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Sp1 Specific Binding Sites Within the Human H-*ras* Promoter: Potential Role of the 6 bp Deletion Sequence in the T24 H-*ras*1 Gene

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Abstract. *Transcriptionally active domains have been identified and located within the 5' -region of the human normal and mutant T24 H-rasl promoters, and have been characterised by linkage to the coding sequences of the bacterial chloramphenicol acetyltransferase (CAT) gene or by using DNaseI footprinting analysis of the promoter sequence. It has been shown, using the latter method, that Sp-1 transcription factor binds to six GC sequences within the H-ras promoter. In the present study we have used unfractionated nuclear protein preparations from HeLa cells and a gel retardation assay to analyse specific binding of nuclear protein preparations from HeLa cells and a gel retardation assay to analyse specific binding of nuclear factors to several oligonucleotide sequences of the human H-rasl promoter. Our data demonstrate the presence of three Spl specific binding sequences in the T24 promoter, one of them containing a Sp-1 consensus GGCGGC absent in the normal H-rasl promoter.*

Ras genes play an important role in the development of human cancer, since members of the *ras* oncogene family have been implicated in a variety of naturally occurring tumors (for a review see ref. 1). These genes are often activated by point mutations (2,3), elevated expression of *ras* RNA transcripts (4,5) and *ras* p21 protein (6,7).

The location of transcriptional start sites of the H-*rasl* gene has been determined using primer extension (8). No obvious TATA box exists in the promoter region, but there are several GC regions. GGGCGG and its inverted complement CCGCCC bind the human HeLa transcription factor Spl and this has been shown to occur in the H-*rasl* promoter by footprinting analysis (9) (for the structure of the promoter sequence see Fig. 1).

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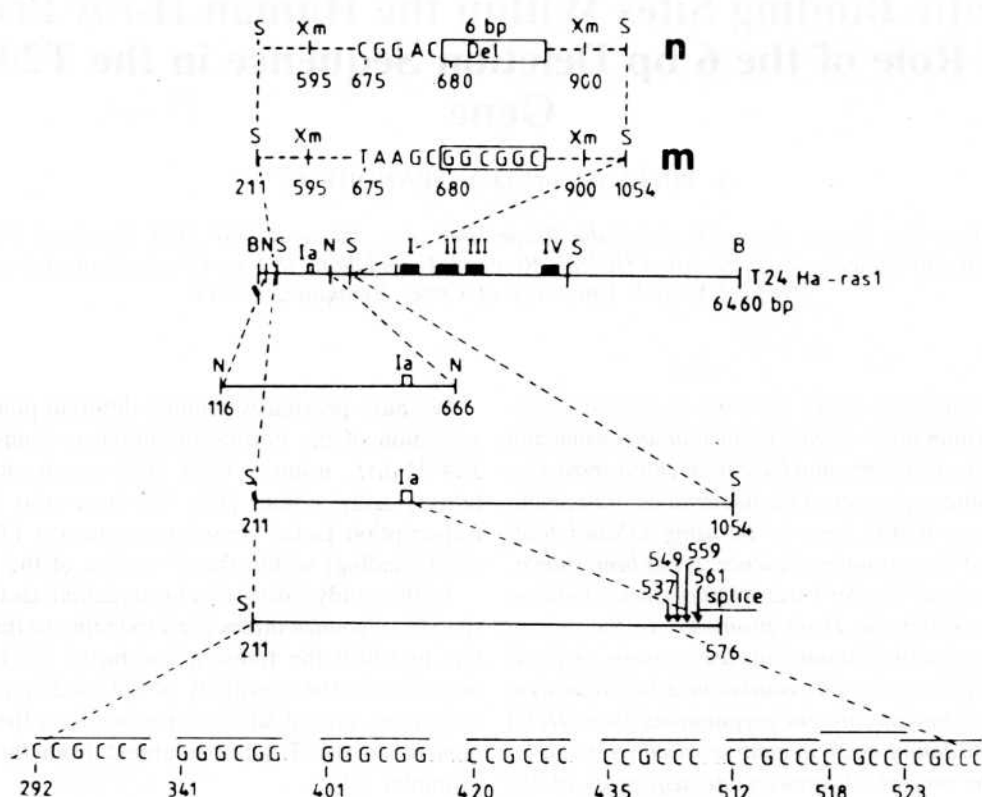
We have previously found a different potency and *trans* -activation of the normal promoter as compared to mutant T24 H-*rasl*, using a CAT (chloramphenicol acetyl-transferase) assay system (10). We have also identified other transcription factor consensus sequences (TPA responsive/ AP-1 binding) within the 5' -region of the promoter (11).

