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Expression of Carbonic Anhydrase II (CA II) Promoter–Reporter Fusion Genes in Multiple Tissues of Transgenic Mice Does Not Replicate Normal Patterns of Expression Indicating Complexity of CA II Regulation *in Vivo*

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Although the proximal, 5' 115 bp of the human carbonic anhydrase II (CA II) gene was sufficient for expression of a reporter gene in some transfected cell lines, we found previously that 1100 bp of this promoter (or 500 bp of the mouse CA II promoter) was not sufficient for expression in transgenic mice. We have now studied the expression of linked reporter genes in mice transgenic for either (1) 11 kb of the human 5' promoter or (2) 8 kb of the human 5' promoter with mouse sequences from the first exon, part of the first intron (since a CpG island spans this region), and the 3' sequences of the gene. Expression was found in both cases, but the tissue specificity was not appropriate for CA II. Although there was a difference in the sensitivity of the assays used, the first construct led to expression in many tissues, while the second construct was expressed only in spleen. These findings indicate considerable complexity of DNA control regions for in vivo *CA II expression.*

KEY WORDS: transgenic mice; carbonic anhydrase; promoter analysis; transcription; DNA control regions.

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INTRODUCTION

Carbonic anhydrase (CA) catalyzes the reversible hydration of carbon dioxide. Nine CA or CA-like isozymes have so far been described in the multigene family (Tashian, 1989, 1992; Pastorek *et al.,* 1994), of which CA II provides the most enzymatic activity. The human and mouse CA II genes have been cloned and sequenced (Venta *et al.,* 1985a,b, 1991). In mice, the CA I, CA II, and CA III genes are tightly linked in the centromeric region of chromosome 3 (Eicher *et al.,* 1976; Beechey *et al.,* 1989), and in man these three genes are closely linked on the long arm of chromosome 8 (Davis *et al.,* 1987; Nakai *et al.,* 1987; Lowe *et al.,* 1991). An interesting feature of the CA II gene in mammals is that it is expressed in almost all tissues, but only in a limited subset of cells (Tashian *et al.,* 1984). Thus a variety of controlling elements may be expected to participate in the mechanism of regulation of CA II expression. In addition, CA II is inducible by 1,25-dihydroxy vitamin D_3 in HL60 cells (Shapiro *et al.,* 1989) as they differentiate into osteoclasts. Thus, increased understanding of this promoter is relevant to osteoclast function and the osteoporosis of aging. CA II is also androgen inducible—an induction studied in the rat lateral prostate, where CA II makes up 15% of the soluble protein (Harkonen and Vaananen, 1988).

The nucleotide sequences surrounding the first and second exons of the human and mouse CA II genes have been compared (Venta *et al.,* 1985a), with an overall percentage identity of 81% and an even higher identity of 88% in the first exon. The region surrounding the TATA box is moderately conserved (60%, −243 to TATA; 69%, TATA to ATG). The regions 700–800 bp on either side of the first exon are extremely G and C rich. Such islands have been shown to be associated with the 5′ regions of many mammalian genes. About 90% of methylated cytosines in mammalian DNA are found in the CpG sequence. This methylation is implicated in the control of gene expression in higher eukaryocytes.

An important step in understanding gene function is the elucidation of a gene's control elements. Transfection of cultured cells is an important first step but the results are frequently misleading, e.g., collagen promoters (Krebsbach *et al.,* 1993) and HSP70.1 (Thompson *et al.,* 1994). This is certainly true of CA II. Calcium phosphate-mediated transfection of long-established cell lines showed that as few as 115 bp (mouse Ltk^- fibroblasts) or 200 bp (human HeLa cells) of 5′ promoter region led to abundant expression of the reporter gene (Shapiro *et al.,* 1987). A finer analysis of this promoter in NIH-3T3 and HepG2 cells, also using $CaPO_4$ transfection, confirmed the effectiveness of short segments (down to 90 bp) in promoting transcription (Marino, 1993). A more recent study of the CA II promoter in renal cells and using lipofection showed that 1.1 kb of proximal 5′ sequence

was optimal (Lai *et al.,* 1995). As a control, lipofection was used as the mode of transfection for HeLa cells, and 1.1 kb was optimal in HeLa cells with this mode of delivery (Lai *et al.,* 1995). Importantly, the latter results employed a control for transfection efficiency which was not employed in the other two studies.

It has frequently been found that the control elements needed for proper *in vivo* regulation are different than those needed *in vitro.* Thus, it is not surprising that even 1.1 kb of proximal 5′ sequences did not result in any expression in transgenic mice (Erickson *et al.,* 1990). We have now studied mice transgenic for constructs containing much larger amounts of 5′ sequence with or without the gene sequences needed to include all of the CA II, 5′ CpG island (Venta *et al.,* 1985a) and 3′, potential regulatory sequences in order to determine if these sequences would lead to the normal pattern of expression. Abundant expression, but not in an appropriate pattern, was found.

