1242–1246 Nucleic Acids Research, 1994, Vol. 22, No. 7

Activity of the rat liver-specific aldolase B promoter is restrained by HNF3

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Received November 29, 1993; Revised and Accepted March 7, 1994

ABSTRACT

Although it contains binding sites for HNF1, NFY and C/EBP/DBP, the proximal promoter of the aldolase B gene is surprisingly weak when tested by transient transfection in differentiated hepatoma cells. This low activity could be due to overlapping between HNF1 and HNF3 binding sites in element PAB, from −127 to −103 bp with respect to the cap site. Replacement of the PAB region by a consensus HNF1 binding site unable to bind HNF3, results in a 30 fold activation of the promoter, in accordance with the hypothesis that activity of the wild-type promoter is normally restrained by HNF3 binding to PAB competitively with HNF1. Consistently, transactivation of the wild-type promoter by excess HNF1 is very high, most likely due to the displacement of HNF3, while the construct with the exclusive HNF1 binding site is weakly transactivated by HNF1. The inhibitory effect of HNF3 on HNF1-dependent transactivation is clearly due to competition between these two factors for binding to mutually exclusive, overlapping sites; indeed, when HNF1 and HNF3 sites are contiguous and not overlapping, the resulting promoter is as active as the one containing an exclusive HNF1 binding site. A construct in which PAB has been replaced by an exclusive HNF3 binding site is weakly expressed and is insensitive to HNF3 hyperexpression. DBP-dependent transactivation, finally, is independent of the nature of the element present in the PAB region.

INTRODUCTION

Aldolase B is an enzyme involved in both glycolytic and gluconeogenic pathways. Expression of the aldolase B gene occurs in the adult liver, proximal tubules of the kidney and small intestine. In the rat liver, it is subjected to a nutrient and hormonal regulation: its transcription is activated by glucose and insulin and inhibited by glucagon and cyclic AMP (1). A 200 bp (−194 to +14) proximal promoter fragment is sufficient to confer a tissue-specific but low expression on a reporter gene in transient transfection assays (2). This promoter can be stimulated, *ex vivo* (2) and *in vivo* (unpublished result) by a DNA element located in the first intron of the rat aldolase B gene. Different *in vitro* protein binding sites have been mapped along the 200 bp promoter fragment and their respective contributions to the promoter activity has been investigated by transient CAT assays in Hep G2 hepatoma cells (3–5). From 5′ to 3′, we found a negative distal element [DE, from position −190 to −170 bp]; a C/EBP-DBP binding site [from −170 to −140]; a NFY binding site [CCAAT box, from −137 to −126] ; overlapping hepatocyte nuclear factors 1 (HNF1) and 3 (HNF3) binding sites [PAB region, from −127 to −103]; and, finally, a proximal element [from −99 to −81] whose cis-effect and cognate DNA binding proteins have not been investigated yet. We previously demonstrated that DBP (in hepatoma cells) and HNF1 were potent transactivators of the aldolase B promoter while HNF3 competitively antagonized HNF1-dependent transactivation (5). Consequently, we hypothesized that the low activity of the aldolase B proximal promoter in hepatocytes and hepatoma cells could be due to the abundance of HNF3 in those cells, competing with HNF1 for binding to the PAB region. Indeed, we found that, in *in vitro* DNase 1 foootprinting experiments using liver nuclear extracts, HNF3 was preferentially bound to the PAB element (4). However, this hypothesis implied that HNF3, reported to be a transactivator of several hepatic genes (6–9), behaved as an 'anti-activator' in the context of the aldolase B promoter. In this paper we present evidence that, indeed, replacement of the region PAB by an exclusive HNF1 binding site, unable to bind HNF3, resulted in a strong stimulation of the aldolase B promoter activity in Hep G2 cells, while replacement of this region by a consensus HNF3 binding site did not significantly modify the low activity of the wild-type promoter. The distal transactivator DBP similarly activated the promoters whatever factor they bind in the region of the PAB element. These results indicates that the aldolase B promoter is normally restrained in liver-type cells by HNF3 independently of the transactivation by proteins of the C/EBP-DBP family.

