Characterization of the Aldolase B Intronic Enhancer*

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The aldolase B gene is transcribed at a high level in the liver, kidney, and small intestine. This high level of gene expression results from cooperation between a weak but liver-specific promoter and an intronic activator. A deletional study of this activator present in the first intron allowed us to ascribe the maximal enhancer function to a 400-base pair (bp) fragment (+1916 to +2329). This enhancer is highly liver-specific and enhances the activity of heterologous minimal promoters in a position and distance-independent fashion in transiently transfected Hep G2 hepatoma cells. The aldolase B enhancer is composed of two domains, a 200-bp module (Ba) inactive by itself but which synergizes with another 200-bp module (Bb) that alone retains 25% of the total enhancer activity. The Bb sequence is 76% homologous between human and rat genes and contains several binding sites for liver-enriched nuclear factors. By electrophoretic motility shift assays, we demonstrated that elements 5 and 7 bind hepatic nuclear factor 1 (HNF1), whereas element 2 binds hepatic nuclear factor 4 (HNF4). A functional analysis of the enhancer whose elements have been mutated demonstrated that mutation of any of the HNF1 sites totally suppressed enhancer activity, whereas mutation of the HNF4-binding site reduced it by 80%.

Aldolase B, one of the three known aldolase isoenzymes, is the only expressed isoenzym in highly differentiated hepatocytes (1) and is also found in kidney and small adult intestine where it is associated with aldolases A or C (2). Aldolase B catalyzes the reversible cleavage of fructose 1-phosphate into dihydroxyacetone phosphate and glyceraldehyde; therefore, it is involved in both glycolytic and gluconeogenic pathways. In human, hepatic nuclear factor 1 (HNF1), which stimulates promoter activity, or HNF3 that, on the contrary, restrained the aldolase B promoter activity (6). In transgenic mice transgenes directed by the −232-bp proximal promoter fragment were totally silent. The addition of 1.8 kb of sequences located in the first intron of the aldolase B gene (+685 to +2514) led to a 50-fold stimulation of the promoter activity ex vivo in Hep G2 cells and allowed for a correct, tissue-specific expression in transgenic mice (7).

The purpose of this work was to delineate the minimal intronic fragment responsible for the enhancer activity and to characterize DNA elements and cognate trans-acting factors involved in both activity and tissue specificity of this enhancer. Among various DNA elements detected in a 400-bp fragment endowed with a liver cell-specific enhancer activity, two HNF1-binding sites were shown to be indispensable for this activity. In addition, a conserved HNF4-binding site also behaved as a positive cis-acting element of the enhancer.

MATERIALS AND METHODS

Plasmid Constructions—For generation of the internal deletion, we started with the −232 A100B/CAT construct previously described (7). The −232 A100B1200/CAT and −232 A100B600/CAT plasmids were obtained by excision of a fragment between sites StuI (located within the B fragment) and RsaI or BamHI (located within the plasmid linkers). The −232 A100 Ba/CAT, −232 A100 Bl/CAT, −232 A100 Bo/CAT, and −232 A100 Ba+/b/CAT plasmids were obtained by cloning in both orientations, into the Smal site of the −232 A100/CAT plasmid (7), the fragment of interest generated by polymerase chain reaction. The a+b polymerase chain reaction fragment was also subcloned in the AIIII site (located upstream from the promoter) or in the ClaI site (located downstream of the CAT gene) of the previously described pECAT vector (4). Then the various promoter fragments −232 A100 (7), −194 to +14 of the aldolase B gene, or −183 to +11 of the pyruvate kinase gene (8), or −105 to +51 of the herpes simplex thymidine kinase (9) gene were excised and subcloned in one or both of these two plasmids.

Plasmids with block mutations in elements 5 and 7 or deletions in elements 2 and 4 were constructed by inserting the mutated fragments, obtained by a two-step polymerase chain reaction procedure (5, 10), in the Smal site of the −232 A100/CAT plasmid. Sequence details on the block mutations are given in Fig. 4.

All constructs were checked by DNA sequencing. The primer sequences used are available upon request.

