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Transcriptional enhancer activity in the variable tandem repeat DNA sequence downstream of the human Ha-*ras*1 gene

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A short term transfection technique using the chloramphenicol acetyltransferase gene as an assay system has been employed to examine the presence of transcriptional regulatory sequences within the variable tandem repeat (VTR) DNA sequence located downstream of the human Ha-*ras*1 gene. Here we find that the VTR sequences possess an endogenous enhancer activity of both the normal and the T24 mutant Ha-*ras*1 gene.

Transcriptional enhancer; Ha-*ras*1 gene; (Human)

1. INTRODUCTION

The *ras* genes constitute an important eukaryotic family of genes which has attracted the interest of scientists in a variety of disciplines including cancer, cell proliferation and differentiation (review [1]). The *ras* genes have been shown to be activated by at least four different mechanisms in vivo or in vitro: (i) mutation of *ras* proto-oncogenes, i.e. in codons 12 [2–4] or 61 [5]; (ii) transcriptional activation in vitro [6–8] and in vivo [9–12]; (iii) gene amplification [13]; (iv) retroviral insertional mutagenesis [14,15]. Elevated levels of the *ras* encoded protein p21 have also been observed in some normal tissues [16]. Moreover, the fact that different *ras* genes are activated in different tumors, i.e. Ha-*ras* [17] and Ki-*ras* [18] in carcinomas and N-*ras* in hemopoietic [19,20] or mesenchymal [21] tumors indicates some tissue specificity in the expression of these genes.

The *Bam*HI fragment containing the normal or the T24 Ha-*ras*1 genes has a region of repetitive DNA located approx. 1.5 kb from the 3′-terminus of the coding sequences of the Ha-*ras*1 gene [22]. This region consists of a variable tandem repeat (VTR) of a 28-bp consensus sequence (fig.1). Previous studies have also suggested that an observed *Bam*HI restriction fragment length polymorphism (RFLP) arises through changes in the number of repeat units of this 28 bp sequence [23,24].

In the present study we have analyzed the VTR region at the 3′-end of the Ha-*ras*1 gene for the presence of transcriptional enhancer activity. Our results demonsrate the presence of such an activity in this region.

2. MATERIALS AND METHODS

2.1. *Constructions of recombinant plasmids*

The location of the VTR sequences with respect

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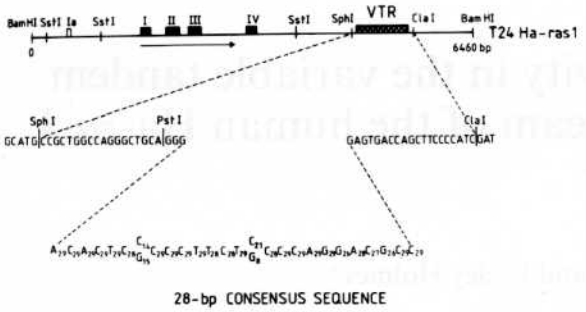

Fig.1. Organization of the human T24 Ha-*ras*1 gene and sequence of the variable tandem repeat (VTR) region. The coding sequences are represented by black boxes, the non-coding 5′-sequences by an open box and the VTR sequences by a crosshatched box. The direction of transcription is indicated by an arrow. The nucleotide sequence of the VTR region with the 28 bp consensus sequence is taken from [22].

to the human T24 Ha-*ras*1 gene and its nucleotide sequence including the 28 bp consensus are shown in fig.1. The normal Ha-*ras*1 allele contains a shorter VTR sequence by approx. 100 bp. The 0.8 or 0.9 kb *Sph*I-*Cla*I fragments carrying the VTR sequence from the normal or the T24 Ha-*ras*1 gene were first recloned (by Dr J. Lang) in plasmid pUC18 to generate plasmids p105 and p106, respectively. The VTR sequences were then excised and recloned into the *Sph*I-*Eco*RI sites of plasmid plC20H [25]. The VTR sequences now surrounded by *Hin*dIII sites were excised and inserted into the single *Hin*dIII site of each of the following plasmids as shown in fig.2. (i) Plasmid pB30 contains the chloramphenicol acetyltransferase (CAT) gene driven by the human ϵ-globin promoter (fig.2a) (obtained from B. Whitelaw). (ii) Plasmid pCAT122A contains the CAT gene driven by the Ha-*ras*1 promoter and it has been described [28] (fig.2b). (iii) Plasmid pB9 contains a promoterless CAT gene (fig.2c) (obtained from B. Whitelaw).

