Volume 203, number 2 FEBS 3839 July 1986

Promotor and enhancer like activity at the 5′-end of normal and T24 Ha-*ras*1 genes

Demetrios A. Spandidos*+ and Marcello Riggio*

**The Beatson Institute for Cancer Research, Garscube Estate, Switchback Road, Bearsden, Glasgow G61 1BD, Scotland and +Hellenic Institute Pasteur, 127 Vas. Sofias Ave, Athens 11521, Greece*

Received 23 May 1986

We have used a short-term transfection technique, in which we monitor the ability of DNA fragments to induce the expression of the chloramphenicol acetyltransferase (CAT) gene in rat 208F fibroblast cells. Using appropriate vectors we have assayed for promoter or enhancer activity of the 0.8 kb *Sst*I fragment located within the 5′-flanking sequences of the first coding exon of the human T24 and normal Ha-*ras*1 genes. We find that this fragment contains promoter and enhancer activities in both the normal and the T24 Ha-*ras*1 gene.

*Ha-*ras1 *oncogene* *Enhancer* *Promoter* *Enzyme assay* *Chloramphenicol acyltransferase*

1. INTRODUCTION

The *ras* gene family play an important role in the development of cancer (review [1]) and have therefore recently been the subject of extensive structural studies. The nucleotide sequences of the Ha-*ras*, Ki-*ras* and N-*ras* structural genes, introns and extragenic regions have been previously reported [1]. Nevertheless, the structural studies by themselves cannot explain the subtleties of gene expression and regulation during growth and development. The identification of regulatory sequences, such as promoters, enhancers, transcriptional initiation sites, binding sites for regulatory proteins, and RNA processing sites require the use of in vivo or in vitro assays for gene expression. One type of DNA sequence, the transcriptional regulatory elements termed enhancers, are *cis*-acting elements which have a positive effect on the transcription of nearby genes (review [2]). Enhancers might be expected to be found in genes that are constitutively expressed at high levels or genes that can be readily induced. The *ras* genes are known to be constitutively expressed in all tissues examined [3,4] and at high levels in some normal tissues [5] and tumors [6–9].

The location of transcriptional start sites has recently been determined in Ha-*ras*1 [10] and N-*ras* [11] genes using primer extension and S_1 mapping techniques, respectively. A non-coding exon at the 5′-end of the Ha-*ras* [10] and N-*ras* [11] gene has been found, but no obvious TATA box exists in the expected promoter region although there are several CCGCCC and their inverted complement GGGCGG sequences nearby [10]. The sequence GGGCGG represents the core consensus for the eukaryotic RNA polymerase Sp1 binding sites [12].

Here, we have used an in vitro short-term transfection assay to analyze the 5′-region of human T24 and normal Ha-*ras*1 genes for the presence of promoter or transcriptional enhancer sequences. Our data demonstrate the presence of promoters and enhancers within the 0.8 kb *Sst*I fragments at the 5′-end of the structural Ha-*ras*1 gene.

2. MATERIALS AND METHODS

2.1. *Construction of recombinant plasmids*

The organization of the human Ha-*ras*1 gene with particular emphasis on the 5′- and flanking sequences is shown in fig.1. The 853 or the 847 bp

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/86/$3.50 © 1986 Federation of European Biochemical Societies