In this study, using a gel-retardation assay, we identified specific oligonucleotides corresponding to the H-*rasl* promoter, to which the transcription factor Spl binds. Our data demonstrate the specificity of Spl binding to the consensus sequences, one of which corresponds to the 6 bp sequence present on the T24 but is absent from the normal H-*rasl* promoter (10).

Materials and Methods

Cell extracts. Nuclear extracts from HeLa S3 cells were prepared from suspension cultures grown to high density in modified Eagle's Minimal Essential Medium with 10% foetal calf serum and 5% CO_2. The cells were harvested by centrifuging at 1000×g, washed with phosphate buffered saline and stored as a dry pellet at -70°C until required. Frozen pellets were thawed in hypotonic buffer containing 25 mM Tris HCl pH 7.5, 5 mM KCl, 0.5 mM $MgCl_2$, 0.5 mM dithiothreitol (DTT) and 0.5 mM phenylmethylsulfonyl fluoride (PMSF) at 5ml/g. Packed cells were left for 30 mins at 4°C, dounce homogenised with a B-type dounce and the nuclei pelleted by spinning at 25 K rpm in a Sorvall SS 34 rotor. The nuclear pellet was washed extensively with buffer containing 25 mM Tris HCl pH 7.5, 5mM KCl, 0.5 mM $MgCl_2$, 0.1 M sucrose, 0.5 mM DTT and 0.5 mM PMSF and resuspended in nuclei extraction buffer containing 25 mM Tris pH 7.5, 1 mM EDTA, 0.1% NP40, 0.5 DTT, 0.5 mM PMSF and 0.6 M KCl. Nuclei were inverted slowly at 4° for 60 min and the extract clarified by centrifugation at 10^5g for 60 min at 4° C. Nuclear extracts were stored at -70°C. Protein estimations were performed as described by Bradford (16).

Synthetic oligonucleotides. The 248/277 and 411/443 *ras* probes were the synthetic double-stranded oligonucleotides 5' -CCGCGGCCCTACTGGCTCCGCCTCCCGCGT-3' and 5'-TGCGCAGGCCCGCCCGAGTCTCCGCCGCCCCGTG-3' representing the regions between 248 and 277 and 411 and 443 of the human H-*rasl* promoter, respectively (for complete sequence of H-*rasl* see ref. 17). The 607/702 *ras* probe was the synthetic oligonucleotide 5'-GGGCGTAAGCGGCGGCGGCGGCGGCGGCGGCGG-3'. The probes used for competition experiments at a concentration 100×



Fifure 1. *Organization of the human H-rasl gene and its promoter. The coding sequences are designated by black boxes and the non-coding 5'-sequences by open boxes. The locations of the sequences GGGCGG and each complementary CCGCCC contained between nucleotids 220 and 576 of the map and situated at the 5' -end of the H-rasl gene are shown. The H-rasl gene extra sequence GGCGGC of the T24 nucleotide 680 is shown. Transcriptional start sites are indicated by arrows. DEL, deletion: B, BamHI S, SstI: Xm XmlI: N, NaeI: I, II, III and IV, Coding exons; Ia, non-coding exon; n, normal; m, mutant T24.*

higher were the following: The E_3AP-1 synthetic oligonucleotide 5' -CCGAAGTTCAGATGACTAACTCAGGG-3' representing the region between nucleotides -81 and -103 of the EIA inducible E_3 promoter (12). The NFI probe 5'-GATCTTATTTTGGCTTGAAGCCAATATG-3' (13). The NFI probe 5'-GATCTTGGTCCGCGCCCCGCCCCCGCATGGCG-3'. The *jun* AP-1 and HSV AP-1 probes represent the AP-1 binding sites in the *jun* and HSV-2 ribonucleotides reductase RR_2 subunit genes respectively and the ATF probe 5'-AGCTTCGGGCGGCTTTCGTCACAGGGTCGGGTC-3' represents the binding site for activating of adenovirus transcription factor.

