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EXPRESSION AND CHROMATIN STRUCTURE OF THE RAT ALDOLASE C GENE AND TRANSGENES*

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Aldolase C mRNA is detected by Northern blot in all fetal tissues in rat; it is very abundant in the adult brain and undetectable in the other adult tissues. However, reverse transcriptase polymerase chain reaction amplification indicates that this gene is not totally repressed in these tissues. A DNase-I hypersensitivity site located in a 115-base pair proximal promoter fragment is detectable in the brain as well as in other adult tissues. Two *MspI/HpaII* restriction sites located at -3800 and -450 base pairs are demethylated in the brain and totally or partially methylated in other tissues.

In transgenic mice, a 12.5-kilobase genomic fragment is strongly and tissue specifically expressed in different lines, with conservation of a methylation pattern similar to that of the endogenous gene. A chloramphenicol acetyltransferase gene directed by either 800 or 115 base pairs of aldolase C 5'-flanking sequences is tissue specifically expressed in transgenic mice, but the level of expression is very low. This level is greatly increased when the transgene consists of a chloramphenicol acetyltransferase hybrid gene directed by 5.5 kilobases of aldolase C 5'-flanking sequences.

We propose therefore that the chromatin structure around the aldolase C promoter is accessible in fetal tissues, then remains open in the adult brain, where the gene is very active, as well as in tissues in which it is practically inactive. The specificity of expression in the brain is conferred by a short 115-base pair proximal promoter fragment that needs more upstream sequences to be fully active.

Fructose-1,6-bisphosphate aldolase is a glycolytic enzyme that exists as three types: aldolase A (muscle type), aldolase B (liver type), and aldolase C (brain type). Expression of these isoenzymes in various tissues is modified during development and carcinogenesis (Lebherz and Rutter, 1969; Numazaki *et al.*, 1984). Aldolase C is strongly expressed in adult and fetal brain and at a lower level in all fetal tissues and in proliferating cells (Schapira *et al.*, 1970; Skala *et al.*, 1987; Takashi *et al.*, 1990). In contrast, this isoenzyme is undetectable in normal non-brain adult tissues.

In a previous work (Vibert *et al.*, 1989) we have shown that although its promoter displays unusual housekeeping characteristics for a tissue-specific gene, the aldolase C gene is regulated at the transcriptional level. However, these characteristics of the promoter are consistent with the low but ubiquitous expression of aldolase C in fetal tissues. Several questions arise from these observations.

1. Is the expression of the aldolase C gene actually abolished in non-brain adult tissues, or is it slowed down to a very low level that could be increased again in proliferating cells, especially in cancer?

2. It has been reported that contrary to specifically expressed genes, ubiquitous genes are associated, in all tissues, with constitutively open and hypomethylated chromatin (Bird, 1986, 1992). So, what is the chromatin structure and the methylation pattern of aldolase C in tissues that express aldolase C actively and in tissues that do not express it detectably?

3. What are the *cis*-acting regions important for a qualitatively and quantitatively normal expression of aldolase C transgenes *in vivo*?

Here we report that the aldolase C gene behaves differently from "classical" tissue-specific genes in that its chromatin structure is open even in tissues in which aldolase C is undetectable. In particular, a DNase-I hypersensitivity site (HSS)[1] located in the vicinity of the start sites of transcription is as strong in adult brain as in liver. The persistent hypersensitivity at the promoter is in line with a persistent low level presence of aldolase C mRNA in adult non-brain tissues, detected only by reverse transcriptase PCR. However, a clear brain preference of expression is conferred on a reporter gene by a short 115-bp promoter fragment in transgenic mice. More upstream sequences increase the level of transgene expression without modifying its specificity.

MATERIALS AND METHODS

Aldolase C Isoenzymes—Aldolase C isoenzymes were separated by cellulose-acetate electrophoresis with specific staining (Penhoet *et al.*, 1966; Susor and Rutter, 1971).

Northern blots and Reverse Transcriptase PCR Amplification—Total cellular RNA from different tissues was prepared as described (Kahn *et al.*, 1981). The Northern blots were performed with 10 μg of total RNAs fractionated on formaldehyde (1.2% w/v) agarose gels and transferred to GeneScreen nylon membranes. The membranes were hybridized with a single-stranded probe complementary to the 3'-noncoding extension of the aldolase C mRNA (Skala *et al.*, 1987). Reverse transcriptase PCR

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[1] The abbreviations used are: HSS, hypersensitive site; PCR, polymerase chain reaction; bp, base pair(s); kb, kilobase(s); CAT, chloramphenicol acetyltransferase.

