Sp1 Specific Binding Sites Within the Human H-ras Promoter: Potential Role of the 6 bp Deletion Sequence in the T24 H-ras1 Gene

A. PINTZAS1 and D.A. SPANDIDOS2,3

1The Beatson Institute for Cancer Research, Carscrue Estate, Bearsden, Glasgow G61 1BD, Scotland, UK; 2Institute of Biological Research and Biotechnology, National Hellenic Research Foundation, 48 Vas. Constantiou Ave., Athens 116 35, 3Medical School, University of Crete, Heraklion, Greece

Abstract. Transcriptionally active domains have been identified and located within the 5'-region of the human normal and mutant T24 H-ras1 promoters, and have been characterised by linkage to the coding sequences of the bacterial chloramphenicol acetyltransferase (CAT) gene or by using DNase I 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 unfraccionated 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-ras1 promoter. Our data demonstrate the presence of three Sp1 specific binding sequences in the T24 promoter, one of them containing a Sp-1 consensus GGGCGGCA absent in the normal H-ras1 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-ras 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 Sp1 and this has been shown to occur in the H-ras1 promoter by footprinting analysis (9) (for the structure of the promoter sequence see Fig. 1).

We have previously found a different potency and trans-activation of the normal promoter as compared to mutant T24 H-ras1, using a CAT (chloramphenicol acetyltransferase) 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-ras1 promoter, to which the transcription factor Sp1 binds. Our data demonstrate the specificity of Sp1 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-ras1 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% CO2. The cells were harvested by centrifuging at 1000 x 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 MgCl2, 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 MgCl2, 0.1 M sucrose, 0.5 mM EDTA 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 mM DTT, 0.5 mM PMSF and 0.6 M KCl. Nuclei were inverted slowly at 4°C for 60 min and the extract clarified by centrifugation at 10,000 x g 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'-CGGCCGCTAATGCTCCGCTTCGCCCGCTGT-3' and 5'-TGGCGCAGGCCGCGCCAGTCTCCGGCGCGCCGTG-3' representing the regions between 248 and 277 and 411 and 443 of the human H-ras1 promoter, respectively (for complete sequence of H-ras see ref. 17). The 607/702 ras probe was the synthetic oligonucleotide 5'-GGGCCCTAACGCCGCCGCGCGCGCGCGCGCGCGCGCGCG-3'. The probes used for competition experiments at a concentration 100x

Correspondence to: Professor D.A. Spandidos, Institute of Biological Research and Biotechnology, National Hellenic Research Foundation, 48 Vas. Constantiou Ave., Athens 116 35, Greece.

Key Words: Sp1 binding sites, H-ras1 oncogene.
higher were the following: The \( E_{\alpha} \)AP-1 synthetic oligonucleotide 5' -CGGAAACTGCAGATCTAAGCTCAGG-3' representing the region between nucleotides -81 and -103 of the EIA inducible \( E_{\alpha} \) promoter (12). The NFI probe 5' -CATCTTTTGTGATAGTCGGCTAAATGCAGA-3' (13). The NFI probe 5' -CATCTTTTGTGATAGTCGGCTAAATGCAGA-3'. The \( \mu \alpha \) AP-1 and HSV AP-1 probes represent the AP-1 binding sites in the \( \mu \alpha \) and HSV-2 ribonucleases reductase \( R_{\alpha} \) subunit genes respectively and the ATF probe 5' -GGCTTTCGGGCAGCCGTCGCTGAGATCTCAGG-3' represents the binding site for activating of adeno virus transcription factor.

Preparation of double stranded oligonucleotides. Single stranded DNA oligonucleotides were made on an Applied Biosystems 381A DNA synthesizer. The oligonucleotides were removed from the synthesis column by elution with 3x2 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 1X T.E. The solution was then heated to 90°C. This resulted 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 \( ^{32} P \)-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 (20 μl) contained the following: 10 mM Hepes (pH 7.9), 0.2 mM EDTA, 94 mM NaCl, 0.1 mM PMSF, 0.1 μg/ml bovine serum albumin, 4% (w/v) glycerol, 3 μg poly(dI)-poly(dC), 0.2-1.0 ng \( ^{32} P \)-labelled DNA and 1 μl (5 μg) 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% acrylamide; N,N'-methylene bisacrylamide) containing 45 mM Tris-borate and 1.25 mM EDTA (0.5X 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
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-rasf 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-rasf gene.

References


Received July 24, 1991
Accepted October 2, 1991

Figure 4. Specific binding of Spl-1 on the 670/702 H-ras 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 Spl-1 binding oligonucleotide E3Pjun/AP-1 and NF-1 do not compete respectively.
binding to labelled synthetic oligonucleotides corresponding to the various sequences of the human H-ras1 promoter was tested by incubating nuclear 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, lane 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-ras1 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-ras1 promoter. Such interactions are thought to be