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Analysis of Promoter Elements for Transcriptional Expression of Rat p53 Gene in Regenerating Liver

Minhyung Lee, Haisun Song, Sunhee Park, Jinhee Choi, Sunhee Yu and Jong-sang Park*
Department of Chemistry, Seoul National University, Seoul, Korea

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We previously found three transcription factor-binding motifs in the rat p53 promoter. They are two recognition motifs of NF1-like protein (NF1-like element 1: −296 ~ −312, NF1-like element 2: −195 ~ −219) and a bHLH protein binding element (−142 ~ −146). In this study, we investigated the DNA-protein complex formation of the three elements with nuclear extracts from both normal and regenerating liver to find the element involved in the induced transcription of p53. The level of each DNA-protein complex on NF1-like and bHLH motifs was not changed. Instead, a new element located at −264 ~ −284 was detected in the DNase I footprinting assay with regenerating nuclear extract. This element has partial homology to the AP1 consensus motif. However, the competition studies with diverse oligonucleotides suggest that the binding protein is not AP1. An *in vitro* transcription assay shows that this element is important for the transcriptional activation of the rat p53 promoter. Therefore, for the induced transcription of the rat p53 promoter, the −264 ~ −284 region is required in addition to two NF1-like and one bHLH motif.

Keywords: AP1, bHLH, Liver regeneration, p53 gene, Transcription regulation.

Introduction

The p53 tumor suppressor protein plays a major role in maintaining the integrity of the genome (Hartwell, 1992). One of its roles is as a checkpoint regulator at the G1-to-S phase transition. In response to various DNA damaging reagents, p53 induces growth arrest at the G1/S checkpoint and attempts to repair the damaged DNA before it is replicated (Kastan *et al.*, 1991; Kuerbitz *et al.*, 1992; Lane, 1992; Nelson *et al.*, 1994). It also functions as a transcription factor (Ko *et al.*, 1996). It can induce the expression of the genes involved in cell cycle regulation (el-Deiry *et al.*, 1993), DNA repair, and apoptosis (Kastan *et al.*, 1992; Wu and Lozano, 1994; Haupt *et al.*, 1995).

The p53 promoter has been reported to have several transcription factor-binding motifs in humans, rats, and mice. In the mice p53 promoter, USF (Upstream Simulating Factor; Hale and Braithwaite, 1995), NF1 (Nuclear Factor 1; Ginsberg *et al.*, 1990), and PF2 (p53 factor 2; Hale and Braithwaite, 1995) binding motifs were identified. NF-κB (Wu and Lozano, 1994), Myc/Max (Roy *et al.*, 1994), NF1, and YY1 (Furlong *et al.*, 1996) play important roles in the human p53 expression. Most of these motifs are highly conserved across the species including humans, rats, and mice. In fact, the NF1 binding site, located at −195 ~ −219 (NF1-like element 2), and bHLH motif were previously found in the rat p53 promoter (Lee *et al.*, 1997; 1998). Moreover, the rat p53 promoter has one more NF1 recognition site at −296 ~ −312 (NF1-like element 1). This NF1 recognition motif is not conserved among the species and may be a rat-specific element (Lee *et al.*, 1998). bHLH motif is also conserved in the rat p53 promoter. This bHLH motif binds a protein, which is not USF or Myc/Max, based on the difference of their molecular weights (Lee *et al.*, 1997). Therefore, it is possible that the expression of the rat p53 gene is regulated by different mechanisms to that of the human and mouse genes.

In the process of liver regeneration after partial hepatectomy, several cell cycle related genes are transiently expressed in a sequential manner (Thompson *et al.*, 1986). These genes include proto-oncogenes such as Ras, Myc, and p53. The level of the p53 mRNA increases between 5 h and 12 h after partial hepatectomy and has its peak at around 9 h. In this study, we tested the DNA-protein complex formation of the known *cis*-regulatory elements and tried to find a transcription factor binding site involved

* To whom correspondence should be addressed.
Tel: 82-2-880-6660; Fax: 82-2-889-1568
E-mail: pfjspark@plaza.snu.ac.kr

in the induced transcription of the rat p53 gene. Electrophoretic mobility shift assays (EMSA) and DNase I footprinting assays were carried out with nuclear extracts from normal and regenerating liver. As a result, a new *cis*-regulatory element located at −264 ~ −284 was identified.

