The helix-loop-helix containing transcription factor USF binds to and transactivates the promoter of the p53 tumor suppressor gene

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ABSTRACT

Expression of the wild-type p53 tumor suppressor gene has been found to play an important role in the regulation of cellular proliferation and differentiation. In addition, in many transformed cells and primary tumors, the gene has undergone allelic deletions and mutant forms of the p53 gene are expressed at elevated levels. In defining transcriptional regulatory regions of the p53 gene, we have previously shown that both the human and murine p53 promoters contain a conserved consensus recognition sequence for the basic-helix-loop-helix (bHLH) containing family of DNA-binding proteins. In the murine p53 promoter this element is required for full promoter activity and contains the sequence CACGTG, a sequence identical to the recognition site for the bHLH containing transcription factors c-Myc, USF and TFE3. Here we examine the ability of one of these factors, USF, to bind to the p53 promoter. By assaying the binding activity of in vitro translated USF as well as factors present in nuclear extracts, we conclude that the transcription factor USF binds in a site-specific manner to a CACGTG motif within the murine p53 promoter and represents the major DNA-binding activity observed in nuclear extracts. Elevated levels of USF, generated upon transfection of a vector expressing USF, lead to enhanced activity of the p53 promoter. These findings indicate that USF may play a central role in regulating p53 expression.

INTRODUCTION

It is now fairly well established that the wild-type p53 gene encodes a protein having properties of a negative growth regulator which can also function as a tumor suppressor. Allelic deletions and mutations in the gene, thought to in part inactivate its tumor suppressing activity, have been associated with a wide range of tumors (1,2,3,4). Furthermore, while mutant p53 can transform primary rat embryoid fibroblasts in cooperation with the activated ras oncogene, wild-type p53 inhibits transformation in this assay (5,6), consistent with its classification as a tumor suppressor.

The sole inactivation of the tumor suppressing activity of wild-type p53 apparently may not always be sufficient for transformation. While transgenic mice deficient in p53 expression develop tumors by six months of age (7), a number of studies indicate that certain p53 mutants may acquire novel activities and function as dominant transforming genes (8,9,10). In many transformed cells, mutant p53 proteins are detected at elevated levels (3,4). In fact, in addition to allelic deletions in the gene, high level expression of mutant p53 is now held to be among the most common genetic alterations yet identified in human cancers (3). Both mutations in the gene and their overexpression appear to be required for its transforming capability. Overexpression of mutant p53 results in both tumor formation in transgenic mice (11) and transformation of normal cells in vitro in cooperation with activated ras (12,13).

In addition to its role in transformation, expression of p53 has been found to undergo cell cycle related modulations. Expression of the gene is induced by mitogens prior to the onset of S-phase (14,15) and the protein is phosphorylated in a cell cycle dependent fashion by the cell cycle regulator p34cdc2 (16,17). A number of recent observations indicate that p53 may be regulated by and control DNA synthesis in response to DNA damage (18,19). While much evidence indicates that p53 may function as a DNA-binding transcription factor (20-24), its precise role in growth control is not yet known.

Since de-regulated expression of mutant p53 appears to be a central feature of malignant transformation and since the basis for this de-regulated expression in not well defined, we have set out to understand the mechanisms controlling p53 expression and the cause of its altered level of expression in tumor cells. We have focused our efforts on analyzing the transcriptional regulatory sequences of both the human (25) and murine (26) p53 genes. We have found that both the human and murine promoters contain a conserved recognition sequence for the basic-helical-loop-helix (bHLH) containing family of DNA-binding proteins (26,27), whose members are often involved in regulating

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cell growth and differentiation (28). In the murine promoter this element maps 70-bp downstream from the transcription initiation site, is required for maximal promoter activity and was shown to bind a nuclear factor present in many in transformed cells (26). Interestingly, the protein binding site requires the sequence CACGTG, identical to that demonstrated to be a specific binding site for the oncogene product c-Myc (29,30). Studies on the potential role of c-Myc in expression of the p53 gene indicate that c-Myc can bind to this p53 promoter element when present as a heterodimeric complex with another bHLH-containing factor, Max (29,31) as well as enhance expression of the promoter (31). In addition to the CACGTG sequence being a binding site for c-Myc, it has also been demonstrated to be a specific binding site for additional cellular bHLH-containing transcription factors such as USF (32,33) and TFE3 (34). That more than one transcription factor can interact with apparently the same DNA sequence indicates that they may influence the expression of overlapping sets of genes. It was therefore important to examine their interactions with the p53 regulatory sequences. Here we demonstrate by electrophoretic mobility shift assays that USF binds to the p53 downstream promoter element. This binding by USF constitutes the major nuclear DNA-binding activity with respect to this promoter element and requires the CACGTG motif.