2.2. *DNA-mediated gene transfer and CAT assays*

Transfer and expression of recombinant plasmids into rat 208F fibroblast cells were carried out using a modification [29] of the calcium phosphate technique [30]. Transfected cells were harvested 45 h post-addition of DNA to the cells and the CAT assay was performed as described [28,31].

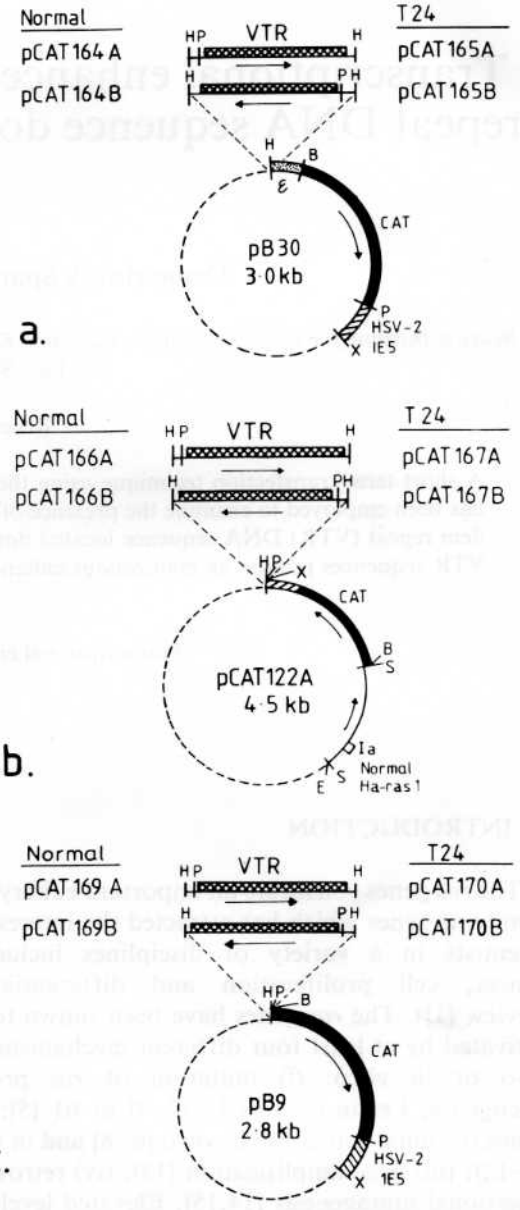

Fig.2. Schematic representation of CAT recombinant plasmids. (a) Structure of the recombinant plasmids used to assay for enhancer function. (b) Structure of the recombinant plasmids used to assay for enhancer function using the Ha-*ras*1 promoter. (c) Structure of the recombinant plasmids used to assay for promoter function using the human ϵ-globin promoter. Dashed line, pUC12; thin line, human DNA; crosshatched box, VTR sequence; black box, CAT gene; hatched box, HSV-2 1E5 gene polyadenylation signal; open box, the non-coding 5′-sequences of the Ha-*ras*1 gene; dotted box, human ϵ-globin promoter [26,27].

3. RESULTS

To examine the presence of transcriptional regulatory sequences in the VTR sequences downstream of the human normal and T24 mutant Ha-*ras*1 genes we investigated the ability of these sequences to provide enhancer or promoter activity using the CAT assay system.

The structures of recombinant plasmids used to assay for enhancer function are shown in fig.2a. Plasmid pB30 contains the human ϵ-globin promoter [26,27] in front of the CAT gene. Recombinants pCAT164A and pCAT164B carry the VTR sequence from the normal Ha-*ras*1 gene in the same or opposite orientation to the CAT gene, respectively. Recombinants pCAT165A and pCAT165B carry the VTR sequence from the T24 mutant Ha-*ras*1 gene in the same or opposite orientation to the CAT gene, respectively. As a positive control we used plasmid pLW4 which carries the HSV-2 gene promoter/enhancer sequences in front of the CAT gene [32]. Recombinant plasmids were introduced into rat 208F fibroblasts by the calcium phosphate technique. Representative results are shown in fig.3a and table 1. The VTR sequences from both the normal and T24 Ha-*ras*1 genes stimulated CAT activity significantly (2.5–2.9-fold) in both orientations.