Cell Culture and Transient Transfection—Hep G2 cells were grown in Dulbecco’s modified medium in the presence of 10% (v/v) fetal calf serum, 1 μM 1-trisiodothyronin, 1 μM dexamethasone, 10 nM insulin, at 37 °C in 5% (v/v) CO2. Mouse 3T3 cells were grown under the same conditions without hormones.

Transfection were carried out by the calcium phosphate method (11), in experimental conditions previously described (5). In each experiment 7.5 μg of the CAT plasmid and 2 μg of the luciferase plasmid were cotransfected. The pRSV luciferase standardization plasmid was used to monitor variations in transfection efficiency. Chloramphenicol acetyl-type pyruvate kinase.

* This work was supported in part by grants from La Ligue Nationale Contre le Cancer, l’Association de Recherche sur le Cancer and le Ministère de l’Education Nationale, and la Technologie et de la Recherche. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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The abbreviations used are: bp, base pair(s); kb, kilobase pair(s); CAT, chloramphenicol acetyltransferase; tk, thymidine kinase; HNF, hepatic nuclear factor; COUP-TF, chicken ovalbumin upstream promoter-transcription factor; C/EBP, CAAT/enhancer binding protein; L-PR, L-5'-flanking fragment was always very low in these cells. Recently we explained this result by a dominant restriction of the transcriptional activity due to binding of the hepatic nuclear factor 3 (HNF3) to the PAB element of the promoter (5). This PAB element binds in a mutually exclusive fashion either hepatic nuclear factor 1 (HNF1), which stimulates promoter activity, or HNF3 that, on the contrary, restrained the aldolase B promoter activity (6). In transgenic mice transgenes directed by the −232-bp proximal promoter fragment were totally silent. The addition of 1.8 kb of sequences located in the first intron of the aldolase B gene (+685 to +2514) led to a 50-fold stimulation of the promoter activity ex vivo in Hep G2 cells and allowed for a correct, tissue-specific expression in transgenic mice (7).
transferease (CAT) assay (12) and luciferase assay (13) were performed as described (5).

**Gel Shift Assays**—Nuclear extracts from adult rat liver and brain were purified according to Gorski et al. (14). The double-stranded oligonucleotides used as probes or competitors were as follows: element 2, 5′-TAAAGGAGTAAAGTCATTATGTATAATGTACTCCAGGCT-3′, +218; element 4, +2195, 5′-TCCCAGTGACAAACATGTGACTGTA-3′, 2220; element 5, +2212, 5′-GACGTGTCAGCTGTTATATTTGAGTTAATGATCTACTCAGGACTCATCTCA-3′, +2246; element 7, +2275, 5′-TTAAGGTTAATGATCTACTAAGAGCTGACTGTA-3′, +2304; HNF1, rat L-PK L1 (15), −106 AAGAGGAGGGAAGCATGTTACTTTAACCAGGACTCATCTCA-3′, +2329; HNF4, rat L-PK L3 (15), −150 TGGTTCTCGAGCATCTGTTACTTTAACCAGGACTCATCTCA-3′, +2514; parts B(a+b) of this subfragment were detected active in 8 out of 10 lines studied and were specifically expressed in the liver and kidney but not in the spleen and brain. As previously reported (7), transgene expression is highly dependent on a position effect, thus explaining the various levels of transgene activity and their total inactivity in two lines (once with each construct). In any case, these results confirm in vivo the delineation of the enhancer region of the aldolase B gene established from ex vivo experiments.

The B(a+b) Fragments Constitute a Transcriptional Liver Cell-specific Enhancer—The next question was whether the B(a+b) fragment had all canonical properties of an enhancer and whether it was liver cell-specific. To answer this question we placed the a+b fragment in both orientations in its normal intronic position or in a distal position, 1.2 kb upstream from the promoter or 1 kb downstream of the CAT gene. The activity

