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The functional activity of the rat c-Ha-ras promoter requires the coordinate involvement of multiple elements

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Promoter activity in the rat c-Ha-ras promoter region was assessed in NIH 3T3 cells using the chloramphenicol acetyltransferase (CAT) reporter gene. Promoter activity was orientation dependent. Deletion of the GC box closest to the transcriptional start site, or the upstream GC box and the two CAAT boxes, greatly diminished promoter activity. The GC boxes in the SV40 early promoter could not functionally replace the downstream c-Ha-ras GC box, but specifically competed for expression of rat c-Ha-ras promoter activity. Serum-stimulated growth of 3T3 cells stably transfected with p035-ras-CAT was not associated with increased promoter activity. These data indicate that the GC and CAAT boxes in the rat c-Ha-ras gene are related to constitutive, and not regulatory, promoter activity, and that these elements are involved in a coordinate manner.

c-H-ras promoter; Proto-oncogene; Promoter

1. INTRODUCTION

The product of the c-ras proto-oncogene, the protein p21, plays an important role during cellular growth [1]. In both in vivo and in vitro systems, it has been shown that cellular growth-stimulating factors increase c-Ha-ras mRNA expression [2–5]. Little is known about the regulation of transcriptional activity of the c-Ha-ras proto-oncogene. In the human c-Ha-ras gene a promoter element has been demonstrated upstream of the transcriptional start site [6], and this region also contains an enhancer element [7]. We have previously reported the cloning and structural characterization of the rat c-Ha-ras 1 gene 5′-flanking sequence [8]. Unlike in the human gene [6], the rat promoter region contains a CAAT box in its characteristic position [8]. As in the human gene a number of 10 bp GC box consensus sequence elements [9], important in the regulation of gene expression, are also present in the rat [8]. We now report data on the functional activity of the rat c-Ha-ras promoter region.

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2. MATERIALS AND METHODS

2.1. *Cell culture*

NIH 3T3 mouse fibroblasts were cultured in DMEM H_{21} medium containing 10% FCS.

2.2. *Plasmid constructions*

pSV40-CAT (kindly provided by the laboratory of Dr John Baxter) contains the SV40 early promoter and the chloramphenicol acetyltransferase (CAT) gene in pUC13. PSV40-comp was generated by deleting the CAT gene by digestion with *Bgl*II and *Bam*HI. The following plasmids were also constructed (fig.1). Plasmid 055-ras-CAT was generated by ligating in place of the SV40 early promoter the *Pst*I-*Pst*I 0.55 kb fragment of the rat

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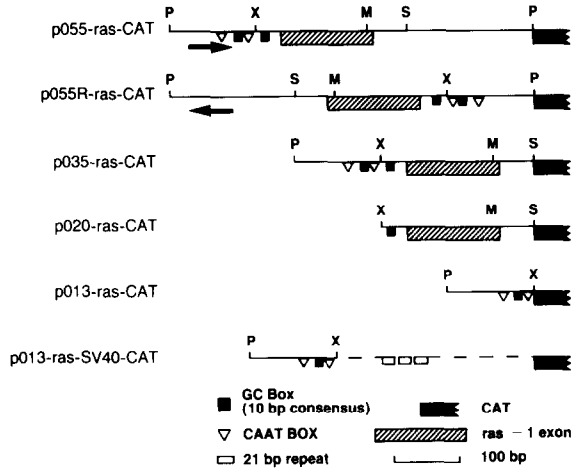

Fig.1. Plasmid constructs in pUC13 of various regions of the rat c-Ha-ras 5′-flanking region containing promoter activity. Construction of these plasmids is described in section 2. The heavy horizontal arrows in the p055-ras-CAT and p055R-ras-CAT constructs indicate the orientation of the DNA insert.