MATERIALS AND METHODS

Constructs

pHCAIICAT-12 kb. This construct (Fig. 1) contains nearly 12 kb from the flanking region of the human CA II (Venta *et al.,* 1991) gene fused to the chloramphenicol acetyltransferase gene derived from pSV2CAT (Gorman *et al.,* 1982). The parent plasmid to pHCAIICAT-12kb was the pHCAIICAT-

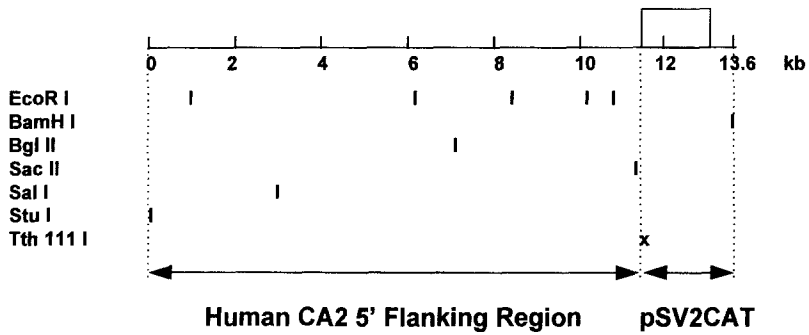

Fig. 1. Schematic diagram of pHCAIICAT-12 kb. The box shows the location of the chloramphenicol acetyltransferase (CAT) gene. The arrowheads show the regions derived from the 5′ region of the human carbonic anhydrase II gene and the pSV2CAT plasmid. The "x" indicates the region of the *Tth111I* site that was destroyed during the cloning procedure. This *Tth111I* site directly overlies the CA II translation start codon in the *CA-2* gene. The gene construct could be conveniently removed from the vector (not shown) by digesting the DNA with *Stu*I and *Bam*HI. See Methods and Materials for a more detailed description of the generation of this construct.

1400 plasmid previously described (Shapiro *et al.,* 1987). Briefly, pHCAII-CAT-1400 was derived by end-filling the *Hin*dIII site of pSV2CAT and the *Tth111*I site of H25-3.8 (which contains the first exon and promoter region of the human CA-2 gene) followed by blunt-end ligation (Shapiro *et al.,* 1987). The *Tth111*I site directly overlies the initiation codon for CA II protein (Venta *et al.,* 1985a) and the *Hin*dIII site is 35 bp upstream of the start of translation of the CAT protein (Gorman *et al.,* 1982; Genbank Accession No. G77788). pHCAIICAT-1400 contains 1288 nucleotides (originally estimated as 1400 bp by gel electrophoresis) upstream of the start of transcription of the human CA-2 gene (Venta *et al.,* 1991). Fusion of an additional 10 kb of 5′ flanking region to the parent plasmid was accomplished as follows: pHCAIICAT-1400 was digested with both *Stu*I and *Sac*II. This removed the enhancer–promoter region of SV40 originally contained in the parent plasmid as well as about 1 kb of the *CA-2* promoter region (which was subsequently returned in the next step). In a separate reaction, 11 kb of 5′ flanking region of the human *CA-2* gene was removed from the lambda bacteriophage clone H25 (Venta *et al.,* 1991) by digestion with *Stu*I and *Sac*II. These two fragments were ligated to produce pHCAIICAT-12kb. The correct order and size of the restriction fragments contained within the clone (Fig. 1) were confirmed by appropriate restriction enzyme digestions.

pMHCAII-8 kb. This construct (Fig. 2) contains about 8 kb of 5′ flanking region from the human *CA-2* gene fused to 378 bp of the mouse *Car-2* promoter [counting to the putative transcription start site (Venta *et*