To investigate whether the enhancer of the VTR sequence functions from a distance and on its own promoter hybrid CAT genes carrying the Ha-*ras*1 promoter and the VTR sequences at the 3′-end of the CAT gene were constructed as shown in fig.2b. Plasmid pCAT122A contains the human Ha-*ras*1 promoter in front of the CAT gene and it has been described [28]. Recombinant pCAT166A carries the VTR sequence from the normal Ha-*ras*1 gene in the same orientation to the CAT gene. We did not obtain plasmid pCAT166B. Recombinants pCAT167A and pCAT167B carry the VTR sequence from the T24 Ha-*ras*1 gene in the same or opposite orientation to the CAT gene, respectively. Recombinant plasmids were assayed in 208F cells as above using the calcium phosphate technique. Representative results are shown in fig.3b and table 2. The VTR sequence from the normal or the T24 Ha-*ras*1 genes enhanced CAT activity at low levels (1.7–2.0-fold). This could be due to a distance effect or to the different tissue specificity of the two distinct promoters. The ϵ-globin promoter carries ATA and CAAT boxes [27] whereas the Ha-*ras*1 promoter lacks these consensus sequences but is rich in GC content, a landmark of the promoters of house-keeping genes [28].

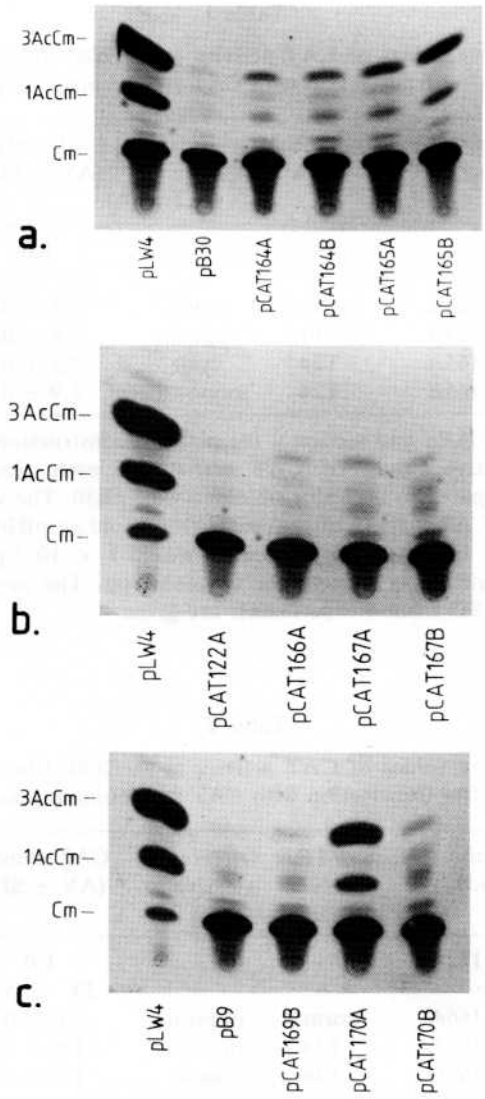

Fig.3. Chromatograms for typical CAT assays upon transfection of rat 208F cells with CAT recombinants. Experiment using recombinants described in fig.2a (a); fig.2b (b); and fig.2c (c).

Since enhancer elements also carry some promoter function [26] we have investigated this possibility by employing an assay for promoter function. The structures of recombinant plasmids used are shown in fig.2c. The CAT gene lacks a

Table 1

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

Recombinant plasmid[a]	VTR DNA	Orientation relative to CAT	CAT activity (AV ± SD)[b]
pB30	–	–	1.0
pLW4	–	–	37 ± 7.3
pCAT164A	normal	same	2.5 ± 0.5
pCAT164B	normal	opposite	2.8 ± 0.8
pCAT165A	T24	same	2.5 ± 0.7
pCAT165B	T24	opposite	2.9 ± 0.9

[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 pB30. The value for CAT activity after transfection of 40 μg pB30 per 2×10^6 208F recipient cells was 5.3×10^{-2} pmol acetylated/μg protein per h incubation. The average and SD 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]	VTR DNA	Orientation relative to CAT	CAT activity (AV ± SD)[b]
pCAT122A	–	–	1.0
pLW4	–	–	29 ± 5.3
pCAT166A	normal	opposite	2.0 ± 0.6
pCAT167A	T24	opposite	1.8 ± 0.4
pCAT167B	T24	same	1.7 ± 0.5