c-Ha-ras gene located upstream of the +1 exon [8]. This plasmid contains the −1 exon as well as 177 bp upstream of the first cap site and 274 bp downstream of the −1 exon. Plasmid 055R-ras-CAT was obtained by ligating this fragment in the reverse orientation. Plasmid p035-ras-CAT was constructed by inserting the *Pst*I-*Sau*3a fragment of the rat c-Ha-ras gene, containing the −1 exon and 177 bp upstream of the first start site [8]. Deletion of 160 bp at the 5′-end of p035-ras-CAT with *Pst*I-*Xho*I generated the plasmid p020-ras-CAT. Plasmid 013-ras-CAT contains the *Pst*I-*Xho* fragment of 135 bp ligated upstream of the CAT gene. The construct p013-ras-SV40-CAT was obtained by ligating the same *Pst*I-*Xho*I fragment upstream of the SV40 component of pSV40-CAT. p5kb-ras-CAT (not shown in fig.1) was constructed by ligating a 5 kb *Eco*RV-*Hin*dIII fragment from λrr5f1 [8] upstream of the CAT gene into pUC18. This fragment contains 3.5 kb upstream of the rat c-Ha-ras −1 exon.

2.3. *Cell transfection and CAT assay*

Transfections were performed by electroporation [1] with an ISCO 494 power supply using $4–7 \times 10^6$ cells and 10–20 μg plasmid DNA in a volume of 0.5 ml. Transfection efficiency was assessed by cotransfection with 20 μg pRSV-βgal that expresses β-galactosidase activity (also provided by Dr John Baxter). 48 h after plating, cells were harvested by trypsinization and cell cytosol was obtained by three cycles of freeze-thawing followed by centrifugation for 10 min in a microfuge. The CAT assay [11] was performed using [^{14}C]chloramphenicol (New England Nuclear, Boston, MA) (4 mM acetyl-CoA, 3 h incubation). The results were normalized for β-galactosidase activity measured in the same cytosol according to Hall et al. [12]. For permanent transfections, cotransfection was with pSV2neo (1–2 μg) followed by selection in 500 μg/ml G418 (Gibco).

2.4. *Thymidine uptake*

Adherent cells were rinsed twice with PBS and then incubated for 3 h in Hank's balanced salt solution (HBSS) containing 1 μCi [^{3}H]thymidine (New England Nuclear). After rinsing twice with ice-cold PBS and three times with cold 10% trichloroacetic acid, the cells were dissolved in 0.1 N NaOH and radioactivity was counted.

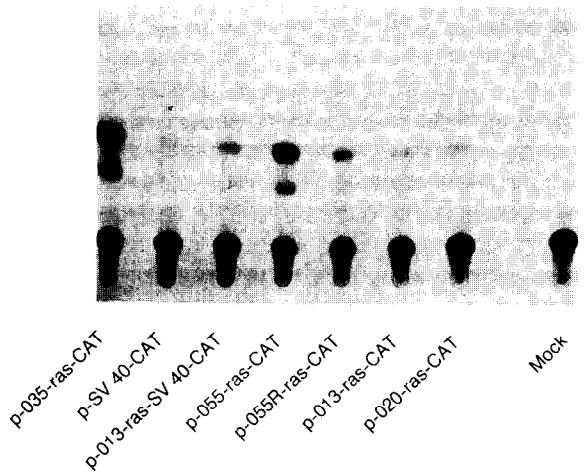

Fig.2. Autoradiograph of CAT activity in NIH 3T3 cells transfected with the plasmid constructs shown in fig.1. Transfection by electroporation and assay of CAT activity were as described in section 2. This figure is representative of three separate experiments. Mock transfections were 3T3 cells subjected to the same procedure without plasmid DNA. CAT activity was quantitated by direct liquid scintillation counting of the acetylated chloramphenicol. None of the plasmids generated more than 7% of the activity of p055-ras-CAT or p035-ras-CAT.

3. RESULTS

NIH 3T3 mouse fibroblasts transfected with p055-ras-CAT (fig.1) express CAT activity (fig.2) indicating that the 0.55 kb *Pst*I-*Pst*I fragment contains a functional promoter of the rat c-Ha-ras gene. Ligation of the same fragment in the reverse orientation (p055R-ras-CAT) greatly diminished enzymatic activity. Strong promoter activity was also present in p035-ras-CAT that lacks the tract downstream of the −1 exon. The 177 bp fragment located upstream of the rat c-Ha-ras −1 exon contains two CAAT boxes in positions −66 and −110 and two decanucleotide GC boxes [8]. Both p013-ras-CAT (lacking the downstream GC box) and p020-ras-CAT (lacking the upstream GC box as well as the two CAAT boxes) had very low promoter activity when compared with p035-ras-CAT. Replacement of the downstream GC box with the SV40 21 bp repeats containing six GC boxes [13] (p013-ras-SV40-CAT) was also associated with low activity, although activity was 5-fold higher than the control pSV40-CAT that lacks the c-Ha-ras element (fig.2).

