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Phorbol ester-responsive H-*ras*1 gene promoter contains multiple TPA-inducible/AP-1-binding consensus sequence elements

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We have constructed recombinant DNA plasmids which carry both the aminoglycoside phosphotransferase (*aph*) gene and the chloramphenicol acetyl-transferase (CAT) gene linked to the human normal or mutant T24 H-*ras*1 promoter. We have transfected these plasmids into rat 208F fibroblasts using the calcium phosphate technique and selected for stable transformants by geneticin resistance. These transformants expressed CAT activity at low levels. However, when treated with the phorbol ester TPA, CAT levels increased substantially. Cells transfected with recombinant plasmids carrying a promoterless CAT gene did not respond to TPA. We have noted four motifs in the H-*ras*1 promoter region which resemble TPA-inducible and AP-1-binding consensus sequences. We suggest that AP-1-like proteins may play a role in control of H-*ras*1 transcription.

H-*ras*1 Promoter; Phorbol ester

1. INTRODUCTION

The development of cancer is widely accepted to be a multistage process (for review see [1]). Moreover, it has been suggested that the specific biological change that takes place during tumor initiation and progression may be a consequence of the activation of cellular proto-oncogenes by genetic or epigenetic events [1].

Phorbol esters such as TPA (12-*O*-tetradecanoylphorbol-13-acetate) are tumor promoters which are capable of potentiating the effect of an initiating carcinogen [2]. It is thought that they exert their biological effect by altering gene expression through a process which involves the activation of protein kinase C [3]. Relevant to this hypothesis is the finding that TPA induces transcription of cellular proto-oncogenes, e.g. c-*fos* [4,5], c-*myc* [4,6] and c-*sis* [7]. TPA can also induce the expression of collagenase [8] and stromelysin [8,9]. These genes have been implicated in tumor invasiveness and metastasis [10] and in angiogenesis [11] and are known to carry TPA-inducible elements in their promoter [12].

It has been shown that quantitative and/or qualitative changes in *ras* gene expression are responsible for triggering various steps of cell transformation, i.e. immortalization, tumorigenicity and metastasis (for review see [13]). In addition *ras* expression can trigger *fos* expression [14] and can induce a number of growth factors including transforming growth factors (for review see [15]) and IL-3 like activity [16].

The regulation of H-*ras*1 gene expression is not completely understood although a number of transcriptional regulatory elements have been described at both the 5′- [17–19] and 3′-end [20] of the gene. Regulatory sequences have also been found in the first [21] and fourth [22] intron of the H-*ras*1 gene. In the first case a six base pair deletion in the first intron of the T24 H-*ras*1 gene increases transcriptional activity from the H-*ras*1

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promoter [21] whereas in the latter case a point mutation in the fourth intron is responsible for increased expression and transforming activity of the oncogene [22]. Moreover, it has been suggested that the regulation of H-*ras*1 transcription may involve the Sp1 transcriptional factor since a number of Sp1-binding sites have been found in the promoter region [23].

The transcription factor AP-1 (activator protein 1) stimulates transcription in vitro and binds specifically to the sequence TGACTA in both the human metallothionein (hMTIIA) and the SV40 enhancer regions [24]. Several TPA-inducible elements contain a common sequence TGAGTCAG [12]. Recent studies suggest that AP-1 interacts with TPA-inducible elements in hMTIIA, SV40 and collagenase genes as determined by direct DNA-binding studies of wild type and mutant templates [24]. Thus AP-1 appears to be one of the cellular transcription factors whose activity may be modulated by TPA.

We have noted four sequences in the H-*ras*1 promoter which resemble the consensus sequence for TPA-inducible and AP-1-binding sequences. We therefore tested if this promoter could be activated by TPA. Our results showed that TPA can indeed induce expression of a CAT gene linked to the human H-*ras*1 promoter region.