**RESULTS**

**Delineation of the Activating Sequence in the Intronic B Element of the Aldolase B Gene**—In our previous studies we identified a 1.8-kb B region ( +685 to +2514) localized in the first intron of the aldolase B gene that was absolutely required for transgene expression in the liver of transgenic mice (7). In transient transfection experiments in hepatoma Hep G2 cells, this B fragment stimulated about 50-fold the basal activity of a 232-bp proximal aldolase B promoter (4). To determine the cis-active sequences in this region, a series of deleted mutants were constructed, and their activity was tested in transient transfection experiments in Hep G2 cells (Fig. 1). Taking advantage of a unique StuI restriction site in the B fragment, we first analyzed the effects of the upstream 1200-bp ( +685 to +1915) and of the downstream 600-bp ( +1916 to +2514) parts of the B fragment. The B1200 subfragment did not change expression of the reference construct devoid of the B element (−232A100CAT construct) whereas, in contrast, the B600 downstream subfragment led to a 120-fold stimulation of the basal activity. This subfragment seemed to be more efficacious than the complete B fragment, perhaps due to its closer position with respect to the minimal promoter. Further subdivision of these 600 bp in three short DNA fragments of 200 bp each, designated fragments Bb, Bb, and Bc, showed that fragments Bb and Bc were totally inactive, whereas the Bb fragment (+2118 to +2329) retained 25% of the activation observed with the B600 subfragment. Finally association of the fragments B(a+b) restored the full activation reached with the B600 fragment (Fig. 1). These results indicated that the 400-bp region, spanning from +1916 to +2329, is able to recapitulate the enhancer activity of the intronic B element of the aldolase B gene. This 400-bp enhancer can be divided into downstream 200 bp, conferring by themselves part of the enhancer activity, and upstream 200 bp by themselves inactive but cooperating with the downstream part to confer a full enhancer activity.

To verify in vivo the relevance of results obtained ex vivo, we generated transgenic mice harboring the constructs studied above (Table I). The transgene bearing the upstream B1200 subfragment was totally inactive in all 7 lines obtained. In contrast, transgenes including either the downstream B600 subfragment or parts B(a+b) of this subfragment were detectably active in 8 out of 10 lines studied and were specifically expressed in the liver and kidney but not in the spleen and brain. As previously reported (7), transgene expression is highly dependent on a position effect, thus explaining the various levels of transgene activity and their total inactivity in two lines (once with each construct). In any case, these results confirm in vivo the delineation of the enhancer region of the aldolase B gene established from ex vivo experiments.
of all these constructs was tested by transient transfections in Hep G2 cells, and the results are reported in Table II. The fold activation observed was totally independent of the forward or backward orientation of the (a+b) fragment and almost independent of its position with respect to the cap site, either upstream from the promoter or downstream of the CAT gene or in its natural intronic position, in agreement with canonical enhancer properties (25). However, when the enhancer strength was tested on a construct consisting of the aldolase B promoter spanning from −194 to +14 bp, and lacking the first 100 bp and the last 120 bp of the intronic sequence (i.e., both splice sites), the level of activation was reduced. We do not know if this reduction results from a specific cooperation between the enhancer and intronic sequences located in the extreme 5’ and 3’ parts of the first intron or from a general increase in the level of transgene expression linked to the presence of a functional intron, already documented in mice but not in transient transfection experiments (26).

The activity of the B(a+b) enhancer fragment was also tested on heterologous promoters, either the liver-specific −183-bp proximal promoter of the L-type pyruvate kinase gene (8) or the ubiquitous 105-bp promoter of the thymidine kinase (tk) gene (9). The B(a+b) fragment enhanced the activity of these promoters by 18- and 15-fold, respectively (Table II). It is noteworthy that stimulation of the L-type pyruvate kinase promoter by the aldolase B enhancer was approximately similar to that by the SV40 enhancer, previously reported (8).

To determine whether the enhancer activity of the a+b fragment was by itself specific to liver cells, the constructs containing either the aldolase B or the L-type pyruvate kinase or the tk promoter, with or without the B(a+b) enhancer, were transiently transfected in mouse 3T6 cells that do not express the aldolase B gene. The enhancer was unable to turn on the liver-specific aldolase B or L-type pyruvate kinase promoters as well to activate the ubiquitous tk promoter. These results indicate that the aldolase B(a+b) enhancer was clearly cell-specific.

