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Inverted Repeats in the TATA-Less Promoter of the Rat Catalase Gene[1]

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The rat catalase gene promoter lacks a TATA box, and has eight initiation sites of transcription as well as several GT and CCAAT boxes. To elucidate the mechanism of transcription in this TATA-less promoter, we analyzed nuclear factors binding to this regulatory region of the catalase gene. Functional analysis of the promoter region revealed that a pair of inverted repeat motifs located on both sides of the transcription initiation sites played an important role in the gene expression. The core sequence of this element is GYCMGGCCCKCTCYKG (M=A/C, K=G/T, Y=T/C), and four species of complex were observed to bind to this sequence. Furthermore, this element in the chimeric promoter negatively interacted with the initiator element of the terminal deoxynucleotidyl transferase gene. These results suggested that the rat catalase gene is regulated by a novel mechanism involving the inverted repeated structure of the promoter and characteristic binding factors.

Key words: catalase gene, *cis*-acting element, DNA binding protein, initiator, TATA-less promoter.

Catalase [EC 1.11.1.6] is an antioxidant enzyme which catalyzes the decomposition of hydrogen peroxide produced by β-oxidation of fatty acids or by oxyradical metabolism. The enzyme has a central role in defense against oxidants, together with other enzymes such as superoxide dismutase and glutathione peroxidase (*1*). Catalase activity is found in the liver at a high level, as well as in the kidney and erythrocytes, but scarcely in connective tissues. The rat catalase gene is a single-copy gene with 13 exons spanning 33 kb, and has multiple transcription initiation points. The promoter region of the gene was shown to have at least eight CCAAT boxes and five GT boxes, but to lack a TATA box and an initiator consensus sequence (*2*). When compared with the human catalase gene (*3*), the nucleotide sequence of the rat catalase gene promoter is highly conserved, except for the insertion of a B1-like repetitious sequence at -880 to -670 bp upstream from the translational initiation codon.

In eukaryotic cells, most transcriptional initiation complexes containing RNA polymerase II are assembled on TFIID binding to a TATA box. Accordingly, the TATA box, as well as the CCAAT box (*4*), is an essential element for the gene expression. A TATA-less promoter often has a pyrimidine-rich initiator element (*5*). It is, however, hard to find a distinct sequence homologous to any known initiator element in the rat catalase promoter. TATA-less promoters are classified into two types according to the presence or absence of the initiator element in the transcription start site. Transcription initiation at multiple sites is frequently observed with a promoter lacking a definite initiator sequence (*6*). This is likely to be the case in the rat catalase gene.

The liver catalase activity is known to be markedly reduced in some transplantable hepatoma cells and cultured hepatoma cell lines (*7-9*). Decrease of catalase activity has also been noted in tumor-bearing hosts, in a tumor size-dependent fashion, and the activity is restored to the normal level by tumor removal (*10, 11*). It is therefore of interest to examine the above biological phenomena from the viewpoint of regulation of the catalase gene expression. We previously observed negative regulatory elements upstream of the rat catalase gene (*12*). In this study, we found unique structure of the promoter region in the gene, and analyzed its involvement in transcriptional regulation.

MATERIALS AND METHODS

Plasmids and Site-Directed Mutagenesis—Plasmids bearing truncated promoter fragments were prepared by digestion with restriction enzymes, *Bal*31 and mung bean nuclease, from a DNA fragment (-187 to -26) of the rat catalase gene. Chloramphenicol acetyltransferase (CAT) reporter plasmids were constructed by insertion of the truncated segments into the polylinker sites of pUC0CAT (*13*). Site-directed mutagenesis was performed by mismatched-primer polymerase chain reaction (PCR) using 100 fmol of -126CAT plasmid DNA as the template. Oligonucleotides, *mut1* (GCTGCAGCAAGAGGTTCGGACGAG), *mut2* (GCTGCAGCAAGAGTTCCGGACGAG), and *mut3* (GCTGCAGCAAGTTGGCCGGACGAG) were

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Abbreviations: CAT, chloramphenicol acetyltransferase; EMSA, electrophoretic mobility shift assays; TdT, terminal deoxynucleotidyl transferase.

used in PCR as mismatched primers. The PCR products were inserted into pUC0CAT. All plasmids were sequenced to check for unexpected mutation. CAT plasmid containing the initiator element from the terminal deoxynucleotidyl transferase (*TdT*) gene was constructed by insertion of a synthetic oligonucleotide (AGCTTCAGAGCCCTCATTC-TGGAGA) into the *Hin*dIII sites of pUC0CAT, −58CAT and −58mut3CAT.

