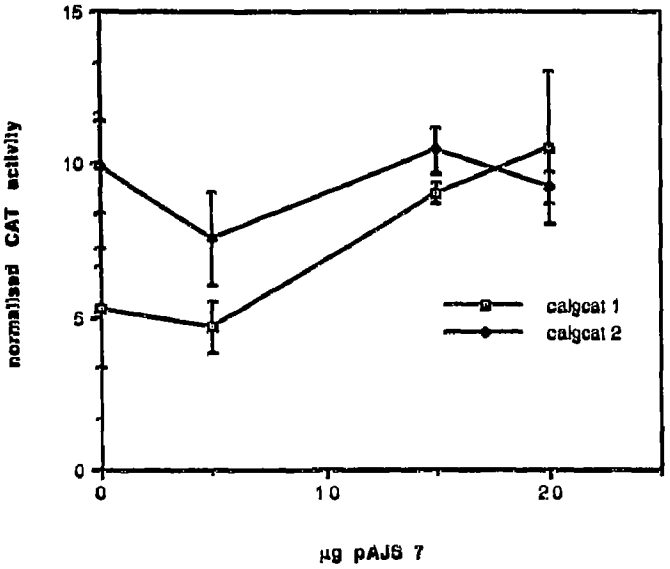

Fig. 3. Cotransfection of increasing amounts of pAJS7 with calgcat 1 relieves the repression in HeLa cells. HeLa cells were transiently transfected with 5 µg of either calgcat 1 or calgcat 2, together with increasing amounts of pAJS 7, as indicated. Cells were harvested 48 h after transfection and the extracts assayed for CAT activity. The total amount of DNA transfected, 25 µg, was kept constant by the addition of carrier plasmid DNA. Data are shown as mean ± SEM (n=3) from a representative experiment.

4). Constructs containing sequences from −724 (calgcat 2) or −292 (calgcat 3) nucleotides upstream of the transcription start site both directed 5–8-fold greater expression than the 150 bp construct. This indicates the existence of multiple, *cis*-acting, positive regulatory sequences in the upstream region of the CT/CGRP gene. In HeLa cells, constructs containing 292 (calgcat 3) and 724 bp (calgcat 2) of upstream information also directed greater levels of CAT expression (approximately 15-fold) than the 150 bp construct (Fig. 2B). However, HeLa cells contrasted with BEN cells in that the presence of sequences between −724 and −1670 did not further raise CAT levels, as in BEN cells, but reduced them by over 50%. These data indicate that sequences located between −724 and −1670 mediate repression of CT/CGRP promoter activity in HeLa cells, but not in BEN cells.

To ascertain whether a *trans*-acting factor was involved in this repression, the 946 bp fragment between nucleotides −724 and −1670, was subcloned into pGem-Blue 4 to form pAJS 7 (Fig. 1). As shown in Fig. 3, transfection into HeLa cells of increasing amounts of pAJS 7, together with a constant amount of calgcat 1, was able to relieve the repression. A 7-fold excess of the 946 bp fragment was sufficient to restore the level of CAT activity generated by calgcat 1 to that of calgcat 2. However, transfection of increasing amounts of pAJS 7 had no significant effect on the CAT activity directed by calgcat 2, demonstrating that this effect is specific to calgcat 1. The ability of this fragment to lift repression strongly suggests that repression is mediated by a *trans*-acting factor.

To localise the putative repressor binding site further, plasmids calgcat 5 and 6 were constructed (Fig. 1). Fig. 4 shows the CAT activity resulting from the transfection of these constructs into BEN and HeLa cells. In both cell types, calgcat 6 directs a higher level of CAT expression than calgcat 2, reflecting the presence between nucleotides −1670 and −724 of positively acting elements, as previously reported [6]. In BEN cells, calgcat 5 directs expression of even greater levels of CAT than does calgcat 6. However, in HeLa cells, CAT expression from calgcat 5 is almost abolished. These data indicate that the 53 bp between nucleotides −1472 and −1525 (Fig. 5A) contain sequences which repress expression in HeLa cells, but which positively regulate expression in BEN cells.