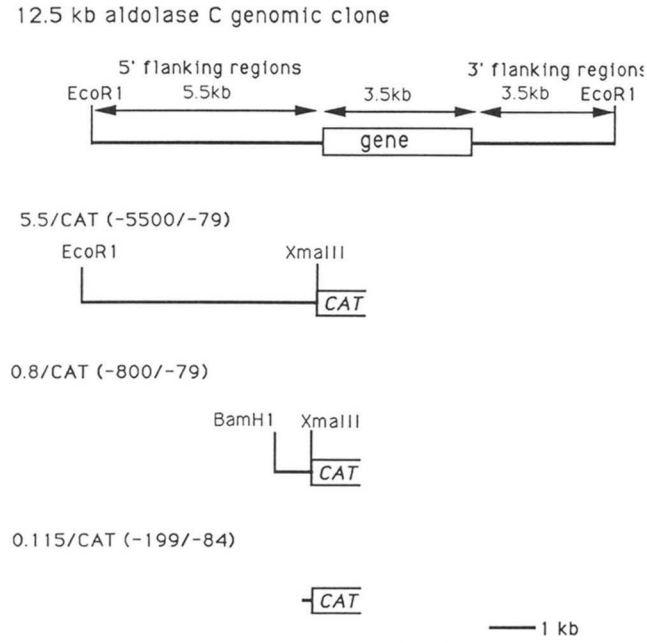

FIG. 1. **Scheme of the different analyzed transgenes.** The 5′ *Eco*RI site of the 12.5-kb adolase C genomic fragment (12.5/aldC) is a cloning site. The 5′-flanking sequences cloned in front of the CAT gene (5.5/CAT, 0.8/CAT, and 0.115/CAT) are numbered with respect to the ATG translation initiator (position +1). The 115-bp fragment obtained by PCR was cloned at the site *Sma*I.

amplification of fragments from aldolase C and aldolase B transcripts and from a ubiquitous reference transcript (cytochrome b_5 reductase) (Zenno *et al.*, 1990) was performed as described (Noonan and Roninson, 1988). Reaction products were subjected to polyacrylamide gel electrophoresis, transferred to a GeneScreen membrane, and hybridized to a labeled internal oligoprobe (Chelly *et al.*, 1989). Nucleotide sequence of the primers complementary and identical to mRNA sequences are indicated in the legend to Fig. 2.

DNase-I Hypersensitivity and Methylation Analysis—Nuclei were isolated essentially as described by Villeponteau *et al.* (1984). Tissues were removed, cut into pieces, washed in ice-cold nuclear isolation buffer (20 mM Hepes, pH 8, 50 mM NaCl, 1 mM EDTA, 0.25 mM EGTA, 0.15 mM spermine, 0.5 mM spermidine, 0.5 M sucrose, 1 mM phenylmethylsulfonyl fluoride, 3.5 mM 2-mercaptoethanol) and then homogenized in a Dounce homogenizer. The homogenate was filtered through gauze and centrifuged at 4 °C for 10 min. The nuclear pellet was resuspended in the nuclear isolation buffer in the presence of 0.5% (v/v) Nonidet P-40, centrifuged, and then washed in the same buffer without Nonidet P-40. This step was repeated once, and then the nuclei were resuspended in a small volume of nuclear isolation buffer containing 25% (v/v) glycerol. Nuclei samples, with 100 units of DNase-I added, were incubated at 25 °C from 0 to 20 min. Reactions were stopped by the addition of 12 mM EDTA and 0.5% (w/v) sodium dodecyl sulfate. Nuclei representing each point were digested overnight at 37 °C with 100 μg/ml (w/v) proteinase K. DNA from these nuclei was then phenol-chloroform extracted and cleaved with the relevant restriction enzymes. Southern blots were hybridized with genomic fragments labeled by random priming. The level of methylation of *Msp*I/*Hpa*II sites was investigated by cutting 10 μg of genomic DNA with a 10-fold excess of the corresponding restriction enzymes. Fragments were electrophoresed in a 0.8% (w/v) agarose gel, in the presence of ^{35}S radioactive size markers provided by Amersham Corp. were then blotted on hybond N+ membrane. The 1.3-kb genomic *Bam*HI fragment described previously (Vibert *et al.*, 1989), encompassing 5′-flanking sequences, first exon, and part of the first intron, was digested with *Sma*I, generating three subprobes that where separated and isolated from a low melting point agarose gel.

DNA Constructs—The different DNA constructs used as transgenes are described in the Thomas *et al.* (1993) and Vibert *et al.* (1989) and schematized in Fig. 1. The 12.5/aldC transgene consists of an unmodified rat aldolase C gene with about 5.5 kb of 5′- and 3.5 kb of 3′-flanking sequences. The 5.5/CAT, 0.8/CAT, and 0.115/CAT transgenes consist in upstream sequences spanning, with respect to the ATG translation initiator, from −5500 to −64 bp, −800 to −64 bp, and −199 to −84 bp, respectively, which are cloned in front of the chloramphenicol acetyltransferase (CAT) reporter gene.

