Cis-acting elements in the promoter region of the human aldolase C gene

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We investigated the *cis*-acting sequences involved in the expression of the human aldolase C gene by transient transfections into human neuroblastoma cells (SKNBE). We demonstrate that 420 bp of the 5'-flanking DNA direct at high efficiency the transcription of the CAT reporter gene. A deletion between −420 bp and −164 bp causes a 60% decrease of CAT activity. Gel shift and DNase I footprinting analyses revealed four protected elements: A, B, C and D. Competition analyses indicate that Sp1 or factors sharing a similar sequence specificity bind to elements A and B, but not to elements C and D. Sequence analysis shows a half palindromic ERE motif (GGTCA), in elements B and D. Region D binds a transactivating factor which appears also essential to stabilize the initiation complex.

Aldolase C; Gene expression; Neuroblastoma cell (SKNBE), *Cis*-acting elements; Sp1 transacting factor

1. INTRODUCTION

Three distinct types of mammalian fructose-1,6-bisphosphate aldolase have been characterized by a variety of structural, biochemical and immunological methods [1]. These closely related isoenzymes, designated aldolase A, B and C, are expressed in a tissue-specific fashion and are regulated during ontogenesis and cancerogenesis [2,3]. Three genes, A, B and C, localized on chromosomes 16, 9 and 17, respectively, encode for the three isoenzymes [4,5,6]. We and others have cloned and sequenced the human genes and cDNAs for the three aldolases [7–17].

Aldolase C is characteristic of brain, where it is mainly localized in neurons and astroglial cells [18,19]. The protein sequence of aldolase C has been deduced from the nucleotide sequence of mouse and rat complementary cDNA clones [20,21] and from human and rat genomic clones [13,14,17,22]. The 5' non-coding region has been analyzed in rat and man. Multiple start sites of transcription have been reported in rat [22], and one major start site has been described in man [17].

In this paper we report the analysis of the promoter region performed by transfection of fusion constructs with a reporter gene into human neuronal (SKNBE) cell line and the identification of the *cis*-elements involved in the regulation of the human aldolase C gene expression.

2. MATERIALS AND METHODS

2.1. *Plasmid construction*

The aldolase C promoter region was excised from the gene as an *XbaI/PstI* fragment, spanning from −1190 bp to +20 relative to the major transcriptional start site [17], and cloned into the M13 mp19 vector. The deletions were made by cutting the 5'-end with *XbaI* and subsequently digesting with *ExoIII* nuclease (Boehringer Mannheim) [23]. The digested fragments were flush-ended by Klenow polymerase and ligated into the M13 mp19 vector. The deleted fragments were checked by sequencing with the chain termination method [24], excised from the vector with the appropriate restriction enzyme, and then cloned in *SmaI/PstI* sites upstream from the CAT reporter gene in the pEMBL-8-CAT expression vector [25]. Chimeric aldolase C-CAT plasmids were purified on a cesium chloride gradient.

2.2. *Cell culture, transient transfections and CAT assays*

Human neuroblastoma (SKNBE) cultured in DMEM plus 10% fetal calf serum (IGNI Flow) were plated at a density of about 250,000 cells per 60 mm Petri dish 16 h before transfection. DNA transfections were carried out by the calcium phosphate precipitation method [26]. Cultures were cotransfected with 10 μg of test plasmid and 1 μg of a vector carrying the luciferase gene under the control of an SV40 enhancerless promoter element [27] as internal standard for transfection efficiency. 2.5 h after transfection, SKNBE cells were exposed to 15% glycerol in HEPES-buffered saline solution for 1 min and then re-fed with growth medium. Cell extracts were prepared 32 h after transfection by several cycles of freezing and thawing. CAT assays were performed as described by Gorman et al. [26]. Protein concentration was determined by the BioRad assay.

Luciferase activity in cell extracts was measured essentially according to de Wet et al. [27], using a Berthold Biolumat LB 9501 luminometer. CAT activity in the extracts of the transfected cells was normalized to the luciferase activity. Each transfection experiment was repeated at least four times and gave reproducible results.

