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A 100 kDa Protein Binding to bHLH Family Consensus Recognition Sequence of RAT p53 Promoter

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Abstract : p53 tumor suppressor plays an important role in the regulation of cellular proliferation. To identify proteins regulating the expression of p53 in rat liver, we analyzed p53 promoter by electrophoretic mobility shift assay (EMSA) and DNase I footprinting assay. We found that a protein binds the sequence CACGTG, bHLH consensus sequence in rat p53 promoter. Southwestern blotting analysis with oligonucleotides containing this sequence shows that the molecular weight of the protein is 100 kDa. This size is not compatible with the bHLH family such as USF or c-Myc/Max which is known to regulate the expression of the human and mouse p53 gene. Therefore this 100 kDa protein may be a new protein regulating basal transcription of rat p53. We purified this 100 kDa protein through sequence-specific DNA affinity chromatogaphy.
Key words : bHLH family, p53 gene, promoter analysis, regulation of expression, transcription factor

The expression of p53 tumor suppressor plays an important role in the regulation of cellular proliferation and malignant transformation (Hollstein *et al.*, 1991). Alteration or loss of p53 is associated with a wide variety of tumor cells. Various mutant forms of the protein can exhibit oncogenic activity in a number of *in vitro* and *in vivo* systems (Jenkins *et al.*, 1985, Eliyahu *et al.*, 1988, Jenkins and Sturzbecher, 1988, Lavigueur *et al.*, 1989).

The mechanism of p53 growth suppression is still undefined. It can suppress the promoters containing the TATA box through interaction with TATA binding protein (Ginsberg *et al.*, 1991, Santhanam *et al.*, 1991, Kley *et al.*, 1992, Mack *et al.*, 1993). p53 can also bind DNA in a sequence-specific manner (Kern *et al.*, 1991). It has a very strong transcriptional activation domain near its amino terminus (Fields and Jang, 1990, Raycroft *et al.*, 1990) and can stimulate the expression of genes down-stream from its binding site. The sequence-specific transcriptional activation by p53 has led to the hypothesis that p53-induced genes may mediate its biological role as a tumor suppressor (Vogelstein and Kinzler, 1992). To date, several genes containing p53-binding sites have been identified. These include muscle creatine kinase (Weintraub *et al.*, 1991), MDM2 (Barak *et al.*, 1993, Chen *et al.*, 1994), a GLN retroviral element (Zauberman *et al.*, 1993), WAF1/p21/CIP1 (El-Deiry *et al.*, 1993), and cyclin G (Okamoto and Beach, 1994). Each of these genes can be rationalized to be functional in a DNA damage-initiated pathway.

p53 accumulates in cells treated with DNA-damaging agents such as UV light, UV-mimetic compounds and γ-radiation, through a posttranslational stabilization mechanism (Mailman and Czyzyk, 1984, Kastan *et al.*, 1991). This accumulation of p53 mediates either growth arrest at the G1/S boundary (Kastan *et al.*, 1991, Kuerbitz *et al.*, 1992) or apoptotic cell death (Clacke *et al.*, 1993, Lowe *et al.*, 1993).

Studies on the transcription regulatory region of p53 show that p53 promoter contains no TATA-like sequence. It has been shown that human and mouse p53 promoters contain conserved consensus recognition sequences for the basic helix-loop-helix (bHLH) family that influence the activity of the promoter (Ronen *et al.*, 1991, Reisman and Rotter, 1993, Roy *et al*, 1994).

To gain insight on the role of the transacting factor in the regulation of the rat p53 gene, we have identified an element of rat p53 promoter required for basal transcription by electrophoretic mobility shift assay (EMSA) and DNase I footprinting assay. Protein binding to the p53 promoter was purified through sequence-specific DNA affinity chromatography. Also, the molecular weight of this protein was determined by Southwestern blot

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analysis.

Materials and Methods

Animals, partial hepatectomy (PH) and preparation of nuclear extracts

Female wistar rats weighing 120-140 g were used for all experiments. The animals were kept in temperature-controlled rooms with 12 h alternating light and dark cycles. Partial hepatectomy was performed under ether anesthesia, with removal of the main lobes (67% of the liver was excised) as described previously (Higgins *et al*, 1931). Nuclear extracts were prepared by the methods de- scribed previously (Lim *et al*, 1992).

Electrophoretic Mobility Shift Assays (EMSAs)

The 455bp fragment (Fig. 1) of the 5' end of the rat p53 gene amplified by PCR was cloned into the pTZ 19U plasmid. The primers for PCR are shown.

