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CHARACTERIZATION OF A SILENCER THAT MODULATES TRANSCRIPTION OF THE HUMAN DISTAL ALDOLASE A PROMOTER

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Summary: Transcription from the distal promoter (pL) of the human aldolase A gene is driven by both positive and negative cis-acting elements. With footprinting and gel mobility assays we defined: (i) the position of the negative regulatory cis-element (AldA-NRE), which spans a GA-rich sequence; (ii) the sequence of AldA-NRE, which is critical for protein specific binding and is homologous to other silencer-like motifs. Removal of the negative cis-element fully restores the expression of the distal promoter. Insertion of the AldA-NRE upstream from a heterologous promoter results in a 6-fold decrease in CAT reporter gene expression, suggesting that AldA-NRE might be involved in a more general type of mechanism that mediates repression of gene transcription.

Using three alternative promoters (distal or pL, middle or pM and proximal or pF) and alternative leader exons (1 plus 2 or 3 or 4), multiple transcripts (L-, M- and F-type), containing the same coding region but a different untranslated 5'-end, arise from the human aldolase A gene (1-3). Two of these mRNAs constitute ubiquitous species, L-type (also called N-type) and F-type (also called H-type) mRNAs, the third one (M-type mRNA) being muscle-specific (4-6). We recently demonstrated that L-type mRNA is expressed also in rodent cell lines and that its concentration is greatly increased when growth stops and cells differentiate (7). The genomic region (pF) upstream from leader exon 4, and that (pL) upstream from leader exon 1, act autonomously as promoters, at least in transfection experiments, pF being the most powerful (8-10). Regulatory cis-

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elements reside near the transcription start sites of all three promoters in man and rodents (8-13). We previously showed that positive and negative elements regulate the transcription from the most distal promoter region (pL), upstream from exon 1 (9).

We have investigated the functional role of the putative silencing element, and have identified a GA-rich sequence between -555 and -220 bp upstream from the longer L-type mRNA start site. This sequence binds a nuclear protein present in the human hepatoma cell line Hep3B. Transcription data indicate that this element is involved in the negative regulation of the human aldolase A pL promoter, as well as in the regulation of other genes.

Materials and Methods

Nuclear extract preparation: Nuclear protein extracts were prepared from Hep3B cells grown in DMEM medium supplemented with 10% fetal calf serum (9).

DNase I footprinting and gel shift assays: DNase I footprinting assays were performed (9). DNA fragments used as probes were obtained by restriction endonuclease digestion of clones LΔ-555 and LΔ-292, previously described (9). Probes 1, 2 and 3 were obtained with EcoRI/TaqI(-555/-347), Bsu36I/HincII(-450/-220) and EcoRI/PstI (-292/-220) digestions, respectively. Labeling reactions were performed using Klenow DNA polymerase (Amersham) and 3000 Ci/mmole α-(^{32}P)dATP (Amersham). For gel retardation assays (9), specific and unrelated competitors were incubated with 5 μg of nuclear extracts for 10 min on ice; 3 fmol of 5' end-labeled double-strand oligonucleotides (about 50,000 cpm) were added to the mixture and the reaction was performed at room temperature for a further 10 min. For competition experiments, a 100-fold molar excess of competitor double-strand oligonucleotides was added to the reaction.

Plasmid construction: The construction of recombinant clones LΔ-811, LΔ-555, LΔ–292 and LΔ-220 is described elsewere (9). The internal deletion -528/-215 was obtained by digesting clone LΔ–555 with StuI, releasing the 313-bp fragment, by filling-in and ligation reactions. Clone RBP9 includes the basal promoter (about 150 bp upstream from the initiation transcription site of retinol-binding protein mRNA) of the human retinol-binding gene cloned upstream from the CAT reporter gene in the pEMBL8CAT vector (14). RBP9+sil (-528/-215) was obtained by cloning a -528/-215 fragment of the human aldolase A distal promoter upstream from the minimal length promoter of the retinol-binding protein gene in SmaI site.