MATERIALS AND METHODS

Cell culture and transient transfections. Monolayers of human hepatoma (Hep G2) cells were maintained in Dubellcco's modified medium (DMEM) supplemented with 10% (vol/vol)

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fetal calf serum, 1 μM L-triiodothyronin, 1 μM dexamethasone, 10 nM insulin. Cells were plated at a density of 10^6 per 28 cm^2 dish.

Transfection was carried out by the calcium phosphate method (10). Each dish received a total of 12 μg of DNA including 2 μg of the pRSV luciferase standardization plasmid that was used to monitor variations in transfection efficiency. Calcium phosphate precipitate was removed at the 18th hour, the cells were fed fresh medium, cultured further for 40 hours and then harvested for chloramphenicol acetyltransferase (CAT) assay (11) and luciferase assay (12) as described.

Cotransfection experiments

The DBP expression vector (pRSV-DBP) was kindly provided by U.Schibler (13), the HNF1 expression vector (pRSV-HNF1) by M.Yaniv (14) and the HNF3α expression vector (pRSV-HNF3α) by R.Costa (15,16). Cotransfections were performed with 7.5 μg of the tested plasmid, 2.5 μg of the expression vector and 2 μg of the pRSV luciferase standardization plasmid, for a total DNA amount of 12 μg per dish.

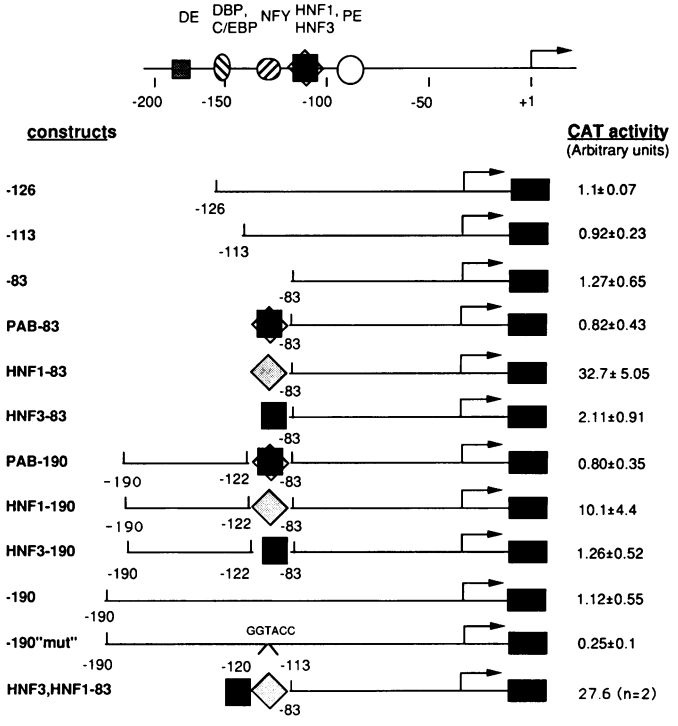

Figure 1. Map and CAT activity of the various aldolase B CAT constructs. All constructs, described in 'Materials and Methods' and in ref (2,5) contain the −83/+14 aldolase B fragment cloned in front of the CAT gene, plus more upstream aldolase B sequences, from −83 to the indicated position (−126, −113). The −126 construct contains the element PAB while the −113 construct contains the element PE, but not PAB. The HNF1-, HNF3-, HNF3,HNF1-, PAB-83 series was obtained by placing the corresponding double stranded oligonucleotides upstream of the −83 construct. The −190 series was obtained by cloning a −122/−190 aldolase B fragment upstream of the constructs of the −83 series. The −190 construct possesses the wild-type promoter fragment (2). the −190 'mut' fragment is mutated by replacement of 6 bp of the element PAB by a linker (5). Transient transfection experiments in Hep G2 cells were performed with 7.5 μg of the aldolase B promoteur constructs and 2 μg of plasmid RSV luciferase. CAT activity was normalized with respect to the transfection efficiency by measuring the luciferase activity. The results presented are the mean of at least 3 independent assays, and are given ± SD; activity of the HNF3,HNF1-83 plasmid has been measured only twice.