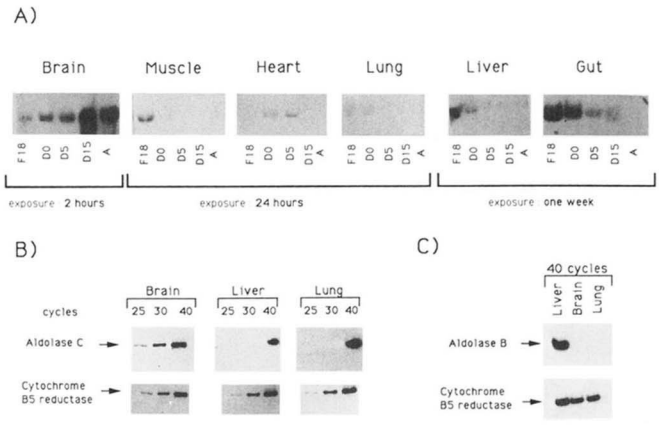

FIG. 2. *Panel A*, Northern blot analysis of the aldolase C mRNA in different tissues during pre- and postnatal development. *F18*, 18 day-old fetus; *D0*, newborn rats; *D5* and *D15*, 5- and 15-day-old rats; *A*, adult rats. 10 μg of total RNA was electrophoresed on a 1.2% formaldehyde (w/v) agarose gel, blotted, and the filters were hybridized with a single-stranded probe complementary to the 3′-untranslated region of the aldolase C mRNA. *Panels B* and *C*, reverse transcriptase PCR analysis of the aldolase C, aldolase B, and cytochrome b_5 reductase mRNAs in adult rat tissues. Amplification primers: for aldolase mRNA, CAACTCCTTCTTCTGCTC (3′ primer spanning from +31 to +48 bp) and TCTGTTGCTAACCAGACC (5′ primer, spanning from −75 to −57 bp). For aldolase B mRNA, TGTTTGTTCCTGCAAGCG (3′ primer spanning from +372 to +391 bp) and CTGATACCTTGGAGCACTC (5′ primer spanning from −44 to −25 bp). For cytochrome b_5 reductase mRNA, GGGGTTCTCGAGGGTGATGGC (3′ primer spanning from +99 to +120 bp) and GGTGTAGAACGGTGACACCACTG (5′ primer spanning from −37 to −16 bp). Each cDNA is numbered with respect to the ATG translation initiator (+1); 1 μg adult RNAs was reversed transcribed, and cDNA was amplified for 20, 25, 30, or 40 cycles, as reported under "Materials and Methods." Amplified DNA fragments were electrophoresed on polyacrylamide gel, blotted, then hybridized with labeled oligonucleotides corresponding to internal sequences of the different fragments (AGTGAGCTGTGCCTGTG) for aldolase C, (TGGAAAAGGAATCACTCCG) for aldolase B, and (GCTTCATGAAGAGGCTGTAGACG) for cytochrome b_5 reductase mRNAs.

Production and Detection of Transgenic Mice—Inserts were isolated from agarose gels and were further purified on Elutip-d columns according to the instructions of the supplier (Schleicher & Schuell). The fragments diluted in 10 mM Tris-HCl, pH 7.5, 0.1 mM EDTA were microinjected using established procedure into fertilized eggs resulting from mating between B6D2F1 hybrids (Gordon and Ruddle, 1983). The animals were screened for the insertions of the transgenes by Southern blot analysis of tail DNA preparations after hybridization with a random primed CAT or aldolase C probe. Transgenic RNAs were detected by S1 nuclease protection analysis using radiolabeled single-stranded probes (Keyser *et al.*, 1985). CAT activity generated by transgene expression in different tissues was measured on 1–100 μg of proteins (Gorman *et al.*, 1982).

RESULTS

Expression of the Aldolase C Gene in Fetal and Adult Tissues—Fig. 2*A* confirms (Mukai *et al.*, 1991; Skala *et al.*, 1987) the dual specificity of aldolase C expression; mRNAs were detected by Northern blot in all fetal tissues tested (brain, muscle, heart, lung, liver, gut) but not in adult tissues, except in brain where its level was much higher than in fetal tissues. However, a minute amount of aldolase C mRNA was detectable after 40 cycles of reverse transcriptase PCR amplification in adult liver and lung, whereas it was detected in brain after 25 cycles (Fig. 2*B*). This low expression of the aldolase C gene in the lung and liver cannot be considered equivalent to the illegitimate transcription phenomenon, that is to say to the very low level transcription of any tissue-specific gene outside its cognate expression sites (Chelly *et al.*, 1989); indeed, under the same reverse transcriptase PCR conditions, the liver-specific aldolase B mRNA (Tsutsumi *et al.*, 1985) was not detected in the lung and

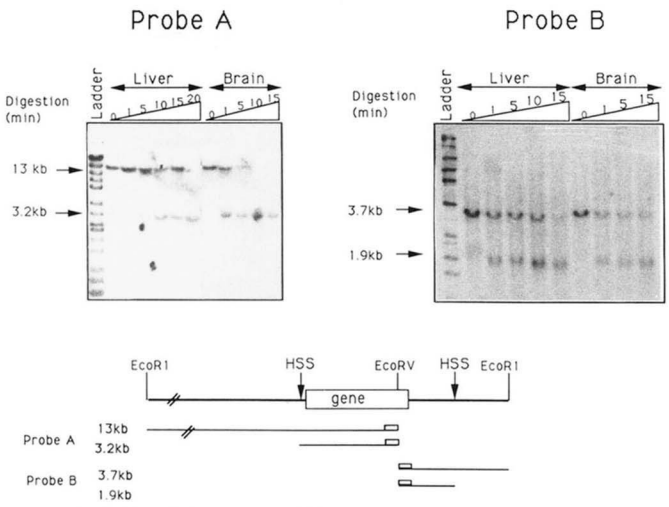

FIG. 3. **DNase-I hypersensitivity analysis of the aldolase C gene.** Nuclei from adult rat liver and brain were incubated with 100 units of DNase-I for 1–20 min at 25 °C. DNA was then purified, digested with *Eco*RI and *Eco*RV enzymes, electrophoresed, and then blotted. The blots were hybridized with either probe A or probe B located here and there on the *Eco*RV site. Southern blot analysis and scheme of the HSSs with respect to the gene (*open box*): the length of the generated fragments is summarized in the *lower part* of the figure.

brain, whereas, as expected, it was very abundant in the liver (Fig. 2*C*). In contrast, the messenger for cytochrome b_5 reductase, a ubiquitous enzyme (Zenno *et al.*, 1990), was amplified in all tested tissues. Therefore, the aldolase C gene is transcribed in fetal tissues and is strongly repressed, but not totally extinguished, in the corresponding non-brain adult tissues. In contrast, the gene is strongly activated during pre- and postnatal development of the brain. This raises the question of whether these differences between aldolase C gene expression in the brain and other tissues occurs in parallel with changes in the chromatin structure of the gene.