The 53 bp putative repressor binding site fragment was inserted upstream of an HSV-tk promoter in a chimaeric CAT construct and transfected into HeLa cells. Fig. 5 shows that transcription was not repressed from the HSV tk promoter by the addition of this 53 bp fragment, relative to the activity of the parent vector, Blcat 2. Thus the putative 53 bp repressor binding site is unable to repress transcription from a heterologous promoter.

4. DISCUSSION

Our results indicate that there is a repressor protein acting on the CT/CGRP promoter in HeLa cells, which binds to a site between 1472 and 1525 nucleotides upstream of the transcription start site. The action of this repressor may contribute to the non-expression of the CT/CGRP gene in HeLa cells. We have shown that the repressor protein is inactive in BEN cells, and that this may explain the ectopic expression of the CT/CGRP gene in these cells.

The inability of the putative repressor binding site to mediate represson of transcription from a heterologous promoter in HeLa cells suggests that repression may operate by interaction between the putative repressor protein and other proteins which specifically bind to the CT/CGRP promoter region. Alternatively, the repressor could act by preventing the binding of positively-acting factors, which themselves act synergistically with other proteins binding to the promoter region. This would be analogous to the repression of the β-interferon gene, which is lifted upon viral infection [12]. Upon induction, the repressor protein dissociates, allowing two positively-acting factors to interact with an adjacent region. Supporting the view that repression of the CT/CGRP gene may be mediated through a similar mechanism, is our finding that the 53 bp fragment, which mediates repression in HeLa cells, stimulates CAT activity in BEN cells, which do not possess an

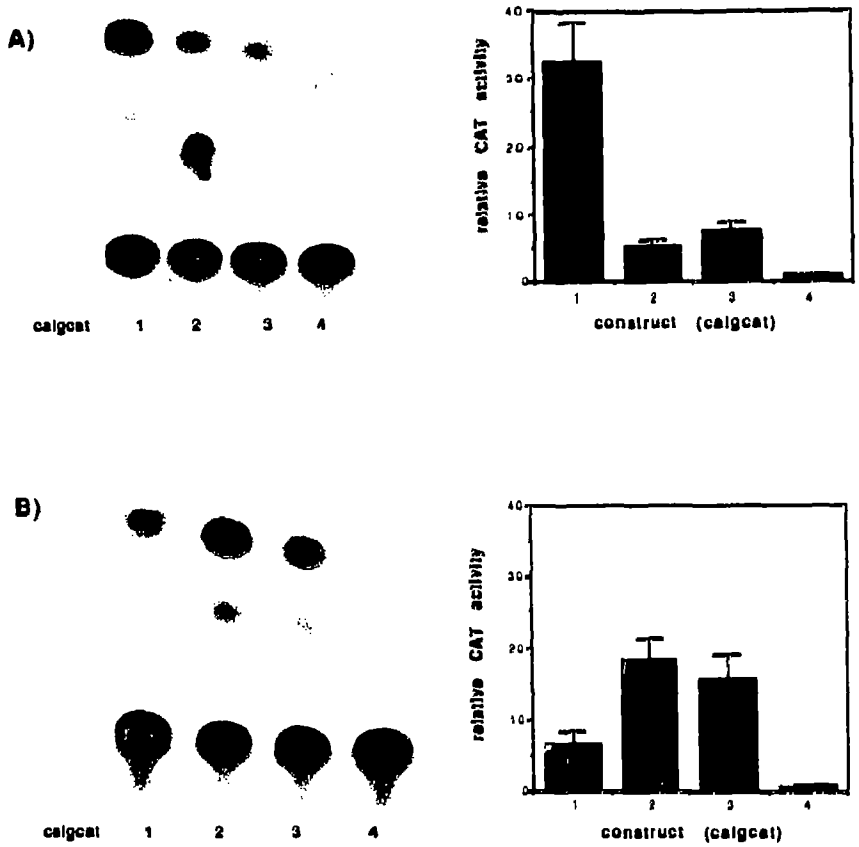

Fig. 4. Transient expression of calgcat 1, 5, 6 and 2 in (A) BEN and (B) HeLa cells. Cells were transfected with 10 μg plasmid DNA, and harvested 72 h after transfection. Resultant CAT activity was determined in protein equalised extracts and is shown expressed relative to that of calgcat 2 in each cell line for a representative experiment.