DNA transfection and CAT assay: Transfections in Hep3B cells were performed with 20 μg of supercoiled plasmid DNA using the calcium phosphate precipitation method (15). Differences in transfection efficiencies were normalized by cotrasfecting 2 μg of cytomegalovirus luciferase construct. In all transfections, pEMBL8CAT and pSV2CAT were used as negative and positive controls, respectively. After transfection for 48h, cell extracts and CAT assay were as described by Gorman et al.(16); luciferase activity was determined as reported (17). The acetylated and unreacted forms were separated by thin-layer chromatography, excised and counted in a Tri-Carb Liquid Scintillation Analyzer, Mod.1600TR (Packard). At least three different CAT plasmid preparations were tested in three independent assays.

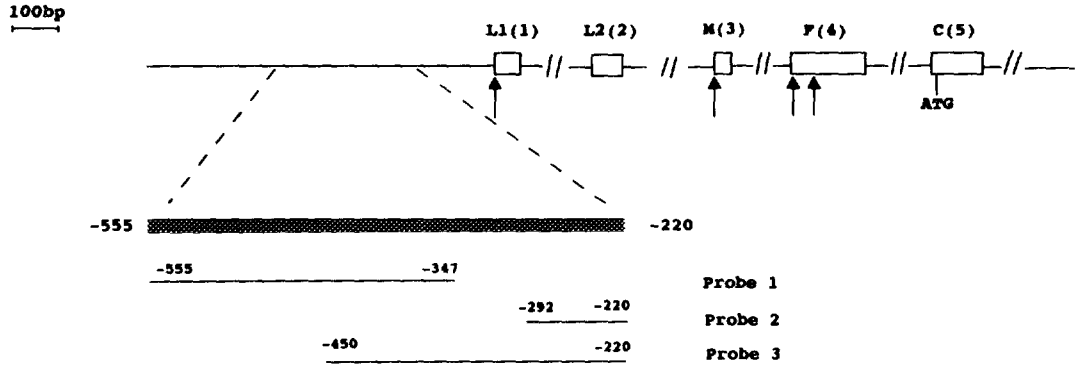


Figure 1. The 5'-end of the human aldolase A gene. Exons 1 to 5 are boxed. Vertical arrows indicate the major transcription start sites of the L-, M- and F-type mRNAs. ATG indicates the first Met codon. C is the common translated exon. Fragment -555/-220, containing the negative elements, is indicated by the solid bar. The location and the length of the probes used in the footprinting experiments are indicated by thin lines.

Results

Identification and characterization of the silencer element (AldA-NRE)

The genomic organization of the 5'-flanking region of the human aldolase A gene, the location of the DNA fragment containing the putative negative cis-element(s) (AldA-NRE) and that of the probes used in DNase I footprinting experiments are schematically depicted in Fig.1. The footprinting patterns generated by the binding of nuclear proteins from Hep 3B cells to the DNA fragments extending from -555 to -347 (probe 1 in Fig.1), from -292 to -220 (probe 2 in Fig.1) and from -450 to -220 (probe 3 in Fig.1) are shown in Fig.2. Two protected elements from -504 to -490 and from -446 to -443 were detected with probe 1, and two from -241 to -239 and from -229 to -227 with probe 2. A fifth pyrimidine-rich protected region, from -367 to -349, was detected with probe 3.

AldA-NRE binding protein

In gel mobility shift assay we used as probe a double-strand oligonucleotide Neg1 (25 bp, see Fig.3), based on the sequence from -504 to -490 that is protected by probe 1 in the footprinting. This sequence contains a GA-rich motif that is repeatedly present in the entire region from -555 to -220. A protein factor present in nuclear extract from Hep 3B formed an abundant retarded complex (Fig.3, lane 1) that was sequence-specific because it could be competed for by an excess of unlabeled oligonucleotide (Fig.3, lane 2), but not by an unrelated oligonucleotide (Fig.3, lane 5). In addition, the specific binding of a nuclear protein to the Neg1 oligonucleotide strictly depended upon the core-sequence AGAGAG of the oligonucleotide; in fact, the MutNeg1 oligonucleotide, mutated in the core-sequence, did not compete with the wild type oligonucleotide in the interaction

