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Deletion analysis of the c-Ha-*ras* oncogene promoter

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Using transient assays of chloramphenicol acetyltransferase expression in CV-1 cells, we have localized the human c-Ha-*ras* oncogene promoter to a 550 bp fragment located about 1 kb upstream from the *ras* coding sequence. Deletion analysis has revealed that a 100 nucleotide region 200 base pairs upstream of the putative initiation sites for transcription is essential for high levels of expression. Within this sequence lie two *Sp1* binding sites and a consensus CCAAT box.

c-Ha-*ras* oncogene; Promoter; Deletion analysis; Chloramphenicol acetyltransferase; Enzyme assay; Consensus box; (Human)

1. INTRODUCTION

The oncogenic potential of the normal *ras* gene can either be activated by single point mutations [1] or by enhanced expression [2]. In certain cases, modulation of *ras* levels can be demonstrated during cellular transformation and tumor progression [3] and during liver regeneration [4] and can have profound effects on cell phenotype [5–7]. It is, therefore, important for our understanding of carcinogenesis as well as our knowledge of gene expression in general to delineate the *cis*-acting elements responsible for *ras* expression.

With the exception of rapidly dividing cells, expression of the *ras* gene remains relatively constant and low in most tissue types and throughout development [8]. Hence, the *ras* genes would appear to fall into the broad class of ubiquitously expressed genes generally termed 'housekeeping genes'. Recent experiments [9] have localized the promoter region of the c-Ha-*ras* gene to a 550 base pair (bp) *Nae*I fragment near the 5′-end of the genomic clone. Like other housekeeping genes studied to date [10], the *ras* promoter is devoid of sequences traditionally observed in highly expressed tissue-specific genes, but contains six functional binding sites for the transcription factor *Sp1* [11]. In this study we have used deletion analysis to characterize further the sequences essential for c-Ha-*ras* expression using transient gene transfer techniques.

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2. MATERIALS AND METHODS

2.1. *Plasmid constructions*

The plasmid pBR-CAT was constructed by subcloning the *Hin*dIII–*Bam*HI fragment of pSV2-CAT [12] into the *Hin*dIII and *Bam*HI sites of pBR322. Promoter fragments were tested by subcloning into the *Hin*dIII site of pBR-CAT after addition of *Hin*dIII linkers.

Deletions were constructed as follows: the 550 bp *Nae*I fragment of c-Ha-*ras* (nucleotides 116–666) was subcloned into the *Sma*I site of pUC18 in an orientation such that the pUC polylinker *Kpn*I site was upstream of the promoter. This plasmid, called pUCNaeI, was partially digested with *Hae*III or *Tha*I and ligated with *Bgl*II linkers. The linkers could then be precisely positioned by restriction analysis. 5′-deletions were constructed by isolating *Bgl*II–*Hin*dIII

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fragments from distinct linker insertions and subcloning these into the *Hin*dIII site of pBR-CAT. 3′-deletions were constructed by subcloning *Kpn*I–*Bgl*II fragments from these linker insertions. Internal deletions were made by digesting the linker-containing pUCNaeI plasmids with *Bgl*II and *Sca*I, each of which will cut only once, and the two fragments obtained from each plasmid were recombined and ligated to generate a large variety of deletions joined by a *Bgl*II linker. These modified promoters were isolated as *Kpn*I–*Hin*dIII fragments and subcloned into pBR-CAT. pSVO-CAT based deletions were constructed by isolating *Bgl*II–*Bam*HI fragments from pUCNaeI linker insertions and subcloning these into pSVO-CAT.

2.2. *Chloramphenicol acetyltransferase assays*

Gene transfer was performed by calcium phosphate co-precipitation [13]. Chloramphenicol acetyltransferase (CAT) assays were performed as described [12]. Portions of the silica gel corresponding to acetylated chloramphenicol were cut out, dissolved in aqueous counting scintillant (Amersham) and counted on a Rackbeta 1211 liquid scintillation counter (LKB). All assays were performed under conditions which were linear for both DNA concentration and time of assay. Equivalent results were obtained from independent plasmid preparations.

