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PRODUCTION AND ANALYSES OF TRANSGENIC MICE WITH ECTOPIC EXPRESSION OF CELLULAR RETINOIC ACID-BINDING PROTEIN

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Transgenic mice with ectopic expression of bovine cellular retinoic acid-binding protein (CRABP) under the control of human metallothionein IIA promoter have been generated. From a total of 10 independent transgenic live borns, six lines have expressed the bovine CRABP messages in a variety of tissues. Among the six lines, three appear to be normal and healthy in the founders as well as their offspring, one has generated transgenic offspring with retarded growth and two have produced only female transgenic mice that are all sterile.

Retinoic acid (RA) has profound effects on biological systems (1), as seen in differentiation and proliferation of cultured cells (2, 3) as well as pattern formation in developing embryos (4, 5) and regenerating limbs (6, 7). The spectacular effects of RA on embryonic development, limb regeneration and maintainence of cellular differentiation/growth are believed to be mediated through two distinct classes of proteins. The one consisting of a family of nuclear receptors for RA is believed to regulate gene expression through binding to specific DNA sequences of target genes (8, 9, 10). The other class contains at least two members of cytosolic proteins, referred to as cellular retinoic acid-binding protein (CRABP) I and II. Both CRABPs have been cloned (11, 12, 13, 14) and shown to be expressed only in

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restricted adult tissues and embryos (12, 13, 15, 16). However, the precise function of CRABPs remained to be elucidated.

To facilitate the understanding of the role CRABPs may play in mediating RA effects, we have produced transgenic mice with ectopic expression of bovine CRABP I under the control of the human metallothionein IIA (MTIIA) promoter (17). Among the six independent lines that express bovine CRABP messages, three appear to be phenotypically normal and healthy, one has generated offspring of apparent illness and retarded growth only in the transgenic animals, and the last two have been able to produce only female transgenic progenies that are all sterile.

MATERIALS AND METHODS

Construction of the fusion gene

The PvuII/HpaII DNA fragment from the full length bovine CRABP cDNA clone (12) was inserted at the NcoI site of the human MTIIA gene (17). Thus, bovine CRABP message containing the complete coding region could be transcribed from the MTIIA promoter and poly-adenylated, whereas the reading frame of MTIIA gene was disrupted. Cloning procedures were according to the standard protocols (18), and DNA sequences of the junctions were confirmed by double-strand DNA sequencing with synthetic oligonucleotides as specific primers.

Production of transgenic mice

DNA fragments containing the complete fusion gene were excised from the vector sequences and microinjection was carried out using a Carl Zeiss micromanipulator system. Fertilized eggs were obtained from superovulated C57BL6/DBA2 females mated with C57BL6/DBA2 males. Mice were obtained from Charles River Japan Inc. The procedures for generating transgenic mice were according to the laboratory manual (19). Transgenic mice were detected by Southern blot analyses on mouse tail DNA samples, with the bovine cDNA fragment as the probes. Polymerase chain reaction (PCR) (20) was used to confirm the fusion gene with oligonucleotides of the human MTIIA promoter region (5'AGTCCCAGCGAACCCGCGTG3') as well as the bovine CRABP coding region (5'CTGCACGAGGATTTATGTTCGGG3'), as the 5' and 3' primers, respectively.

Detection of bovine CRABP expression in transgenic mice

RNA samples from animal tissues were prepared using LiCl precipitation method (21) and assayed for the presence of bovine CRABP messages by specific RNase protection assays (22).

