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In vivo activity of the most proximal promoter of the human aldolase A gene and analysis of transcriptional control elements

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The genomic region upstream from exon F (exon IV) of the human aldolase A gene has been studied for its ability to direct the transcription of a reporter gene in vivo. Transfection experiments in human hepatoma cells (Hep 3B) followed by CAT assay, and S_1 mapping analysis, demonstrated that: (i) this region is able to drive CAT gene transcription; (ii) all the transcriptional control elements of this promoter are downstream from nucleotide −384 of the longer ubiquitous RNA start site and the sequences between −384 and −262 play a crucial role in transcriptional efficiency; (iii) initiation starting points for two mRNAs exist 61 bp apart. Gel retardation and footprinting assays demonstrated the presence of DNA-protein complexes mainly in the region between −384 and −262 and such ubiquitous binding factors as Sp1 and AP-1.

Transcriptional control; DNA-binding factor; Aldolase A promoter; Footprinting analysis

1. INTRODUCTION

The human aldolase A gene is a useful system with which to study the molecular mechanisms that regulate both tissue-specific and constitutive gene expression. In fact, using 3 putative promoter regions, multiple transcripts containing the same coding segment with alternative 5′ leader exons arise from the human aldolase A gene [1–3]. One of the alternative leader exons is muscle specific (M), and the others (L_1, L_2 and F) are of the 'housekeeping' type. The first two exons (L_1 and L_2) are present in aldolase A mRNA, which was first isolated and sequenced from liver [4] and then also detected in lymphoblasts, muscle and spleen [2]. The proximal leader exon F (exon IV) has been found in two mRNA species of slightly different lengths that are expressed in all human and rat tissues examined so far [2,3,5,6]. The putative promoter region responsible for the expression of the latter ubiquitous mRNAs, and the relative initiation sites have been suggested by structural analysis and conventional S_1 mapping and primer elongation experiments [2,3,5].

Here we demonstrate the in vivo activity of the most proximal promoter region and the presence of regulatory elements at this ubiquitous promoter.

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Abbreviations: DMEM, Dulbecco medium Eagle's modified; bp, base pair

2. MATERIALS AND METHODS

Chemicals were from Sigma, Merck or Serva. Restriction enzymes, T_4 polynucleotide kinase and M13 primer were from Amersham International (UK). Deoxy- and dideoxy-nucleotides were from Pharmacia Inc. (Uppsala, Sweden). DNase I, poly(dI-dC) and *Bal*31 exonuclease were from Boehringer, Ingelheim. Thin-layer chromatography paper was purchased from Macherey-Nagel, FRG. X-ray films were from Fuji. [^{14}C]Chloramphenicol was purchased from NEN Du Pont (De Nemours, France).

2.1. *Plasmid construction*

The recombinant clone pA5′CAT was constructed by cloning an *Eco*RI-*Bam*HI fragment, which contains the complete 5′ region (3500 bp) of the human aldolase A gene, in the *Hin*dIII-*Sma*I sites of pEMBL8-CAT polylinker (see fig.1). Clone pAΔ-384 includes, in the *Hin*dIII/*Sma*I site of pEMBL8-CAT [7], a *Sma*I fragment of about 500 bp containing 120 bp of exon F and 384 bp of its upstream region. Progressive deletions of pAΔ-384 (see constructs in fig.1) were obtained by *Bal*31 exonuclease.

DNA fragments, used in bandshift and footprinting assays, were obtained using internal restriction sites of pAΔ-384 and cloned in the *Sma*I site of pUC12 plasmid.

2.2. *Cell culture, DNA transfection and CAT assay*

Human hepatoma cell line (Hep 3B) [8], HeLa cells and human fibroblasts were grown as monolayers in DMEM medium supplemented with 10% (v/v) fetal calf serum. Cells were plated at a density of 1×10^6 per 10 cm tissue culture plate and were transfected by the calcium phosphate method [9]. 20 μg of supercoiled plasmid DNA was added to each plate and 2 μg of pSVβ globin were cotransfected as internal marker. CEll extracts were prepared as described by Gorman et al. [10].