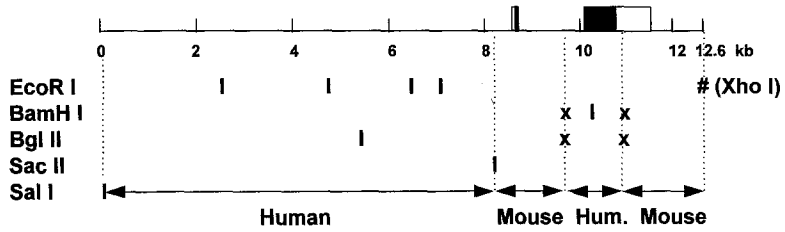

Fig. 2. Schematic diagram of pMHCAII-8 kb. The boxes show the location of the two exons of the construct. The white areas of the boxes indicate the untranslated regions, whereas the black areas indicate the protein-coding regions. The first exon is derived from the mouse carbonic anhydrase II gene and the second exon is a minigene construct derived from the human carbonic anhydrase II gene and its cDNA. The arrowheads at the bottom show the locations of human- and mouse-derived sequences. The # sign shows the location of the *Eco*RI site that was destroyed and replaced by an *Xho*I site by ligation of an *Xho*I linker. The four "x's" show the location of the missing *Bam*HI and *Bgl*II sites that were ligated by their complementary ends to fuse the human and mouse sequences at these points [see Curtis *et al.* (1983) and Venta *et al.* (1985a,b) for exact sequences]. The gene construct could be conveniently removed from the plasmid vector (not shown in this figure) by digesting the DNA with *Sal*I and *Xho*I. See Methods and Materials for a more detailed description of the generation of this construct.

al., 1985a)]. It uses human CA II as the reporter gene to eliminate all prokaryote sequences and uses mouse sequences where possible (although mouse human sequence differences have generally been irrelevant; see Discussion). The fusion between these two regions is at a *Sac*II site which occurs in a nearly identical position in both the human and the mouse genes [(Venta *et al.,* 1985a) from the transcription start site, −356 for the human and −378 for the mouse]. The two parent plasmids involved in the construction of pMHCAII-8kb are pMHCAII (described next) and pHCAIICAT-12 kb (described above). Briefly, pMHCAII was constructed from pBS$^-$ and four DNA fragments (in their order in the clone): the 1.9-kb *Bam*HI–*Bgl*II piece from A6-2.7 (Venta *et al.,* 1985b), the 0.6-kb *Bam*HI–*Bam*HI piece from H25-3.8 (Venta *et al.,* 1991), the 0.7-kb *Bam*HI–*Bgl*II piece from pHCAII 38.3 (Montgomery *et al.,* 1987), and the 1.7-kb *Bam*HI–*Eco*RI piece from A6-3.5 (Venta *et al.,* 1985b). These fragments were ligated in the appropriate order to produce pMHCAII. The *Eco*RI site in pMHCAII was cut and end-filled using standard procedures (Maniatis *et al.,* 1982) and this was followed by the addition of *Xho*I linkers. Cohesive ends were generated by digestion of the ligated *Xho*I linkers and the plasmid was recircularized by ligation. This plasmid was cut at the *Sac*II site and *Sal*I site [contained in the polylinker of the pBSM13-vector (Stratagene, San Diego)]. The plasmid was gel purified away from the small "linker" piece. In a separate reaction, the *Sal*I–*Sac*II fragment of pHCAIICAT-12 kb was isolated by a similar procedure. These two DNA pieces were ligated to produce pMHCAII-8kb. Appropriate restriction digestions were performed (Fig. 2) to confirm the identity of the plasmid. The protein produced from this construct should be identical to the human CA II except for a single conservative and non-charge-changing amino acid substitution of serine for glycine at amino acid position 7. Thus, the product of the transgene and the endogenous mouse CA II are electrophoretically distinguishable due to the charge differences between the human and the mouse CA II isozymes.

Transgenics

The fragments described above were excised, purified by Schleicher and Shuell Elutip, and suspended in 10 m*M* Tris, 0.25 m*M* EDTA for injection. Injection was performed by the National Transgenic Development Facility into C57BL/6J × SJL/J F_2 zygotes by standard techniques (F_1 parents are used to avoid the two-cell block to development). Positive transgenics were identified by the polymerase chain reaction (PCR). In the case of pHCAIICAT-12 kb, a reaction buffer (Promega—Madison) containing 50 m*M* KCl, 10 m*M* Tris–HCl, pH 9.0, 1.5 m*M* $MgCl_2$, 0.1% gelatin (w/v), 1% Triton X-100, 100 μmol of each dNTP, and a 0.5 μM concentration of each

primer per 50 μl of reaction mixture was covered with light mineral oil and 2.5 U of *Taq* polymerase (Promega-Madison) was added. Genomic mouse DNA (50–100 ng) was added to the reaction mixture at a temperature above the annealing temperature. The samples were amplified for 30 cycles consisting of denaturation at 95°C for 1.3 min, annealing at 55°C for 1.3 min, and extension at 72°C for 1.3 min, with a final extension at 72°C for 10 min on the 30th cycle. The 5′ primer is located at 335–365 bp (designation of Ohtsubo and Ohtsubo, 1978) in the CAT sequence (5′CCA GAC CGT TCA GCT GGA TAT TAC GGC CTT 3′) and the 3′ primer is located at 542–568 bp in the CAT sequence (5′ AGT GAA TAC CAC GAC GAT TTC CGG CAG 3′). The primers bracket a target sequence 233 bp in length. A 9-μl aliquot was electrophoresed on a 4% Nusieve agarose–Tris–acetate–EDTA gel. To detect the pMHCAII-8 kb transgene, primers were made at sites with significant variation between human and mouse CA II. The primers were 5′ GAGGTTCTCAGACCTGTTAT 3′ and 5′ GTAAAGCTCTTAG-ATCACTG 3′, which generate a 295-bp product. The conditions used were as described above but with 33 1.3-min cycles of 95, 53, and 72°C followed by 9.9 min of extension at 72°C.