USF is a cellular encoded transcription factor involved in the transcriptional regulation of a number of viral and cellular genes (32,33). The factor binds in a site specific manner to the sequence CACGTG and results in a 3- to 25-fold stimulation of transcription from the adenovirus major late promoter (35). Stimulation of transcription by USF occurs via two transcriptional activation regions near the amino terminal portion of the protein (36) and has been shown to be mediated through an interaction with the initiator protein TFII-I (37). In addition to USF having the same DNA-binding specificity as c-Myc, the cloning and sequencing of the cDNA for USF revealed a conserved structural similarity with c-Myc as well as other c-Myc related transcription factors (38). These factors all contain bHLH and leucine zipper domains that have been shown to be required for protein dimerization and DNA binding (27).

The identity between the USF binding site and the conserved promoter element in the murine p53 gene raised the possibility that USF may bind to the p53 promoter thereby influence expression of the p53 gene. In principle, during oncogenic transformation, either deregulated expression of members of this family of transcription factors or mutations in the p53 promoter resulting in enhanced protein binding, could lead to altered expression of p53. In co-transfection studies, USF was found to trans-activate the p53 promoter controlling expression of a reporter gene. Since this element was shown to be required for activity of the p53 promoter, these findings indicate that USF may play a central role in expression of p53 gene.

**MATERIALS AND METHODS**

**Cell culture and preparation of nuclear extracts**

All cell lines, SVT2, NIH-3T3, MethA, and Cos, were maintained in Dulbecco’s modified Eagle’s minimal medium supplemented with 10% fetal bovine serum. For preparation of nuclear extracts, cells from two sub confluent 100mm culture dishes were washed twice with cold phosphate-buffered saline (PBS), collected and resuspended in 0.8ml lysis buffer (10mM Tris pH 7.9, 0.1mM EDTA, 10mM KCl, 1mM DTT and 1mM PMSF). The cell suspension was incubated on ice for 15 min, brought to 0.5% NP-40 and mixed briefly. The nuclei were pelleted (30 sec in microfuge) and resuspended in 200µl extraction buffer (20mM Tris pH 7.9, 0.4M NaCl, 1mM EDTA, 1mM DTT, 1mM PMSF). The suspension was mixed at 4°C for 15 min and centrifuged for 5 min in the cold. The nuclear extract was stored at -70°C.

**Electrophoretic mobility shift assay**

Binding reactions contained 10 µg (protein) of nuclear extract, 20 fmole end-labeled double-strand oligonucleotide (5'-TTT-CCCTCCCAGTGCTCACCCTGGC-3'), 1 µg poly (dI-dC), 25mM Tris-HCl (pH 7.9), 50 mM KCl, 6.25 mM MgCl₂, 0.5mM EDTA, 1mM DTT and 10% glycerol in a volume of 30 µl. The mixture was incubated on ice for 15 min followed by 20°C for 15' and electrophoresed through 4% polyacrylamide gels (29, acrylamide:bisacrylamide) in the presence of 0.045 M Tris-borate, 1 mM EDTA (0.5XTBE). Gels were dried and subjected to autoradiography for 24 to 72 hrs. For competition experiments, oligonucleotides were added at a 50- or 100-fold molar excess to the binding reactions prior to addition of nuclear extract. For incubation of the binding reactions with antibody, 0.5µl of rabbit serum containing anti-USF antibody (provided by M. Sawadogo) or the control anti-p53 antibody was added after DNA-binding reactions. Antibodies were incubated with nuclear extracts at 20°C for 10 min followed by 4°C for 10 min prior to electrophoresis.

**In vitro transcription and translation**

The pBluescribe (Stratagene) derived vector carrying the USF cDNA (31) was linearized and transcribed using T7 RNA polymerase. The synthesis of RNA was monitored by agarose gel electrophoresis in the presence of EtBr. The RNA was purified by phenol/CHCl₃ extraction and ethanol precipitation. One-tenth of the transcription reaction was translated in vitro using rabbit reticulocyte lysates (Stratagene) with non-radioactive gel electrophoresis in the presence of EtBr. The RNA was purified by phenol/CHCl₃ extraction and ethanol precipitation. One-tenth of the transcription reaction was translated in vitro using rabbit reticulocyte lysates (Stratagene) with non-radioactive methionine at 30°C for 1hr in a 50µl reaction. DNA-binding reactions contained 5µl rabbit reticulocyte lysate, 20 fmole end-labeled double-strand oligonucleotide and were carried out as described above.