For the CAT assay [10], we used 100 μg of protein extract, 0.2 μCi [^{14}C]chloramphenicol, 20 μl of 4 M acetyl-CoA, in Tris 250 mM, in a total volume of 150 μl. After incubation at 37°C for 1 h, the reaction was stopped with ethylacetate. The acetylated and unreacted forms, separated by thin-layer chromatography, were excised and counted in a scintillation counter.

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2.3. *RNA extraction and S_1 mapping assay*

Total RNA was extracted by the guanidine thiocyanate procedure [11]. In the S_1 mapping assays [12] with total RNA extracted from transfected cells, a *Kpn*I-*Eco*RI fragment, including about 500 bp of the human aldolase A gene and 220 bp of the CAT gene, was cloned in M13mp19 and used as probe (see fig.2). For the cotransfected SVβ globin internal marker, a *Bam*HI fragment, including about 700 bp of the β globin gene cloned downstream the SV40 promoter, was used as probe (see fig.2).

2.4. *Preparation of nuclear extracts and gel retardation assay*

Nuclear protein extracts were prepared from HeLa cells grown in suspension to a density of approximately 5×10^5 cells/ml, according to Shapiro et al. [13]. For the gel retardation assays HeLa cell nuclear extracts (about 10 μg of protein) were incubated in a total volume of 20 μl containing: 10000 cpm end-labelled probe, 8% Ficoll, 20 mM Hepes (pH 7.9), 50 mM KCl, 1 mM EDTA, 0.5 mM DTT, 10% glycerol, 2 mM $MgCl_2$, 4 mM spermidine, 3 μg poly(dI-dC) and 0.5 μg salmon sperm DNA. Samples were incubated at 25°C for 10 min. Free DNA and DNA-protein complexes were resolved on a 4% polyacrylamide gel in 0.5 × TBE (1 × TBE is: 90 mM Tris-borate, 90 mM boric acid, 4 mM EDTA). After electrophoresis at 10 V/m in a cold room, the gel was dried and autoradiographed.

For competition experiments, from 10- to 100-fold molar excess of competitor DNA was added to the reaction.

2.5. *DNase I footprinting*

DNase I footprint assays were performed as described by Lichtsteiner et al. [14]. An end-labelled DNA fragment (20000 cpm) was added to a 20 μl reaction (final volume), containing 25 mM Hepes (pH 7.6), 60 mM KCl, 7.5% (v/v) glycerol, 0.1 mM EDTA, 0.75 mM DTT, 5 mM $MgCl_2$ and 1 μg double-stranded poly(dI-dC) competitor DNA. Nuclear extract was added last and, after 90 min in ice, DNase I (1 μl) and 100 μg/ml BSA were added to the reactions. After 5 min in ice, the reactions were stopped, extracted and loaded onto 8% polyacrylamide 10 M urea sequencing gel, as previously described [14].

3. RESULTS AND DISCUSSION

3.1. *Transient expression analysis of the proximal promoter region*

A restriction fragment of about 3500 bp, which includes the 5′ flanking region of the human aldolase A gene, was cloned in pEMBL8-CAT, in the correct 5′ to 3′ orientation, to yield the recombinant plasmid pA5′-CAT (fig.1A). The recombinant plasmid pAΔ-384 contains 120 bp of exon F and 384 bp of its upstream putative promoter region. *Bal*31 exonuclease was used to delete the pAΔ-384 clone to produce pAΔ-262, pAΔ-178, pAΔ-144 recombinant clones (fig.1A).

These constructs were assessed for expression of CAT enzyme activity after transient transfections into human hepatoma cells (Hep 3B). Fig.1B shows the CAT activity of each of the constructs depicted in fig.1A. The CAT activity observed with both pA5′-CAT and pAΔ-384 was very high: 80–88%, expressed as a relative ratio to the activity of pSV_2-CAT that contains the promoter and enhancer of SV40.