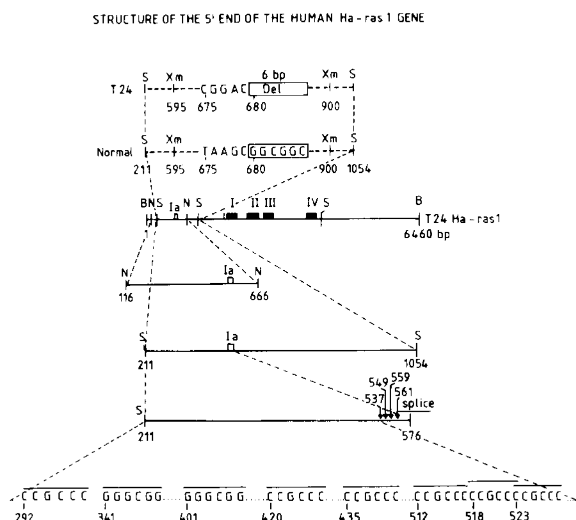

Fig.1. Organization of the human Ha-*ras*1 gene. The coding sequences are designated by black boxes and the non-coding 5′-sequences by open boxes. The locations of the sequence GGGCGG and its complementary CCGCCC contained between nucleotides 220 and 576 of the map and situated at the 5′-end of the Ha-*ras*1 gene are shown. Transcriptional start sites are indicated by arrows. DEL, deletion; B, *Bam*HI; S, *Sst*I; Xm, *Xma*III; N, *Nae*I; I, II, III and IV, coding exons; Ia, non-coding exon.

*Sst*I fragment from the normal or the T24 Ha-*ras*1 gene respectively [13,14] containing the untranslated exon [10] was assayed.

Plasmid pCAT12 was constructed by inserting a 0.9 kb *Bam*HI-*Xba*I fragment from plasmid pLW4 [15] into a *Bam*HI-*Xba*I digest of plasmid pUC12 (obtained from BRL). The 0.9 kb *Bam*HI-*Xba*I fragment carries the structural CAT gene and the 3′ Herpes simplex virus immediate early 5 gene polyadenylation sequences. Plasmids pCAT122A and pCAT122B carry the 0.8 kb *Sst*I fragment from the normal Ha-*ras*1 and plasmids pCAT123A and pCAT123B the T24 equivalent fragment from the mutant in one or other orientation as indicated in fig.2a. Plasmid pTKCMO-11 contains a 726 bp *Eco*RI/*Sma*I fragment carrying the Moloney murine sarcoma virus enhancer and mink sequences obtained from an integrated provirus [16] in front of the CAT gene. The Moloney sequences are proximal to the CAT sequences and detailed construction of this plasmid has been reported in [17].

Plasmid pTKCAT3 was constructed by R. Miksicek. The CAT gene and the 3′-polyadenylation sequences from plasmid pSV2-CAT [18] were linked to the HSV-1 *tk* promoter and the hybrid gene inserted into the pUC8 vector. Plasmids pTKCAT117A and pTKCAT117B carry the normal and plasmids pTKCAT118A and pTKCAT118B the T24 0.8 kb *Sst*I fragment in one or other orientation as indicated in fig.2b. The 0.8 kb *Sst*I fragment was first cloned into the *Sst*I site in the polylinker of plasmid pIC-20H [19] and then excised as a *Hin*dIII fragment and inserted into the *Hin*dIII site of pTKCAT3.

2.2. *DNA transfections and CAT assay*

Short-term transfections into rat 208F fibroblast cells [20] were performed using a modification [21] of the calcium phosphate technique [22]. Cells were harvested 45 h post-addition of DNA to the cells and the CAT assay was performed from the cell extracts as described [18]. Briefly, each assay contained in a volume of 0.15 ml, 0.25 μCi [^{14}C]chloramphenicol (Amersham) and soluble extract containing 60 μg protein as estimated by the method of Bradford [23]. After incubation at 37°C, the reaction products were isolated and separated by thin-layer chromatography on silica plates using chloroform:methanol (95:5) as solvent. After autoradiography, the acetylated and non-acetylated forms of [^{14}C]chloramphenicol were scraped off and quantitated by scintillation counting.

3. RESULTS

To identify putative control signals in the 5′-flanking sequences of the human Ha-*ras*1 gene, we investigated the ability of a 0.8 kb fragment located in the 5′-non-coding region of the Ha-*ras*1 gene to provide a promoter or enhancer in convenient vector systems employing the CAT assay.