CAT Assay—Transfection of plasmid DNA was performed by the calcium phosphate co-precipitation method (*14, 15*), using 8 μg of CAT plasmid and 4 μg of pSVLgal carrying the mouse β-galactosidase gene (gift from Dr. Nanba, Tottori University). The Fischer rat embryonic fibroblast cell line 3Y1 used in this assay was obtained from the Japanese Cell Resource Bank, and maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. The CAT activities were determined by separation of acetylated chloramphenicol by high-performance liquid chromatography (*16*), and normalized with respect to β-galactosidase activities measured as described (*17*) to compensate for variations in transfection efficiency. Each transfection experiment was carried out in triplicate.

Electrophoretic Mobility Shift Assay (EMSA)—Nuclear extract from the rat liver was prepared as described (*18*). DNA segments were obtained from the promoter region of the CAT plasmids by restriction enzyme digestion, and end-labeled with T4 polynucleotide kinase and [γ-^{32}P]-ATP. EMSAs were performed using a modification of the reported method (*19*). Briefly, 1 μg of nuclear extract was bound to 1 fmol of probe in a mixture containing 25 mM HEPES-KOH pH 7.9, 100 mM KCl, 0.1 mM EDTA, 10 mM $MgCl_2$, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 0.2 μg/μl poly d(I-C), and 7.5% glycerol. DNA-protein complexes were resolved in 5% acrylamide: bis-acrylamide (39:1)-TGE (50 mM Tris, 380 mM glycine, 2 mM EDTA pH 8.2) gel containing 2.5% glycerol, and detected by autoradiography.

DNase I Footprinting—DNase I footprint analysis was carried out as previously described (*20*). Briefly, the binding mixtures contained 20 μg of nuclear protein and 5×10^4 cpm of end-labeled probes (−187 to −26 bp of the catalase gene) in 50 μl of binding buffer [10 mM HEPES (pH 7.9), 50 mM KCl, 6.5 mM $MgCl_2$, 1 mM EDTA, 10% glycerol, 1 μg of poly d(I-C), 6 mM 2-mercaptoethanol]. After incubation for 15 min on ice and for 2 min at 20°C, 50 μl of 5 mM $CaCl_2$-10 mM $MgCl_2$ solution was added. Digestion with 1 μl of freshly diluted DNase I solution was then allowed to proceed at 20°C for 1 min. The reaction was stopped, and the resulting DNA fragments were analyzed after electrophoresis on a 5% sequencing gel.

UV Crosslinking Analysis—The nuclear extracts (5 to 15 μg) were incubated in standard EMSA reaction, containing 10 fmol of probe (1×10^5 cpm). After complex formation, the mixtures were irradiated with ultraviolet light at 254 nm (10 W germicidal lamp) at 2 cm distance for 20 min, and crosslinked complexes were separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) according to Laemmli (*21*).

RESULTS AND DISCUSSION

Transcriptional Regulation in the Rat Catalase Promoter—The absence of both an initiator element and a TATA box in the promoter region was reported to result in multiple transcription initiation sites in the case of the mouse thymidylate synthetase gene (*6*). Likewise, the rat catalase gene lacks both a TATA-box and an initiator consensus, resulting in multiple start sites of transcription (*2*). We therefore sought to elucidate the transcriptional mechanism of the catalase gene.

First, to determine the *cis*-acting elements in the promoter region involved in transcriptional regulation, CAT assay in 3Y1 rat fibroblast cells was carried out using various promoter deletion mutants of the catalase gene (Fig. 1). Previously, the CAT activity of the 3Y1 cell line was demonstrated to have a similarity with that from a rat hepatoma cell (*12*). The promoter region (−187 to −26 bp counted from the initiation codon of the catalase) was partitioned enzymatically into four regions, I, II, III, and IV. The CAT plasmids, −187CAT, −164CAT, and −126CAT, which contained DNA fragments from −187, −164, and −126 bp to −26 bp, respectively, were found to generate CAT activity at a high level. The CAT plasmids −187⊿A CAT, −164⊿A CAT, and −126⊿A CAT, which