These data demonstrate, for the first time, the existence of functional promoter sequences upstream from exon F, and provide indirect evidence for the existence of other functional distal promoters. Moreover, the 384 bp upstream from exon F contain all the elements necessary for the optimal activity of this proximal ubiquitous promoter; sequences further upstream appear to be unrelated to this activity.

A deletion of about 122 bp (clone pAΔ-262), and another larger deletion (pAΔ-178) decreased promoter activity by about 66%; a larger deletion producing the pAΔ-144 construct further decreased the promoter activity. These results indicate that the region between −384 and −262 contains sequences crucial for efficient promoter activity, whereas less efficient *cis*-elements are contained in the region between −178 and +1. No regulatory elements were found between −262 and −178. The same results were obtained in transfected

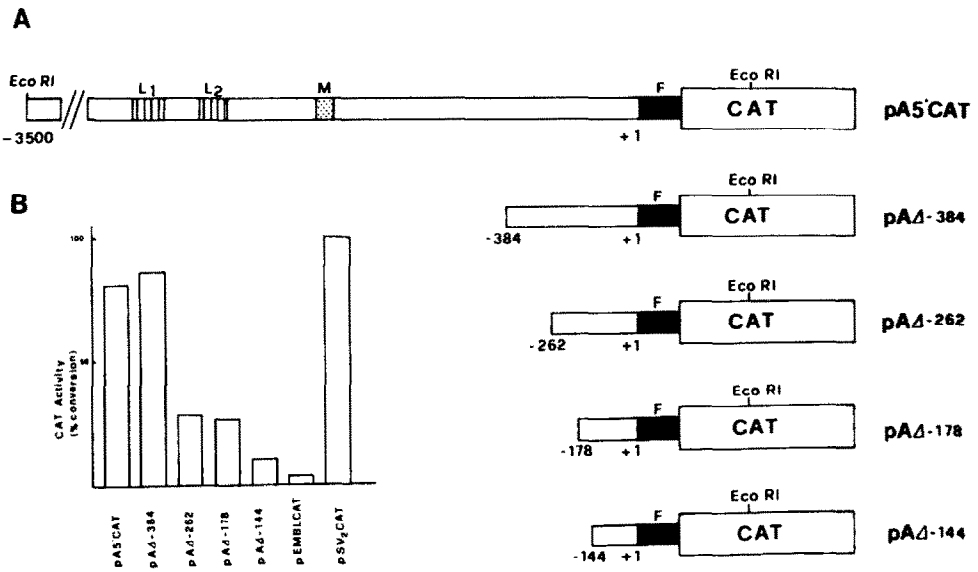

Fig.1. (A) Map of plasmids containing deletions of the 5′ flanking region of the human aldolase A gene linked to the CAT gene. L_1, L_2, M and F are the non-coding exons of the gene. (B) Expression of CAT activity in Hep3B cells transfected with deletion plasmids. The values shown are the average of 4 independent experiments. CAT activities are expressed as relative ratio to pSV_2-CAT activity.

HeLa cells and human fibroblasts (data not shown). Therefore, these findings are further confirmation of the ubiquitous expression of the proximal promoter region of the human aldolase A gene.

Fig.2 shows an S_1 nuclease analysis on total RNA isolated from Hep 3B cells transfected with the recombinant clones pAΔ-384 and pAΔ-262. Two protected fragments of about 370 bp and 310 bp, respectively, were observed. The initiation sites of these transcripts correspond to those previously indicated by structural analysis, S_1 mapping and primer elongation experiments as being the starting points for the ubiquitous mRNAs [3].

The deletion of 122 bp (clone pAΔ-262), which caused CAT activity to decrease by about 66% compared with clone pAΔ-384, affects the transcriptional efficiency of both mRNAs. As shown in fig.2, lane 2, the level of the two transcripts decreases to the same extent, whereas the cotransfected β globin transcript showed the same efficiency in both transfection experiments (fig.2, lanes 1 and 2). This suggests that the region between −384 and −262 contains *cis*-elements essential also for the transcriptional modulation of both ubiquitous mRNAs.

