Vol. 171, No. 1, 1990 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
August 31, 1990 Pages 243-249

TISSUE-SPECIFIC EXPRESSION OF RAT PYRUVATE KINASE L/CHLORAMPHENICOL ACETYLTRANSFERASE FUSION GENE IN TRANSGENIC MICE AND ITS REGULATION BY DIET AND INSULIN

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Received July 16, 1990

SUMMARY: We produced transgenic mice carrying about 3 kb of the 5'-flanking sequence of the rat pyruvate kinase L gene linked to the chloramphenicol acetyltransferase (CAT) structural gene. Expression of the transgene was observed only in tissues in which the endogenous L-type pyruvate kinase is expressed. Dietary glucose or insulin induced similar increases in the levels of CAT and L-type pyruvate kinase mRNAs in the liver. However, the fructose-induced level of CAT mRNA was about 3- and 6- fold lower than those of endogenous L-type pyruvate kinase mRNA in the liver and kidney, respectively, confirming our previous finding that stabilization of the transcripts of the pyruvate kinase L gene is an important regulatory step in fructose induction, especially in the kidney. Thus we conclude that all the cis-acting elements responsible for tissue-specific expression of the L-type pyruvate kinase and its stimulation by dietary components and insulin are localized in the sequence from about nucleotide -3000 to +37 in the pyruvate kinase L gene.

The rat pyruvate kinase L (PKL) gene encodes both the R- and L-type isozymes of pyruvate kinase (EC 2.7.1.40). It is about 9.3 kilobase pairs long and consists of 12 exons and 11 introns (1). The first (exon R) and second (exon L) exons encode the 5'-terminal sequences specific to the R- and L-

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The abbreviations used are: PKL, pyruvate kinase L; CAT, chloramphenicol acetyltransferase; bp, base pairs; kb, kilobases.

0006-291X/90 $1.50

types, respectively, while the other remaining exons are common to the two types. By use of two alternative promoters, which are located upstream of the type-specific exons, the PKL gene is expressed in a tissue-specific manner; the R-type isozyme is expressed in erythroid cells and the L-type isozyme is expressed in the liver, kidney and small intestine (2-4).

Administration of diet containing glucose to normal rats or of insulin to diabetic rats increases the level of the L-type isozyme mRNA in the liver, mainly due to stimulation of gene transcription (5,6). Dietary fructose also induces increase in the mRNA in the liver, kidney and intestine, but the time courses of increase are much faster than those of the increases induced by dietary glucose and insulin (5,7,8). The fructose-induced increase of the L-type isozyme is dependent on the tissue, and in the liver, also on the plasma insulin level. Dietary fructose acts mainly at the transcriptional level in normal rat liver, but at the post-transcriptional level in diabetic liver and normal and diabetic kidney (5,6,8).

Recently the interactions between *cis*-acting DNA sequences and *trans*-acting proteins have been found to be important in tissue-specific and hormonal regulations of gene transcription (9,10). In the present study, we produced transgenic mice carrying a PKL/chloramphenicol acetyltransferase (CAT) fusion gene and found that the 5'-flanking region of the PKL gene contains all the elements necessary for tissue-specific expression of the L-type isozyme and stimulation of its gene transcription by components of the diet and insulin.

MATERIALS AND METHODS

Materials: Restriction endonucleases and other enzymes were purchased from Toyobo, Takara Shuzo, New England Biolabs and Bethesda Research Laboratories. Sequenase, a sequencing kit, was from United States Biochemicals. Nylon filters (Hybond N) were obtained from Amersham Corp. [α-^{32}P]dATP (3000 Ci/mmol) and [^{14}C]chloramphenicol (60 mCi/mmol) were from Du Pont-New England Nuclear.

Construction of a Plasmid: A plasmid containing an EcoRI/MspI fragment (from about nucleotide -3200 to +37 relative to the cap site of the L-type) of the PKL gene (1) inserted into the EcoRI/HindIII site of pUC18 was constructed by a series of ligations of subfragments, creating a HindIII site at position +37 with HindIII linker. This fragment was isolated after blunt ending the EcoRI site and was inserted into the SmaI/HindIII site of the pUC0cat, a promoter-less CAT vector (11), to produce pLcat3200. The 5'- and 3'-end points of this construct were confirmed by DNA sequencing of denatured plasmid DNA by the chain termination method (12,13).

