A 3 kb sequence from the mouse cellular retinoic-acid-binding protein gene upstream region mediates spatial and temporal LacZ expression in transgenic mouse embryos

LI-NA WEI*1, GEE-JANG CHEN1, YA-SHU CHU1, JEN-LAN TSAO1 and M. CHI NGUYEN-HUU2

1Department of Microbiology and Immunology, Chang-Gung Medical College, Tao-Yuan, Taiwan, Republic of China
2Department of Microbiology, University of Southern California, Los Angeles, CA 90033, USA

*To whom correspondence should be addressed

Summary

A 3233 base pair (bp) sequence of the 5'-flanking region of the mouse cellular retinoic-acid-binding protein (CRABP) gene is determined. From this region, a 3 kb fragment located 150 bp upstream from the transcriptional initiation site is isolated and fused to a LacZ reporter sequence. Transgenic mouse embryos of this fusion gene show spatially and temporally specific expression of LacZ protein and the expression of this fusion gene at the RNA level is confirmed by RNAase protection assays, which detect specific fusion transcripts in RNA samples from tissues of transgenic mouse embryos. In contrast, transgenic mouse embryos of a shorter fusion gene containing only 583 bp from the same upstream region of the mouse CRABP gene fused to the same reporter sequence show no LacZ activities. Thus, it is concluded that the 3 kb sequence, but not the 583 bp sequence, of the mouse CRABP gene contains information for its temporally and spatially specific expression in mouse embryos.

Key words: cellular retinoic-acid-binding protein gene, LacZ, reporter gene, transgenic mice.

Introduction

Retinoic acid (RA) exerts profound effects on biological systems (Roberts and Sporn, 1984). In tissue cultures, it has been shown to affect cellular differentiation and proliferation in many cell types (Edwards and McBurney, 1983; Eriksson et al. 1986; Horton et al. 1987; Kato and Deluca, 1987; Makowske et al. 1988; Preis et al. 1988; Strickland et al. 1980). In developing embryos and regenerating tissues, it exerts teratogenic effects on many targets such as craniofacial areas (Abbott and Birnbaum, 1989; Yasuda et al. 1986), limbs (Crawford and Stocom, 1988; Ide and Aono, 1988; Maden and Keeble, 1987) and the central nervous system (CNS) (Durston et al. 1989; Tibbles and Wiley, 1988; Wiley, 1983). The spectacular effects of RA on regeneration and pattern duplication in chick and amphibian limbs, as well as the discovery of an anteroposterior gradient of RA concentration in chick limb buds (Thaller and Eichele, 1987), has led to the hypothesis that RA could be a natural morphogen conferring positional information for limb development (Eichele, 1989). Support for this hypothesis has been obtained from a recent study showing that anteroposterior transformation of the developing CNS could be induced by RA in frog embryos (Durston et al. 1989).

The molecular mechanisms mediating RA effects are not fully understood. A specific cytosolic binding protein for RA (cellular-retinoic-acid-binding protein, or CRABP) was first discovered and cDNA clones for CRABP were obtained from various species (Chytil and Ong, 1984; Nilsson et al. 1988; Sani and Hill, 1974; Shubeita et al. 1987; Wei et al. 1987, 1989, 1990). In addition, cDNAs of three nuclear receptors for RA (RARs) have recently been cloned and identified as belonging to the steroid/thyroid hormone receptor gene family, which appear to be transcriptional factors activated by RA (Giguere et al. 1987; Petkovich et al. 1987; Zelent et al. 1989). It is generally believed that RA regulates the expression of some target genes through the RA–RAR complexes in the nuclei of target cells. However, it remains inconclusive as to what kind of biological functions CRABP exerts after it binds RA in the cytosol. One possible role CRABP may play in the morphogenetic effects of RA has been suggested from an interesting observation that the concentration gradient of CRABP is opposite to that of endogenous RA in developing chick limb buds (Maden et al. 1988).
It has been proposed that with this reversed concentration gradient, CRABP is able to steepen the effective gradient of free RA in these areas, making it more likely to act as an inducer for different gene regulation in various cell types (Maden et al. 1988; Summerbell and Maden, 1990). The expression of CRABP has been examined in adult tissues (Kato et al. 1985; Wei et al. 1987) as well as embryos (Maden et al. 1990; Milam et al. 1990; Perez-Castro et al. 1989). It is interesting to note that although CRABP expression in adult tissues is relatively low except in certain areas such as eyes, skin and the reproductive system, its expression is much more abundant and specific in embryonic tissues that are known to be the teratogenic targets for RA.

