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AlF-B, a novel CCAAT-binding transcription activator that interacts with the aldolase B promoter

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We describe here a 70 kDa transcription factor AlF-B, which preferentially binds to an element encompassing a CCAAT motif on the rat aldolase B promoter. Comparison of binding specificities, relative molecular masses, and subunit compositions with those of other known CCAAT-binding factors indicated that AlF-B is a novel member of CCAAT-binding factors.

Aldolase B gene; Transcription, CCAAT-binding factor; NF-Y; AlF-B

1. INTRODUCTION

Many eukaryotic gene promoters have a transcription regulatory element containing a CCAAT sequence [1]. Several mammalian factors that recognize a CCAAT motif have been identified, e.g. NF1/CTF [2,3], C/EBP [4], NF-Y/CBF/CP-1/ACF [5], CP-2 [6], YB-1 [7] and its related proteins dbpB [8] and EFIA [9], and CDP [10]. Although CCAAT elements in various promoters have a gross similarity in sequence, a subset of such CCAAT elements seems to be recognized by a particular factor [6]. Here we report a novel CCAAT factor with high affinity to the aldolase B (aldB) gene promoter as compared with the albumin and the adenovirus major late promoters. This factor migrates as a 70 kDa protein on SDS-polyacrylamide gel.

2. MATERIALS AND METHODS

Crude nuclear extracts were prepared from rat livers and partially purified through phosphocellulose column as described previously [11]. Factors that recognize site B in the aldB promoter were further purified by a DNA-affinity column chromatography [12]. Gel retardation assays were carried out as previously described [11]. Methylation interference assays were performed as in [13]. Separation of proteins on a SDS-polyacrylamide gel, extraction from the gel, and renaturation of proteins were carried out as described in [14].

Oligonucleotides used are as follows. aldB site B (B-oligo): GCTGTTCACGCGCCAATCAGAGTTAG [11]; aldB site A (A-oligo): AATCAGAGTTATTGAATAAACACCTC [11], NF-Y box in the adenovirus major late promoter (adY-oligo); ACCTATAAACCAATCACCTTCCTTGATGCC [6], C box in the rat albumin promoter (albC-oligo), GGGGTAGGAACCAATGAAATGAAAGGTTA [15] (only the sequences of the coding strands are shown, and CCAAT sequences are underlined).

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3. RESULTS AND DISCUSSION

In a previous paper, we showed three important *cis*-elements (sites A, B and C) in the rat aldolase B promoter [11]. Site B is an element containing a CCAAT motif which is indispensable for efficient liver-specific transcription in vitro [11]. To detect factors which bind to site B, we carried out a gel retardation assay using crude nuclear extracts (crude NE) from livers. As shown in Fig. 1A, ^{32}P-labeled B-oligo binds to proteins in the crude liver NE. Formation of this complex was reduced in the presence of unlabeled competitor B-oligo but not of A-oligo, showing the specificity of the complex formation. Since site B contains a CCAAT motif, the protein that bound to site B was expected to be one of the known CCAAT-binding factors. One such candidate is NF-Y, because nucleotide sequence around the CCAAT in the aldB promoter is closely related to binding sites for NF-Y in the class II genes of the major histocompatibility complex [7,16]. To see if NF-Y binds to site B, we performed competition experiments with two typical NF-Y binding sequences. One is a CCAAT box in the adenovirus major late promoter (adY-oligo) [6] and the other a CCAAT box in the rat albumin promoter (albC) [15]. The results in Fig. 1A show that the presence of either oligo reduced the formation of the B-oligo complex. However, these oligos did not compete effectively as compared to B-oligo (lanes 4–7). Conversely, complex formation of ^{32}P-labeled adY-oligo with NF-Y in the crude liver NE was strongly interfered with an excess amount of either competitor adY- or alb-C oligo, but not with B-oligo (Fig. 1B, lanes 11–17).