In order to determine whether the GC boxes in p035-ras-CAT were functionally important, we attempted to neutralize available Sp1 in 3T3 cells by co-transfecting pSV40 comp DNA together with

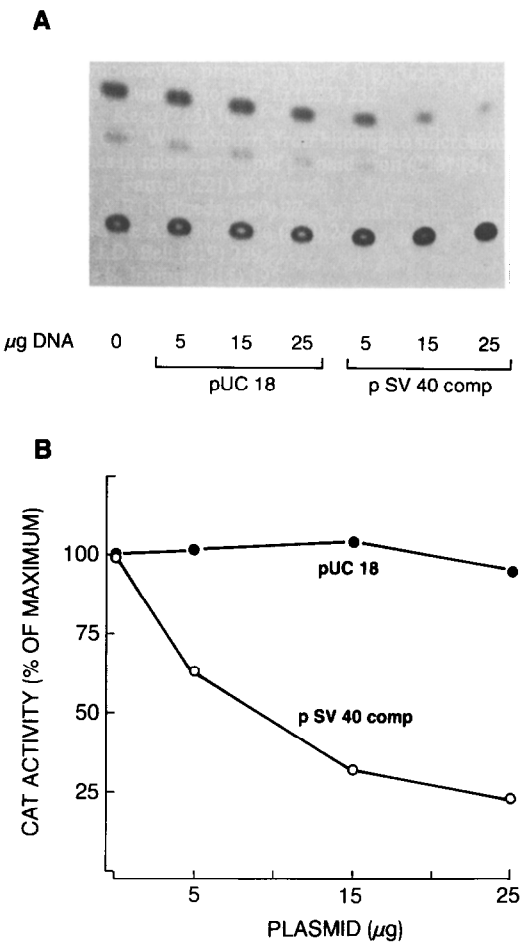

Fig.3. Competition for Sp1 interaction with the rat c-Ha-ras promoter. 5 μg p035-ras-CAT were transfected by electroporation into 3T3 cells together with the amounts of DNA of pUC18 and pSV40 comp as shown in (A). Electroporation and assay of CAT activity were as described in section 2. (B) Densitometric analysis of the data shown in (A). CAT activity (% of maximum) refers to activity relative to p035-ras-CAT transfected in the absence of competing plasmid DNA.

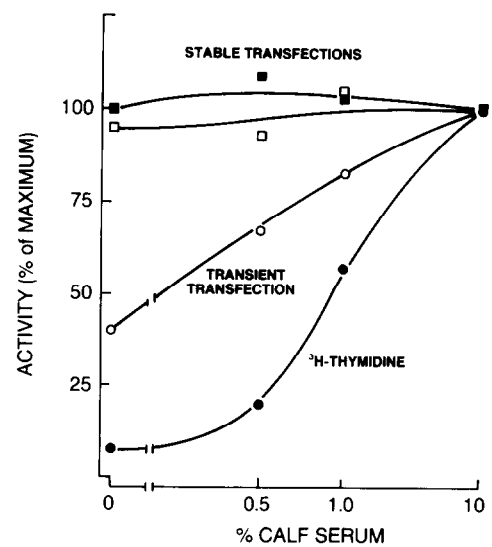

Fig.4. Relationship between serum-stimulated cell growth and rat c-Ha-ras promoter activity. NIH 3T3 cells were transfected with 20 μg p035-ras-CAT or p5kb-ras-CAT (section 2). CAT activity was determined after 48 h of cell culture (transient transfection with p035-ras-CAT; ○—○) or after selection of stable transfectants [(■) p035-ras-CAT, (□) p5kb-ras-CAT]. After serum deprivation for 2 days, the indicated concentration of calf serum was added for 12 h. [^{3}H]Thymidine incorporation was assayed in parallel cultures of cells (section 2). CAT activity was expressed as a percent of maximum activity (CAT or thymidine incorporation) in cells growing in 10% calf serum.

p035-ras-CAT. The former plasmid contains the GC boxes of the SV40 early promoter. Only pSV40 comp, and not pUC18 transfected as a control, decreased the expression of p035-ras-CAT (fig.3).

