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Upstream Elements Involved *in Vivo* in Activation of the Brain-specific Rat Aldolase C Gene

ROLE OF BINDING SITES FOR POU AND WINGED HELIX PROTEINS*

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The rat aldolase C gene encodes a glycolytic enzyme strongly expressed in adult brain. We previously reported that a 115-base pair (bp) promoter fragment was able to ensure the brain-specific expression of the chloramphenicol acetyltransferase (CAT) reporter gene in transgenic mice, but only at a low level (Thomas, M., Makeh, I., Briand, P., Kahn, A., and Skala, H. (1993) *Eur. J. Biochem.* 218, 143–151). Here we show that *in vivo* activation of this promoter at a high level requires cooperation between an upstream 0.6-kilobase pair (kb) fragment and far upstream sequences. In the 0.6-kb region, a 28-bp DNA element is shown to include overlapping *in vitro* binding sites for POU domain regulatory proteins and for the Winged Helix hepatocyte nuclear factor-3 β factor. An hepatocyte nuclear factor-3 β -binding site previously described in the short proximal promoter fragment is also shown to interact *in vitro* with POU proteins, although with a lower affinity than the 28-bp motif. Additional binding sites for POU factors were detected in the upstream 0.6-kb sequences. Progressive deletion in this region resulted in decreased expression levels of the transgenes in mice, suggesting synergistic interactions between these multiple POU-binding sites. We propose that DNA elements characterized by a dual binding specificity for both POU domain and Winged Helix transcription factors could play an essential role in the brain-specific expression of the aldolase C gene and other neuronal genes.

Molecular events underlying specific gene expression in the brain still remain poorly elucidated compared with those governing gene transcription in other differentiated tissues, in part due to the complexity of this organ, which appears as a mosaic of various cell types. Some genes are highly specific to certain neuron subpopulations (1, 2), whereas others, especially isoenzymes of the general metabolism, are expressed in any types of neurons, although at different levels (3, 4). The aldolase C gene, coding for the brain-specific form of the glycolytic enzyme aldolase, belongs to this last class and, in addition, is also transcribed in glial cells (5, 6). Like other genes broadly

expressed in the brain, the aldolase C gene exhibits several features of housekeeping genes, namely the absence of canonical TATA and CAAT boxes, GC-rich sequence, and multiple transcription start sites (3, 4, 7–9). We have previously reported that brain-specific expression of the CAT¹ reporter gene in transgenic mice could be directed by a short (<115 bp) promoter fragment of the aldolase C gene (10); however, this short fragment was not able to direct the high level transgene expression observed with the full-length 5.5-kb 5'-flanking region (11, 12). To identify DNA regulatory elements required to ensure high level gene expression in the brain, we have now defined *in vivo*, in transgenic mice, activating sequences of the aldolase C gene and some of the cognate DNA-binding transcription factors. We found that cooperation between an upstream 0.6-kb region and a far upstream 3.8-kb region is required. Several DNA motifs exhibiting overlapping binding sites for both POU domain proteins (13, 14) and the Winged Helix HNF-3 β factor (15, 16) were identified in the 0.6-kb region. Since this type of dual motif is also present in the brain-specific 115-bp promoter of the aldolase C gene and in regulatory elements of several genes specifically expressed in secretory neurons, we proposed that it could constitute an important determinant for regulated expression of brain-specific genes, both broadly expressed and neuron-type restricted genes.

MATERIALS AND METHODS

Plasmid Constructions—The 5.5/CAT, 0.8/CAT, and 0.115/CAT constructs have been described previously (10, 11). The transgenes 3.8+0.8/CAT, B+C+D+0.8/CAT, A+0.8/CAT, A+B+0.8/CAT, A+C+0.8/CAT, A+D+0.8/CAT, and 3.8+0.5/CAT contain different restriction fragments of the aldolase C gene 5.5-kb 5'-flanking region inserted upstream of the CAT reporter gene. The following restriction sites were used: *Xba*I, *Sac*I, *Bam*HIa, *Bam*HIb, and *Sma*I (see Fig. 1). The transgene 3.8+115/CAT was obtained by cloning the 3.8-kb fragment upstream of the 0.115/CAT construct. The transgene 3.8+0.7/CAT was obtained by inserting a polymerase chain reaction fragment (subsequently sequenced) between the *Bam*HIa and *Sma*I sites.² All constructs were cloned in the sense orientation, except the transgene A+C+0.8/CAT, in which fragment C was cloned in the antisense orientation.

Production and Analysis of Transgenic Mice—Inserts were isolated from agarose gels and purified on Elutip-d columns (Schleicher & Schuell). The fragments (5–10 ng in a 10 mM Tris-HCl (pH 7.5), 0.1 mM EDTA buffer) were microinjected into fertilized B6D2 mouse eggs (17). Transgenic founders and offspring were identified (18) by Southern blotting using a random-primed CAT probe. CAT activities generated by transgene expression were assayed on 0.5–500 μ g of liver, heart, lung, and brain extract proteins (19).

¹ The abbreviations used are: CAT, chloramphenicol acetyltransferase; kb, kilobase pair(s); bp, base pair(s); GST, glutathione S-transferase; HNF, hepatocyte nuclear factor; EMSA, electrophoretic mobility shift assay; WH, Winged Helix.

² Further details of the cloning procedures are available on request.

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Nuclear Extracts and Recombinant Proteins—Nuclear extracts were prepared from rat brain according to Piette *et al.* (20) and from rat liver according to Gorski *et al.* (21). Bacterial expression vectors for the different glutathione *S*-transferase (GST) fusion proteins were constructed by inserting an entire coding region or a POU-coding region of mouse class III POU genes into the vector pGEX2T (Amersham Pharmacia Biotech). For GST-Brn-1, the 2-kb *Sma*I fragment was subcloned at the *Sma*I site of pGEX2TAB (in which the *Bam*HI site is modified by the fill-in reaction to alter the coding frame). The resulting fusion protein possesses part of Brn-1 (from amino acids 257 to 498) including the POU domain. For GST-Brn-2, the *Bam*HI-*Eco*RI fragment derived from a Brn-2 partial cDNA clone was subcloned in frame in pGEX2T. This vector can produce a fusion protein with part of the Brn-2 protein (from amino acids 131 to 486) including the POU domain. For GST-Brn-4, the 2.3-kb *Bam*HI-*Eco*RI fragment was subcloned in pGEX2T. The fusion protein contains the entire Brn-4 amino acid sequence plus three extra residues added at the junction. For GST-Oct-6, the 1-kb *Sma*I fragment was subcloned at the *Sma*I site of pGEX2T. This expression vector can produce a fusion protein containing the Oct-6 POU domain (from amino acids 229 to 448). The recombinant proteins were produced and purified as recommended by Amersham Pharmacia Biotech.