-535 -516
Left primer : 5'-CGGAATTCCGCCTTGGCTACAAAGACT-3'
EcoRI

-91 -110
Right primer : 5'-CGGGATCCAGCAAGGAAAGTCCCAATGA-3'
BamHI

The 455bp fragment of the p53 promoter was digested with HinfI restriction enzyme and treated with CIP and labeled by using [γ-^{32}P] ATP and T4 polynucleotide Kinase. The resulting fragment (Fig. 1) was isolated on a 15% acrylamide gel, and recovered by electroelution. The binding reaction mixture contained 5 μg of nuclear extract, 2 μg of poly(dI-dC) and binding buffer (5X: 300 mM KCl, 60 mM HEPES, pH 7.9, 25 mM $MgCl_2$, 1 mM EDTA, 1 mM DTT, and 60% glycerol) in a volume of 20 μl. For the competition assay, cold oligonucleotides were added. The sequence of the oligonucleotides are shown.

5'-TTCCCCTCCCACGTGCTCACACTGG-3'

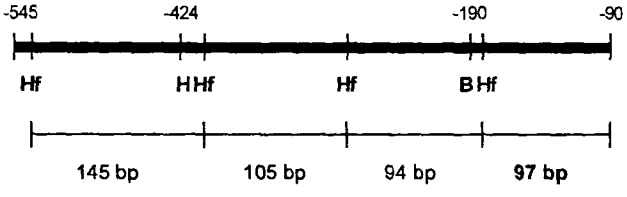

Fig. 1. Diagrammatic representation of rat p53 promoter. The 455 bp fragment of rat p53 promoter amplified by PCR was cloned into the EcoRI-BamHI site of the pTZ 19U vector. As the 455 bp fragment of rat p53 promoter is digested with HinfI restriction enzyme, the resulting fragments are indicated by their size. Hf, HinfI; H, HindIII; B, BstNI.

The mixture was allowed to stand at room temperature for 10 min and radiolabeled 97 bp DNA fragment was added to the binding reaction and incubated at room temperature for a further 20 min. Then, the mixture was electrophoresed through 6% polyacrylamide gel in the presence of 0.25X TBE.

DNase I footprinting

The 455bp fragment of the p53 promoter was digested with HindIII and the resulting fragment was isolated on an 8% polyacrylamide gel. After electroelution, it was treated with CIP and labeled using [γ-^{32}P] ATP and T4 polynucleotide Kinase. To generate a single end-labeled fragment, the ^{32}P-labeled DNA was digested with BstNI and isolated on an 8% polyacrylamide gel. For a DNase I footprinting assay, the binding reaction mixture contained the same materials for EMSA as described above except for a 50-80 μg nuclear extract in a volume of 50 μl. After incubation, samples were treated with DNase I, purified, and separated by electrophoresis through 8% acrylamide/ 8 M Urea gels.

Preparation of sequence-specific DNA affinity resin

The preparation of sequence-specific DNA affinity resin was done following the method of Kadonaga and Tjian (Kadonaga and Tjian, 1986). Chemically synthesized complementary oligonucleotides (5'-gatcTTCCCC TCCACGTGCTCACACTG-3',5'-gatcCAGTGTGAGCACGTGGGAGGGGAA-3') were anealed and ligated for 10~15 repeats of specific binding units. The ligated oligonucleotides were covalently attached to Sepharose CL-2B by a slight modification of the method of Arndt-Jovin *et al.* (Arndt-Jovin *et al*, 1975).

Purification of p53 promoter binding protein

Crude rat liver nuclear extracts were applied to a 3 ml DNA-cellulose column (Phamacia). The column was washed with 15 ml of N buffer (20% v/v glycerol, 5 mM $MgCl_2$, 0.1 mM EDTA, 20 mM HEPES (pH 7.9), 0.05% Brij-35, 1 mM DTT, 5 mM PMSF) containing 100 mM NaCl. The binding proteins were eluted with the 250 mM NaCl in N buffer. Active fractions were pooled and incubated with poly(dI-dC) on ice for 20 min. After centrifugation at 13,000 rpm for 10 min, the supernatant was applied to a 2 ml DNA affinity column. The column was washed with 15 ml of 200 mM NaCl in N buffer and the binding proteins were eluted with 400 mM NaCl in N buffer. The eluted proteins were assayed through EMSA with radiolabeled oligonucleotides. The active fractions were pooled and reapplied to a DNA affinity column. The active fractions from a second passage were assayed by EMSA and Southwestern blot analysis.

