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Transgenic Mice Reveal Novel Sites of Calcitonin Receptor Gene Expression during Development

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To characterize the tissue and developmental-specific transcriptional activity of the human calcitonin receptor (hCTR) gene *in vivo*, transgenic mice containing a 4.9-kb hCTR promoter/β-galactosidase (lacZ) construct were generated. Between 8.5 and 10.5 days of development, lacZ-positive cells were observed on the lateral side of cervical and occipital level somites and in the lateral myotome. LacZ-positive cells also appeared to be migrating from the dermomyotome into the adjacent limb buds, suggesting that the hCTR promoter is active in hypaxial muscle progenitors. By 11.5–16 days of development, novel hCTR expression sites were identified that included limb buds, cornea, retina, skin, intercostal muscles, muscles of the limbs, face, and dorsal root ganglion. hCTR promoter activity in a number of these tissues was repressed at adult stages of development. RT-PCR demonstrated endogenous mCTR mRNA in all lacZ-positive tissues assayed. The developmental regulation of hCTR gene expression in the above tissues suggests that CTRs are likely to play an important role in their morphogenesis.

Key Words: **human calcitonin receptor promoter; development; transgenics.**

Calcitonin (CT) is a 32 amino acid peptide hormone produced primarily by the parafollicular cells of the thyroid gland in response to elevated levels of extracellular calcium (1, 2). CT contributes to calcium homeostasis by direct inhibition of osteoclast-mediated bone resorption and by enhancing calcium excretion by the kidney (3–5). High-affinity receptors for CT (CTRs) have been found in a variety of tissues that include brain (6, 7), adult skeletal muscle (8), testis (9) placenta (10), osteoclasts (11, 12), and kidney (13, 14). The CTR is a member of the seven transmembrane domain G-protein coupled receptor superfamily that includes PTH/PTH-related peptide, secretin, glucagon, and vasoactive intestinal polypeptide (15). The binding of CT to CTRs activates adenylate cyclase causing a rapid increase in intracellular levels of cAMP (16–18).

Evidence suggests a role for CT/CTRs in development. Burgess (19) demonstrated that CT modifies interocular distance and influences neural crest and neural plate development in *Xenopus* embryos. Recently, Wang *et al.* (20) demonstrated that CTRs, when bound with CT, increase intracellular levels of calcium in preimplantation embryos and accelerates their development. To elucidate the role CTRs plays in postimplantation embryonic and fetal development, studies on the temporal and tissue-specific transcriptional activity of the CTR gene must first be undertaken. In this report, we employed the transgenic mouse as a model system to study the tissue and developmental specific transcriptional activity of the human CTR (hCTR) gene *in vivo*. By linking a 4.9 kb human CTR (hCTR) gene promoter fragment to a β-galactosidase (lacZ) reporter gene, hCTR transcriptional activity in transgenic mice can be assayed employing simple histochemical procedures.

MATERIALS AND METHODS

Generation and analysis of hCTR/lacZ transgenic mice. A 4.9 bp hCTR promoter fragment as described in Hebden *et al.* (21) (EMBL Accession No. AJ271672) was blunt ended with Klenow and cloned into the blunt-ended *Pst*I cut vector pSDKlacZ/*Bgl*II (Fig. 1). hCTR/lacZ transgenic lines were generated and hCTR/lacZ copy number determined by methods outlined in Pondel *et al.* (22). To analyze hCTR/lacZ expression, transgenic males from each line were mated to wild-type F1 mice. The appearance of a vaginal copulation plug was considered day 0.5. At days 8.5–9, 9.5–10, 11.5–12, and 15.5–16, whole embryos and fetuses were fixed and stained with X-gal as previously described (22). In some cases 9.5- to 10-day-old embryos and 15.5- to 16-day-old fetal limbs were fixed, stained with X-gal as above, and embedded in paraffin followed by sectioning (15 μM) and counterstaining with eosin.

To analyze lacZ expression in neonatal and adult transgenics, a variety of tissues that included skin, eye, kidney, brain, spinal cord, anterior naris, liver, spleen, bone, kidney, testis, limb, and ribs with intercostal muscles attached were removed, fixed, and stained with X-gal as indicated above.