Preparation of double stranded oligonucleotieds. Single stranded DNA oligonucleotides were made on an Applied Biosystems 381A DNA synthesizer. The oligonucleotides were removed from the synthesis column by elution with 3×2 ml of ammonia. This solution was incubated at 55 ° overnight to deprotect the oligonucleotides. To purify the oligonucleotide further, an Applied Biosystems oligonucleotide purification cartridge (OPC) was used. To anneal two single stranded complementary oligonucleotides were incubated together at a concentration of 0.05 M in 1× T.E. The solution was then heated to 90°C. This results in the formation of double stranded oligonucleotides at a concentration of 0.05 M. To check that the annealing had worked, the double stranded oligonucleotides (along with single-stranded oligonucleotides for comparison) were run on a 8% polyacrylamide gel.

Double stranded oligonucleotides were 5' –end labelled using $\gamma-^{32}$P-ATP and T4 polynucleotide kinase and end filled using the Klenow fragment of DNA polymerase according to Maniatis *et al* (18).

Complex formation and analysis. Reaction mixtures (20μl) contained the following: 10mM Hepes (pH 7.9), 0.2mM EDTA, 94mM NaCl, 0.1mM PMSF, 0.1ng/ml bovine serum albumin, 4% (w/v) glycerol, 3μg poly(dI)-poly(dC), 0.2-1.0 ng ^{32}P –labelled DNA and 1.0μl (5μg) HeLa nuclear extract added last to initiate the binding reaction. 500 mM stock solutions of DTT were used to add the required concentration to the binding reaction. Specificity was determined by competition with an excess of unlabelled competitor DNA. Incubation was on ice for 30 min, and reaction mixtures were loaded directly onto a 4% polyacrylamide gel (30:1 acrylamide: N,N'-methylene bisacrylamide) containing 45 mM Tris-borate and 1.25 mM EDTA (0.5× TBE). Gels were run at 4° in 0.55X TBE, dried and exposed to X-ray film for autoradiography.

Results

The affinity of the Spl transcription factor for specific

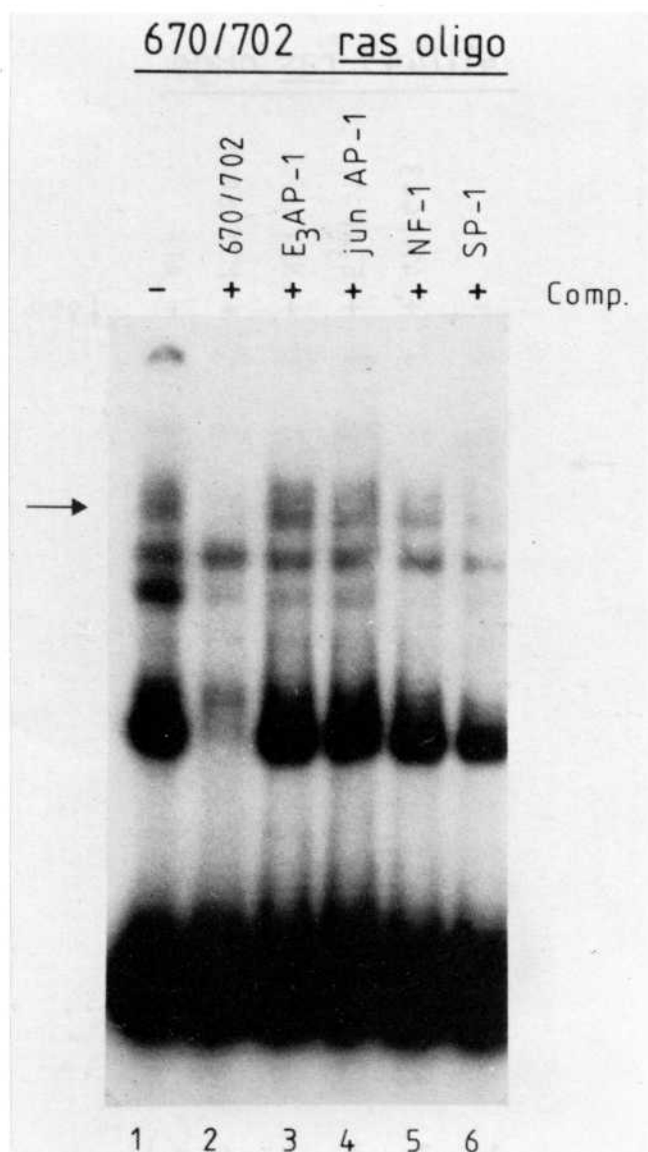


Figure 4. *Specific binding of Sp-1 on the 670/702 H-*rasl *oligonucleotide. Lane 1 shows the binding of the factor(s) to the oligo. Lanes 1 and 6 show the specificity of the binding by competition with itself and a cold Sp-1 binding oligonucleotides E_3P_1*jun/*AP-1 and NF-1 do not compete respectively.*

