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Regulation of gene expression by the CYP27B1 promoter—study of a transgenic mouse model☆

Ivanka Hendrix[a,b,*], Paul Anderson[a,b], Brian May[b], Howard Morris[a]

[a] *Hanson Institute, Adelaide 5000, Australia*
[b] *School of Molecular and Medical Bioscience, University of Adelaide, Adelaide 5005, Australia*

Abstract

The enzyme 25-hydroxyvitamin D 1-hydroxylase (CYP27B1) is the rate limiting enzyme in the two-step activation process of Vitamin D to its active form 1,25-dihydroxyvitamin D (1,25D) and is located in the mitochondrial fraction of the proximal tubular cells of the kidney. More recently CYP27B1 activity and expression have also been identified in a number of non-renal cells, which is suggestive of new, previously unidentified roles for Vitamin D in the human body. Although the regulation of CYP27B1 activity and expression has been a major focus of interest over the past decades, the exact molecular mechanism behind the regulation of CYP27B1 activity and expression and the role of the CYP27B1 promoter, herein, are still poorly understood. In this study, we created a transgenic mouse model that expresses the luciferase reporter gene under the control of the full-length, 1.5 kb, human CYP27B1 promoter. This animal model allows us to study in vivo the tissue-specific, CYP27B1 promoter-controlled, regulation of the expression of the CYP27B1 gene.

Keywords: Vitamin D; 25-Hydroxyvitamin D 1-hydroxylase; Transgene

1. Introduction

The enzyme 25-hydroxyvitamin D 1-hydroxylase or CYP27B1, is the key enzyme in the two-step activation process of Vitamin D to its biologically active form 1,25-dihydroxyvitamin D (1,25D) [1]. It is located in the proximal tubular cells of the kidney. The activity of the CYP27B1 enzyme is apparently tightly regulated by a number of physiological and dietary factors such as parathyroid hormone (PTH), calcium, phosphate, 1,25D, and factors involved in growth and differentiation [2–4].

Although the kidney was originally believed to be the sole producer of the active form of 1,25D, more recently, CYP27B1 enzyme activity and mRNA expression have been identified in a number of extra-renal tissues. These include the intestine, brain, and bone [5–7]. These findings raise the possibility that, besides its classical roles in mineral homeostasis and bone metabolism, 1,25D has other, so far unidentified roles in the human body. Evidence from dietary and knock-out animal studies has lead to the suggestion of a potential role for 1,25D in a wide range of physiologocal and patho-physiological processes, including autoimmune diseases, psoriasis, and cancer.

Although the metabolism of 1,25D has been the focus of intensive research for a number of decades, the molecular mechanisms underlying the regulation of CYP27B1 expression and activity in the kidney and in extra-renal cells are still poorly understood. In vitro studies, using promoter constructs of the CYP27B1 promoter, have suggested regulatory regions in this promoter for factors known to affect 1,25D synthesis such as PTH and 1,25D; the precise sequences through which these factors regulate gene transcription are, however, unknown.

To fully understand the regulation of CYP27B1 gene expression in vivo, we established a transgenic mouse model that expresses the luciferase reporter gene under the control of the full-length, 1.5 kb, human CYP27B1 promoter. This animal model allows us to identify factors that modulate CYP27B1 expression through response elements located within the 1.5 kb CYP27B1 promoter.

2. Materials and methods

2.1. DNA construct

The pGL3-pCYP27B1-luciferase (pGL3-pCYP27B1-luc) construct contained the luciferase reporter gene flanked by

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* Corresponding author. Tel.: +61-8-8222-3514; fax: +61-8-8222-3518.
E-mail address: ivanka.hendrix@adelaide.edu.au (I. Hendrix).

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the 1541 bp (−1497 to +44 relative to the transcriptional start site) promoter region of the human CYP27B1 gene. The complete 1541 *Kpn*I–*Xho*I fragment was inserted upstream of the luciferase reporter gene in the pGL3-Basic vector (Promega Corp., NSW, Australia). The 3513 bp pGL3-pCYP27B1-luc construct was excised by *Kpn*I and *Bam*HI restriction enzyme digestion.