The structure of the recombinant plasmids used to assay for promoter function is shown in fig.2a. The CAT gene lacks a promoter in pCAT12. Recombinants pCAT122A and pCAT122B carry the 0.8 kb *Sst*I DNA fragment from the normal Ha-*ras*1 gene in the same or opposite orientation to the CAT gene respectively. Recombinants pCAT123A and pCAT123B carry the 0.8 kb *Sst*I DNA fragment from the mutant T24 Ha-*ras*1 on-

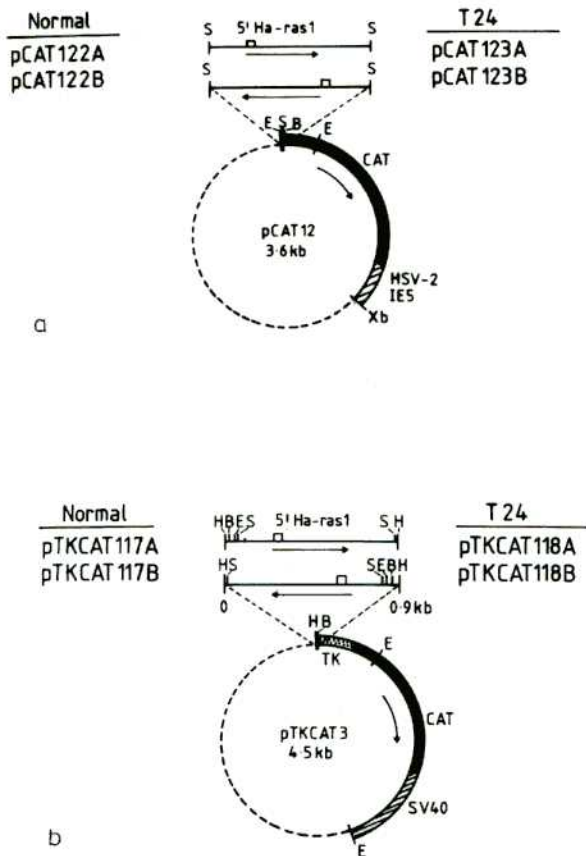

Fig.2. Schematic representation of CAT recombinant plasmids. (a) Structure of the recombinant plasmids used to assay for promoter function. Dashed line, pUC12; thin line, human DNA; open box, non-coding exon Ia of Ha-*ras*1; black box, CAT gene; hatched box, HSV-2 1E5 gene polyadenylation signal. The maps are not drawn to scale. S, *Sst*I; E, *Eco*RI; B, *Bam*HI; Xb, *Xba*I. (b) Structure of the recombinant plasmids used to assay for enhancer function. Sequence representation as in a with the following exceptions: hatched box, SV40 polyadenylation signal; dotted box, HSV-1 *tk* promoter. S, *Sst*I; E, *Eco*RI; B, *Bam*HI; H, *Hin*dIII.

cogene in the same or opposite orientation to the CAT gene, respectively. As positive control we used plasmid pTKCMO-11 which contained the enhancer from Moloney MSV in plasmid pTKCAT3 [17].

Recombinant plasmids were introduced into rat 208F fibroblasts by the calcium phosphate technique [22]. 45 h after transfection CAT activity was measured in cell extracts. We first optimized conditions for efficient measurements of CAT activity. The amount of cell protein per assay was adjusted to 60 μg so that the CAT activity was linear for at least 1 h with the recombinants used here. Representative results with the plasmids described in fig.2a are shown in fig.3a and table 1. Both the normal and T24 Ha-*ras*1 sequences stimulated CAT activity significantly (11–16-fold) in both orientations.