important for the control of *ras* expression in normal and tumor tissues. Spl has been shown to bind specifically in the H-*ras* promoter region (9). In the present study we have examined regions 248/277, 411/443 and 670/702 for Spl binding and we have found specific Spl-like activity in all three regions of the H-*rasl* sequence. Ishii *et al* (9), who analysed binding of purified Spl to the H-*rasl* promoter by footprinting experiments, did not find obvious binding of the factor to the region covered by the 411/443 oligonucleotide, although it is shown to contain two potential Spl binding sites. The difference beween the results of this group and ours may be due to the better sensitivity of the gel retardation assay we have employed or most possible, due to factors present in nuclear extracts that activate Spl proteins, an activity which cannot be observed in experiments with purified Spl. It is of interest that the T24 promoter has an extra GGCGGC box in the region 670/702, which, as shown in Figure 4, binds to Spl-like factor. This may be important for the biological properties of the T24 H-*rasl* oncogene. It has also been found that the oligonucleotide corresponding to the 670/702 region of the normal H-*ras* promoter did not bind Spl-like factors (data not shown). This is consistent with the view that the extra 6 bp sequence on the T24 promoter plays an important role in the biological function of this oncogene. Further studies are in progress to determine the nature of the protein(s) and their role in the regulation of the T24 H-*rasl* gene.

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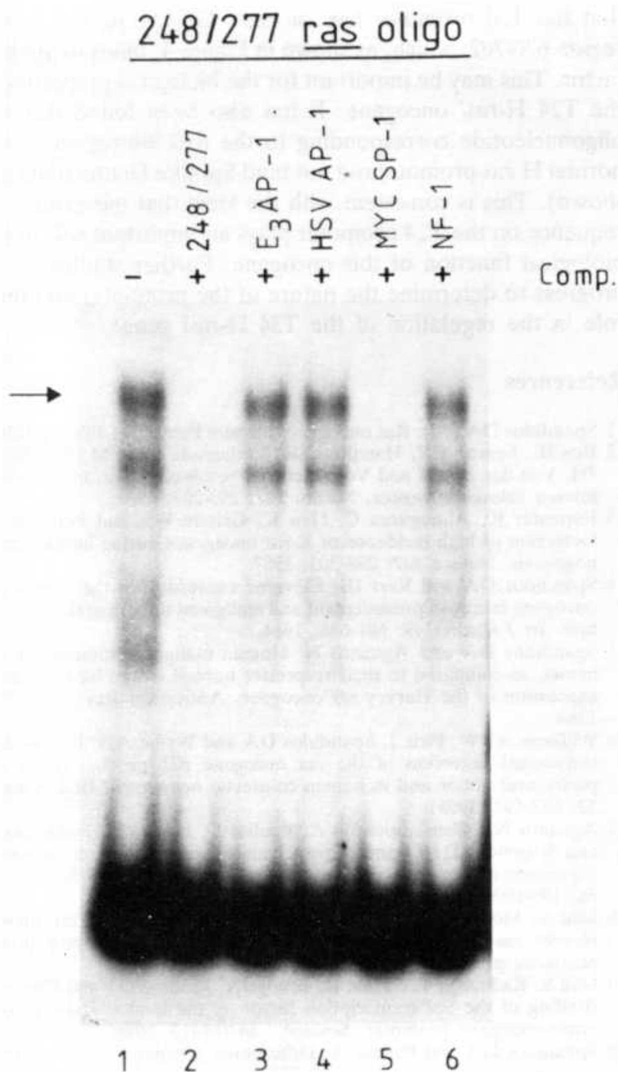