2. MATERIALS AND METHODS

2.1. *Recombinant plasmids*

The organization of the human H-*ras*1 gene with emphasis on the promoter region contained within the 843 bp *Sst*I fragment is shown in fig.1. Construction of plasmids p201A and p202A carrying the *aph* gene and the CAT gene linked to the human H-*ras*1 promoter region (843 bp *Sst*I fragment) is described in fig.2. The figure also shows the control plasmid p220A which carries a promoterless CAT gene linked to the *aph* gene.

2.2. *DNA transfection and CAT assays*

Transfections of rat 208F cells with plasmid DNA were performed using a modification [26] of the calcium phosphate technique [27]. Stable transfectants were isolated in the presence of 200 μg/ml geneticin. For CAT assays, cells grew exponentially in the absence of geneticin. TPA was purchased

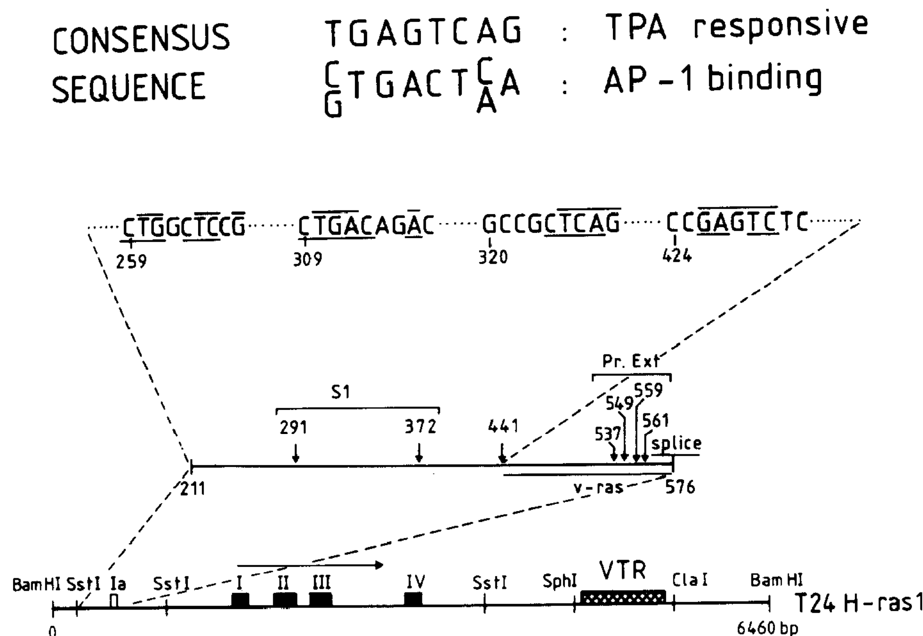

Fig.1. Organization of the human H-*ras*1 gene. The nucleotide sequence is taken from Capon et al. [25]. The coding sequences are designated by black boxes and the non-coding 5′-sequences by open boxes. The variable tandem repeat (VTR) is indicated by a hatched box and the transcriptional orientation by an arrow. The transcriptional start sites at positions 291 and 372 were determined by S_1 mapping [19] and at positions 537, 549, 559 and 561 by primer extension [17]. Position 441 indicates the start of homology with the v-*ras* oncogene. The sequences resembling the TPA-responsive and AP-1-binding consensus sequence at positions 259, 309, 320 and 424 are shown. Homologies with TPA-responsive sequences are shown with lines above and with AP-1-binding sequence with lines below the nucleotides. I, II, III and IV, coding exons; Ia, non-coding exon.

