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# The First Exon of the Rat Aldolase C Gene Is Essential for Restoring the Chromatin Structure in Transgenic Mice

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**A 13-kb fragment of the rat aldolase C gene contains sufficient information for gene expression. Transgenic mice carrying the 13-kb fragment showed restoration of chromatin structure and tissue-specific, copy number-dependent expression. To localize the regulatory elements responsible for restoring chromatin structure, several mutated constructs were used to produce transgenic mice. Three activities were examined: recreation of DNase hypersensitive sites, restoration of methylation status, and copy number-dependent expression. Deletions of the 3′-flanking region did not affect those activities. Deletion of seven introns affected the mRNA levels but not the restoration of the chromatin structure. The insertion of the LacZ gene into the first exon of the transgene interfered with both the restoration of the chromatin structure and the copy number-dependent expression in transgenic mice. DNase I footprinting assays revealed that brain-specific factors bind to the sequence disrupted by the LacZ insertion. These results suggest that the sequence in the first exon is essential for restoring the chromatin structure of the rat aldolase C gene.**

**Key words: aldolase C, central nervous system, chromatin structure, copy number-dependent expression, transgenic mice.**

In higher eukaryotes, multiple specialized cells are derived from a single fertilized egg. This variation of cells is achieved by the selective expression of genomic information, mainly by transcriptional regulation, which is complicated due to the complexity of the chromatin structure involved. Thus, besides the regulatory elements such as the promoter, enhancer, and silencer, regulatory elements for opening the chromosomal domain are necessary for eukaryotic gene expression. The most extensively studied regulatory region of the chromosomal domain is the locus control region (LCR) of the human $\beta$-globin gene cluster (*1*). Such regulatory regions of several genes have been reported to date (*1-15*). However, the mechanism for opening chromosomal domains remains to be elucidated.

Aldolase C is one of the aldolase isozymes, expressed mainly in the brain (*16*). This enzyme is expressed mostly in Purkinje cells and to some extent in other neurons and glial cells (*17-19*). Analyses of the promoter region of the aldolase C gene have demonstrated that the promoter consists of non-TATA and TATA-like sequences, and that there are multi-initiation sites (*20, 21*). A constitutive enhancer element was recently found in a (G+C)-rich motif of the aldolase C promoter (*22*). Analyses of transgenic mice showed that the 5′-flanking region is responsible for the brain-specific expression of aldolase C (*9, 23, 24*). We have also suggested that the 13-kb region of the intragenic and flanking sequences of the rat aldolase C gene comprises sufficient information for gene expression (*9*). The 13-kb transgene was insulated from position effects and showed copy number-dependent, high-level expression as well as correct regional expression in the central nervous system (CNS). The methylation status of the transgene was also restored to that observed in the rats.

In the present study, to localize the regulatory element(s) responsible for insulating the rat aldolase C transgene, we used several constructs to produce transgenic mice. We examined the activity for insulating the transgene by following three criteria: (i) the recreation of DNase I hypersensitive sites (DNase HSSs), (ii) the restoration of methylation status, and (iii) copy number-dependent expression. Deletions of the 3′-flanking region did not affect the three activities. Deletion of seven introns did not affect the restoration of chromatin structure, but altered the mRNA levels. In contrast, the insertion of the LacZ gene into the first exon interfered with these activities. The sequence disrupted by the insertion was recognized by brain-specific factors. These results suggest that the disrupted sequence in the first exon is essential for the organization of the chromatin structure, as well as for conferring copy number-dependent expression on the transgene.

## MATERIALS AND METHODS

*Plasmids*—The construct, 6-7RAC, includes a 13-kb fragment of the rat genomic sequence and an insertion of the 95-bp *Sma*I-*Pvu*II fragment from pUC9 into a unique *Eco*RV site in the ninth exon (see Fig. 2A). The construct

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Abbreviations: DNase HSS(s), DNase hypersensitive site(s); CNS, central nervous system; LCR, locus control region.

was cloned into *Sal*I and *Eco*RI sites of pBluescript (Stratagene, La Jolla, CA, USA), which generated the plasmid designated pRACXR13$\alpha$ (*9*). A unique *Kpn*I site in the vector sequence of the plasmid pRACXR13$\alpha$ was replaced by *Not*I linker, and the plasmid was designated as p6-7RAC. To construct three different rat aldolase C transgenes with a series of 3′-flanking deletions (see Fig. 2, B-D), a 3.7-kb *Eco*RI fragment that contains a portion of the ninth exon and the 3′-flanking region (from positions +3300 to +7036: relative to the initiation site [+1]) was purified from p6-7RAC and subcloned into the *Eco*RI site of pBluescript (designated pRACR4). The 2.7-kb *Eco*RI-*Bam*HI (from positions +3300 to +5994), 1.4-kb *Eco*RI-*Hinc*II (from positions +3300 to +4694), and 0.4-kb *Eco*RI-*Bgl*II (from positions +3300 to +3737) fragments were purified from the plasmid pRACR4 by digesting with appropriate restriction enzymes. These fragments were substituted for the 3.7-kb *Eco*RI fragment of the p6-7RAC by using the *Eco*RI linker, and were designated p6-6RAC, p6-5RAC, and p6-4RAC, respectively. In plasmid p6-7RAC(cDNA), seven introns of p6-7RAC were removed by replacing sequences from the *Eco*52I site (position +69) in the first exon to the *Apa*I site (position +2927) in the eighth exon with the corresponding cDNA fragment (*25*) (see Fig. 2E). The eighth intron was left intact in this plasmid. Plasmid p6RAC-LacZ includes a 5.6-kb fragment of the 5′-flanking region and the 74-bp first exon of the rat aldolase C fused to the bacterial LacZ reporter gene between *Hin*dIII and *Bam*HI sites of pBluescript (*9*). Plasmid p6-7RAC(LacZ) was constructed by inserting the 7-kb *Eco*52I fragment (from positions +69 to +7036) of the p6-7RAC into the *Not*I site of p6RAC-LacZ with the same orientation. The 6-bp direct repeat (from positions +69 to +74) was created at both sites of the LacZ gene by the construction of this transgene (see Figs. 2F and 9). The plasmid pRACS558 is comprised of the 558-bp *Sma*I fragment (from positions −457 to +101) of the rat aldolase C gene in the *Sma*I site of pBluescript.

*Production and Identification of Transgenic Mice*—The 6-7RAC, 6-6RAC, 6-5RAC, 6-4RAC, 6-7RAC(cDNA), and 6-7RAC(LacZ) constructs were isolated from corresponding plasmids by removing vector sequences with appropriate restriction enzymes. These DNA fragments were purified from agarose gel and suspended in 10 mM Tris-HCl (pH 7.5), 0.1 mM EDTA. Fertilized eggs (BDF1; [C57BL/6×DBA2]F1) were recovered and microinjected as described previously (*26*). The surviving embryos were transferred to the oviducts of pseudopregnant ICR mice. Transgenic founder mice were identified by Southern blot analyses and were used to generate transgenic progenies.

