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Multiple Elements for Negative Regulation of the Rat Catalase Gene Expression in Dedifferentiated Hepatoma Cells[1]

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Like such hepatic genes as those for albumin and aldolase B, the rat catalase gene shows markedly reduced expression in carcinogenesis of hepatocytes. Strong silencer activity has been widely observed in the 5′-flanking region of the gene, downstream from the G-rich sequence identified in a previous study. In this study, we identified and characterized multiple elements involved in negative regulation of catalase gene expression by reporter assay and gel shift assay. One of the silencer elements is located 3 kb upstream of the gene and has GATATCCCGATATC as core sequence. The observation that protein binding to the element is abundantly expressed in dedifferentiated hepatoma cell lines, but scarcely in well-differentiated cell lines suggests that this element is involved in negative regulation of the catalase gene expression in hepatocarcinogenesis. This element was targeted by a novel 20-kDa nuclear protein, which is designated HNRF (hepatocarcinogenesis-related negative regulatory factor).

Key words: catalase gene, core sequence, hepatocarcinogenesis, silencer, transcriptional regulation.

Some hepatic genes, for instance, albumin, ornitin transcarbamylase, transthyretin, and aldolase B genes, are down-regulated in carcinogenesis of hepatocytes (*1*, *2*). This down-regulation is mainly attributed to diminishing of some of liver-enriched transcription factors (LETF), such as hepatocyte nuclear factor–1 (HNF-1) family, HNF-3 isomers, HNF-4, and CCAAT enhancer binding protein (C/EBP) family (*3–5*). An important aspect of the down-regulation, however, is the involvement of a *trans*-repressor bound to a silencer element in the negative regulatory region of these hepatic genes. Recently, hepatocarcinogenesis-related transcription factor (HTF) was isolated, and its expression was found to be enhanced in hepatocellular carcinomas (HCC) (*6*). This factor is structurally similar to human X-box–binding protein–1 (hXBP-1) and is expressed in regenerating liver and HCC (*7*, *8*), but its role remains to be elucidated.

Expression of the rat catalase gene is markedly reduced in carcinogenesis of hepatocytes. Previously, negative regulation of the catalase gene was extensively analyzed in the rat hepatoma cell line, and a G-rich sequence (GGGGGAG) located at –3402 bp from initiation codon ATG of the gene was identified as a silencer element (*9*). Thereafter, we examined in detail the role of this sequence with deletion mutants, and suggested presence of negative regulatory elements distinct from the G-rich sequence. Strong silencer activity was also observed in a wide region (–3269 to –1701 bp) downstream from the G-rich sequence in the previous study (*9*). The aim of this article is to identify and characterize the negative regulatory element in this region of the catalase gene.

MATERIAL AND METHODS

Cell Lines—Reuber cells, a rat minimal deviation hepatoma cell line, were given by Dr. M. Nanba, Okayama University. Fao, C2, and C2rev7 cells, derivations of the H4IIEC3 line adapted to growth *in vitro* from the rat hepatoma cell line Reuber, were obtained from Dr. Mary C. Weice, Institute Pasteur, France (*1*, *10*, *11*). AH66 cells are a subline derived from Yoshida ascites hepatoma (*12*), and 3Y1 cells are an immortalized cell line from Fischer rat embryonic fibroblast. AH66 and 3Y1 cell lines were obtained from the Japanese Cancer Research Resources Bank (JCRB). All cell lines were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 200 units/ml of penicillin, and 100 μg/ml of streptomycin.

Plasmids—Truncated fragments from the 5′-flanking region of the rat catalase gene (–3269 to –1701 bp) were prepared with restriction enzymes and amplified in pUC18 vector. CAT plasmids were constructed by insertion of the truncated segments into the polylinker sites of pCCAT-P11 (*9*), which contained the rat catalase gene promoter (–126 to –26 bp). All the CAT plasmids were purified by two cycles of CsCl equilibrium centrifugation.

CAT (Chloramphenicol Acetyltransferase) Assay—3Y1

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Abbreviations: CAT, chloramphenicol acetyltransferase; EMSA, electrophoretic mobility shift assay.

and AH66 cells were transfected with 10 μg of CAT constructs by calcium phosphate precipitation (*13*) and incubated for 48 h after transfection. Cell extract was prepared by sonication and centrifugation at 12,000 rpm for 5 min at 4°C, then treated at 65°C for 5 min to inactivate chloramphenicol deacetylase. The CAT activities were determined by separation of acetylated chloramphenicol by high-performance liquid chromatography (*14*). Each transfection experiment was carried out in triplicate, and the values of CAT activities that deviated by within 5% were considered valid and are reported here.