Materials and Methods

Partial hepatectomy and preparation of nuclear extracts Female Wistar rats weighing 120~140g were used for all experiments. Partial hepatectomy was performed under ether anesthesia with removal of the main lobes (67% of the liver was excised). Nuclear extracts were prepared from normal and regenerating liver by the method described previously (Lee *et al.*, 1997). Briefly, rat liver was homogenized in 4 vol of buffer A [10 mM Tris, pH 7.9, 5 mM $MgCl_2$, 1 mM EDTA, 1 mM spermidine, 1 mM DTT (1,4-Dithiothreitol), and 0.1 mM PMSF (Phenylmethanesulphonyl Fluoride)] containing 0.32 M sucrose. Homogenates were layered over buffer A containing 2 M sucrose and centrifuged for 50 min at 40,000 × *g*. The nuclei were suspended in lysis buffer [15 mM Hepes (N-2-Hydroxyethylpiperazine-N′-ethanesulphonic acid), pH 7.9, 100 mM KCl, 3 mM $MgCl_2$, 1 mM DTT, 0.1 mM EDTA, and 0.1 mM PMSF], to which 0.1 vol of 4 M $(NH_4)_2SO_4$ was added and stirred at 4°C for 30 min. After being centrifuged for 1 h at 130,000 × *g*, 0.3 g of $(NH_4)_2SO_4$ per ml of supernatant was added and stirred for 30 min. Nuclear extract was centrifuged for 15 min at 16,000 × *g*, dissolved in dialysis buffer (20 mM Hepes, pH 7.9, 100 mM KCl, 20% glycerol, 2 mM $MgCl_2$, 1 mM DTT, 0.2 mM EDTA, and 0.5 mM PMSF), and dialyzed overnight at 4°C against the dialysis buffer.

Electrophoretic Mobility Shift Assay (EMSA) The sequences of the synthesized oligonucleotides are as follows. Oligo 1 (NF1-like element 1: 5′-TTCGTGCTGGCGGCAACATCTCAAG-3′, 5′-CTTGAGATGTTGCCGCCAGCACGAAA-3′), oligo 2 (NF1-like element 2: 5′-TTGTTATGGCGACTATCCAGCTTTG-3′, 5′-CAAAGCTGGATAGTCGCCATAACAA-3′), oligo 3 (bHLH motif: 5′-TTCCCCTCCCACGTGCTCACACTGG-3′, 5′-CCA GTGTGAGCACGTGGGAGGGGAA-3′), oligo 4 (5′-GAACC CTGACTCTGCAAGTCCCCCG-3′, 5′-CGGGGGACTTGCA GAGTCAGGGTTC-3′), oligo 5 (5′-CTCAAGCAGAACCCT GACTCT-3′, 5′-AGAGTCAGGGTTCTGCTTGAG-3′).

Each oligonucleotide was end-labeled by T4 polynucleotide kinase (New England Biolabs, Inc., Beverly, USA) and [γ-^{32}P] ATP (Amersham Life Science, Buckinghamshire, UK). The binding reactions were carried out with normal and regenerating nuclear extracts as described previously (Lee *et al.*, 1997). For the com-petition study, oligo 4, oligo 5, CRE (5′-TGCTGACG TCAAAC-3′, 5′-GTTTGACGTCAGCA-3′), and AP1 consensus oligonucleo-tides (5′-CGCTTGATGAGTCAGCCGGAA-3′, 5′-GCGAACTACTCAGTCGGCCTT-3′) were added to the binding reaction mixtures. The reaction mixtures were electrophoresed through a 6% polyacrylamide gel in the presence of 0.25× TBE (22.25 mM Tris, 22.25 mM Boric Acid, 0.5 mM EDTA).