### Analysis of Protein-DNA Interactions

A computer analysis of the a+b enhancer sequence using the recently published MatInspector program (27) was performed. Only the analysis of the Bb (+2118 to +2329) enhancer fragment gave relevant information indicating potential binding sites for liver-enriched nuclear factors such as HNF1, HNF3, HNF4, and CAAT/enhancer binding protein (C/EBP) and for the ubiquitous AP1 complex. Since this Bb short fragment alone also retained part of the enhancer function and is 76% conserved between human and rat aldolase B genes, we focused our attention on these 200 bp. To confirm that elements of the Bb fragment actually interact with DNA-binding proteins, we first used in vivo DNase I footprinting experiments. The in vivo footprint revealed protein occupancy all over the fragment (not shown), such that it was rather difficult to deduce from this pattern well delineated windows. However, we used this experiment together with the identification of potential binding sites to design seven oligonucleotides that were used for gel shift assay experiments. Fig. 2 summarizes features of sequence analysis and in vivo footprinting experiments and shows the elements whose binding activity was then individually analyzed by gel shift assays. We found that elements 1 and 3 bind factors present in both liver and brain nuclear extracts; in contrast, elements 2, 4, 5, and 7 have a different binding activity in liver and brain (Fig. 3). To determine whether elements 2, 4, 5, and 7 bind previously identified liver-specific transcription factors, we used oligonucleotides of known binding specificity as competitor in the gel retardation experiments. Binding of element 2 was only competed for by the HNF4 oligonucleotide (Fig. 3, c), whereas binding to elements 5 and 7 were highly competed for by the HNF1 oligonucleotide and by each other (Fig. 3, c and d).

In addition, binding activity of element 2 was specifically supershifted by anti-HNF4 antibodies (Fig. 3, c), whereas binding activities of elements 5 and 7 were both supershifted by anti-HNF1 antibodies (Fig. 3, c and d).
The binding activity of element 4 was more difficult to identify. Competition experiments using oligonucleotides with different affinity for either HNF4 or COUP-TF were performed (Fig. 3, b), and none of them totally competed for the binding to element 4. The element 4 binding activity was also insensitive to anti-HNF4 antibodies. In contrast, element 4 as well as element 2 were effective in displacing HNF4 bound to the L3 L-PK site (Fig. 3, b). Therefore element 4 could bind factor(s) of the nuclear receptor superfamily different from HNF4 and could bind HNF4 with a low affinity. These element 4-binding factors are not likely to correspond mainly to COUP-TF (28), which is expressed in the brain as well as in the liver. Moreover element 4, whose sequence was reminiscent of an HNF3 recognition site, failed to bind this nuclear factor in our experiments as judged from competition experiments with an authentic HNF3-binding oligonucleotide. These results establish that the aldolase B enhancer is modular in nature, possessing binding sites for at least two liver-specific transcription factors, HNF1 and HNF4.

**Mutational Analysis of the Function of the Liver-specific Protein-binding Sites Present in the Aldolase B Enhancer**—The relative contribution of elements 2, 4, 5, and 7 to the enhancer strength in Hep G2 cells was tested by transient transfection of mutant constructs in which each of these element was mutated separately or in combination. The mutations were obtained as described under “Materials and Methods,” and the a+b-mutated fragments were introduced into an intronic position of the −232 A100 CAT aldolase B vector. We verified by electrophoretic mobility shift assay that mutated element 5 did not bind HNF1 (not shown). Mutation in this element as well as deletion of the other HNF1-binding site, element 7, rendered the enhancer totally inactive. Surprisingly, when both HNF1-binding sites were deleted, we observed an activity of the reporter below the basal level for the enhancerless promoter (Fig. 4). Deletion of element 2, bearing sequences binding HNF4, resulted in an 80% decrease in the enhancer activity. Deletion of element 4 had a minor effect upon the enhancer activity as the construct with this mutation retained 50% of the activity of the native enhancer. These results imply that the aldolase B enhancer activity is dependent on binding of the liver-enriched nuclear factors HNF1 and HNF4 to their cognate DNA sequences. A striking feature of the aldolase B enhancer is the absolute requirement of both intact HNF1-binding sites to be functional.