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Abbreviations: CAT, chloramphenicol acetyl transferase; DMEM, Dulbecco's modification of Eagle's medium; MMLV, murine mammary leukemia virus. *Enzymes:* fructose-1,6-bisphosphate aldolase (EC 4.1.2.13).

2.3. RNA isolation and primer extension

SKNBE cells were seeded at a density of 1×10^6 in a 100 mm Petri dish and re-fed with 10 ml of fresh medium 4 h before transfections. 32 h after transfection, cells were scraped and the total cytoplasmic RNA was isolated by the phenol–chloroform method [28].

Primer extension on RNA from cells transfected with aldolase C-CAT chimeric plasmids was carried out as described by Ham et al. [29]. The elongation reactions were performed in 20 μl (final volume) using 1 μl (20 U) of MMLV reverse transcriptase (Boehringer Mannheim) and 4 μl of the 5 × reverse transcriptase buffer The reaction was carried out at 42°C for 120 min and stopped by the addition of 1 μl of 0 5 M EDTA and DNase-free RNase to 30 μg/ml; the RNA was digested at 37°C for 30 min. The mixture was extracted once with phenol/chloroform and then ethanol precipitated; the pellet was dissolved in formamide loading buffer and loaded on 6% denaturing polyacrylamide gel.The gel was fixed in 10% acetic acid, dried and autoradiographed at −80°C with intensifying screens.

2.4. Nuclear extract, DNase I footprint and gel retardation assays

Nuclear protein extracts were prepared as described by Shapiro et al. [30]. A 184 bp long fragment, including the region from −164 to +20, and a fragment spanning from −269 to −196 (+1 being the major transcription start site) of the aldolase C gene promoter were used as probes for the binding reactions. Labeling reaction was performed using Klenow polymerase (Amersham) and α-(^{32}P)dATP (DuPont) or polynucleotide kinase (Promega) and [γ-^{32}P])dATP (DuPont). Synthetic complementary oligonucleotides, used as probes or as competitors in the gel retardation and footprint assays, were synthesized on an Applied Biosystem 951 synthesizer, purified on acrylamide gels and 5'- end labeled using T4 polynucleotide kinase (Promega). Gel shift assays were performed as described elsewhere [31], in the presence of 3 μg poly(dI/dC) per sample, as non-specific competitor. Samples were loaded on 5% non-denaturing polyacrylamide gel. DNase I footprints were performed as described by Lichtsteiner et al. [32].

The following double-stranded oligonucleotides were used as competitors or probes:

(1) oligo(a), 24mer (aldolase C protection A) 5'-ATGCAGCCACG-CCCCCGGAGGAGT-3';

(2) oligo(b), 30mer (aldolase C protection B). 5'-GAGGGGGTGT-GGTCAGGGCGGGCATGCAGC-3';

(3) oligo(c), 23mer (aldolase C protection C): 5'-GCCTCTGAGGG-CGTGGTCTTGCC-3';

(4) oligo(d), 39mer (aldolase C protection D): 5'-TAAAGGGATT-GTGGCAGAAATGAACGCAAGGTCATGGCC-3';

(5) oligo(Sp1) [33], 11mer: 5'-CGGGGCGGGGC-3';

(6) Mutant Sp1, 20mer (aldolase C): 5'-GGTCATTTCTTTCATGC-AGC-3';

(7) Unrelated oligo, 20mer. 5'-GACATCCGCAGCCTCATTTA-3'.

3. RESULTS

To localize cis-elements that regulate the efficiency of the aldolase C gene promoter, a series of 5'-deleted promoter fragments were fused to the CAT gene in the pEMBL-8-CAT vector and used in transient transfections into the human neuroblastoma cell line (SKNBE). Plasmids pEMBL-8-CAT wild type and pGM2-CAT (containing the Rous Sarcoma virus long terminal repeat) were used as negative and positive control, respectively. CAT activity is thus expressed as percentage of the activity obtained by transfection of the pGM2-CAT construct that was arbitrarily assigned 100% and normalized to the luciferase activity of the extracts.