Electrophoretic protein blotting and subsequent DNA hybridization [Southwestern(DNA-protein) blot analysis]

The proteins were subjected to electrophoresis on a 10% sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE). For blotting, proteins were transferred from 10% SDS-PAGE to a PVDF membrane at 300 mA for 50 min. The blot was gently washed in TNE-50 (10 mM Tris, pH 7.5, 50 mM NaCl, 1 mM EDTA, 1 mM DTT) and blocked overnight at 4 C in blocking buffer (2.5% (w/v) dried milk powder, 25 mM HEPES, pH 8.0, 1 mM DTT, 10% glycerol, 50 mM NaCl, 0.05% Nonidet P-40, 1 mM EDTA). Hybridization was performed in TNE-50 buffer for 2 h at room temperature. In addition to the specific radiolabeled oligonucleotides (250,000 cpm/ml), poly(dI-dC) (5 μg/ml) was added as a nonspecific competitor. The blot was washed three times with TNE-50 buffer for 20 min and subjected to autoradiography.

Results and Discussion

Potential regulatory motif in rat p53 promoter for basal transcription

The 455 bp fragment of rat p53 promoter cloned into the EcoRI-BamHI site of the pTZ 19U vector (Fig. 1) contains a functional promoter (between position -535 and -214) and a non-coding first exon (between position -213 and -90). The nucleotide position is indicated as described previously (Bienz-Tadmor *et al*, 1985). The 455 bp fragment of the rat p53 promoter shows a remarkably similar sequence to the regulatory region of the human and mouse p53 gene.

It has been shown that CANNTG (consensus bHLH protein-binding motif) plays an important role in the expression of the human and mouse p53 gene (Reisman and Rotter, 1993; Roy *et al*., 1994; Hale and Braithwaite, 1995). In previous studies on the regulation of p53 transcription, others found that the bHLH family proteins were associated with the human or mouse p53 transcription. This bHLH family contains USF(Reisman and Rotter, 1993) and c-Myc/Max (Roy *et al*., 1994). In the human p53 transcription, c-Myc can bind this p53 element in a heterodimeric form with Max. It has been reported that this heterodimeric binding induces human p53 expression (Reisman and Rotter, 1993). Another bHLH family, USF can bind the consensus sequence CANNTG in murine or in the human p53 gene. These two transcription factors can recognize the same target points, and, under certain conditions, compete for binding and thus influence expression of the p53 gene.

Rat p53 promoter also has the CACGTG sequence, which is the consensus bHLH family binding motif. To verify the role of this motif, first we carried out EMSA with a 97bp DNA fragment containing the CACGTG motif (Fig. 1). Lane N in Fig. 2A shows that there are binding proteins to this DNA fragment. In a competition experiment with specific oligonucleotide containing CACGTG motif, the protein binding disappeared even when a small quantity of competitor was added (Fig 2B). To find out the binding sequence, a DNase I footprinting assay was carried out. In Fig. 3, the protected region contains the CACGTG sequence as expected. These results mean that there is a protein binding to the bHLH family binding motif, and that this protein

