Bidirectional activity and orientation-dependent specificity of the rat aldolase C promoter in transgenic mice

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Received 19 April 2001; revised 17 May 2001; accepted 18 May 2001

First published online 31 May 2001

Edited by Ned Mantei

Abstract We previously reported that the rat aldolase C 115 bp promoter is sufficient to ensure the brain specific expression of the chloramphenicol acetyltransferase reporter gene in transgenic mice. We identify in a further reduced 84 bp promoter several putative binding sites for the transcriptional factors Sp1, USF, AP1, and AP2. Deletion or mutation of these partially overlapping binding sites results in inactivation of the cognate transgenes. Moreover, we show that the 115 bp sequence is able to direct bidirectional transcription in vivo but, surprisingly, transcriptional activity in the opposite direction is no more brain specific.

Key words: Brain specific transcription; Bidirectional promoter; Transgenesis

1. Introduction

Fructose 1,6-diphosphate aldolase C is a glycolytic enzyme strongly and specifically expressed in adult brain. We previously reported that (i) in spite of its tissue specificity, the rat aldolase C gene promoter displays features of housekeeping genes (absence of TATA and CAAT sequences, presence of G+C rich elements and multiple transcription start sites dispersed over about 100 nucleotides [1], (ii) a short 115 bp promoter fragment is able to ensure, at a low level, the specific expression of the chloramphenicol acetyltransferase (CAT) reporter gene in the brain of transgenic mice [2]. Moreover, the two GC rich regions (A/A′ and B boxes) defined by footprinting experiments in this 115 bp fragment being functionally redundant in vivo [3], the minimal brain specific promoting region was further reduced to 84 bp. Therefore, we considered the rat aldolase C promoter as an appropriate tool to investigate the still poorly understood molecular events underlying the brain specific expression of neuronal genes. In this paper, we report the identification of several partially overlapping transcription factor binding sites in the 84 bp promoter and the in vivo analysis of various modified transgenes containing mutation, deletion, or multimerization of some of these sites. All analyzed mutant transgenes were found to be inactive in vivo. We also cloned the initially described 115 bp promoter in an inverted orientation upstream of the CAT reporter gene and obtained active transgenic lines demonstrating that the TATA-less rat aldolase C promoter displays a bidirectional activity. However, while this promoter fragment confers a brain specificity on the reporter gene in the forward orientation, it behaves nearly as an ubiquitous promoter in the backward orientation. This suggests that the spatial arrangement of the different regulatory proteins on the promoter could constitute an additional level of complexity in brain specific gene expression.

2. Materials and methods

2.1. Plasmid constructions

The 115/CAT, 84/CAT (−167/−84, previously noted B/C/CAT), and hepatocyte nuclear factor (HNF)mut/CAT constructs have been described previously [2,3]. The 76 bp fragment (−159/−84) was prepared by polymerase chain reaction (PCR) and cloned upstream of the CAT reporter gene, resulting in the 76/CAT construct. Box B mut/84/CAT and Homol mut/84/CAT constructs correspond to the 84/CAT transgene where sequences CGCCC (−150/−146) and GGAGT (−141/−137) were mutated by PCR to GAATT and AATTC, respectively. The 6($Sp1_B$)/76/CAT was obtained by inserting a multimer of the $Sp1_B$ site upstream of the 76 bp fragment in the 76/CAT construct. The transgene 115 inv/CAT was prepared by cloning the 115 bp fragment in the inverted orientation upstream of the CAT reporter gene.

2.2. Brain nuclear extracts

Nuclear extracts were prepared from rat brain according to Piette et al. [4] and from HeLa cells according to Dignam et al. [5].

2.3. Electrophoretic mobility shift assays (EMSAs)

Oligonucleotides used as probes or competitors were: Intermediate, 5′-CCAGGATGGGAGGTGTCTGTCACGC-3′ (−172/−148), Box B, 5′-GATGGGAGGTGTCTGTCACGCCCCCAGGGAGTC-3′ (−168/−136), Box B mut, 5′-GATGGGAGGTGTCTGTCA**GAATTC**CAGGGAGTC-3′ (nucleotides −150/−146 mutated), Homolog, 5′-CAGGGAGTCACGTAGCTCTGCGGCAT-3′ (−144/−119), Homol mut 5′-CAG**AATTC**CACGTAGCTCTGCGGCAT-3′ (nucleotides −141/−137 mutated) derived from the rat aldolase C gene; Sp1cons, 5′-CTAACTGGGCGGAGTTATGC-3′ (derived from the simian virus 40 enhancer) [6]; MLP, 5′-GGAACACCCGGTCACGTGGCCTACACCT-3′ (derived from the Adenovirus major late promoter (MLP)) [7]; hMtII 5′-AACTGACCGCCCGCGGCCCGT-3′ (derived from the human metallothionein IIa gene) [8].