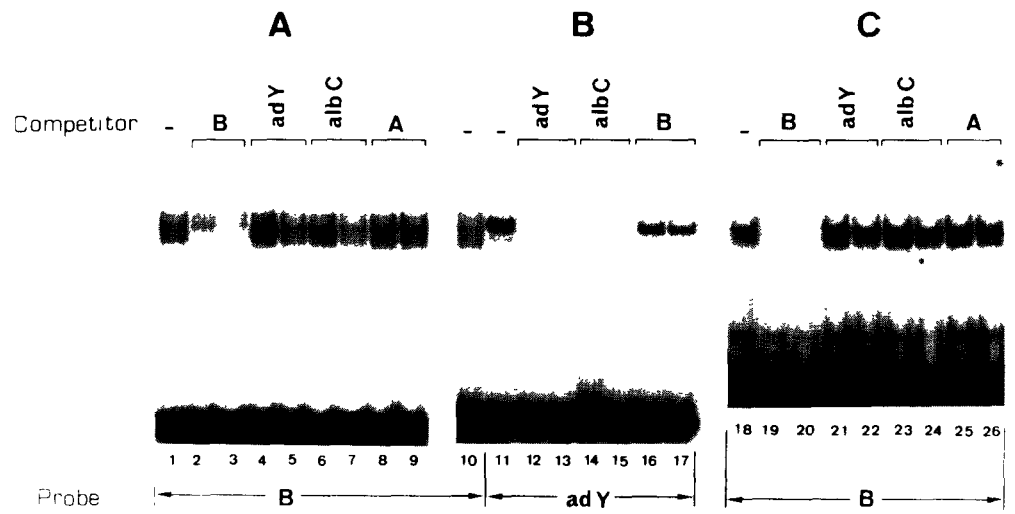

Fig. 1. Detection of site B-binding activities in the liver NE. ^{32}P-labelled B- (panel A) or adY-oligo (panel B) was incubated with the crude liver NE in the presence of either competitor adY-oligo (CCAAT box in the adenovirus major late promoter), albC-oligo (CCAAT box in the rat albumin promoter), or A-oligo (site A in the aldolase B promoter) Panel C shows a gel retardation assay using affinity-purified AlF-B and ^{32}P-labeled B-oligo in the presence of competitors indicated on top of the autoradiogram Competitors used were 10- and 30-fold excess amounts of the probes

These results implied that B-oligo preferentially binds a factor(s) distinct from NF-Y. To confirm this, we purified the site B-binding factor. Crude liver NE was passed through a phosphocellulose column equilibrated with a buffer containing 0.1 M KCl. Site B binding activity was recovered in flow-through fraction, while NF-Y was adsorbed to the column [11]. The flow-through fraction was then subjected to a binding site affinity column chromatography on a B-oligo-bound Sepharose 4B [12]. Using the purified factor (termed AlF-B), we carried out similar competition experiments as in Fig. 1A. In this case, formation of AlF-B complex was inhibited in the presence of B-oligo, but not in the presence of either competitor adY-, albC- or A-oligo (Fig. 1C). Thus, AlF-B has a very low affinity to the NF-Y sites.

We next wished to determine the relative molecular mass of AlF-B. After AlF-B preparation (phosphocellulose fraction) was run on a SDS-polyacrylamide gel, proteins were extracted from the gel slices, renatured

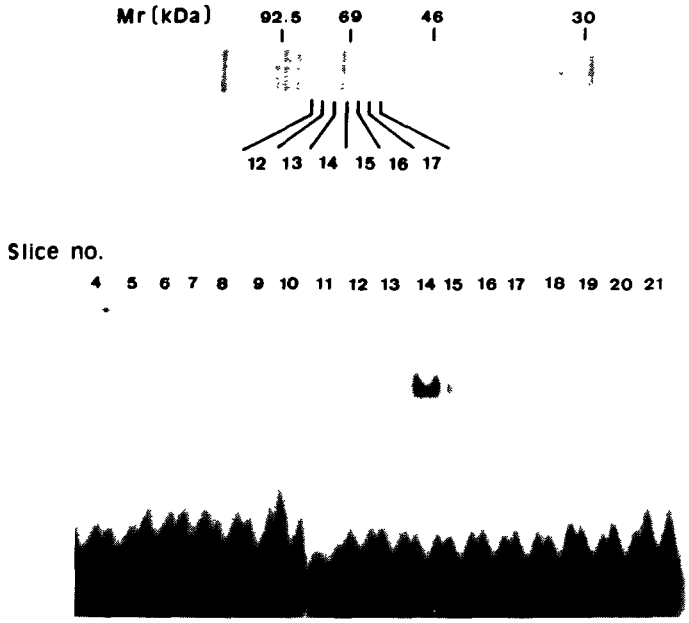

Fig. 2. Estimation of relative molecular weight of AlF-B. AlF-B preparation (phosphocellulose fraction, [11]) was run on an SDS-polyacrylamide gel. After electrophoresis, the gel was cut into 3 mm slices from the top, and proteins were extracted, renatured and assayed for B-oligo-binding activity as in Fig. 1. Upper panel shows CBB-staining of the gel