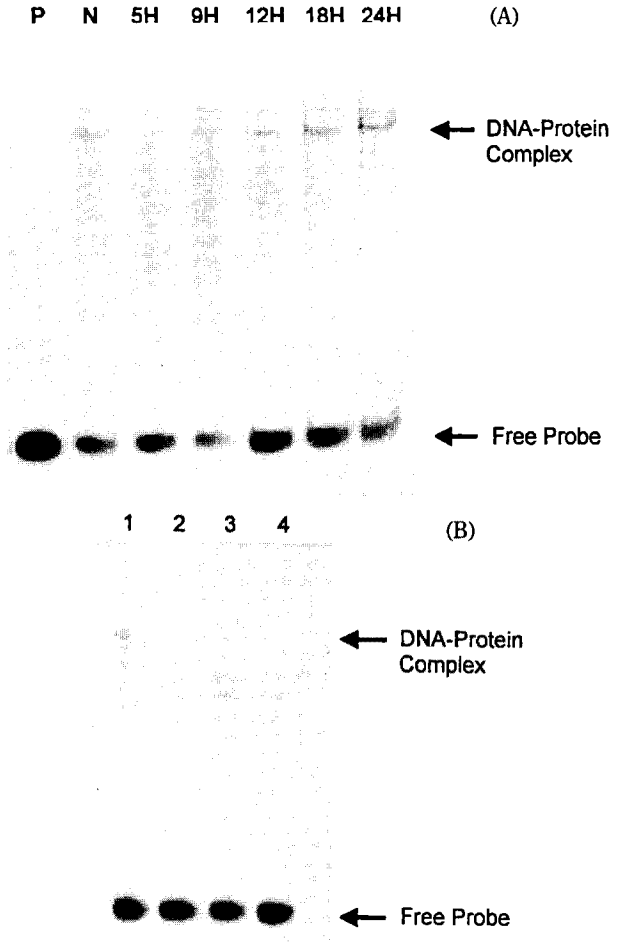

Fig. 2. (A) EMSA with nuclear extract from normal and regenerating rat liver. Nuclear extracts prepared from normal(N) and regenerating (5-24h) liver cells were assayed for protein binding to a radiolabeled 97 bp DNA fragment. 97 bp DNA fragment of rat p53 promoter was described in Fig. 1. After partial hepatectomy, nuclear extracts were prepared at the indicated time (H: hour). P indicates EMSA without nuclear extract. (B) Competition assay with oligonucleotides. Specific oligonucleotides were added as competitor in EMSA. The sequence of the oligonucleotides is shown. 5'-TTCCCCTCCCACGTGCTCACACTGG-3' 1: EMSA without competitor; 2: with 10pM competitor, 3: with 100pM competitor; 4: with 1nM competitor.

may be involved in the basal expression of rat p53.

The binding activity of the CACGTG binding protein in the rat regeneration system

Adult rat liver does not divide under normal physiological conditions. After partial hepatectomy in rats, the remaining intact lobes rapidly grow and the growth ceases in about 7 days when the original organ mass is regained (Grisham, 1962). The study on the alteration of gene expression during liver regeneration shows p53 expression increases in the course of sequential expression of proto-oncogene proteins. It has been reported that the mRNA of the p53 gene reached its peak 8 to 12 h after the partial hepatectomy (Thomson *et al.*, 1986).

To investigate the role of the CACGTG binding protein in liver regeneration EMSA and DNase I footprinting assay were carried out with nuclear extract prepared from regenerating liver. In Fig. 2A, the binding activity of the CACGTG binding protein seems to decrease 5 h after partial hepatectomy. But the activity begins to increase at 9 h and shows almost same binding intensity at 12 h compared to normal nuclear extract. In a DNase I footprinting assay with nuclear extract from rat regenerating liver 9h after partial hepatectomy, the region containing the CACGTG sequence was protected (lane 3 in Fig. 3). In regenerating liver, we found that three other regions of the p53 promoter were bound by some proteins (data not shown). The study for verification of the relation between these proteins and CACGTG binding protein is in progress.

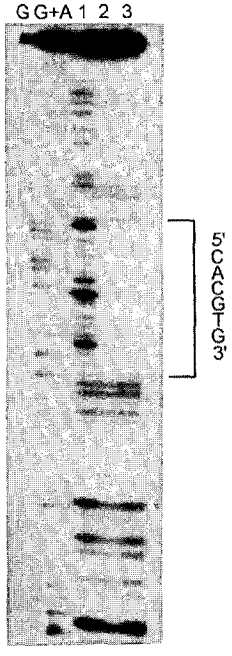

Fig. 3. DNase I footprinting of the protein binding site on DNA fragment of the rat p53 promoter. The ^{32}P-labeled noncoding strand DNA of p53 promoter fragment, containing the consensus recognition site (CACGTG), was assayed for digestion by DNase I in the absence (lane 1) or presence of the nuclear extract prepared from normal (lane 2) and regenerating (lane 3) liver. The lanes indicated A+G or G are the free probe digested at adenine and guanine or guanine residue.

Purification of CACGTG binding protein

We used sequence-specific DNA affinity chromatography to purify 100 kDa protein from the nuclear extract of rat liver. For preliminary purification, DNA-cellulose chromatography was carried out. In the course of