3. RESULTS

Initial promoter mapping involved subcloning the entire 5′-region of the c-Ha-*ras* genomic clone from the *Bam*HI site to the *Sma*I site within the 5′-untranslated sequence (5′UT) (nucleotides 0–1646 as numbered in [14]) to generate vector pBR-BSCAT. As shown in fig.1, the *Nae*I fragment (nucleotides 116–666) in plasmid pBR-NaeCAT had equivalent promoter activity to the entire fragment and must, therefore, contain the control sequences sufficient for expression of the ras gene. All subsequent studies then focused on this region.

In order to delineate the sequences controlling c-Ha-*ras* expression, a series of deletions were constructed in this 550 bp fragment. These were generated as described in section 2, and then subcloned into the CAT expression vectors to measure their relative promoter strength. The results of these experiments are shown in fig.2. Deletions from the 5′-end of the insert to nucleotide 196 consistently led to a small increase in CAT activity which was lost with further deletions beyond nucleotide 248. Deletion to nucleotide 375 led to a dramatic drop in CAT activity to background levels. Deletions from the 3′-end to nucleotide 321 or beyond abolish transcription from the promoter and internal deletions further support these results. Taken together, these results localize sequences necessary for transcription to a 100 bp region between nucleotides 275 and 375. In the opposite orientation, the *Nae*I fragment gives rise to approximately a quarter as much CAT activity as in the correct orientation.

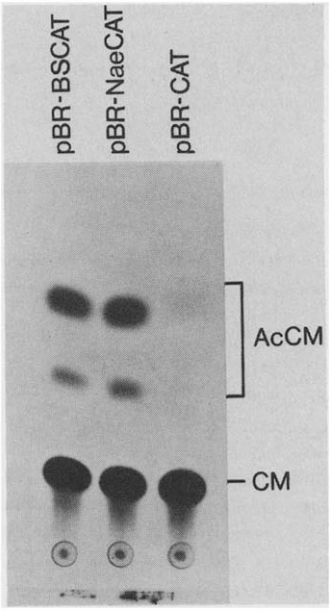

Fig.1. Localization of promoter activity. 10 μg of each plasmid was transfected into CV-1 cells and the CAT activity of cell extracts was measured 48 h later as described [12]. The autoradiogram of a thin layer chromatography plate is shown. The mobilities of the acetylated (AcCM) and unacetylated (CM) forms of [^{14}C]chloramphenicol are shown.

Reports from another group have confirmed that the *Nae*I fragment utilized here does in fact contain sequences responsible for *ras* transcription [9]. In addition, this group went on to begin dele-