The fusion constructs with their relative CAT activity are shown in Fig. 1. Construct Δ-1190, containing 1190 bp upstream from the initiation start site [17], is able to direct the transcription of a reporter gene with an efficiency of about 65% with respect to the pGM2-CAT positive control. Higher activity, of about 90%, is generated with Δ-820 and Δ-420 constucts. The reduction in activity seen with construct Δ-1190 suggests that sequences between −1190 and −820 may contain negative regulatory elements or that distal sequences may form bent DNA that decreases the promoter activity. A decrease of activity was observed with constructs Δ-164 and Δ-86, which contain 164 bp and 86 bp, respectively. These data indicate the presence of important positive regulatory elements in the region from −420 to −164 bp. However, constructs Δ-164 and Δ-86 still show 30% of CAT activity over the background levels of expression obtained with the pEMBL-8-CAT negative control.

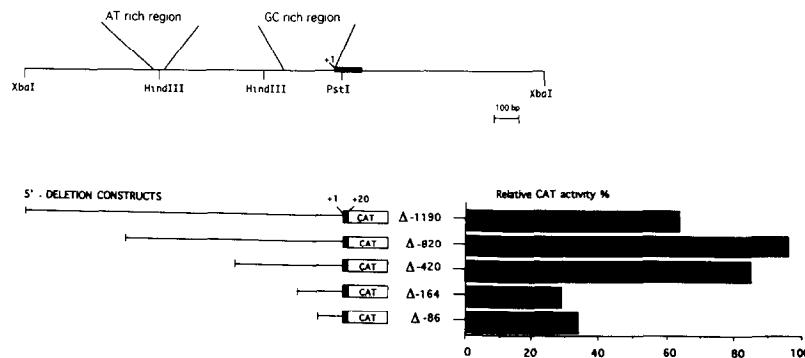To determine the correct transcription start site of the

Fig. 1. Transient expression of human aldolase C gene-CAT fusion constructs. Schematic diagram of the aldolase C 5'-flanking region. The first exon (black box), the start site of transcription (+1) and some restriction sites are indicated. 5'-deleted clones contain 20 bp of the first exon; relative CAT activity was normalized to the pGM2-CAT construct which was arbitrarly assigned 100% activity. All extracts were adjusted to identical luciferase activities.

aldolase C-CAT fusion constructs, primer extension experiments were performed on RNA from SKNBE cells transfected with constructs Δ-420, Δ-164 and Δ-86 (Fig. 2). The Δ-420 construct is able to initiate transcription (see Fig. 2, lane 1 thick arrow) at the major start site, which in the genomic sequence lies 1342 bp upstream from the methionine codon, previously determined on the endogenous RNA [17]. A displacement of the major start site and also of some minor sites was observed (thin arrows) with constructs Δ-164 and Δ-86 (lanes 2 and 3). This indicates that the region from −420 to −164 bp is important not only for the enhancement of transcriptional activity of the promoter, but also for the precise and specific start of transcription.