DNase-I Hypersensitivity Analysis of the Aldolase C Gene in Expressing and Nonexpressing Tissues—Nuclei from adult brain and liver were purified and digested by DNase-I for different times, and then DNA was purified and digested with *Eco*RI and *Eco*RV enzymes. The digestion products were analyzed by Southern blotting (Fig. 3) using probes recognizing either the ends of a 13-kb 5′ fragment (probe A) or of a 3.7-kb 3′ fragment (probe B). Fig. 3 shows that two strong HSSs were similarly detected in the liver and in the brain. The upstream HSS, detected with probe A, is located nearby the major start sites of transcription (Vibert *et al.*, 1989). The downstream HSS, detected with probe B, is located downstream from the gene, at about 1.5 kb from the polyadenylation site. In fact, this downstream HSS is located in a DNA fragment that appears by zooblotting to be a conserved region in different mammals and birds (not shown) and could, therefore, correspond to the presence of another gene unrelated to aldolase C. In any case, these results indicate that a strong HSS is detected in the promoter of the aldolase C gene, in tissues that transcribe this gene strongly as well as in tissues that transcribe it only minimally. Since the difference of gene expression in these two tissues could not be ascribed to differences in chromatin accessibility, we addressed the question of a possible relationship between gene activity and methylation of CG dinucleotides.

Methylation Analysis—Methylation of CG dinucleotides belonging to *Msp*I/*Hpa*II restriction sites (CCGG) in and around the aldolase C gene was analyzed by comparison of the restriction patterns obtained with *Msp*I that is not inhibited by methylation and with *Hpa*II that is inhibited. Digests were analyzed by Southern blotting, and hybridization was performed successively with three types of probes: probe 1 was the *Bam*HI/*Sma*I fragment spanning from −800 to −450 bp; probe 2, the *Sma*I/*Sma*I fragment located between −450 and +70 bp; and probe 3, the *Sma*I/*Bam*HI fragment spanning from +70 to +400 bp. To standardize the results and facilitate the positioning on the genomic fragment of the different described sites, we arbitrarily attributed the position +1 at the beginning of the cDNA described by Mukai *et al.* (1991), which is located in the major start sites of transcription (Vibert *et al.*, 1989).

Fig. 4*A* shows that probe 1 revealed different DNA fragments when digestion was performed with *Hpa*II or *Msp*I, using either liver or brain DNA, indicating that a CCGG site located at position −3.8 kb was methylated in the liver and demethylated in the brain, whereas the site at −1.7 kb was methylated in both tissues. Probe 2 revealed that the CCGG site located at position −450 bp was partially methylated in the liver and demethylated in the brain, whereas the site at +70 bp remained demethylated in both tissues. Finally, probe 3 detected two additional downstream *Msp*I/*Hpa*II sites, inside (+2.7 kb) and outside (+4.6 kb) the aldolase C gene; the former was always methylated and the latter always demethylated. It is worth noting that this downstream demethylated site is in the immediate vicinity of the downstream DNase-I HSS (located at +5.1 kb) and conserved region described above and might correspond to a downstream gene, as already discussed.

In Fig. 4*B* we took advantage of the presence of two *Pst*I restriction sites upstream and downstream of the +1 site to locate more accurately some of the *Msp*I/*Hpa*II sites and to demonstrate that the site at −450 bp was totally demethylated in the brain only, whereas it was partially methylated in the adult liver, spleen (data not shown) and fetal liver that express the aldolase C gene minimally or weakly. Therefore, although the chromatin conformation of the aldolase C gene appears to be open in all tissues, a clear difference in methylation of upstream CG dinucleotides exists between the brain, where the gene is very actively transcribed, and the other tissues. To gain insight into the function of the flanking regions possessing either the DNase-I HSS or differentially methylated CG dinucleotides, we subsequently created transgenic mice carrying either the aldolase C gene, or constructs in which a reporter gene was directed by aldolase C 5′-flanking sequences (Fig. 1).

Expression of the Rat Aldolase C Gene in Transgenic Mice—We introduced into fertilized mouse eggs the entire rat aldolase C gene, an *Eco*RI/*Eco*RI genomic fragment (Vibert *et al.*, 1989) including 5.5 kb of 5′-flanking region, the gene (3.5 kb), and 3.5 kb of the 3′-flanking region (Fig. 1). We produced four male founder lines (37, 39, 41, and 42) carrying from 3 to 30 copies of the transgene per haploid genome. The founders were outbred to generate independent mouse transgenic lines. The expression of the transgene in different tissues was determined by S1 nuclease mapping. Fig. 5 shows that the expression of the aldolase C gene was very high in the brain of F1 animals, much higher than the expression of the endogenous gene in rat brain. A considerably lower expression was detected in some non-brain tissues, *e.g.* liver, gut (especially in line 37), and lung (investigated in line 42). The high expression of the transgene in the brain was confirmed by detection of the activity of the different isozymic forms after cellulose acetate electrophoresis (Fig. 6); the brain aldolase activity is generated by the various tetramers resulting from the combination between subunits A (predominant form in muscle) and C. In line 39, harboring three copies of the transgene, C4 homotetramers were reinforced; they were largely predominant in line 41 harboring 30 copies. From these results, we can suppose that the enzyme activity generated by the 12.5/aldC transgenes is more or less correlated with the number of integrated copies.