The structures of the recombinant plasmids used to assay for enhancer function are shown in fig.2b. Plasmid pTKCAT3 contains the HSV-1 *tk* promoter in front of the CAT gene and is an appropriate vector for assaying enhancer activity [17]. Recombinants pTKCAT117A and

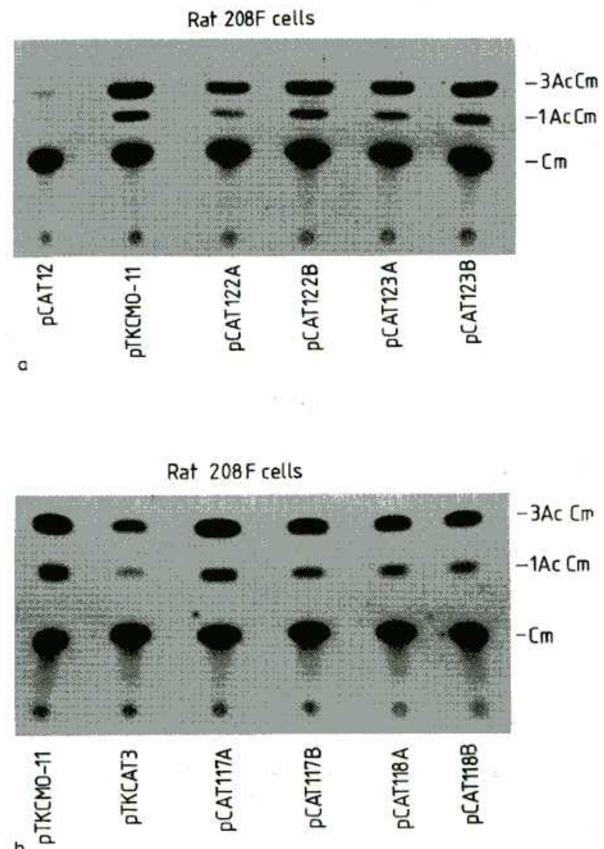

Fig.3. Chromatograms for typical CAT assays upon transfection of rat 208F cells with CAT recombinants. (a) Experiment using the recombinants described in fig.2a. See section 2 for details. (b) Experiment using the recombinants described in fig.2b. See section 2 for details.

Table 1

Relative values of CAT activity in rat 208F fibroblast cells after transfection with CAT recombinant plasmids

Recombinant plasmid[a]	Ha-*ras*1 DNA	Orientation relative to CAT	CAT activity (av ± SD)[b]
pCAT12	–	–	1.0
pTKCMO-11	–	–	38.1 ± 5.9
pCAT122A	normal	same	12.0 ± 5.9
pCAT122B	normal	opposite	16.5 ± 3.7
pCAT123A	T24	same	13.4 ± 3.6
pCAT123B	T24	opposite	11.0 ± 5.7

[a] See fig.2a and section 2 for plasmid constructions
[b] Relative values of CAT activity of each plasmid compared to the value obtained with pCAT12. The value for CAT activity after transfection of 40 μg pCAT12 per 2×10^6 208F recipient cells was 6.9×10^{-4} pmol acetylated/μg protein per 1 h incubation. The average and standard deviation from 4 experiments are given

Table 2

Relative values of CAT activity in rat 208F fibroblast cells after transfection with CAT recombinant plasmids

Recombinant plasmid[a]	Ha-*ras*1 DNA	Orientation relative to CAT	CAT activity (av ± SD)[b]
pTKCAT3	–	–	1.0
pTKCMO-11	–	–	7.6 ± 2.2
pCAT117A	normal	same	6.0 ± 0.1
pCAT117B	normal	opposite	3.2 ± 0.8
pCAT118A	T24	same	4.3 ± 1.1
pCAT118B	T24	opposite	2.7 ± 0.2

[a] See fig.2b and section 2 for plasmid constructions
[b] Relative values of CAT activity of each plasmid compared to the value obtained with pTKCAT3. The value for CAT activity after transfection of 40 μg per 2×10^6 208F recipient cells was 2.5×10^3 pmol acetylated/μg protein per 1 h incubation. The average and standard deviation from 4 experiments are given

pTKCAT117B carry the 0.8 kb *Sst*I DNA fragment from the normal Ha-*ras*1 gene in the same or opposite orientation to the CAT gene, respectively. Recombinants pTKCAT118A and pTKCAT118B carry the 0.8 kb *Sst*I DNA fragment from the mutant T24 Ha-*ras*1 oncogene in the same or opposite orientation to the CAT gene, respectively. Recombinant plasmids were introduced into rat 208F fibroblasts by the calcium phosphate technique as above. Representative results are shown in fig.3b and table 2. Both the normal and T24 Ha-*ras*1 sequences stimulated CAT activity significantly (2.7–6.9-fold) in both orientations.