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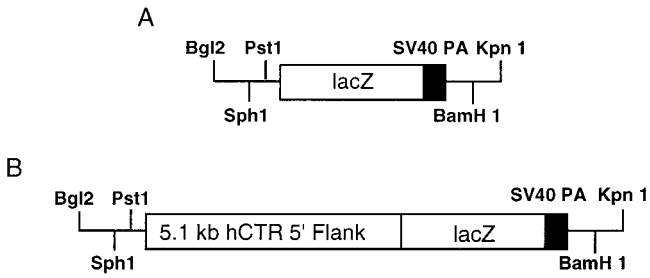

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Line	Copy no.	lacZ expression
1	20	-
2	2	+
3	8	+
4	8	+

FIG. 1. LacZ expression constructs. (A) Promoterless expression construct pSDKlacZpa/Bgl2 containing an SV 40 polyadenylation site. (B) Expression construct phCTR/lacZ containing a 4.9 kb hCTR promoter fragment. (C) hCTR/lacZ copy number (per haploid genome) in transgenic lines 1–4 and presence (+) or absence (−) of lacZ expression in transgenic embryos, fetuses, neonates, and adult mice.

RT-PCR on fetal, neonatal and adult mouse RNA. To determine if the endogenous mCTR gene and the hCTR/lacZ transgene were coexpressed in the same tissues, a variety of tissues demonstrated to express lacZ in transgenics were isolated from wild-type embryonic/fetal/neonatal and adult mice (see Table 1) and extracted for total RNA. RT-PCR was then carried out as described in Jagger *et al.* (23). RNA from fetal, neonatal and adult spleen was employed as a negative control.

Semiquantitative RT-PCR analysis on RNA from adult/fetal limb, facial, and intercostal muscle was carried out as above except that PCR products were removed after 10, 15, 20, 30, and 35 cycles. PCR products were then analyzed as above.

RESULTS/DISCUSSION

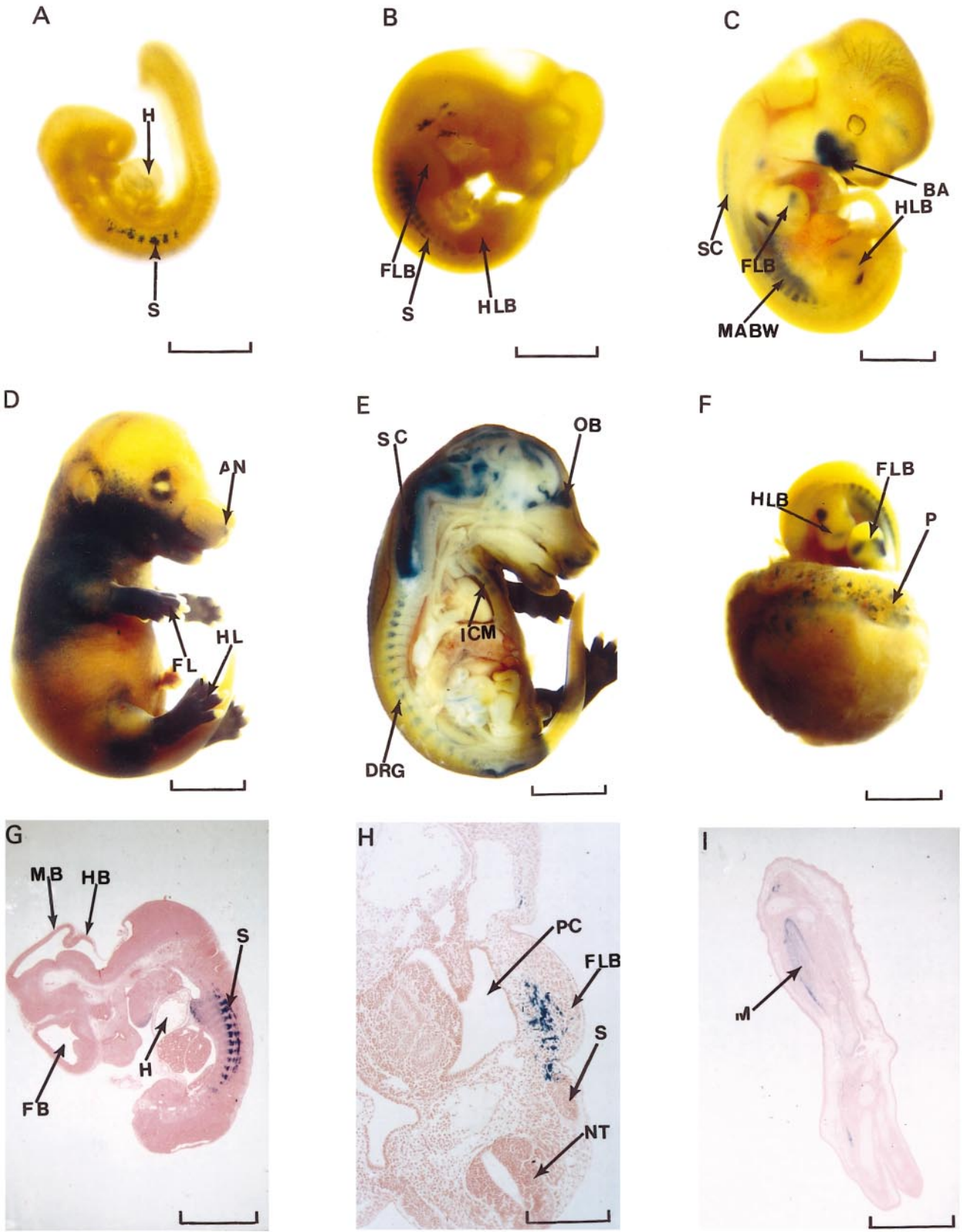
hCTR Promoter Activity in Transgenic Embryos, Fetuses, and Neonates