Electrophoresis Mobility Shift Assay (EMSA)—Nuclear extracts from rat hepatoma cell lines were prepared as described (*15*) with a slight modification with an isotonic Nonidet P-40 lysis procedure. Probe DNA fragments obtained from plasmids were end-labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase. EMSAs were performed using a modification of a previous method (*16*). Briefly, 2 μg of nuclear extract was preincubated for 30 min on ice with 1 μg of poly d(I-C) in binding buffer containing 25 mM HEPES-KOH pH 7.9, 40 mM KCl, 0.1 mM EDTA, 10 mM $MgCl_2$, 10 μM $ZnSO_4$, 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride (PMSF), 7.5% glycerol, and 10 mg of BSA. The binding mixture with 1 fmol of the labeled probe (1×10^5 cpm) was incubated for 30 min on ice, then subjected to 5% PAGE in TGE buffer (50 mM Tris, 380 mM glycine, and 2 mM EDTA pH 8.2).

Northern Blot Analysis—Total RNA was isolated from hepatoma cell lines and liver as described previously (*17*). Northern blot analysis was carried out as described by Goldberg (*18*). Rat catalase cDNA (1.1-kb *Pst*I fragment) was labeled with [α-^{32}P]dCTP by the random primer labeling method and used as probe.

DNase I Footprinting—DNase I footprint analysis was carried out as described previously (*19*). Briefly, the binding mixtures contained 20 μg of nuclear protein and 1×10^5 cpm of end-labeled probes in 40 μl of binding buffer similar to that for EMSA were incubated for 30 min on ice. After by addition of 1 μl of 15 mM $CaCl_2$–15 mM $MgCl_2$ to the mixture, the DNA-protein complex was digested with freshly diluted DNase I solution (0.005, 0.01, and 0.05 units) at 18°C for 1 min. The reaction was stopped by addition of 0.3 M NaCl, 25 mM EDTA, and 1.5 μg of tRNA, and DNA was extracted with phenol-chloroform. The extracted DNA fragments were precipitated with ethanol, and analyzed by a denaturing 5% PAGE.

Southwestern Blot Analysis—Southwestern blot analysis was performed according to Bowen *et al.* (*20*) with minor modification. Portions of 10 and 30 μg of nuclear extract were separated on 10% SDS-PAGE containing 4 M urea according to Laemmli, U.K. (*21*). The protein in the gel was renatured with 10 mM Tris-HCl pH 7.5, 50 mM NaCl, 2 mM EDTA, 0.1 mM DTT, and 4 M Urea, then electrotransferred to nitrocellulose membrane. The membrane was pretreated with 10 mM Tris-HCl pH 7.5, 50 mM NaCl, 10 mM $MgCl_2$, 0.1 mM EDTA, 5 mM 2-mercaptoethanol, 1 mM PMSF, 1× Denhardt solution, and 150 μg of tRNA as a nonspecific competitor. End-labeled DNA probe (1×10^6 cpm) was applied to the membrane in the same buffer at room temperature for 1 h. The membrane was then washed in excess volume of renaturing buffer and autoradiographed.