Because c-Has-ras mRNA levels increase in cells stimulated to divide [2–5], we examined whether c-Ha-ras promoter activity correlated with the growth activity of 3T3 cells. Cells were transfected with p035-ras-CAT, either stably or transiently, and were subsequently cultivated in different concentrations of calf serum. As expected, [^{3}H]thymidine uptake increased as cells were incubated in increasing concentrations of calf serum (fig.4). Surprisingly, although CAT activity correlated positively with thymidine uptake in transient transfections, activity in stable transfectants was unaltered in cells in different states of growth. A similar lack of relationship between cell growth and c-Ha-ras promoter activity was seen in cells stably transfected with p5kb-ras-CAT.

4. DISCUSSION

There are similarities as well as major differences between the rat and human c-Ha-ras promoter regions [6,8,15]. To these we now add that the rat, unlike the human, promoter is orientation dependent and therefore lacks an enhancer element, at least in the corresponding *Pst*I-*Pst*I 0.55 kb fragment. This conclusion is strengthened further by the inability of the *Pst*I-*Xho*I 135 bp fragment to drive a heterologous SV40 promoter (p013-ras-SV40-CAT).

The functional importance of Sp1 binding to the GC boxes in the human c-Ha-ras promoter has been demonstrated [14]. Our data support the concept that Sp1 is an important factor in the activation of the c-Ha-ras promoter. This is because, as reported for the human gene [14], competition for available Sp1 by SV40 GC boxes inhibits promoter activity. However, the presence of only one of the two decanucleotide GC boxes (p020-ras-CAT and p013-ras-CAT) is not enough for full promoter activity. This finding also holds for the human c-Ha-ras promoter [14]. It is therefore likely that more than one Sp1 molecule binding to multiple GC recognition sites must interact with each other in order to generate full promoter activity. The reason why the GC boxes in the SV40 promoter could not effectively substitute for the endogenous ras promoter GC boxes (p013-ras-SV40-CAT) is presumably because of steric changes that result from alterations in the distances between the different elements [15]. Our data also support the concept that Sp1 moleules binding to one or more GC boxes in the rat c-Ha-ras promoter also require the cooperative interaction of CAAT box binding factor (CTF) [16].

We explored the relationship between cell replication and rat c-Ha-ras promoter activity. Surprisingly, contradictory results were obtained with stable or transient transfections with c-Ha-ras promoter CAT constructs. Thus, when the cells were induced to replicate in the presence of serum, c-Ha-ras promoter activity was unaltered in stably transfected, but enhanced in transiently transfected, 3T3 cells. The reason for this difference is unknown, but could relate to a difference in CAT enzyme stability that may be masked at higher levels of expression as occurs in the stable transfectants. Irrespective of the reason, these data indicate the hazard of interpreting results when only one form of transfection is used. However, it seems more likely that the permanent transfection condition reflects the true promoter activity because all cells contain the same number of copies of the transfected gene. In addition, because the transfected cells were from a large pool of individual colonies, it is unlikely that lack of regulation occurred because of insertion into a unique site in the genome.

We have previously observed that TSH stimulation of growth of FRTL5 rat thyroid cells is associated with an elevation in c-Ha-ras steady-state mRNA levels [5], but does not involve increased c-Ha-ras promoter activity (Damante, G. and Rapoport, B., submitted). Our present finding of a similar lack of effect of serum-stimulated growth of 3T3 fibroblasts on rat c-Ha-ras promoter activity suggests that the CAAT/GC complex functions as a constitutive promoter and is not directly involved in the regulation of gene expression. Indeed, there is no direct evidence that we are aware of that indicates regulation of a gene via a CAAT/GC complex. Instead, other regulatory elements will presumably be found that may modulate c-Ha-ras transcriptional activity. One possible candidate, at the 3′-end of the human c-Ha-ras gene, has recently been identified [17].

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