DNase I footprinting analysis The 455 bp fragment of the 5′ end of the rat p53 gene was amplified by PCR (polymerase chain reaction). Rat liver chromosomal DNA was used as a template for PCR. The sequences of the primers are 5′-CGGAATTCCGCCTTGGCTACAAAGACT-3′ and 5′-CG GGATCCAGCAAGGAAAGTCCCAATGA-3′. The primers have *Eco*RI and *Bam*HI sites, respectively. The enzyme recognition sites are underlined. The amplified 455 bp fragment was cloned into the *Eco*RI-*Bam*HI site of pUC19 vector. The fragment contains functional promoter (−544 ~ −115) and non-coding first exon (−114 ~ −90). The 455 bp fragment was digested with *Hin*dIII. The resulting *Hin*dIII-*Bam*HI fragment was isolated by 8% polyacrylamide gel electrophoresis. After electroelution, it was treated with CIP (Calf intestinal phosphatase, Promega, Madison, USA) and end-labeled using [γ-^{32}P]ATP (Amersham Life Science) and T4 polynucleotide kinase (New England Biolabs, Inc.). This ^{32}P-labeled DNA was digested with *Bst*NI and isolated from 8% polyacrylamide gel. The binding reaction mixture contained 40 μg of nuclear extract, 40 ng of poly(dI-dC) and ^{32}P-labeled DNA fragments. The reaction was allowed to proceed for 20 min at room temperature after mixing. Then, the mixtures were treated with 0.2 ~ 1 unit DNase I (Promega) for 30 sec, extracted with phenol and chloroform, and separated in 8% acrylamide/7 M urea gel by electrophoresis.

***In vitro* transcription assay** The transcription reaction mixture (40 μl) contained transcription buffer (10 mM Hepes, pH 7.6, 3.5% glycerol, 25 mM KCl, and 6 mM $MgCl_2$), 1 mM each of ATP, CTP, GTP, and UTP, 2 μl of RNasin (80 units; Promega), 1 μg of circular DNA template, and 100 μg of nuclear extract. pUC19 vector containing the p53 promoter at the *Eco*RI-*Bam*HI sites was used as a DNA template. For blocking of protein binding to the −288 ~ −295 region, 10 pmole or 100 pmole of double-stranded oligonucleotide (oligo 4) was added to the transcription reaction. After 45 min incubation at 30°C, the reaction was terminated by the addition of 560 μl of transcription stop mixture (0.25 M NaCl, 1% SDS, 20 mM Tris·HCl, pH 7.5, and 5 mM EDTA) containing 80 μg of proteinase K (Boehringer Mannheim GmbH, Mannheim, Germany) and 40 μg of yeast tRNA (Boehringer Mannheim GmbH). The mixtures were incubated for 30 min at 37°C and extracted with phenol and chloroform. The RNAs were precipitated by the addition of 750 μl ethanol and resuspended in 10 μl water. Ninety μl DNase I solution (20 mM Hepes, pH 7.6, 50 mM NaCl, 10 mM DTT, 5 mM $CaCl_2$, and 5 mM $MgCl_2$) containing 5 units of DNase I and 40 units of RNasin was added to each sample. After 15 min of incubation at 37°C, DNase I reaction was stopped by the addition of 2× DNase I stop solution (20 mM Tris·HCl, pH 8.0, 50 mM EDTA, 1% SDS, and 0.3 M NaCl) and heated at 65°C for 5 min. Each sample was extracted with phenol and chloroform and precipitated by the addition of 500 μl ethanol. The precipitated RNA was resuspended in 5 μl water and analyzed by primer extension analysis.

Primer extensions were carried out as described in the user manual from Promega. A 25-base oligonucleotide was used as a primer, which was complementary to the coding strand of the pUC19 vector from nucleotides 26 to 50 downstream of the *Bam*HI site. The primer extension products were separated in a 10% acrylamide/8 M urea gel and subjected to autoradiography.

Results

The levels of the DNA-protein complexes on NF1-like element 1, NF1-like element 2, and bHLH motif were not changed during the liver regeneration It was previously reported that the two NF1-like and bHLH motifs were involved in the basal level transcription of the rat p53 gene (Lee *et al.*, 1997; 1998). To assay the alteration of the protein-binding affinities to these motifs in the rat p53 promoter, EMSAs with specific oligonucleotides were carried out with normal and regenerating liver nuclear extract. In the EMSA with oligo 1 containing NF1-like element 1, the level of protein-DNA complex was not changed (Fig. 1A, lanes N and R). The level of protein-oligo 2 complex was not changed either (Fig. 1B, lanes N and R). Furthermore, the complex between a factor and oligo 3 containing bHLH motif had almost the same intensity in normal and regenerating liver extract (Fig. 1C, lanes N and R). The unchanged levels of the DNA-protein complexes on the motifs suggest that they may not play a major role in the induced transcription of the p53 gene.