Production of Transgenic Mice: An EcoT22I/ApaLI fragment containing the PKL/CAT fusion gene and a part of the pUC18 vector (about 220 bp) at the 3'-end was isolated from pLcat3200. The 5'-end of this fragment corresponded to about position -3000 relative to the cap site of the L-type. This fragment was microinjected into fertilized eggs of C57BL/6 mice as described (14). Transgenic mice were identified by Southern blot analysis of extracted tail DNA (15), using a ^{32}P-labeled CAT probe. The founder mice were then mated with C57BL/6 and their offsprings were mated with each other. For line 49, homozygous transgenic mice were produced by mating heterozygous litter-mates. The copy number was estimated by Southern blot analysis (16).
Treatments of Animals: About 7-week-old transgenic mice were used for experiments. Normal transgenic mice were starved for 32 h and then given synthetic diet containing 10 % casein, and 81 % glucose or fructose (5). Mice were killed 16 hours after the start of feeding and control mice were killed at zero time. Transgenic mice were made diabetic by intraperitoneal injection of streptozotocin (7). Diabetic animals were given the above synthetic glucose diet for one day before treatment with insulin. Insulin (one unit/mouse) was injected subcutaneously and mice were killed 16 h later.
CAT Assays: Various tissues from glucose-fed transgenic mice were homogenized in 10 mM Tris-HCl, pH 7.8, heated at 60°C for 10 min (17), and centrifuged at 10,000 x g for 5 min. The CAT activities of one μg samples of extracted proteins in the supernatants were assayed as described by Gorman et al. (18).
Isolation of Total RNA from Tissues and Northern Blot Analysis: Total RNA was isolated from tissues by the acid guanidine-phenol-chloroform method (19). Total RNA (20 μg) was separated by electrophoresis in 0.8% denaturating agarose gel, transferred to a nylon filter and fixed by UV irradiation (16). The filter was hybridized with ^{32}P-labeled probe as described previously (5,8), except that hybridization and washing were performed at 42°C and 65°C for CAT mRNA, and at 50°C and 65°C for the L-type mRNA. The filter was then exposed to Kodak XAR-5 film at -80°C.
Preparation and Labeling of DNA Probes: The probes used were a 1900 bp PstI/DraI fragment of pLPK57 for the L-type isozyme (8) and a 666 bp HindIII/ScaI fragment of pUC0cat for CAT. The probe for β-actin was a 443 bp human genomic fragment, as described before (8). Hybridization probes were labeled with [α-^{32}P]dATP with random oligonucleotide primers (20).

RESULTS AND DISCUSSION

Production of Transgenic Mice: We constructed a transgene that contains the 5'-flanking region of the PKL gene from about nucleotide -3000 to +37 (relative to the cap site of the L-type isozyme) linked to the CAT gene as shown in Fig.1 and introduced into fertilized mouse eggs. In this way, we obtained 10 lines of transgenic mice. Of these founder mice, we selected line 49, which contained about 10 copies of the entire transgene per haploid genome in tandem array and obtained homozygous transgenic mice of this line by mating heterozygous litter-mates.

Tissue Distribution of the Transgene Product: We examined the tissue-specific expression of the CAT gene in transgenic mice. As shown in Fig.1, CAT activity was detected in the liver, kidney, and small intestine, but not in other tissues. The tissues expressing CAT activity coincided with those expressing