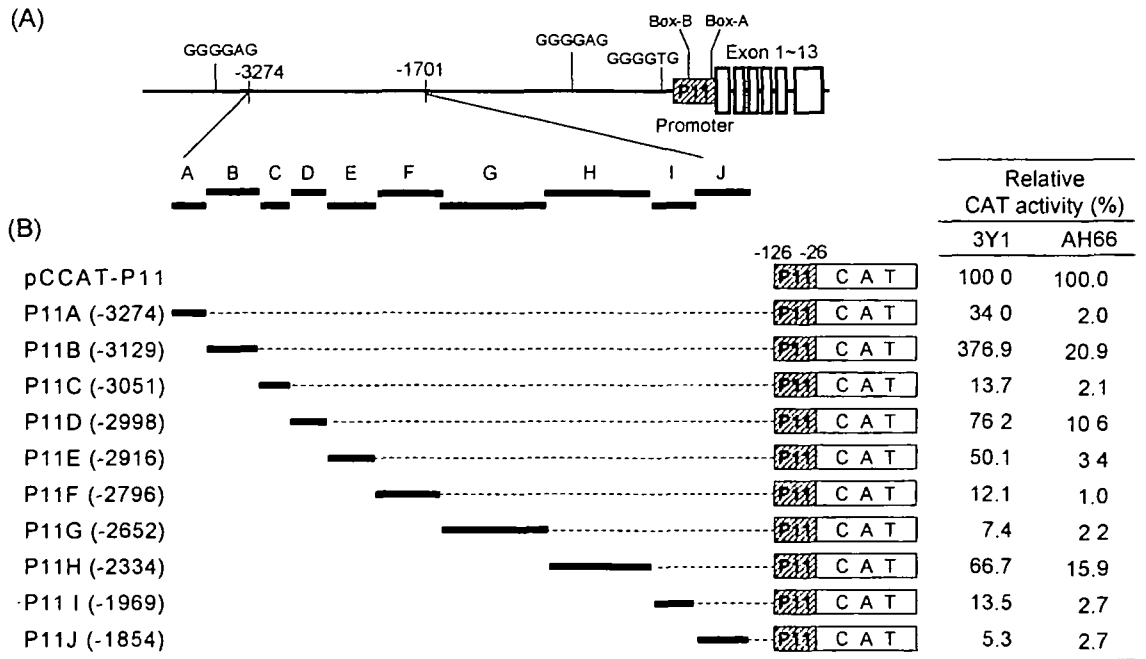

Fig. 1. **Relative CAT activities of plasmids bearing DNA fragments from 5′-flanking region of the rat catalase gene.** (A) The 5′-flanking region of the rat catalase gene is shown by a solid line, and 13 exons are indicated by open boxes. P11 promoter (–126 to –26 bp from ATG) is mapped in front of the first exon, and contains boxes A and B. Sub-fragments (A to J) obtained by restriction digestion of negative regulatory region (–3274 to –1701 bp) are indicated in lower panel. G-rich silencer elements are also mapped in the 5′-region. (B) CAT constructs containing DNA segments (A to J) are shown in the left panel. The designations of CAT plasmids, P11A to P11J, are followed by the position of the 5′ end of the inserted fragments in parentheses. Relative CAT activities derived from these constructs are given in percent based on the activity generated by pCCAT-P11 parental plasmid.

RESULTS

(1) Negative Regulatory Elements of Transcription in the Rat Catalase Gene—We found a negative regulatory region located at –3269 to –1701 bp of the rat catalase gene. The regulatory element is distinct from the G-rich silencer described in our previous study (*9*). To examine transcriptional regulation in the region, small segments generated by use of restriction enzymes were analyzed for transcriptional activities by CAT assay. P11 promoter, which is a TATA-less promoter of the catalase gene located at –126 to –26, contained boxes A and B and retained a basic transcription activity in hepatoma cells (*22*, *23*). The restriction fragments from the negative regulatory region were adjoined to the P11 promoter in pCCAT-P11 reporter plasmid, and their CAT activities were determined in the rat hepatoma AH66 and fibroblast cell line 3Y1, which are deficient in the catalase gene expression. As shown in Fig. 1, CAT plasmids containing fragments C, F, G, I, and J markedly reduced CAT activities relative to pCCAT-P11 parental plasmid, while the A, D, E, and H fragments showed small effects. These results suggested that the C, F, G, I, and J fragments retained strong transcriptional silencer elements. On the other hand, the B fragment showed strong enhancer activity, suggesting that it contains a CTF/NF1 enhancer element.