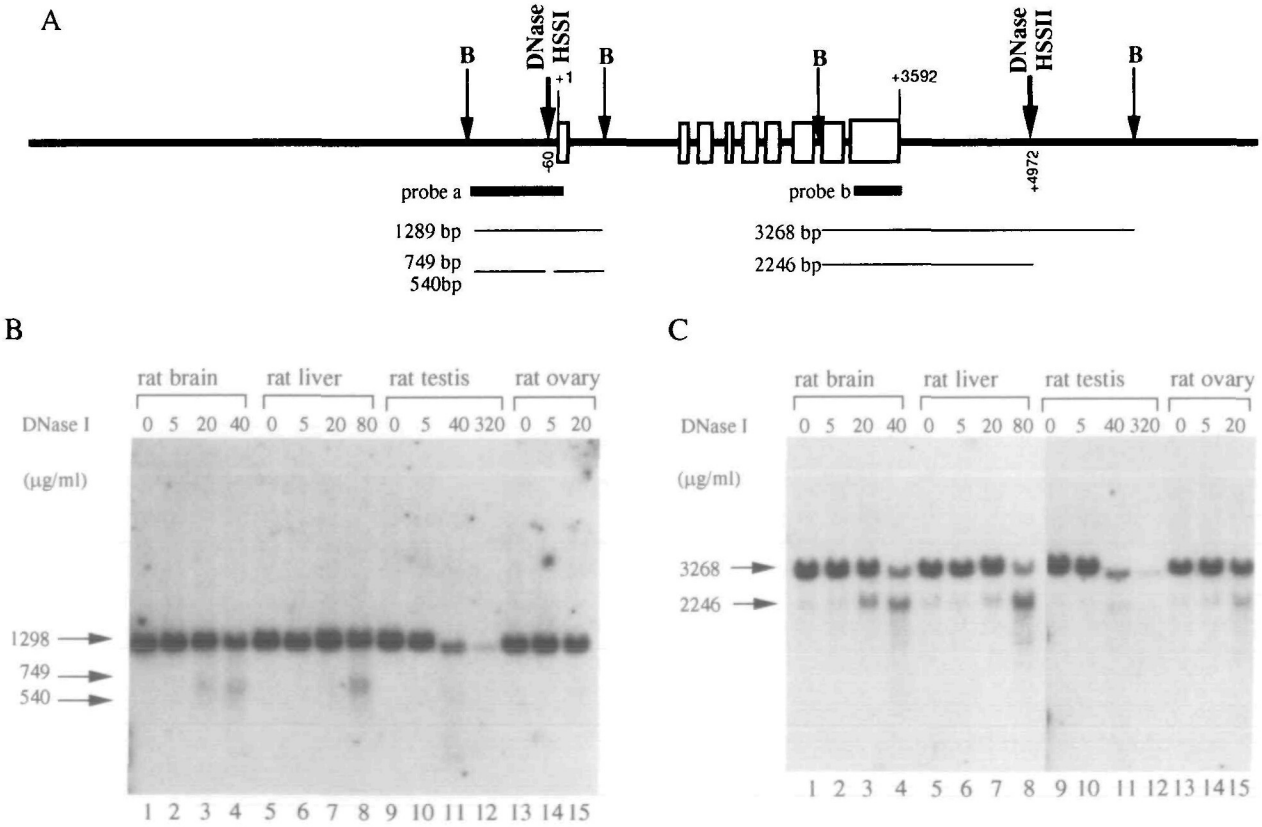


Fig. 1. **DNase I hypersensitive sites (DNase HSSs) of the rat aldolase C gene.** (A) Schematic representation of the rat aldolase C gene and two DNase HSSs detected in the present study. Thick lines indicate flanking regions and introns. Open boxes indicate exons of the rat aldolase C gene. Numbers with plus (+) or minus (−) indicate relative distances from the transcriptional start site (+1). Thick vertical arrows with DNase HSSI or II indicate DNase HSSs. Thin vertical arrows with B indicate *Bam*HI sites. Thick horizontal bars with probe a or b indicate probes for detecting DNase HSSs upstream or downstream, respectively. Thin horizontal bars with numbers indicate the detected sizes of fragments digested with *Bam*HI in the absence or presence of DNase I. (B and C) Analyses of upstream and downstream DNase HSS, respectively. Nuclei were digested with the amounts of DNase I ($\mu$g/ml) indicated at the top of the figure. Sizes of fragments are indicated on the left next to the arrows.

*Southern Blot Analyses*—Genomic DNA (5 $\mu$g) was digested with appropriate restriction enzymes and separated by agarose gel electrophoresis. When genomic DNA was digested with each restriction enzyme, pBluescript or $\lambda$ DNA was added to aliquots of the reaction mixture, and complete digestion was verified in each reaction. Southern blot analyses were performed as described (*9*). Hybridization was carried out in a solution of 7% polyethylene glycol containing 10% sodium dodecyl sulfate (SDS), 5×Denhardt's solution, 100 $\mu$g/ml of heat-denatured salmon sperm DNA and $^{32}$P-labeled probe DNA. After hybridization, the membrane was washed at a final stringency of 0.1×standard saline citrate and 0.1% SDS at 65°C. The transgene copy number was determined by hybridizing an appropriate probe(s) to the transgene and endogenous mouse gene in blots as described (*9*). The copy number standards contain the equivalent of 1, 5, 10, 20, 40, and 100 copies of each construct in 5 $\mu$g of genomic DNA from a C57BL/6 mouse. The radioactivity obtained by autoradiography was quantified using a Fuji Bio-Image Analyzer.

*Determination of DNase I Hypersensitive Sites*—Minced tissues were brought up to 10 ml/g-tissue with homogenization buffer (50 mM Tris-HCl [pH 8.0], 100 mM KCl, 5 mM $MgCl_2$, 50% glycerol, 0.05% saponin, 200 mM $\beta$-mercaptoethanol) and homogenized with a motor-driven Teflon-glass homogenizer. The homogenate was layered over 20-ml cushions of the same buffer in 50-ml centrifuge tubes and centrifuged at 2,000×*g* for 15 min at 4°C. The nuclear pellet was resuspended with digestion buffer (50 mM Tris-HCl [pH 8.0], 100 mM NaCl, 3 mM $MgCl_2$, 0.1 mM phenylmethylsulfonyl fluoride [PMSF]), then repelleted by centrifugation at the same speed for 5 min at 4°C. The nuclear pellet was then resuspended at the concentration of 10 $A_{260}$ with digestion buffer. Differing concentrations of DNase I (40 $\mu$l) were added to 360 $\mu$l aliquots of nuclear suspension. These reaction mixtures were incubated for 5 min at 37°C and the reaction was terminated by the addition of 40 $\mu$l of stop solution (0.25 M EDTA, 2.5% SDS, 1 mg/ml proteinase K). The samples were incubated for 3 h at 60°C with gentle shaking, and nucleic acids were extracted with phenol/chloroform and chloroform, followed by ethanol precipitation. The precipitate was resuspended in TE8 (10 mM Tris-HCl [pH 8.0], 0.1 mM EDTA) containing 100 $\mu$g/ml of RNase A and incubated for 3 h at 37°C. After incubation, DNA was re-extracted and resuspended in TE8. The sensitivity of nuclei to DNase I was