RESULTS

The fusion gene construct, as shown in Fig. 1, contains the complete bovine CRABP coding region and the truncated MTIIA gene

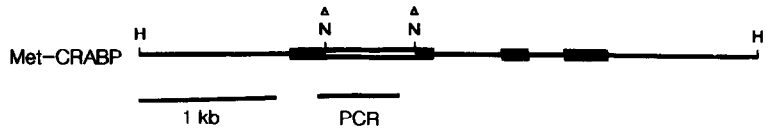

Figure 1. The structure of MTIIA-CRABP fusion gene. The PvuII-HpaII fragment from the bovine full length CRABP cDNA (11) was inserted into the human MTIIA gene (17) at the NcoI site of the first exon. Open box indicates bovine cDNA sequence, filled boxes indicate MTIIA exonic sequences, and the thin line indicates the untranscribed region of MTIIA genomic sequence between the two HindIII sites. PCR indicates the 513bp DNA fragment of the PCR product that covers 58bp from the MTIIA sequence and 455bp from the CRABP sequence.

which will not be translated due to the lack of translation initiation codon. A total of 10 independent transgenic live borns have been generated. Two have died before weaning and 2 lines do not express the fusion gene. The remaining 6 lines have been bred for analyses. In order to confirm the presence of fusion gene at low copy numbers as detected by Southern blot analyses, PCR has been used to monitor line 15 and line 30. Figure 2 shows a picture of an agarose gel with PCR products of 513 bp (58 bp from MTIIA promoter, 455 bp from bovine CRABP) DNA fragments amplified from DNA samples of several line 15 and line 30 mice.

Bovine CRABP RNA expressed in transgenic mice is detected by RNase protection assays as shown in Fig. 3, in which a bovine CRABP-specific fragment of 396 nucleotides is detected in all the tissues examined, including liver, kidney, spleen, lung and pancreas, of transgenic lines 6, 7, 15, 23 and 26 (data for lines 15, 23, and 26 not shown). Lines 6, 7 and 15 appear to be normal and healthy, and express the fusion transcripts at very similar levels. The transgene has been stably transmitted in these lines. The founders of line 23 (male) and line 26 (female) are both healthy. However, during a breeding period of 6 months, they have generated only 2 and 4 transgenic offspring, respectively, and all of these mice are sterile female. For line 40, the founder appears healthy, however, it has generated only 3 transgenic F_1 progenies that grew poorly (about only half of the normal body

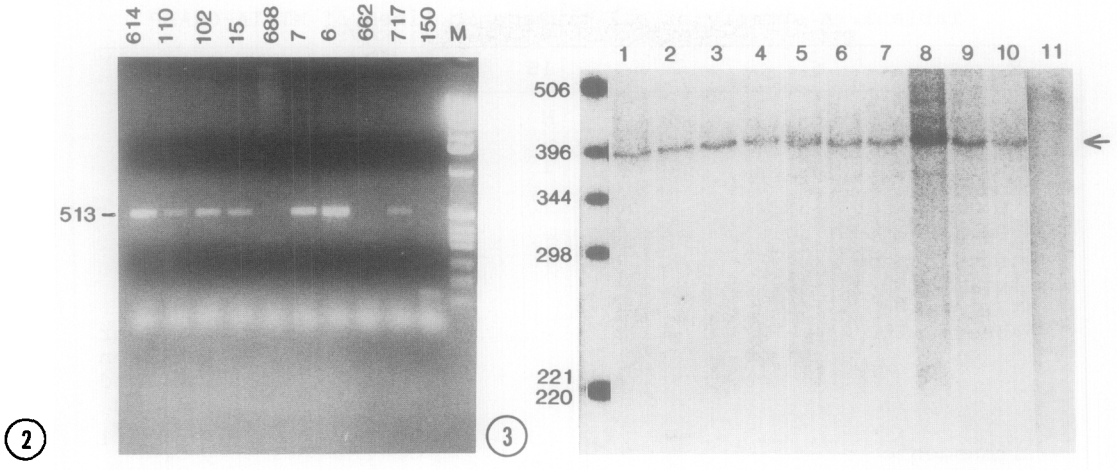

Figure 2. An agarose gel showing PCR products of the fusion gene present in DNA isolated from mouse tails. The MTIIA- and CRABP-specific oligonucleotide sequences used as the 5'- and 3'-primers, respectively, are shown in MATERIALS AND METHODS. DNA amplification was carried out in a final volume of 50μl using 0.1 μg genomic DNA isolated from mouse tail. The dNTPs were present in a final concentration of 200 μM. Taq polymerase was purchased from Cetus. Thirty cycles of denaturation at 94° C for 2 minutes, annealing at 55° C for 1 minute, and extension at 72° C for 3 minutes were carried out in a Perkin-Elmer Cetus DNA Thermal Cycler for each sample. The molecular weight marker of BRL 1 kb ladder is indicated as M.
6: the founder of line 6; 7: the founder of line 7; 15: the founder of line 15; 102 and 110: two F_1 of line 15; 717: one F_3 of line 15; 614: one F_3 of line 6. 150, 662 and 688: three non-transgenic mice generated from lines 15, 6 and 7, respectively.