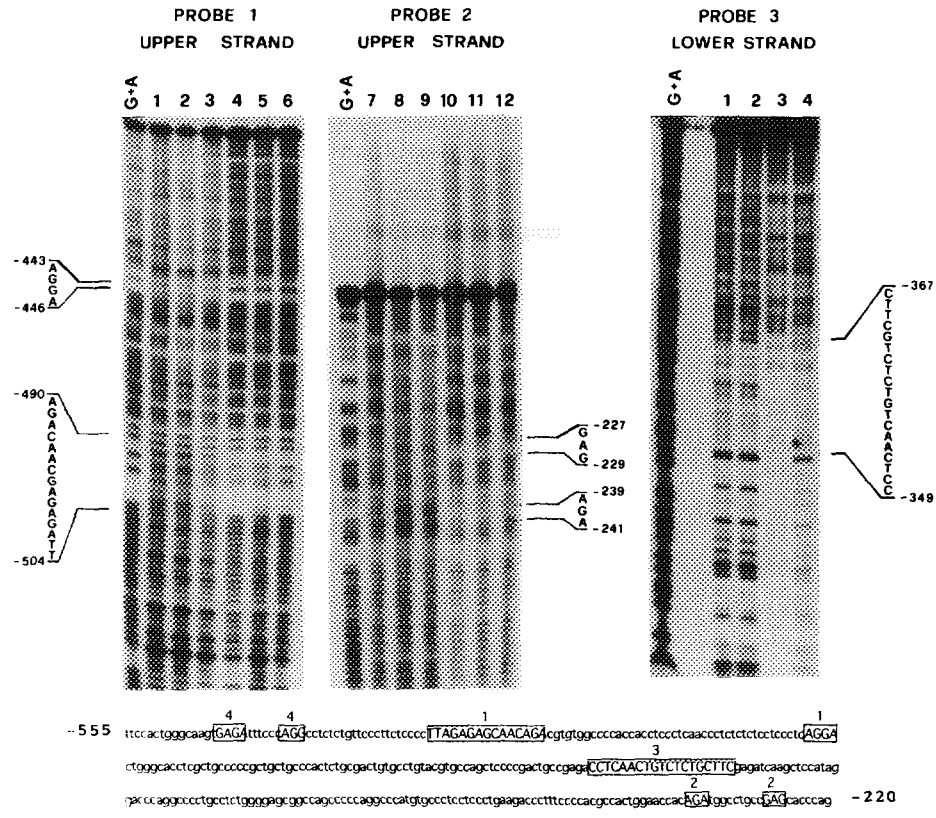


Figure 2. In vitro DNase I footprinting analysis of a segment of the 5'-flanking region of the aldolase A distal promoter. The radiolabeled probes (probe 1 from -555 to -347, and probe 2 from -292 to -220) were incubated with 20, 30 and 40 µg/ml of DNaseI with (lanes 4, 5, 6, 10, 11, 12) or without (lanes 1, 2, 3, 7, 8, 9) 30 µg of nuclear extracts from Hep3B cells. On the right is the footprinting obtained with the radiolabeled probe 3 from -450 to -220, incubated with 20 and 40 µg/ml of DNaseI with (lanes 3 and 4) or without (lanes 1 and 2) 30 µg of nuclear extracts from Hep3B cells. The G+A sequence ladder after the Maxam-Gilbert reaction is shown for each probe. The DNA sequence from -555 to -220 is shown in the lower part of the figure. Protected sequences (capital letters) are boxed. Boxes 1, 2 and 3 include the protections obtained with probe 1, 2 and 3, respectively. Boxes 4 refer to the protection previously described (9).

with the nuclear protein (Fig.3, lane 4). Furthermore, the oligonucleotide Neg3 (34 bp, see bottom Fig.3), which includes the sequence from nucleotide -367 to nucleotide -349, was protected by probe 3 in footprinting experiments. The Neg3 oligonucleotide contains a core-sequence CTGTCTCT, which is complementary to the GA-rich sequence of the Neg1 oligonucleotide (see bottom Fig.3). As shown in Fig.3, lane 3, Neg3 competed with Neg1 in the interaction with nuclear proteins. In addition, Neg3, used as probe, formed a relatively abundant complex with nuclear protein from Hep 3B (Fig.3, lane 6); the formation of the complex was prevented by a 100-fold molar excess of either Neg3 (lane 7) or Neg1 (lane 8)

