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Wild-type but not mutant p53 activates the hepatocyte growth factor/scatter factor promoter

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ABSTRACT

p53 transactivates the expression of a variety of genes by binding to specific DNA sequences within the promoter. We have investigated the ability of wild-type p53 and a non-DNA binding p53 mutant to activate the hepatocyte growth factor/scatter factor (HGF/SF) promoter using chloramphenicol acetyltransferase reporter constructs. We also used deletion sequences of the HGF/SF promoter to identify which regions, if any, were responsible for p53 binding. Our results show that wild-type but not mutant p53 activates the HGF/SF promoter when using –3000 and –755 bp upstream of the HGF/SF gene. This activation is lost when promoter sequences covering –365 and –239 bp are used. Analysis of the DNA sequence between –365 and –755 bp shows one putative p53 half-site with 80% homology to the consensus sequence and another half-site 3 bases downstream of this with 100% homology to the consensus sequence. In contrast to previously identified p53 binding DNA sequences, the downstream half-site is inverted. We propose that the HGF/SF promoter can be activated by wild-type p53 *in vivo* and that this could be as a result of a novel form of sequence-specific DNA binding.

INTRODUCTION

Wild-type p53 is a transcription factor that serves a dual role in the control of cellular proliferation. It is probably best recognized for its tumour suppressor function whereby p53 can induce either a G_1/G_2 arrest (1,2) or apoptosis (3) in cells that have sustained DNA damage. The nature, extent and site of DNA damage all relate to the type of p53-mediated response that ensues, as reviewed by Gottlieb and Oren (4), indicating that this tumour suppressor is a highly versatile and response-selective 'guardian of the genome'.

p53 can also positively regulate cell division and differentiation by specific induction or repression of promoters for growth-associated factors and growth factor receptors (5). The cloning and sequencing of p53 cDNAs from several species has led to a detailed understanding of structural features of the protein. The ability of p53 to activate gene expression by sequence-specific DNA binding has been attributed to amino acid residues 102–292 (6), which lie in the core domain of the protein. To date, the number of growth-related genes which are known to be transcriptionally activated by p53 in this manner are relatively few.

When p53 is mutated, as it is in ~50% of all human cancers (7), it is usually a missense mutation occurring in the core domain of the protein. Depending on the nature of the mutation, it can give rise to loss of wild-type p53 function, thus allowing cell cycling to proceed unchecked and inappropriate gene expression to occur. Wild-type p53 has been observed to stimulate activation of the epidermal growth factor receptor (EGF-R) promoter (8) and could be an important regulator of its expression in normal development. Notably, stimulation shows increased sensitivity to certain forms of mutant p53, suggesting that cell proliferation could be enhanced by overexpression of the EGF-R in cancers involving these p53 mutants.

Growth factors act in a paracrine, autocrine or endocrine fashion and interact with specific cell surface receptors. p53 may influence the growth factor network by regulating the expression of certain growth factor receptors, such as the EGF-R and c-met (9,10), or other growth-related molecules, such as transforming growth factor-α (11) and insulin-like growth factor binding protein 3 (12).

HGF/SF is derived from a single chain molecule which is proteolytically cleaved to form a biologically active heterodimer consisting of an α chain (69 kDa) and a β chain (34 kDa) (13). It is produced by a wide variety of tissues, including the liver, pancreas, salivary glands, thyroid, duodenum and kidney, and is thought to exert its effects mainly by paracrine interaction with the cell surface receptor c-met (14). Studies indicate that expression of HGF/SF is restricted to cells of mesenchymal origin (15) in a manner that is highly controlled and developmentally regulated.

The biological effects of HGF/SF are numerous. It is the most potent stimulator of DNA synthesis for mature hepatocytes (16). It also stimulates the invasiveness and movement of epithelial cells, but is cytostatic to others (17–19). Because of its significant effect on the proliferation and invasive capacity to some cell types, HGF/SF may be an important candidate for enhancing tumour development and metastasis formation. Indeed, several tumour types have been reported to show elevated expression of HGF/SF (20–24). It is of great interest to elucidate the mechanisms responsible for controlling the expression of both HGF/SF and c-met with the aim of understanding neoplasia formation and progression associated with abnormal HGF/SF and c-met expression.

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In order to decipher positive and negative regulatory elements involved in controlling HGF/SF expression, the promoter regions of human, mouse and rat HGF/SF have been cloned and sequenced (25–27). Cell lines have also been used to measure HGF/SF expression in response to various ligands. These include epidermal growth factor (28), platelet-derived growth factor (28), fibroblast growth factor (28), IL-1 (29), injurin (30) and injurin-like factor (31), all of which increase the expression of HGF/SF in human skin fibroblasts or MRC-5 cells. Transforming growth factor-β and some glucocorticoids down-regulate HGF/SF production in leukaemia cell cultures and human lung fibroblasts (32).