**Transfections and CAT Assays**

DNA transfections were carried out by calcium phosphate coprecipitation. SVT2, NIH-3T3 and Cos cells at approximately 30% confluence in 100mm dishes were transfected with 10µg of reporter plasmid expressing the CAT gene from the intact murine p53 promoter (p0.7CAT; ref. 25) and with vectors expressing either the USF cDNA (38) or the MyoD cDNA (40) from the Mo-MuLV LTR. A derivative of the p53 promoter,
pΔHLH-CAT lacks 46bp of the minimal promoter containing the CAGCAG motif (25). After 8 hrs in the presence of the precipitate, the cells were treated with 20% glycerol in complete medium for 1 min. The cells were washed twice and maintained in complete medium. After 48 hr, extracts were prepared and assayed for CAT activity (39) using equivalent amounts of protein in a one hour reaction.

RESULTS

In vitro synthesized USF binds to the p53 downstream promoter element

The murine p53 promoter has been shown to contain a number of potential regulatory motifs (Fig. 1). These include a site with homology to a NF-1 recognition recognition sequence, near the transcription start site (+5 to +17). Binding of a cellular factor to this site overlaps the transcription start site. Sequences upstream of the transcription start site contain a GC-rich region with a potential Sp1 binding site (−44 to −50), an AP-1 like binding site, designated PF-1 (−57 to −64). In previous studies of the murine p53 promoter, we identified a downstream regulatory element that was required for maximal activity of the promoter (26). This element maps between +70 and +75 relative to the transcription initiation site and contains the sequence CAGCAG.

Since the transcription factor USF has been shown to bind site-specifically to sequences containing CAGCAG, we wanted to determine whether USF could bind to this element and thus account for the nuclear DNA-binding activity. In order to determine whether USF can in principle bind to the p53 downstream element, we translated USF in rabbit reticulocyte lysates and assayed its ability to bind to this element by electrophoretic mobility shift analysis. Incubation of control unprogrammed lysates with the oligonucleotide that extends over the p53 CAGCAG motif (+60 to +86) resulted in the formation of little or no detectable specific DNA-binding activity (Fig. 2). Incubation with lysates programmed with in vitro transcribed USF mRNA resulted in the generation of an abundant DNA-protein complex. This binding was specific and required the USF binding site, CAGCAG, since it was completely competed by a 50-fold molar excess of an unlabeled oligonucleotide containing this sequence but not by a 100-fold molar excess of the mutant oligonucleotide (GGTGTG). Furthermore, this complex contained USF since incubation of the DNA-binding reaction with affinity purified anti-USF antibodies resulted in the formation of a super-shifted complex that migrated close to the origin. The control antibody, anti-p53, had no effect on the DNA-binding activity or upon migration of the complex. We conclude from these results that USF can specifically bind to the p53 downstream CAGCAG containing element. Furthermore, the ability of the anti-USF antibodies to alter the mobility of the USF-DNA complex provided us with an assay for the activity of USF in nuclear extracts.

Nuclear USF binds to the p53 downstream promoter element

Knowing that in vitro synthesized USF can bind to the p53 CAGCAG motif and that we could assay for this binding by the use of anti-USF antibodies, we next asked whether the DNA-protein complex that we have previously identified (26) contains USF. In order to test whether binding to the p53 downstream element involves USF, we asked whether the addition of the anti-USF antibodies to nuclear extracts would disturb the DNA-binding activity. If USF were to be present in the DNA-protein complex, treatment with antibodies that recognize it might be expected to either alter the mobility of the complex as was seen with the in vitro synthesized protein or to disrupt its formation. For these experiments we prepared extracts from the transformed murine cell lines SVT2 and MethA. Consistent with our previous results, we found that incubation of nuclear extracts from both SVT2 and MethA (Fig 3) cells with the oligonucleotide containing the p53 CAGCAG motif (+60 to +86), resulted in the generation of an abundant DNA-protein complex observed in electrophoretic mobility-shift assays. This binding was specific and required the CAGCAG motif since binding was competed by a 50-fold molar excess of an unlabeled oligonucleotide containing this sequence, but not by the mutant oligonucleotide. When these nuclear extracts were incubated with the antibody that recognizes USF we observed a 'supershifted' DNA-protein complex having a decreased electrophoretic mobility. This effect was specific in that only the CAGCAG dependent binding activity was supershifted. Additional complexes, seen most prominently with the MethA extracts (Fig. 3), were unaltered in their mobility. As additional controls for the specificity of the reaction, we tested the effect of antibodies that recognize p53. No effect on DNA binding was observed when the extracts were treated with these control antibodies. Furthermore, non-immune rabbit antiserum or 10% fetal bovine serum also had no effect on DNA-binding or mobility (data not shown). These results indicate that either USF or a protein antigenically similar to USF is bound to the p53 downstream element.