3.2. *Binding of ubiquitous* trans-*acting factors to regulatory sequences*

Gel retardation and footprinting assays were performed to detect and identify nuclear factors that interact with the essential proximal (downstream nucleotide −384) promoter region of the human aldolase A gene. Using internal restriction endonuclease sites, several small fragments of the region between −384 and +1 were cloned in the *Sma*I site of plasmid pUC12. Fig.3A shows a scheme of these subclones.

Nuclear extracts from HeLa cells were tested for DNA-protein binding using 5′ end-labelled probes. After electrophoresis on low-ionic strength 4% acrylamide gels, several retarded bands were detected for each probe (fig.3, lane 2, B–E). To test the specificity of the binding, competition experiments were performed in the presence of 10-, 50- and 100-fold molar excess of either alien DNA fragments (ferritin DNA, etc.; data not shown) or the same unlabelled probes (fig.3, lanes 3–5 in panels B and D). Several specific DNA-protein complexes were detected for each probe used; no competition was found in the presence of alien non-specific DNA.

Sequence analysis of the region upstream from exon F indicates the presence of several Sp1 binding sites (see fig.4). To determine whether Sp1 factors were specifically responsible for the DNA-protein interactions observed (fig.3B and D), competition experiments were performed using as competitor a *Sph*I-*Nco*I DNA fragment of about 90 bp of the SV40 promoter region containing six Sp1 binding sites [15]. With the pFH1

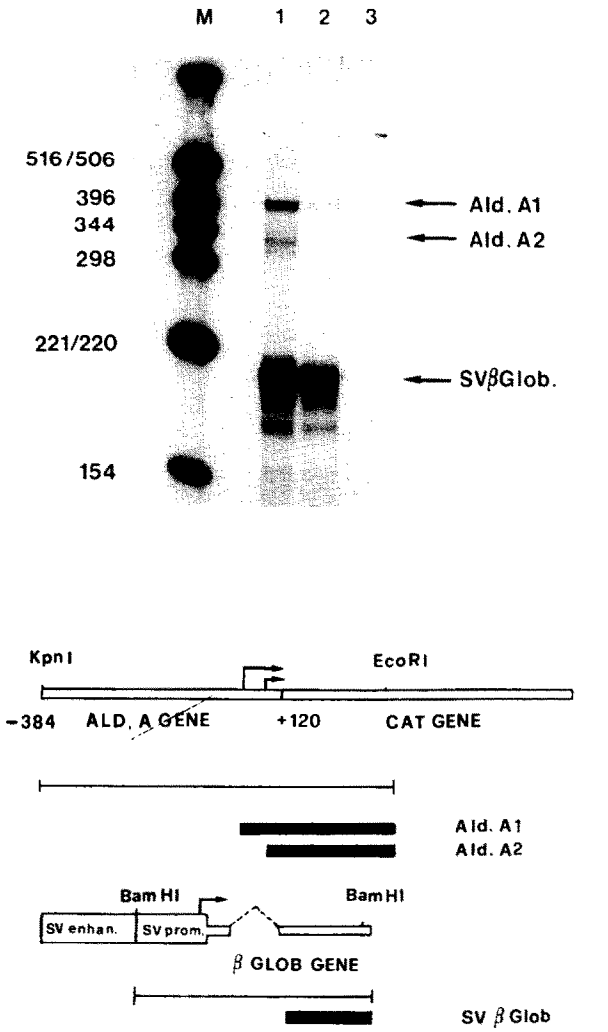

Fig.2. S_1 mapping of the RNAs extracted from Hep3B cells transfected with pAΔ-384 (lane 1) and pAΔ-262 (lane 2). In lane 3, *E. coli* RNA was used as negative control. In all transfection experiments (lanes 1 and 2) the SVβ globin plasmid was used as an internal control. The arrows indicate the positions of the aldolase A and β globin protected bands. The lower part shows the position of the probes (thin lines) and that of protected bands (solid bars) with respect to the CAT and β globin transcription units. The arrows indicate the two initiation transcription sites of ubiquitous aldolase A mRNAs.

probe all the specific DNA-protein complexes observed were competed for by the *Sph*I-*Nco*I DNA fragment (fig.3C). The pFH6 probe, which includes the region essential for promoter activity, revealed two specific major complexes (fig.3D). The upper band was efficiently competed for by the *Sph*I-*Nco*I DNA fragment of the SV40 promoter (fig.3E), whereas the lower specific band was unaffected. This indicates that nuclear factors other than Sp1 could be involved in the DNA-protein complex interaction seen in the lower band.