The -264 ~ -284 region is specifically protected in the regenerating liver nuclear extract in DNase I footprinting assay To determine whether a *cis*-element that may be involved in the induced transcription of the p53 gene existed, we carried out a DNase I footprinting assay with normal and regenerating liver nuclear extracts. The −264 ~ −284 (ACCCTGACTCTGCAAGTCCCC) region was protected by the regenerating liver nuclear extract (Fig. 2A). EMSA with oligo 4 containing the protected region showed that a factor binds to the element more strongly in regenerating nuclear extract than in normal extract (Fig. 2B), suggesting that this region may be specifically involved in the induced transcription of the rat p53 gene.

This protected region (ACCCTGACTCTGCAAG TCCCC) has partial homology to the AP1 consensus motif (TGACTCA). To verify whether this region is an AP1 recognition site, a DNA oligonucleotide competition assay was carried out. The sequences of the protected region and the oligonucleotides are presented in Fig. 3A. In Fig. 3B, the radiolabeled oligo 4, containing the whole protection region, was used as a probe. Non-labeled oligo 4 itself and oligo 5 were used as competitors. Oligo 5 contained the 5′-half of the protected region including the AP1-like sequence. Oligo 4-protein complex was abolished by 10 and 100 molar excess of oligo 4 itself, but even a 100 molar excess of oligo 5 did not reduce the intensity of the complex. On the contrary, radiolabeled oligo 5-protein complex was abolished more effectively by oligo 4 than by oligo 5 itself (Fig. 3C). These results revealed that the whole protection region was required for full recognition of the binding protein and the AP1-like sequence in oligo 5 was not enough for the full binding activity. Therefore,

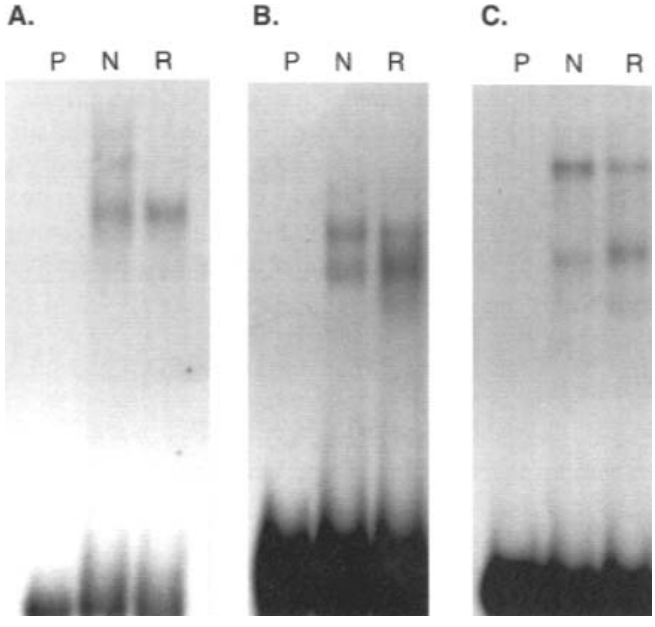

Fig. 1. Electrophoretic mobility shift assay with the radiolabeled oligo 1, oligo 2, and oligo 3. Nuclear extracts prepared from normal and regenerating liver were assayed for protein binding to oligo 1 (A), oligo 2 (B), and oligo 3 (C). N, normal nuclear extract; R, regenerating nuclear extract; P, EMSA without nuclear extract.