**DISCUSSION**

We have previously described the promoter of the aldolase B gene which is liver-specific but needs the presence of an intronic enhancer to be strongly active in transiently transfected hepatoma cells (4). In transgenic mice, any transgenes devoid of this enhancer were totally inactive, whereas transgenes possessing this element were expressed in the liver, kidney, and small intestine (7). Since the active intronic region was previously ascribed to a large 1.8-kb fragment whose cis-acting elements and cognate transcription factors had not been reported, we described here the modular structure of this enhancer, characterized liver-specific nuclear proteins interacting with its cis-active elements, and functionally investigated its properties, its tissue specificity, and the role of elements binding liver-enriched transcription factors, HNF1 and HNF4.

First, deletional studies allowed us to ascribe the active enhancer to a 400-bp fragment spanning from nucleotides +1916 to +2329 with respect to the start site of transcription. This 400-bp active fragment can be subdivided into two parts called Bb and Ba; the former is totally inactive by itself but appears to cooperate with the latter since activity mediated by Bb fragment alone is 25% only of the activity generated by the B(a+b) 400-bp enhancer fragment. This 400-bp aldolase B enhancer fulfills the requirement of a typical enhancer; it is functional in both orientations and in any position, either upstream or downstream from the promoter as well as in its natural intronic position. Finally, the aldolase B enhancer is able to stimulate heterologous promoters, either liver specific or ubiquitous. However, this stimulation only occurs in liver-specific cells (Hep G2 cells), and not in fibroblasts, indicating that this enhancer is by itself tissue-specific, as is the aldolase B promoter. This tissue specificity has been recently confirmed in transgenic mice harboring a chimeric transgene directed by a promoter silent in the liver (29) plus, in front of it, the 400-bp aldolase B enhancer; this construct was strongly expressed in the liver as well as in the kidney and small intestine tissue structures normally synthesizing aldolase B. Therefore, the 400-bp intronic enhancer of the aldolase B gene appears to play an essential role in both liver and tissue specificity of aldolase B gene expression.

We have determined the cis-acting elements and cognate transcription factors involved in these properties.