Expression of Aldolase/CAT Chimeric Genes in Transgenic

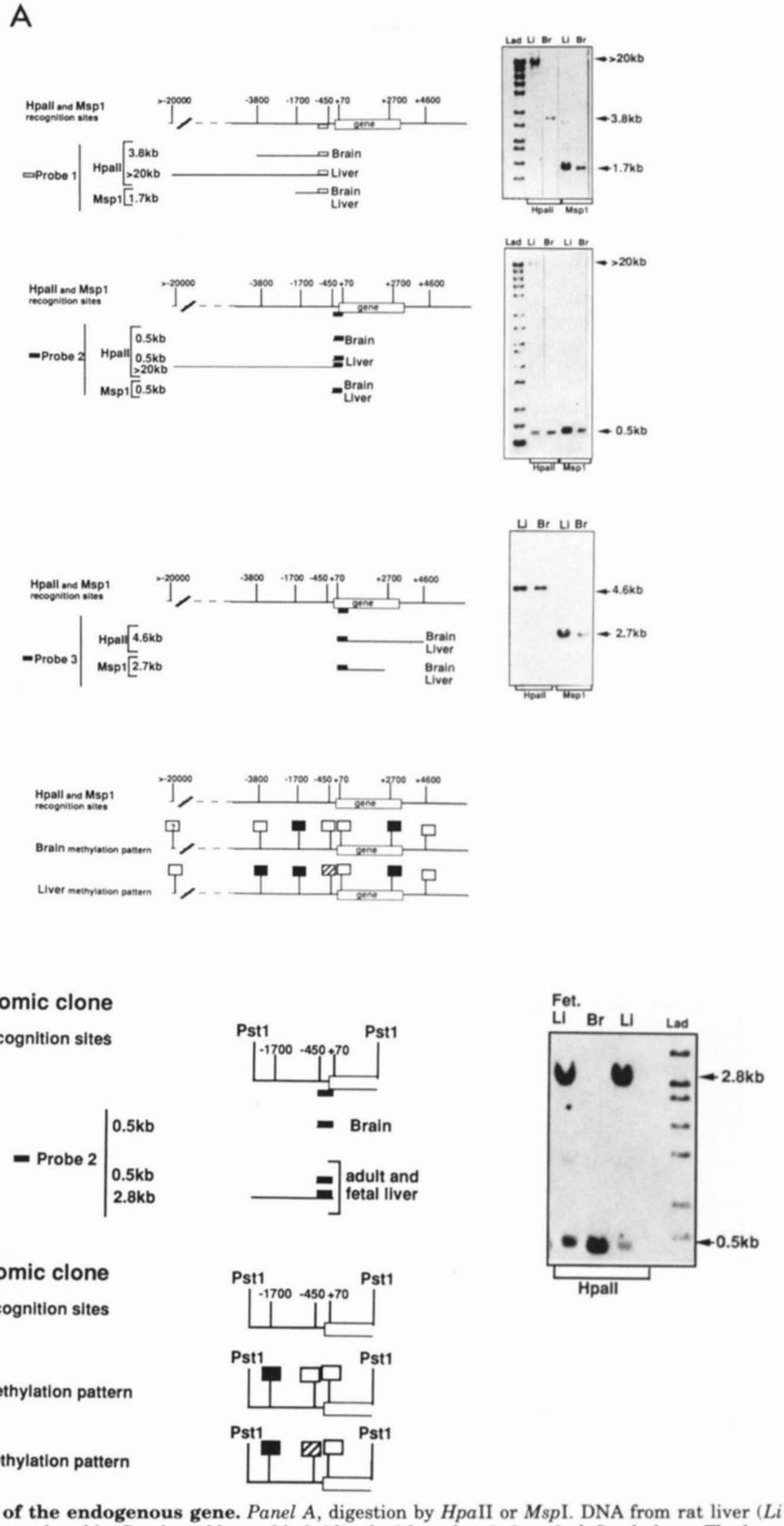

FIG. 4. **Methylation analysis of the endogenous gene.** *Panel A*, digestion by *Hpa*II or *Msp*I. DNA from rat liver (*Li*) and brain (*Br*) was digested with *Hpa*II or *Msp*I, then analyzed by Southern blot and hybridized with probes 1, 2, or 3, defined above. The length and the position of the fragments obtained after hybridization with each probe are presented near the corresponding Southern blots. The *lower part* of the figure summarizes the methylation pattern of the aldolase C gene: the methylated *Msp*I/*Hpa*II sites are represented by *black squares*, the demethylated sites by *open squares*, and the partially methylated sites by *hatched squares*. *Lad*, molecular weight markers. The *question mark* (*?*) signifies that a upstream site cannot be studied in brain because of the downstream complete cleavage. *Panel B*, digestion by *Hpa*II and *Pst*I. To focus on the region surrounding the promoter, double digestions of DNA from rat liver (*Li*), brain (*Br*), and fetal liver (*Fet. Li*) were performed. The Southern blot was hybridized with probe 2.