Four transgenic lines containing the expression construct hCTR/lacZ (Fig. 1B) were generated and lacZ expression analyzed in transgenic progeny. Only progeny from transgenic lines 2, 3, and 4 demonstrated lacZ expression (Fig. 1C). All lines demonstrated the same developmental and tissue specific lacZ expression pattern. In 8.5- to 9-day-old embryos lacZ-positive cells were observed on the lateral side of somites at occipital/cervical levels (Fig. 2A, Table 1). By 9.5–10 days of development, lacZ-positive cells were observed at interlimb level in the elongating lateral myotome (Fig. 2B and 2G and Table 1). Detailed histological analysis at limb levels demonstrated the presence of lacZ-positive cells that appeared to be migrating from the dermomyotome of somites into the adjacent limb buds (Fig. 2H, Table 1). LacZ-positive cells were also observed in syncytial trophoblast cells within the placenta (Table 1). At occipital/cervical levels lacZ-positive cells appeared anterior to the somites (Fig. 2B, Table 1). Studies have shown that cells from the lateral lip of the dermomyotome at occipital/cervical and limb levels delaminate and undergo long range migration to form limb and tongue (hypaxial) muscles. At interlimb levels, the lateral dermomyotomal lips remain epithelially arranged during elongation and deposit cells which will give rise to the hypaxial muscles of the anterior body wall (24, 25). Our data therefore suggests that the hCTR gene is transcriptionally active in muscle cell progenitors that give rise to the hypaxial muscles. By 11.5–12 days of development the lacZ-positive lateral myotome continued to elongate anteriorly (Fig. 2C, Table 1). LacZ-positive cells were now clearly visible in limb buds, spinal cord, brain, anterior nares (nostrils) and maxillary component of the first branchial arch (Fig. 2C, Table 1). LacZ-positive cells were also present on the developing cornea, the inner neural layer of the optic cup (future nervous layer of retina) (Table 1) and placenta (Fig. 2F, Table 1). By 15.5–16 days of development, intense lacZ expression was observed on skin (Fig. 2D, Table 1). Sagittal sectioning of 15.5- to 16-day-old transgenic fetuses demonstrated hCTR promoter activity in spinal cord, brain and olfactory bulbs. LacZ-positive cells were also observed in dorsal root ganglia, cornea, intercostal muscles, muscles in the face and limbs, nerve fiber layer of the retina and placenta (Figs. 2E and 2I and Table 1). An identical expression pattern was seen in 1- to 2-day-old neonatal transgenic mice with the exception that no lacZ staining was observed on the anterior nares.