(2) Binding Protein to the Negative Regulatory Region—To determine whether these silencer elements are involved in negative regulation of the catalase gene expression in hepatocarcinogenesis or dedifferentiation of hepatocytes, we performed CAT assay in Reuber cells, which is a well-differentiated hepatoma cell line expressing the catalase gene (*24*, *25*). The results were inconclusive, however, be-

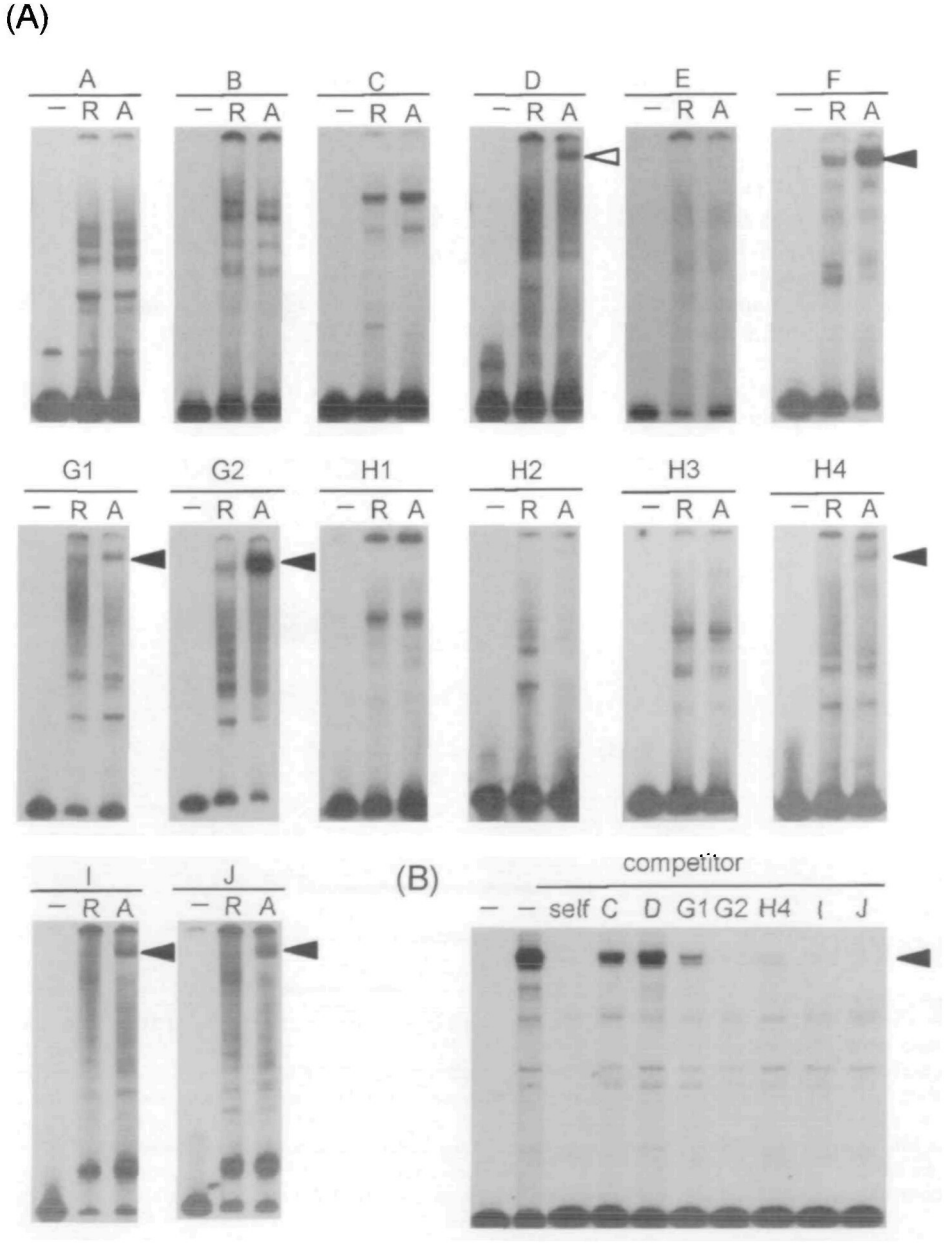

Fig. 2. **EMSA of subfragments from 5′-flanking region of the catalase gene.** (A) Each DNA segment was end-labeled (10,000 cpm/lane), and EMSAs were carried out with nuclear extracts from Reuber (R) and AH66 (A) cells, and without extract (–). The probes are indicated at the top of each panel. G and H fragments were split into smaller fragments as follows: G1 (–2652 to –2495 bp), G2 (–2495 to –2335 bp), H1 (–2335 to –2255 bp), H2 (–2255 to –2197 bp), H3 (–2197 to –2062 bp), and H4 (–2062 to –1969 bp). Filled arrowheads indicate the large complex provisionally named F-complex, and the open arrowhead shows a large complex with the D-fragment distinct from F-complex. (B) Competitive EMSA using AH66 nuclear extract and F-fragment probe were performed in the presence of 50-fold excess of C, D, F (self), G1, G2, H4, I, and J fragments as competitors.

cause of the very low efficiency of DNA-transfection in the cells. Therefore, DNA–binding proteins that bound to these negative regulatory regions were examined by EMSA with nuclear extracts from AH66 (a dedifferentiated hepatoma cell line) and Reuber cells. To prepare probes suitable for EMSA, the G and H regions were segmented by further digestion with restriction enzymes.