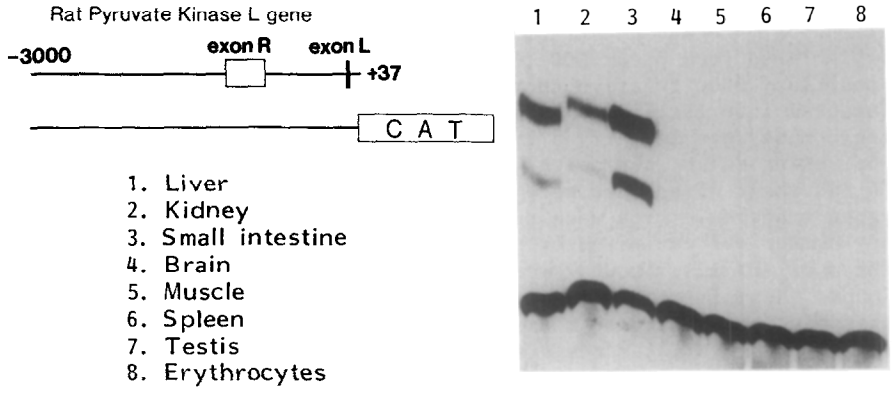
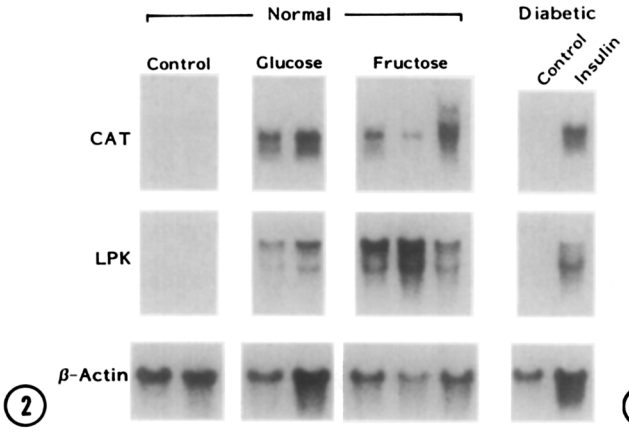


Fig.1. **CAT activities in various tissues of transgenic mice.** The structure of the transgene is shown schematically on the left. Protein extracts were prepared from the indicated tissues of transgenic mice and their CAT activities were determined. The upper two bands in the autoradiogram show acetylated forms of chloramphenicol. Lane numbers correspond to tissue numbers examined.

the endogenous L-type isozyme. However, no CAT activity was detected in erythrocytes in spite of the presence of the cap site of the R-type isozyme in the transgene. This can be explained by two possibilities. The transgene may not contain all the *cis*-acting elements necessary for expression in erythrocytes. Alternatively, active CAT enzyme may not be produced from the transcript from the R-type promoter of the transgene since its transcript contain multiple translation initiation codons and introns. Use of the first initiation codon present in exon R results in no production of the CAT enzyme due to the translation termination codons present in the intron sequence between exons R and L. Alternative splicing of the transcript using the 5'-end of intron between exons R and L, and the 3'-end of small t intron eliminates the CAT coding sequence. Northern blot analysis of reticulocytes mRNA will be required to examine these possibilities. In any case, these results indicated that the region within 3000 bp upstream of the PKL gene contains all the *cis*-acting elements to direct tissue-specific expression of the L-type isozyme.

Regulation of Transgene Expression by Components of the Diet and Insulin: Next, we examined the expression of the transgene in the liver and kidney under various nutritional and hormonal conditions in comparison with that of the endogenous PKL gene. On Northern blot analysis, two bands of CAT mRNA of about 1.5 and 1.9 kb were detected although the transcript is expected to be



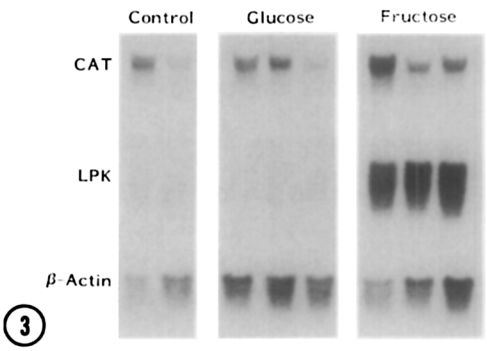


Fig. 2. **Northern blot analysis of liver RNA of transgenic mice after administration of glucose or fructose diet or insulin.** Samples of 20 μg of total RNA from the liver of each mouse were separated by electrophoresis on agarose gel containing 2.2 M formaldehyde, transferred to a nylon filter, and hybridized with labeled probes as indicated. LPK, L-type pyruvate kinase.