Electrophoretic Mobility Shift and Methylation Interference Assays—Oligonucleotides used as probes or competitors were as follows: 28 bp (bp -862 to -835 of the rat aldolase C gene (7)); for the rat aldolase C sequences, base pairs are numbered with respect to the ATG translation initiator), 5'-ATGCTATTTAAATAAAGTGTATTTAATG-3'; 28mut (representing the 28-bp sequence mutated at positions -854 (T → G) and -853 (A → C)), 5'-ATGCTATTGCAATAAAGTGTATTTAATG-3'; box C site (bp -117 to -96 of the rat aldolase C gene), 5'-TGCTGCTGCCTTATTACTCCA-3'; site 524 (bp -524 to -497 of the rat aldolase C gene), 5'-GAAACTCAAATCCATTATTCATGCCCTTGAA-3'; site 824 (bp -824 to -803 of the rat aldolase C gene), 5'-GTCCACTGAATCTAATTTTGGG-3'; Oct (representing the Oct-1-binding site of a herpes simplex virus immediate-early gene promoter (22)), 5'-GCATGCTAATGATATTCCTTT-3'; CRH II (bp -134 to -113 of the corticotropin-releasing hormone gene promoter (23)), 5'-TGCTCCTGCATAAATAATAGG-GCCCT-3'; HNF-3 (bp -111 to -90 of the mouse transthyretin gene promoter (24)), 5'-GTTGACTAAGTCAATAATCAGA-3'; and Sp1 (sequence derived from the SV40 enhancer (25)), 5'-GCATAACTCCGCC-CAGTTAG-3'.

Electrophoretic mobility shift assays (EMSA) were performed as described previously (12, 26). Binding reactions contained 0.1–0.5 ng of T4 kinase-radiolabeled double-stranded probe and 7–10 μ g of rat brain nuclear extract proteins with 300 ng of poly(dI-dC) or of rat liver nuclear extract proteins with 1 μ g of sonicated salmon sperm DNA or 10–50 ng of recombinant protein with 100 ng of poly(dI-dC). Various amounts of specific or unrelated competitors were included or not. For supershift assays, binding reactions were incubated for 30 min at 0 °C, and then 1 or 2 μ l of antibodies were added and further incubated for 30 min at room temperature. DNA complexes were separated on 6% (w/v) non-denaturing polyacrylamide gels. Anti-HNF-3 β polyclonal antibodies were kindly provided by J. E. Darnell (Rockefeller University, New York); anti-Oct-1, anti-Brn-1, and anti-Brn-2 polyclonal antibodies were supplied by Santa Cruz Biotechnology, Inc.

For methylation interference assays (27), binding reactions included partially methylated 5'- or 3'-labeled 28-bp probe. Bound and free DNA fractions separated by gel shift electrophoresis were cleaved with piperidine at modified guanine and adenine residues (28) and loaded on a 8 M urea, 15% (w/v) polyacrylamide gel.

RESULTS

In Vivo, an Upstream 0.6-kb Fragment and a Far Upstream 3.8-kb Fragment Are Both Necessary for High Level Expression of the Aldolase C Transgenes—Using the CAT reporter gene, we previously showed that the transcriptional activity of the rat aldolase C gene in transgenic mice was ensured by a short 115-bp promoter that was brain-specific, but that was considerably more active when present in a construct containing 5.5 kb of aldolase C upstream sequences (10, 11). To localize activating sequences in this 5.5-kb fragment, we analyzed, in transgenic mice, various combinations of aldolase C gene 5'-noncoding sequences fused to the CAT reporter gene. Potential regulatory elements are usually correlated with the presence of DNase I-hypersensitive sites in the chromatin. However, in the

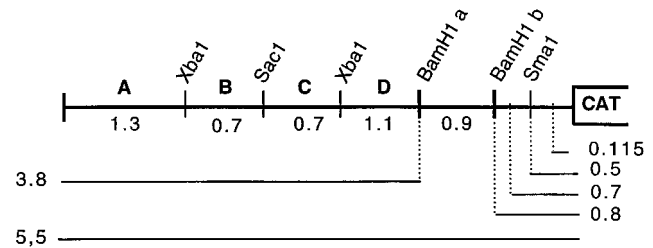


FIG. 1. Scheme of the different aldolase C/CAT transgenes. The restriction sites used for the construction of the different transgenes and the positions of the 0.7- and 0.115-kb fragments generated by polymerase chain reaction are indicated. The approximate sizes and the designations of the subfragments deleted or cloned upstream of the CAT reporter gene are also noted.

TABLE I
CAT activity of aldolase C/CAT transgenes in brains of transgenic mice

For each transgenic mouse, three other tissues were tested (heart, lung, and liver); CAT activities were at least 100–1000-fold lower than in brain.

transgene	Founder or line ^a	CAT activity cpm/min/ μ g
5.5/CAT	9	436
	23	393
	29	203
	24	87
	1	62
0.8/CAT	30	2.4
	3	0.8
	24	0.6
3.8+0.8/CAT	5	4800
	12	1900
	28	1445
	24	1310
	1	878
3.8+0.115/CAT	22	32
	16	4.6
	2	0.3
	15	0.03

^a Copy numbers ranged from 2 to 40. No correlation between copy number and CAT activity level was observed.

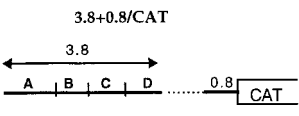
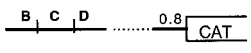
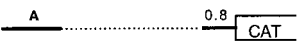
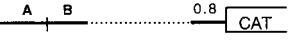
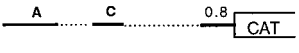
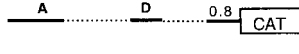
case of the aldolase C gene, we reported only one hypersensitive site located in the vicinity of the transcription start sites in the 115-bp promoter (11). So, we used naturally occurring restriction sites to perform arbitrary deletions in the 5.5-kb fragment (Fig. 1). All constructs contained the 115-bp promoter and exhibited brain-specific expression, with CAT activities being at least 100–1000-fold higher in the brain than in the heart, lung, and liver of transgenic animals.