The nuclear factors that are mostly responsible for the tissue-specific transcription in differentiated liver cells include HNF1 (30), HNF3 (17), HNF4 (31), HNF6 (32), C/EBP (33), and DBP (34). The experiments with the aldolase B enhancer were first carried out in the Hep G2 hepatoma cells in which the relative amounts of HNF1, HNF3, and HNF4 proteins are close to those found in the fully differentiated liver but which lack C/EBP and DBP (35). In the enhancer sequence a computer search for the presence of putative binding sites for the above-mentioned transcription factors was performed, resulting in the identification in fragment Bb of such consensus sequences for factors HNF1, HNF3, and HNF4. Band shift experiments clearly demonstrated that elements 5 and 7 do indeed bind HNF1 nuclear factor, whereas element 2 binds HNF4. The sequence of element 4 could suggest binding of both HNF3 and HNF4/COUPTF factors. However, the retarded bands observed in a gel shift assay with an element 4-specific probe, detected in the presence of liver but not of brain nuclear ex-
tracts, were specifically displaced neither by an excess of HNF3-specific oligonucleotide nor by anti-HNF4α antibodies. COUP-TF factors are known to be ubiquitous, particularly abundant in the brain (36), so that it is unlikely that the complex with element 4 corresponds to these factors. Since the binding activity of element 4 was partly competed for by some HNF4-binding sites and by synthetic DR1 motif, whereas the element 4 itself was able to displace authentic HNF4-containing complexes, we speculate that this element could mainly bind in liver extracts, as yet, unknown members of the nuclear receptor superfamily. Therefore we explored the role of all of the above-mentioned elements, including element 4, in the strength of the aldolase B enhancer by deleting or mutating each of them. Both HNF1-binding sites (elements 5 and 7) were also deleted. Point mutation or deletion of either HNF1-binding site abolishes completely the enhancer function, whereas deletion of both sites leads to restriction of the basal aldolase B promoter transcriptional activity. The requirement of both intact HNF1-binding sites in order to maintain the enhancer function in a hepatoma cell line is a striking feature of this enhancer. A common feature of several liver-specific enhancers is the cooperation between two or more different liver-enriched nuclear factors (17, 37–40). The stringent dependence of the aldolase B enhancer activity upon HNF1-binding sites seems in discrepancy with the report that the aldolase B gene is active in the liver of HNF1-α-deficient knock-out mice (41). However, transcriptional activity of the aldolase B gene in these mice could be explained by the presence of a compensatory increased amount of HNF1-β in the liver of the HNF1-α-deficient mice, both of these factors binding to the same target and being transcriptional activators (38, 42). It is noteworthy that interchangeability of HNF1-α and -β for sustaining the function of
enhancers possessing HNF1-binding sites is not a general phenomenon since knock-out HNF1-α-deficient mice are totally defective in expression of the phenylalanine hydroxylase gene whose distal enhancer (HSS-III), located 3500 bp upstream from the promoter, contains two HNF1-binding sites (43). The phenylalanine hydroxylase gene also contains two additional putative HNF1-binding sites around position −1200 (HSS-II), and its chromatin structure is closed in HNF1-α-deficient mice (44). Therefore, it could be that the hypothetical role of HNF1-α in remodeling chromatin structure of the phenylalanine hydroxylase gene is not ensured by residual HNF1-β, at least at its concentration in the liver. In the kidney, by contrast, the amount of HNF1-β is higher than in the liver, and the phenylalanine hydroxylase gene remains active in HNF1-α −/− mice. Therefore it could be that relatively high concentrations of either HNF1-α or β are required to open chromatin structure of the phenylalanine hydroxylase gene, whereas residual HNF1-β in the liver of HNF1-α −/− mice would be sufficient to sustain activity of the aldolase B enhancer whose open chromatin structure could depend on other factors.

The HNF4-binding site (element 2) appears also to be essential for the aldolase B gene enhancer activity since its deletion results in an 80% reduction of transcriptional activity, which is fully consistent with the report by Stoffel et al. (45) that the aldolase B gene is silent in HNF4-deficient knock-out murine embryos. In contrast, deletion of element 4 binding non-identified ubiquitous factors, the aldolase B enhancer seems to be a novel strong and highly tissue-specific enhancer useful for target gene expression to the liver, proximal tubules of the kidney, and enterocytes of the small intestine.

Acknowledgments—We are very grateful to Mireille Cognet for useful advice and to Michel Raymondjean for stimulating discussions.

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In conclusion, specificity and activity of the intronic aldolase B gene enhancer strongly relies on binding sites for liver-specific HNF1 and HNF4 factors. Surprisingly, either of the two HNF1-binding sites is crucially required for the enhancer activity. Although the aldolase B gene proximal promoter includes binding sites for HNF1 (element PAB from −126 to −104 bp (46)) and, as recently demonstrated, for HNF4 (element PE, located from −99 to −81 bp), a specific cooperation between these factors bound to the promoter and the enhancer does not seem to be crucial since the enhancer also works very well with the ubiquitous −105 tk promoter. Probably through its multiple elements for both liver-specific and non-identified ubiquitous factors, the aldolase B enhancer seems to be a novel strong and highly tissue-specific enhancer useful for target gene expression to the liver, proximal tubules of the kidney, and enterocytes of the small intestine.

FIG. 4. Functional analysis of different elements of the Bb enhancer fragment by transient transfection in Hep G2 cells. The transfected constructs are described under “Materials and Methods.” Maps of the non-mutated plasmid (wild) and of the various mutants are recalled with precise indication of the deleted or mutated elements. The relative CAT activity, standardized by the luciferase activity as indicated in Fig. 1, is given as the percentage of residual activity with respect to the wild-type construct. The data are presented as the means ± S.E. of four separate experiments.

Acknowledgments—We are very grateful to Mireille Cognet for useful advice and to Michel Raymondjean for stimulating discussions.

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