Promoter sequence analysis has further characterized potential response elements and identified a high degree of sequence conservation between species (95% between mouse and rat and ~90% between rodent and human), implying common regulatory mechanisms of HGF/SF gene expression. Whilst one putative p53 half-site has previously been identified in the rat HGF/SF promoter (27), no experimental evidence for transcriptional activation of the HGF gene by p53 has been provided to date.

We observed several putative p53 half-sites within 1000 bp of the HGF/SF promoter that have not been previously identified. Here we have investigated the ability of p53 to activate different HGF/SF promoter constructs. We show that wild-type human p53, but not mutant p53, activates murine HGF/SF promoter–*CAT* constructs which contain the novel p53 half-sites. This suggests that wild-type p53 can regulate cell proliferation indirectly, via HGF/SF promoter activation.

MATERIALS AND METHODS

Plasmids

Wild-type and mutant human p53 expression plasmids (kindly provided by Jo Milner, YCRC p53 Laboratory, Department of Biology, York University, York, UK) used the human cytomegalovirus (CMV) major immediate-early promoter–enhancer (bases 209–864) in the vector pRc/CMV (Invitrogen). pRc/CMV wt hp53 contains wild-type human p53 cDNA, whilst pRc/CMV M237I contains mutant human p53 with a Met→Ile substitution at amino acid 237 (33).

CAT plasmids containing the HGF/SF promoter sequences (–3000, –755, –365 and –291 bp) were made by ligating restriction fragments into the promoter-less plasmid pCAT-basic (Promega) as previously described by Plaschke-Schlutter *et al.* (26).

Cell culture and transfection

The cells used in these experiments were clone D4 *ras*-transformed NIH 3T3 mouse fibroblasts (34) which were propagated in Dulbecco's modified minimum essential medium containing 10% foetal bovine serum. In a typical experiment, 1×10^6 cells were plated into a 100 mm diameter Petri dish and co-transfected with 5 µg HGF/SF–*CAT* construct and 5 µg p53 expression plasmid. Transfections were carried out using the calcium phosphate method as previously described (35). A control plasmid containing the Rous sarcoma virus promoter and the *Escherichia coli lacZ* gene was co-transfected in each experiment.

CAT assay

Cells were harvested 48 h post-transfection and lysed by three cycles of freeze-thawing. Cell extract volumes used in the CAT assay were adjusted according to the β-galactosidase activity in each transfection. CAT enzyme activity was then measured using ^{14}C-labelled chloramphenicol (Dupont) as described previously (36). Each transfection was repeated three times.

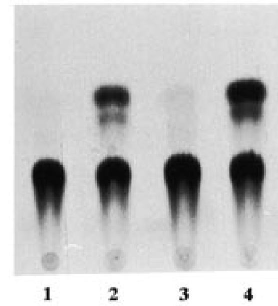

Figure 1. Transcriptional activity of the –3000 and –755 bp HGF/SF promoter constructs in the presence of p53. Ras 3T3 cells were co-transfected with either of the HGF/SF constructs with wild-type or mutant p53 expression plasmids. Lane 1, transfection of the –3000 bp HGF/SF promoter with the vector alone; lane 2, –3000 bp with wild-type p53; lane 3, –3000 bp with mutant p53; lane 4, –755 bp of the HGF/SF promoter with wild-type p53. The amount of cell extract used in each CAT assay was adjusted according to β-galactosidase activity as described in Materials and Methods.

RESULTS AND DISCUSSION

Wild-type p53 transactivates –3000 and –755 bp HGF/SF promoter constructs

Figure 1 shows that ras 3T3 fibroblasts had strong CAT activity when co-transfected with –3000 bp of the HGF/SF promoter and wild-type p53. When analysed quantitatively using a phosphor-imager, the percentage conversion of total [^{14}C]chloramphenicol ('CAT activity') was ~20% (20-fold higher than controls using plasmid vector alone). The CAT activity increased to 30% when transfection was performed using the –755 bp HGF/SF promoter construct with wild-type p53. When cells were co-transfected with mutant p53 and either of the HGF/SF–*CAT* constructs (–3000 or –755 bp) very low CAT activity was observed (1%). A similar result was obtained when the HGF/SF plasmids were transfected in the absence of exogenously added p53 (here the vector alone was used). The low CAT activity (1%) indicates that endogenously expressed p53 in ras 3T3 fibroblasts is minimal and has a negligible effect on the *CAT* constructs. We also attempted to transfect p53-null mouse embryo fibroblasts (a kind gift from Larry Donehower, Division of Molecular Virology, Baylor College of Medicine, Houston, TX, USA) to remove any risk of interference from endogenous p53, but these cells transfected with very low efficiency and were therefore unsuitable for use in these experiments. It is important to note that p53 is highly conserved in evolution and that it recognizes a common DNA sequence. Our results demonstrate cross-species reactivity between human p53 and the murine HGF/SF promoter.