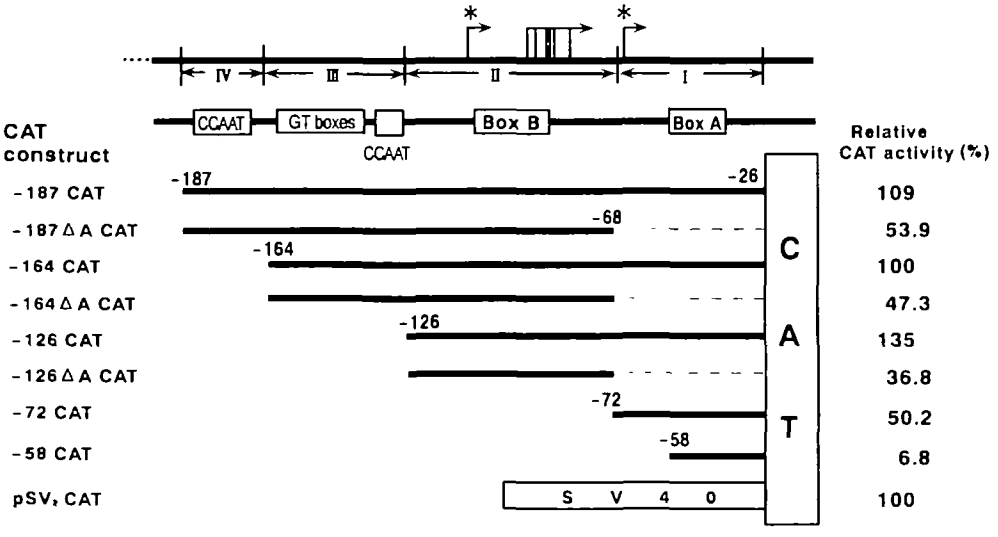

Fig. 1. **Relative CAT activities expressed by constructs carrying various promoter regions of the rat catalase gene.** The transcription initiation sites are shown with hook-shaped arrows, and frequent initiation sites are denoted with asterisks at the top of the figure. Under the map, regions protected by footprinting are schematically illustrated. The DNA fragments inserted into CAT plasmids are shown by bars with the nucleotide positions of the 5′ ends relative to the initiation codon. Relative CAT activities derived from these constructs are given in percent, based on the activity generated by pSV2-CAT as 100%.

lack region I (−68 to −26 bp) from each of the promoters, showed CAT activities of 30 to 50% of those of the respective parental plasmids. The plasmid −72CAT, which consisted of only region I, exhibited CAT activity similar to that of −126⊿A CAT. A mutant with a further deletion, −58CAT almost lacked CAT activity, due to the absence of a transcriptional initiation site. These results suggested that the minimum span holding the promoter activity is located in regions I and II.

The GT box sequence in region III (−164 to −126 bp) is similar to the β-globin enhancer motif (*22*); therefore, this region would appear to be responsible for expression of the catalase gene in erythrocytes.

Factors Binding to the Rat Catalase Promoter—The next objective in this study was to determine the nuclear factors binding to the regulatory element of this promoter. DNase I footprinting in the promoter region revealed that protected areas were observed in the region represented as boxes A and B in Fig. 1, as well as in the putative GT and CCAAT boxes (Fig. 2). Therefore, to define the nuclear factors and the binding sites, EMSAs were performed with this region. As shown in Fig. 3, four complexes designated as **a**, **b**, **c**, and **d** were observed, when the DNA segment of region I including box A was used as the probe, and all the bands were competed out by a DNA fragment from region II, where box B was mapped. A nonspecific competitor segment failed to remove these bands. EMSA with region II as the probe showed the same result as that with region I. As a result of sequence analysis in this region (Fig. 4), the