Furthermore, the *Sph*I-*Nco*I DNA fragment also competed with the pFH2 probe for binding with 2 out

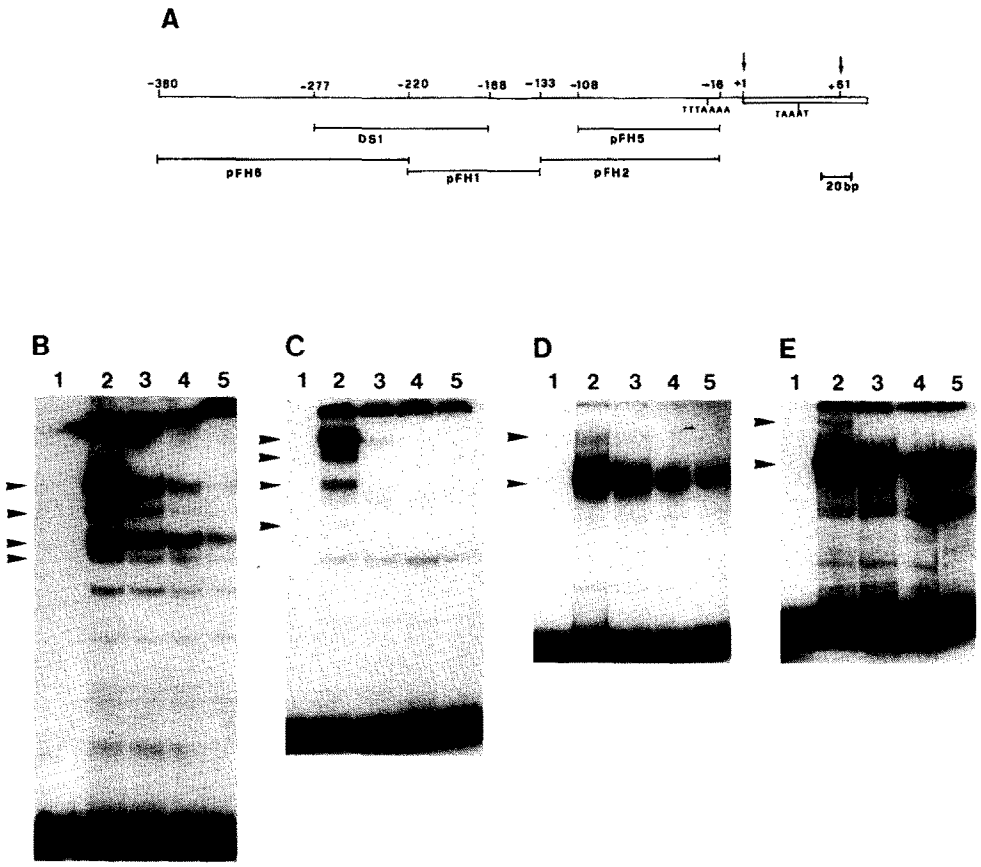

Fig.3. (A) Schematic representation of subclones used in the bandshift assay. In the top line is shown the proximal promoter region of the human aldolase A gene. The open bar represents about 80 bp of exon IV. The arrows indicate the two initiation transcription sites of ubiquitous mRNAs. Small fragments (DS1, pFH5, pFH6, pFH1, pFH2) obtained with *Hae*III, *Dde*I and *Sau*3A restriction enzymes were cloned in the *Sma*I site of pUC12 and the resultant plasmids were used to generate end-labelled probes. (B) Bandshift assay of the clone pFH1. Lane 2 shows the presence of DNA-protein complexes. The results shown in lanes 3, 4, 5 were obtained with a 10-, 50- and 100-fold molar excess of unlabelled pFH1 as competitor: the arrows indicate complexes that are affected by the competitor DNA. (C) Competition analysis of the pFH1 probe by a 10-, 50- and 100-fold molar excess (lanes 3, 4 and 5, respectively) of the *Sph*I-*Nco*I fragment of SV40 promoter element. Lane 2 = control without competitor. (D) Gel mobility assay with the pFH6 probe. The DNA-protein complexes are shown in lane 2. The results shown in lanes 3, 4 and 5 were obtained with a 10-, 50- and 100-fold molar excess of unlabelled pFH6 fragment as competitor; the arrows indicate complexes which are affected by competitor DNA. (E) Competition analysis of the pFH6 probe with a 10-, 50- and 100-fold molar excess (lanes 3, 4 and 5) of the *Sph*I-*Nco*I fragment of SV40 promoter element. Lane 2 = control without competitor. Lane 1 from B to E = probes alone.

of 3 complexes (data not shown). This suggests that the third complex could involve TATA-like binding factors, located at the 3′ end of the pFH2 fragment (see fig.3A).