hCTR/LacZ Expression in Adult Transgenic Mice

In contrast to fetal and neonatal hCTR/lacZ transgenics, no lacZ expression was detected in skin, cornea, anterior nares and limb/facial/intercostal muscles of adult (4–6 weeks old) transgenics suggesting hCTR promoter activity was silenced in these tissues. However, the hCTR promoter was still transcriptionally active in the brain. LacZ expression was observed in the olfactory bulbs, hippocampus, cerebellum and cerebrum (Fig. 3A). LacZ expression was also observed in the ventral roots and dorsal horn of the spinal cord (Figs. 3B and 3C). In male transgenics, lacZ activity was also present in the testis (Table 1). Finally, the hCTR promoter was still transcriptionally active in the nervous layer of the retina (Fig. 3D). No lacZ expression in kidney and osteoclasts was detected.

The results above demonstrated the hCTR promoter was active in a variety of tissues not previously reported to express CTRs that include: (1) dermomyotome/lateral myotome (2) limb buds (3) maxillary component of the 1st branchial arch (4) embryonic, fetal and neonatal cornea/retina (5) fetal intercostal, limb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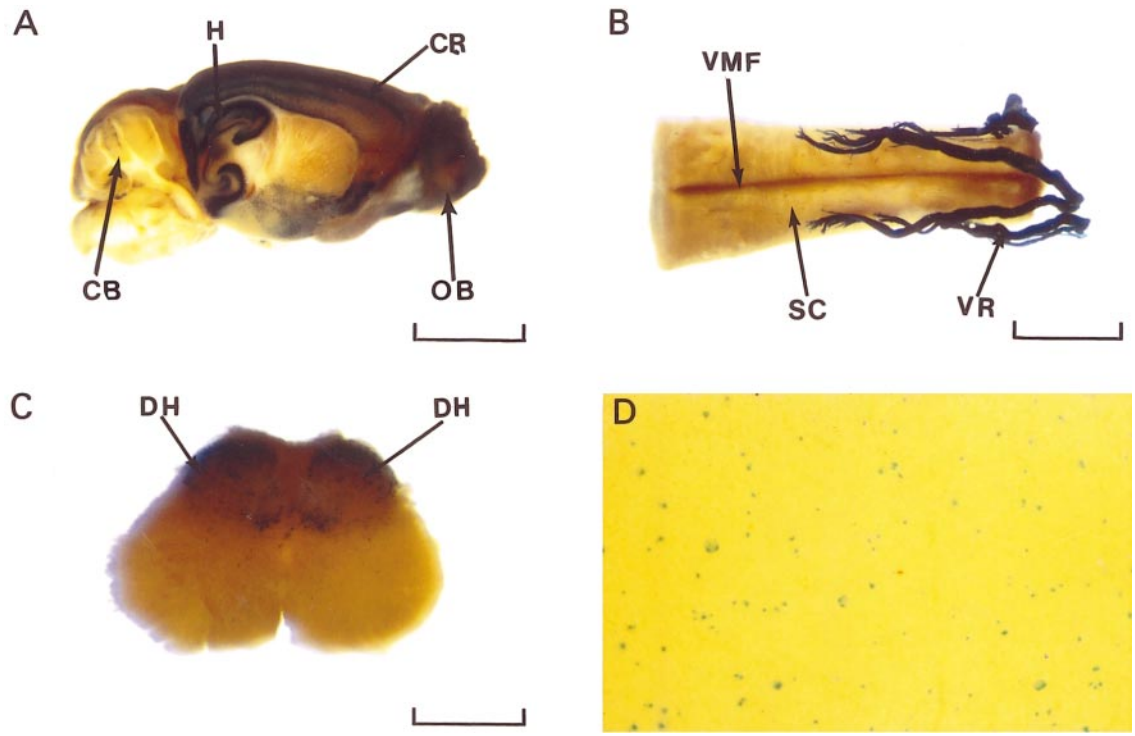

FIG. 3. LacZ expression in hCTR/lacZ adult transgenics. (A) Brain: CB cerebellum; H, hippocampus; CR, cerebrum; OB, olfactory bulb. (B) Adult spinal cord: VMF, ventral medial fissure; SC, spinal cord. (C) Adult spinal cord: DH, dorsal horn; (D) neural fiber layer of the retina. LacZ analysis was performed on a minimum of 3–5 adults per transgenic line.