As shown in Fig. 2A, large DNA-protein complexes shown by filled and open arrowheads were observed abundantly in AH66 cells, but scarcely in Reuber cells, when EMSAs were carried out with the D, F, G1, G2, H4, I, and J fragments as probes. Interestingly, all except the H fragment showed strong silencer activities by CAT assay in AH66 cells. One of these DNA-protein complexes, temporarily named "F-complex," seems to be targeted to similar DNA sequences to the F fragment, because formation of the complex was competitively reduced by the G1, G2, H4, I, and J fragments in F fragment-probed EMSA (Fig. 2B). The D fragment seems to be targeted by distinct factor(s), since the D fragment hardly interfered with the F-complex formation. The C fragment, which also exhibited silencer activity, was not involved in the F-complex formation. Silencer activity of H fragment might be counteracted by an enhancer.

EMSA probed with the B fragment, which showed enhancer activity in CAT assay, indicated a similar pattern of binding proteins in both Reuber and AH66 cells. One of the factors bound to the B fragment was thought to be CTF/NF1 and to be equally expressed in these cells. DNA segments such as the A, E, H1, H2, and H3 fragments were not likely to be involved in regulation of transcription, according to the pattern of EMSA and the activity in CAT assay.

(3) Expression of the F-Complex in Hepatocarcinogenesis—To examine whether the expression of the F-complex is associated with hepatocarcinogenesis, the expression was determined in several hepatoma cell lines by EMSA. Fao was established as a well-differentiated hepatoma cell line from H4IIEC3, a derivative of Reuber cells. C2 and C2rev7 cell lines were isolated as a dedifferentiated cell type from Fao and as a revertant from C2, respectively (*5*). As shown in Fig. 3B, the catalase gene was expressed in Reuber, Fao, and C2rev7, as well as rat liver. Interestingly, the F-complexes were abundantly produced in malignant hepatomas, AH66 and C2 (Fig. 3A). Conversely, little production was observed in well-differentiated hepatoma cell lines, Reuber, Fao, and C2rev7, as well as the control rat liver. These results indicated that formation of the F-complex was closely associated with hepatocarcinogenesis.

(4) Binding and Silencer Function in the Subsegment of F Fragment—To examine the binding site of the F-complex and its involvement in regulation of gene expression, the F fragment was analyzed as a representative of these silencer fragments. Segments F1 (–2749 to –2721 bp) and F2 (–2721 to –2651 bp) were generated by digestion of the F fragment with restriction enzyme *Alu*I. In the F fragment-probed EMSA, the F-complex formation was reduced greatly by competition with F2, but only slightly by competition with F1 (Fig. 4A). Moreover, CAT plasmids contain-

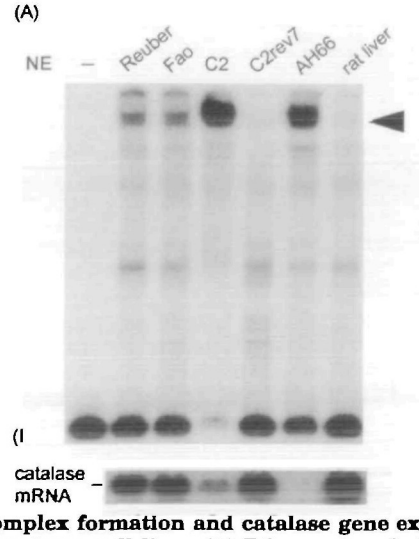

Fig. 3. **F-complex formation and catalase gene expression in various hepatoma cell lines.** (A) F-fragment probed EMSA was conducted with nuclear extracts from Reuber, Fao, C2rev7 cell lines and control rat liver as differentiated cells, and with that from AH66 and C2 cell lines for dedifferentiated cells. Filled arrowhead shows F-complex. (B) Catalase gene expression in various hepatoma cell lines and liver were determined by Northern blot analysis with 10 μg of total RNA from these cells.