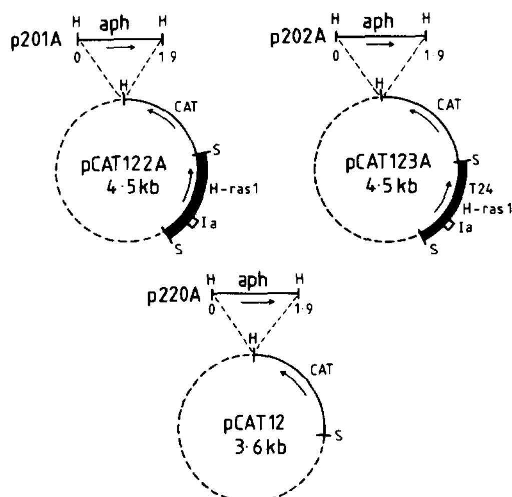

Fig.2. Structure of *aph* recombinant plasmids used to transfect the rat 208F cells. The 1.9 kb *Hin*dIII fragment carrying the *aph* gene under the HSV-1 thymidine kinase promoter and 3′-polyadenylation signal was derived from plasmid p25 (obtained from Jas Lang) was inserted into the single *Hin*dIII site of plasmids pCAT122A and pCAT123A. The junction between *aph* and CAT genes occurs between the HSV-1 *tk* and the HSV-2 immediate early 5 gene 3′-polyadenylation sequences linked to the *aph* and CAT genes. Plasmids pCAT122A and pCAT123A carry the CAT gene linked to the normal and mutant T24 H-*ras*1 gene promoter, respectively, and they have been previously described [18]. Plasmid p210A was constructed by inserting the 1.9 kb *Hin*dIII fragment carrying the *aph* gene into the single *Hin*dIII site of plasmid pCAT12 [18]. Dashed line, plasmid sequences; thin line, CAT and *aph* genes; thick line, H-*ras*1 sequences; open box, non-coding H-*ras*1 exon; H, *Hin*dIII; S, *Sst*I. Arrows indicate transcriptional orientation. Maps are not drawn to scale.

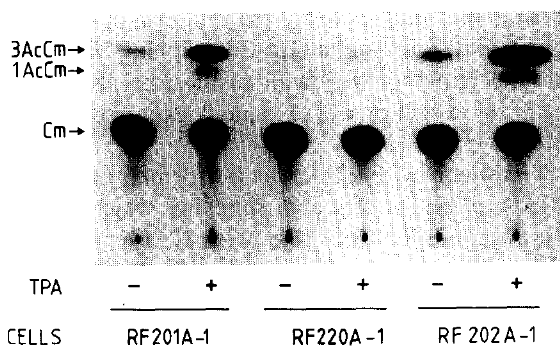

Fig.3. Chromatograms for representative CAT assays with extracts from recipient 208F and transfectant cell lines with (50 ng/ml) and without treatment with TPA.

from Sigma and 1000× stocks were made in DMSO. Cells in the presence or absence of TPA were harvested and assayed for CAT activity as previously described [18].

3. RESULTS

Since the *ras* gene and phorbol esters are known to co-operate in cell transformation in vivo [28] or in vitro [29], it was of interest to examine the responsiveness of H-*ras*1 promoter to TPA. The possibility that the human H-*ras*1 promoter could respond to the phorbol ester TPA was examined by treating transfectants carrying a CAT gene linked to the *ras* promoter.

Transfectant cell lines were obtained by trans-

Table 1

Relative values of CAT activity in transfectant rat 208F fibroblasts

Cells[a]	Transfected plasmid[b]	Promoter[b]	TPA[c]	CAT activity (average ± SD)[d]	Fold increase
RF220A-1	p220A	–	–	1.0	–
RF220A-1	p220A	–	+	1.0 ± 0.2	–
RF201A-1	p201A	H-*ras*1	–	2.5 ± 0.3	1.0
RF201A-1	p201A	H-*ras*1	+	38 ± 2.5	15.2
RF202A-1	p202A	T24 H-*ras*1	–	4.2 ± 0.4	1.0
RF202A-1	p202A	T24 H-*ras*1	+	51 ± 3.2	12.1