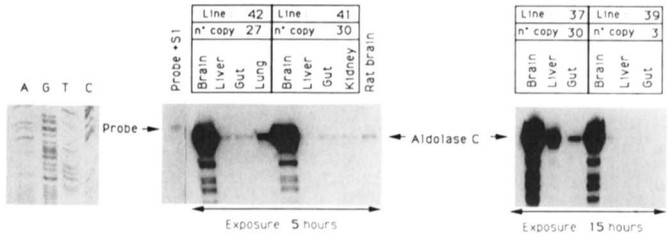

FIG. 5. **Nuclease S1 protection assay of the transgenic rat aldolase C mRNA in mice.** 10 μg of total RNA (brain, liver, gut, kidney, and lung) from adult transgenic mice were hybridized with a radiolabeled single-stranded 160-bp aldolase C cDNA probe and then digested with 200 units of S1 nuclease for 30 min at 37 °C. The protected cDNA fragment (120 bp) was analyzed on a sequencing polyacrylamide gel. The positions of the undigested probe and a rat brain control are indicated. The different lines of transgenic mice harboring the 12.5/aldC transgene and the number of integrated copies estimated with respect to the signal generated on Southern blots by the endogenous gene are precised.

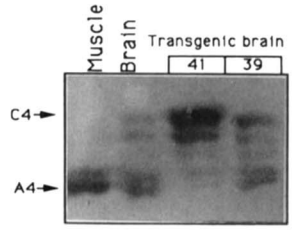

FIG. 6. **Isozymic pattern of aldolase C in transgenic and nontransgenic tissues.** Tissue extracts were electrophoresed on cellulose acetate strips that were then stained for aldolase activity. Nontransgenic animals: A4, homotetramer present in muscle; A4, A3C, A2C2, AC3, and C4, tetramers present in brain. Transgenic mice: brain extract from line 41 F1 (30 integrated copies) and from line 39 F1 transgenic mouse (3 integrated copies).

TABLE I
CAT activity in different tissues of aldolase/CAT transgenic mice

Transgene	Mouse line	Copy no.	CAT activity, cpm/min/μg of protein						
			Brain	Liver	heart	kidney	spleen	lung	intestin
5.5/CAT	32	1	1.3	<0.01	<0.01	<0.01	<0.01	0.1	<0.01
5.5/CAT	9	2	436	<0.01	0.2	<0.01	0.08	<0.01	0.2
5.5/CAT	29	3	203	<0.01	<0.01	<0.01	<0.01	0.4	<0.01
5.5/CAT	1	5	62	<0.01	<0.01	0.6	<0.01	<0.01	<0.01
5.5/CAT	23	7	393	<0.01	0.15	<0.01	<0.01	0.4	0.08
5.5/CAT	24	7	87	<0.01	<0.01	<0.01	<0.01	0.07	0.02
0.8/CAT	24	2	0.6	<0.01	<0.01	<0.01	<0.01	<0.01	N.D
0.8/CAT	30	2	2.4	<0.01	<0.01	<0.01	<0.01	<0.01	N.D
0.8/CAT	3	4	0.8	<0.01	<0.01	<0.01	0.06	<0.01	<0.01
0.115/CAT	15	1	1.5	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
0.115/CAT	19	4	1.1	<0.01	<0.01	0.06	0.02	<0.01	<0.01
0.115/CAT	13	10	1.4	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
0.115/CAT	9	15	0.04	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01

Mice—Different transgenic lines harboring the 5.5/CAT, 0.8/CAT, and 0.115/CAT transgenes were obtained (Table I). A significant CAT activity was detected in the brain of the transgenic mice only, which signifies that a short promoter fragment limited to 115 bp upstream of the major start sites of transcription is sufficient to confer a brain specificity of expression. However, CAT activity in the brain ranged from 62 to 436 arbitrary units for five lines harboring the 5.5/CAT transgene, this activity being especially low only in line 32 harboring a single copy of the transgene. In contrast, the CAT activity was considerably lower in all lines with the 0.8/CAT and the 0.115/CAT transgenes, ranging from 0.04 to 2.4 arbitrary units, even in animals harboring a relatively high copy number (lines 13 and 9; Table I). In addition, no clear correlation was observed for either type of transgene between the number of integrated copies and the CAT activity. These results suggested that elements located upstream of the tissue-specific proximal promoter of the aldolase C gene are necessary for stimulating its activity in the chromosomal context.

Since the upstream 5′-flanking sequence that seems to be important for a quantitatively correct expression of the transgenes contains the differentially methylated *MspI/HpaII* sites, we investigated the methylation of these sites in the transgenes.

Methylation Analysis of the Transgenes—The methylation status of the transgenes was analysed as reported for the endogenous gene. The methylation pattern of the *MspI/HpaII* sites was identical in the endogenous gene and in each copy of the 12.5/aldC transgene (Fig. 7*A* shows the methylation pattern of the line 41 bearing 30 copies), indicating that this pattern depends on information intrinsic to the aldolase C fragments and not on the transgene chromosomal location. Moreover, we can conclude from this analysis that these copies are integrated head to tail. The DNA methylation pattern of the other lines (42, 37, and 39) gave similar results. In contrast, all *MspI/HpaII* sites of the 5.5/CAT transgene were similarly partially methylated in both brain and liver of the different transgenic mouse lines. Fig. 7*B* shows the results obtained for the line 29 after hybridization with probe 1. A similar pattern was obtained with probe 2 with, as expected, an additional fragment of about 550 bp corresponding to the partial digestion of the *HpaII* sites located at −450 bp and in the CAT gene (data not shown).

