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Organization of Mouse DNA Polymerase β Gene Silencer Elements and Identification of the Silencer-binding Factor(s)

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Different portions of the 5′-upstream region of the mouse DNA polymerase β gene were combined with bacterial chloramphenicol acetyltransferase (CAT) gene of the CAT vector. Transfection of these recombinant plasmids into mouse NIH/3T3 cells has revealed that each of the previously identified two negatively acting regions (silencers I and II) of this gene consists of multiple sub-domains. The distal silencer (silencer I) at around −1.5 kb consists of four sub-domains (−1852 to −1667, −1663 to −1616, −1564 to −1525 and −1355 to −1257). The promoter-proximal silencer (silencer II) at around −0.5 kb consists of two functional domains (−681 to −523 and −490 to −447) separated by a neutral region of 33 base pairs. Silencer II functioned efficiently when silencer I was deleted. Conversely, the distal silencer I functioned efficiently when silencer II was deleted. Thus, these silencers functioned redundantly to each other in NIH/3T3 cells. Nucleotide sequence analysis revealed no extensive sequence similarity between these two silencers. Significant sequence similarity is present between a distal portion of silencer II and the c-*myc* gene silencer, and also between a proximal portion of silencer II and the mouse F9 cell-specific silencer. A protein factor(s) that specifically bound to the silencer elements was detected in nuclear extracts of NIH/3T3 cells and mouse liver in which DNA polymerase β was expressed at a rather low level. The same binding factor(s) can bind to both silencer I and II regions, although its affinity for silencer II is much higher than that for silencer I.

Key words: DNA polymerase β gene — Transcriptional silencer — CAT-assay — Gel mobility shift assay

Mammalian DNA polymerase β consists of a single polypeptide of about 38,000 daltons, and is the simplest in structure among prokaryotic and eukaryotic DNA polymerases so far known.[1,2] This enzyme has been believed to be involved in DNA repair[1] and also DNA synthesis accompanied with a DNA recombination event.[1,3] Mouse DNA polymerase β gene has been cloned[4] and functional analysis of its 5′-upstream region has revealed the existence of two negatively acting regions and the promoter.[5,6] We have localized the promoter of this gene at nucleotide positions −48 to +33. This region contains two major transcription initiation sites (nucleotide positions +1 and +10), a so-called GC-box which is known as the transcription factor Sp1-binding consensus sequence, and a 10-base-pair (bp)[3] palindromic sequence similar to the binding consensus for the transcription factor USF (MLTF) or ATF.[5] The involvement of Sp1-like mouse cell factor in the expression of the DNA polymerase β gene has been suggested by *in vivo* DNA competition studies.[5] The promoter of human DNA polymerase β gene also consists of three Sp1-binding consensus sequences and a 10 bp palindromic sequence.[7]

We have been interested in the analysis of two negatively acting regions (here we call them silencer elements) located upstream of the mouse DNA polymerase β gene promoter, since the elements play an important role in the regulation of cell type-specific expression of mouse DNA polymerase β gene,[8] and possibly in the regulation of DNA replication.[9] The present study was carried out to define the sequences involved in transcriptional repression. We constructed libraries of deletion mutants extending from 5′ to 3′ or 3′ to 5′ across the silencer regions, and examined their capacity to repress the gene expression. Analysis of the phenotype of the deletion mutants suggests that each of two silencers of DNA polymerase β gene consists of dispersed multiple sub-domains. Furthermore, we have detected a protein factor(s) that specifically binds to the silencer elements in nuclear extracts of NIH/3T3 cells and mouse liver. This factor(s) bound to regions in both silencers I and II, but its affinity for silencer II was much higher than that for silencer I.

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[3] Abbreviations: bp, base pair(s); kb, kilobase(s); CAT, chloramphenicol acetyltransferase.

MATERIALS AND METHODS

Plasmid constructions The plasmid p5′−2190βCAT,[6] which was created by ligating the DNA fragment encompassing nucleotide positions +33 to −2190 of the mouse DNA polymerase β gene (+1 denotes the transcription initiation site) to the upstream side of the bacterial chloramphenicol acetyltransferase (CAT) gene (Fig. 1), was cut with *Xba*I, *Kpn*I and then exonuclease III to create a series of 5′-end unidirectional deletion derivatives.[10] Since there was a relatively strong stop on progress of exonuclease III digestion at around the nucleotide position −100 region, some of the deletion derivatives further downstream were created by digesting p5′−827βCAT with nuclease Ba131. Deletion break-points of deletion derivatives were determined by the dideoxy-sequencing methods[11, 12] using either a synthetic 17mer (5′-GTACAATCTGCTCTGAT-3′) which hybridized to the region close to the *Nde*I site of CAT vector DNA or a 17mer (5′-TGTCTGACTTAACTAAA-3′) which hybridized to the nucleotide positions −456 to −440 of DNA polymerase β gene. In total, 32 5′-deletion mutants were obtained.