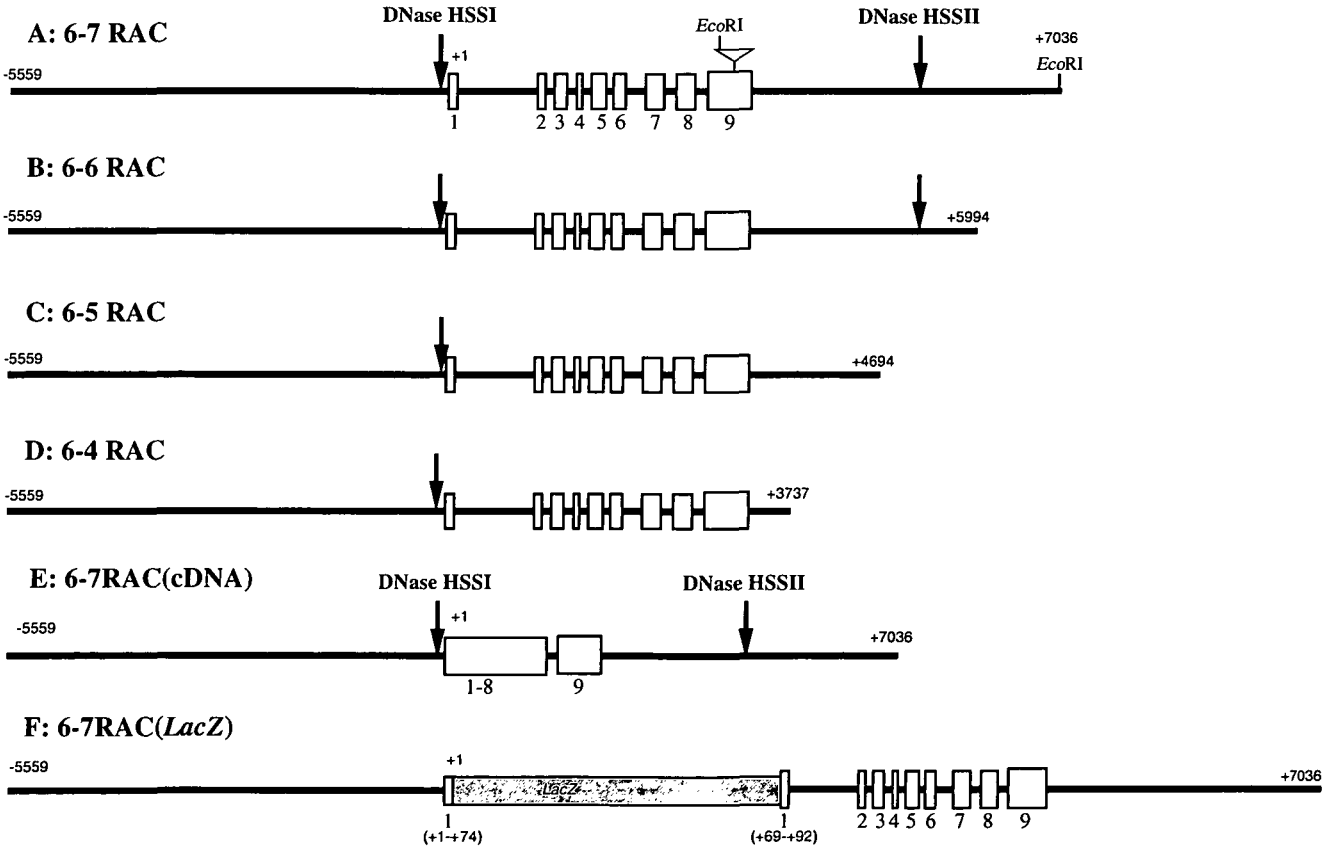


Fig. 2. **Schematic representation of the rat aldolase C constructs.** Symbols, abbreviations, and numbers are as shown in Fig. 1A. Numbers under the exons in panels A, E, and F indicate exon numbers. The open box with 1-8 in panel E indicates a fusion exon from exons 1 to 8 ("MATERIALS AND METHODS"). Open boxes with 1 (+1-+74) and 1 (+69-+92) in panel F indicate two divided parts of exon 1. The 6-bp direct repeat (positions +69 to +74) was created at both sides of the LacZ reporter gene in the 6-7RAC(LacZ) construct (see Fig. 9). The shaded box in panel F indicates the LacZ reporter gene. In panels E and F, the numbers for relative distance allow positions to be referred to in terms of intact genomic sequences, even for constructs with deleted introns or an inserted LacZ gene. The triangle on exon 9 of the 6-7RAC construct in panel A indicates the inserted DNA derived from pUC9 to distinguish the transgene from the endogenous mouse gene (*9*). *Eco*RI sites are also indicated on the 6-7RAC. All constructs contain the same insert at the identical site. Vertical arrows indicate two DNase hypersensitive sites at expected sites.

different among tissues. The extent of DNA digestion was examined by electrophoresis on an agarose gel, stained with ethidium bromide.

*S1 Nuclease Analyses*—RNA was prepared by the acid guanidium method (*27*). Total RNA (40 $\mu$g) was hybridized with a 5′-labeled cDNA probe (see Fig. 7A) at 50°C in 10 $\mu$l of hybridization solution (40 mM PIPES [pH 4.6], 1 mM EDTA, 400 mM NaCl, 80% deionized formamide) for 3 h. After hybridization, 300 $\mu$l of digestion solution (280 mM NaCl, 50 mM sodium acetate [pH 4.6], 4.5 mM $ZnSO_4$, 20 $\mu$g of heat-denatured salmon sperm DNA, 300 units of S1 nuclease [Takara Shuzo, Kyoto]) was added to the hybridization solution, and the mixtures were incubated for 40 min at 40°C. This condition is suitable to detect products from both the transgene and endogenous mouse gene (see Fig. 7B) (*9*). After extraction, samples were analyzed on a 5% polyacrylamide-7.3 M urea gel. To estimate the detected intensity, the radioactivity obtained by autoradiography was quantified using a Fuji Bio-Image Analyzer.

*Measurement of β-Galactosidase Activity*—Brains from mice were suspended in buffer-I (250 mM Tris-HCl [pH 8.0], 5 mM dithiothreitol, 15% glycerol, 0.1 mM PMSF) and homogenized with a motor-driven Teflon-glass homogenizer. The homogenates were sonicated for 4 min (intensity 4, percent duty cycle 50) on ice-water. The samples were centrifuged at 10,000×*g* for 10 min at 4°C. One milliliter of reaction buffer (60 mM $Na_2HPO_4$, 40 mM $NaH_2PO_4$, 10 mM KCl, 1 mM $MgCl_2$, 50 mM β-mercaptoethanol) and 0.1 ml of *o*-nitrophenyl-β-D-galactoside (4 mg/ml in 60 mM $Na_2HPO_4$ and 40 mM $NaH_2PO_4$) were added to 0.1 ml of the resulting supernatants. The reaction was carried out at 37°C, and the change of absorption at 420 nm was monitored with a DU-65 spectrophotometer operating with a Kinetics Soft-Pac module (Beckman Instruments, Fullerton, CA, USA).

*DNase I Footprinting*—Nuclear extracts from rat brain and liver were prepared as described (*28*).

The plasmid pRACS558 was digested with *Hin*dIII. The fragment was labeled at the 3′ ends with Klenow fragment and [$^{32}$P]dCTP. The end-labeled fragment was digested with *Bam*HI and resulting fragments were separated by electrophoresis through a 2% agarose gel. The probe DNA was collected on a sliver of DEAE-cellulose membrane by electrophoresis and was eluted from the membrane in a buffer of high ionic strength (*29*).

