An Intronic Enhancer Essential for Tissue-specific Expression of the Aldolase B Transgenes*

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Expression in mice of transgenes directed by regulatory regions of the rat aldolase B gene requires the presence of a B element located in the first intron, while constructs devoid of this intronic enhancer are silent. Histo- and immunochemical staining of transgenic tissue sections showed that the longer transgene was expressed in the proximal tubular cells of the kidney, enterocytes located in small intestine villi and liver parenchymal cells. In the liver, a maximal expression was observed in perivenous hepatocytes, while the transgene was weakly active in periportal hepatocytes, which reproduced the pattern of functional zonation already reported for other glycolytic and gluconeogenic genes in the liver. We also established that the transgene retained the necessary elements for a correct chronological expression during development but was lacking elements necessary for activation by high carbohydrate diet. Instead, transgene expression was paradoxically stimulated in fasted animals, suggesting that the endogenous gene, which must be active under both glycolytic and gluconeogenic conditions, could possess distinct elements activating it in fasted as well as in carbohydrate-fed animals; the former element might be conserved in the transgene and the latter one might be lost.

Aldolase B is the isoform of fructose 1,6-bisphosphate aldolase, which is specific to hepatocytes, proximal tubular cells of the kidney, and enterocytes, where it plays an essential role in fructose metabolism. Indeed, aldolase B is much more active on fructose 1-phosphate resulting from fructose phosphorylation by fructokinase than the other two aldolase isoforms, aldolase C (specific to the brain) and aldolase A (ubiquitous and very active in the muscle). In addition, aldolase B activity is required in gluconeogenic organs for both glycolysis (hydrolysis of fructose 1-phosphate and fructose 1,6-bisphosphate into trioses) and gluconeogenesis (condensation of triosephosphates into fructose 1,6-bisphosphate). In humans, aldolase B deficiency is responsible for hereditary fructose intolerance, a recessive autosomal disease characterized by hypoglycemia and clotting disorders upon fructose feeding. In the liver, aldolase B progressively replaces aldolase A (and, to a lesser extent, aldolase C) during fetal development, becoming practically the only isoform in postnatal hepatocytes (Schapira et al., 1975; Numazaki et al., 1984). Aldolase B gene expression is regulated at the transcriptional level during development and cell differentiation and is also subjected to a transcriptional regulation by diet and hormones; transcription is activated about 4-fold by fasting rats fed a high fructose diet and is inhibited by fasting, glucagon, and cyclic AMP (Munnich et al., 1985; Weber et al., 1984).

The 200-base pair proximal promoter fragment of the aldolase B gene contains binding sites for ubiquitous and liver-enriched transcriptional factors, especially for proteins of the CAAT/enhancer binding protein family, hepatocyte nuclear factors 1 and 3 (HNF1 and HNF3). The HNF1 and HNF3 binding sites overlap each other, and their occupancy is mutually exclusive, HNF1 being a transcriptional activator whose effect is counteracted by HNF3 (Grégori et al., 1993, 1994). This competition between HNF1 and HNF3 could explain why the activity of this promoter tested by transient expression is very weak in hepatocytes and hepatoma cells (Grégori et al., 1991, 1994). In HepG2 hepatoma cells, activity of the aldolase B promoter is stimulated about 50-fold by an intronic element, termed element B, located in a fragment spanning from nucleotides 650 to 2448 (Grégori et al., 1991).

In the present paper, we show that this element is absolutely required for the expression in transgenic mice of constructs in which the choramphenicol acetyltransferase (CAT) gene is put under the control of aldolase B regulatory regions (aldolase B/CAT constructs). This tissue-specific expression has been more precisely analyzed by in situ detection of the CAT protein using immunological and enzymatic methods whose performances are compared. The expression of the transgene was localized to proximal tubules of the kidney, enterocytes in the upper region of intestine villi, and in the liver to pericentral and centrolobular hepatocytes.