and facial muscles (5) fetal/neonatal skin (6) adult retina. Recently, Jagger *et al.* (23) linked a 2.1 kb porcine CTR (pCTR) promoter to a lacZ reporter gene and studied its transcriptional activity in transgenic mice. While the pCTR/lacZ construct and endogenous mCTR gene were transcriptionally active in tissues such as anterior nares, brain, testis and spinal cord, no lacZ expression was detected in hypaxial muscle precursors, lateral myotome, fetal and neonatal limb, facial and intercostals muscles, placenta, skin and dorsal root ganglia. Since both pCTR and hCTR promoters are highly homologous (>85%) (26), the wider range of tissue-specific hCTR promoter activity in transgenic mice is likely due to its greater amount of 5′ flanking region rather than species differences. Deletional analysis of the hCTR promoter in transgenic mice is currently underway to identify specific sequences that direct hCTRs transcriptional activities in the above tissues. It is likely that such sequences interact with transcriptional regulatory factors that may play a role in the differentiation of these CTR expressing tissues.

Although the cloning of the mouse CTR (mCTR) promoter has not been reported, data from our laboratory has demonstrated that a 4.0 kb mCTR promoter (gift of P. Sexton, U. of Melbourne. Australia) displayed similar tissue and developmental specific transcriptional activity to that of the hCTR promoter in transgenic mice (i.e., lacZ-positive cells in hypaxial muscle precursors, fetal but not adult skeletal muscle, brain, spinal cord, etc.). However, the 4.0 kb mCTR promoter was not active in mCTR-positive tissues such as skin, placenta and cornea. Like the pCTR and hCTR promoter, mCTR/lacZ was not expressed in kidney and osteoclasts (unpublished results). It is likely that additional positive regulatory elements (i.e., enhancer) further 5′, in the body of the CTR genes or in their 3′

FIG. 2. LacZ expression in hCTR/lacZ transgenic embryos and fetuses. (A) 8.5- to 9-day embryo: S, somite; OP, otic pit; H, heart. (B) 9.5- to 10-day embryo: LM, lateral myotome; FLB, forelimb bud; HLB, hindlimb bud. (C) 11.5- to 12-day embryo: SC, spinal cord; BA, maxillary component of first branchial arch; HLB, hindlimb bud; FLB, forelimb bud; LM, lateral myotome. (D) 15.5-day-old fetus: AN, anterior nares; HL, hindlimb; FL, forelimb. (E) Sagittal section 15.5-day-old fetus: DRG, dorsal root ganglion; ICM, intercostal muscles; SC, spinal cord; OB, olfactory bulb. (F) 11.5-day-old fetus attached to placenta: HLB, hindlimb bud; FLB, forelimb bud; ST, lacZ-positive syncytial trophoblast cells. (G) Sagittal section of 9.5-day-old embryo: FB, forebrain; MB, midbrain; HB, hindbrain; H, heart; LM, lateral myotome. (H) Transverse section of 9.5- to 10-day-old transgenic embryo: NT, neural tube; S, somite; FLB forelimb bud; PC, peritoneal cavity. (I) Thin section of hindlimb from a 15.5- to 16-day-old fetal limb. M, muscle; CPF, cartilage primordium of fibula. Scale bars: A, 500 μm; B, 1.0 mm; C, 2 mm; D, E, 3.5 mm; F, 3.5 mm; G, 1.0 mm; H, 200 μm; I, 100 mm.