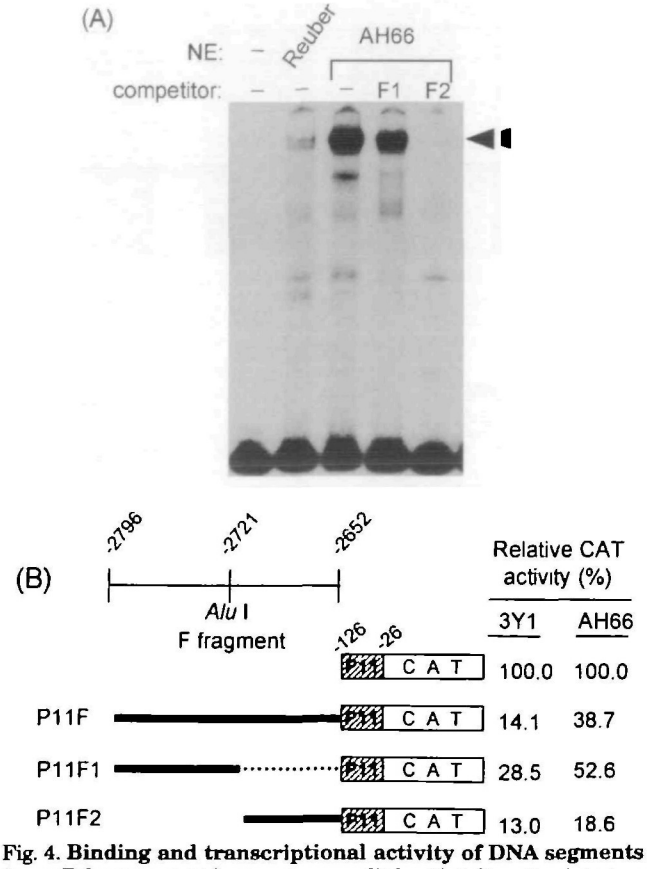

Fig. 4. **Binding and transcriptional activity of DNA segments from F fragment.** F fragment was split by *AluI* digestion into two segments, F1 (–2749 to –2721 bp) and F2 (–2721 to –2652 bp). (A) Competitive EMSA was carried out using AH66 nuclear extract and F fragment probe in the presence of 50-fold excess of F1 and F2 segments as competitors. Filled arrowhead shows F-complex. (B) CAT assay. CAT constructs containing F, F1, and F2 fragments are shown at the left of the panel. Relative CAT activities are shown as in Fig. 1.

ing F2 showed greatly reduced CAT activities (Fig. 4B). These results suggested that the F2 region was involved in transcriptional repression mediated by the F-complex formation.

(5) Core Sequence of Binding Site of the F-Complex—To identify the core sequence of the binding site of the F-complex, DNase I footprinting was performed with the F fragment and the nuclear extract from AH66 cells. As shown in Fig. 5, the F2 region was protected from DNase I digestion in upper and lower strands of the F fragment. Posterior portion (–2689 to –2659 bp) of F2 showed particularly strong footprinting. Similarly, the F1 region also showed some protein binding, but these DNA protein complexes will be analyzed in future.

To focus on the core sequence, synthetic oligonucleotides were subjected to competitive EMSA probed with the F fragment. As shown in Fig. 6, the F-complex formation was not influenced by addition of oligo 22 (corresponding to –2676 to –2655 bp, lane 4 to 6). Conversely, the complex completely disappeared by competition with wild-type oligomer (wt: –2691 to –2652 bp, lane 7 to 9), suggesting that