As already reported (11), there was no correlation between the number of integrated copies and the CAT activity of the aldolase C/CAT chimeric transgenes. For most constructs, we therefore analyzed a great number of independent transgenic lines or founders to overcome integration site-dependent variations of transgene expression.

A large deletion leaving ~0.8 kb of upstream sequences (transgene 0.8/CAT) was previously shown to drastically reduce transcription in the brain (11), compared with the construct containing 5.5 kb of 5'-noncoding sequences (transgene 5.5/CAT) (Fig. 1 and Table I). CAT activities directed by the

TABLE II
Dissection of the 3.8-kb fragment

See the legend to Table I for details.

transgene	Founder or line ^a	CAT activity cpm/mn/μg
	5	4800
	12	1900
	28	1445
	24	1310
	1	878
	1	40
	31	35
	27	28
	3	22.7
	29	0.9
	4	0.74
	12	0.7
	19	0.4
	4	1180
	12	996
	29	420
	15	13
	19	1.7
	16	0.9
	31	1250
	21	1200
	53	353
	61	81
	46	1.06
	9	0.59
	22	0.25
	51	0.05
	56	3467
	31	2850
	1	2600
	30	1363
	19	794
	7	351
	34	547
	8	234
	59	238
	53	55
	4	854
	30	326
	26	253
	17	180
	8	125
	14	11

^a Copy numbers ranged from 2 to 40. No correlation between copy number and CAT activity level was observed.

0.8/CAT transgene were in the same range as those obtained with the 0.115/CAT transgene containing the short promoter alone (10, 11).

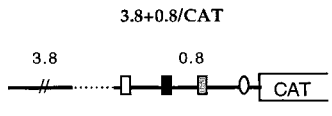
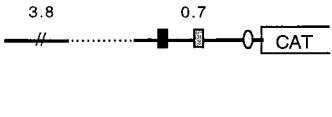
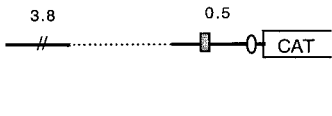
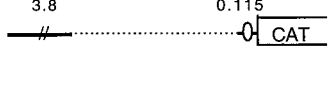
The transgene 3.8+0.8/CAT, containing an internal deletion of 0.9 kb (*Bam*HIa-*Bam*HIb; Fig. 1), was then analyzed. CAT activities ranged between 400- and 1000-fold the activities observed with the transgene 0.8/CAT (Table I), being even higher and less dispersed than CAT activities directed by the original 5.5/CAT construct. This latest observation could be explained either by the occurrence of a negative element in the deleted 0.9-kb fragment or by a position effect. These results indicate that the far upstream 3.8-kb fragment contains elements able to induce transcriptional activation.

However, when cloned directly upstream of the 115-bp promoter (transgene 3.8+0.115/CAT; Fig. 1), the 3.8-kb element directed low CAT activities in the brains of transgenic mice, not exceeding 32 cpm/min/μg of protein (Table I). The 3.8-kb fragment alone is thus unable to ensure activation. Cooperation between upstream elements, present in the 0.6-kb sequences located immediately upstream of the 115-bp promoter, and far upstream elements, present in the 3.8-kb region, is necessary to activate the aldolase C promoter *in vivo*.

The Far Upstream 3.8-kb Activating Fragment Is Composed

TABLE III
Dissection of the 0.8-Kb fragment

See the legend to Table I for details. □, 28-bp element; ■, site 824 oligonucleotide; ▣, site 524 oligonucleotide; ○, box C oligonucleotide.

transgene	Founder or line ^a	CAT activity cpm/mn/μg
	5	4800
	12	1900
	28	1445
	24	1310
	1	878
	30	792
	2	666
	56	346
	31	91
	32	90
	25	50
	17	43
	59	1.9
	28	103
	11	55
	4	52
	36	51
	6	24
	19	13
	7	9.8
	22	32
	16	4.6
	2	0.3
	15	0.03

^a Copy numbers ranged from 2 to 40. No correlation between copy number and CAT activity level was observed.

of Several Discontinuous cis-Acting Subfragments—The 3.8-kb fragment was then subdivided into four subfragments, A–D, which were cloned in various combinations upstream of the 0.8/CAT construct (Fig. 1 and Table II). The fully active transgene 3.8+0.8/CAT, which contained subfragments A, B, C, and D, was again reported in this table. Deletion of element A (transgene B+C+D+0.8/CAT) decreased the brain CAT activities below 40 cpm/min/μg of protein in the nine transgenic lines analyzed, with five lines exhibiting CAT activities below 1 cpm/min/μg of protein. These results indicate that element A is necessary for transcriptional activation. However, when element A was cloned alone upstream of the 0.8/CAT construct (transgene A+0.8/CAT), we observed a binary distribution of the CAT values, with only three transgenic lines out of six expressing high activities in the brain. This dispersion of the CAT values, which probably reflects the position effect already reported in transgene expression (29), contrasts with the high activities obtained in all lines with the 3.8+0.8/CAT transgene. We then hypothesized that element A contains sequences able to activate transcription (see lines 4, 12, and 29), but that depending on the integration site, it could need sequences present in element B or/and C or/and D to overcome a negative influence of the chromatin environment. We therefore cloned each of elements B–D between the element A and 0.8/CAT sequences (Table II). When element B was present in addition to element A (transgene A+B+0.8/CAT), a bimodal distribution of the CAT values was observed again, as was the case with element A alone. In contrast, the A+C+0.8/CAT transgene directed high levels of CAT expression in the brain for

nine out of ten transgenic lines, with activities ranging from >200 to 3500 cpm/min/ μ g of protein. Consequently, fragment C seems to contain elements allowing, in cooperation with element A, for a high transcriptional activity more or less independent of the chromatin environment. The A+D+0.8/CAT transgene directed CAT activities intermediate between those obtained with transgenes containing A or A+B elements and transgenes containing A+B+C+D or A+C elements. Thus, the 1.3-kb fragment A and the 0.7-kb fragment C were the most efficient far upstream elements to ensure a high transcriptional level, in cooperation with the 0.6-kb upstream region.