The standard reaction consists of the following compo-

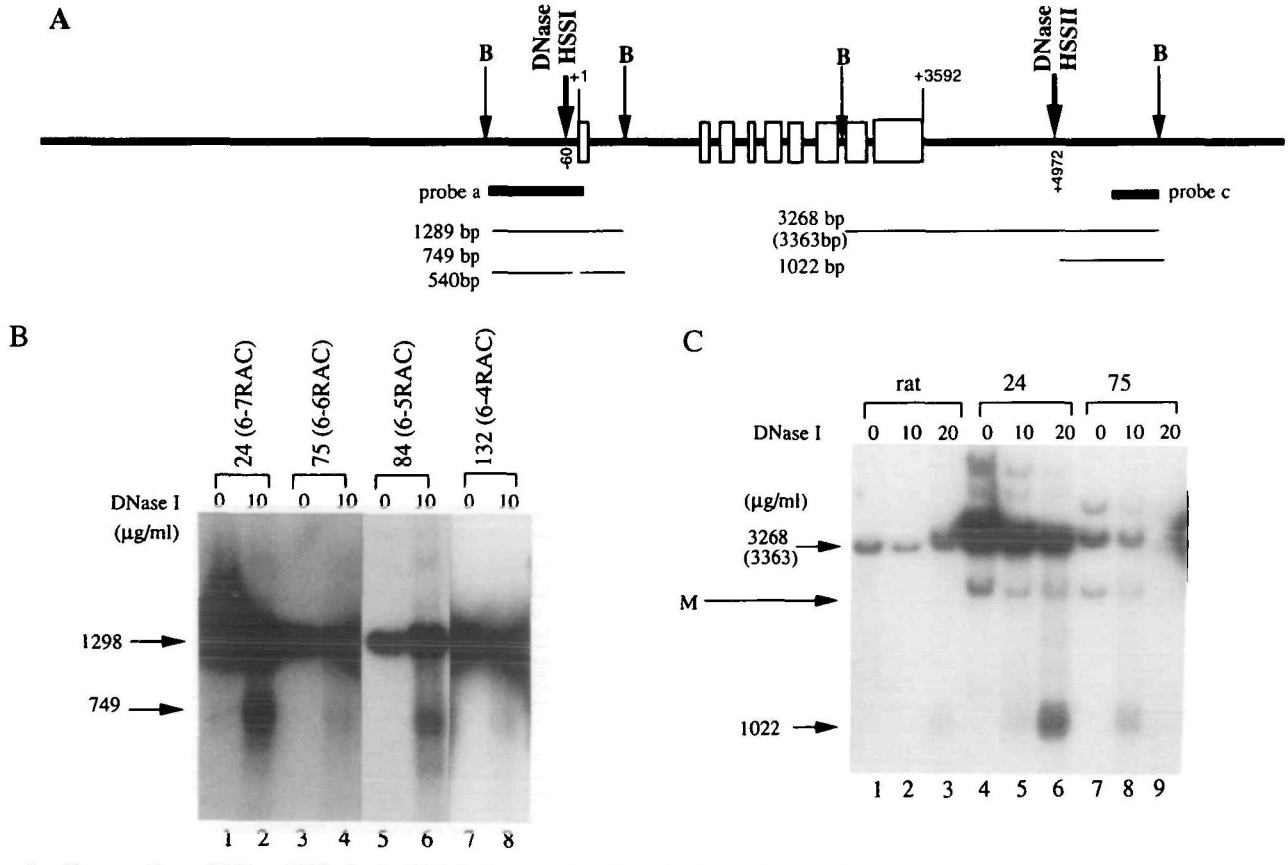


Fig. 3. **Restoration of DNase HSSs in the 3′-deletion constructs.** (A) Schematic representation of DNase HSSI and II at expected sites. Symbols, abbreviations, and numbers are as in Fig. 1A. The bar with probe a or c indicates the probe used in panels B or C, respectively. Thin horizontal bars with numbers indicate the expected sizes of fragments digested with *Bam*HI in the absence or presence of DNase I when DNase HSSs were recreated. The size of the *Bam*HI fragment derived from the transgene was estimated as 3,363 bp because there is a 95-bp insert in the ninth exon ("MATERIALS AND METHODS"), indicated in parentheses. (B and C) Examinations of DNase HSSI and II, respectively. Brain nuclei of animals were digested with DNase I as indicated at the top of the panels. The sizes of fragments are indicated next to the arrows at the left of the panels. In panel B, the length of exposure was varied to facilitate visualization of bands. In panel C, the band derived from the endogenous mouse gene is indicated by the "M" arrow. Rearranged fragments (high molecular weight) are also observed in the lanes of transgenic mice.

nents in a final volume of 20 $\mu$l: 10 mM HEPES (pH 7.5), 50 mM KCl, 0.1 mM EDTA, 0.5 mM DTT, 0.5 mM PMSF, 4 mM $MgCl_2$, 4 mM spermidine, 10% glycerol, 500 ng of salmon sperm DNA, 1-2 ng of end-labeled DNA fragment, and up to 6 $\mu$l of nuclear extract. The extract was preincubated with 500 ng of salmon sperm carrier DNA in the reaction mixture for 15 min at 0°C, after which the end-labeled fragment was added and the mixture was incubated for an additional 15 min at 0°C. Freshly diluted DNase I (12 ng) was added and digestion was allowed to proceed for 1 min at room temperature. The reactions were stopped by the addition of 2 volumes of 50 mM EDTA, 0.2% SDS, 100 $\mu$g/ml of tRNA, and 100 $\mu$g/ml proteinase K. The mixtures were incubated for 45 min at 42°C. Nucleic acids were extracted with 1 volume of phenol : chloroform (1 : 1), ethanol-precipitated, dissolved in 90% formamide and 10 mM EDTA with tracking dyes, heated at 90°C for 5 min, and loaded on 8% polyacrylamide-7.3 M urea gels.

## RESULTS

*Two DNase I Hypersensitive Sites Surround the Rat Aldolase C Gene*—Since several chromosomal domain regulatory regions are associated with tissue-specific DNase I hypersensitive sites (DNase HSSs) (*30, 31*), we examined DNase HSSs surrounding the rat aldolase C gene (Fig. 1). Nuclei were isolated from rat tissues and digested with different concentrations of DNase I to obtain similar extents of digestion, as described in "MATERIALS AND METHODS." Purified DNAs were subsequently digested with *Bam*HI and assayed by Southern hybridization (Fig. 1). Two DNase HSSs (DNase HSSI and II) were found in the 5′- and 3′-flanking regions, respectively. They were mapped at the indicated sites by combinatorial analyses with probes and restriction enzymes (Fig. 1A). The DNase HSSI was located at the promoter region of the gene (surrounding position −60). With increasing amount of DNase I, a fragment of about 750 bp was observed in both the brain and liver (Fig. 1B, lanes 3, 4, and 8). A fragment of about 540 bp was also detected, but was faint due to the small extent of hybridization with the probe (Fig. 1, A and B). In the testis and ovary, DNase HSSI was not found under the conditions in which DNase HSSII could be detected (Fig. 1, B and C, lanes 11 and 15). The DNase HSSII was located at the 3′-flanking region around position +4972. A digested fragment (about 2,246 bp) was detected in all tissues examined (Fig. 1C). Faint fragments (about 2,250 bp) were observed in some tissues treated with a low concentration of DNase I (Fig. 1C, lanes 1, 2, 5, 6, 13, and 14). In these tissues, endogenous nucleases probably digested DNase HSSII to some extent. This site was also confirmed by the detection of a 1,022-bp fragment

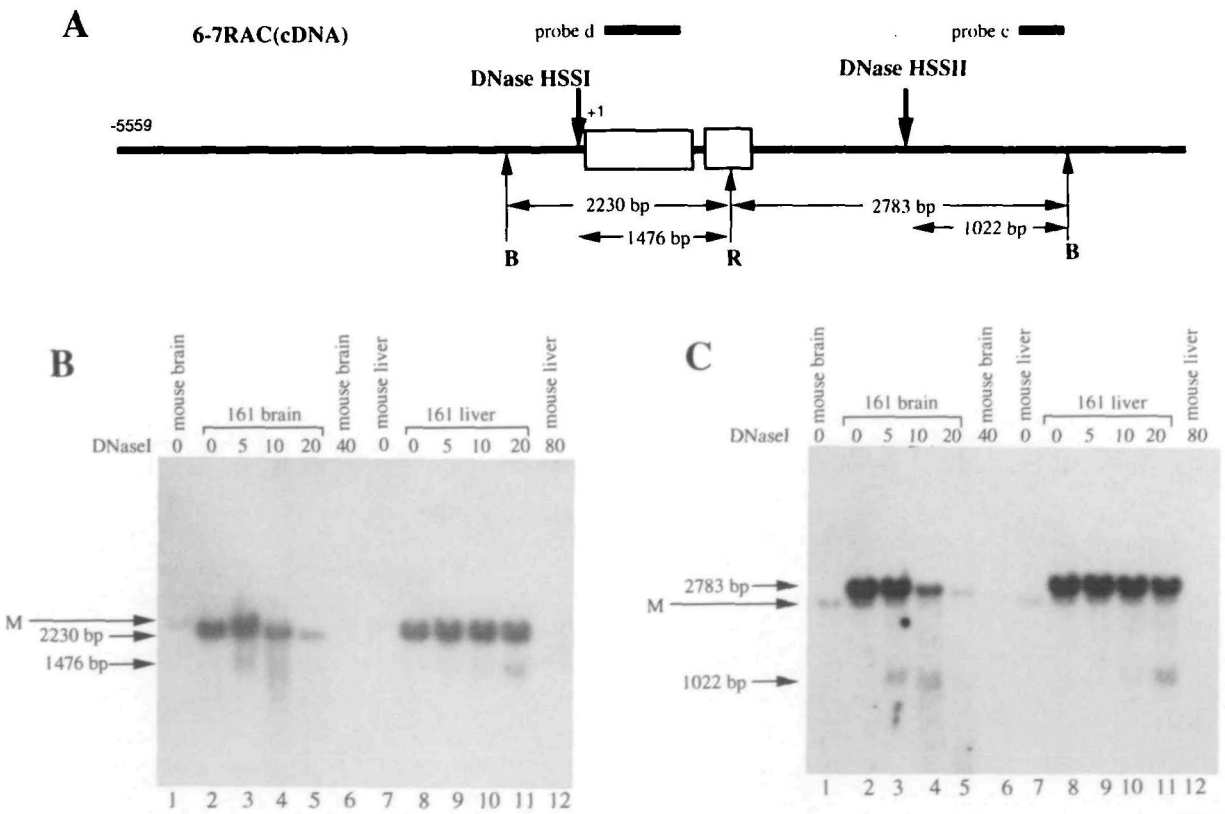


Fig. 4. **Restoration of DNase HSSs in Tg161 carrying the 6-7RAC(cDNA).** (A) Schematic representation of DNase HSSI and II at the expected sites. Open boxes are the same as shown in Fig. 2E. Bars with probe d or c indicate probes used in panels B or C, respectively. Thick downward and thin upward pointing arrows indicate DNase HSSs and the recognition sites of enzymes (*Eco*RI or *Bam*HI), respectively. Sizes of expected fragments are indicated between sites. (B and C) Analyses of DNase HSSI and II, respectively. Brain and liver nuclei of animals were digested with DNase I as indicated at the top of the panels. Purified DNAs were digested with *Eco*RI and *Bam*HI. Fragments are indicated by arrows with sizes at the left of the panels. The bands derived from the endogenous mouse gene are indicated by the "M" arrows.

hybridized with a downstream probe (Fig. 3C, lane 3). There was no detectable DNase HSS in the intragenic region (data not shown).