DISCUSSION

Aldolase C is a brain-specific isoenzyme that is synthesized with aldolase A throughout the brain, although some brain regions appear to be richer in this enzyme than others (Popovici *et al.*, 1990; Thompson *et al.*, 1982). In addition to this absence of a narrow specificity for a particular neuronal population, aldolase C is also characterized by its low expression in any actively proliferating cells, fetal tissues, or cancers. This strange dual specificity, expression in proliferating cells and in nonproliferating brain cells, is not unique to the aldolase C gene; the same phenomenon is shared with other brain-specific isoenzymes, *e.g.* phosphofructokinase (Kahn *et al.*, 1980, 1981), creatine kinase B (Delain *et al.*, 1973; Gazdar *et al.*, 1981), and glycogen phosphorylase (Sato *et al.*, 1972). Therefore, these genes, and in particular the aldolase C gene, are ubiquitously active, although sometimes at a relatively modest level, in all fetal tissues, while their expression is repressed in adult non-brain tissues and stimulated in developing and adult brains. In fact, we show in this paper that the repression of the aldolase C gene in adult non-brain tissues is not total, a very low amount of aldolase C mRNA being detectable by reverse transcriptase PCR. Consistent with this finding, the chromatin conformation in and around the aldolase C gene appears to remain open, as judged from DNase-I hypersensitivity analysis, in adult non-brain tissues as well as in the brain. In fact, conservation of an open chromatin structure in genes expressed in fetal tissues and secondarily extinguished in adult tissues could be the rule. The α-fetoprotein gene, for instance, is ac-