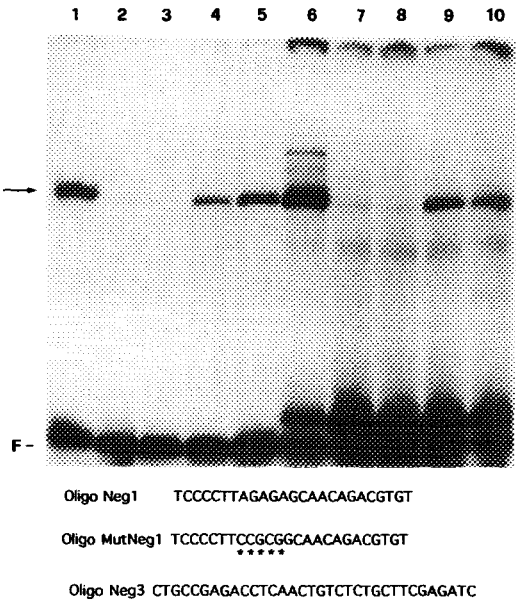


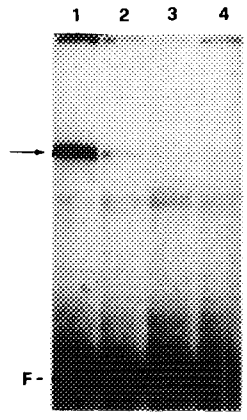
Figure 3. Gel-retardation analysis. The 5'-end-labeled probes, *i.e.*, oligonucleotides Neg1 (lanes 1-5) and Neg3 (lanes 6-10), were incubated with 5 µg nuclear extracts from Hep3B. Homologous and crosswise competition experiments were performed with a 100- fold molar excess of Neg1 (lanes 2 and 8), Neg3 (lanes 3 and 7), MutNeg1 (lanes 4 and 9) and unrelated (lanes 5 and 10) unlabeled oligonucleotides. The arrow indicates the specific DNA-protein complex. F indicates the free DNA probe. In the lower part the sequences of Neg1, MutNeg1 and Neg3 are indicated. Asterisks indicate the site of mutated nucleotides. In lanes 1 and 6 no competitors were used.

unlabeled oligonucleotides, but not by an unrelated oligonucleotide (lane 10). The MutNeg1 did not prevent either the binding with Neg1 probe (lane 4) or that with probe Neg3 (lane 9).

The putative AldA-NRE was homologous with the sequence of mammalian and avian ubiquitous silencers (18-23). Therefore, we next determined if the protein factor that binds to AldA-NRE interacts with other silencer elements. In a gel mobility shift assay we used as competitors the synthetic oligonucleotides (CYP2C11 OVII and β-IFN) of the silencer elements found in the rat cytochrome P450 and human β-IFN genes (20, 21), homologous to the AldA Neg1 and AldA Neg3, respectively. Both CYP2C11 OVII (Fig.4, lane 3) and β-IFN (Fig.4, lane 4) competed with the Neg1 probe for the binding with a protein factor, as also found for Neg1 (Fig.4, lane 2). Thus the same protein factor recognizes similar negative regulatory cis-elements of various genes.

Transcriptional regulation by AldA-NRE

To determine whether the binding sites of the region from -555 to -220, which includes the negative cis-elements, were important for the promotion of transcription initiation, we performed transient transfection experiments in



Oligo Neg1 TCCCCTTAGAGAGCAACAGACGTGT

Oligo CYP2C11 O VII ACTTGAGAGGAAAGAGAGAGAGGGTAAGAGGGACTGAGGGAG

Oligo Neg3 CTGCCGAGACCTCAACTGTCTCTGCTTCGAGATC

Oligo β-IFN TGAGATGGTCCTCTCTCTATTCAGAGGAA

Figure 4. Competition binding analysis. Complex between the 5'-end-labeled Neg1 oligonucleotide and nuclear protein from Hep3B cells, indicated by the arrow (lane 1), was competed with a 100- fold molar excess of Neg1 (lane 2), CYP2C11 (lane 3), β-INF (lane 4) unlabeled oligonucleotides. F indicates the free DNA probe. Sequences of AldA Neg1, AldA Neg3, the silencer motifs of the rat CYP2C11 (20) and human β-IFN (21) genes are shown on the bottom. The "AGAGAG" core sequence and its complementary "CTCTCT" are underlined.