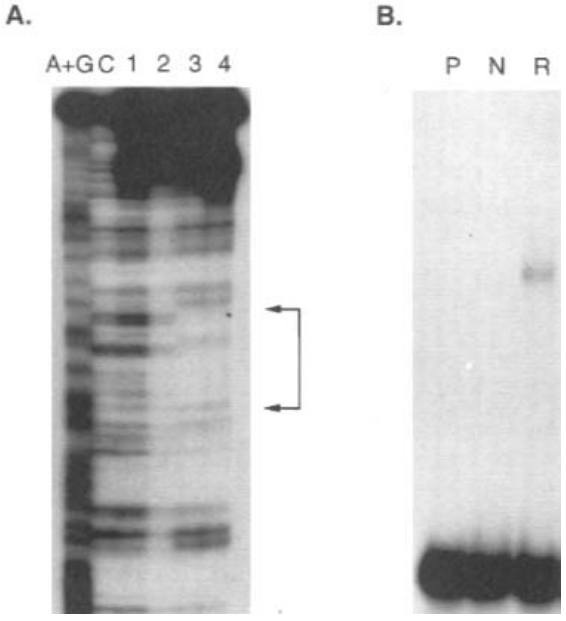

Fig. 2. DNase I footprinting and mobility shift assay. A. DNase I footprinting assay with normal and regenerating extract. The ^{32}P-labeled non-coding strand DNA fragment was assayed for digestion by DNase I in the absence of nuclear extract (lane C), the presence of 40 μg normal nuclear extract (lanes 1 and 2), or the presence of 40 μg regenerating nuclear extract (lanes 3 and 4). A+G indicates the free probe digested at adenine and guanine residues. B. EMSA with radiolabeled oligo 4. Nuclear extracts were assayed for protein binding to the radiolabeled oligo 4. P, free probe without nuclear extract; N, probe with normal nuclear extract; R, probe with regenerating nuclear extract.

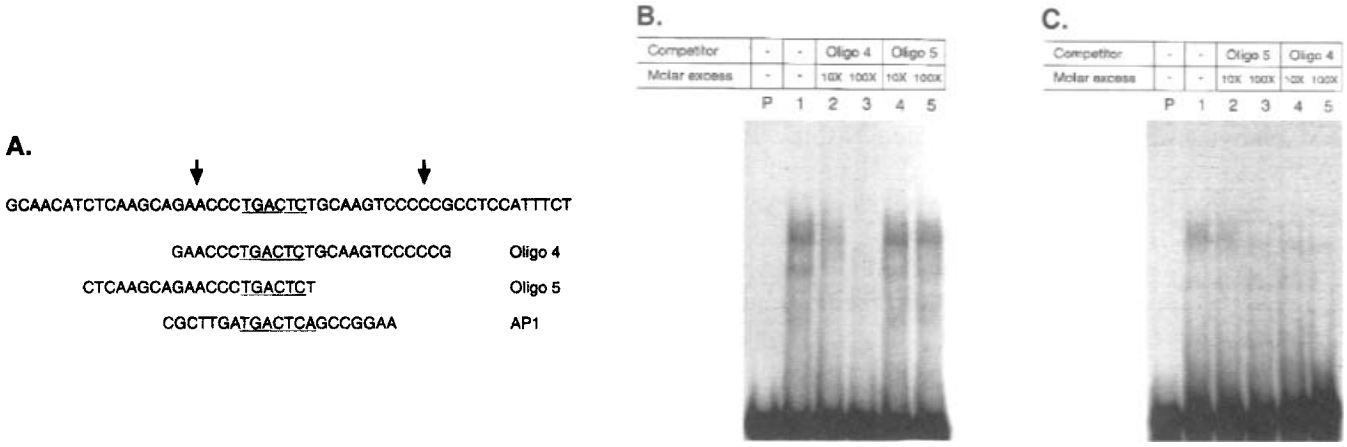

Fig. 3. DNA sequence around the promoter and mobility shift assay. A. The sequences of the promoter around the −264 ~ −284 region. The diagram shows the alignment of the sequence around −264 ~ −284. The boundary of −264 ~ −284 footprint on the rat promoter is indicated by arrows. The core sequence of the AP1 recognition motif is underlined. The sequences of oligo 4 and 5 are also presented. B. Oligonucleotides competition assay in EMSA with radiolabeled oligo 4. Regenerating nuclear extracts were assayed for protein binding to a radiolabeled oligo 4 without competitor (lane 1) or in the presence of the competitor; lanes 2 and 3, oligo 4; lanes 4 and 5, oligo 5. P indicates EMSA without nuclear extract. C. Oligonucleotides competition assay in EMSA with radiolabeled oligo 5. Regenerating nuclear extracts were assayed for protein binding to a radiolabeled oligo 5 without competitor (lane 1) or in the presence of the competitor; lanes 2 and 3, oligo 5; lanes 4 and 5, oligo 4. P indicates EMSA without nuclear extract.