The plasmid p5′−100βCAT,[5] which contains the DNA fragment encompassing nucleotide positions +33 to −100 of DNA polymerase β gene, was digested with *Kpn*I, then blunt-ended by S1-nuclease digestion. The polylinker portion excised from the plasmid pUC19 was ligated to the blunt-ended *Kpn*I site of p5′−100βCAT[5] to create the plasmid p5′−100βRLCAT. The DNA fragment encompassing nucleotide positions −1852 to −88 of the DNA polymerase β gene was isolated from one of the 3′-end deletion derivatives of pUC19-MGβ01,[4] and was inserted between the *Kpn*I and *Xba*I sites of p5′−100βRLCAT. The obtained plasmid, p3′−88βCAT (Fig. 1) was digested with *Sal*I and *Sph*I then with exonuclease III to create a series of 3′-end unidirectional deletion derivatives, leaving the intact promoter which contained the −100 to +33 portion of DNA polymerase β gene and 9 additional nucleotides derived from polylinker of pUC19. Deletion break-points of obtained deletion mutants were determined by the dideoxy-sequencing method using either a synthetic 17mer (5′-GGTGGTATATCCAGTGA-3′) which hybridized to the CAT-coding region or a 17mer (5′-TCACGCGCCAGGCTACG-3′) which hybridize to the nucleotide positions −38 to −22 of DNA polymerase β gene. In total, 22 3′-deletion mutants were isolated and characterized.

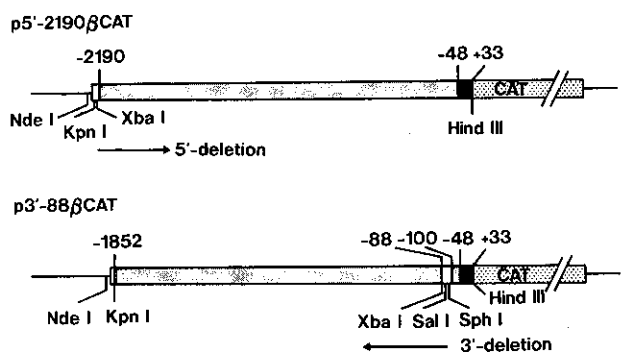

Fig. 1. Constructs of plasmids used for making 5′- and 3′-deletion mutants of mouse DNA polymerase β gene silencer region. The shaded bars in plasmids indicate the DNA polymerase β gene sequence cloned into CAT-vector DNA whose break points are indicated by nucleotide numbers relative to the 5′-most proximal major cap-site of the DNA polymerase β mRNA. The minimum region required for promoter activity is indicated by a closed box. Details of the procedures for the construction of 5′- and 3′-deletion derivatives are described in "Materials and Methods."

All plasmids were propagated in *E. coli* HB101 and isolated by the standard procedures.[13] Isolated plasmid DNAs were further purified through two cycles of ethidium bromide-CsCl density gradient centrifugation.

Cell culture, DNA transfection and CAT assay Mouse NIH/3T3 cells were cultured in Dulbecco's modified minimal essential medium (DMEM) supplemented with 10% (v/v) calf serum.

The DEAE-dextran method with chloroquine treatment[14] was used for DNA transfection into cells at 20 h after seeding 4×10^4 cells per 100 mm dish. Each transfection contained 5 μg of plasmid DNA per dish. Cells were harvested at 48 h after transfection. Extracts were prepared and CAT activity was measured as described.[6, 14] In order to quantify the CAT activity, radioactive spots corresponding to acetylated chloramphenicols were taken from the thin layer plates, and radioactivity was measured in a toluene-based scintillator by using a liquid scintillation counter. CAT activities were normalized to protein amounts[6] or the amounts of plasmid DNA in cells at the time of harvest.[6, 15] Transfections were performed multiple times to avoid errors owing to fluctuation in transfection efficiency.

Gel-mobility shift assay Nuclear extracts were prepared from NIH/3T3 cells and mouse livers according to the methods of Dignam *et al.*[16] and Gorski *et al.*,[17] respectively as described previously.[18] The final concentration of protein in NIH/3T3 nuclear extract and that in liver nuclear extract were 2.4 mg/ml and 5.3 mg/ml, respectively.

The DNA-protein binding buffer for the gel-mobility shift assay contained 4 m*M* Tris-HCl (pH 8.0), 12 m*M* HEPES (pH 7.9), 40 m*M* NaCl, 7.5 m*M* $MgCl_2$, 0.46 m*M* EDTA, 1.2 m*M* dithiothreitol and 10.2% glycerol. The binding reaction mixture (10 μl) contained 2 μg of

poly(dI-dC), the indicated amount of competitor DNA, 5 μg of nuclear extract and double-stranded probe DNA (0.4 ng) that was end-labeled with ^{32}P. The incubation was carried out for 20 min at room temperature. The DNA-protein complex was resolved in a 4% polyacrylamide gel in the electrophoresis buffer containing 50 m*M* Tris, 380 m*M* glycine (pH 8.5), and 2 m*M* EDTA.[19] The gel was then dried and exposed to X-ray film for autoradiography.