Figure 2. USF binds site-specifically to the p53 CAGCAG motif. Rabbit reticulocyte lysates either unprogrammed or programmed with USF mRNA were incubated with a 3P-labeled double-strand oligonucleotide that contains sequences from the murine p53 gene between nucleotides +60 to +86. Complex formation was assayed by electrophoretic mobility shift. The specificity of binding and the requirement of the CAGCAG motif were assayed by competition experiments using a 50-fold molar excess of the homologous wild-type oligonucleotide (wt) or a 100-fold molar excess of a mutant (mut) containing the sequence GGTGTG. The arrow indicates the position of the USF generated DNA-protein complex. Anti-USF or anti-p53 antibody was added to the binding reaction after formation of the protein-DNA complex.
USF transactivates the p53 Promoter

The results that we have presented indicate that USF, either translated in vitro or present in nuclear extracts derived from SVT2 and MethA cells, can bind to the downstream CACGTG motif within the p53 promoter. We have shown previously that the sequences containing this motif are required for maximal activity of the p53 promoter. Deletion of these sequences resulted in a 5-fold decrease in promoter activity (26). These results indicated that whatever factor or factors bind to this element are likely to play a role in regulating expression from the promoter. In order to test whether USF could alter expression from the p53 promoter, we expressed USF in SVT2 cells and measured the response of the intact p53 promoter expressing the *E. coli* chloramphenicol acetyltransferase (CAT) gene (p0.7CAT). p0.7CAT was transfected onto SVT2 cells either alone or in the presence of increasing amounts of pLTR-USF. Forty-eight hrs after transfection, cell extracts were prepared and equal amounts of extracts were tested for CAT activity. As shown in Figure 4, co-transfection of the vector expressing USF with the intact murine p53 promoter driving expression of CAT resulted in an increase in the expression of the CAT gene that was dependent on the amount of pLTR-USF. At the highest level of pLTR-USF tested we observed an approximately 13-fold increase in the level of expression from the p53 promoter. These results indicate that binding by USF to the p53 promoter has functional consequences leading to elevated promoter activity. Deletion of the element containing the USF binding site (pΔHLH-CAT) lead to a lower basal level of activity of the p53 promoter as well as to a significant reduction in its ability to be transactivated by USF (Figure 4). At the higher concentrations of pLTR-USF (15μg), the activity of a p53 promoter lacking the downstream CACGTG motif was induced approximately 2- to 3-fold (data not shown). This residual effect could be due to at least two non-mutually exclusive mechanisms: either an indirect mechanism whereby USF may induce the level of other transcription factors or a direct mechanism involving the binding by USF to additional sites on the p53 promoter. Further experiments will determine the basis for this residual transcriptional activity.

We also tested additional cell types for their ability to support USF mediated transactivation of the p53 promoter. Results shown in Figure 5 and Table 1 demonstrate that both NIH-3T3 and Cos cells, cotransfection of the plasmid expressing USF lead to
enhanced activity of the p53 promoter. The highest relative enhancement was seen with NIH-3T3 cells where the basal level activity of the p53 promoter is quite low. This transactivation was not observed when transfections were carried out in either SVT2 or Cos cells with a vector that expresses another bHLH-containing transcription factor, MyoD, indicating that transactivation by USF is specific.