Southern Analyses

Ten micrograms of genomic DNA was digested overnight with 40 U of *Eco*RI, electrophoresed on a 0.9% agarose gel, and transferred by capillary action, using 10× SSC, to a nylon membrane after depurination in 0.25 *N* HCl and denaturation in 0.5 *N* NaOH. The filter was hybridized with the ^{32}P-labeled 1800-bp *Nco*I–*Hin*dIII fragment of pSVOCAT.

Cell Separations

To prepare nucleated blood cells for analysis of CAT activity, two transgenic females were injected i.p. with 0.1 ml of a 1/5 dilution of India Ink in PBS to help localize lymph nodes. The animals were warmed and bled (the source of peripheral blood leukocytes; PBLs). They were then sacrificed and the thymi, mesenteric lymph nodes, and spleens dissected. These were teased apart in PBS (separately for tissue, pooled for the two animals). The tissue fragments were allowed to settle, the supernatants counted in a hemocytometer, and the suspensions adjusted to 10^6/ml of PBS, frozen, and assayed for CAT as described above. The heparinized blood was diluted 10-fold in cold, 0.83% NH_4Cl to lyse RBCs and centrifuged at 500*g* for 5 min. The cell pellet was resuspended in NH_4Cl, then centrifuged, and the white pellet suspended at 10^6/ml in PBS, frozen, and assayed for CAT as described above.

Chloramphenicol Acetyl Transferase Assays

CAT assays were performed essentially according to the previously described protocol (Gorman *et al.,* 1982). Tissues were homogenized 1:1 (w/v) with 0.75 *M* Tris, pH 7.8. Insoluble debris was pelleted, the supernatant was heated to 60°C for 10 min to inactivate endogenous acetylases, and coagulated proteins were pelleted. Fifty micrograms of soluble protein was incubated in the standard assay (Gorman *et al.,* 1982) for 2 hr at 37°C, and the amount of radiolabeled acylated chloramphenicol was analyzed by thin-layer chromatography (TLC). The TLC plates were autoradiographed for 12–16 hr. In some cases, a CAT enzyme immunoassay was performed, using the CAT-ELISA[c] kit from Boehringer Mannheim and was used according to their instructions. This assay was found to be less sensitive and was used only to compare white blood cell activities.

Electrophoreses and Histochemistry of CA II

Tissue samples (0.1–0.2 g) were homogenized in 0.5 ml PBS, then centrifuged, and the supernatant (up to 5 μl) was applied to 57 × 140-mm Cellogel strips (Crescent Chemical, Hauppauge, NY). Electrophoresis was carried out at 300 V for 2.5–3 hr in 0.09 *M* Tris–HCl buffer, pH 9.1, containing 2.8 m*M* EDTA and 10 m*M* boric acid. Electrophoretically separated mouse and human CA isozymes were tested for CA activity by staining strips with 0.1 *M* Veronal buffer, pH 9.0, containing 0.15% bromthymol blue. Carbonic anhydrase activity (yellow spots) was visualized when stained strips were flooded with CO_2 (Tashian, 1969).

RESULTS

pHCAIICAT-12 kb Construct

Nine transgenic founders were identified, of which seven were fertile. Southern analyses after digestion with *Eco*RI demonstrated lack of internal rearrangements and variable dosages of the construct in several lines (Fig. 3). Abundant expression was found in many tissues (Table I, Figs. 4 and 5). Although there was some variation in expression of the CAT reporter gene from line to line, e.g., sterile animal 1618 had relatively high basal brain and kidney expression and sterile founder 34 had high expression in stomach, most of the transgenic animals had a very similar pattern of reporter gene expression. Levels of expression were very high in spleen (normally a moderately expressing tissue) and testes (normally a high expressing tissue) but low in many tissues in which CA II is normally highly expressed: blood, kidney, and lung. Since imprinting of transgenes occurs frequently, we