RESULTS

Fine mapping of the transcriptional silencer elements located upstream of the mouse DNA polymerase β gene

We previously identified in the 5′-flanking region of the mouse DNA polymerase β gene two negatively acting regions which functioned in mouse NIH/3T3 cells.[6] The promoter-distal region was roughly estimated to lie within 300 bp at around nucleotide position −1700 and the proximal one within 400 bp at around nucleotide position −650.[6] These regions appear to have the properties of so-called transcriptional silencer elements, including position- and orientation-independent negative functions on heterologous promoters as well as on their own promoter.[6, 8, 20]

A series of 5′-deletion derivatives in and around these regions were constructed in CAT gene-containing plasmids, and the plasmid DNA was transfected into mouse NIH/3T3 cells. The CAT expression level was determined in the cell extracts prepared at 48 h after transfection. Examples of the experimental results are shown in Fig. 2A. These results, together with those of several other independent experiments, were quantified, and averaged values with standard deviations are presented in Fig. 3A. A deletion from −1852 to −1633 caused no significant change in the CAT expression level. When deletion was extended to −1616, about 1.5-fold increase of CAT expression was observed. This result indicates that a weak silencer element is present between −1633 and −1616. Seven deletion derivatives with

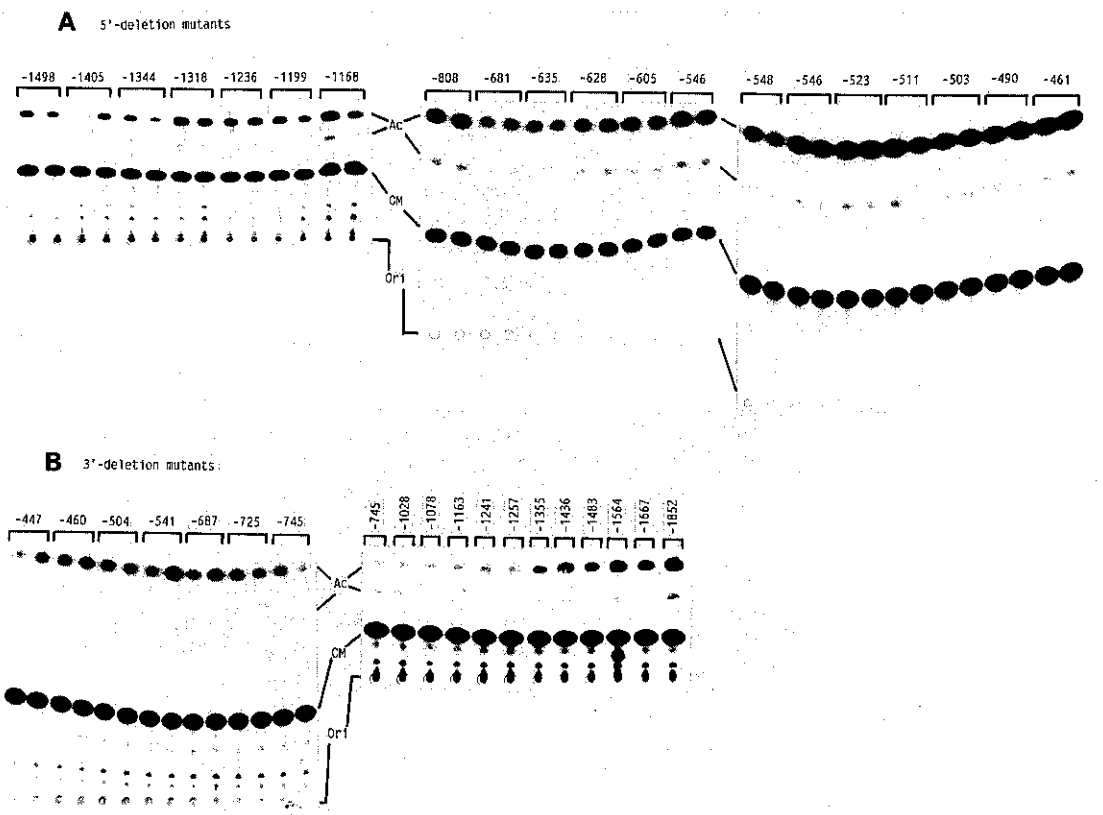

Fig. 2. Mapping of the silencer region of the DNA polymerase β gene. A: CAT activity in NIH/3T3 cells transfected with 5′-deletion mutants. 5′-Deletion break points of DNA polymerase β gene inserts of these mutants are given at the top of each lane. Acetylated and non-acetylated forms of ^{14}C-chloramphenicol are marked by Ac and CM, respectively. B: CAT activity in NIH/3T3 cells transfected with 3′-deletion mutants. 3′-Deletion break points are given at the top of each lane.

break points localized between −1616 and −1344 did not show any significant difference in CAT expression. A further deletion of 26 bp from −1344 to −1318 caused an increase of the CAT expression of about two-fold. Thus, the previously identified upstream silencer region can be divided into two sub-domains (Fig. 3B, upper).

Since no difference was seen in CAT expression of deletion mutants whose break points were −1091, −971, −910 and −828,[6] we focused our attention on the deletion derivatives whose break points reside between −808 to −342. The deletion from −808 to −681 resulted in the reduction of the CAT expression by about 60%, indicating the existence of a positively acting element, presumably an enhancer, within this region (Fig. 3A). Further deletion of the 5′-terminus up to −456 caused a gradual but large increase of the CAT expression (about 5-fold finally). Thus, the promoter proximal silencer can be mapped within the region from −681 to −456, which is further split into two domains by 34 bp of relatively neutral region between −523 and −490 (Fig. 3B).