Progressive 5'-Deletions of the Upstream 0.6-kb Fragment, Removing Putative POU Domain Protein-binding Sites, Reduce Transgene Expression—Computer analysis of the 0.6-kb sequence and *in vitro* DNA/protein interaction studies (see below) revealed the presence of several DNA-binding sites for regulatory proteins of the POU domain family. To determine which sequences are able to cooperate with the far upstream activator, we compared the CAT activities directed by the 3.8+0.8/CAT transgene and by transgenes progressively deleted in the upstream 0.6-kb region, including the 3.8+115/CAT construct (Fig. 1). The results are presented in Table III. Four protein-binding sites (described below) are indicated on the schemes of the transgenes. Analysis of the 3.8+0.7/CAT transgene showed very dispersed expression levels, with three out of nine transgenic lines (lines 2, 30, and 56) still exhibiting high CAT activities in the brain (from 350 to 800 cpm/min/ μ g of protein) and the others expressing the reporter gene at markedly decreased levels (from 90 to <1 cpm/min/ μ g of protein). CAT activities directed by the 3.8+0.5/CAT transgene were similarly dispersed, but remained at or below 100 cpm/min/ μ g of protein in all ten lines, *i.e.* in the same range as activities observed with the 3.8+115/CAT transgene. Thus, the main decrease in the transgene expression level was observed when the 100 bp located at the 5'-end of the 0.6-kb fragment were deleted, although an additional decrease also seemed to occur when an additional 200 bp were removed.

A Conserved 28-bp Element in the 0.6-kb Fragment Binds Both POU Domain and Winged Helix Factors—Comparison between the 5'-flanking sequences of the human (30) and rat (7) aldolase C genes revealed a perfect homology over an AT-rich, 28-bp motif located at the 5'-end of the rat gene 0.6-kb fragment (bp -862 to -835). Binding of nuclear factors on the 28-bp labeled probe was investigated by EMSA. Using rat brain nuclear extracts, three specific complexes (*x*, *y*, and *z*) were observed, which were abolished by competition with the 28-bp unlabeled oligonucleotide and not by the unrelated Sp1 competitor (Fig. 2A, lanes 1-3 and 7). Rat liver nuclear extracts displayed two 28-bp binding activities (Fig. 2B, lanes 1-3): complex *x'*, exhibiting the same electrophoretic mobility (data not shown) as the brain complex *x*, and the retarded band *u*. Complex *x/x'* was also detected with other non-neuronal tissue or cell extracts, whereas complexes *y* and *z* were only detected with brain extracts (data not shown).

The AT-rich sequence of the 28-bp motif was reminiscent of binding sites for homeodomain proteins. We focused on the POU protein family, whose several members are specifically expressed in nervous tissues and have been involved in regulatory events concerning development and differentiation of the nervous system (31). Indeed, the ubiquitous complex *x/x'* and the brain-specific complexes *y* and *z*, but not the liver complex *u*, were totally displaced by the Oct oligonucleotide (Fig. 2, A and B, lanes 4), containing the Oct-1-binding site TAATGARAT of a herpes simplex virus immediate-early gene promoter (22, 32). The CRH II oligonucleotide, matching with the preferential affinity consensus sequence CATnTAAT for the class III

POU proteins (23), was also able to abolish formation of complexes *x*, *y*, and *z* with brain extracts (Fig. 2A, lane 5).

A methylation interference analysis performed on the major brain-specific complex *y* revealed strong interference at adenine -854 on the noncoding strand and adenine -853 on the coding strand of the 28-bp element (Fig. 2C). Accordingly, the 28mut oligonucleotide, bearing T \rightarrow G and A \rightarrow C transversions at positions -854 and -853, respectively, was unable to prevent formation of complex *y* with the labeled 28-bp probe (Fig. 2A, lane 6); it also failed to compete for formation of the brain complexes *x* and *z* and of the liver complexes *x'* and *u* (Fig. 2, A and B, lanes 6), suggesting that the binding sites for each of these interactions overlap and that they share at least 1 nucleotide required for DNA/protein contacts.

Adenines -854 and -853 are located at the center of a palindromic motif (-858 to -849) presenting homologies in both orientations to several consensus binding sites for POU domain proteins (Fig. 3A). This aldolase C motif matches at only six out of eight bases with the canonical octamer ATGCAAAT, but the most highly conserved positions (A⁺¹ and A⁺⁷) are respected, and the T at position -1 is favorable (32). The aldolase C motif also matches at seven out of nine positions with the TAATGARAT consensus sequence, which represents an alternative binding site for Oct-1 (22, 32), and at eight out of nine positions with one of the Brn-2 consensus binding sites, MATnWAAT (*n* = 0, 2, or 3) (23).

In supershift experiments with commercial polyclonal antibodies, complexes *x* (brain) and *x'* (liver) were abolished by antibodies directed against the ubiquitous factor Oct-1 (Fig. 2, A, lane 8; and B, lane 7). Complexes *y* and *z* were abolished by antibodies directed against the brain-specific Brn-1 and Brn-2 factors, respectively (Fig. 2A, lanes 9 and 10).

Screening of data banks for homology to known protein-binding sites revealed that the 28-bp sequence also matched at eight out of eleven positions in antisense orientation with the consensus binding site for the WH protein HNF-3 β (Fig. 3A) (15, 33). Accordingly, in liver extracts, complex *u* was specifically abolished by the HNF-3 oligonucleotide, consisting of the HNF-3 β -binding site of the transthyretin gene, and by a polyclonal antibody directed against the HNF-3 β protein (Fig. 2B, lanes 5 and 8). Anti-HNF-3 α and anti-HNF-3 γ antibodies failed to supershift or abolish complex *u* (data not shown).