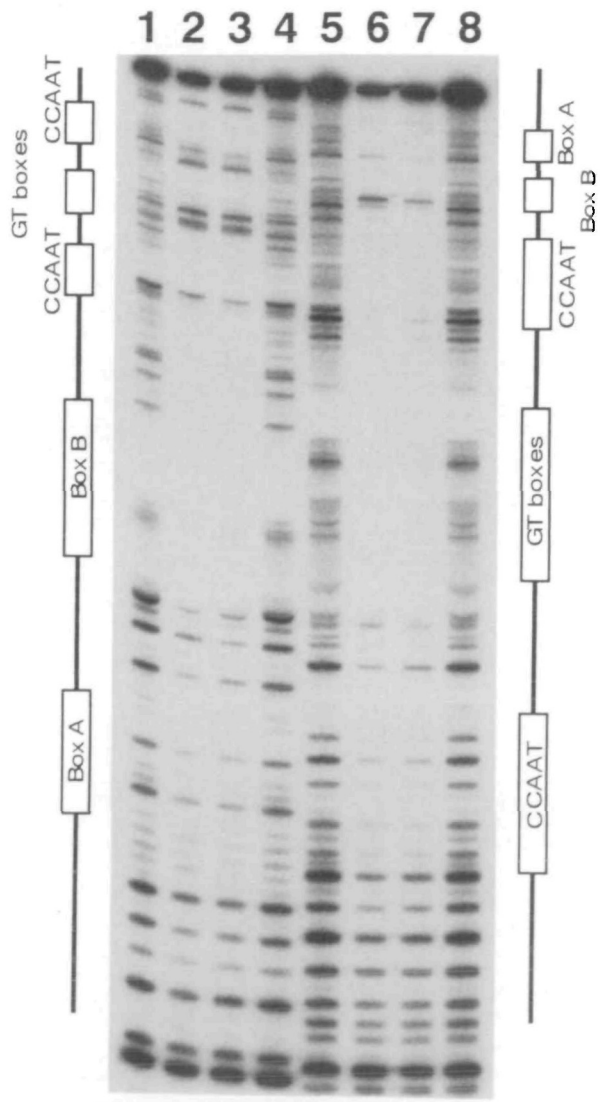

Fig. 2. **DNase I footprinting analysis performed on the promoter region of the rat catalase gene.** DNA fragments from −187CAT were end-labeled at the −26 bp site (upper strand: lanes 1-4) and at the −187 bp site (lower strand: lanes 5-8), and mixed with 20 μg of the nuclear extract. The complexes were digested with DNase I for 1 min (lane 2 and lane 6) or 2 min (lane 3 and lane 7), followed by electrophoresis. DNA probes treated with the enzyme were applied on the gel (lanes 1, 4, 5, and 8).

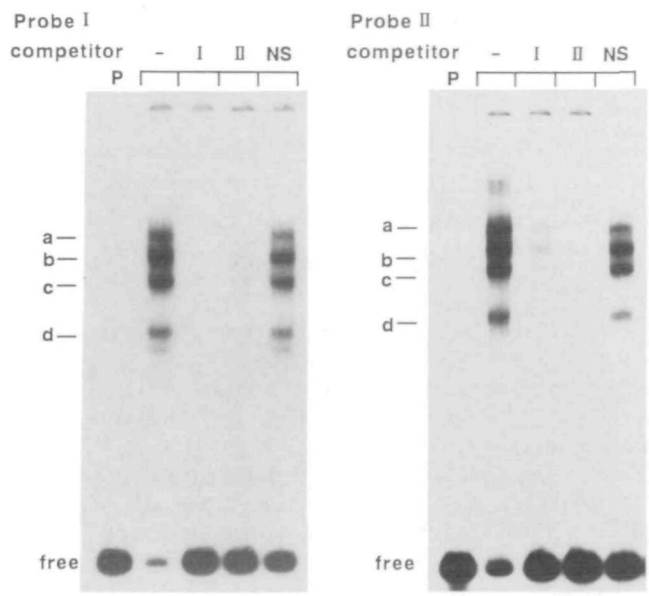

Fig. 3. **Detection of factors binding to catalase gene promoter by EMSA.** Probe I and probe II, representing regions I and II in Fig. 1, were labeled at the 5′ ends, and incubated with 1 μg of a rat liver nuclear extract with or without a 50-fold molar excess of a competitor such as a non-labeled DNA segment of region I, region II or NS (nonspecific competitor). The reaction products were analyzed as described in "MATERIALS AND METHODS." Four major complexes are denoted as **a**, **b**, **c**, and **d**. Lanes P show free probes.

Rat (Box A)	-54 gctc GTCCGGCCCTCTTG cct	-41
Rat (Box B)	-91 gtca GTCAGGCCCGCTGG agt	-104
Mouse (Box A-like)	-54 gctgc GTCCGTCCCTGCTG tct	-41
Mouse (Box B-like)	-95 tgttg GCCAGGCCCGCTGG agt	-108
Human (Box A-like)	-42 cga GCCGAGGCCTCCTG c	-29
Consensus	5′ $G^{T}_{C}C^{A}_{C}GGCCC^{T}_{G}C^{TT}_{CG}G$	3′

Fig. 4. **Alignment of the sequences present on both sides of a transcription initiation site in catalase genes.** Numbers indicate positions from the translation initiation codon of each catalase gene. Core sequences are shown by upper case letters.