EMSAs were performed as previously described[3,9]. Binding reactions contained 7–10 μg of nuclear extract proteins or 0.125 footprint unit of the recombinant human Sp1 protein (Promega) with 300 ng of poly(dIdC) or 1 μg of sonicated salmon sperm DNA and 0.1–0.5 ng of T4 kinase-radiolabeled double-stranded probe. When added, competitors were present at a 200-fold molar excess. For supershift assays, binding reactions were incubated for 30 min at 4°C as usually, then supplemented with 1 or 2 μl of antibodies and further incubated for

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Abbreviations: CAT, chloramphenicol acetyltransferase; HNF, hepatocyte nuclear factor; PCR, polymerase chain reaction; EMSA, electrophoretic mobility shift assay; USF, upstream stimulatory factor

0014-5793/01/$20.00
PII: S0014-5793(01)02540-6

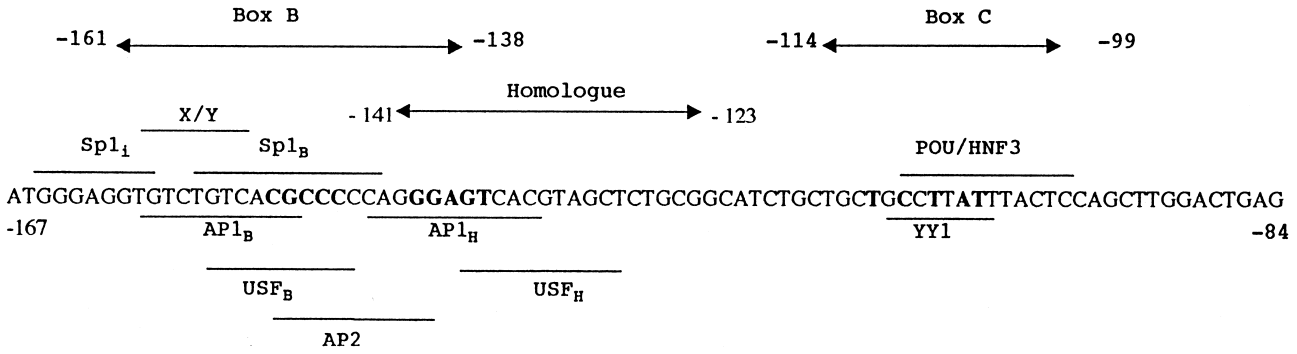

Fig. 1. Protein binding sites in the 84 bp aldolase C promoter. The B and C footprinted boxes described in [3] and the 19 bp homolog between the rat and human genes are indicated by arrows. Bases mutated in Box B mut/84/CAT, Homol mut/84/CAT, and HNF mut/CAT transgenes are indicated by boldface letters. Already reported and newly identified protein binding sites are presented above and under the aldolase C sequence, respectively.

30 min at 4°C. Free and bound DNAs were separated on non-denaturing 6% (w/v) polyacrylamide gels. Polyclonal anti-upstream stimulatory factor 1 (USF1) antibodies were kindly provided by M. Raymondjean [10]. Polyclonal anti-AP2 antibodies were supplied by Santa-Cruz Biotechnology, Inc.

2.4. Production and analysis of transgenic mice

Inserts were isolated from agarose gels and purified on Elutip-d columns (Shleicher and 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 [11]. Transgenic founders and offspring were identified [12] by Southern blot using a random-primed CAT probe. CAT activities generated by transgene expression were assayed on 200–500 μg of brain, liver, heart and lung extract proteins [13].