Construction of vectors

The −83 aldolase B/CAT construct was obtained by subcloning a PCR fragment of the aldolase B promoter spanning from nt −83 to +14 into the previously described Pe CAT plasmid (2); the 3′ primer [5′ TTGAGCTCAAATAGGATGGAT 3′] contained a *Sst*I restriction site while the 5′ primer [5′ TTGGTACCAGGAGGAGGGCAAAAGT 3′] contained a *Kpn*I restriction site.

The constructs HNF1-83, HNF3-83 and PAB-83 were obtained by a second step of subcloning of the appropriate double stranded oligonucleotides into the *Sma*I site of the −83 construct. The HNF1 oligonucleotide [5′ CTAGCTGGTTATACTTTAACCAGGACTCA 3′] spanned nt −96 to −68 of the L-type pyruvate kinase gene promoter (17). The HNF3 oligonucleotide [5′ GTTGACCTAAGTCAATAATCAGA 3′] spanned nt −111 to −90 of the transthyretin gene promoter (18). The PAB oligonucleotide reproduced the PAB region of the aldolase B gene promoter, from nt −126 to −104 [5′ CAGGAGTTATTGAATAAACACCTC 3′].

The construct HNF3,HNF1-83 was obtained by subcloning a PCR fragment in between the *Sma*I and *Sst*I sites of the PeCAT vector. The template used for amplifying this fragment was the HNF1-83 plasmid and the primers were: 5′ primer, [5′ GTTGACCTAAGTCAATAATCAGACTAGCTGGTTATACTTTAACCAGGACTCA 3′]; 3′ primer, [5′ TTGAGCTCAAATAGGATGGAT 3′].The 5′ primer is composed of the transthyretin HNF3 sequence as a non-matched 5′ tail followed by the pyruvate kinase HNF1 sequence hybridizing with the complementary sequence of the HNF1-83 plasmid. The 3′ primer contains a *Sst*I site.

The constructs HNF1-190, HNF3-190 and PAB-190 were obtained by subcloning a PCR fragment of the aldolase B promoter spanning from nt −190 to −122 into the filled-in *Bam*HI restriction site of the constructs HNF1-83, HNF3-83 and PAB-83, respectively. The primers used for amplifying this fragment were: 5′ primer, [5′ TTGTCGACTCTGACATTCTACGAG 3′]; 3′ primer, [5′ CTCTGATTGGCGCG 3′].

The other constructs used have been previously described (2,5). All constructs were checked by DNA sequencing.

RESULTS

The HNF1-dependent transactivation of the aldolase B promoter is restrained by HNF3 in HepG2 cells

We previously suggested that the low efficiency of the proximal aldolase B promoter in transiently transfected hepatocytes or hepatoma cells was due to the dual binding activity of the PAB region, able to bind on a mutually exclusive mode either HNF1 or HNF3 (4,5), as demonstrated by gel shift assays using either liver or Hep G2 cell nuclear extracts (4). Indeed, in the context of this promoter, HNF1 is a strong transactivator while HNF3, abundant in the liver and differentiated hepatoma cells, is not, and thus impairs the HNF1-dependent transactivation (5). We also reported that the proximal promoter incubated *in vitro* with liver nuclear extracts bound HNF3 rather than HNF1 on the PAB region (4). This hypothesis implies that replacing the PAB region by an exclusive HNF1 binding site should strongly stimulate the promoter activity while replacing this region by an exclusive

HNF3 binding site should be without any effect, except to unable HNF1 to transactivate the promoter.