A series of 3′-end deletion derivatives which have the DNA polymerase β gene promoter region (−100 to +33) in an intact form were also constructed to determine the 3′-borders of the silencer elements. Examples of CAT-transient expression assay in NIH/3T3 cells are shown in Fig. 2B. These results, together with several other independent experiments, were quantified, and averaged values with standard deviations are presented in Fig. 3C. The 3′-deletion mutants having break points localized between −88 and −447 did not show a change of CAT expression level. An increased level of CAT expression was observed with the deletion from −447 to −460. This 13 bp region between −447 and −460 lies close to the promoter-proximal border of the negatively acting region revealed by the analysis of 5′-deletion mutants (Fig. 3B). Although we did not succeed in making a 3′-deletion between −541 and −687, the CAT expression level did not change until −703. Deletion from −703 to −745 caused a significant decrease in CAT expression, and this positively acting region lies within the −808 to −681 region identified from the analysis of 5′-deletions (Fig. 3B). A slight increase of CAT expression was observed with the deletion between −1525 and −1564. The deletion from −1667 to −1852 also caused a further 50% increase of CAT expression level, indicating the presence of a silencer element in this region.

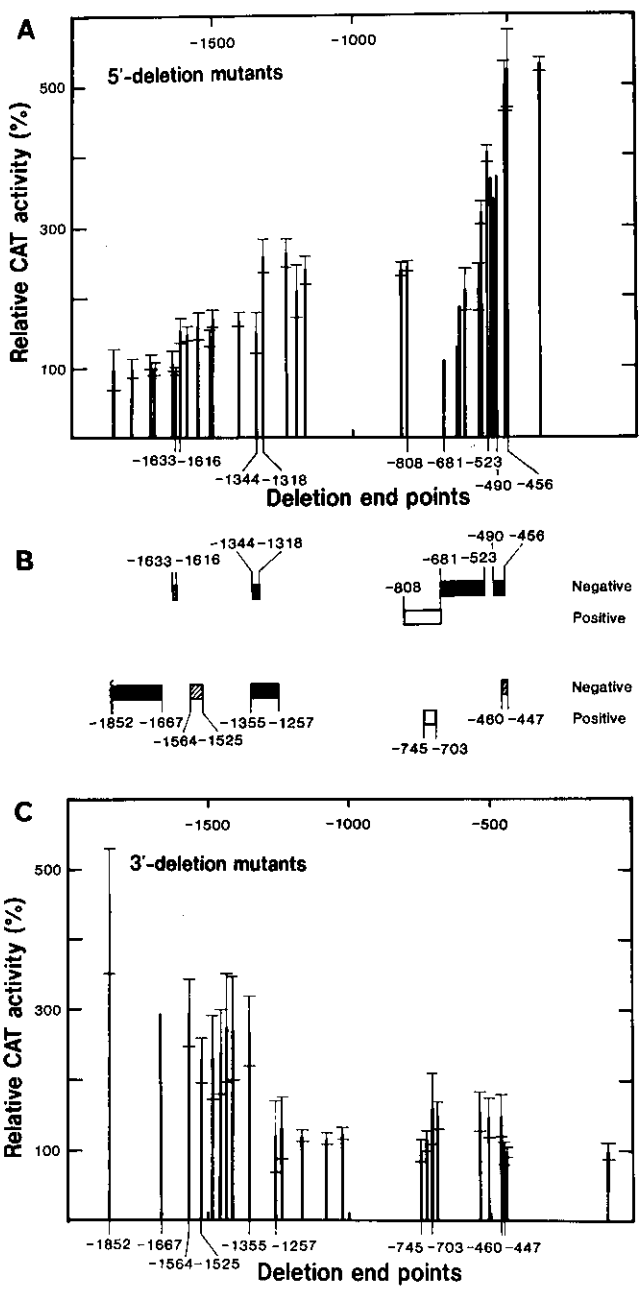

Fig. 3. Quantitative analysis of CAT-expression of deletion mutants. The radioactivities of spots corresponding to acetylated ^{14}C-chloramphenicol on thin layer chromatographic plates shown in Fig. 2 and others not shown were quantified. Values obtained from several independent experiments using 5′-end deletion derivatives (A) or 3′-end deletion derivatives (C) were averaged and are shown as fractions of the CAT expression with the original p5′−1852βCAT. The standard deviation is also shown with each mean value. B: Summary deduced from the data shown in A (upper) and C (lower). The regions which have a strong negative effect on CAT expression are shown with closed boxes and those which have weak negative effect are shown with shaded boxes. The regions which have positive effect are shown with open boxes. The nucleotide position of each border relative to the transcription initiation site is also shown.