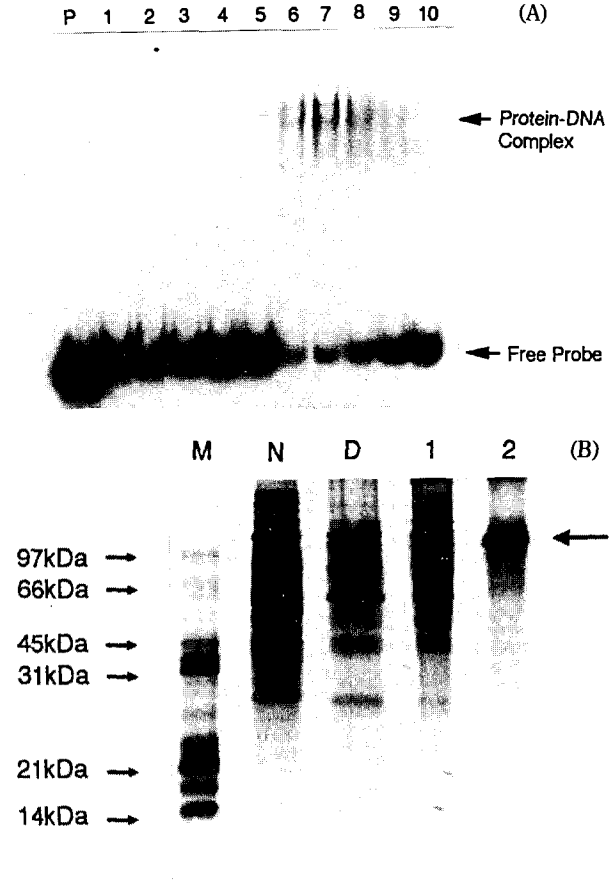

Fig. 4. EMSA and SDS-PAGE with purified 100 kDa protein. (A) During the purification steps, the elution of CACGTG binding protein from affinity chromatography was monitored through EMSA with radiolabeled oligonucleotides. Lane 1 and 2 are 200 mM washing fractions and lane 3-10 are 400 mM fractions. Protein-DNA complex is indicated with an arrow. P indicates EMSA without nuclear extract. (B) Purified p100 was separated in SDS-PAGE and silver stained. N: crude nuclear extract; D: Active fraction from DNA-Cellulose chromatography; 1: active fraction from 1st passage through sequence-specific DNA affinity chromatography; 2: active fraction from 2nd passage through sequence-specific DNA affinity chromatography. Purified p100 is indicated with an arrow. Molecular weights are indicated on the left.

sequence-specific DNA affinity chromatography, EMSA was executed with radiolabeled oligonucleotides to monitor elution of the target protein (Fig. 4A).

After all the purification steps, the eluted proteins were separated in SDS-PAGE and silver stained (Fig. 4B). Fig. 4B shows that 100kDa protein was enriched after a second passage through sequence-specific DNA affinity chromatography.

Molecular weight of the CACGTG binding protein.

To confirm the molecular weight of this bHLH family protein, Southwestern blot analysis was carried out with the active fractions from sequence-specific DNA affinity chromatography. For specific binding we used short synthetic oligonucleotides instead of a 97 bp DNA fragment. According to the results, the molecular weight of the protein is about 100 kDa (Fig. 5). The size of this protein is different from that of known bHLH family proteins such as USF (43 and 44 kDa) or c-Myc (55 kDa)/Max (20 kDa) which are known to regulate the transcription of human and mouse p53 gene (Reisman and Rotter, 1993, Roy *et al.*, 1994).

Rat p53 promoter lacks a TATA box, but it was shown that the p53 promoter contains a number of potential regulating motifs. In our study, EMSA and DNase I footprinting assay indicate that the bHLH protein binding motif plays an important role in rat p53 transcription. Through purification of the protein binding to this motif and Southwestern blot analysis, we confirmed that the molecular weight of the protein is 100 kDa. The size of the protein is not compatible with bHLH proteins which are known to regulate human and mouse p53 expression. Thus 100 kDa protein may be a new protein regulating rat p53 transcription.

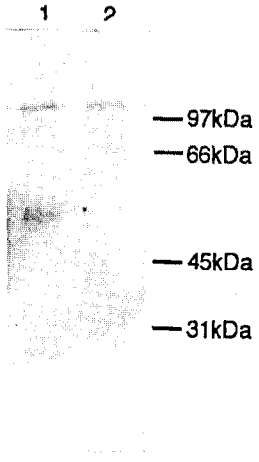

Fig. 5. Southwesten blot analysis. Proteins of each purification step were separated by electrophoresis, transferred to the PVDF membrane and assayed by incubation with the radiolabeled oligonucleotides containing binding sequence. Molecular masses are indicated on the right. 1: active fraction from 1st passage through sequence-specific DNA affinity chromatography; 2: active fraction from 2nd passage through sequence-specific DNA affinity chromatography.

Acknowledgments

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