Fig. 3. **Northern blot analysis of kidney RNA of transgenic mice given glucose or fructose diet.** Samples of 20 μg of total RNA from the kidney of each mouse were analyzed as described for *Figure* 2. LPK, L-type pyruvate kinase.

about 1.55 kb long excluding poly(A) tail (Figs.2 and 3). The smaller species was major, but the mechanism of production of multiple mRNA species remains to be determined. Multiple bands of the L-type isozyme mRNA were also detected in mice, as observed in rats (7), and their sizes were similar to those of the rat mRNAs. In rats, multiple species of the L-type isozyme have been shown to be generated by use of alternative polyadenylation sites (22), and this may also be the case in mice. In any case, the levels of the CAT and L-type isozyme mRNAs were barely detectable in the liver of transgenic mice that had been starved for 32 h or made diabetic (Fig.2). Administration of diet containing glucose or fructose caused marked increases in the levels of these mRNAs. Insulin administration also induced the CAT and L-type mRNAs. Analyses of the levels of CAT and L-type isozyme mRNAs by densitometry of autoradiograms, however, indicated significant differences between the magnitudes of the increases in the two mRNAs by the inducers: dietary glucose or insulin caused comparable increases in the levels of CAT and L-type isozyme mRNAs, whereas fructose induction of CAT mRNA was about 3-fold less than that of the L-type mRNA.

On the other hand, dietary glucose did not affect the expression of the transgene or of the endogenous L-type isozyme in the kidney (Fig.3). In contrast, dietary fructose resulted in about 18-fold (18.1 ± 3.3, mean ± SEM) increase in the L-type isozyme mRNA level, but only 2.9-fold (2.9 ± 1.3, mean ± SEM) increase in the CAT mRNA level. None of these treatments appreciably affected the levels of β-actin mRNA in the liver and kidney, as described before (8).

These results indicate that the sequence of about 3 kb upstream of the PKL gene contained all the *cis*-acting elements necessary for transcriptional stimulation of the L-type isozyme by dietary carbohydrates and insulin as well as for tissue-specific expression of this isozyme. The transgene included a fragment of about 220 bp of pUC vector in addition to the PKL and CAT gene sequences, but this vector sequence is unlikely to be responsible for regulation of the transgene. Very recently, Tremp et al. produced two series of transgenic mice carrying either the entire rat PKL gene (13 kb) or a minigene (8.36 kb) devoid of exons three to ten (two to nine in their numbering of exons), with 3.2 kb of the 5'-flanking region and 1.4 kb of the 3'-flanking sequence (21). They showed that transgene expression from the cap site of the L-type is tissue-specific and is stimulated by dietary sucrose. In the present work, we extended their observations by showing that the upstream region from about -3000 to +37 relative to the cap site of the L-type isozyme of the rat PKL gene contains all the sequences that direct the tissue-specific expression, as well as the responses to dietary components and insulin, of a linked structural gene. In addition, we examined the effects of glucose and fructose separately since these carbohydrates induce L-type isozyme mRNA by different mechanisms (5-8). Previous studies indicated that induction of L-type isozyme mRNA in normal liver by fructose could be fully explained by stimulation of gene transcription (6,8). But, this was not the case in transgenic mice: induction of transcription of the transgene by fructose was significantly lower than that of the endogenous L-type isozyme mRNA, suggesting that stabilization of the transcripts of the PKL gene, which is

replaced by the CAT sequence in the transgene, plays a significant role in the fructose induction even in normal liver. This post-transcriptional regulation was suggested to be a major factor in fructose induction in the kidney and diabetic liver (5,8). The present results clearly confirmed this. Thus, the transcript of the PKL gene contains the sequence(s) responsible for stabilization. Further studies are required to identify the cis-acting elements necessary for stabilization of the transcript as well as for transcriptional regulation of the PKL gene.

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