To understand the mechanisms involved in regulating CRABP gene expression, which may shed light on the role CRABP plays in mediating RA effects, the gene for mouse CRABP has been cloned and analyzed previously (Wei et al. 1990). It has been shown to contain no TATA box and proposed to belong to the housekeeping gene family. In search of any possible regulatory elements for its highly specific expression during embryonic stage, we have used a fusion gene approach with the E. coli LacZ structural gene as the reporter in a transgenic mouse system. As an initial step, we have found that an approximately 3 kb long DNA fragment located 150 bp upstream from the site for transcriptional initiation of the mouse CRABP gene contains regulatory information for most of its temporally and spatially specific expression in mouse embryos.

**Materials and methods**

**DNA sequence determination**

DNA fragments isolated from the mouse CRABP genomic clones (Wei et al. 1990) were restriction mapped and digested with various restriction enzymes for subcloning into M13mp18 and M13mp19. DNA sequences were determined using the dideoxy chain-termination method (Sanger et al. 1977). For DNA fragments isolated from the mouse CRABP genomic sequence determination (Hogan et al. 1988), CRABP transcripts were derived from the 1.37 kb long HindIII–PvuII fragment of the fusion gene, which covered 930 bp from the CRABP genomic sequence (HindIII to NaiI), 360 bp from the Hox1.3 gene (XhoI to ATG for translation initiation), and 84 bp from the E. coli LacZ structural gene. This probe would protect a specific 156 nt long RNA fragment covering 72 nt from the 5'-untranslated region of the mouse CRABP mRNA. CRABP-specific probe derived from the AluI fragment (Wei et al. 1990) of the mouse genomic clone was included in the same RNA samples, which protected a specific 147 nt long RNA fragment from mouse CRABP mRNA.

**Construction of hybrid genes**

DNA fragments from the 5'-flanking region of the mouse CRABP gene were fused to the 5'-end of the reporter sequence, Hox1.3–LacZ at the XhoI site (Zakany et al. 1988). To include as much regulatory information as possible in the CRABP–LacZ hybrid gene, the longest 5'-upstream sequence from the mouse genomic CRABP clone, containing approximately 3 kb, was released by EcoR1 and NaiI double digestions and fused to the 5'-end of the Hox1.3–LacZ sequence. This long construct was designated as L-CRABP–LacZ. A short construct was made to contain only a 583 bp PstI to NaiI fragment from the same upstream region of the mouse genomic clone fused to the same XhoI site of the Hox1.3–LacZ sequence and designated as S-CRABP–LacZ. Thus, S-CRABP–LacZ is approximately 2.4 kb shorter than L-CRABP–LacZ in the 5'-end (EcoR1 to PstI). These DNA fragments were blunt-ended by Klenow enzyme and T4 DNA polymerase and ligated to the blunt-ended XhoI site of the Hox1.3–LacZ vector. Linear DNA fragments containing these fusion genes were excised from the vector sequences, isolated from agarose gel and purified from CsCl gradients for microinjection.