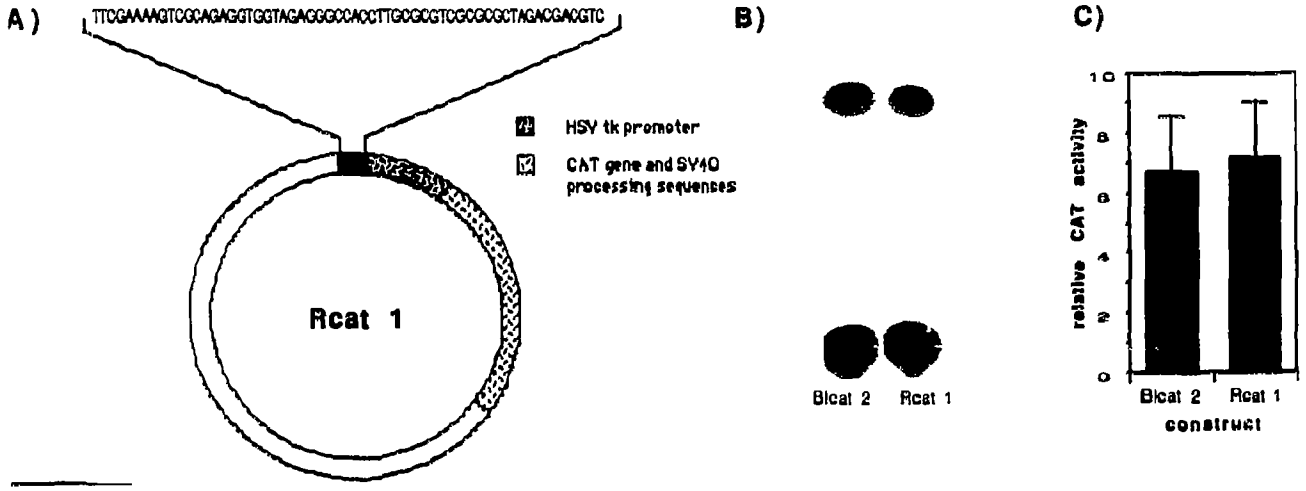

Fig. 5. The effect of insertion of the repressor element upstream of a heterologous promoter in HeLa cells. (A) The 53 bp *Pst*I/*Hin*dIII fragment was inserted upstream of the HSV-tk promoter in the plasmid Bleat 2, to form plasmid Rcat1. A representative autoradiograph (B) and graph (C) of the resultant CAT activity after transfection of Rcat 1 and the parent vector Bleat 2 into HeLa cells is shown. Values shown are the mean ± SEM of triplicate determinations in this experiment. There is no significant difference in the CAT activities produced by either plasmid in HeLa cells.

active repressor. This suggests that positively-acting factors could interact with this region when no repressor is present.

We have previously shown that the CT/CGRP gene possesses a CpG island towards the 5′ end of the gene that is unmethylated in all normal tissues [7]. The theory of CpG islands argues that their unmethylated status not only allows binding of transcription factors to the DNA without interference from methyl groups, but also leads to a more open chromatin structure so that the same factors can gain access more easily to the DNA [13]. Genes whose expression is tissue-specific and which possess CpG islands must therefore be actively repressed in non-expressing tissues. Such a mechanism has been shown to operate in the case of the retinol binding protein [14]. Our demonstration of repression of CT/CGRP promoter activity in HeLa cells provides a second example.

This paper suggests a possible model to explain the ectopic expression of the CT/CGRP gene. In non-expressing tissues, represented here by HeLa cells, the CT/CGRP gene is repressed through the action of a repressor protein which binds upstream of the transcription start site. In the process of neoplastic transformation this repressor protein is lost or inactivated. Once the repressor is inactive the CT/CGRP gene may be ectopically expressed immediately, or it may require further activation by positively-acting factors. Thus derepression of the CT/CGRP gene may lead to its ectopic expression in certain forms of neoplasia.

Acknowledgements: We thank the Cancer Research Campaign for supporting this work. We are grateful to Dr. D. Kioussis for the gift of HeLa cells.

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