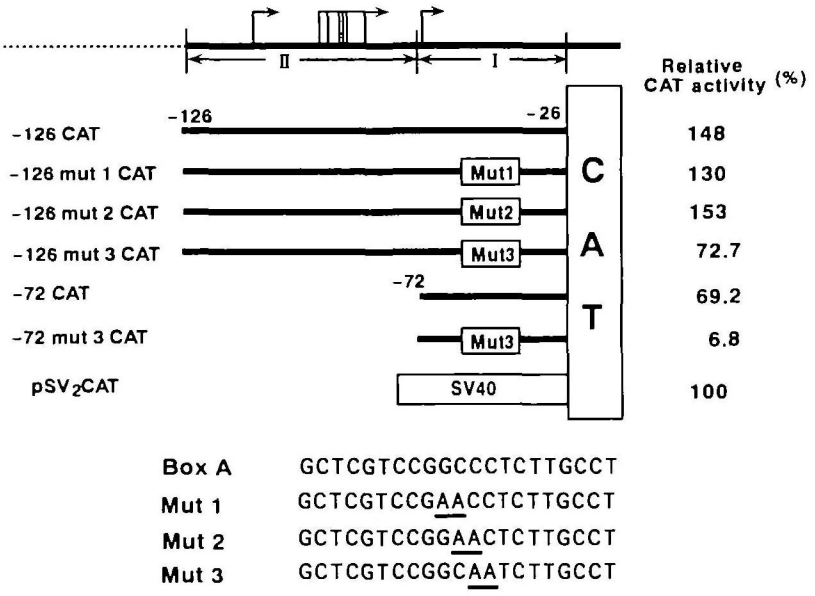

Fig. 5. **Effect of mutations in box A on the CAT activity.** CAT assay was performed as in Fig. 1. The mutation sites in the sequences are indicated by underlines.

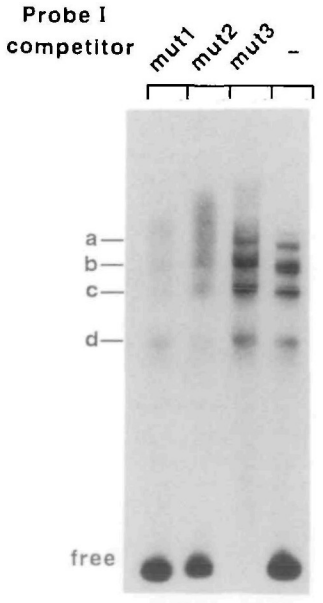

Fig. 6. **Effect of mutations in box A on binding of the factors.** Binding activity of a mutated box A fragment was determined by competitive EMSA using the region I fragment as the probe and non-labeled DNA fragment containing a mutation (*mut1*, *mut2*, and *mut3*) as the competitor. −: EMSA without competitor.

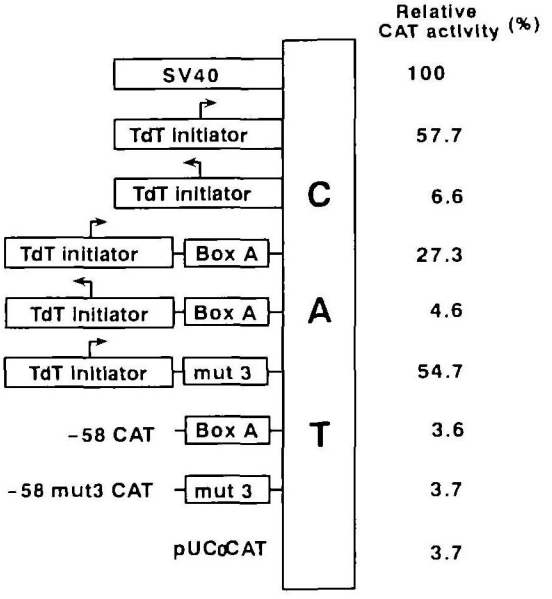

Fig. 7. **Interaction of box A with the initiator element of *TdT* gene.** CAT assay was performed as in Fig. 1. The hook-shaped arrows show the orientation of transcription in the initiator element.

sequence of box A (GTCCGGCCCTCTTG) was found to be similar to that of box B in an inverted orientation. The same promoter structure was also found in the mouse catalase gene (*23*). Likewise, the human catalase gene harbors a similar sequence downstream from the initiation sites (*3*). These results indicated that a common mechanism of transcription regulation of the catalase gene is utilized among rat, mouse, and human, and the consensus sequence is GYCMGGCCCKCYKG (M=A/C, K=G/T, Y=T/C). Furthermore, this consensus sequence of box A was confirmed by the observation that all the bands disappeared in EMSA using region I cleaved at GGCC with *Hae*III as the probe (data not shown).