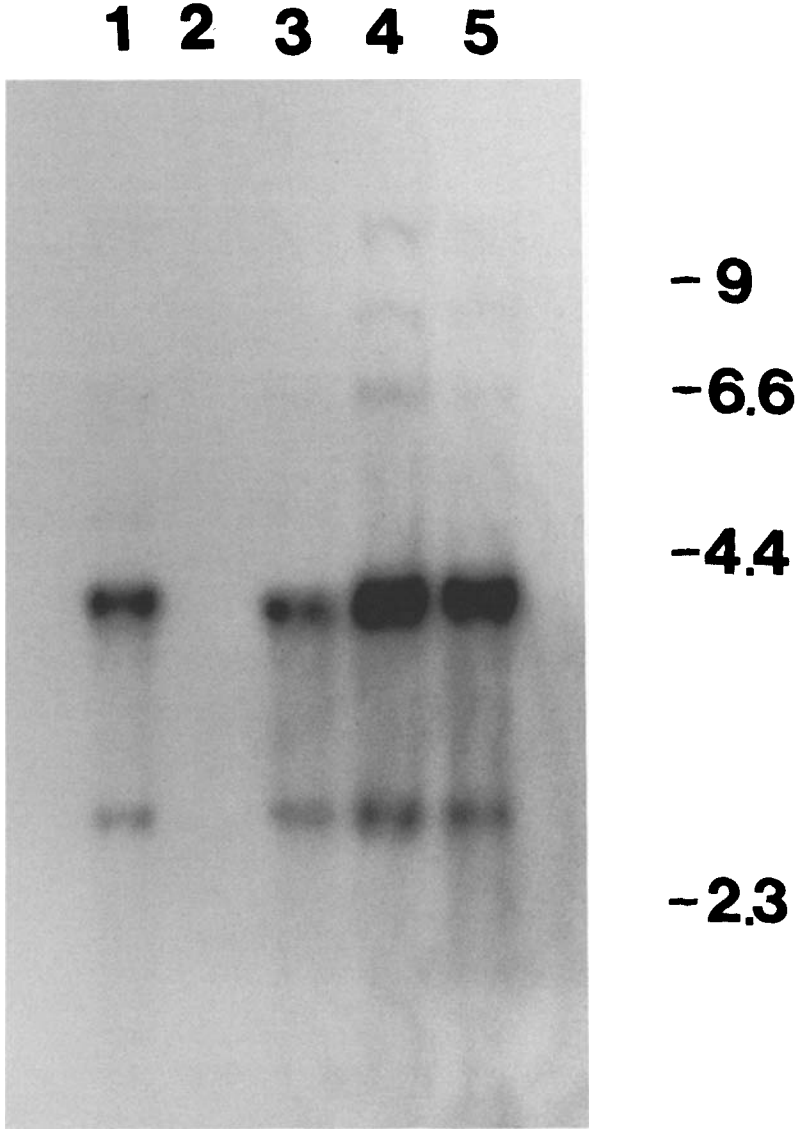

Fig. 3. Southern analysis of genomic DNA from representative positive and negative pHCAIICAT-12 kb transgenics after digestion with *Eco*RI and hybridization to a probe from pSVOCAT. Lane 1, individual mouse from line 33, PCR positive; lane 2, individual mouse from line 33, PCR negative; lane 3, another line 33, PCR-positive mouse; lane 4, individual from line 36, PCR positive; lane 5, another line 36, PCR-positive mouse.

Table I. Chloramphenicol Acetyl Transferase (CAT) Recorder Gene Activity in Various Tissues of Transgenic Mice Expressed as Percentage Conversion by 50 μg/120 min Exposure (Reaction Time)

Transgenic line	Animal No.	Sex	Transmitted by female/male	Basal brain	Cortex	Blood	Heart	Kidney	Liver	Lung	Spleen	Stomach	Coagulating gland	Testes
27	234	F	—[a]	1.57	0.94	1.73	0.72	0.85	1.10	1.12	11.01	0.88	—	—
27	236	M	—	—	—	—	—	—	—	—	—	—	0.52	8.5
28	87	F	Female	2.2	1.42	9.20	1.13	3.31	7.27	2.68	44.80	.87	—	—
29	33	M	Male	—	—	—	—	—	—	—	—	—	0.61	33.56
29	19983	F	Female	0.87	0.60	0.89	0.52	0.83	1.05	0.74	42.45	0.52	—	—
29	19985	F	Female	0.80	2.30	0.84	0.67	1.41	1.06	0.99	42.09	0.75	—	—
29	19986	M	Female	1.81	3.33	1.13	0.60	0.91	1.01	0.98	38.42	0.70	—	—
31	1601	F	Male	0.61	0.70	3.79	0.53	0.61	0.71	0.67	0.76	0.68	—	—
31	1607	M	Male	1.28	0.93	1.91	0.89	0.65	0.95	0.51	0.72	0.34	0.36	10.33
32	1618	M	Male	9.35	17.47	3.57	1.25	6.28	14.0	1.39	36.74	1.96	—	—
33	111	M	Male	0.57	0.69	3.58	0.71	0.62	1.08	0.65	55.61	0.48	—	—
33	1652	M	—	—	—	—	—	—	—	—	—	—	0.38	27.34
33	1999	F	Female	0.69	1.15	1.12	0.48	0.97	1.57	0.60	49.41	0.41	—	—
34	34	M	—	—	0.64	0.53	0.64	0.57	0.67	0.52	21.22	12.23	—	—
35	63	F	Male	0.82	0.69	1.23	0.47	1.34	0.89	0.96	13.50	0.64	—	—
35	1697	M	—	0.87	1.80	0.64	0.45	0.87	0.76	0.55	8.50	0.40	0.38	21.65
36	80	F	Female	2.44	3.23	1.84	0.64	1.24	1.33	1.13	61.73	0.71	—	—

