Analysis of a Brain-specific Isozyme

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 MapI/Hpal II 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 chrompheromonal 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 chrompheromonal 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 (Leberher 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.

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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.

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
amplification of fragments from aldolase C and aldolase B transcripts and from a ubiquitous reference transcript (cytochrome c oxidase subunit I) isolated essentially as described by Villeponteau et al. (1989). 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.

**Results**

Expression of the Aldolase C Gene in Fetal and Adult Tissues—Fig. 2A 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. 2B). 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 (Tsutsu et al., 1985) was not detected in the lung and liver.
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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 EcoRI and EcoRV 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 MspI/HpaII restriction sites (CCGG) in and around the aldolase C gene was analyzed by comparison of the restriction patterns obtained with MspI that is not inhibited by methylation and with HpaII that is inhibited. Digests were analyzed by Southern blotting, and hybridization was performed successively with three types of probes: probe 1 was the BsmHI/SmaI fragment spanning from -800 to -450 bp; probe 2, the SmaI/SmaI fragment located between -450 and +70 bp; and probe 3, the SmaI/BamHI 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. 4A shows that probe 1 revealed different DNA fragments when digestion was performed with HpaII or MspI, 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 MspI/HpaII 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. 4B we took advantage of the presence of two PstI restriction sites upstream and downstream of the +1 site to locate more accurately some of the MspI/HpaII 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 EcoRI/EcoRI 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/salC transgenes is more or less correlated with the number of integrated copies.

Expression of Aldolase/CAT Chimeric Genes in Transgenic
Expression of the Rat Brain-specific Aldolase C Gene

Fig. 4. Methylation analysis of the endogenous gene. Panel A, digestion by HpaII or MspI. DNA from rat liver (Li) and brain (Br) was digested with HpaII or MspI, 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 MspI/HpaII sites are represented by black squares, the demethylated sites by open squares, and the partially methylated sites by hatched squares. Lead, 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 HpaII and PstI. 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.
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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 radio-labeled 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).

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<th>Transgene</th>
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<th>Liver</th>
<th>Heart</th>
<th>Kidney</th>
<th>Spleen</th>
<th>Lung</th>
<th>Intestine</th>
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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 transcrip-

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 glycoenergy 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-
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A 12.5/aldC transgene methylation pattern

**Head to tail integration**

| -3800 | -1700 | 450 | +70 |
| -3800 | -1700 | 450 | +70 |
| -3800 | -1700 | 450 | +70 |
| -2700 | +4600 |

**Probe 2**

- HpaII recognition sites
- Cytosine methylation sites

**Brain methylation pattern**

**Liver methylation pattern**

B 5.5/CAT transgene methylation

**HpaII recognition sites**

| -3800 | -1700 | 450 | +70 |
| -3800 | -1700 | 450 | +70 |
| -3800 | -1700 | 450 | +70 |
| -2700 | +4600 |

**Probe 1**

- Brain methylation pattern
- Liver methylation pattern

**Cat methylation pattern**

**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 (Br) DNA from line 41 (30 integrated copies) was cut by HpaII or MspI. 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 HpaII. 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 a-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 (Mi-querol 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...
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(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′/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 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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