3.3. *Localization of DNA-protein complexes along the promoter region*

To localize the position of the DNA-protein complexes, the pFH6 DNA probe (fig.3A) was used in DNase I footprinting analyses (see fig.5). Two protected regions were observed in the upper strand (lanes 1–4) between −334 and −311 (A region), and between −309 and −285 (B region). In the lower strand (lanes 5–8) the protected regions were found between −258 and −292 (A′ region), and between −290 and −303 (B′ region). Since the DNA fragments protected by the DNase I cleavage are very long on both strands, the binding of various factors may be inferred.

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CGCTGCAGCCGCGAAGACCGGAAGCTGGGGCCCCGGGCCGCGCGCGCTGGCCCTGGGAGGCGAAACTCAGCTTCCTTCGTTTCCGACTTTTCCATCCGCGTCCTCCACTTCCCCGTTCCG 2400
CCCTCCCCCATTGCCAACATTCTGGCTGAGTCACGGCGCCCCAGAGCCGGCCAGGCTGGGGGAAAGGAGCAGAAGGGAGGGCCCTAGCGACCCGCGGGATGTGGTCCGAGTCACGTCCGA 2520
GGGGGGTGGGGAGGGATCGTGTTCTCGGCGCCCGCCCCTTCCTAGCGCGGCCTCTGGGCTGGCTCTCGGGGGCGGCCCGTAGCCCAGTCCGTCGCCTGCCATTGGACGCCGCCCGCTCCT 2640
CGTAAAGGAAAAAGCTCGGCCGAGGGCGGAGTGGTGCCTTTAAAAGGCCGGGCGCCGCCTTCCGCCTGCACCGCCTCCTGCGCCGCCCCTTCCGAGGCTAAATCGGCTGCGTTCCTCTCG 2760
GAACGCGCCGCAGAAGGGGTCCTGGTGACGAGTCCCGCGTTCTCTCCTTGAATCCACTCGCCAGCCCGCCGCCCTCTGCCGCCGCACCCTGCACACCCGCCCCTCTCCTGTGCCAGGTGA 2880
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Fig.4. Sequence of the human aldolase A gene including exon F (exon IV) and its upstream region. The two transcription start sites (+1 and +61) are indicated by arrowheads. The 176 nucleotides including exon F are underlined. Dots indicate the Sp1 binding sites and triangles the Ap1 binding site. The TTTAAAA sequence is boxed.