Figure 3. An RNase protection assay showing expression of the bovine CRABP messages. Total RNAs were prepared from mouse tissues (21) and 30 μg of each sample was analyzed with the bovine CRABP specific RNA probe (16) which protected a specific 396 base RNA fragment in RNAs from various tissues of transgenic mice. An arrow on the right indicates the specifically protected RNA fragment. Samples 1-5 are from line 6 transgenic mouse tissues, 6-10 are from line 7 transgenic mouse tissues.
1 & 6: heart; 2 & 7: kidney; 3 & 8: liver; 4 & 9: lung; 5 & 10: pancreas; 11: yeast tRNA. On the left the molecular weight marker derived from HinfI digested pBR322 DNA is shown. Exposure time is for 2 days.

weight of non-transgenic litter mates at the age of four weeks). All the three F_1 mice of line 40 have died before they were sexually mature and they all have very small liver and spleen as compared to their normal littermates. Preliminary data has shown bovine CRABP expression particularly high in the liver, kidney and lung of this line (data not shown). These three lines are currently under further investigation.

Table 1. A summary of all transgenic lines of MTIIA-CRABP

F_0	6	7	15	23	26	40
Sex	[a]M	M	M	M	[b]F	F
Copy # of transgene	>30	>30	1	20-30	>30	1-2
Expression of transgene in liver	+	+	+	+	+	+++
Abnormality	-	-	-	-	- small liver small spleen and retarded growth in transgenic F_1	
Transgenic ratio (M:F) in						
F_1	12:20	4:2	19:15	[c]0:2	[c]0:4	[d]1:2
F_2	17:10	5:11	10:18	0	0	0
F_3	11:9	30:27	17:14	0	0	0

[a] Male.
[b] Female.
[c] All transgenic progenies are female and sterile.
[d] No transgenic progenies have been obtained due to the death of all F_1 transgenic mice before they were sexually mature.

The six lines that express the MTIIA-CRABP transgen are either normal (line 6, 7 and 15) or show defects in their offsprings (line 23, 26 and 40). Table 1 summerizes the results of these six lines generated from a total of approximately 5000 microinjected mouse embryos which have produced 60 F_0 live borns.

DISCUSSION

This is the first report to show ectopic expression of CRABP in transgenic mice. The human MTIIA promoter is able to express bovine CRABP cDNA in a variety of mouse tissues. With the exception of line 40, the basal levels of expression in different organs is relatively similar in the different lines which have various copy numbers of the transgene (Table 1). In an attempt to induce the expression of the transgene by either Zn or Cd ions, we have not been able to observe elevated levels of fusion

transcripts in lines 6, 7 or 15. Induction by heavy metals has not been done in lines 23, 26 or 40 due to their poor efficiency in produce transgenic offspring.

In the three lines, 6, 7 and 15, no phenotypical abnormality has been observed in the founders, their offspring or the homozygotically transgenic progenies. Lines 23, 26 and 40 appear healthy in the founders, yet have a degree of defects in their offspring (Table 1). Preliminary data has shown relatively higher level of transgene expression in the liver, kidney and lung of line 40. It remains to be determined if the expression of bovine CRABP has caused the defects in these mice.

It can be concluded that, ectopic expression of CRABP messages at low levels in many different organs which do not nomally express detectable CRABP has no significant effects in the adult mice. However, from the preliminary results of lines 23, 26 and 40, it has been suggested that ectopic expression at higher levels in specific tissues may be related to defects in growth and reproduction.

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