Dual Binding Sites for POU and WH Proteins Also Occur in the Brain-specific Promoter of the Aldolase C Gene and in Other Neuronal Genes—We have already reported an HNF-3-binding site in the brain-specific rat aldolase C gene 115-bp promoter (termed box C in Ref. 12). Raynal *et al.* (34) found that this proximal box C was also a binding site for recombinant Brn-2 protein. Homologies of the box C site oligonucleotide to both a Brn-2 consensus sequence (*n* = 0) and the HNF-3 consensus sequence are indicated in Fig. 3B. Using recombinant GST fusion proteins, we observed that both the 28-bp element and the proximal box C were able to bind the POU domains of Brn-1, Brn-2, and Oct-6 and the entire Brn-4 protein (data not shown). However, the affinity of the Brn-2 POU domain was much lower for the proximal box C than for the 28-bp site, as shown by competition experiments (Fig. 4A). The binding of Brn-2 to the 28-bp probe was almost completely abolished by a 50-fold excess of the 28-bp or Oct competitor, whereas it was only partially competed for by a 800-fold excess of the box C site oligonucleotide (no competition was observed with a 800-fold excess of the unrelated Sp1 oligonucleotide). Similar results were obtained for competition of Oct-1, Brn-1, and Brn-2 binding in brain extracts and of GST-Brn-1 POU domain binding (data not shown). Using liver extracts, despite an apparently better matching of the proximal box C sequence than of the

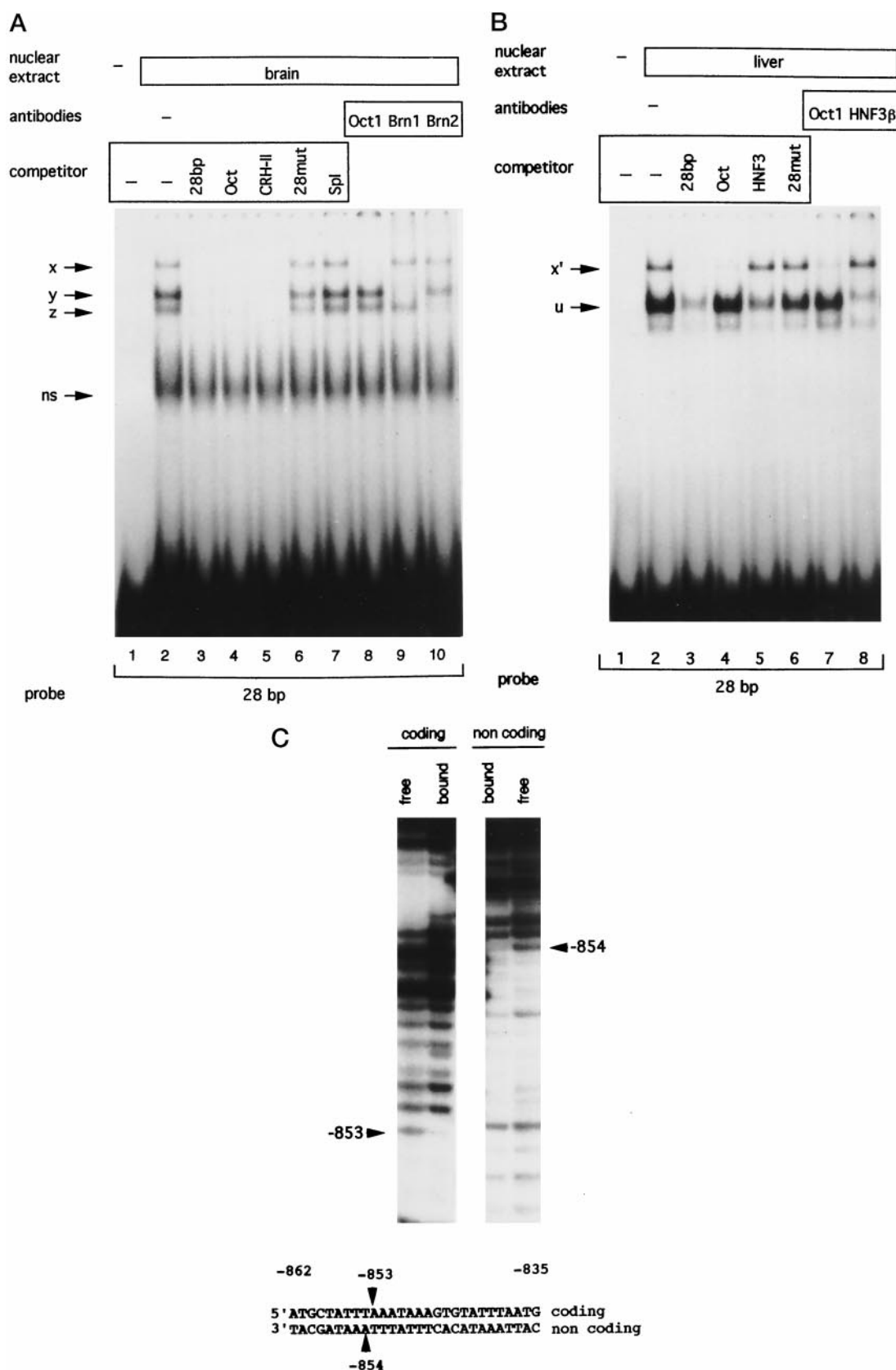


FIG. 2. Analysis of the 28-bp element binding activities in brain and liver nuclear extracts. **A**, EMSA was performed using the 28-bp radiolabeled probe in the presence (lanes 2–10) or absence (lane 1) of rat brain nuclear extract. The indicated unlabeled competitors were added (lanes 3–7) at a 50-fold molar excess; the indicated antibodies were added (1 μ l) after completion of the binding reaction (lanes 8–10). Complexes x, y, and z are indicated by arrows. ns, nonspecific complexes. **B**, EMSA was performed using the 28-bp radiolabeled probe in the presence (lanes 2–8) or absence (lane 1) of rat liver nuclear extract. The indicated competitors were added (lanes 3–6) at a 50-fold molar excess; the indicated antibodies were added (1 μ l) after completion of the binding reaction (lanes 7 and 8). Complexes x' and u are indicated by arrows. **C**, methylation interference assay was performed on the brain-specific complex y prepared on a partially methylated 28-bp probe end-labeled on the coding or noncoding strand. Residues at which methylation interfered with protein binding are indicated by arrows and are summarized at the bottom.