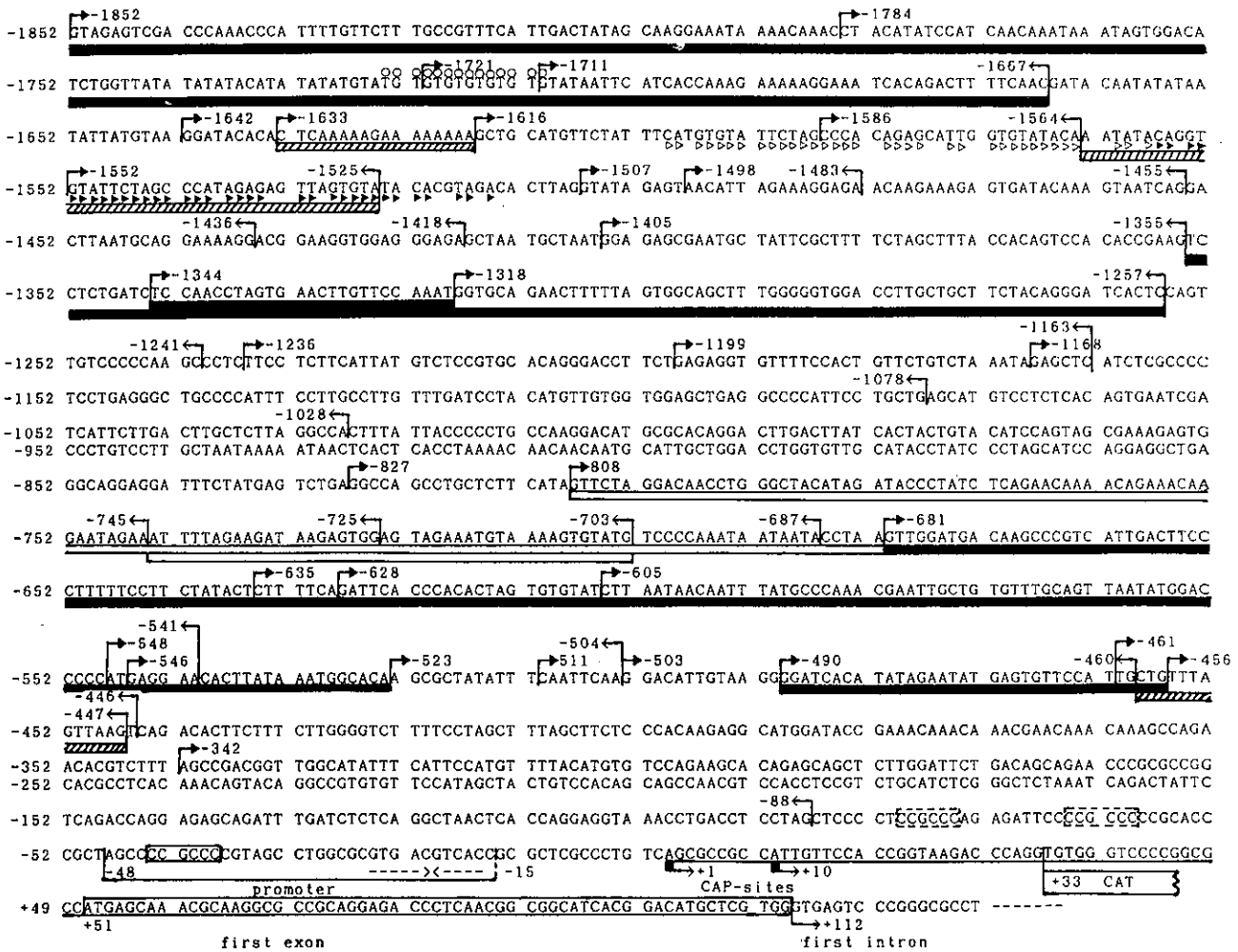

Fig. 4. Nucleotide sequence in and around DNA polymerase β gene silencer. Nucleotide +1 denotes the 5′-most proximal major cap site, and residues preceding it are indicated by negative numbers. Two major cap sites determined previously[4] are indicated by arrows at +1 and +10. The first exon is indicated by an underline and the protein coding region is indicated by an open box. The first intron starts at position +112. The numbered vertical lines with arrows indicate the positions of the 5′-break points of 5′-deletion mutants (┌→) or those of the 3′-break points of 3′-deletion mutants (←┐). Negatively acting regions are marked with closed bars (strong) or shaded bars (weak) under the sequence. Positively acting regions are marked with open bars under the sequence. The TG repeat is marked with an open circle and 43 bp direct repeats are shown by closed triangles. So-called GC-boxes are enclosed with dotted or solid boxes. The 10 bp palindromic sequence is shown by a pair of arrows. The promoter region is also indicated.

The negatively acting region together with the positively acting region deduced from 5′-deletion analysis and 3′-deletion analysis are summarized in Fig. 3B (upper and lower, respectively). From these results, we conclude that the distal silencer (silencer I) consists of four sub-domains and the promoter-proximal silencer (silencer II) consists of two sub-domains.