To test this hypothesis we designed truncated aldolase B promoter fragments consisting of either the L-type pyruvate kinase gene HNF1 binding site, or the transthyretin gene HNF3 binding site, or both HNF3 and HNF1 binding sites placed in front of the −83 construct. Activity of these plasmids was compared to that of a construct in which the PAB region itself was ligated upstream of the −83 construct. The constructs of the −83 series are devoid of the proximal element (PE) identified by *in vitro* DNAse 1 footprinting experiments in the wild-type promoter (4).

Fig. 1 shows that the activity of the −126, −113 and −83 aldolase B CAT constructs in transiently transfected Hep G2 cells was similarly low. As expected, ligation of the aldolase B PAB region upstream of the −83 fragment did not modify this activity (note that −126 and PAB-83 constructs only differ by the presence or the absence of the element PE). Ligation of the exclusive HNF3 binding site in front of the −83 sequence did not significantly change the promoter activity, while ligation of the exclusive HNF1 binding site stimulated this activity by about 30 fold, in full accordance with our hypothesis. Activity of HNF3,HNF1-83 construct was similar to that of the HNF1-83 plasmid.

These results were confirmed with constructs in which the binding sites for NFY, C/EBP-DBP and the element DE have been added upstream of the HNF1-83, HNF3-83 and PAB-83 constructs, giving the HNF1-, HNF3- and PAB-190 series (Fig. 1). However, the HNF1-190 construct was 2.5 fold less active than the HNF1-83 construct, probably because of the presence of negative elements, especially DE and, perhaps, the NFY binding site (5).

Fig. 2 shows the results of transactivation experiments using cotransfection of the different aldolase B constructs with HNF1 and HNF3 expression vectors. The −113 and −83 constructs, although lacking the PAB regions, remained stimulated about 20 fold by large excess of HNF1; this suggests that cryptic site(s) in the construct are potential target(s) for binding HNF1 when over-expressed under the direction of the pRSV-HNF1 expression vector. Indeed, a −83/+14 fragment was able to bind recombinant HNF1α produced in a baculovirus-dependent system (a gift from F.Tronche and M.Yaniv), but not endogenous HNF1 from liver nuclear extracts (data not shown), indicating the presence in this fragment of a cryptic low affinity HNF1 binding site. Accordingly, the HNF3-83 construct was also slightly stimulated by HNF1. The PAB-83 construct was strongly stimulated (about 120 fold) by HNF1, as this has been reported for the −190 wild-type construct. This is probably due to the ability of a high HNF1 concentration to displace HNF3 bound to PAB. The HNF1-83 construct, that is by itself 30 fold more active than the other constructs, is much less stimulated by HNF1 (about 5 fold only), probably because the L-type pyruvate kinase gene exclusive HNF1 binding site of this construct is already saturated by the endogenous HNF1 of Hep G2 cells. However, the stimulation by HNF1 over-expression was 2−3 fold higher for the HNF1-190 than for the HNF1-83 plasmid (not shown), suggesting that the negative elements contained in the −190/−122 fragment could interfere with HNF1 binding at physiological concentration. Indeed, we have previously suggested that NFY could impair binding of HNF1 on the immediately downstream binding site (5).

DBP-dependent transactivation is independent of the binding activity on the PAB region

The −170/−140 element binds proteins of the C/EBP-DBP family and, in Hep G2 cells, mediated a 25 fold transactivation by over-expressed DBP (5). Fig. 3 shows that a similar 15−37 fold activation by DBP was observed for the wild-type −190 plasmid, the HNF1-, HNF3 and PAB-190 series and the −190 'mut' plasmid whose PAB element is mutated by replacement of six bases (5). These results confirm our previous features that HNF1 and DBP-dependent transactivations of the −190 construct are additive and indicate that the DBP action is independent of the complex assembled on the PAB region.