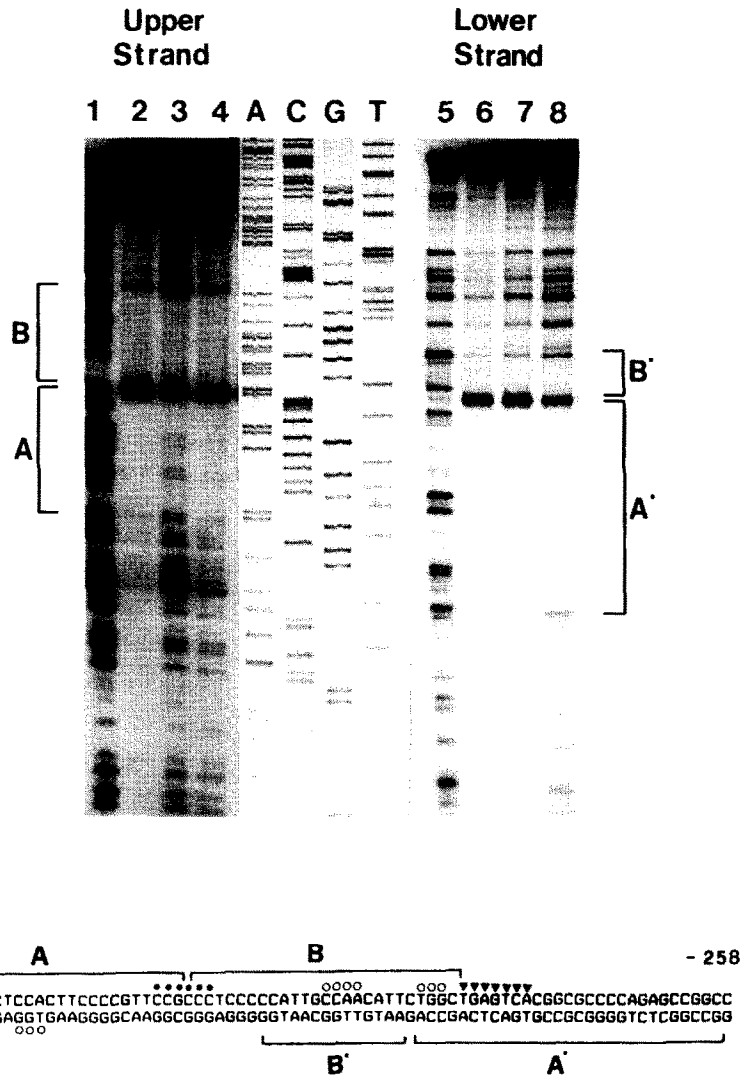

Fig.5. DNase I footprinting assay. pFH6 (see fig.3, panel A) was used as probe: it was 5′ end-labelled on the upper (lanes 1–4) or on the lower (lanes 5–8) DNA strand. Lanes 1 and 5 show DNase I digested probe in the absence of cell extract. The results shown in lanes 2 and 6, 3 and 7, 4 and 8 were obtained with 100, 200 and 400 μg of DNase I, respectively, in the presence of 20 μg of HeLa cell nuclear extract. ACGT is a Sanger sequence ladder. Brackets show the footprint regions. The location of footprints together with the nucleotide sequence of the pFH6 segment is shown in the lower part. Brackets above and below the sequence indicate the regions of footprints on the upper and lower strands, respectively. Filled circles represent the nucleotides involved in the Sp1 binding site. Open circles indicate the homology with the sequence motif of the NF1 binding site. Triangles show the sequence motif of AP-1 binding site.

DNA sequence analysis of clone pFH6 showed a canonical Sp1 binding site [15], a consensus sequence for the nuclear factor AP-1 [16] and high homology with the sequence recognized by the NF1-like factor [17] (see fig.4 and the bottom of fig.5).

These nuclear factors are ubiquitous proteins and are probably responsible for the constitutive expression of many genes [18,19]. Moreover, the recognition sequences for Sp1, AP-1 and NF1 are adjacent suggesting that these factors could act in conjunction to modulate transcription [20,21]. Therefore, the binding of Sp1 factors and presumably also of AP-1 and NF1 in the 5′ region upstream exon IV could account for the constitutive expression and the modulation of transcription of the human aldolase A gene. However, the much longer DNA stretch protected in footprinting assay analysis suggests the possible existence of other protein-interacting factors besides the above-mentioned ubiquitous ones.

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