MATERIALS AND METHODS

DNA Constructs—The maps of the different transgenes analyzed are shown in Fig. 1. The 232ABC/CAT and 232A600/CAT transgenes were derived from previously described constructs (2200ABC/CAT and 2200A/CAT) (Grégori et al., 1991) by XbaI digestion at position −232. The transgene 232A100/CAT was derived from 232A600/CAT by PstI digestion at position +197 and subcloning into the V88 CAT vector. The transgene 232A100B/CAT was derived from 232A100/CAT by cloning blunt-ended HindIII fragment B into the SalI site of this construct.

Production and Detection of Transgenic Mice—The 1600ABC/CAT, 1600AB/CAT and 1600 AC/CAT fragments were isolated from the previously described (Grégori et al., 1991) 2200ABC/CAT, 2200AB/CAT and 2200AC/CAT constructs, respectively, by a ClaI digestion (Fig. 1). The 232ABC/CAT, 232A600/CAT, 232A100/CAT, and 232A100B/CAT fragments were isolated from 232ABC, 232A600, and 232A100, and

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1 The abbreviations used are: HNF, hepatocyte nuclear factor; CAT, choramphenicol acetyltransferase; D1G, digoxigenin; PBS, phosphate-buffered saline.
then rinsed in PBS, cryoprotected by immersion in sucrose (10% wt/vol) sucrose in PBS for 1 h, then 30% sucrose in PBS overnight, both at 4°C, and then frozen and stored in liquid nitrogen until use. Tissue blocks were cross-sectioned at 7 μm in a cryostat, and sections were mounted on slides (Super Frost/Plus, CML, Nemours, France), air dried, and processed for immunohistochemical staining with any further fixation.

Immunohistochemical Staining for CAT—Rabbit polyclonal affinity-purified antibody to CAT (Miskimins et al., 1992) was purchased from 5 Prime → 3 Prime, Inc. (Padu, PA). Anti-rabbit IgG-digoxigenin (DIG), F(ab)2 fragments from sheep, anti-DIG-peroxidase, F(ab) fragments, and anti-DIG-fluorescein isothiocyanate were purchased from Boehringer Mannheim (Meylan, France). Tissue sections were exposed to a 1/1000 dilution for anti-CAT antibodies at room temperature for 1 h and rinsed three times in PBS. Sections were then treated with anti-rabbit-DIG antibody (1/400 dilution) for 30 min and rinsed three times in PBS; this was then followed by either anti-DIG-fluorescein isothiocyanate or anti-DIG-peroxidase (1/400 dilution). Sections labeled with fluorescein isothiocyanate were mounted with Vectashield (Vector, Biotys, Compiegne, France) and immediately observed with an epifluorescence microscope. Anti-DIG-peroxidase antibody was revealed with diaminobenzidine solution (10 mg/15 ml in presence of H2O2 (1/1000)) for 15 min, rinsed in PBS, and mounted after dehydration and clarification. Control sections were obtained by omission of the anti-CAT antibody and by using complete technique on wild type tissues.

Histochemical Staining for CAT—CAT activity was revealed using the Donoghue technique (1991, 1992). Briefly, sections were incubated for 4–24 h with a mixture that contained 0.3 mM acetyl-CoA (lithium salt; Pharmacia Biotech, Paris), 4 mM chloramphenicol (Sigma, Saint Quentin Fallavier, France), 5 mM potassium ferricyanide, 5 mM sodium citrate, 3 mM copper sulfate, and 63 mM of Sorensen's phosphate buffer, pH 6.0. They were then rinsed in distilled water, dehydrated in alcohol, cleared in xylene, and mounted. Control slides were obtained by omission of acetyl-CoA or chloramphenicol in the mixture or by using the complete mixture on non-transgenic tissues.