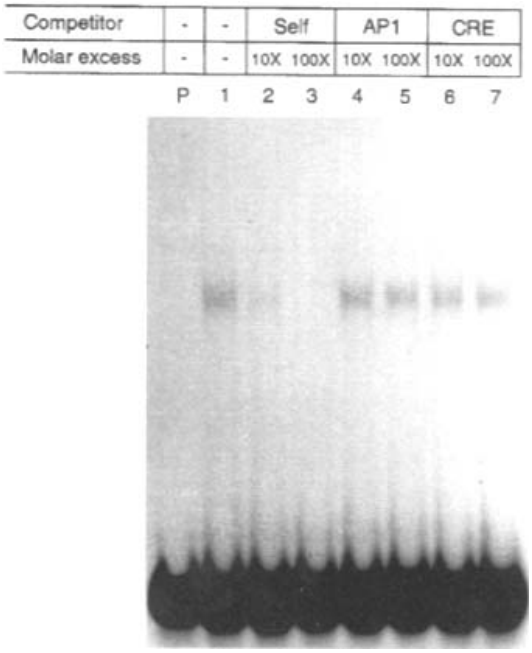

Fig. 4. Oligonucleotides competition assay in EMSA with radiolabeled oligo 4. Regenerating nuclear extracts were assayed for protein binding to radiolabeled oligo 4 without competitor (lane 1) or in the presence of the competitor. Lanes 2 and 3, oligo 4; Lanes 4 and 5, AP1 consensus; Lanes 6 and 7, CRE. P indicates EMSA without nuclear extract.

it is possible that the binding protein is not AP1. To clarify this, we carried out the competition study with AP1 consensus oligonucleotides. In Fig. 4, AP1 and CRE oligonucleotides did not compete with the binding site for the binding protein. These results confirmed again that the protein binding to the −264 ~ −284 region is not AP1.

The protein binding to the −264 ~ −284 region can induce transcription of the rat p53 gene The transactivation effect of the binding protein to the −264 ~ −284 region in the rat p53 promoter was assayed by an *in vitro* transcription reaction. In this assay, we tried to determine the transactivation effect by the *in vitro* transcription reaction in the presence or absence of oligo 4 which contains the full binding region as a competitor. To detect the p53-specific transcript, a primer extension method was used. The oligonucleotide complementary to 50 ~ 75 bases downstream from the *Bam*HI site was used as a primer.

About 10 and 100 molar excess of competitor was added to lanes 3 and 4, respectively. As the quantity of the competitor increased, the quantity of transcription product was reduced (Fig. 5). This revealed that the factor binding to the motif was a transcription activator for the p53 promoter.

Discussion

The transcription level of the p53 gene can be regulated by various mechanisms. The previous report showed that the transcription of the human p53 gene could be increased by NF-κB binding to the promoter in response to genotoxic stress (Wu *et al.*, 1994). In some tumor cell lines, high level expression of c-Myc, which is a member of bHLH family, could induce the expression of the human p53 gene (Roy *et al.*, 1994). The rat p53 promoter has two NF1-like protein binding elements (Lee *et al.*, 1998). One is located at −195 ~ −219 (NF1-like element 2) and is highly conserved among the mouse, rat, and human promoters. The other is located at −296 ~ −312

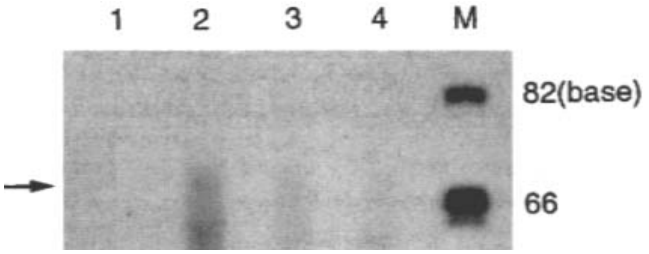

Fig. 5. *In vitro* transcription assay with competitor. Transcription reaction with 100 μg of regenerating nuclear extract was carried out with the template of the p53 promoter inserted at the *Eco*RI-*Bam*HI sites of pUC19 in the absence (lane 2) or the presence of competitor; lane 3, 10 pmol oligo 4; lane 4, 100 pmol oligo 4. Lane 1 is the control lane without the template DNA. Lane M contains DNA size markers.