Hep3B cells with different reporter recombinant plasmids containing various lengths or internal deletions of the L-type promoter segment (see Fig.5). Functional analysis of clones LΔ-555, LΔ-292 and LΔ-220 previously indicated a down-regulation exerted by a negative cis-element located between -555 and -220 (9). To demonstrate that the putative negative cis-element caused the dramatic decrease of CAT activity in clones LΔ-555 and LΔ-292, we deleted about 300 bp from clone LΔ-555, to obtain clone LΔ-555(Δ-528/-215). After this internal deletion CAT activity returned to 80% of that of clone LΔ-811. Thus -555/-220 fragment in its own configuration represses transcription of this gene and behaves as a true silencer. In addition, the DNA-protein interactions in the -555/-220 region, revealed by footprinting and bandshift assays, are responsible for the negative regulation of the L-type promoter.

To test the effect of AldA-NRE on a heterologous promoter, we cloned the DNA fragment from -528 to -215 upstream from the minimal length promoter of the retinol binding protein gene (14). AldA-NRE decreased the level of CAT activity by about 6-fold compared to that of the retinol-binding protein promoter alone (Fig.5). This result demonstrates that AldA-NRE is a more general type of silencer element, able to decrease promotion of transcription initiation also at the level of other genes.

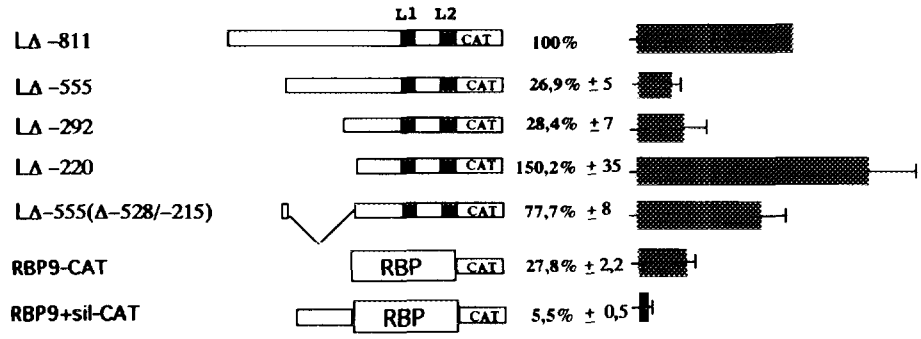


Figure 5. Expression of the recombinant aldolase A-CAT fusion gene in Hep3B cells. Left: map of the recombinant constructs of the 5'-flanking region upstream from exon L1. Right: CAT activities (relative to LΔ-811, which includes the minimal length AldA promoter (9), was taken as 100%) of the chimeric clones. The values are the average of three experiments with three different DNA preparations. RBP9: retinol-binding protein minimum length promoter; sil: 300 bp of the silencer sequence (i.e., from -528 to -215 segment of the L-promoter DNA sequence).

Discussion

We have mapped within the human aldolase A gene a negative regulatory cis-element (AldA-NRE) and detected a protein factor from Hep 3B cells that binds to this element. This factor bound to AldA-NRE down-regulates to one-fifth the transcription of the distal promoter in transfected Hep 3B cells, as in other cell types (HeLa and HepG2) tested. The negative cis-element acted as a true silencer: an internal deletion of about 300 bp from clone LΔ-555, which removed the entire region of negative control, restored CAT activity to 80% (clone LΔ-555(Δ-528/-215)). In addition, the negative element repressed by about 6-fold the expression of a CAT gene driven by a heterologous promoter. This suggests that AldA-NRE can regulate transcription of other cellular genes and that it might belong to a family of negative elements common to several genes.

DNase I protection analysis of the negative cis-element from -555 to -220 revealed a series of GA-rich elements. We also show the AGAGAG motif is the target for the binding with a nuclear factor present in Hep3B extracts, as well as in HeLa and HepG2 cells (data not shown). In fact, site-directed mutagenesis of five bp within this sequence eliminated the specific DNA-protein interaction, which indicates that the binding is highly specific. The AGAGAG motif and its complementary sequence CTCTCT are highly homologous to several previously described negative elements (18-23). We found that the same nuclear factor, or factors belonging to the same protein family, bind(s) to the silencer sequences present in the pL promoter of the human AldA gene, in the human β–INF and the rat cytochrome P450 genes. In addition, the AGAGAG sequence is highly repetitive along the 300 bp analyzed. The repetition of these sequences, as reported for such other regulatory elements as the silencer elements of the

chicken vimentin gene (24), and the glutathione transferase P element (25), could cooperate with the protein factors to repress promoter activity and may prove to be a general rule in genes utilizing this type of negative transcriptional control.

Acknowledgments

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