RESULTS AND DISCUSSION

Expression of the Different Aldolase B-CAT Transgenes in Vivo—Between two and four lines of each of the seven aldolase B/CAT transgenes tested were obtained, except for the 232A100/CAT transgene present in one line only (Table I). CAT activity was determined in tissue extracts from 3-month-old transgenic mice harboring between 2 and 100 copies of the transgenes per cell. The main result of these studies in transgenic mice is that the intronic element B is indispensable to the expression of the transgenes; 1600AB/CAT, 232ABC/CAT, and 232A100B/CAT transgenes are expressed in the liver and kidney of most transgenic lines while no 1600AC/CAT, 232A600/CAT, and 232A100/CAT transgenes are active in any line. These latter results confirmed previous, non-published data from our laboratory, namely that different types of transgenes directed by up to 2000 base pairs of 5'-flanking sequence of the aldolase B gene were totally inactive in transgenic mice. Therefore, the intronic element B, which is able to stimulate about 50-fold the activity of the promoter in transiently transfected HepG2 cells (Grégori et al., 1991) is also an indispensable element for tissue-specific and high level expression of the aldolase B transgene in vivo. Such a cooperation between a proximal promoter and a distal enhancer to ensure a correct expression of transgenes has been described for other genes, e.g. the albumin gene (Pinkert et al., 1987) and the apolipoprotein gene (Brooks et al., 1994).

However, the element B is clearly insufficient to confer on the transgenes an expression dependent on the number of integration copies and independent of site, which is to say to behave as a locus control region (Grosved et al., 1987; Fraser et al., 1990). Indeed, the CAT activity directed by a same transgene is highly variable in different lines and is not dependent on the transgene copy number. In two lines, the expression of the 232ABC/CAT (line 14) and 232A100B/CAT (line 52) transgenes is even nil in the liver and ectopically stimulated in the spleen. Therefore, even in the presence of the element B, ex-
pression of the transgenes is highly influenced by the integration site, which indicates that they contain neither locus control region nor so-called “insulators” (Bode and Maas, 1988; Kalos and Fournier, 1995; Phi-Van et al., 1990) able to protect transgenes from the influence of neighbor regulatory elements. Nevertheless, except for the two lines mentioned above, the transgenes with a B element are at least 100-fold more expressed in the liver and kidney than in the brain and spleen. The CAT activity in small intestine extracts seemed to be in the nonspecific range, but this could be due to artifacts, for instance to proteolytic degradation of the CAT protein in extracts contaminated with pancreatic secretions or to dilution of expressing cells by abundant non-expressing tissue, because the CAT protein was well detected in some enterocytes by immunohistochemical and histological techniques (see below).

Developmental Regulation of the Aldolase B 1600ABC/CAT Transgene in the Liver—Expression of the aldolase B gene in the liver is developmentally regulated; the mRNA is undetectable before day 14 of gestation, and from that stage to adult it is detectable in perivenous hepatocytes and in rare periportal hepatocytes (Fig. 2, and Schapira et al., 1975). Using the line 7 expressing the 1600ABC/CAT transgene at a high level, we observed that developmental regulation of transgene expression mimics that of the endogenous aldolase B gene (Table II). Therefore, the transgene contains the elements required for a correct regulation of aldolase B gene expression during development.

In Situ Detection of the CAT Protein in Transgenic Mice Harboring the 1600ABC/CAT Transgene—Since the 1600ABC/CAT transgene was that containing most potentially regulatory sequences of the aldolase B gene, we used mice of line 7, expressing this transgene at a high level, for an immunohistochemical analysis of CAT transgene expression.

CAT Immunohistochemical Staining—Fluorescence or diaminobenzidine staining was equivalent on all transgenic tissues examined with a cytoplasmic and occasionally nuclear positivity. No positivity was noted in controls.

Histochemical Stain for CAT—Positivity of transgenic tissues consisted of a cytoplasmic granular brown precipitate. In addition, some nuclei were strongly stained in transgenic tissue. Nevertheless, in transgenic and wild type tissues, some nuclei were occasionally weakly stained, indicating that this nuclear staining is not specific, as already described (Donoghue et al., 1991, 1992). No cytoplasmic positivity was noted in controls.

Both immunohistochemical and histochemical techniques showed exactly the same cellular and tissue pattern of positivity.

In the liver (Fig. 2, a and b), positive hepatocytes were localized in the central region with a perivenular ring distribution. Very rare positive hepatocytes were also seen in the perportal area. No positivity was observed in vascular cells, Kupffer cells, epithelial bile duct cells, and portal connective tissue.