This is the first report of a direct role for p53 in controlling expression of HGF/SF. Whilst one putative p53 half-site, based on DNA sequence analysis, has been reported (27), our data are the first *in vivo* evidence to show functional activity of p53 on HGF/SF transcription.

Table 1. Gel shift analysis of p53 binding to HGF/SF promoter sequences.

Oligonucleotide sequence (site in HGF/SF promoter)	Wild-type p53 murine (supershift)	Mutant p53 murine (supershift)	Wild-type p53 human (supershift)
GGCCATGTCTGGCCATGTCT (–810 to –819 bp)	Yes (Yes)	No (No)	Yes (Yes)
ACACATGCATACACATGCAT (–381 to –390 bp)	Yes (Yes)	No (No)	Yes (Yes)

Summary of gel mobility shift analysis results. The ability of two 20 bp oligonucleotide sequences, each a tandem repeat of previously unidentified putative p53 half-sites within the rat and mouse HGF/SF promoter, to bind to human and murine wild-type p53 and murine mutant p53 is shown. The effect of adding p53-specific monoclonal antibodies to supershift the complexes is given in brackets (monoclonal antibody 248 for murine p53 and D01 for human p53). The exact position of the site relative to the proposed start site of gene transcription (25) is provided in the first column. Whilst both of these sequences were found to bind to human and murine wild-type p53, no complexing was observed with murine mutant p53.

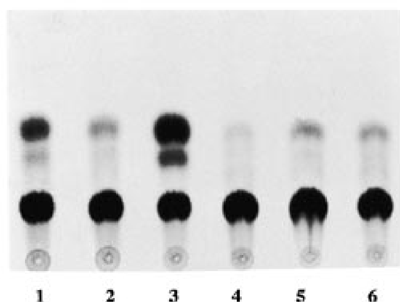

Figure 2. Transcriptional activity of truncated HGF/SF promoter constructs in the presence of p53. The activities of various HGF/SF deletion constructs were compared with those of –3000 bp (with wild-type p53, lane 1; with mutant p53, lane 2) and –755 bp (with wild-type p53, lane 3; with mutant p53, lane 4). The –365 bp HGF/SF promoter plasmid was co-transfected with wild-type p53 (lane 5) and mutant p53 (lane 6).

Importantly, we have identified several novel DNA sequences within the HGF/SF promoter that could be critical in the transcriptional activity of p53. These show between 80 and 100% homology to the p53 consensus sequence, in contrast to the previously reported half-site, which only shows 70% homology. Initial studies using mobility shift analysis were performed to investigate the ability of several oligonucleotide sequences derived from the HGF/SF promoter to bind to p53. These oligonucleotides were 20 bp constructs each representing a tandem repeat of the putative p53 half-site as it exists within the HGF/SF promoter. Whilst mutant p53 failed to bind to any of these sequences, wild-type p53 showed DNA binding that was confirmed by supershifting with a p53-specific monoclonal antibody. A summary of these results is given in Table 1. Taken together with the data presented here, it is likely that the previously published p53 half-site in the HGF/SF promoter is not the sole p53 response element required for transcriptional activation of this gene.

To date, wild-type p53 is known to transactivate some 20 gene promoters, but only a few of these are growth factors, one example being transforming growth factor-α (10). Activation of HGF/SF expression by wild-type p53 may be a normal function in growth and development. It is of interest to note that although some p53-null mice develop to maturity, many show growth abnormalities (37). HGF/SF-null mice all die at ~13 days gestation (38). Taken together, these observations imply that the role of p53 in HGF/SF regulation is modulatory rather than absolute.