Figure 2. *Specific Sp-1 binding on the 248/277 H-*rasl *oligonucleotide. Arrow indicates the position of Sp-1 complex. Lane 1 shows the binding of the HeLa cells factor to the oligo. Lanes 2 and 5 show the specificity of the binding by competition with a cold Sp-1 binding oligonucleotide and the* myc *Sp-1 oligonucleotide respectively. Lanes 3, 4 and 6 show that the E_3P_1 HSV AP-1 and NF-1 do not compete with the 248/277 oligonucleotide.*

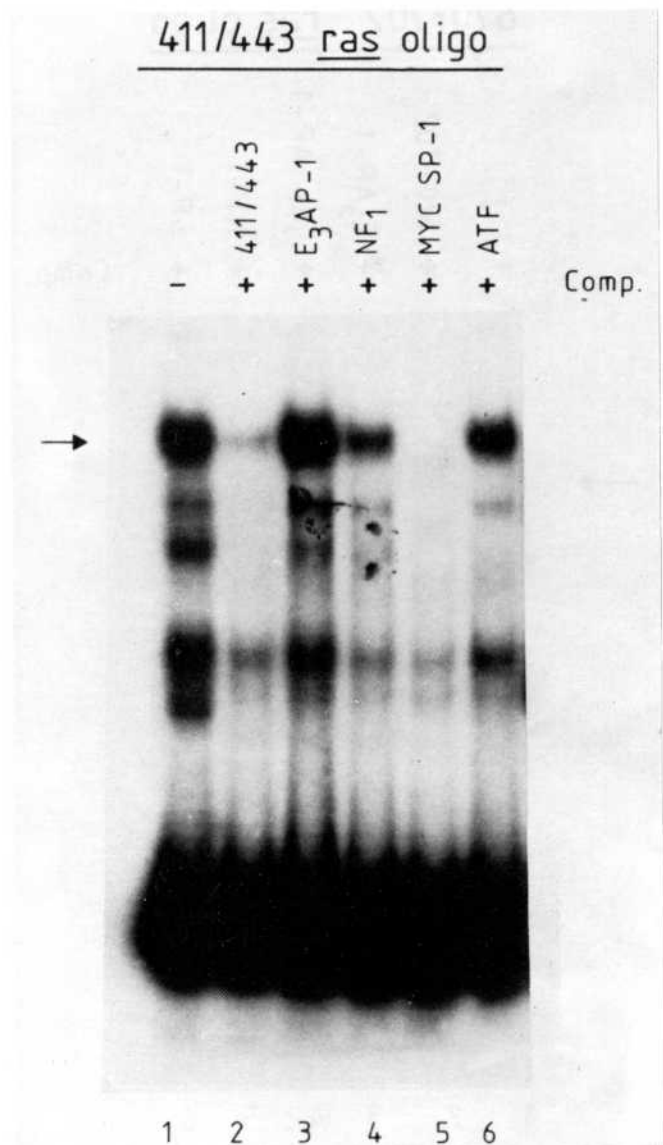

Figure 3. *Binding of Sp-1 on the 411/443 H-*rasl *oligonucleotide. The specificity of the binding is shown in lanes 2 and 5 by competing with itself and the myc Sp-1 oligonucleotide respectively. Lanes 4 and 6 show partial competition with NF_1 and ATF binding oligonucleotides respectively whereas in lane 3 the E_3 P-1 does not compete.*

binding to labelled synthetic oligonucleotides corresponding to the various sequences of the human H-*rasl* promoter was tested by incubating muclear protein extracts from HeLa cells with the labelled DNA and determining protein-DNA interaction by gel retardation assay. The results are shown in Figures 2-4. We can draw the following conclusions from these data.(i) There is at least one factor binding to each of the three oligonucleotides.

(ii) Interaction between protein and DNA is specific, as shown in lane 2 of Figures 2-4 by competing with cold oligonucleotides.

(iii) There is a Spl-like activity in the nuclear extracts as shown by competition with a cold Spl binding oligonucleotide (Figure 2, lane 5; Figure 3, line 5; Figure 4, lane 6).

(iv) Cold oligonucleotides representing binding sites of AP-1, NF1 and ATF transcription factors do not compete.

The above points show clearly that we have an Spl-like activity bound to three different consensus sequences of the human H-*rasl* promoter, one of which is absent from the normal promoter.

Discussion

We have used synthetic oligonucleotides and a gel retardation assay in order to investigate specific interactions of factors existing in protein nuclear extracts with consensus sequences in the H-*rasl* promoter. Such interactions are thought to be