*Two DNase HSSs Were Recreated in the 3′-Deletion and cDNA Constructs, but Not in the Construct Comprising the Disrupted First Exon by a LacZ Insertion*—Several chromosomal domain regulatory regions are hypersensitive to cleavage by DNase I (*31*), and our previous study suggested that such a regulatory region of the rat aldolase C gene is located downstream of the initiation site (*9*). Therefore, DNase HSSII seems to be one of the candidates for the chromosomal domain regulatory region. To investigate whether DNase HSSII is responsible for the regulation of chromosome structure, we produced transgenic mice using three constructs with a series of 3′-flanking deletions (Fig. 2, B-D) and examined the chromatin opening activity (Fig. 3). As shown in Fig. 3B, the expected DNA fragments were detected by digestion with DNase I, indicating that DNase HSSI was recreated in all the integrated transgenes with a series of 3′-deletions (6-7RAC, 6-6RAC, 6-5RAC, and 6-4RAC). In the analysis of DNase HSSII, the expected fragments were also detected in transgenic mice carrying 6-7RAC or 6-6RAC (Fig. 3C). Two constructs (6-5RAC and 6-4RAC) did not contain DNase HSSII (Fig. 2, C and D), but these constructs showed position-independent and copy-dependent expression (Tables I and III). These results demonstrated that the deleted sequence is not responsible for restoring DNase HSSs, suggesting that the DNase HSSII is not the chromosomal domain regulatory region of the gene.

To examine the intragenic region, we used rat aldolase C cDNA and LacZ as reporter genes (Fig. 2, E and F). In 6-7RAC(cDNA), seven introns were removed by replacing the exon-intron structure of the genome with the corresponding region of the cDNA (Fig. 2E). In the mouse carrying the 6-7RAC(cDNA) construct, DNA was digested with *Eco*RI and *Bam*HI to examine the restoration of DNase HSSs (Fig. 4A). In the analysis of the DNase HSSI and II, the expected fragments were detected in both the brain and liver when nuclei were treated with several doses of DNase I (Fig. 4, B and C, lanes 3, 4, 10, and 11).

The above results suggest that the regulatory elements for opening the chromatin domain are located in the exons, last intron and 145-bp 3′-flanking region that remains in the 6-4RAC. To examine these regions, we made the 6-7RAC(LacZ) construct that has the bacterial LacZ gene in the first exon of the 6-7RAC as a reporter (Fig. 2F). We used this construct and its serial 3′-deletion mutants to produce transgenic mice. DNase HSSs were then examined in the transgenic mice. In the transgenic mice carrying the 6-7RAC(LacZ), there was no discrete DNase HSS (Fig. 5). In the case of the serial 3′-deletion mutants of the 6-7RAC(LacZ), the same result was observed (data not shown).

*Restoration of Methylation Status Was Also Prevented by the Insertion*—The above results indicate that the insertion of the LacZ gene interfered with the chromatin opening activity. DNA methylation is involved in the

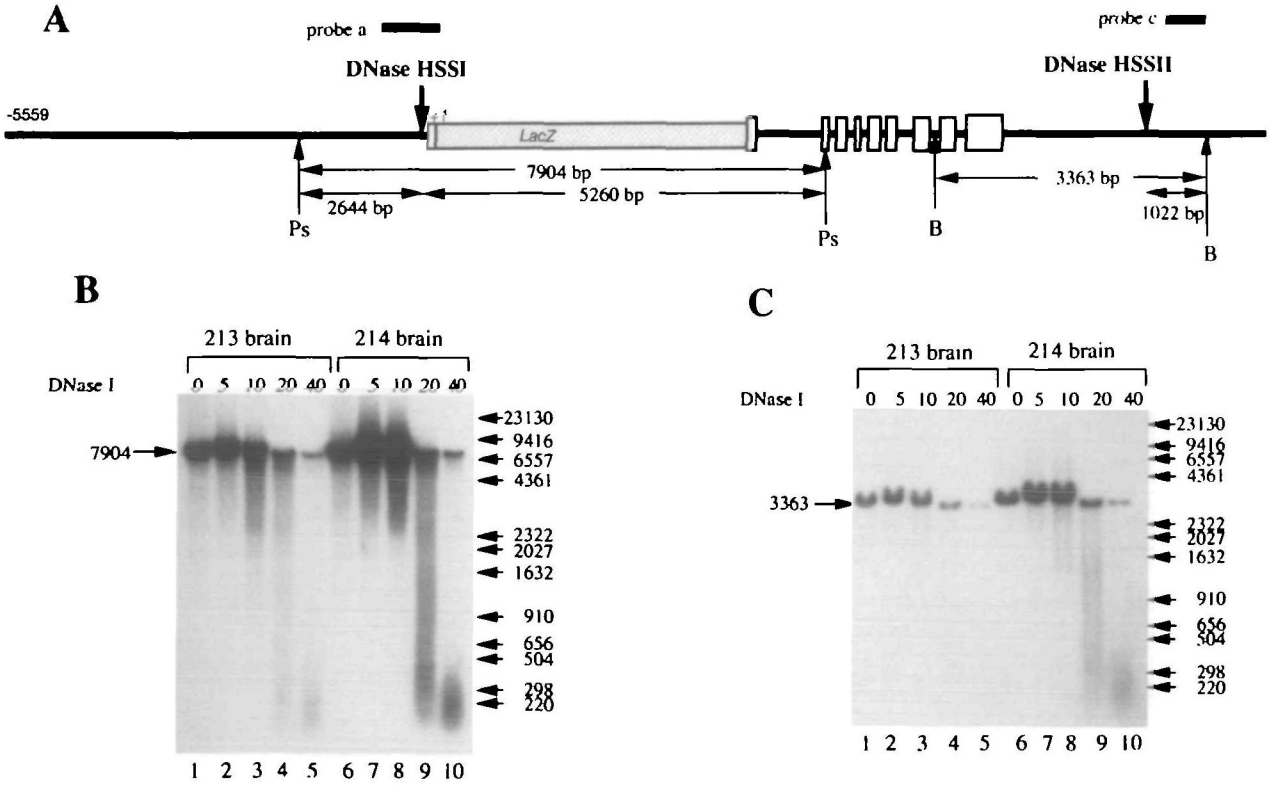


Fig. 5. **No discrete DNase HSS on the 6-7RAC(LacZ) in the transgenic mice.** (A) Schematic representation of the DNase HSSI and HSSII at the expected sites and the probes. Upward pointing arrows with Ps or B indicate *Pst*I or *Bam*HI sites, respectively. Sizes of expected fragments are indicated between sites. Symbols are the same as those in Fig. 2F. (B and C) Analyses of DNase HSSI and II in transgenic mice. Nuclei from brains of two lines (Tg213 and 214) were digested with the indicated amounts of DNase I. After extraction, purified DNAs were digested with *Pst*I (B) or *Bam*HI (C). Size markers are on the right in base-pairs.