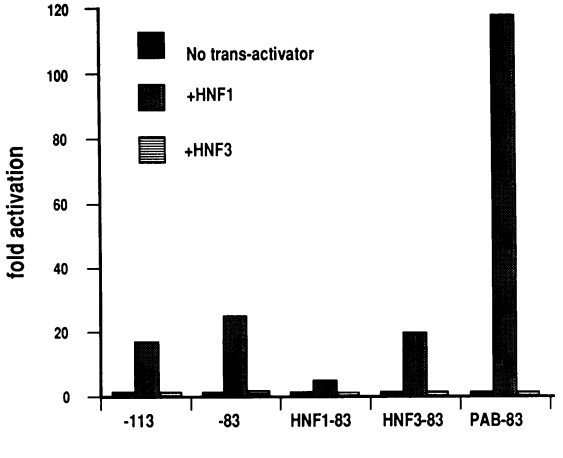

Figure 2. Transactivation of various aldolase B/CAT constructs by HNF1 and HNF3. Hep G2 cells were cotransfected with 7.5 μg of each construct and 2.5 μg of expression vectors RSV-HNF1 and RSV-HNF3. The CAT activity was normalized with respect to the luciferase activity in the same cell lysate. The results are the mean of at least 3 independant experiments. In the inset are indicated the expression vector co-transfected with the aldolase B/CAT constructs.

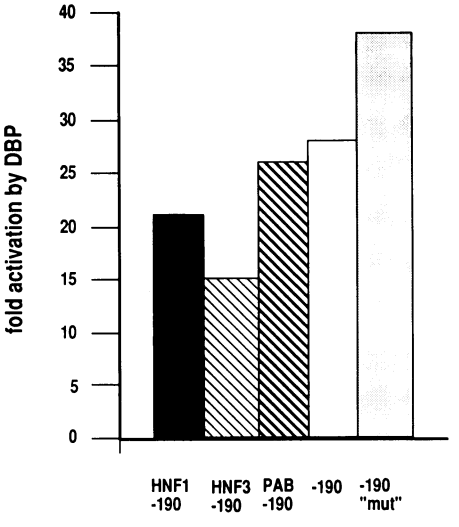

Figure 3. Transactivation of various aldolase B/CAT constructs by DBP. Hep G2 cells were transfected with 7.5 μg of each construct and 2.5 μg of CMV-DBP expression vector (+DBP). The CAT activity is expressed as fold activation with respect to the same constructs in the absence of CMV-DBP expression vector.

DISCUSSION

All results reported in this paper are consistent with a model in which the unique features of the PAB motif, binding either HNF1 or HNF3, play a major role in the activity of the aldolase B gene promoter. Since HNF3 proteins appear before HNF1a in endodermal tissues (18), a high HNF3/HNF1 ratio in fetal liver could contribute to repress aldolase B gene expression while the major aldolase isozyme synthesized is aldolase A. HNF1α, a homeodomain transcription factor, has been reported to be a major transactivator of numerous liver-specific genes (7,19–23), and is also a strong activator of the aldolase B promoter. However, in the liver and differentiated hepatoma cell lines, HNF3, in fact corresponding to several monomeric proteins of the growing *Forkhead* family (16), competes with HNF1 for binding to PAB.