Nucleotide sequence analysis The nucleotide sequence of the upstream regulatory region of mouse DNA polmerase β gene containing transcriptional silencer elements was determined and is presented in Fig. 4. Nucleotide sequence analysis revealed several structural features of DNA polymerase β gene silencer elements. (1) There are 36 bases of alternating purine-pyrimidine repeats including 14 bases of alternating T and G (−1724 to −1711) in the distal silencer region. This repeat was reported to be the potential Z-DNA-forming sequence[21,22] and has been found in several transcriptional regulatory elements.[23,24] Thus, secondary structure of DNA, such as Z-form, might have a role in

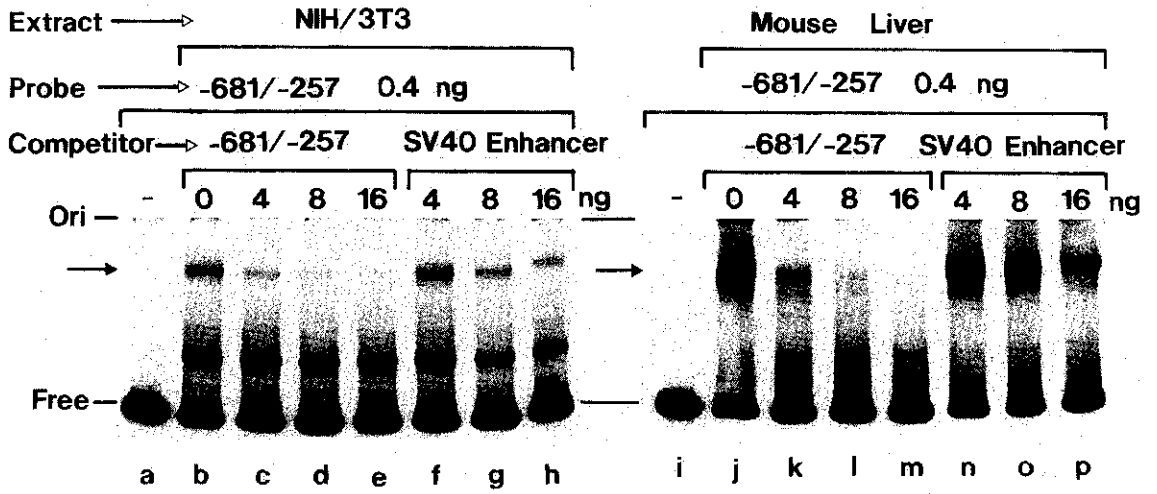

Fig. 5. Specific binding of NIH/3T3 cell and mouse liver protein(s) to the DNA fragment containing DNA polymerase β gene silencer II. The DNA fragment −681 to −257 (0.4 ng) end-labeled with ^{32}P was incubated with NIH/3T3 cell nuclear extract (lanes b to h) or mouse liver nuclear extract (lanes j to p) in the presence of indicated amounts of the unlabeled fragment −681 to −257 (lanes b to e and j to m) or the SV40 enhancer fragment (lanes f to h and n to p). DNA-protein complex was resolved by electrophoresis in a 4% polyacrylamide gel. Lanes a and i represent the radioactive probe fragment applied to the gel without nuclear extract. The arrows indicate the band representing sequence-specific DNA-protein complex.

silencer function. (2) There are 43 bp direct repeats in the distal silencer region (−1600 to −1515), although deletion of this region caused only a slight increase of CAT expression. (3) There are no extensive sequence similarities between distal silencer elements and promoter-proximal ones.

Detection of binding activity to DNA polymerase β gene silencer in NIH/3T3 cell and mouse liver nuclear extracts In order to detect a binding factor(s) to DNA polymerase β gene silencer sequence, NIH/3T3 cell nuclear extract was prepared and used in a gel-mobility shift analysis. When the 425 bp DNA fragment (−681 to −257) containing the promoter-proximal silencer was used as a probe, several slowly migrating bands were detected (Fig. 5, lane b). The addition of the unlabeled 425 bp fragment in the binding reaction progressively abolished the formation of the most slowly migrating band, but no other band disappeared. In contrast, a 187 bp DNA fragment containing SV40 enhancer (nucleotide positions 108 to 294 of SV40 DNA), whose nucleotide sequence is not extensively related to that of the DNA polymerase β silencer, showed only marginal competition to the DNA-protein complex formation. Therefore, the most slowly migrating band represents a specific complex formed by protein factor(s) and the promoter-proximal silencer sequence.

In the previous studies,[3,8] we observed that the level of DNA polymerase β mRNA in mouse liver was about 20% of that in NIH/3T3 cells and was the lowest among mouse tissues so far examined. This fact suggests that the binding protein to DNA polymerase β gene silencer is more abundant in liver than in NIH/3T3. The nuclear extract from mouse liver generated the DNA-protein complex with the 425 bp DNA fragment (Fig. 5, lane j). As is the case with NIH/3T3 cell nuclear extract, the most slowly migrating band represents the specific complex formed by protein factor(s) and the promoter-proximal silencer, which was proved by the competition analysis (Fig. 5, lanes j to p). The intensity of the band representing the specific complex relative to those of non-specific complexes in the low-molecular-weight region is much higher with the liver extract than with the NIH/3T3 cell extract, suggesting that the concentration of the silencer-binding protein is higher in liver extract than in NIH/3T3 cell extract.