4. DISCUSSION

In this study we have used a gene transfer technique to examine for the presence of transcriptional regulatory sequences 5′ to the coding region of the Ha-*ras*1 gene. With the short-term transfection assay employed here using CAT expression as an indicator system, we found that the 0.8 kb *Sst*I fragment located about 1 kb at the 5′ of the Ha-*ras*1 gene contains both promoter and enhancer activities. It is notable that both promoter and enhancer functions are exerted when the 0.8 kb *Sst*I fragment is inserted in either orientation relative to the CAT gene. Further, there is no significant difference in promoter and enhancer functions between the DNAs from the normal or the mutated T24 Ha-*ras*1 gene.

Our results agree with those of Ishii et al. [10] who have found that a 551 bp *Nae*I fragment (fig.1), which apart from 95 bp falls within the 5′-end of the 853 bp *Sst*I fragment used in our studies, has promoter activity employing a similar CAT assay system. However, Ishii et al. [10] have not examined the promoter activity in the opposite orientation or from the T24 Ha-*ras*1 corresponding fragment. Our results are also the first to demonstrate an enhancer activity in the 0.8 kb *Sst*I fragment. It has been reported previously, although no data were provided to support the claim, that the 305 bp *Xma*III fragment (position 595–900 in the map, fig.1) may contain a transcriptional enhancer since deletion of this fragment reduced the transforming activity of the T24 oncogene [1]. Since the 305 bp *Xma*III fragment is contained within the 853 bp *Sst*I fragment used in our studies this is consistent with our findings. Our results, using the CAT assay, clearly demonstrate that the enhancer is present in both the T24 and normal DNA fragments. However we cannot exclude the possibility that the slight differences in

enhancer activity may reflect the small differences in the nucleotide sequences between the normal and T24 DNAs. The presence of the positive regulatory element found in our studies is also consistent with previous findings which showed that deletion of the 0.8 kb *Sst*I fragment abolished the biological activity of the Ha-*ras*1 gene, but replacement by the HSV-1 enhancer fully restored it [24].

Although the nucleotide sequence of the coding and extragenic regions of some of the *ras* genes is known, the regulation of their expression has not been elucidated. Recently, Ishii et al. [10] using primer extension analysis have defined the transcriptional start sites for the Ha-*ras*1 gene. As shown in fig.1 they found four start sites and a non-coding exon (40 bp long) located approx. 1.0 kb upstream of the known first coding exon. Using a CAT assay they also demonstrated a promoter activity in the 551 bp *Nae*I DNA fragment which contains about 420 bp of DNA upstream of the putative transcriptional initiation sites as well as 40 bp of the first exon and 90 bp of the first intron (fig.1). However, no TATA box or CAAT box could be found in their expected locations (about −30 and −80 bp respectively). On the other hand, several copies of the GC box GGGCGG or its complementary sequence CCGCCC were found in the *Sst*I fragment (see fig.1 and [13,14]).

A similarity in high GC content and absence of a TATA box has been found between the Ha-*ras*1 promoter region with the promoter of some other genes involved in cellular growth control such as the human EGF receptor, hypoxanthine phosphoribosyltransferase, 3-hydroxy-3-methylglutaryl-CoA reductase and adenosine deaminase [10]. Although the significance of this similarity is not known, it is tempting to speculate that expression of these genes may be regulated in a similar manner. The significance of these findings is hard to assess completely at present.