DNase I digestion of a 184 bp fragment, spanning from −164 to +20, in the presence of SKNBE nuclear extracts revealed three protected elements, A, B and C (Fig. 3). Element A spans from −37 to −54 bp relative to the major start site. This is a GC-rich region and contains a C stretch that has been found to be the recognition site for the trans-acting factor ETF [34]. Element B (from −56 bp to −82 bp) contains the consensus core sequence for the ubiquitous trans-acting factor Sp1 [35] and a sequence similar to the AP3 consensus sequence [36]. Since only two nucleotides divide elements A and B, it may be considered one large protected segment. Element C, which shows a protection that covers the sequence between −103/−114 bp, appears not crucial either for the transcriptional activity or for the correct initiation of transcription (compare in Figs. 1 and 2, the CAT activity and the initiation of transcrip-
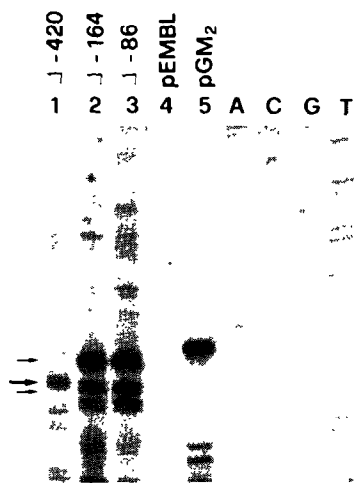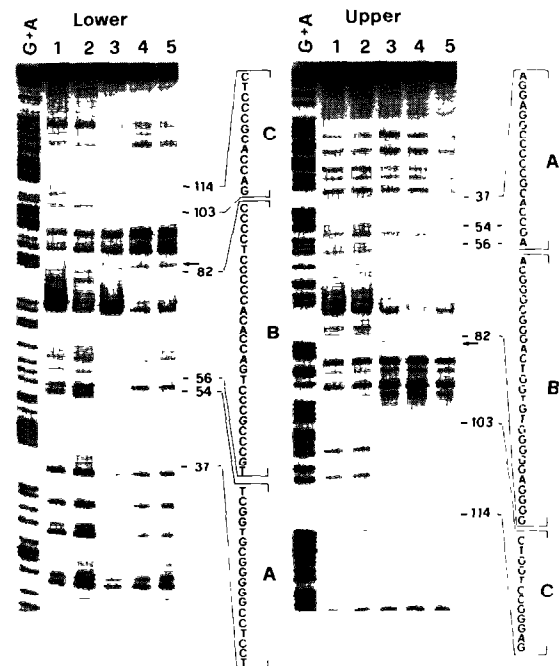

Fig. 3. DNase I protection analysis of the aldolase C promoter region. The human aldolase C gene promoter, from −164 to +20 bp, lower and upper strand, were end-labeled and incubated on ice with (lanes 3, 4 and 5) or without (lanes 1 and 2) 20 μg of SKNBE nuclear extract for 90 min. The DNA was digested with 50 ng of DNase I for 1 min and 2 min (lanes 1 and 2, respectively) or 200 ng for 1 min, 2 min and 3 min (lanes 3, 4 and 5, respectively) and the products were analyzed on 6% sequencing gel. Maxam-Gilbert sequencing reactions obtained from the same fragment were used as markers. The position and the sequences of the protected regions are schematically depicted. An arrow indicates a DNase I hypersensitivity site

Fig. 2. Primer extension analysis. 50 μg of total cytoplasmic RNA from SKNBE cells transfected with the chimeric constructs and with the positive control plasmid pGM2-CAT were used for primer extension analysis. Elongated products corresponding to the correct initiation start site of the human aldolase C gene chimeric constructs (lanes 1 to 3) are indicated by a thick arrow. Thin arrows indicate a displacement of the major start site obtained with constructs Δ-164 and Δ-86 (lanes 2 and 3). Size markers were obtained by sequencing Δ-420 DNA annealed to the same primer used for the extension reaction.

tion using constructs Δ-164 and Δ-86). To analyze the trans-acting factors involved in the binding to elements A and B, we performed a footprinting competition analysis using 50- and 100-fold molar excesses of the oligonucleotide for Sp1. This oligonucleotide was able to remove both protection A and B (see Fig. 4, lanes 5 and 6). No competition with protections A and B was observed using a mutated Sp1 consensus sequence or an unrelated oligonucleotide as competitor (Fig. 4, lanes 7–10). These results indicate that Sp1 and/or similar factors may bind to the GC-rich A and B elements. Since element B contains a consensus sequence for AP3, we used a 100-fold molar excess of an oligonucleotide containing the homologous AP3 sequence as competitor. Under our conditions we were not able to detect any competition (data not shown).