When the 341 bp fragment (−1504 to −1164), which contains the proximal sub-domain of the distal silencer element, was used as a probe in the gel-mobility shift analysis, a faint, slowly migrating band was detected with both NIH/3T3 cell and liver extracts (Fig. 6). The SV40 enhancer fragment did not compete (data not shown), indicating that this band also resulted from a specific interaction between a protein factor(s) and the distal silencer sequence. Interestingly, the unlabeled DNA fragment containing the promoter-proximal silencer sequence (−681 to −257) competed more effectively than the distal silencer sequence itself (−1504 to −1164) (Fig. 6). Conversely, the −1504/−1164 fragment

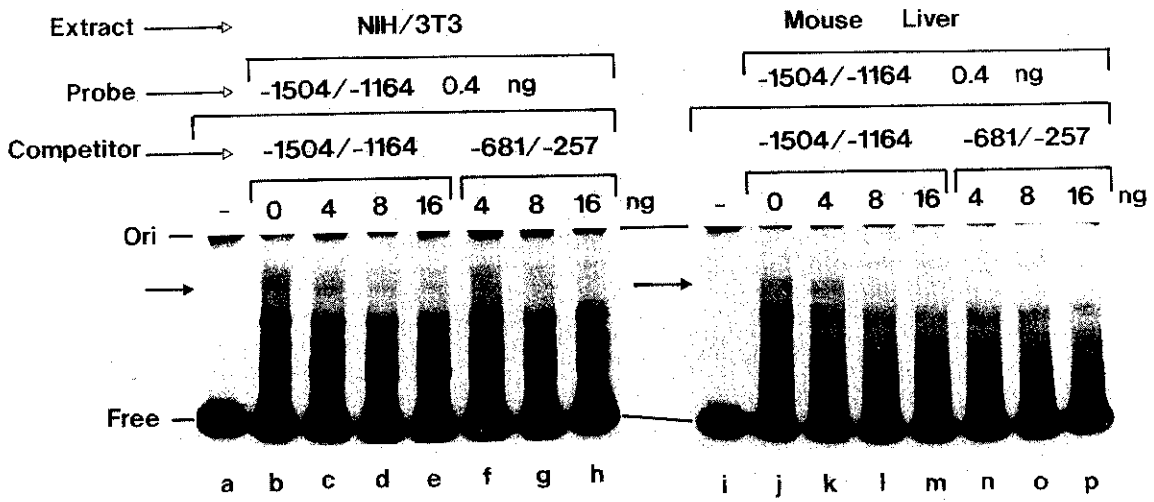

Fig. 6. Effect of silencer II fragment on the complex formation between silencer-binding factor(s) and the silencer I fragment. The DNA fragment −1504 to −1164 (0.4 ng) end-labeled with ^{32}P was incubated with NIH/3T3 cell nuclear extract (lanes b to h) or mouse liver nuclear extract (lanes j to p) in the presence of the indicated amounts of the unlabeled fragment −1504 to −1164 (lanes b to e and j to m) or the fragment −681 to −257 (lanes f to h and n to p). The DNA-protein complex was resolved by electrophoresis in a 4% polyacrylamide gel. Lanes a and i represent the radioactive probe fragment applied to the gel without nuclear extract. The arrows indicate the band representing sequence-specific DNA-protein complex.

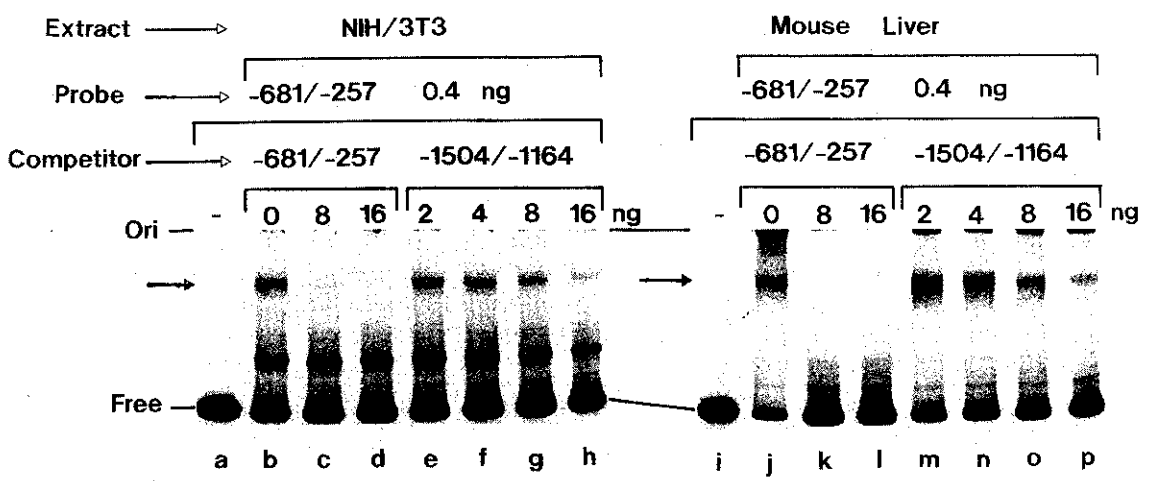

Fig. 7. Effect of silencer I fragment on the complex formation between silencer-binding factor(s) and the silencer II fragment. The DNA fragment −681 to −157 (0.4 ng) end-labeled with ^{32}P was incubated with NIH/3T3 cell nuclear extract (lanes b to h) or mouse liver nuclear extract (lanes j to p) in the presence of the indicated amounts of the unlabeled fragment −681 to −257 (lanes b to d and j to l) or the fragment −1504 to −1164 (lanes e to h and m to p). DNA-protein complex was resolved by electrophoresis in a 4% polyacrylamide gel. Lanes a and i represent the radioactive probe fragment applied to the gel without nuclear extract. The arrows indicate the band representing sequence-specific DNA-protein complex.

hardly interfered with the formation of the complex between the protein factor(s) and the $-681/-257$ fragment at the concentration where the interference by the own $-681/-257$ fragment was observed (Fig. 7). These results suggest that the same factor(s) can bind to both DNA fragments containing sequences of distal and proximal silencers, but the affinity for the latter is much higher than that for the former.