In the kidney (Fig. 2, c and d), the epithelial cells of the first two convoluted parts of the proximal tubule (S1 and S2) and the capsular epithelium (Bowman’s parietal cells) of the glomerulus were positive for CAT with either histochemistry or immunohistochemistry. Nuclei staining was particularly strong in the S2 segment. No other kidney cell was positive for CAT.

Intestine immunostaining revealed a 50% positivity of villi enterocytes, with no staining of Lieberkühn crypts (Fig. 2, e and f). There was no positivity in the lamina propria. CAT histochemistry disclosed the same pattern of positivity in a weaker manner.

These patterns of immunohistochemical labeling of the aldolase B/CAT transgene product are consistent with previous results using anti-aldolase B specific antibodies for detecting the enzyme in various tissues (Schapira et al., 1975). However, the pattern of extreme zonation of transgene expression, detected in perivenous hepatocytes and in rare periportal hepato-
activity was detected by brown Fe$_{2}$phenaolpyruvate carboxykinase (Miethke et al., 1980). However, as a gluconeogenic enzyme, it could also be present in periportal hepatocytes synthesizing phosphoenolpyruvate carboxykinase (Welsh, 1972), and 6-phosphogluconate dehydrogenase (Hildebrand, 1980). The apparent discrepancy between zonation of regulatory regions in the observed restriction of transgene expression to perivenous hepatocytes. Alternatively, we cannot exclude that the special pattern of expression of the transgene in the liver lobule reflects the lack of some important cis-acting element(s) in the transgene, as evidenced by the strong dependence on the site of integration.

In any case, further analyses of the aldolase B promoter and enhancers will be required to determine the role of these regulatory regions in the observed restriction of transgene expression to perivenous hepatocytes.

Paradoxical Response of the Aldolase B/CAT Transgene to Fasting and Carbohydrate-rich Diets—Fig. 3 shows that the response of endogenous aldolase B mRNA and transgenic CAT mRNA to fasting and refeeding glucose were practically inverse; while, as expected (Weber et al., 1984; Munnich et al., 1985), the abundance of the aldolase B messenger increased when fasted rats were fed a high carbohydrate diet, the abundance of the transgenic CAT mRNA decreased under the same conditions. The first hypothesis for explaining this paradoxical response of the transgene relies on the absence of some cis-acting element(s) in the transgene and on the duality of the aldolase B functions in the opposite pathways, glycolysis, and gluconeogenesis.

In other words, it seems that the transgene lacks a positive glucose response element, perhaps similar to that characterized in the promoter of the L-type pyruvate kinase gene (Bergot et al., 1992; Diaz-Guerra et al., 1993), or in a distal upstream region of the spot 14 gene (Shih and Towle, 1994). However, it could remain an element whose role is to ensure a persistence of aldolase B synthesis under gluconeogenic dietary conditions, while purely glycolytic genes, such as the L-type pyruvate kinase gene, are totally extinguished (Weber et al., 1984; Vaulont et al., 1986). This hypothesis is in line with the observation that the L-pyruvate kinase glucose response element (L-PK GIRE) behaved, in transfected hepatocytes, as a positive element in the presence of glucose but also as a negative element in the absence of glucose or in the presence of glucagon (Bergot et al., 1992). If such an element exists in the endogenous aldolase B gene but not in the transgene, this could account for both absence of positive response to glucose and sustained expression in fasted animals, while a different element could account for the stimulation of transgene expression during fasting.

CONCLUSION

The aldolase B gene needs to be expressed in vivo with an intronic activator cooperating with its tissue-specific promoter.

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2 A. Hatzfeld, personal communication.
These elements are sufficient to confer on a CAT reporter transgene a correct tissue-specific expression in the kidney, small intestine, and liver plus (in the liver) a drastic restriction of the expression to hepatocytes around the centrallobular vein. However, the physiological stimulation of the gene by a carbohydrate-rich diet was replaced by the opposite phenomenon, that is to say a stimulation in fasted animals. This paradoxical dietary response of the transgene suggests that the aldolase B gene, which must be expressed under both glycolytic and gluconeogenic conditions, could contain different elements stimulating transcription in either conditions. The glucose response element would be lacking in the transgene while the element enhancing transcription during fasting would be present.

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