[a] Rat 208F recipient and transfectant RF201A-1, RF201B-1 and RF220A-1 cell lines were derived as described in section 2
[b] See fig.2 for plasmid constructions
[c] Cells treated with 50 ng/ml TPA for 12 h at 37°C
[d] Relative values of CAT activity in each cell line vs that obtained in RF201A in the absence of TPA. The value for CAT activity in RF201A cells was 8.5×10^{-4} pmol acetylated chloramphenicol/μg protein per h incubation. Average ± SD from three experiments

fecting plasmids p201A, p202A and p220A (see figs 1 and 2 for details) into recipient 208F cells and selecting for geneticin resistance. As seen in fig.3 the exogenous CAT gene is expressed at low levels.

Treatment with TPA was performed at various TPA concentrations and time intervals. At optimum conditions, that is, after 12 h exposure to 50 ng TPA/ml, CAT expression in RF201A and RF202A transfectants which carried CAT plasmids linked to the normal and T24 H-*ras*1 promoters, respectively, was substantially increased (15.2-fold increase with the normal and 12.1-fold increase with the mutant T24 promoter, respectively) over untreated cells (fig.3 and table 1). On the other hand cells transfected with recombinant plasmid p220A, which carries a promoterless CAT gene did not respond to TPA (table 1). Moreover, the response of the H-*ras*1 promoter to TPA was the same when cell lines transfected with recombinant plasmids p201B, p202B and p220B carrying the *aph* gene in the opposite orientation relative to the CAT gene were employed (not shown).

4. DISCUSSION

The mechanism by which the various control elements of H-*ras*1 promoter operate to trigger RNA synthesis still remains to be determined. However, it is obvious that an important step towards understanding the function of these transcription regulatory elements is the biological characterization of the cellular proteins which interact with them.

It is possible that TPA-inducible elements are regulated by posttranslational modification of preexisting transcriptional factors which are already present in the cell [30]. Nishizuka [3] has suggested that alteration of gene expression by TPA involves a cascade of events triggered by the action of protein kinase C.

When v-*ras* genes from Harvey and BALB murine sarcoma viruses are introduced into epidermal cells in vivo they can act as initiators of two-stage mouse skin carcinogenesis. Treatment with TPA induced benign papillomas some of which progressed to invasive carcinomas [28].

The complete dependence on TPA treatment for tumor development suggested that a critical interaction must take place between the Ha-MSV-initiated cell and TPA [28]. Several explanations have been put forward for the observed synergism between *ras* gene activation and TPA treatment. Stimulation of cell growth by TPA occurs through direct interaction with protein kinase C [31]. Alternatively, both *ras* p21 and TPA could be involved in signal transduction perhaps through phosphoinositide turnover [32] or the epidermal growth factor receptor system [33,34].

TPA treatment is also known to cause increased cellular proliferation and elevated *myc* expression [35,36]. A co-operative effect between TPA and transfected *ras* genes in the transformation of primary fibroblasts in vitro has also been observed [29]. In this paper we investigate if TPA actually affects expression of the H-*ras*1 gene. We show that in stably transfected rat, fibroblasts, TPA induces expression of the reporter CAT gene covalently linked to the H-*ras*1 promoter. It is of interest that the H-*ras*1 promoter region carries 4 motifs resembling the consensus sequences of TPA-inducible and AP-1-binding elements (fig.1). Although these experiments do not prove that the effect of TPA is mediated through protein/DNA interactions at these motifs, this is a possibility that we are currently testing.

It has been found recently that the transcription factor AP-1 forms a complex with the *fos* encoded protein and they share a common DNA-binding site [37]. Moreover, the transcription factor AP-1 is homologous to the proto-oncogene *jun* [38]. Thus, it is possible that both the fos and jun protein products bind to the H-*ras*1 promoter and regulate transcription of the H-*ras*1 gene. This is also being tested. Finally, it is also of interest that *fos* can be induced by microinjecting *ras* p21 [39] and that truncated *ras* genes can still transform cells [40].

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