DISCUSSION

Detailed analysis of the 5′-flanking region of DNA polymerase β gene has revealed that the silencer of this gene consists of long-dispersed multiple sub-elements. Yeast silencer involved in repression of the mating-type loci also consists of three sub-elements.[25] One of these elements functions as an ARS (autonomous replication sequence) and another has an ability to activate transcription (UAS: upstream activating sequence). It might be interesting to examine the properties of each DNA polymerase β gene silencer element, such as replication ability in mammalian cells, although no extensive sequence homology with the yeast silencer was observed. Complex sub-domain structures were observed in various viral and cellular enhancers,[25-27] and might represent general properties of distal transcription regulatory elements of eukaryotic genes. Deletion of distal silencer elements (-1852 to -1257) in the presence of promoter proximal silencer elements (-681 to -456) only moderately increased the CAT expression. Conversely, the deletion of proximal silencer elements, leaving distal silencer elements and promoter intact, only slightly increased the CAT expression. Thus, these two silencer regions function redundantly to each other in NIH/3T3 cells.

In addition to NIH/3T3 cells, we have observed a similar extent of DNA polymerase β gene silencer function in HeLa cells.[20] However, none of these silencer elements functions efficiently in mouse neuroblastoma N18TG2 cells, in which a much higher level of DNA polymerase β mRNA is expressed than that in NIH/3T3 cells.[8] A clear inverse correlation between expression level of DNA polymerase β mRNA and the extent of silencer function suggests that silencer elements play an important role in controlling the cell type-specific expression of DNA plymerase β gene.

Computer-assisted DNA sequence searching was employed to compare the silencer region of DNA polymerase β gene with the yeast silencer region,[28] the c-*myc* silencer region,[29] the chicken lysozyme gene silencer region,[30] and the undifferentiated F9 cell-specific silencer.[31] No extensive homology has so far been detected with yeast silencer or chicken lysozyme gene silencers. However, significant similarity was observed between the promoter proximal portion of the DNA polymerase β gene silencer II (-470 to -458) and the silencer element (5′-GCATTCCATTGTT) derived from mouse F9 cell chromosomal DNA.[31] Ten out of thirteen nucleotides are identical (Fig. 8).

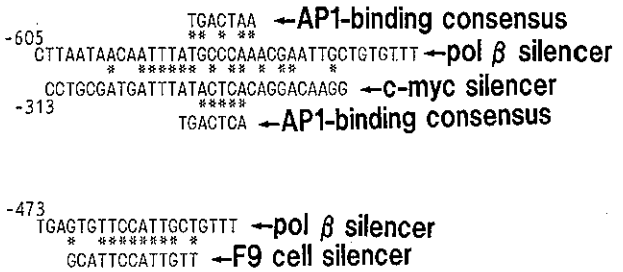

Fig. 8. Homology between mouse DNA polymerase β silencer sequence and human c-*mcy* gene silencer of F9 cell silencer. Sequences similar to the consensus sequence for the transcription factor AP1 (5′-TGACTA/CA-3′)[34] are also indicated.

Significant similarity was also observed between the distal portion of the silencer II region (-604 to -575) of DNA polymerase β gene and the minimum silencer sequence of the human c-*myc* gene (-243 to -313 with respect to the cap site of c-*myc* mRNA)[29] (Fig. 8). Fourteen out of 30 nucleotides are identical, and in particular 7 nucleotides of AT-rich sequence (5′-ATTTATA-3′) are almost completely conserved between these two sequences. Flanking this AT-rich sequence there are seven nucleotides which are not identical but closely related to the AP-1 (c-Fos/c-Jun) binding consensus[32] in both c-*myc* and DNA polymerase β gene. Although only 5 out of 7 nucleotides match the AP-1-binding consensus, the c-Fos/c-Jun complex has been reported to bind to the c-*myc* gene sequence.[32,33] Thus, c-Fos protein in combination with c-Jun may participate in the DNA-protein complex with the DNA polymerase β gene silencer sequence as in the case of the c-*myc* gene. Slight inhibition of the complex formation by SV40 enhancer (Fig. 5) might support this idea, since it contains AP-1 (c-Jun) binding site. We have detected a binding protein(s) which has an affinity to both distal and proximal silencer regions. Our preliminary data using anti-c-Fos antibody suggested the involvement of c-Fos-related protein in the DNA-protein complex formation with the DNA polymerase β gene silencer (our unpublished data). Since this silencer-binding factor(s) appears to exist more abundantly in mouse liver than in NIH/3T3 cells, the factor(s) may be related to but distinct from the c-Fos protein. Further studies on this binding factor would be important for understanding the molecular mechanisms of silencer functions.

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