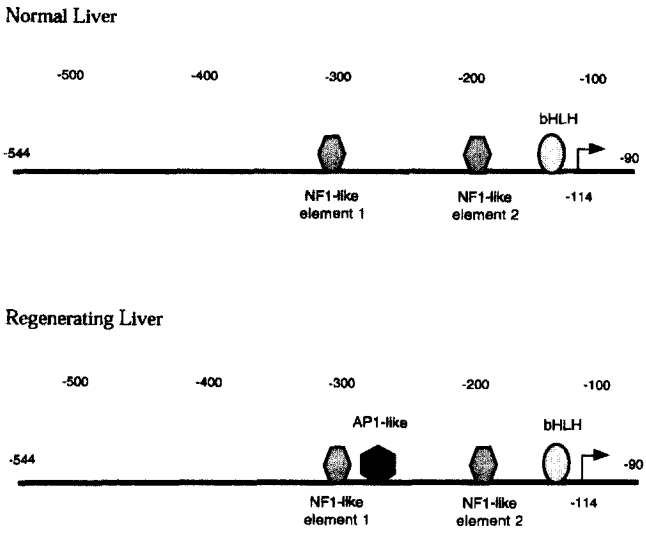

Fig. 6. Regulatory motifs of the rat p53 promoter in normal and regenerating liver. The diagram shows the position of transcription factor-binding motifs to the major start site for transcription in normal or regenerating liver. The rat p53 promoter is numbered by the convention adopted by Tuck and Crawford (Tuck and Crawford, 1989).

(NF1-like element 1), and has not been detected in the human and mouse p53 promoters. It was proven that a 40 kDa protein binds to the two NF1-like motifs. These two NF1-like and bHLH motifs seem to be essential for the basal transcription of the rat p53 gene (Lee *et al.*, 1997; 1998). From our unpublished data, the addition of the purified 40 kDa NF1-like protein into the transcription reaction can activate the p53 gene. Therefore, it is possible that the quantitative increase of the transcription factors may activate the p53 promoter after partial hepatectomy. To clarify this theory, the protein-DNA complex levels of the motifs were tested with normal and regenerating liver nuclear extracts. As NF-κB binding to the rat p53 promoter was not detected in normal and regenerating liver, the NF-κB motif was excluded in this study. In EMSAs with normal and regenerating liver nuclear extracts, the protein binding affinities of the bHLH and NF1-like motifs were not changed during the liver regeneration (Fig. 1). The unchanged levels of DNA-protein complexes suggest that the motifs may not play a critical role in the induced transcription of the p53 gene. However, it is possible that the bHLH and NF1-like motifs and their binding proteins participate in the induced transcription of the p53 gene.

Instead of bHLH and NF1-like motifs, a AP1-like sequence at $-264 \sim -284$ may be involved in the rat p53 transcription during the liver regeneration. This motif has partial homology to the AP1 consensus recognition motif, but this AP1-like sequence was not enough for the full recognition of the binding protein. Moreover, the AP1 oligonucleotide did not compete with the motif for the binding protein. This suggests that the binding protein is not AP1. An *in vitro* transcription assay showed that the reduced binding of a factor to the $-264 \sim -284$ region decreased the promoter activity, suggesting that the factor binding to the motif can increase the transcription activity of the rat p53 promoter.

In summary, the transcription factor binding motifs in the rat p53 promoter are presented in Fig. 6. The NF1-like and bHLH protein recognition motifs may participate in the induced transcription as well as basal transcription of the rat p53 gene. In addition to the motifs, it is postulated that a factor binding to the $-264 \sim -284$ region may be required for the maximal increase of the p53 transcription. Studies for the activation mechanism of the binding protein are in progress.

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