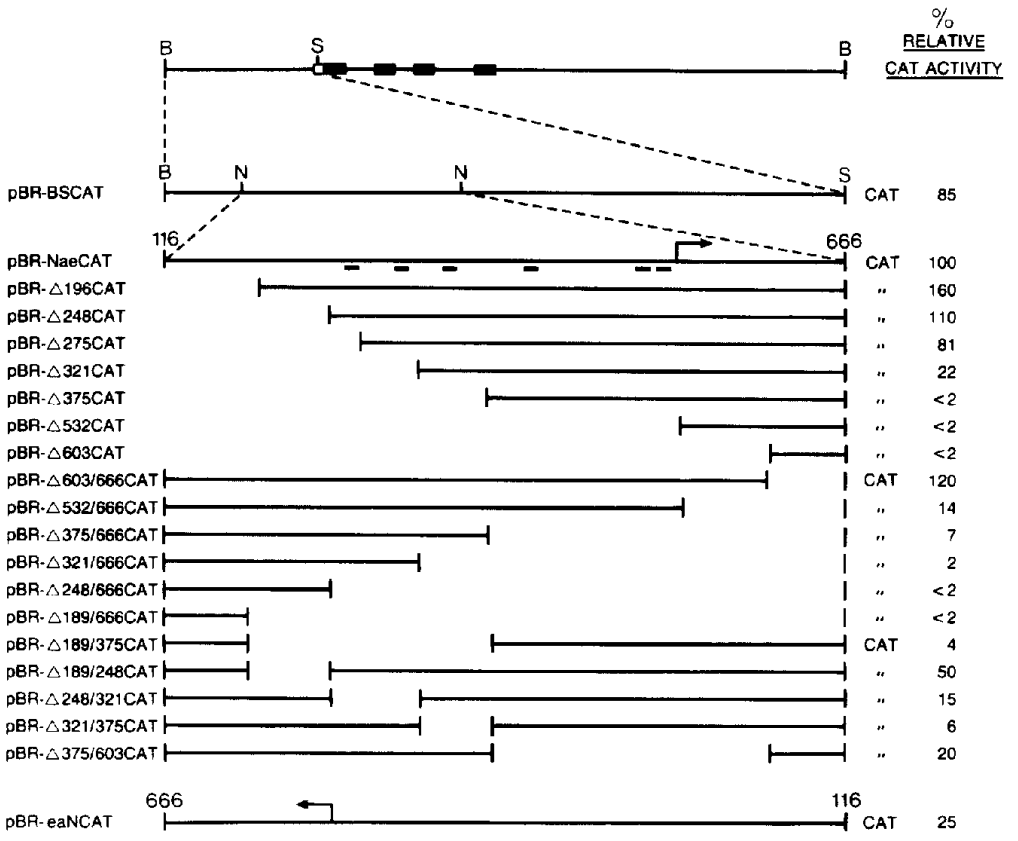

Fig.2. Deletion analysis of the c-Ha-*ras* promoter. The organization of the human c-Ha-*ras* gene is shown in the top line with the non-coding and coding exons represented by open and solid boxes respectively. Plasmid pBR-BSCAT contained the 1.6 kb *Bam*HI/*Sma*I fragment encompassing the entire 5′-portion of the gene, while pBR-NaeCAT contains only 550 bp from nucleotide 116 to nucleotide 666. Shown on this segment is the location of the putative transcription initiation sites (shown as an arrow) and the in vitro *Sp1* binding sites [11] (shown as thick underlines). Beneath are deletion constructs in which the thin solid line represents the portion of the promoter remaining in the vector and vertical lines represent the position of *Bgl*II linkers. Vector pBR-eaNCAT contains the *Nae*I fragment in the inverted orientation relative to the CAT gene. The relative CAT activity of these constructs represents the percentage of CAT activity obtained from the complete *ras* promoter in pBR-NaeCAT. All values represent the means of two separate transfections which showed less than 10% variation.

tion analyses of this fragment subcloned into the vector pSVO-CAT. They observed that a 5′-deletion from nucleotide 116 to nucleotide 351 had no effect on promoter activity and that deletion to nucleotide 427 reduced *ras* promoter activity by only three quarters in CV-1 cells [11]. In contrast, we had found that deletions to nucleotide 375 virtually abolished CAT activity. In order to better understand this discrepancy, we have reconstructed a number of the 5′-deletions of the *ras* promoter in pSVO-CAT. A direct comparison of pBR-CAT and pSVO-CAT based promoter-deletion vectors is shown in fig.3. Surprisingly, when these pSVO-CAT based vectors are transfected into CV-1 cells, CAT activity remains high even when the deletion to nucleotide 530 (removing virtually all of the promoter sequence) is tested. Furthermore, the level of *ras*-promoted CAT activity in this vector is only 3–4-fold above the vector background.