[a]Not determined.

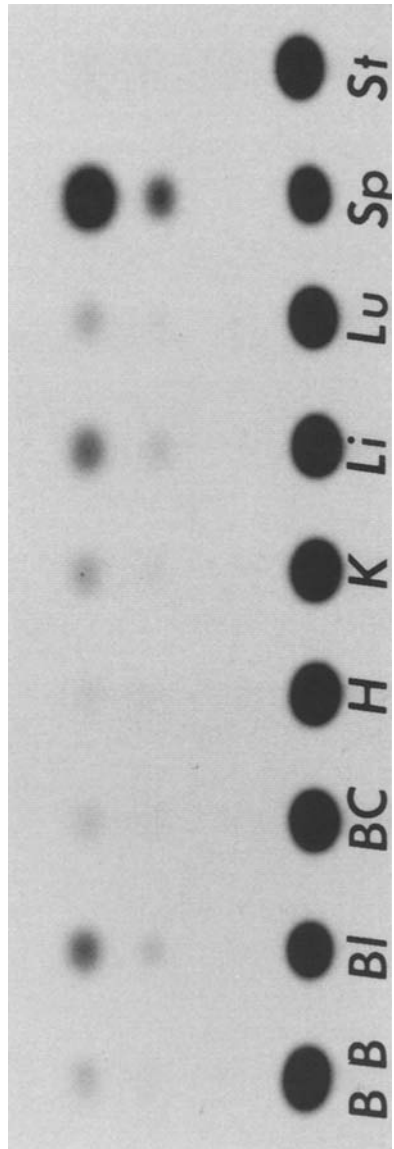

Fig. 4. Autoradiogram of TLC of CAT reporter gene activity in various tissues of a pHCAIICAT-12 kb transgenic mouse. BB, basal brain; Bl, blood; BC, brain cortex, H, heart; K, kidney; Li, liver; Lu, lung; Sp, spleen; St, stomach.

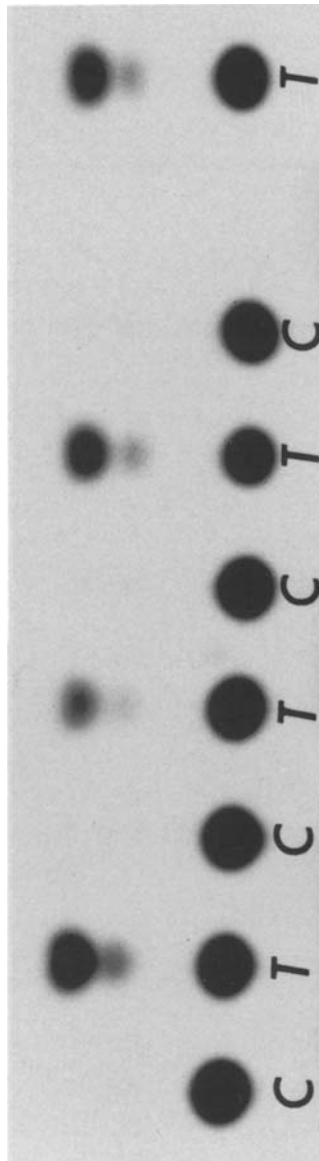

Fig. 5. Autoradiogram of TLC of CAT reporter gene activity in testes (T) and coagulating gland (C) of four mice from four lines transgenic for the pHCAIICAT-12 kb construct.

Table II. ELISA Determination of Chloramphenicol Acetyl Transferase (CAT) Recorder Gene Activity in Purified Cell Fractions

Cells	CAT (pg/10^6 cells)
Splenocytes	844 ± 125[a] (3)
Thymocytes	116 ± 13.5 (3)
Lymphocytes	100
Peripheral blood leukocytes	19.5

[a]Mean ± SE (*N*).

compared expression when the transgene was transmitted by males compared to females. The variation in expression was not correlated with the transmitting parent but was correlated with the different transgenic lines.