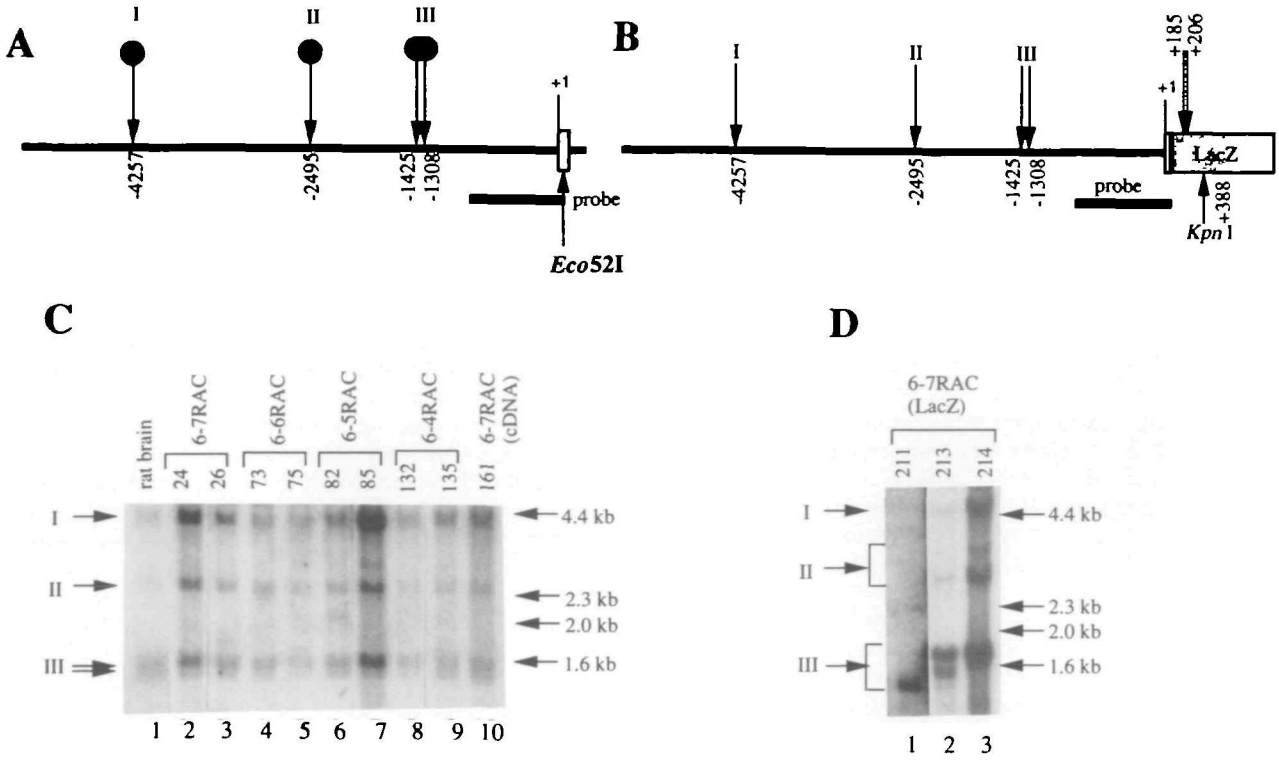


Fig. 6. **Effects of mutations on methylation status.** (A) Schematic representation of *Hha*I sites and their methylation status in the 5′-flanking region of the rat aldolase C gene in the brain. Downward and upward pointing arrows indicate *Hha*I sites and an *Eco*52I site, respectively. Arrows I, II, and III indicate the sites examined here. Vertical arrows with shaded circles indicate partially methylated sites. The thick bar with "probe" indicates the probe. (B) Schematic representation of *Hha*I sites in the 5′-region of the 6-7RAC(LacZ). Downward and upward pointing arrows indicate *Hha*I sites and a *Kpn*I site, respectively. The thick bar with "probe" indicates the probe. (C) Methylation status of the rat aldolase C gene and transgenes with an intact first exon. Brain DNA was digested with *Hha*I, followed by *Eco*52I. Numbers in the upper part of the figure indicate transgenic lines. Arrows I, II, and III along the left side correspond to the fragment sizes from positions +1217 to site I (−4257), site II (−2495), and site III (−1308 and −1425) in panel A, respectively. (D) Methylation status of the 6-7RAC(LacZ). Brain DNAs from transgenic mice were digested with *Hha*I, followed by *Kpn*I. The length of exposure was varied to facilitate visualization of bands. Size markers are on the right in panels C and D.

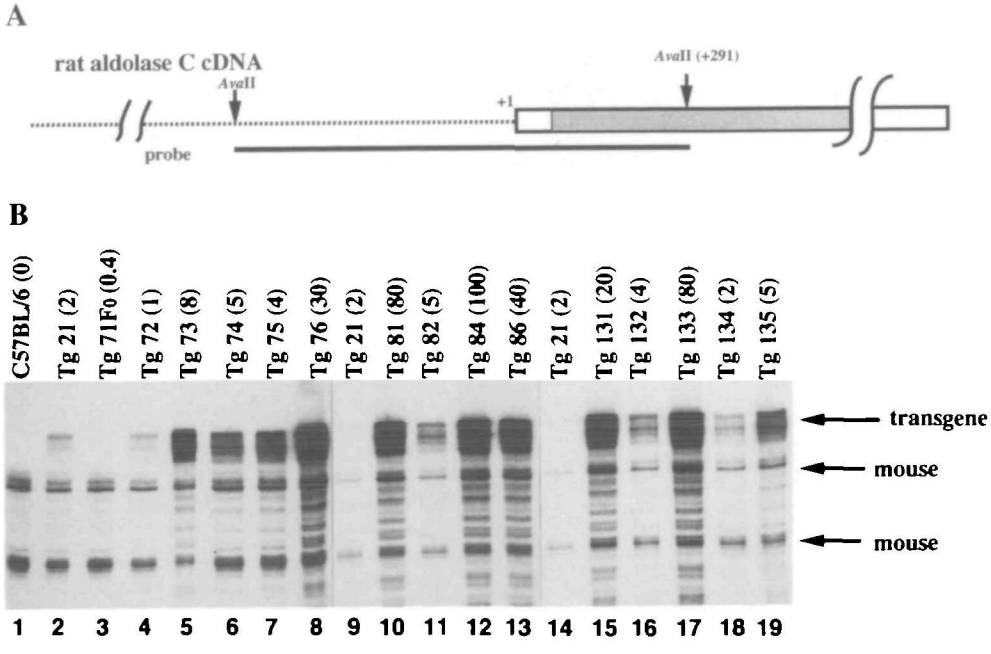


Fig. 7. **Expression levels of the rat aldolase C transgenes.** (A) Schematic representations of rat aldolase C cDNA and probe. The broken bar indicates vector sequences. Open and shaded boxes indicate non-coding and coding regions of the cDNA, respectively. The horizontal line indicates a probe DNA. (B) S1 nuclease protection assay. Protected fragments derived from endogenous mouse gene and transgene are indicated by arrows. Numbers in the upper part of the figure indicate transgenic lines from which RNAs were prepared. Numbers in parentheses indicate copy numbers of the transgene. Tg21, 71-76, 81-85, and 131-135 carry 6-7RAC, 6-6RAC, 6-5RAC, and 6-4RAC transgenes, respectively.

regulation of tissue-specific genes in somatic cells (*32*) and methylation status was restored in transgenic mice carrying the 13-kb fragment of the rat aldolase C gene (*9, 23*). We examined the methylation status of the 5′-flanking region of each transgene by using methylation-sensitive *Hha*I (Fig. 6). There are four *Hha*I sites in the 5′-flanking region of the gene, and they were partially methylated in rat brain (Fig. 6A). Although *Eco*52I is also a methylation-sensitive enzyme, an *Eco*52I site in the first exon of the gene is completely demethylated and is sensitive to the enzyme in rat brain. Therefore, four discrete fragments could be detected by digestion with *Eco*52I and *Hha*I (Fig. 6C).

To examine the methylation status of the rat aldolase C gene and transgenes comprising an intact first exon, double digestion with *Eco*52I and *Hha*I was performed, because *Hha*I sites downstream of the first exon are different between the genomic and cDNA clones (Fig. 2). In these transgenic mice, the methylation pattern (sites I, II, and III) was restored and four bands were observed (Fig. 6C, lanes 1 to 10).

To examine methylation status in the 5′-flanking region of the 6-7RAC(LacZ), double digestion with *Kpn*I and *Hha*I was performed. In this case, two additional *Hha*I sites in the LacZ reporter were also involved (Fig. 6B). In transgenic mice carrying the construct, the methylation status differed among lines (Fig. 6D). In Tg211, *Hha*I sites surrounding the probe were completely demethylated and only a 1.4-kb fragment was detected (Fig. 6D, lane 1). In Tg213 and 214, *Hha*I sites in the 5′-flanking region were partially methylated, but the extent of methylation in each site was different (Fig. 6D, lanes 2 and 3).