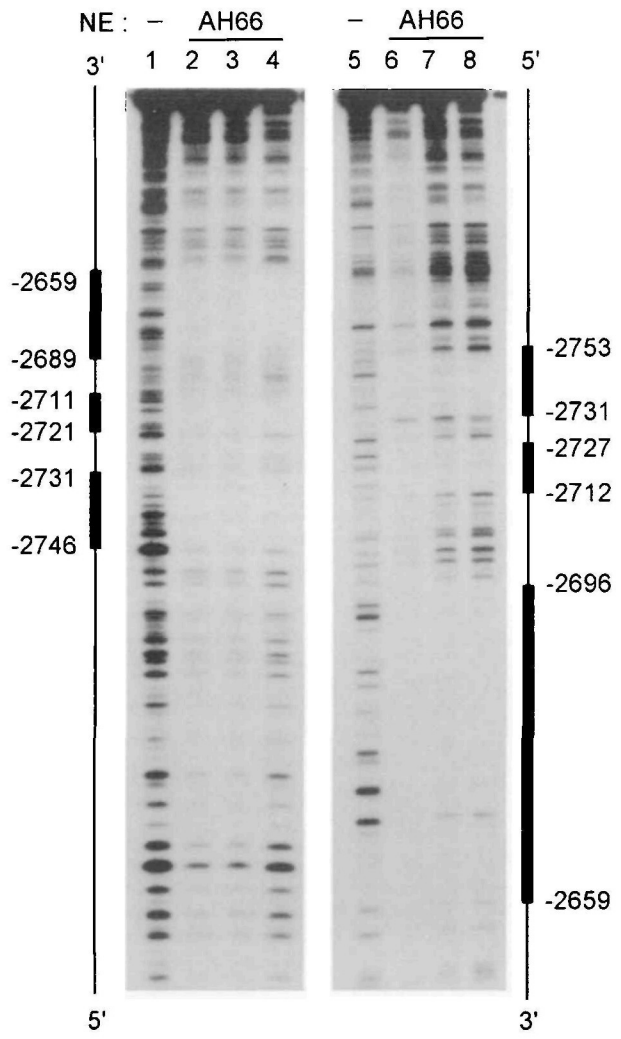

Fig. 5. **DNase I footprinting.** F fragment cloned *Sma*I site of pUC18 was labeled at the 5'-end, following digestion with *Eco*RI or *Hin*dIII. Fragments were excised by restriction digestion at opposite site of labeling and recovered from PAGE. Footprinting of the F fragment with nuclear extract from AH66 cells was performed with various DNase I concentrations: 0.005 unit (lanes 1, 2, 5, and 6), 0.05 unit (lanes 3 and 7), and 0.1 unit (lanes 4 and 8).

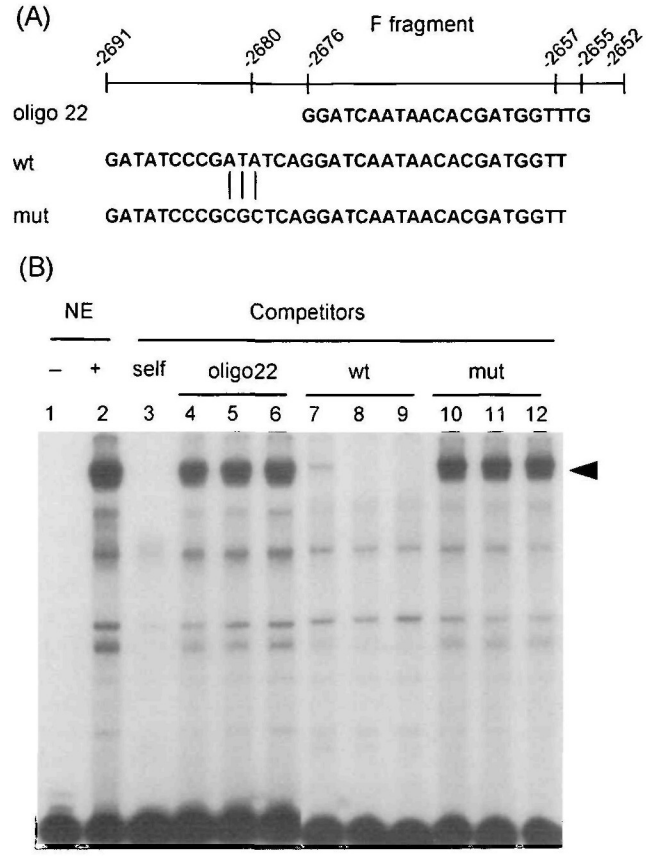

Fig. 6. **Core sequence for binding site of the F-complex.** (A) Sequences of the oliogonucleotide used in competitive EMSA proved F fragment are shown under the map of F fragment. Mutant oligomer (mut) has ATA in the wild-type oligomer (wt) replaced by CGC. (B) Competitive EMSA was carried out using AH66 nuclear extract and F fragment probe in the presence of 5-, 25-, and 50-fold excess of double-stranded oligomers.