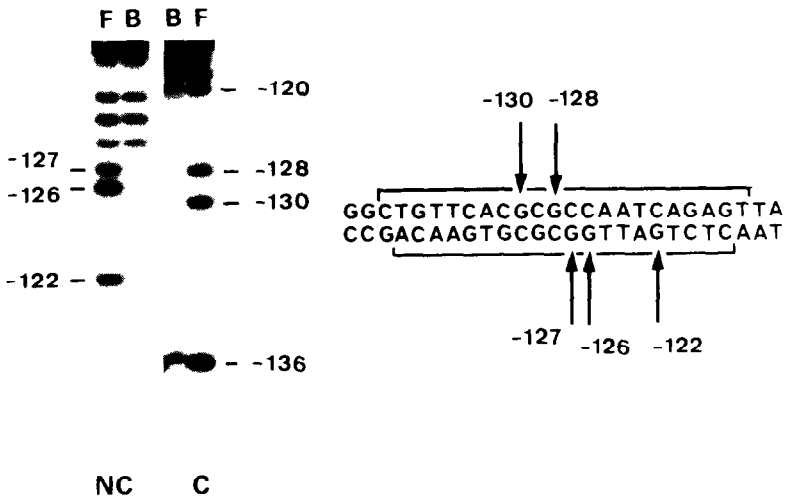

Fig. 3. Methylation interference analysis. ^{32}P-end-labelled B-oligo was partially methylated with dimethylsulfate, and added to the binding reaction containing AlF-B (phosphocellulose fraction). Free (F) and bound DNAs (B) were isolated by native polyacrylamide gel as in Fig. 1. DNAs were purified, cleaved with piperidine, and run on a polyacrylamide-urea sequencing gel. C, coding strand; NC, noncoding strand. Arrows indicate guanine residues whose methylation inhibited binding, and brackets indicate a region protected from DNase I footprinting [11].

and assayed for site B-binding activity. The activity was recovered mostly from a single gel slice corresponding to about 70 kDa (Fig. 2, slice no. 14). Similar result was obtained from the experiments using affinity-purified AlF-B (data not shown). This observation, together with chromatographic behaviour described as above, would discriminate AlF-B from other factors that need assembly of heterologous subunits for their activities. For example, human CP2 is composed of two different subunits that can be separated through phosphocellulose chromatography [6]. Furthermore, the size of AlF-B, 70 kDa, differed from those of known factors such as C/EBP family (25–42 kDa) [16,17], CTF/NF-1 (52–66 kDa) [2,3], NF-Y/CBF/CP1/ACF (37 kDa and 40–43 kDa subunits) [5], YB-1 and the related factors (about 35 kDa) [9], and CDP (180 kDa and 190 kDa) [10].

Fig. 3 shows the results of dimethylsulfate-methylation interference assays for the identification of contact sites of AlF-B. Methylation of guanine residues at −130 and −128 sites on the coding strand, and at −122, −126, and −127 sites on the noncoding strand of site B strongly inhibited the binding of AlF-B. Distribution of these contact sites is somewhat different from those of other characterized factors (e.g. CTF/NF-1 [3] and CP2 [6]).

Given the binding specificity and relative molecular mass, we think that AlF-B is a novel member of CCAAT-binding factors. This factor binds more strongly to site B in the aldB promoter than to the NF-Y binding sites in the albumin and the adenovirus major late promoters. AlF-B is expressed in various cell types, although its concentration varies considerably (data not shown).

Previously, we [11] and Raymondjean et al. [18] observed a marked inhibition of transcription from the aldB promoter using titration with B-oligo in an in vitro transcription assay with liver NE. However, little inhibition of transcription from the adenovirus major late promoter was observed in such a titration assay with B-oligo. These observations strongly support our data presented here.

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