3. Results and discussion

3.1. In vitro and in vivo analysis of the 84 bp promoter

In a previous study concerning functional dissection of the 115 bp rat aldolase C promoter, we reported that the two GC rich A/A′ and B boxes were functionally redundant in vivo and that a promoter including either the A/A′ or the B box linked to the C box (characterized by a dual specificity for both POU domain regulatory proteins and the Winged Helix HNF3β) [14] is sufficient to ensure a brain specific CAT activity in transgenic mice (transgene B/C/CAT in [3], denoted 84/CAT in this paper). We then focused our study on the 84 bp promoter encompassing boxes B and C (Fig. 1). This 84 bp sequence retained two already described binding sites for the factor Sp1, $Sp1_i$ (located in the sequence Intermediate, between A/A′ and B boxes) and $Sp1_B$ (located in the B box) (Fig. 1 and Fig. 2A, lanes 1–4), and a binding site for two widespread expressed unidentified proteins X and Y [2]. In addition, computer analysis and EMSA studies allowed us to identify other DNA/protein interactions. Overlapping homologies with consensus binding sites for the factors USF and AP1 are present both in box B (USF_B and $AP1_B$) and in a short sequence of 19 bp perfectly conserved between the rat and human aldolase C genes, called Homolog (USF_H and $AP1_H$) (Fig. 1). The binding of USF to oligonucleotides Box B and Homolog was confirmed by EMSA using as competitor the Adenovirus MLP binding site [7] and anti-USF1 antibodies (Fig. 2B, lanes 6–10); the same result was obtained with anti-USF2 antibodies (not shown). Binding of AP1 to so-called $AP1_B$ and $AP1_H$ sites was also confirmed using the oligonucleotides Box B and Homolog which were found able to compete weakly in EMSA with the binding of AP1 complexes from a brain extract to the specific AP1 binding element of the human collagenase gene [15] (not shown). The predicted interaction with the embryonic protein AP2 could not be detected with adult brain extracts; however, the oligonucleotide Box B was able to compete with the binding of the factor AP2 from a HeLa cell extract to the DNA probe hMtII [8] representing the AP2 binding site of the human metallothionein gene (Fig. 2C, lanes 13–16). This AP2 motif could be involved in the weak but ubiquitous expression of aldolase C in embryonic tissues [16]. Finally, a low affinity binding site for the factor YY1 [17] was also observed in vitro on box C (not shown).

In order to identify critical DNA elements required for the brain specificity of the 84 bp promoter, various mutant versions of the 84/CAT wild-type transgene were analyzed in vivo in transgenic mice (Table 1). The Box B mut/84/CAT transgene, where mutation of five nucleotides in Box B suppressed the binding of Sp1 (Fig. 2A, lane 5), USF (Fig. 2B, lane 12), AP1, and AP2 (not shown), was totally inactive. Deletion of the upstream $Sp1_i$ binding site in the 76/CAT construct also

Table 1
Mutations in the 84 bp promoter

Transgene	Altered binding sites	Range of CAT activity in brain
84/CAT[a] [3]	wild type	4–39
Box B mut/84/CAT	$Sp1_B$, USF_B, $AP1_B$, AP2 mutated	<0.02
76/CAT	$Sp1_i$ deleted	<0.02–0.03
6($Sp1_B$)/76/CAT	$Sp1_i$ deleted, 6($Sp1_B$) inserted	<0.02–0.05
Homol mut/84/CAT	$AP1_H$, USF_H mutated	<0.02–0.05
HNF mut/CAT[3]	HNF3β mutated	<0.02

[a]The 84/CAT transgene corresponds to the previously described B/C/CAT construct in [3]. CAT activities are expressed in cpm min^{-1} μg $protein^{-1}$. For each transgene, 6–15 lines or founders were analyzed.