The −420 to −164 region that appeared important for the enhanced transcriptional activity of clones Δ-820 and Δ-420 was analyzed by DNase I digestion assay. No DNase I protected sequences were observed between sequences from −420 to −269. Using as probe a fragment from −269 to −164 (Fig. 5, panel A), a protected element from −203 to −196 (element D) was mapped.
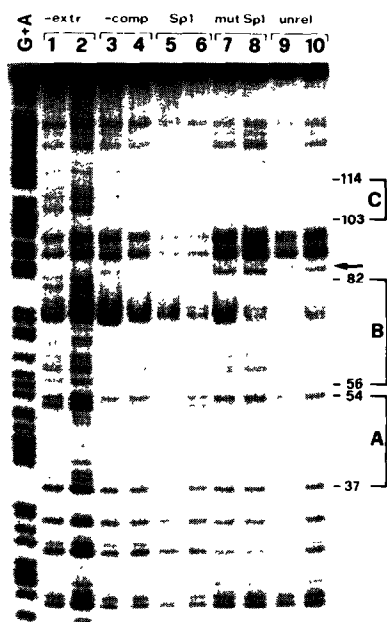
Fig. 4. DNase I footprinting competition analysis. The lower strand of the aldolase C promoter (−164 to +20) was end-labeled and incubated on ice with (lanes from 3 to 10) or without (lanes 1 and 2) 20μg of SKNBE nuclear extract for 90 min on ice. DNase I digestions were carried out on ice using 50ng of the enzyme for 1 min, 2 min (lanes 1and 2) or 200 ng for 2 min (lanes 3–10). Unlabeled competitors at 50- and 100-fold molar excesses were used: oligo Sp1 (lanes 5 and 6), mutant Sp1 (lanes 7 and 8) or unrelated oligonucleotide (lanes 9 and 10). The protected areas are schematically depicted An arrow indicates a DNase I hypersensitivity site

Gel mobility assays, using labeled oligonucleotides, encompassing the protected regions A and B of Fig. 3, show two major retarded bands for each oligonucleotide (Fig. 6, lanes 1 and 6). Competition with a 50-fold molar excess of cold oligonucleotide for Sp1 is able to displace both DNA–protein complexes formed with elements A and B (lanes 3 and 9). Using an oligonucleotide containing a mutated sequence for Sp1 (lane 8) or an unrelated oligonucleotide (lanes 4 and 10), the bands were not competed. These data strongly suggest that Sp1 or a factor with the same sequence specificity is involved in the binding to elements A and B. The oligonucleotide that includes protection C of Fig. 3 also gives rise to two major DNA–protein complexes (Fig. 6, lane 12). The specificity of the binding was assessed by competing the bandshift with itself or with unrelated oligonucleotides. Binding was competed by 25- and 50-fold molar excesses of the same unlabeled oligonucleotide (lanes 13 and 14). Although also this element is GC-rich, no competition was observed using a 50-fold molar excess of the Sp1 oligonucleotide (lane 15) or of an unrelated oligonucleotide (lane 16). Gel shift analysis of the region activating the transcription indicates that the oligonucleotide d from −230 to −190 bp displays a specific bandshift (see Fig. 5, panel B lane 1) that is removed using a 50-fold molar excess of the same cold

oligonucleotide (lane 2). No competition was observed using the same molar excess of the oligonucleotide containing the Sp1 binding site or an unrelated oligonucleotide (lanes 3 and 4). The −230 to −190 region is included in clones Δ-1190, Δ-820 and Δ-420, all of which show enhanced activity, and excluded from clones Δ-164 and Δ-86, which show a decreased activity. Therefore, the binding of a protein to the −230 to −190 sequences strongly indicates that an activating DNA element, designated 'element D' (Fig. 5), is responsible for the full activity of the promoter.

4. DISCUSSION

The 5'-flanking region of the brain-specific aldolase C gene possesses unusual features for a tissue-specific gene. In fact, it is characterized by an AT-rich region and a 68% GC-rich region respectively localized −700 bp and −228 bp upstream from the major transcription start site. A canonical CAAT box is found at nucleotide −736; however this is not the expected position of a functional CAAT box relative to the transcription start site [17].