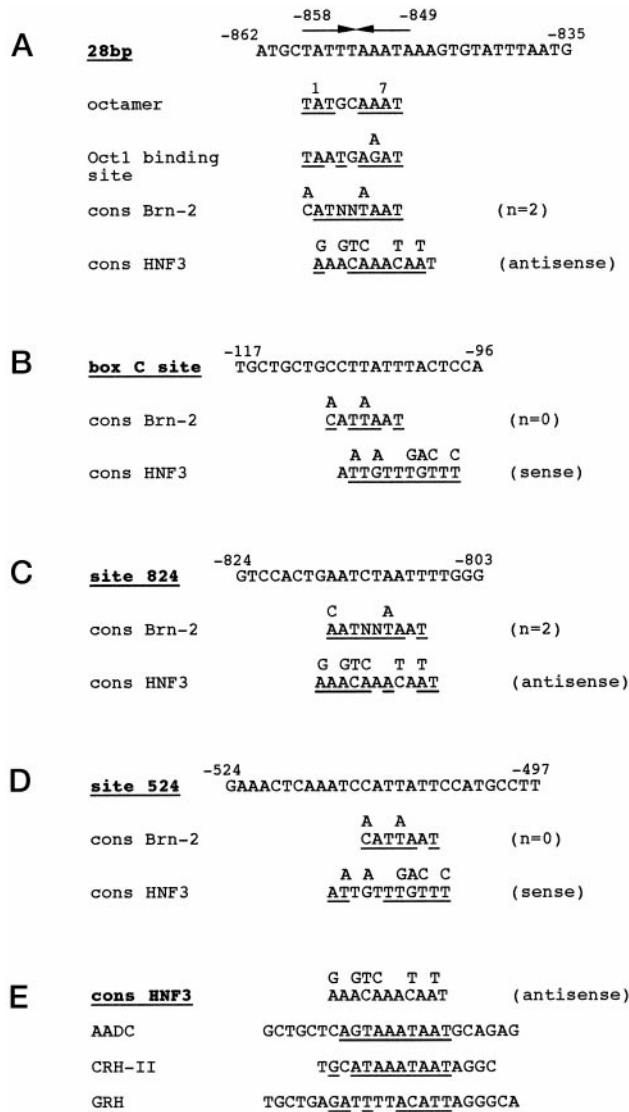


FIG. 3. Sequence homologies between different DNA elements and consensus binding sites for POU domain proteins and HNF-3 β . DNA elements from the aldolase C gene are analyzed in A–D. The palindromic sequence (–858 to –849) of the 28-bp element is indicated. The TAATGAGAT Oct-1-binding site is from Ref. 32; the Brn-2 consensus (cons) sequence MATnWAAT (with $n = 0, 2, \text{ or } 3$) is from Ref. 23; and the HNF-3 consensus sequence is from Ref. 33. In E, the –96/–74 fragment of the aromatic L-amino-acid decarboxylase (AADC) gene (34), the CRH II motif of the corticotropin-releasing hormone (CRH) gene (23), and the AT-a motif of the gonadotropin-releasing hormone (GRH) gene (35) are compared with the HNF-3 consensus binding site (33). The matching nucleotides are *underlined* in the consensus sequences in A–D and in the gene sequences in E. When 2 bases are acceptable in consensus sequences, both letters are indicated one upon the other.

28-bp sequence with the HNF-3 consensus motif (Fig. 3, A and B), we also observed a considerably lower affinity of the box C site oligonucleotide compared with the 28-bp element for HNF-3 β : Fig. 4B shows that the 28-bp oligonucleotide·HNF-3 β complex was displaced by a 50-fold excess of the 28-bp unlabeled probe *versus* a 200-fold excess of the transthyretin HNF-3 site oligonucleotide and a 800-fold excess of the box C site oligonucleotide. In this experiment, Oct-1 binding was also efficiently competed by the 28-bp oligonucleotide, but not by the other competitors used. Therefore, *in vitro*, the proximal box C is a binding site for both POU proteins and HNF-3 β , although with a weaker affinity for these factors than the 28-bp motif.

This type of dual DNA motif able to bind both POU and WH proteins has also been reported in the neuronal promoter of the

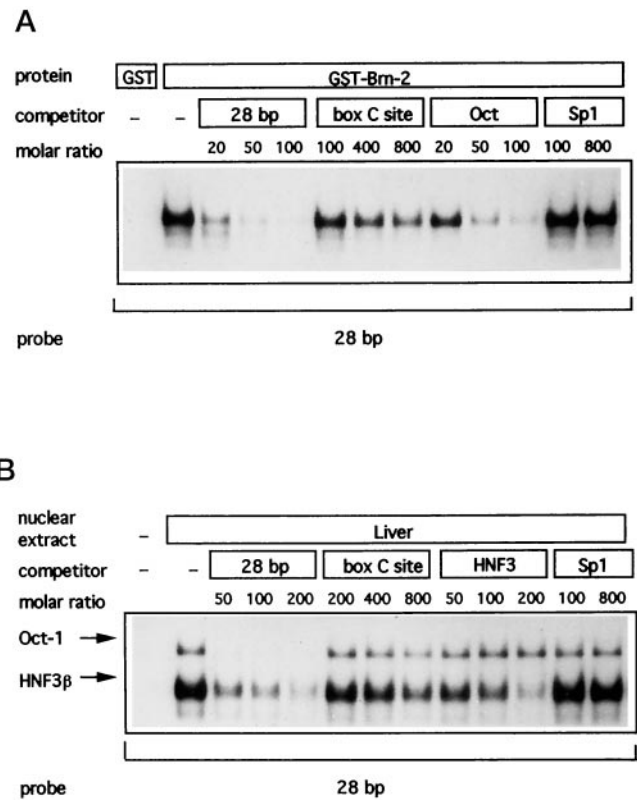


FIG. 4. Compared binding properties of the 28-bp element and the box C site oligonucleotide. EMSA was performed using the 28-bp radiolabeled probe and the GST protein or the GST-Brn-2 POU domain fusion protein (A) or rat liver extract (B). The different competitors were present at the indicated molar excess. The faint higher mobility band observed in A probably represents a degradative product of the recombinant fusion protein. In B, Oct-1 and HNF-3 β complexes are indicated by *arrows*.

aromatic L-amino-acid decarboxylase gene (34). Moreover, we show in Fig. 3E that two other *cis*-acting POU-binding sites described in neuronal genes present sequence homologies to the HNF-3 consensus motif (33): the strong Brn-2-binding site CRH II of the corticotropin-releasing hormone gene (23) and the Oct-1-binding site AT-a of the gonadotropin-releasing hormone gene (35) match the HNF-3 consensus sequence at ten out of eleven and at eight out of eleven nucleotides, respectively.