[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 pCAT122A. The value for CAT activity after transfection of 40 μg pCAT122A per 2×10^6 208F recipient cells was 7.1×10^{-2} pmol acetylated/μg protein per h incubation. The average and SD from 3 experiments are given

promoter in plasmid pB9. Recombinant pCAT169B carries the VTR sequence from the normal Ha-*ras*1 gene in the opposite orientation to the CAT gene. We did not obtain plasmid pCAT169A. Recombinants pCAT170A and pCAT170B carry the VTR sequence from the T24 Ha-*ras*1 gene in the same or opposite orientation to

Table 3

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

Recombinant plasmid[a]	VTR DNA	Orientation relative to CAT	CAT activity (AV ± SD)[b]
pB9	–	–	1.0
pLW4	–	–	24 ± 4.3
pCAT169B	normal	opposite	2.9 ± 1.6
pCAT170A	T24	normal	5.2 ± 2.4
pCAT170B	T24	opposite	3.5 ± 2.0

[a] See fig.2c and section 2 for plasmid constructions
[b] Relative values of CAT activity of each plasmid compared to the value obtained with pB9. The value for CAT activity after transfection of 40 μg pB9 per 2×10^6 208F recipient cells was 3.0×10^{-2} pmol acetylated/μg protein per h incubation. The average and SD from 3 experiments are given

the CAT gene, respectively. Recombinant plasmids were assayed in 208F cells as above. Representative results are shown in fig.3c and table 3. The VTR sequences from both the normal and the T24 Ha-*ras*1 genes stimulated CAT activity significantly in both orientations (2.9–5.2-fold). However, the VTR from the T24 mutant Ha-*ras*1 gene in the same orientation (pCAT170A) as the CAT gene gave consistently higher (5.2-fold) as compared to the opposite orientation (pCAT170B) (3.5-fold) CAT activity. The reason for this is not known but is being investigated.

4. DISCUSSION

In the present study we have analyzed the transcriptional regulatory functions of the VTR sequence located downstream of the human Ha-*ras*1 gene. Using a short term transfection technique and the CAT assay system we have found transcriptional enhancer activity in this sequence. Consistent with our findings are the results obtained by Seeburg et al. [33]. These authors have found that sequences containing the variable tandem repeat facilitate the expression of *ras* p21 in in vitro cell transformation studies. Moreover, it has been reported (although no data were presented) that the transforming activity of EJ Ha-*ras*1 DNA lacking the VTR region was 5–10-fold

lower than that of the original DNA and that the *ras* p21 gene product was also reduced [23].

It has been suggested that variation in the size of the VTR region may alter expression or control of the Ha-*ras*1 gene which may in turn predispose certain cell types to malignancy [23]. The observation that several human tumors contain elevated levels of Ha-*ras* transcripts [8–11] taken together with the findings that some tumorigenic cells, i.e. EJ cells have a longer VTR sequence [22] is consistent with this hypothesis.

Transcriptional enhancers have been usually found at the 5′-end or within the gene (review [34]). However, an enhancer element at the 3′-end of the Ha-*ras*1 gene described in this study may not be unique to this gene. A tissue-specific enhancer located at the 3′-end of the chicken adult β-globin gene has also been reported [35].

The consensus sequence $GXTGTGG^{TTT}_{AAA}$ has been found in many transcriptional enhancers [34]. This sequence when compared to the sequence contained between the *Sph*I and *Cla*I sites including the 28-bp consensus sequence of the VTR region (fig.1) shows no sequence homology. However, others have described a separate consensus sequence for the enhancer found in an immunoglobulin gene [36]. Moreover, studies on the polyomavirus enhancer sequence suggest that an enhancer may comprise several types of sequence domain [37].

Our findings raise the question whether the initiation, promotion, progression and maintenance of some tumors depend on overexpression of the Ha-*ras*1 gene. This could be the result of amplification, tissue specificity or mutations in the VTR sequence. Some of these hypotheses may be testable in the near future.