In this study we have shown that the 5'-flanking sequences of the human aldolase C gene, function as a stronger promoter in human neuroblastoma cells. We have identified DNA segments that contain cis-acting elements, localized from −54 to −37 (A), −82 to −56 (B) and −114 to −103 (C) from the major transcription start site, respectively. Furthermore, an upstream segment (element D) spanning from −203 to −196, which we describe as a second region essential for transcription, has been found to be a strong activating element for the promotion of transcription.

Experiments presented in this paper suggest that Sp1 or Sp1-like factors may be involved in the binding to element B, where a consensus sequence for Sp1 is localized. Also element A, although lacking the canonical Sp1 consensus sequence, seems to interact with factors that bind to GC-stretches. A number of studies have established that Sp1 activates promoters of various cellular genes, including mouse dihydrofolate reductase [37], mouse adenine phosphoribosyl-transferase [38], human c-Ha-ras [39], human adenosine deaminase [40], human p120 antigen [41], human amyloid precursor protein [42], human D1A dopamine receptor [43], human neuropeptide Y [44], human Pim-1 proto-oncogene [45], human and rat α subunit of the regulatory protein G0 [46] and human nerve growth factor receptor gene [47].

Like aldolase C, these genes belong to the so-called 'TATA-less promoters' and are characterized by extremely GC-rich regions. Some of them are considered housekeeping genes and are expressed in most cell types, others are tissue-specific genes like aldolase C. The role of Sp1 in the regulation of such a diverse composite set of genes is still unknown, but probably
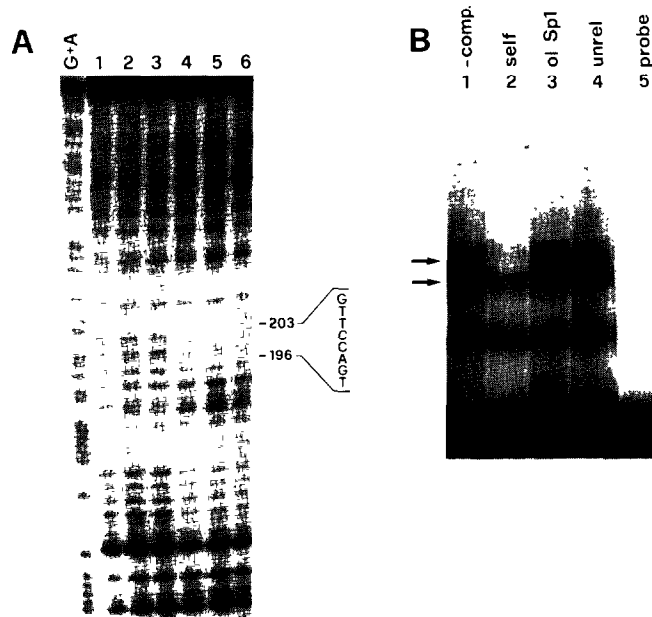
Fig. 5. DNase I footprinting and gel mobility shift analyses. A: The lower strand of aldolase C promoter (from −269 to −164) was end-labeled and incubated 90 min on ice with (lanes 4,5 and 6) or without (lanes 1, 2 and 3) 20 μg of SKNBE nuclear extract. Samples were subsequently digested with 50 ng of DNase I for 1 min, 2 min and 3 min on ice (lanes 1, 2 and 3) or with 200 ng of the enzyme for 1 min, 2 min and 3 min (lanes 4, 5 and 6). The protected region is schematically depicted. B: Gel mobility shift analysis of the protected area D. Labeled oligonucleotide d (from −230 to −190) was incubated with 4 μg of SKNBE nuclear extract. Competitors were added to the reaction at a 50-fold molar excess, prior to the addition of the extract. The oligonucleotides used are listed above each lane. Arrows indicate the specific retarded bands.

requires interaction with other promoter elements, some of which might be tissue-specific. In addition, multiple factors that, besides factor Sp1, recognize GC boxes have been found. These include the transcription factor ETF, that stimulates in vitro the transcription from several promoters without TATA boxes [34], and the brain-derived GC-box-binding factor, present in brain extracts and absent from liver and kidney ex-

tracts, that regulates the transcription of the murine brain tissue plasminogen activator (t-PA) [48]. Further experiments using deletion or mutation of the Sp1 binding sites detected in the −82 to −37 region are required to assess the role of these GC-rich elements in the regulation of the aldolase C promoter.