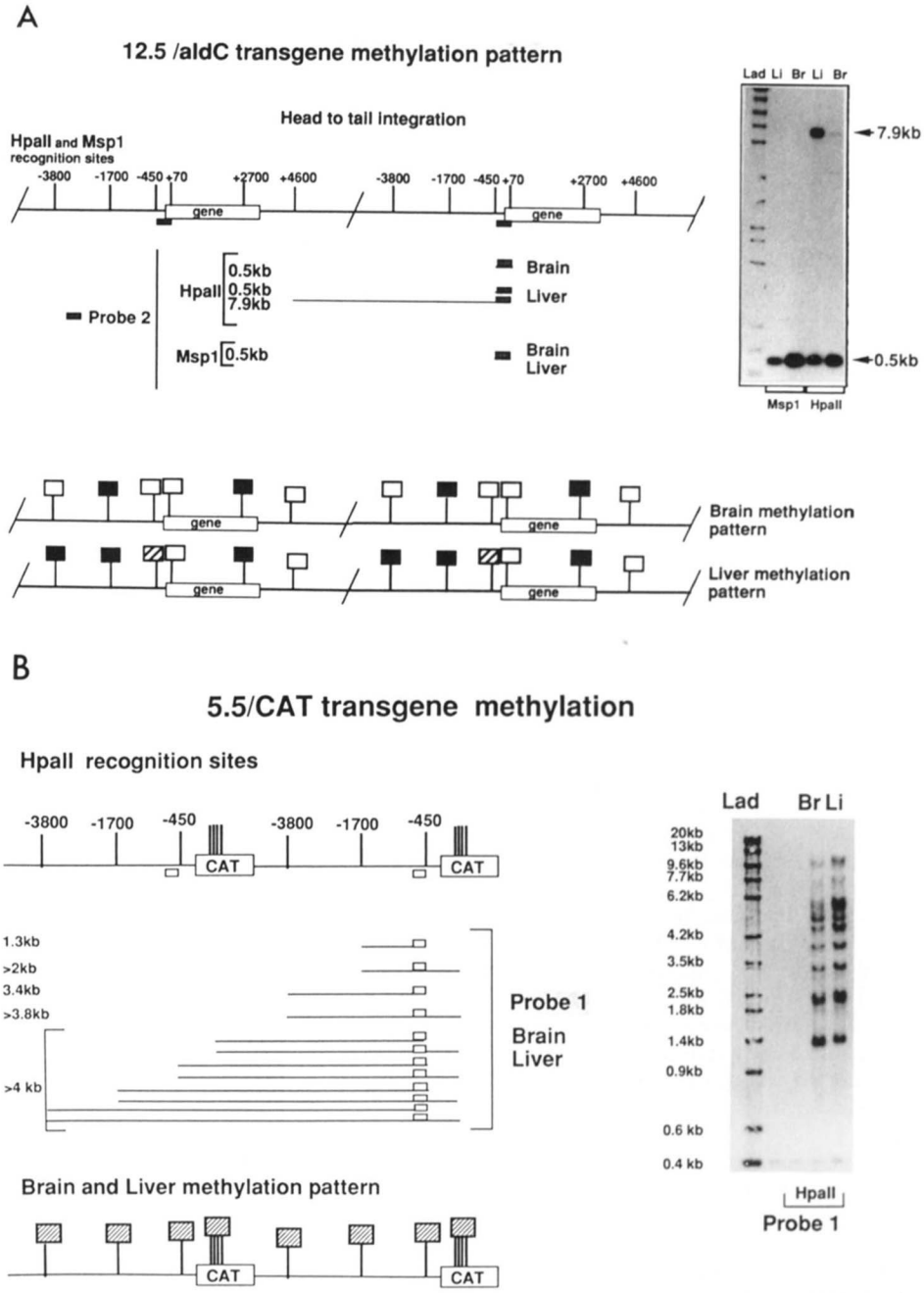

FIG. 7. **Methylation analysis of the transgenes.** DNA was processed as in Fig. 4. The length of the fragments and the position and methylation pattern of the sites were mapped, as indicated in Fig. 4. *Panel A*, 12.5/aldC transgene. Liver (*Li*) and brain (*B*) DNA from line 41 (30 integrated copies) was cut by *Hpa*II or *Msp*I. The Southern blots were hybridized with the three probes described above. Only the hybridization pattern obtained with probe 2 is presented. On the scheme, two transgenes integrated head to tail were represented. *Panel B*, 5.5/CAT transgene. Liver (*Li*) and brain (*Br*) DNA from line 29 (3 copies) was cut by *Hpa*II. The Southern blot was hybridized with probe 1. The methylation pattern analysis shows that all sites are partially methylated in the brain as well as in the liver.

tively transcribed in fetal liver and repressed in adult liver (Tilghman and Belayew, 1982). However, DNase-I HSSs are still present upstream of the α-fetoprotein gene in adult liver, although they are absent in spleen where the gene is never expressed (Godbout and Tilghman, 1988). Similar results have recently been reported in our laboratory, by *in vivo* footprinting, for the L-pyruvate kinase gene that is active in fetal intestine and repressed in the exocrine pancreas derived from an intestinal evagination while its chromatin remains accessible (Miquerol *et al.*, 1994). Importantly, a common characteristic of this type of genes extinguished in differentiated tissues after they have been active during the fetal period is that they can be reactivated, for instance in cancer or in cultured cells in the examples discussed above. In the case of the aldolase C gene, the DNase-I HSS located in the immediate vicinity of the major start sites of transcription is as strong in the liver, where the gene is practically inactive, as in the brain, which suggests that in non-brain tissues the promoter remains accessible to transcription factors but either lacks an essential brain-specific positive factor or is blocked by a negative factor.

The CAT transgenes directed by either 115 or 800 bp of 5′-flanking sequences are tissue specifically but very weakly expressed in the brain. Transgene expression is strongly stimulated by more upstream sequences located between −5500 and −800 bp. This 5′-flanking region does not contain any additional DNase-I HSSs, often associated with remote enhancers

(Elgin, 1988; Gross and Garrard, 1988) but includes the *MspI/HpaII* site at −3800 bp which, in the endogenous aldolase C gene, is differentially methylated in the brain and liver.

Although the association between gene expression and demethylation has been described (Meehan *et al.*, 1992; Peek *et al.*, 1991; Tate and Bird, 1993; Bird, 1992), the exact role of this phenomenon in the control of gene expression remains poorly understood. From various experiments, it seems that modifications in the methylation level are not primarily involved in the rapid changes of gene expression but rather serve to lock an expression pattern (activation or repression) after it has been established (Boyes and Bird, 1992; Opdecamp *et al.*, 1992; Szyf *et al.*, 1990). In the aldolase C gene, the methylation pattern, *i.e.* two *MspI/HpaII* sites demethylated in the brain and totally (−3800 bp) or partially (−450 bp) methylated in other tissues, is conserved in the different lines harboring the 12.5/aldC transgene whose level of expression seems to be more or less correlated with the number of integrated copies. In contrast, the 5.5/CAT transgene, albeit including the same 5′-flanking region as the 12.5/aldC transgene, is expressed without any relationship with the copy number. This absence of tissue-specific methylation pattern and of copy-dependent expression might be related to the lack of aldolase C intragenic or 3′-flanking element(s) or to the own influence of the procaryotic CAT gene.

In contrast with the results of transgene expression, we have observed that sequences upstream of the position −115 bp were inefficient on the activity of aldolase/CAT constructs in transiently transfected PC12 cells (Thomas *et al.*, 1993). Such positive *cis*-acting sequences, active in the chromosomal context (in transgenic animals or in stably transfected cells) but inactive when tested by transient transfection assays, have been reported in different genes such as those for chicken lysozyme (Phi-Van and Stratling, 1988; Stief *et al.*, 1989; Phi-Van *et al.*, 1990) or human interferon-β (Bode and Mass, 1988). It has been suggested that this type of element is important for insulating a gene and protected it from the influence of surrounding chromosomal sequences. However, the 5′-flanking fragment of the aldolase C gene, encompassing from −5500 to −800 bp, although able to stimulate transgene expression, is clearly insufficient to insulate the 5.5/CAT transgene, whose expression remains dependent on the integration site and independent of the copy number.

In conclusion the aldolase C gene represents a paradigm of a family of genes whose expression is ubiquitous in the fetus and restricted to the brain in adults, but which are reactivatable in proliferating non-brain tissues. We have already shown in this paper that the chromatin structure around a brain-specific aldolase C proximal promoter fragment remains accessible outside the brain and that this promoter needs, in order to be strongly active *in vivo*, the presence of more upstream 5′-flanking sequences acting in the chromosomal context but not in transient expression experiments.

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