DISCUSSION

We have presented results of experiments that indicate that the helix-loop-helix containing transcription factor USF binds in a site-specific manner to an essential CACGTG motif within the murine p53 promoter. We conclude that USF, either when synthesized in vitro or when present in nuclear extracts of both SVT2 and MethA cells, binds to this element since the mobility of the major DNA-binding complex is significantly reduced by anti-USF antibodies to yield a super-shifted complex. This binding site maps 70-base pair downstream from the p53 transcription start site and is required for maximal expression of the promoter in transformed murine fibroblast cell lines such as SVT2 and MethA (26). Consistent with USF having a role in regulating p53 expression, elevated levels of USF, generated upon transfection, leads to enhanced expression of the p53 promoter. Since additional factors such as TFE3 (34) may bind to the CACGTG motif, their potential for playing a role in p53 expression cannot be excluded. However, since USF represents the majority if not all of the detectable DNA binding activity in the cells that we assayed, we have focussed our attention on the activity of this factor with respect to the p53 promoter.

USF is a cellular encoded transcription factor, first identified on the basis of its being required for maximal activity of the adenovirus major late promoter (32,33). It has since been found to interact with and influence the expression of several cellular genes including the murine metallothionein I gene (41), the rat gamma-fibrinogen gene (42) and the human growth hormone gene (43). A Xenopus homolog of USF, designated B1, has been implicated in the developmentally regulated expression of the TFIIB gene (44). The binding of USF to the p53 promoter coupled with its ability to transactivate the promoter indicates that the p53 gene may also be regulated, in part, by USF. We are now pursuing these findings in order to determine whether binding by USF plays a role in regulating expression of p53 during the cell cycle or in elevating the level of expression of p53 in transformed cells.

USF is a member of the c-Myc family of DNA-binding transcription factors by virtue of its containing a bHLH domain as well as a leucine zipper domain (38). Both of these domains have been demonstrated for many other well characterized DNA-binding transcription factors to be important for protein-protein interactions and site-specific DNA binding. The c-Myc/bHLH family of DNA-binding transcription factors recognize and bind specifically to DNA containing the consensus sequence CANNTG (27). In addition to sharing structural features with this family, USF specifically binds a subset of the consensus which contains the sequence CACGTG, the same as that shown to be required for DNA-binding by c-Myc/Max heterodimers (29,30). In separate studies, we have found that c-Myc/Max heterodimers, synthesized in vitro, can also bind to the p53 downstream CACGTG motif, although with a lower affinity than shown here for USF (31). That two transcription factors can recognize the same target points to the possibility that USF and c-Myc may under certain conditions compete for binding and thus influence expression of p53. Interestingly, USF has been implicated in possibly regulating expression of the adenovirus major late promoter through competitive interactions with an initiator-binding transcription factor (37). Competition between various transcription factors may be particularly relevant in transformed cells where the expression of c-Myc is elevated and where it plays a causal role in transformation. Under these conditions, c-Myc may be capable of competing for binding with USF. This may be the case for example in Burkitt's lymphomas which express activated c-Myc as a result of chromosomal translocations as well as high levels of the mutant p53 gene (45,46). Experiments to test this hypothesis are now in progress.

In addition to being elevated in transformed cells, the level of activity of the p53 promoter as well the level of p53 mRNA have been shown to be induced in response to mitogens (14,47). Previous studies looking at additional transcription factors that interact with the p53 promoter have revealed a number of candidates that may be involved in regulating these responses. Unlike many promoters transcribed by RNA polymerase II, the p53 promoter appears to lack an upstream TFIID binding site or TATA homology (48). However, the murine p53 promoter contains structures analogous to those found in many other non-TATA box promoters (49) such as the presence of a short GC-rich region between positions -41 and -51 containing a consensus Sp1 binding site and a NF-1 like recognition sequence over the transcription initiation site which may play a role in regulating transcription (47). Ginsberg et al. also identified an AP-1 like recognition site that binds a factor, distinct from AP-1, which appears to confer serum-responsiveness to the promoter (47). The roles of these various elements in modulating p53 expression remains to be determined.

It appears that wild-type p53 must be mutated in order to acquire oncogenic activity, either as a result of the inactivation of a growth suppressing activity of the wild-type protein or as a result of the acquisition of transforming functions (50,51). Mutant forms of the protein are generally overexpressed and this overexpression may be required to facilitate either a transforming activity of the protein or to activate any remaining endogenous wild-type p53 or a de novo dominant transforming activity (3). Our findings that both USF and c-Myc can bind to the p53 promoter indicate that they may play a role in regulating p3 expression. This raises the possibility that, during oncogenic transformation, altered levels of USF and/or deregulated expression of c-Myc may lead to elevated expression of mutant p53.
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