2.2. Generation of transgenic mice and genomic DNA analysis

Transgenic mice were generated by pro-nuclear injection of the purified pGL3-pCYP27B1-luc construct into CBA/C57 embryos, which were re-implanted into pseudo-pregnant mothers. Transgenic founders were identified from genomic DNA, extracted from tail samples. Briefly, tail samples were taken at 10 days of age, digested in a buffer containing 50 mm Tris, pH 8, 10 mM EDTA, 100 mm NaCl, 1% SDS, and 140 μg/ml Proteinase K and incubated overnight at 55 °C. DNA was extracted by phenol/chloroform extraction, precipitated in ethanol and reconstituted in 1× TE (10 mm Tris and 1 mM EDTA, pH 7.5) buffer. Transgene copy number was calculated by Southern blot analysis of genomic DNA, using a luciferase-specific DNA probe (1.6 kb *Xba*I/*Nco*I fragment obtained from the pGL3-Basic vector). Four transgenic founders (2990, 2992, 3039, and 3042) were obtained following pro-nuclear injection of the pGL3-pCYP27B1-luc construct. Transgene copy numbers integrated in the genome of these transgenic animals were 1, 3, 20, and 12, respectively. Transgenic founders were mated with wild-type CBA/C57 mice to obtain heterozygous offspring for analysis. The male founder 2990 was a runt and was unable to reproduce. Genotyping of offspring was performed by PCR amplification of a 246 bp luciferase fragment with the primers 5′ ttctatccgctggaagatggaaccgctg 3′ and 5′ acgcgcccaacaccggcataaagaattg 3′. PCR amplification was carried out in a buffer containing 10× PCR buffer (Perkin-Elmer Applied Biosystems, CA, USA), 5 mm deoxynucleotide triphosphates, 25 mm $MgCl_2$, 0.2 μM of each primer, and 2 U/μl Taq-polymerase (Perkin-Elmer Applied Biosystems) for 100 ng genomic DNA. After 36 cycles (30 s at 94 °C, 1 min at 62 °C, and 2 min at 70 °C), the PCR products were analyzed on a 2% agarose gel and stained for 10 min in ethidium bromide.

2.3. Tissue-specific transgene expression

All animals used were heterozygous CBA/C57 transgenic mice, bred at the Institute of Medical and Veterinary Science for experiments approved by the IMVS Animal Ethics Committee. Throughout the experiment, animals were maintained on a commercial chow diet (Milling Industries Pty Ltd., SA) and supplied with water at libitum. The animals were housed in a 12-h light:12-h dark cycle. The tissue-specific CYP27B1 promoter directed luciferase expression was identified using three transgenic mice from each independent line. Adult mice were sacrificed and the following tissues were removed at the time of death: heart, lung, liver, spleen, proximal small intestine (first 5 cm's of the small intestine), distal small intestine (last 5 cm's of the small intestine), kidneys, ovaries, skeletal muscle, bone, bone marrow, and skin.

2.4. Enzymatic luciferase activity and protein assays

Reporter gene expression was analyzed by measuring enzymatic luciferase activity in tissue samples obtained from transgenic mice. Fresh tissue samples were homogenized in 5× Reporter Lysis Buffer (Promega Corp.), subjected to one freeze-thaw cycle and centrifuged. The supernatant was removed and kept on ice. Twenty microliter of supernatant were used to measure the enzymatic luciferase activity using 50 μl of commercially available Luciferase Assay Reagent (Promega Corp.). The luminescence signal was measured over 10 s using a TD 20/20 luminometer (Turner Design Instrument, Sunnyvale, CA, USA).

The protein content of tissue samples was measured using a commercially available protein assay kit (Bio-Rad, NSW, Australia). Luciferase activity is expressed as light units per microgram of proteins.

3. Results

3.1. Transgene expression

To investigate the tissue-specific transcriptional activity of the 1.5 kb CYP27B1 promoter in vivo, three independent transgenic mouse lines (2992, 3039, and 3042) were generated expressing the luciferase reporter gene under the control of the 1.5 kb CYP27B1 promoter. In all three lines, a very similar pattern of transgene expression was detected, suggesting that the 1.5 kb CYP27B1 promoter controls gene expression in vivo in a tissue-specific manner. The absolute levels of luciferase activity varied greatly among the three transgenic lines. The reporter gene expression found in tissue samples from offspring from line 2992, the highest expressing line, was up to 25- and 200-fold higher than reporter gene expression detected in tissue extracts from animals from lines 3039 to 3042, respectively. In line 2992, high levels of luciferase activity were found in the kidney, testis, brain, and bone extracts (Fig. 1). Lower levels of luciferase activity were detected in heart, lung, liver, distal small intestine, skeletal muscle, spleen, skin, and ovary extracts. No transgene expression was detected in the proximal small intestine or in tissue extracts from non-transgenic animals. Since offspring from line 2992 have only three copies of the transgene integrated in their genome, compared with 12 and 20 in 3042 and 3039, respectively, the pGL3-pCYP27B1-luc construct confers no copy number dependent transgene expression.