*Copy Number-Dependent Expression Was Also Interfered with by the Insertion*—The restoration of the chromatin structure (DNase HSSs and methylation status) was position-independent in transgenic mice when the transgene contained an intact first exon. We examined the effects of the LacZ insertion on position-independent and copy number-dependent expressions of the transgene. We assessed the copy number dependence by means of linear regression analyses on each data set obtained by S1 nuclease analysis and β-galactosidase activity (Fig. 7, Tables I and II). As shown in Fig. 7, a rat aldolase C cDNA probe was used to detect not only products from the transgene, but also that from mouse aldolase C gene, and the expression levels of the transgene were normalized to that of the endogenous mouse gene as an internal reference. We empirically observed that the intensity of the internal reference increased when high-level expression of the transgene was detected (Fig. 7B, lanes 8, 10, 12, 13, 15, and 17). To determine the exact expression levels, total RNAs from transgenic mice were serially diluted with C57BL/6

TABLE I. **Correlation between copy number and expression level of the transgene.**

| Transgene | Line No. | Copy number | Expression level[a] | Expression level per transgene[b] | Average value of expression levels per transgene[c] (normalized value[d]) | Correlation coefficient ($r^2$) |
|---|---|---|---|---|---|---|
| 6-7RAC | 21 | 2 | 0.73 | 0.36 | 0.58 (1.00) | 0.927 |
| | 23 | 1 | 0.40 | 0.40 | | |
| | 24 | 17 | 13.11 | 0.77 | | |
| | 26 | 5 | 4.27 | 0.85 | | |
| | 29 | 33 | 16.70 | 0.51 | | |
| 6-6RAC | 72 | 1 | 0.71 | 0.71 | 0.60 (1.04) | 0.982 |
| | 73 | 8 | 3.72 | 0.47 | | |
| | 74 | 5 | 3.36 | 0.67 | | |
| | 75 | 4 | 3.21 | 0.80 | | |
| | 76 | 30 | 11.18 | 0.37 | | |
| 6-5RAC | 81 | 80 | 26.99 | 0.34 | 0.44 (0.76) | 0.917 |
| | 82 | 5 | 2.23 | 0.45 | | |
| | 84 | 100 | 38.56 | 0.39 | | |
| | 85 | 1 | 0.42 | 0.42 | | |
| | 86 | 40 | 24.81 | 0.62 | | |
| 6-4RAC | 131 | 20 | 21.32 | 1.07 | 0.84 (1.45) | 0.918 |
| | 132 | 4 | 2.54 | 0.64 | | |
| | 133 | 80 | 39.80 | 0.50 | | |
| | 134 | 2 | 1.74 | 0.87 | | |
| | 135 | 5 | 5.62 | 1.12 | | |
| 6-7RAC (cDNA) | 161 | 12 | 0.36 | 0.03 | 0.03 (0.05) | |

[a]Values were calculated by dividing values for the transgene by those for endogenous mouse. Each value represents the average of values obtained in up to seven separate assays. [b]Values were calculated by dividing the value of the expression levels by the number of copies of the transgene. [c]Values were calculated by dividing the sum of the expression levels per gene by the number of results. [d]Values were normalized against that of 6-7RAC.

TABLE II. **Correlation between copy number and expression level of the LacZ construct.**

| Transgene | Line No. | Copy number | Expression level[a] | Expression level per transgene | Correlation coefficient ($r^2$) |
|---|---|---|---|---|---|
| 6-7RAC (LacZ) | 211 | 2 | 0 | 0 | 0.456 |
| | 212 | 3 | 17.22 | 5.74 | |
| | 213 | 8 | 8.61 | 1.08 | |
| | 214 | 10 | 30.70 | 3.07 | |

[a]Arbitrary values (units/mg protein) were calculated from the β-galactosidase activity. The β-gal activity was measured by the reported method (*9*).

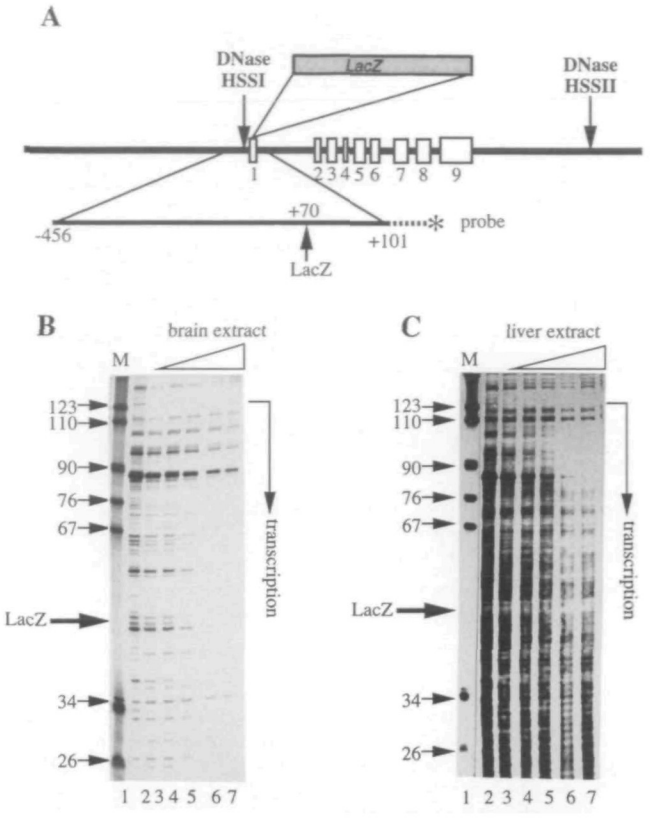


Fig. 8. **DNase I footprinting analyses.** (A) Schematic representation of the probe for footprinting. The triangle with the shaded box and the upward pointing arrows with LacZ indicate the insertion site of the LacZ gene. The broken bar and asterisk indicate the probe. The broken line indicates the vector sequence. The asterisk indicates the labeled *Hin*dIII site. Other symbols are the same as those described in Fig. 2. (B) Footprinting analysis with brain nuclear extract. (C) Footprinting analysis with liver nuclear extract. Ms indicate $^{32}$P-labeled size markers (*Hpa*II-digested pBR322). Horizontal arrows with LacZ indicate the site of the LacZ insertion in the 6-7RAC(LacZ) construct. Downward arrows with transcription indicate the major transcriptional initiation site. In lanes 2 to 7, the probe was incubated with 0, 2, 4, 6, 8, 10, 12 $\mu$g of nuclear protein, respectively. Size markers are indicated on the left in bases.

RNA, and S1 nuclease assays were performed (data not shown). When transgenic mice carried over 20 copies of transgene, the expression levels of the 3′-deletions were calculated from the values of serially diluted assays, where total RNAs were diluted to be equivalent to those of mice with under 10 copies of each transgene. When transgenic mice carried under 20 copies, the expression levels were computed from the values of both non- and serially diluted assays. To eliminate errors among the separated assays, brain RNA of Tg21 was assayed in each series of reactions (Fig. 7B). These data and the results obtained by linear regression analyses are shown in Table I. The correlation coefficients of constructs comprising a series of the 3′-flanking deletions were over 0.917 (a value of $r^2=1$ is a perfect linear correlation). This result confirmed that the deletion of the 3′-flanking region does not affect the position-independent and copy number-dependent expression of the transgene. The average expression levels ranged from 76 to 145% in comparison with that of the 6-7RAC.

In the 6-7RAC(cDNA), we could not determine copy number-dependent expression, because only a single transgenic line was obtained. The deletion of seven introns resulted in a 20-fold decrease in the mRNA per transgene copy (Table I).

In the case of the 6-7RAC(LacZ) construct, $\beta$-galactosidase activity was measured by using whole brain extract. As shown in Table II, the correlation coefficient of the 6-7RAC(LacZ) was markedly decreased (a correlation coefficient of $r^2=0.456$). There was no correlation between the copy number and expression levels. These results indicate that the LacZ insertion into the first exon interferes with the three activities examined here.

*The Sequence Disrupted by the Insertion Was Recognized by Brain-Specific Protein Factor(s)*—There are two possible explanations for the inactivation of the three activities by the LacZ insertion. (i) The LacZ sequence comprises some inhibitory elements and interferes with all three activities. (ii) The LacZ insertion disrupts the genetic information essential for gene regulation; for example, disruption of the regulatory element and/or disturbance of the interactions among regulatory elements. If the latter is the case, transcription factor(s) would interact specifically with the DNA sequence surrounding the disrupted site. To examine this possibility, we performed DNase I footprinting assays by using nuclear extracts derived from brain and liver. Brain-specific protection was observed at the region surrounding the site disrupted by the LacZ insertion in the 6-7RAC(LacZ) construct (Fig. 8B, lanes 5 to 7). Protein factors derived from liver recognized mainly a sequence surrounding the downstream of the transcriptional initiation site (Fig. 8C, lanes 6 and 7). These results indicate that the region disrupted by the LacZ insertion includes *cis*-elements recognized by brain-specific factors.