Since the spleen is populated largely by B and T lymphocytes, we performed preliminary studies on transgene expression in cell fractions from appropriate tissues using the ELISA assay (Table II). Splenocytes showed much higher levels of CAT gene expression than did thymocytes (primarily T cells), lymphocytes from mesenteric lymph nodes (B and T lymphocytes), or peripheral blood leukocytes (granulocytes, monocytes, and B and T lymphocytes). These results suggest that T or B lymphocytes are not the major contributors to high expression in spleen.

pMHCAII-8 kb Construct

Two founders were identified, of which only one was fertile. The histochemical technique was used, after electrophoresis to separate mouse from human CA II. This less sensitive (than CAT activity) assay detected expression of this reporter gene only in spleen (Fig. 6). This activity was readily detected in spleen extracts of the founder of one line and five animals of the other line, when the gene was transmitted both maternally and paternally. No expression was found in blood (4), heart (4), liver (6), lung (4), kidney (6), muscle (2), stomach (4), large intestine (1), testes (4), brain cortex (4), basal brain (1), and cerebellum (3) (the numbers in parentheses indicate the number of animals studied). This pattern of expression, given the lower sensitivity of the detection system, might be compatible with that seen in pHCAIICAT-12 kb except for the results in testes, which were similar to levels in spleen in most lines of pHCAIICAT-12 kb.

DISCUSSION

These results on regulation of CA II expression in transgenic mice provide a sharp contrast to the results found with transfections of cultured cells.

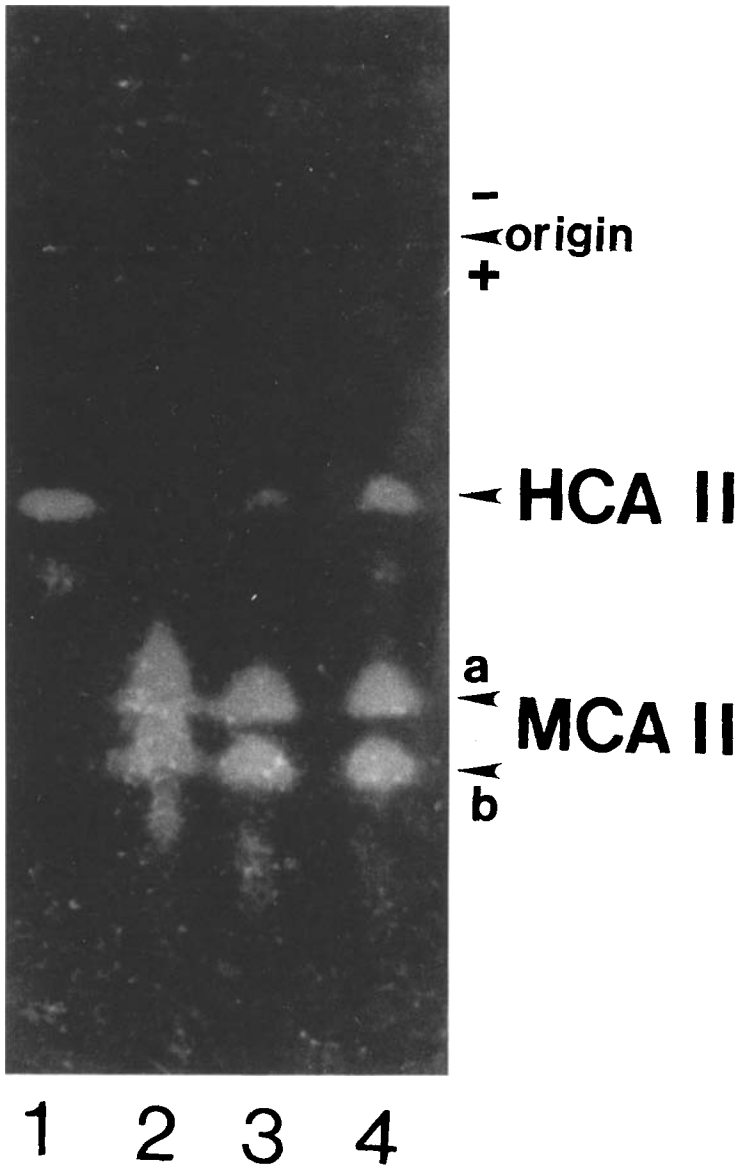

Fig. 6. Electrophoresis of human CA II (HCA II) and mouse CA II (MCA II) isozymes in spleen from mouse with pMHCAII-8 kb transgene. HCA II control (lane 1); normal heterozygous (*Car*-2a,b) mouse control (lane 2); mouse with HCA II transgene (lane 3); mouse with HCA II transgene + HCA II (lane 4).