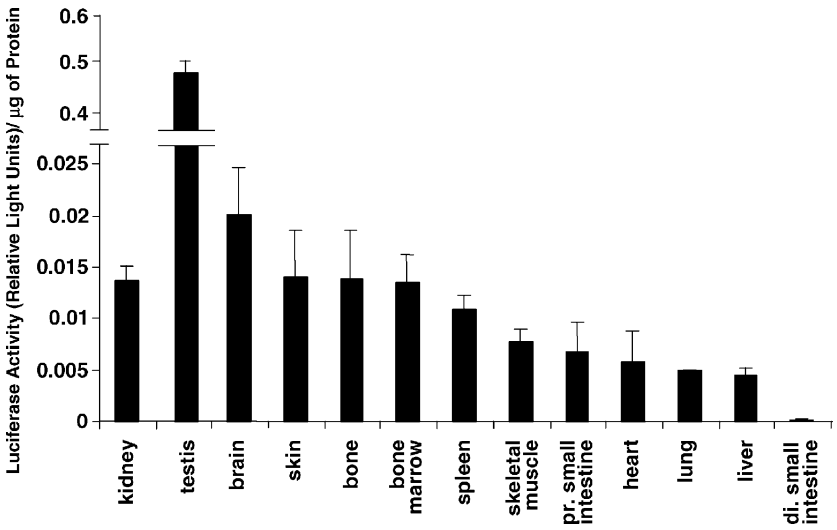

Fig. 1. Relative luciferase activity in different tissue extracts from transgenic offspring of line 2992.

4. Discussion

The 1.5 kb human CYP27B1 promoter directed reporter gene expression in a tissue-specific manner in three independent transgenic mice lines. Although the tissue-specific expression pattern of the transgene did not differ significantly from one line to the other, the absolute levels of luciferase activity varied greatly among the three lines. The variation in transgene expression between independent lines, is a common observation in studies utilizing transgenic animals [8,9]. The introduction of exogenous DNA to the mouse germline by pro-nuclear injection usually results in the integration of tandem transgene arrays at a random site within the genome. Both the site of integration and the tandem repeat nature of the transgene insertion have been associated with transgene silencing and subsequently with variable transgene expression [8,9]. Although an association has been suggested between the extent of transgene silencing and the number of transgene copies in the array, the pGL3-pCYP27B1-luc construct used in these experiments did not confer copy number dependence [10].

The promoter directed reporter gene expression was highest in testis, brain, bone, and kidney extracts from mice of all three lines. Lower levels of expression were detected in skin, heart, spleen, liver, lung, ovary, skeletal muscle, and distal small intestine extracts. No luciferase activity was detected in proximal small intestine extracts from mice of any of these three lines or in tissues from non-transgenic littermates. The pattern of transgene expression closely followed the tissue-specific distribution pattern of the endogenous CYP27B1 gene, which has been identified in a number of tissues and cells including the fetal and adult kidney, brain, testis, keratinocytes, activated pulmonary macrophages, intestine, fetal and adult bone, spleen and liver [5–7,11]. The absence of luciferase activity in the proximal small intestine reported in this study is consistent with work by Theodorpoulos and co-workers who found a significantly higher expression of CYP27B1 mRNA in the distal part of the fetal small intestine when compared to expression levels detected in the proximal part of the fetal small intestine [12]. The lack of local CYP27B1 activity and, therefore, 1,25D synthesis in this organ, suggests that the proximal small intestine is purely an endocrine target organ of circulating 1,25D.

In summary, we have created a transgenic mouse model that expresses the luciferase reporter gene under the control of the full-length, 1.5 kb, human CYP27B1 promoter. The CYP27B1 promoter directed luciferase expression in a tissue-specific manner; luciferase activity was present in a number of tissues, including the kidney, brain, and testis. No luciferase activity was, however, detectable in the proximal small intestine. The pattern of promoter directed reporter gene expression was found to be consistent with the distribution pattern of the endogenous CYP27B1 gene. This transgenic mouse model will be helpful in the identification of the factors that regulate CYP27B1 expression and activity throughout the body and the role of the CYP27B1 promoter, herein, at the molecular level.

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