HNF3 binding sites have been identified as essential *cis*-activating element in the promoters and (or) enhancers of several liver-specific genes, for instance the genes for α1-antitrypsin, transthyretin (19), tyrosine aminotransferase (24), apolipoprotein B (9) and albumin (8). However, the transactivation by HNF3 of an HNF3-dependent minimal promoter is relatively low ; it does not exceed 4 fold (25), as compared to more than 100 fold with HNF1 (14,26,27). Several papers report that an important role of HNF3 could be to cooperate with other factors bound to contiguous DNA elements. For instance, a close cooperation betweenHNF3 and the glucocorticoid receptor has been reported in the glucocorticoid responsive enhancer of the tyrosine aminotransferase gene (24), between HNF3 and nuclear factor 1 (NF1) in the albumin gene enhancer (8), and between HNF3 and proteins of the HNF4/ARP1/COUP-TF family in the apolipoprotein gene enhancer (9). In the aldolase B promoter, we find another exemple of a functional interaction between HNF3 and another transcription factor, namely an antagonistic interaction with HNF1. An aldolase B promoter possessing an exclusive HNF3 binding site instead of the element PAB is poorly active when transiently transfected in Hep G2 cells, and is not transactivated by a HNF3 expression vector, which signifies that HNF3 is not by itself a transactivator in the context ot the aldolase B promoter. Instead, it behaves as an inhibitor since it restrains the promoter of being activated by HNF1. This inhibitory effect of HNF3 on HNF1-dependent transactivation is totally dependent on the competition between these factors for binding to overlapping sites since replacement of the composite PAB site by contiguous non overlapping sites for HNF3 and HNF1 (HNF3,HNF1-83 construct) lead to an activity as high as with the HNF1-83 promoter. An apparent paradox is that the proximal aldolase B promoter is so severely restrained by HNF3 when tested in transiently transfected Hep G2 cells while the aldolase B gene is strongly expressed *in vivo*. In fact, the activator located in the first intron of the aldolase B gene is needed for this high expression *in vivo* (unpublished data). We can hypothesize that the interaction between the promoter and the intronic activator is capable of overriding the HNF3-dependent inhibition of the gene. The question can be raised of whether HNF3 itself, in fact, is important for this interaction. In the transthyretin gene, for instance, a high affinity HNF3 binding site located in the promoter is necessary for the activation by a distal enhancer ; in this gene, however, in contrast with our results on the aldolase B promoter, the HNF3 site is also essential to the promoter activity in the absence of enhancer (7). Very recently, HNF3 has been proposed to play a role in the transition of chromatin from an inactive to an active conformation (28). In the case of the aldolase B gene, binding of HNF3 to the PAB element in some early fetal tissues could contribute to open chromatin around the aldolase B promoter, therefore making it permissive for subsequent HNF1 binding and transcriptional activation.

Cis-acting elements located upstream of the PAB motif can also modulate the promoter activity, as previously reported (5) while the PE box identified by DNAse 1 footprinting and located downstream of PAB seems dipensable in our experimental system. As a whole, the upstream −190/−122 fragment decreases the activity of the strongly active HNF1-83 plasmid, perhaps due to a partial steric hindrance between NFY and HNF1 bound to closely apposed sites (4). Accordingly, HNF1 hyper-expression allows to restore full activity of the promoter.

The C/EBP-DBP site has been shown to be especially sensitive to DBP in Hep G2 cells. In the present paper, we show that the transactivation by DBP is largely independent on the nature of the element at the PAB position, and thus of the factor bound to this element. Rather, proteins of the C/EBP-DBP family could interact with the transcription initiation complex (directly or through auxiliary/bridging factors (29–31), and does not act primarily by modifying the ratio of HNF1 to HNF3 bound to PAB. This explains our earlier observation that transactivations by HNF1 and DBP expression vectors are additive (5).

In conclusion, our results emphasize a new type of functional interaction between HNF3 and another factor, here the antagonism with HNF1 that maintains the aldolase B promoter under a negative constraint in tissues where both HNF1 and HNF3 are present (that is to say, endodermal derivatives of the primitive gut) (32). To determine how this negative constraint is over-come *in vivo* and what is the role of the intronic activator in this phenomenon require further investigations which are currently under way.

ACKNOWLEDGEMENTS

This work was supported by grants from l'Association de Recherche sur le cancer, la Ligue nationale contre le cancer and l'Université Paris V René Descartes. We are especially grateful to Michel Raymondjean for helpful discussion. pRSV-DBP vector was kindly provided by Dr U.Schibler, pRSV-HNF1α vector was kindly provided by Dr M.Yaniv, pRSV-HNF3 vector was kindly provided by Dr R.Costa. Recombinant HNF1α was provided to us by Drs F.Tronche and M.Yaniv.

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