TABLE 1

Developmental and Tissue-Specific Expression of hCTR/LacZ Transgene and Endogenous mCTR Gene

Days P.C.	Tissue	RT-PCR	LacZ+
8.5–9	Lat. somites (occ./cerv.)	na	+
9.5–10	Lat. somites (occ./cerv.)	na	–
	Lat somites (limb)	na	–
	Lat myotome	na	+
	Limb buds	+	+
	Placenta	+	+
11.5–12	Lateral myotome	na	+
	Limb buds	+	+
	1st branc. arch	na	+
	Anterior naris (nostrils)	+	+
	Spinal cord	+	+
	Brain	+	+
	Cornea	na	+
	Optic cup	na	+
	Placenta	+	+
15.5	Skin	+	+
	Anterior naris	+	+
	Cornea	+	+
	Retina	na	+
	Brain	+	+
	Spinal cord	+	+
	Dorsal root ganglion	na	+
	Limb muscles	+	+
	Intercostal muscles	+	+
	Facial muscles	+	+
	Placenta	+	+
	Liver/spleen	–	–
Neonate	Skin	+	+
	Placenta	+	+
	Anterior naris	–	–
	Cornea	+	+
	Retina	na	+
	Brain	+	+
	Spinal cord	+	+
	Dorsal root ganglion	na	+
	Limb muscles	+	+
	Intercostal muscles	+	+
	Facial muscles	+	+
	Liver/spleen	–	–
Adult	Skin	–	–
	Anterior nares	–	–
	Cornea	–	–
	Retina	na	+
	Brain	+	+
	Spinal cord	+	+
	Dorsal root ganglion	na	+
	Limb muscles	+	–
	Facial muscles	+	–
	Intercostals	+	–
	Osteoclasts	+	–
	Kidney	+	–

Note. –, indicates no expression; na, no assay performed.

flanking region are required for their transcriptional activity in these tissues. YAC (yeast artificial chromosome) hCTR constructs containing extensive amounts of both 5′ and 3′ hCTR flanking region are currently being employed for transgenic mouse production in order to identify such positive regulatory elements.

mCTR RNA Expression in Fetal, Neonatal, and Adult Tissues Demonstrating hCTR Promoter Activity

We next wished to determine if the endogenous mCTR gene was transcriptionally active in tissues that demonstrated hCTR promoter activity. Unfortunately, *in situ* hybridization has been proven by many laboratories to be ineffectual in detecting CTRs in developing mouse embryos and fetuses. Although antibodies have recently been developed to detect CTRs on mouse osteoclasts (27) we have found them unable to detect CTRs in a variety of known CTR-positive mouse tissues (unpublished results). Therefore, tissues demonstrated to express lacZ in, fetal, neonatal and adult hCTR/lacZ transgenics were isolated from wild-type mice and RNA extracted. The RNAs were then utilized for RT-PCR employing oligos specific for mCTRs and actin (see Materials and Methods). Endogenous mCTR gene expression was active in all lacZ-positive tissues assayed (Table 1). While RT-PCR detected the presence of mCTR gene transcripts in adult facial, limb and intercostal muscle, no hCTR/lacZ expression was detected in these muscles. Semiquantitative RT-PCR (see Materials and Methods) demonstrated an 8- to 10-fold decrease in mCTR gene expression in these tissues between neonatal and adult stages of development. This suggested that our inability to detect hCTR promoter activity in adult muscle was likely due to a significant postneonatal decrease (as with the endogenous mCTR gene) in its transcriptional activity. Although not all lacZ-positive tissues such as retina, dorsal root ganglion and hypaxial muscle precursors could be tested for endogenous mCTR gene expression (due to difficulties in isolating these tissues), the coexpression of mCTR mRNA in all lacZ-positive tissues assayed and mCTR/lacZ expression in hypaxial muscle precursors in transgenics make it unlikely that hCTR promoter activity in these tissues was ectopic.

While the functional roles CT/CTRs play in kidney and osteoclasts are well known, their role in postimplantation embryonic and fetal development has not yet been investigated. It is well established that cAMP can influence the transcriptional regulation of various genes through distinct promoter-responsive sites (28). The ability of CTRs to activate adenylate cyclase and increase intracellular levels of cAMP make it reasonable to hypothesize that CT/CTRs may play a role in the transcriptional activation of genes regulating the development/differentiation of a variety of tissues. Furthermore, the ability of CTRs to regulate intracellular levels of calcium and the importance of calcium ion concentration in cell aggregation/development (20, 29–31) make it is plausible to hypothesize that the CTR gene may play a role in the process of muscle cell progenitor migration, aggregation and differentiation. mCTR gene "knock-out" and overexpression studies in transgenic mice are currently underway to further elu-

cidate the role(s) CTRs play in mouse embryonic/fetal development.

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