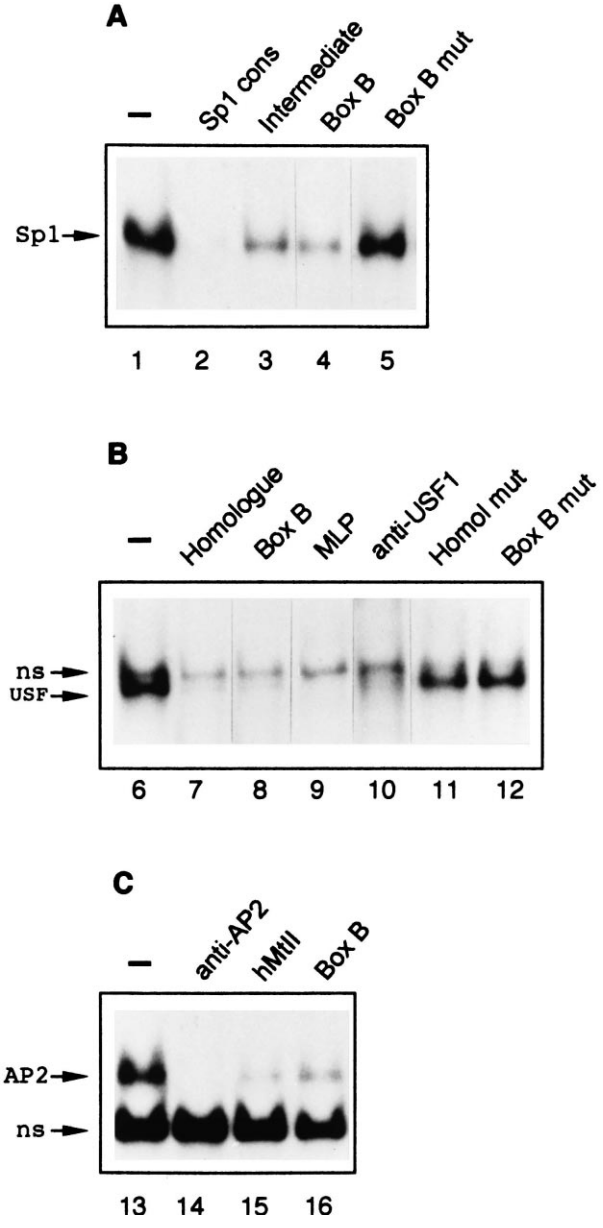

Fig. 2. EMSA analysis of the 84 bp promoter. EMSAs were performed as described in Section 2. A: The radiolabeled Sp1cons oligonucleotide was incubated with recombinant human Sp1 protein. B: The radiolabeled Homolog oligonucleotide was incubated with a rat brain nuclear extract. C: The radiolabeled hMtII oligonucleotide was incubated with a HeLa cell nuclear extract. The arrows indicate position of specific (specifically displaced by cognate competitor) and non-specific (ns) (non displaced) complexes. The indicated competitors were present at a 200-fold molar excess. Addition of anti-USF1 (1 μl, lane 10) and anti-AP2 (2 μl, lane 14) antibodies resulted in reduction of the specific bands without appearance of supershifted complexes. In each case, this inhibition was controlled to be specific since the anti-USF and anti-AP2 antibodies were found unable to inhibit the binding of the unrelated proteins AP1 and Sp1, respectively, to their cognate sites (not shown).

abolished or drastically reduced CAT activity. However, insertion of six $Sp1_B$ sites upstream of the inactive 76 bp fragment, resulting in the transgene 6($Sp1_B$)/76/CAT, did not restore CAT expression in the brain of transgenic mice. Therefore binding of Sp1 molecules is not sufficient for transcriptional activity and DNA interaction with other regulatory proteins probably occurs upstream of and in the B box. Finally, substitution of five nucleotides at the 5′-end of the Homolog element, abolishing the binding of USF (Fig. 2B, lane 11) and of AP1 (not shown) in this region, resulted in the inactive transgene Homol mut/84/CAT. We already reported, in the context of the 115 bp promoter, that destruction of the HNF3β binding site (transgene HNF mut/CAT) also abolished CAT activity [3]. This result was not due to the loss of interaction with POU factors or YY1 since in vitro affinity for these proteins was rather enhanced in the mutated sequence (not shown).

In conclusion, each of the four binding sites $Sp1_i$, $Sp1_B$/USF_B/$AP1_B$/AP2, USF_H/$AP1_H$, and HNF3β/POU/YY1 seems to be critical for transcriptional activity of the rat aldolase C promoter. It was therefore impossible, on basis of these data, to attribute the brain specificity of the 84 bp promoter to precise DNA elements. It is probable that multiple interactions on this short sequence contribute to a complex and 'minimal' array that cannot be altered further without losing its promoter activity.