The biological significance of element C is not yet clear. The deletion of this area, in fact, does not signif-
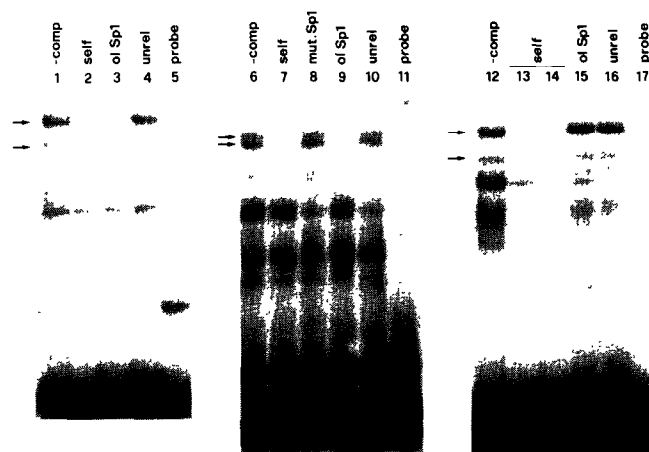

Fig. 6. Gel mobility shift analysis of protected areas A (lanes 1 to 5), (B) (lanes 6 to 11) and (C) (lanes 12 to 17). Reactions were performed using 4 μg of SKNBE nuclear extracts and labeled oligonucleotides a, b and c encompassing the three protections, respectively. Competitors, used at a 50-fold molar excess, are listed above each lane (see also Materials and Methods). Unlabeled oligonucleotide c was used at 25-fold and 50-fold molar excess in lanes 13 and 14, respectively. Arrows indicate the specific DNA–protein complexes observed.

icantly affect CAT activity (see Fig. 1, constructs Δ-164 and Δ-86). Experiments using point mutant clones should clarify its contribution to the transcriptional regulation of this gene.

The second region, which maps from −420 to −164, appears to confer full activity to the promoter (compare Δ-420 and Δ-164 in Fig. 1). This region, as shown by primer extension experiments, also seems to be important for the correct positioning of the transcription initiation site (compare clones Δ-420 and Δ-164 in Fig. 2). Clone Δ-164, which lacks this element, shows not only a decrease of CAT activity, but also a displacement of the major start site of transcription.

DNase I footprint and gel shift experiments enabled us to identify a protected area (called 'element D') mapping from −203 to −196 (Fig. 5, panels A and B). This element contains half the palindromic motif, identified as a functional estrogen responsive element (ERE) [49,50]. Whether this element might also affect the transcription of the aldolase C gene remains to be proved.

Taken together, the expression of the human aldolase C gene seems to be due to interactions of several factors, including the ubiquitous Sp1 or Sp1-like factors, and an upstream activator (element D). Since we found low levels of aldolase C mRNA in non-nerve cell lines, such as human hepatoma cells (Hep3B), human fibroblasts, HeLa cells and human lymphocytes (data not shown), the prevalence of aldolase C transcripts in neuronal tissues may also be due to both transcriptional and post-transcriptional events such as different messenger stabilization in different tissues. In addition, post-translational modifications could account for the efficient transcriptional regulation of tissue-specific genes. In this respect, the Sp1 factor has been shown to be post-translationally modified by O-glycosylation and this modification has been demonstrated to be relevant for its transcriptional activity [51]. However, other hypotheses, including different chromatin structures that in vivo could restrict to specific tissues the binding of transcriptional factors to DNA or the presence of distant control elements outside the 1.2 kb region characterized so far, cannot be excluded.

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