Calcium phosphate-mediated transfections of relatively undifferentiated, long-term cultured cell lines indicated that only short stretches of 5′ CA II sequences were needed for expression. For instance, when transfections of mouse Ltk$^-$ cells were studied, the addition of each GC box resulted in a stepwise increase in the ability of the human CA II promoter to direct transcription of the reporter gene (Shapiro *et al.,* 1987). The addition started at the TATA box and continued through each putative element until maximal levels were reached when all known elements up to position −300 were included. Similarly, in human HeLa cells, transcription above background was not seen until 149 bases of 5′ sequence was included, when the reporter gene activity increased 10-fold, to nearly maximal levels (Shapiro *et al.,* 1987). In contrast, lipofection studies of differentiated renal tubular cells indicated the role of other 5′ sequences. Transcriptional activity up to 10-fold above background levels was found with constructs containing 180 and 270 bp of 5′ sequence but an increase of up to 500-fold was induced by 1.1 kb of 5′ sequence (Lai *et al.,* 1995). The high level of induction by the 1.1-kb 5′ sequence in both LLC-PK 1 cells and mouse primary renal tubular cell cultures suggested that there is a regulatory element(s) located between −420 bp and −1.1 kb which is activated in renal tubular cells. However, the same −1.1-kb construct was ineffective in transgenic mice (Erickson *et al.,* 1990).

We have now used 12 kb of 5′ flanking CA II sequences in transgenic mice and find abundant expression in many tissues but not in the correct relative expression patterns. Testis, which is normally a high-expressing tissue for CA II, was consistently high for the reporter gene, while blood, which normally has the highest CA II activity of any tissue, was relatively low in reporter gene activity. Similarly, liver, which normally has low CA II enzymatic activity, was, in the transgenics, sometimes high, whereas lung and kidney, which are normally high CA II expressors, had low levels. Finally, spleen, which is not normally a highly expressing tissue for CA II, showed very high levels of reporter gene expression. The same 12-kb construct delivered by lipofection to cultured renal tubular cells provided no more effect than the 1.1-kb construct [or decreased expression if correction for number of copies is not performed (Lai *et al.,* 1995)].

The addition of that portion of the CpG island which is included in the first intron, and potential 3′ regulatory sequences, but with only 8 kb (instead of 12 kb) of 5′ sequences in pMHCAII-8 kb, did not further enhance specificity. In fact, expression (corresponding to high levels of expression given the sensitivity of the assay) was detected only in the spleen. Thus, the 4 kb of 5′ sequences from −12 to −8 kb may have enhancing activity which is not highly tissue specific.

We have considered several reasons for the finding that the 12 kb of

flanking DNA allows the expression of the reporter gene but not in a manner analogous to endogenous CA II. The inappropriate high expression is not likely to be due to the insertion sites which can cause chromosomal position effects (Al-Shawi *et al.,* 1990) since it was similar in a number of transgenic lines. Species specificity (since the 12 kb is human sequences) is unlikely to be involved—the chicken α-actin (Petropoulos *et al.,* 1989) and lysozyme (Bonifer *et al.,* 1990) promoters work appropriately in transgenic mice. However, when there are species-specific patterns of gene expression, the promoter reflects its source, e.g., human *Thy-1* retains a human pattern of expression in transgenic mice (Gordon *et al.,* 1987). Locus control regions (LCR) are unlikely to be the explanation since the addition of LCRs to constructs requiring them raises low-level, gene dosage-independent expression to high-level, dosage-dependent expression. These elements were first demonstrated for the human β-globin locus, which required a distal regulatory region located 50–60 kb upstream of the gene for high-level expression in transgenic mice (Grosveld *et al.,* 1987) or mouse erythroleukemia cells (Blom van Assendelft *et al.,* 1989). The three CA genes, I, II, and III, are clustered (Lowe *et al.,* 1991), as are the β-globin loci but differ for tissue of expression rather than time of expression. Perhaps an LCR involved in the expression of such clustered, but diversely expressed, genes would show properties different from those of the β-globin LCR.

A major question is whether the missing regulatory region(s) is (are) within or outside the gene. Regulatory regions have been found in second introns [thymidine kinase (Rotheneder *et al.,* 1991)] which we have not included. An alternative approach for finding the essential elements is suggested by recent success with YAC transgenesis (Schedl *et al.,* 1993). Instead of adding further elements to our current constructs, if a YAC transgene can provide proper expression of CA II, subsequent transgenic constructs could delete portions to identify essential elements.

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