3.2. The rat aldolase C promoter is bidirectional

A number of promoters lacking a TATA box and an initiator element display a bidirectional activity [18–24]. We examined whether the brain specific rat aldolase C promoter exhibits the same characteristic. The 115 bp promoter was cloned in the inverted orientation upstream of the CAT reporter gene and expression of the 115 inv/CAT resulting transgene was analyzed in different tissues for eight transgenic founders (Table 2). Two of them (animals 19 and 24) remained silent (as already reported for CAT/aldolase C chimeric constructs, transgene expression was dependent on the integration site, [25]). Five founders (animals 3, 5, 6, 8, and 34) presented a CAT activity not only in brain but also in lung and heart; transgenes were always inactive in liver. Spleen was also assayed in animal 34 and was found positive for CAT expression. Except for the founder 3, levels of CAT activity were similar in lung and brain, but weaker in heart. Finally, founder 27 was the only one where CAT activity was detected in brain and not in other tissues. We can notice that the 115 bp promoter has approximately the same strength whether inserted in the forward or backward orientation (see Table 2 and legend).

Thus we can conclude that the 115 bp aldolase C promoter is able to drive bidirectional transcription in vivo. A number

Table 2
Bidirectional activity of the 115 bp promoter

Transgene 115 inv /CAT : Box C Box B Box A/A′ CAT
115 bp

founder	brain	lung	heart	liver	spleen
3	9.1	1.14	0.48	<0.02	nd
5	3	2.66	0.67	<0.02	nd
6	1.37	0.98	0.14	<0.02	nd
8	0.56	0.47	0.11	<0.02	nd
19	<0,02	<0,02	<0,02	<0,02	nd
24	<0,02	<0,02	<0,02	<0,02	nd
27	0.7	<0.02	<0.02	<0.02	nd
34	0.05	0.08	0.02	<0.02	0.02

The brain CAT activities directed by the wild type construct 115/CAT in four transgenic lines were: 0.04, 1.1, 1.4 and 1.5 cpm min^{-1} μg $protein^{-1}$ [2]. nd: not determined.

of TATA-less promoters have been reported to direct divergent transcription of two genes located head-to-head on a chromosome [18,21–24]. In some cases, they rather appear as the overlap of two distinct promoters with shared DNA elements but distinct regulation features [21,22]; in other cases, the model of a single integrated bidirectional promoter has been proposed, providing for a mechanism of coregulation as for two chicken genes encoding enzymes of the same biosynthetic pathway [18]. In the case of the aldolase C gene, computer research did not revealed any open reading frame in the opposite direction in the 5.5 kb sequences available upstream of the promoter. For the bidirectional thymidine synthase promoter, transcription of upstream transcripts from the opposite strand of the DNA has been shown but presumably without any functional significance [19]. We can hypothesize that the lack of TATA and initiator elements that would specify transcriptional orientation and the presence of symmetrical binding sites such as GC boxes represent features that allow the transcriptional machinery to function in both orientations. Indeed, in the aldolase C 115 bp promoter as in most bidirectional promoters so far reported, one or several Sp1 binding sites occurred [18,20–24]. The presence of two USF binding sites in the 115 bp fragment could also be involved in the observed bidirectional activity. In the transcobalamin II promoter gene, a USF binding motif alone has been shown to direct bidirectional transcription, presumably by recruiting or stabilizing the binding of components of the basal transcription machinery [20].

The most interesting feature of the aldolase C promoter activity is that inverting its orientation modifies the pattern of expression of the reporter gene in the different tissues analyzed. The rat insulin II gene promoter was also shown to function in both orientations in vivo in transgenic mice but with the same tissue specificity [26]. However, in this latter study, a large fragment (720 bp including enhancer plus promoter sequences) was inserted in the opposite orientation. In contrast, the strict brain specificity of the 115 bp aldolase C promoter in its natural orientation was lost in five out of six active mouse transgenic lines when it was inserted in the reverse orientation. The only case in which the promoter in the backward orientation was active in brain and not in other tissues could correspond to insertion of the transgene in the vicinity of a transcription unit active in the brain.

In conclusion, we have shown by our mutagenesis experiments that brain specificity of the 84 bp minimal active promoter fragment requires integrity of all of its DNA elements and therefore could be combinatorial in nature. The influence of the promoter orientation on its specificity of expression indicates that the brain specificity depends not only on mutual interaction between the different DNA elements and cognate DNA binding proteins but also on the orientation of these elements with respect to the sense of transcription. This information should be taken in consideration for further investigation on molecular mechanisms involved in regulation of brain specific gene expression.

Acknowledgements: We thank Marie-France Szajnert for computer research.

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