## DISCUSSION

Aldolase C is expressed predominantly in the central nervous system (CNS) (*16*). Transgenic mice carrying a 13-kb fragment of the rat aldolase C gene showed copy number-dependent and correct regional expression in the CNS (*9*). Therefore, the 13-kb sequence comprises sufficient information for gene regulation, including a regulatory region for conferring position independence on the transgene. In the present study, to localize the regulatory region, we used several constructs to produce transgenic mice and examined them. As summarized in Table III, deletions of the 3′-flanking region and seven introns did not affect restoration of chromatin structure, but the insertion of the LacZ gene into the first exon interfered with all activities examined here. The sequence disrupted by the LacZ insertion was recognized by brain-specific factors. These results suggest that the sequence in the first exon is essential for restoring the chromatin structure of the rat aldolase C gene.

Only the insertion of the LacZ gene interfered with restoration of chromatin structure. Negative effects of insertional mutagenesis can be explained mainly by two possibilities: (i) inhibitory effects of the inserted sequence and/or (ii) disruption of genomic information for gene regulation by the insertion. Because the sequence disrupted by the LacZ was recognized by brain-specific factors (Fig. 8), we believe that the loss of the activities is attributable to the disruption of genomic information for the gene

TABLE III. **Summary of expression and restoration of chromatin structure of the rat aldolase C transgene.**

| Transgene | Number of mice | Copy-dependent expression | Restoration of chromatin structure | | |
|---|---|---|---|---|---|
| | | | Methylation status | DNase HSSI | DNase HSSII |
| 6-7RAC | 5 | + | + | + | + |
| 6-6RAC | 5 | + | + | + | + |
| 6-5RAC | 5 | + | + | + | n.d.* |
| 6-4RAC | 5 | + | + | + | n.d.* |
| 6-7RAC (cDNA) | 1 | n.d. | + | + | + |
| 6-7RAC (LacZ) | 4 | − | − | − | − |

+ or − indicate results positive or negative in each test, respectively. n.d., not determined. n.d.*, there was no sequence of DNase HSSII in these constructs.

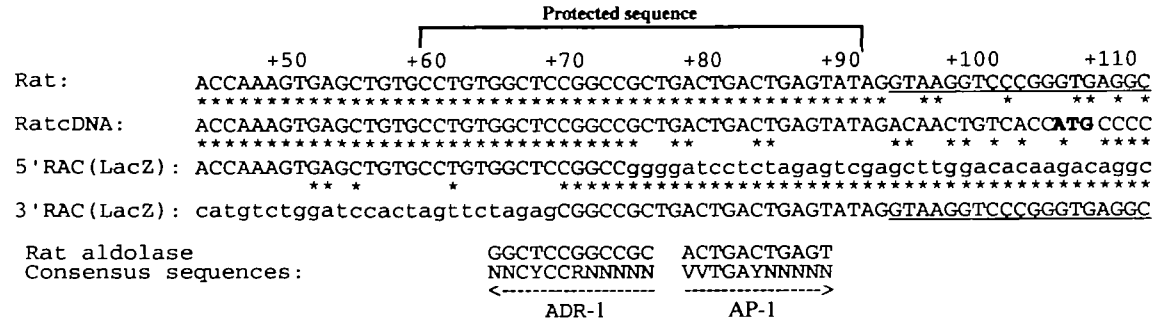


Fig. 9. **Alignment of sequences surrounding the site disrupted by the LacZ insertion.** The bracket indicates the sequence protected by brain-nuclear proteins. The sequence was determined by electrophoresis with chemically cleaved sequencing ladders. Rat and Rat cDNA indicate the rat aldolase C gene and cDNA, respectively. 5′ RAC(LacZ) and 3′RAC(LacZ) indicate 5′ and 3′ boundaries between the rat aldolase C gene and LacZ reporter gene of the 6-7RAC(LacZ), respectively. Numbers at the top of the sequences indicate the relative distance from the major transcriptional initiation site of the rat aldolase C gene. Asterisks indicate the same sequences as the rat aldolase C gene. Underlines indicate first introns. ATG written in bold letters in Rat cDNA indicate a translation initiation codon. Lowercase letters indicate sequences derived from LacZ or vector in the 6-7RAC-(LacZ). The 6-bp direct repeat (from positions +69 to +74) was created by the construction of this transgene. Rat aldolase indicates sequences corresponding to the consensus sequences of ADR-1 and AP-1. Consensus sequences of ADR-1 and AP-1 are shown. Letters in the consensus sequences are as follows: G=G, A=A, T=T, C=C, Y=C/T, R=A/G, V=A/G/C, N=A/G/C/T.

regulation by the insertion. In this case, there are two further possible explanations for the inactivation: (i) disruption of regulatory element and/or (ii) disturbance of the interactions among elements by the large insertion (about 3.9 kb). If the insert disrupted a regulatory element, the element has critical roles and may be novel, because there is no consensus sequence across the inserted site. There are several reports that recognition sequences in regulatory regions for chromosomal domains have critical roles; for example, the c-Myb site for the adenosine deaminase gene (*12*), the MEF3 site for the aldolase A gene (*14*), and the GATA and NF-E2/AP-1 sites for β-globin gene (*33*). Alternatively, if the interactions among regulatory elements are disturbed by the insertion, the elements are separated by the 3.9-kb LacZ fragment. There were consensus sequences of ADR-1 (*34*) and AP-1 (*35*) in the footprinted region (Fig. 9). These regulatory elements were recreated at the 5′- and 3′-side of the LacZ gene, respectively, even when insertion occurred (Fig. 9). The ADR-1 site is found in several yeast genes including alcohol dehydrogenase (ADH) (*34*). This element is required for chromatin remodeling during ADH2 gene activation (*36*). AP-1/NF-E2 is found in the human β-globin locus control region (LCR) (*33, 37*). We do not know whether the disruption of a recognition site or the spatial arrangement of recognition sites affects the activity to insulate a transgene from position effects.

The first exon of the rat aldolase C gene is not DNase HSS itself, and the DNase HSSI close to the first exon was not brain-specific (Fig. 1B). If the first exon includes a regulatory region for insulating the rat aldolase C gene, this property is different from that of other chromosomal domain regulatory regions associated with tissue-specific DNase HSSs (*30, 31*). However, since this region seems to include several tissue-specific elements (Fig. 8), there is a possibility that this region is crowded with factors and nucleosome positioning is disrupted by their binding to create the DNase HSSI. In this case, the feature that a regulatory region has several recognition sites is similar to the human β-globin LCR (*38*). Analyses of *trans*-acting factors that recognize the footprinted sequence will provide important information on chromosomal domain regulation. Alternatively, if the first exon does not include such a regulatory region, how is the first exon of the gene related to the activity? In the first exon, many genes include an initiator element that constitutes a core promoter and some initiators are responsible for tissue-specific expression (*39*). Because the disruption or truncation of the sequence did not abolish CNS-specific expression and basal transcription (*9, 23, 24*), it is not likely that the disrupted sequence constitutes a promoter. Since human β-globin LCR was recently found to achieve sheltering of γ-globin gene expression from position effects by cooperation with the 3′-flanking region (*40*), a fascinating possible model is that the protected region cooperates with the dominant chromosomal domain regulatory region, for example LCR, to form an active chromatin loop.

There is an aberrant feature that different protein factors

recognize different elements in the first exon between brain and liver (Fig. 9), although DNase HSSI is observed in both tissues (Fig. 1). However, this discrepancy can be explained as follows. Because the gene is expressed in the fetal liver (*41*), open chromatin, once formed, may be stably propagated like the chicken vitellogenin gene (*42*). To achieve CNS-specific expression, negative transcription factors may interact with this region to repress the gene in liver. Alternatively, negative transcription factors bind to the region and create DNase HSSI by disrupting the nucleosome positioning surrounding the first exon, because DNase I hypersensitive sites generally map close to the binding sites for *trans*-acting factors (*43*).

In this study, other regulatory elements for mRNA level were also suggested. Some regulatory elements for expression levels could be separable from those for insulating the gene because the expression levels varied among the 3′- and intron-deleted transgenes, in which chromatin structure was restored (Tables I and III). In the case of intron-deletion, the decrease of mRNA level may be intron-dependent because position-independent restoration of chromatin structure was observed (Tables I and III). In this case, there are two possibilities for intron-dependent decrease of mRNA level: (i) decrease of transcriptional efficiency (deletion of positive elements in introns) and/or (ii) post-transcriptional effects (instability of mRNA).

Further work is needed to understand how the first exon is involved in the insulation mechanism of the rat aldolase C gene.

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