Although the inverted repeat structure was found in the promoter, the transcription was not bi-directional (data not shown), suggesting that basal transcription factors asymmetrically recognize the promoter region.

Mutation within Box A—The promoter activity decreased as a result of deletion of region I. It is still unclear whether this decreased transcription was caused by loss of box A or by loss of the transcription start site. To examine this, point mutations were introduced around the *Hae*III site in box A by mismatched primer PCR, as shown in Fig. 5. Plasmid −126mut3CAT, carrying substitution mutation *mut3* within box A, but which still harbored box B and the start site, showed CAT activity reduced to a half of that of

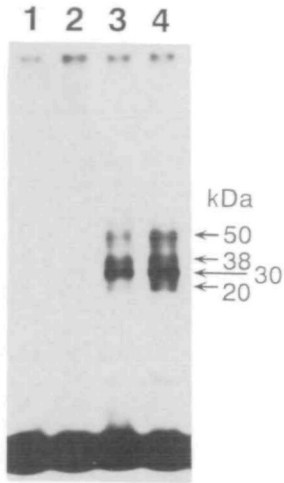

Fig. 8. **UV crosslinking of box A proteins.** The end-labeled region I (1×10^5 cpm) was mixed with 5 μg (lane 3) or 15 μg (lane 4) of the nuclear extract. The complexes were irradiated with UV light, and analyzed by SDS-PAGE. The complex of the probe and 15 μg of protein was electrophoresed without UV irradiation (lane 2), and lane 1 shows the irradiated probe. The estimated molecular weight is presented on the right of the panel.

the parental plasmid (−126CAT). Furthermore, the CAT activity of −72mut3CAT was abolished, in spite of the presence of the initiation site. The other mutants, *mut1* and *mut2* had little effect. These results suggested the importance of box A in the transcription activity of the promoter.

Next, the effects of mutations on the binding activity of the box A factors were examined. The competition in EMSA was carried out with the DNA fragment from region I as the probe, and with the same fragment carrying mutations as competitors. As shown in Fig. 6, the *mut3* fragment failed to compete with the region I fragment for binding of all the box A factors, indicating that DNA segment carrying *mut3* had completely lost affinity for the proteins, while the mutant sequences, *mut1* and *mut2*, still retained the affinities. These results suggested that the box A factors are critical in the transcription of the catalase gene, and that the initiation site in region I might be less important.

Interaction of Box A with Initiator Element—The initiator element was demonstrated to specify the location and direction of transcription initiation independently of the TATA box in adenovirus major late promoter (*5*) and *TdT* gene (*24*). To analyze the interaction between the box A element and the initiator, a hybrid promoter was constructed by insertion of the box A element downstream of the initiator from *TdT* gene. CAT activity generated by this chimeric promoter was significantly reduced compared with the CAT plasmid carrying a single element, either the initiator or box A, while the *TdT* initiator carrying the box A mutant (*mut3*) restored the transcriptional activity (Fig. 7). These results suggested that the initiation site of the catalase gene could not be replaced with the *TdT* initiator, and that these elements negatively interfered with transcription, either by steric hindrance or by cross modulation between the factors binding to each of the elements. Recent studies have shown that the initiator element is associated with proteins such as HIP-1 (*25*), YY1 (*26*), TFII-I (*27*), and USF (*28*). The box A binding proteins in this study should be examined for interaction with these initiator-binding proteins.

Conclusion—Characterization of the box A factors was carried out by determination of molecular weight after ultraviolet light crosslinking (Fig. 8). The molecular weights of these proteins were estimated as approximately 50, 38, 30, and 20 kDa. Furthermore, EMSAs under various conditions revealed that the nuclear factors binding to box A were extremely stable to heat treatment (data not shown). Some transcription factors such as C/EBPα are well known to be stable to heat (*29*). The recognition sequences of box A factors were quite distinct from the sequence for C/EBPα.

The TATA- and initiator-less promoter of the P-glycoprotein gene possesses an intragenic sequence motif called MED-1 (multiple start site element downstream) that appears to play an important role in transcription initiation (*30*). The core sequence of MED-1, GCTCCG, is located at −18 to −13 bp from the initiation codon of the rat catalase, which is outside region I containing box A. Therefore, it appears that the box A element and the factors in this study are novel ones, and that both the element and the factors play a significant role in the transcription of the catalase gene.

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