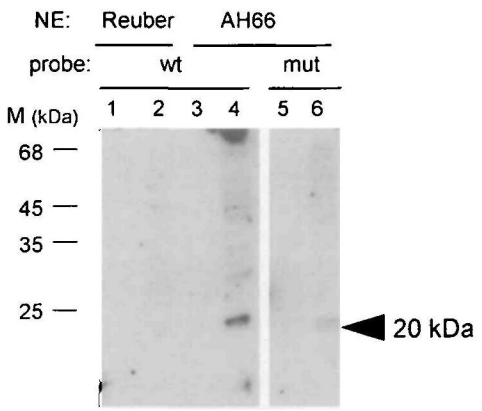

Fig. 7. **Southwestern blot analysis of F fragment binding protein.** Portions of 10 μg (lanes 1, 3, and 5) and 30 μg (lanes 2, 4, and 6) of nuclear extracts from Reuber and AH66 cells were subjected to SDS-PAGE. Southwestern blot analysis was then performed with end-labeled wild-type oligomer (wt; lane 1 to 4) and mut oligomer (mut; lane 5 and 6) as probes.

the 15-bp sequence GATATCCCGATATCA (–2691 to –2676), which is deduced by subtraction of oligo22 sequence from the wt sequence, has strong binding activity to the complex. To confirm the result, mutant oligomer (mut) with transversional mutation in the sequence was prepared by replacement of ATA (located at –2682 to –2680 bp) with CGC. The mutant oligomer almost completely lost the binding activity to the complex (lane 10 to 12). It is conceivable, therefore, that this 15-bp sequence is the core of the binding site of the F-complex. However, this 15-bp sequence failed to show as a common sequence among the F-complex binding sites, F, G1, G2, H4, I and J fragments. Elucidation of this discrepancy cannot be drawn from simple binding of the F-complex.

To identify the component of the F-complex, Southwestern blot analysis of nuclear extracts from Reuber and AH66 cells was carried out with wild-type and mutant oligomers as probes. As shown in Fig. 7, a 20-kDa nuclear protein was observed in AH66, not in Reuber cells. This protein was weakly bound to mutant oligomer. This observation is in accordance with the result of incomplete competition in EMSA probed with mutant oligomer (Fig. 6B, lanes 10–12). These results suggested that the F-complex contained the 20-kDa protein as a component.

DISCUSSION

The findings of the present study demonstrate that transcriptional repression in the catalase gene occurs as follows. The F-complex containing 20-kDa protein as a component, which is abundant in the malignant hepatoma cells but scarce in well-differentiated hepatoma cells, negatively regulates gene expression by binding to multiple sites (at least 6 sites) of the elements in the catalase gene. One of the silencer elements represents with GATATCCCGATATC in the F fragment.

The core sequence resembles the GATA motif, which is indicated with WGATAR and bound by Zn-finger GATA family transcription factors, and which is involved in globin gene expression in erythrocytes (*26*) and albumin gene expression in liver (*27*), and distributed in various tissues (*28*). However, it is not known whether the GATA family protein is induced in hepatocarcinogenesis and negatively regulates the hepatic gene expression. Moreover, these factors have molecular masses of approximately 45 kDa (*29*). These properties of GATA proteins differ from those of the F-complex with 20-kDa protein component.

In a recent study, HTF was isolated as a transcription factor induced in HCC (*6*). This factor binds to CRCGTCA and is closely related to the human XBP-1, which is essential for liver development and growth, and stimulates α-fetoprotein gene expression (*30*). The molecular mass of this protein predicted from the sequence data is 30 kDa (*6*). These properties completely differ from that of the 20-kDa protein associated with the silencer sequence in the rat catalase gene.

The 20-kDa protein is thought to be dimerized with some other factor(s), because its molecular mass is too small for it to compose the large F-complex, and because the core sequence in the F fragment contains an inverted repeat within the span of 13 bp. This protein is obviously induced in hepatocarcinogenesis, and serves as a *trans*-repressor for the catalase gene expression. Therefore, since the F-complex containing 20-kDa protein is thought to be a novel transcription factor, we designated this protein a hepatocarcinogenesis-related negative regulatory factor or HNRF.

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