As we have shown using an inflexible mutant of p53 (M237I) that is unable to bind to DNA (33), activation of the HGF/SF

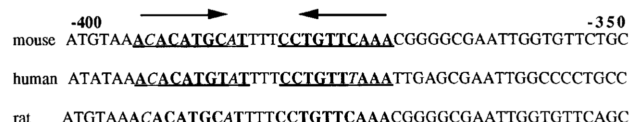

Figure 3. Sequences of the mouse, human and rat HGF/SF promoters between –400 and –350 bp containing putative p53 binding sites. Genomic sequences are aligned and putative p53 binding regions are in bold and underlined. Bases that do not conform to the consensus p53 binding site are in plain type.

promoter is abrogated. It remains to be determined if other types of p53 mutation retain the ability to activate the HGF/SF promoter. To date, there is no direct evidence to suggest that HGF/SF is overexpressed as a result of p53 mutations in cancer, but several independent studies (39–41) have identified a high incidence of p53 mutations in human oesophageal squamous cell carcinomas. Moreover, Takada *et al.* (42) have reported that these carcinomas show a significant elevation of HGF/SF concentration. Also, HGF/SF is secreted by various types of human leukaemia cells (43), a disease which is sometimes associated with p53 mutation, and Nakamura *et al.* have found significant amounts of HGF/SF in the blood and bone marrow plasma of patients with leukaemia and lymphoma (44).

Inability of wild-type p53 to activate –365 and –239 bp of the HGF/SF promoter and identification of putative p53 binding sites

To identify which regions of the HGF/SF promoter sequence are responsible for p53 binding we performed transfections using truncated constructs of the HGF/SF promoter, one being –365 and the other –239 bp upstream of the gene. The CAT activity was greatly reduced using either the –365 bp construct (Fig. 2) or the –239 bp construct (6% conversion of [^{14}C]chloramphenicol in each case) to values almost identical to those obtained with mutant p53 transfections (4 and 8% respectively). This indicates that either all or part of the p53 response element in the HGF/SF promoter exists between –755 and –365 bp of the promoter.

In our experiments, the mouse HGF/SF promoter DNA sequence between –755 and –365 bp included one p53 half-site (a decamer) with 80% homology to the consensus sequence (5′-G/A G/A G/A C A/T A/T G T/C T/C T/C-3′) (45). This putative half-site is situated three thymidines downstream of another half-site (100% homology to consensus sequence) which runs in the opposite direction (inverted). The sequences of these are ACACATGCAT (–394 to –385) and CCTGTTCAAA (–381 to –372) (bases which do not conform to the consensus sequence are underlined). The rat promoter (27) contains identical sequences

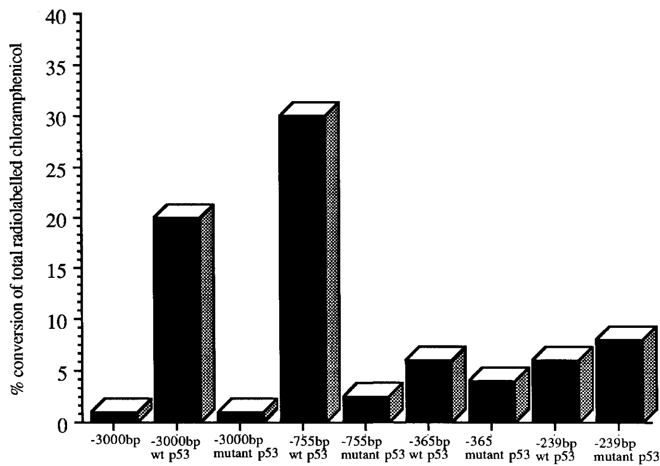

Figure 4. A graphical summary of activity of progressive 5′ deletions of the HGF/SF promoter. Values were obtained by analysis of CAT assay thin layer chromatography plates on a phosphorimager. The HGF/SF construct (–3000, –755, –365 or –239 bp) was either transfected with the vector alone or in combination with either wild-type (wt) or mutant p53 as described on the *x*-axis. Activity was determined by calculating percentage conversion of total [^{14}C]chloramphenicol into [^{14}C]acetylchloramphenicol.

to this and the human sequence (25) has one base change in each half-site (Fig. 3).

CAT activity was lost when a –365 bp construct was used (Fig. 2). Analysis of the sequence between –365 bp and the start site for gene transcription failed to identify any further p53 half-sites. However, we have noted that there is another half-site with 90% homology to the consensus sequence in the HGF/SF promoter at ~–1000 bp. Thus our data imply that this half-site is not essential for p53 binding, as it is not contained within the –755 bp promoter which gave the strongest CAT activity. A graphical representation of our data is given in Figure 4.

In summary, we have shown that wild-type p53 may directly regulate HGF/SF expression. The *in vivo* implications of this may be relevant to both normal growth and development and to oncogenesis, tumour progression and metastasis formation.

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