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REFERENCES

[1] Barbacid, M. (1987) Annu. Rev. Biochem., in press.
[2] Reddy, E.P., Reynolds, R.K., Santos, E. and Barbacid, M. (1982) Nature 300, 149–152.
[3] Tabin, C.J., Bradley, S., Bargman, C., Weinberg, R., Papageorge, A., Scolnick, E., Char, R., Lowy, D. and Chang, E. (1982) Nature 300, 143–149.
[4] Taparowsky, E., Suard, Y., Fasano, O., Simizu, K., Goldfarb, M. and Wigler, M. (1982) Nature 300, 762–765.
[5] Yuasa, Y., Srivastava, S.K., Dunn, C.Y., Rhim, J.S., Reddy, E.P. and Aaronson, S.A. (1983) Nature 303, 775–779.
[6] Chang, E.H., Furth, M.E., Scolnick, E.M. and Lowy, D.R. (1982) Nature 297, 479–483.
[7] Spandidos, D.A. and Wilkie, N.M. (1984) Nature 310, 469–475.
[8] Spandidos, D.A. (1985) Anticancer Res. 5, 485–498.
[9] Slamon, D.J., De Kernion, J.G., Verma, I.M. and Cline, M.J. (1984) Science 224, 256–262.
[10] Spandidos, D.A. and Kerr, I.B. (1984) Br. J. Cancer 49, 681–688.
[11] Spandidos, D.A. and Agnantis, N.J. (1984) Anticancer Res. 4, 269–272.
[12] Spandidos, D.A., Lamothe, A. and Field, J.K. (1985) Anticancer Res. 5, 221–224.
[13] Pulciani, S., Santos, E., Long, L.K., Sorrentino, V. and Barbacid, M. (1985) Mol. Cell. Biol. 10, 2836–2841.
[14] Westaway, D., Papkoff, J., Moscovici, C. and Varmus, H.E. (1986) EMBO J. 5, 301–309.
[15] George, D.L., Glick, B., Trusko, S. and Freeman, N. (1986) Proc. Natl. Acad. Sci. USA 83, 1651–1655.
[16] Spandidos, D.A. and Dimitrov, T. (1985) Biosci. Rep. 5, 1035–1039.
[17] Fujita, J., Yoshida, O., Yuasa, Y., Rhim, J.S., Hatanaka, M. and Aaronson, S.A. (1984) Nature 309, 464–466.
[18] Pulciani, S., Santos, E., Lauver, A.V., Long, L.K. and Barbacid, M. (1982) Nature 300, 539–542.
[19] Eva, A., Tronick, S.R., Gol, R.A., Pierce, J.H. and Aaronson, S.A. (1983) Proc. Natl. Acad. Sci. USA 80, 4926–4930.
[20] Souyri, M. and Fleissner, E. (1983) Proc. Natl. Acad. Sci. USA 80, 6676–6679.
[21] Hall, A., Marshall, C.J., Spurr, N. and Weiss, R.A. (1983) Nature 303, 396–400.
[22] Capon, D.J., Chen, E.Y., Levinson, A.D., Seeburg, P.H. and Goeddel, D.N. (1983) Nature 302, 33–37.
[23] Krontiris, T.G., DiMartino, N.A., Colb, M. and Parkinson, D.R. (1985) Nature 313, 369–374.
[24] Heighway, J., Thatcher, N., Corny, T. and Hasleton, P.S. (1986) Br. J. Cancer 53, 453–457.
[25] Lawrence, M.J., Efle, M. and Wykes, E.J. (1984) Gene 32, 481–485.

[26] Lang, J.C., Wilkie, N.M. and Spandidos, D.A. (1983) J. Gen. Virol. 64, 2679–2696.
[27] Baralle, F.E., Shoulders, C.C. and Proudfoot, N.J. (1980) Cell 21, 621–626.
[28] Spandidos, D.A. and Riggio, M. (1986) FEBS Lett. 203, 169–174.
[29] 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.
[30] Graham, F.L. and Van der Eb, A.J. (1983) Virology 52, 456–467.
[31] Anderson, M.L.M., McColl, L.A. and Spandidos, D.A. (1986) Anticancer Res. 6, 663–666.
[32] Gaffney, D.F., McLauchlan, J., Whitton, J.L. and Clements, J.B. (1985) Nucleic Acids Res. 13, 7847–7863.
[33] Seeburg, P.H., Colby, W.W., Capon, D.J., Goeddel, D.V. and Levinson, A.D. (1984) Nature 312, 71–75.
[34] Lang, J.C. and Spandidos, D.A. (1986) Anticancer Res. 6, 437–450.
[35] Choi, O.-R. and Engel, J.D. (1986) Nature 323, 731–734.
[36] Church, G.M., Ephrussi, A., Gilbert, W. and Tonegawa, S. (1985) Nature 313, 798–801.
[37] Veldman, G.M., Lupton, S. and Kamen, R. (1985) Mol. Cell. Biol. 5, 649–658.