Our findings that the Ha-*ras*1 promoter functions in both orientations is of special interest since this has been thought to be exclusively a property of enhancers [2]. A precedent for such an element has been provided recently by studies on the calcium ionophore-inducible cellular promoter which functions as a bidirectional promoter with enhancer like properties [25]. An enhancer sequence has also been found in the upstream 5′ of the *fos* proto-oncogene [26]. The roles of these regulatory sequences in the induction of cancer by oncogenes such as Ha-*ras* are obviously of great importance.

ACKNOWLEDGEMENTS

I would like to thank Drs Peggy Anderson and Jas Lang for critical reading of the manuscript and Neil M. Wilkie for stimulating discussions. The Beatson Institute is supported by the Cancer Research Campaign of Great Britain.

REFERENCES

[1] Marshall, C.J. (1985) in: RNA Tumor Viruses (Weiss, R. et al. eds) vol.2, pp.486–558, Cold Spring Harbor Laboratory, NY.
[2] Lang, J.C. and Spandidos, D.A. (1986) Anticancer Res. 6, 437–450.
[3] Langbeheim, H., Shih, T.Y. and Scolnick, E.M. (1980) Virology 106, 292–300.
[4] Slamon, D.J. and Cline, M.J. (1984) Proc. Natl. Acad. Sci. USA 81, 7141–7145.
[5] Spandidos, D.A. and Dimitrov, T. (1985) Biosci. Rep. 5, 1035–1039.
[6] Spandidos, D.A. and Kerr, I.B. (1984) Br. J. Cancer 49, 681–688.
[7] Slamon, D.J., Dekernion, J.B., Verma, I.M. and Cline, M.J. (1984) Science 224, 256–262.
[8] Spandidos, D.A. and Agnantis, N.J. (1984) Anticancer Res. 4, 269–272.
[9] Spandidos, D.A., Lamothe, A. and Field, J.L. (1985) Anticancer Res. 5, 221–225.
[10] Ishii, S., Merlino, G.T. and Pastan, I. (1985) Science 230, 1378–1381.
[11] Hall, A. and Brown, R. (1985) Nucleic Acids Res. 13, 5255–5268.
[12] Kadonaga, J.T., Jones, K.A. and Tjian, R. (1986) Trends Biochem. Sci. 11, 20–23.
[13] Capon, D.J., Chen, E.Y., Levinson, A.D., Seeburg, P.H. and Goeddel, D.N. (1983) Nature 302, 33–37.
[14] Reddy, E.P. (1983) Science 220, 1061–1063.
[15] Gaffney, D.F., McLauchlan, J., Whitton, J.L. and Clements, J.B. (1985) Nucleic Acids Res. 13, 7847–7863.
[16] Dhar, R., McClements, W.L., Enquist, L.W. and Vande Woude, G.F. (1980) Proc. Natl. Acad. Sci. USA 77, 3937–3941.
[17] Anderson, M.L.M., McColl, L.A. and Spandidos, D.A. (1986) Anticancer Res., in press.

[18] Gorman, C.M., Moffat, L.F. and Howard, B.H. (1982) Mol. Cell Biol. 2, 1044–1051.
[19] Lawrence, M.J., Erfle, M. and Wykes, E.J. (1984) Gene 32, 481–485.
[20] Quade, K. (1979) Virology 98, 461–465.
[21] Spandidos, D.A. and Wilkie, N.M. (1984) in: Transcription and Translation – A Practical Approach (Hames, B.D. and Higgins, S.J. eds) pp.1–48, IRL Press, Oxford.
[22] Graham, F.L. and Van der Eb, A.J. (1973) Virology 52, 456–467.
[23] Bradford, M.M. (1976) Anal. Biochem. 72, 248–254.
[24] Puga, A., Gomez-Marquex, J., Brayton, P.R., Contin, E.M., Long, L.K., Barbacid, M. and Notkins, A.L. (1985) J. Virol. 54, 879–881.
[25] Lin, A.Y., Chang, S.C. and Lee, A.S. (1986) Mol. Cell. Biol. 6, 1235–1243.
[26] Deschamps, J., Meijlink, F. and Verma, I.M. (1985) Science 230, 1174–1177.