4. DISCUSSION

Using chimeric plasmid constructs we and others

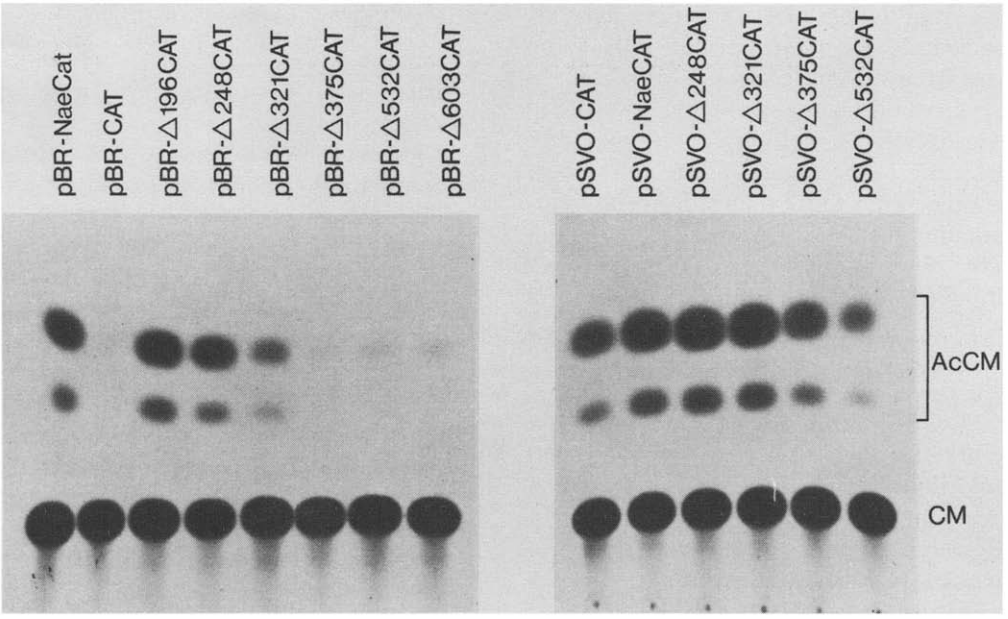

Fig.3. Comparison of 5′-deletions in pBR-CAT and pSVO-CAT. The *Nae*I fragment containing the *ras* promoter and 5′-deletions of it were subcloned in front of the CAT genes in the promoterless vectors pBR-CAT and pSVO-CAT and assayed in CV-1 cells as described in fig.1. The mobilities of acetylated (AcCM) and unacetylated (CM) forms of chloramphenicol are indicated.

[9,11,15] have demonstrated that the 550 bp *Nae*I fragment contains sufficient information to permit expression of the c-Ha-*ras* oncogene. The c-Ha-*ras* promoter has a high GC content and contains 9 consensus sequences for the binding of transcription factor *Sp1* [9]. Six sites have been shown to be functional binding sites for purified *Sp1* in vitro centered around nucleotides 267, 294, 345, 405, 514 and 525 [11]. As expected [16], the lack of a TATA box leads to transcriptional initiation from multiple sites at nucleotides 537, 549, 559 and 561 in A431 cells [9].

Deletions from either end of the promoter, or internal deletions removing nucleotides 275–375 (about 200 bp upstream from the putative start sites of transcription) had the most profound effects on promoter activity. Although this region overlaps slightly with regions previously determined to be sufficient for expression [11], major differences in results have been observed. The high transcriptional background of pSVO-CAT relative to pBR-CAT might have precluded the authors of the previous report from detecting significant differences following deletion of crucial sequences.

The relationship of these deletions to the sequences protected by *Sp1* in vitro indicates that only the two sites centered around nucleotides 295 and 345 would be within the critical region. Interestingly, within this stretch from nucleotide 362 to nucleotide 368 lies the consensus sequence for the CCAAT box, an element normally found about 70–80 nucleotides upstream from eucaryotic transcriptional start sites [17]. Further support for a functional role of this site comes from two recent studies. Jones et al. [18] have found that this element functions as a strong binding site for the transcription factor CTF in vitro. As well, the rat c-Ha-*ras* oncogene has recently been shown to contain two CCAAT boxes located at nucleotides −100 and −65 [19]. Thus, the c-Ha-*ras* oncogene may be an example of a distinct subset of housekeeping genes which utilize CCAAT boxes in conjunction with *Sp1* binding sites.

Although the CCAAT box described herein lies approx. 170 bp upstream from the putative initiation sites of the *ras* gene [9], the mapping of these sites has recently been brought into question [20]. Homology beween c-Ha-*ras* and v-Ha-*ras* extends

upstream beyond the putative transcriptional start sites to nucleotide 440 [20], a region only 80 bp downstream from the consensus CCAAT box. As the v-Ha-*ras* oncogene was probably transduced from a c-Ha-*ras* mRNA molecule, this argues that, at least in some cases, transcription must occur from sites other than those mapped. We are currently examining the sites of transcriptional initiation from the *ras* promoter in vector pBR-NaeCAT and in normal cells to determine if they differ from those previously observed.

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