The 0.6-kb Fragment Contains Additional POU Domain Protein-binding Sites—Removal of the high affinity 28-bp Brn-1- and Brn-2-binding site in the 3.8+0.7/CAT transgene can be related to the important decrease in the CAT expression level observed in the brains of most transgenic mice (Table III). However, in three out of nine transgenic lines, CAT expression was still elevated. Computer screening for homologies to different POU protein binding sites and to the HNF-3 consensus motif revealed several dual DNA motifs scattered all over the upstream 0.6-kb fragment. Two of them are reported in Fig. 3 (C and D). The site 824 (spanning residues –824 to –803, present in the 3.8+0.7/CAT transgene, but not in the 3.8+0.5/CAT transgene) and site 524 (spanning residues –524 to –497, present in the 3.8+0.5/CAT transgene, but not in the 3.8+115/CAT transgene) oligonucleotides were analyzed by EMSA. In liver extracts, both were inefficient in abolishing HNF-3 β binding to the 28-bp probe, even at high molar excess (data not shown). In contrast, in brain extracts, the site 824 and site 524 oligonucleotides were able to compete with the 28-bp probe for Oct-1, Brn-1, and Brn-2 binding (data not shown). *In vitro*, the affinity for the Brn-2 POU domain was in the decreasing order

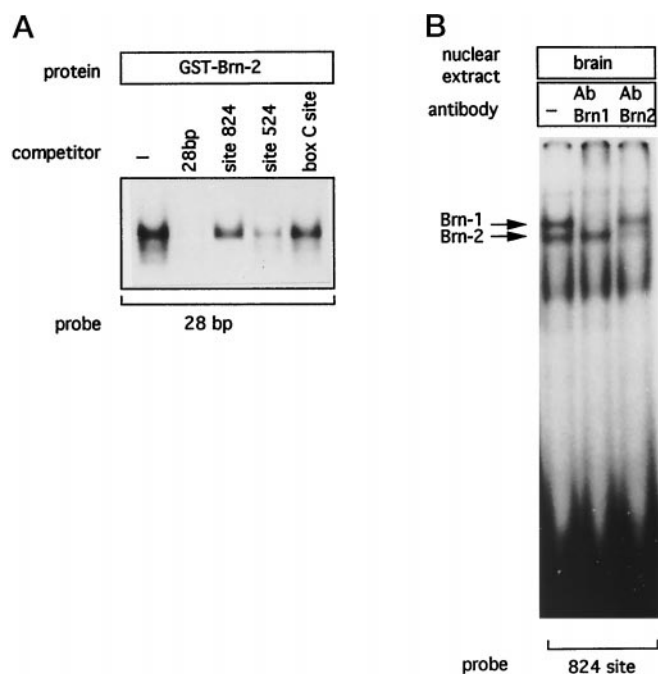


FIG. 5. Additional binding sites for brain-specific POU proteins in the 0.6-kb fragment. A, EMSA was performed using the 28-bp radiolabeled probe in the presence of the GST-Brn-2 POU domain fusion protein. The indicated competitors were present at a 50-fold molar excess. B, EMSA was performed using the radiolabeled site 824 oligonucleotide in the presence of rat brain nuclear extract. Antibodies (Ab) directed against Brn-1 and Brn-2 proteins were added (1 μ l) or not, as indicated, after completion of the binding reaction. Brn-1 and Brn-2 complexes are indicated by arrows. The Oct-1 complex is barely detectable.

28-bp probe/site 524/site 824/box C site as shown by competition assays (Fig. 5A). When used as labeled probes in supershift experiments, both site 824 (Fig. 5B) and site 524 (data not shown) oligonucleotides were able to bind Brn-1 and Brn-2 in brain extracts. Therefore, *in vitro*, the site 824 and site 524 oligonucleotides appear to be POU protein- rather than HNF-3 β -binding sites. *In vivo*, deletion of the site 524 element did not seem to have a great effect on transcriptional activity (Table III). However, deletion of the site 824 element could account for the decrease in CAT activity observed between the 3.8+0.7/CAT and the 3.8+0.5/CAT transgenes. The different *cis*-acting sequences of the aldolase C gene investigated in this study are summarized in Fig. 6.

DISCUSSION

High Level Expression of the Aldolase C Gene Requires the Presence of a Modular Far Upstream Region—A functional synergy between distal and proximal regulatory elements has been reported to be involved in the transcriptional control of numerous genes (36, 37), including neural genes (3, 9, 38, 39), and is well illustrated by the case of the rat aldolase C gene. Indeed, high level expression of the aldolase C/CAT constructs in the brains of transgenic mice required cooperation between a 0.6-kb fragment located immediately upstream of the tissue-specific but weak 115-bp proximal promoter and a far upstream 3.8-kb region (Fig. 6).

In this 3.8-kb region, the more distal 1.3-kb element A is critical for promoter activation. However, additional sequences present mainly in the 0.7-kb element C seem to be necessary to maintain a high stable level of transcriptional activity. *cis*-Acting positive elements can be seen as sequences that increase the probability of formation of an active chromatin domain rather than as sequences that directly increase the rate of

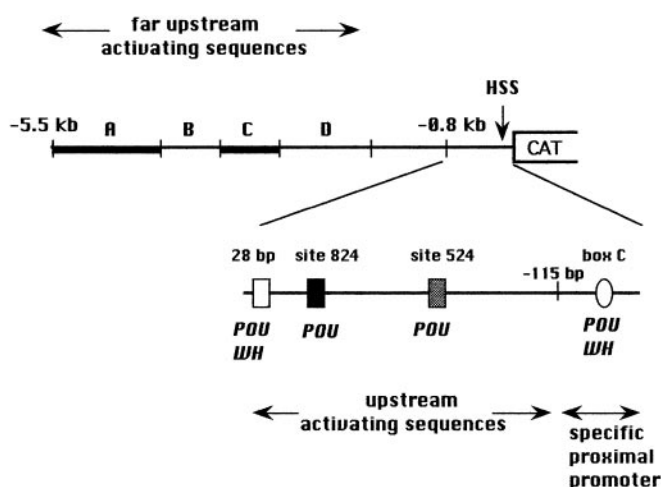


FIG. 6. Rat aldolase C gene 5'-regulatory sequences. In the far upstream 3.8-kb region, the most efficient subfragments A and C are represented by thick lines. In the 0.8-kb fragment, the hypersensitive site (HSS) located in the vicinity of the transcription start sites (11) is indicated. The binding activities for POU domain and/or WH proteins of the four binding sites investigated in this paper are also represented.

transcription (40, 41). In this model, sequences present in element C could act by increasing the number of cells expressing the transgenes in the brain. We will now attempt to delineate more precisely which DNA motifs in elements A and C contribute to the activity of the far upstream 3.8-kb region and could represent potential binding sites for transcription factors. Due to the absence of relevant cultured cell lines for studying regulation of the aldolase C gene (12), this will require creation of novel transgenic mouse lines.

Upstream and Proximal Regions of the Aldolase C Gene Contain Binding Sites for POU Domain and WH Factors—At the 5'-end of the 0.6-kb sequence located immediately upstream of the 115-bp promoter, a 28-bp DNA element, perfectly conserved between the rat and human aldolase C genes, was shown in this study to be an *in vitro* binding site for both ubiquitous and brain-specific homeoproteins of the POU family on one hand and for the liver-expressed Winged Helix HNF-3 β factor on the other hand. Deletion of 100 bp including this 28-bp element leads to a markedly decreased transgene expression. The presence in the aldolase C gene regulatory regions of a binding site for the HNF-3 β factor, classically involved in the differentiation of endoderm-derived tissues (42), has already been discussed in the case of the proximal box C element of the 115-bp promoter (12). Here we show that the HNF-3 β -binding motifs present in this proximal box C and in the 28-bp element overlap with potential binding sites for brain-specific regulatory proteins, suggesting that binding of either protein is exclusive. Whereas liver-restricted in adult tissues, the HNF-3 β factor is expressed and essential in fetal nervous structures (43–45). It could be implicated itself in inducing a permissive chromatin structure of the aldolase C gene (46), allowing for subsequent interactions with brain-specific POU factors. Alternatively, other brain-specific regulatory factors of the WH family (47–49) could be involved in the expression of the aldolase C gene.

In addition to the 28-bp motif and the proximal box C, we report the presence of several supplementary putative binding sites for POU proteins in the upstream 0.6-kb fragment. Deletion of one of them (site 824 element) can also be related to an *in vivo* decrease in transcriptional activity. Different POU proteins are shown in this study to interact *in vitro* with aldolase C DNA elements. One of them, Oct-1, was found to be expressed in most tissues, both adult and embryonic (50). First

identified as the protein binding the octamer motif ATGCAAAT (51), Oct-1 was further shown to interact with a broad repertoire of non-canonical sequences and has been involved in the transcriptional control of ubiquitously expressed, developmentally regulated, and tissue-specific genes (32). In particular, it has been reported to be essential for the activity of the neuron-specific enhancer of the gonadotropin-releasing hormone gene in transfection experiments (35). Several POU domain proteins are specifically expressed in embryonic and adult nervous systems, each in a temporally and spatially specific way (14, 31). Among them, the Brn-1 and Brn-2 factors, which are the most widely expressed POU proteins in the brain (50), also interact with the 28-bp element and with the site 824 and site 524 oligonucleotides. Brn-2 represents a remarkable illustration of the high flexibility of the POU domain specificity (52), allowing for variable spacing (0, 2, or 3 nucleotides) and orientation of the two DNA sites contacting the POU_S and POU_H subdomains (23). From *in vitro* differentiation assays (53) and *in vivo* gene knockout experiments (54, 55), it has been speculated that Brn-2 exerts a critical function both in early and late neural development, especially in determining neuroendocrine phenotypes. However, rather few promoters were shown to be controlled by Brn-2 (23, 56, 57). In the Purkinje cells of the cerebellum, which display the highest level of aldolase C in adult brain (5, 6, 8, 58), Brn-1 and Brn-2 are both expressed, whereas Oct-1 is reported to be absent (50). Other brain cells, namely astrocytes and some large neurons in the cerebral cortex, also express the aldolase C protein (5, 6, 58). It will be interesting to investigate at a cellular level the reciprocal localization of the three POU factors shown in this study to interact with aldolase C DNA motifs and of the endogenous aldolase C isoenzyme.

Such an array of multiple binding sites has frequently been reported in the regulatory regions of genes depending on POU proteins, allowing for cooperative binding and synergistic activation (23, 37, 59). The rather low affinity of some of the aldolase C POU protein-binding sites (*e.g.* the proximal box C and site 824 oligonucleotides) could be compensated either by cooperative interactions through adjacent binding sites for POU factors or for other transcription factors (60, 61) or by association of the POU proteins with a coactivator, as already described (62, 63). The modular structure of enhancers has been reported as a general feature in higher eukaryotes (29, 64, 65). In the case of aldolase C, another level of complexity could be brought by the fact that broad expression in the brain (5, 6) could result from the additive effects of several *cis*-acting elements, each of them being specifically active in a restricted brain cell population. Several studies have investigated the cell-specific expression of aldolase C constructs in the brains of transgenic mice. A large 13-kb rat genomic fragment including 5.5 kb of upstream sequences was shown by Arai *et al.* (66) to mimic expression of the endogenous gene (5, 6). In contrast, Walther *et al.* (58) recently reported that genomic sequences of the mouse aldolase C gene directed expression of a *lacZ* reporter gene exclusively in non-neuronal cells of transgenic mice. However, the transgene used by these authors contained only 1.5 kb of 5'-flanking and 0.5 kb of 3'-flanking regions of the mouse gene. When Arai *et al.* (66) examined the regional expression of a transgene retaining the 5.5-kb 5'-flanking region of the rat gene fused to the *lacZ* gene, they found that the β -galactosidase activity was neuronal and especially strong in Purkinje cells, *i.e.* mimicked expression of the endogenous gene, in three out of seven transgenic lines. However, β -galactosidase localization was different in four other lines, probably due to an integration site effect. Therefore, a relevant study of brain expression patterns directed by different combinations of *cis*-acting sequences of the rat aldolase C gene will require the

inclusion of essential regulatory elements in the transgenes, as has been proposed recently for exon 1 (67).

POU/WH Dual Motifs Could Be Essential for Brain-specific Gene Expression—In conclusion, the aldolase C gene could represent a new target for brain-specific POU domain proteins, binding to different motifs of the regulatory regions. Several of these motifs are also able to bind transcriptional factors of the WH HNF-3 family (Fig. 6). It is noteworthy that one of these dual motifs, the proximal box C, is located in the brain-specific 115-bp promoter of the aldolase C gene (12). The neuronal aromatic L-amino-acid decarboxylase, corticotropin-releasing hormone, and gonadotropin-releasing hormone genes also contain POU protein-binding sites that represent already described (34) or putative (Fig. 3E) HNF-3 β -binding sites in functionally *cis*-active fragments (23, 35). In contrast to the aldolase C gene, these three genes are restricted to specific secretory neuron subpopulations. The POU/WH motifs could therefore constitute important brain-specific *cis*-acting elements in broadly expressed as well as in neuron-type restricted genes.

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