**Production of transgenic mice**

Production of transgenic mice was carried out essentially as described (Hogan et al. 1986). Mice were obtained from Charles River Japan Inc. Fertilized eggs for microinjection were obtained from superovulated C57BL6/DBA2 females mated with C57BL6/DBA2 males. Pseudopregnant females for fosters were produced by matings between C57BL6/DBA2 adult females and vasectomized CD-1 males. Microinjection was conducted using a Carl Zeiss micromanipulator system and embryo transfers were carried out with oviduct transfer techniques. F0 embryos at different days of gestation were obtained by killing the pregnant females, and were analyzed for LacZ activities as described (Zakany et al. 1988). Transgenic lines were established for assaying stable LacZ activities in the embryos. Transgenic mice and embryos were identified by Southern blot analyses on DNA isolated from tails and placenta, respectively, with probes prepared from DNA fragments containing the E. coli LacZ structural gene, which did not show cross hybridization to the mouse endogenous LacZ gene under highly stringent conditions.

**Enzyme histochemical analysis of LacZ expression**

Enzyme histochemical analyses of LacZ activities in embryos were conducted as described (Zakany et al. 1988). Stained embryos were examined and photographed using a Nikon stereoscope SMZ-10 photosystem. To examine internal staining, stained embryos were either sliced in half sagittally with a sharp scalpel or embedded in OCT for thin sectioning with a cryotome. Photomicrographs of sections were taken without further staining. To improve internal staining, embryos older than day 14 were fixed and sagittally sliced first, and then stained.

**RNAase protection assay**

Embryonic RNA isolation and RNAase protection assays were carried out as described (Wei et al. 1989). The probe used for detecting the Hox1.3–LacZ fusion transcripts was derived from the 1.37 kb long HindIII–PvuII fragment of the fusion gene, which covered 930 bp from the CRABP genomic sequence (HindIII to NarI), 360 bp from the Hox1.3 gene (XhoI to ATG for translation initiation), and 84 bp from the E. coli LacZ structural gene. This probe would protect a specific 156 nt long RNA fragment covering 72 nt from the 5'-untranslated region of the mouse Hox1.3 gene and 84 nt from the E. coli LacZ structural gene. As an internal control, the mouse CRABP-specific probe derived from the AluI fragment (Wei et al. 1990) of the mouse genomic clone was included in the same RNA samples, which protected a specific 147 nt long RNA fragment from mouse CRABP mRNA.

**Results**

**DNA sequence of the upstream region of the mouse CRABP gene**

An approximately 2.6 kb EcoR1–SmaI DNA fragment located 540 bp upstream from the site for transcription initiation (indicated by an asterisk under the sequence at 3140 position in Fig. 1) identified previously (Wei et
ATG is underlined. The five G/C boxes identified previously (Wei et al. 1990) are labeled with the 5 boxes. The translational initiation codon underlined and numbered from I to IX. An asterisk under nucleotide 3140 indicates the site for transcriptional initiation. Thin underlines indicate restriction sites used in constructing fusion genes (R, PstI, and Narl), RcoRI; N, PstI terminates at the Smal fragment has been determined and overlapped, for 200nt, with the previously reported genomic sequence that shown here. The nine pairs of inverted repeat sequences are bold-in preparing probe for RNAase protection assays (H, CRABP gene. The complete sequence of the 2.6kb EcoRI to

**Fig. 1. DNA sequence of the upstream region of the mouse CRABP gene.** The complete sequence of the 2.6kb EcoRI to Smal fragment has been determined and overlapped, for 200nt, with the previously reported genomic sequence that terminates at the PsiI site. The continuous 3.2kb DNA sequence immediately upstream of the translational initiation is shown here. Thin underlines indicate restriction sites used in constructing fusion genes (R, EcoRI; P, PsiI; N, NarI), and in preparing probe for RNAase protection assays (H, HindIII). The nine pairs of inverted repeat sequences are bold-underlined and numbered from I to IX. An asterisk under nucleotide 3140 indicates the site for transcriptional initiation. The five G/C boxes identified previously (Wei et al. 1990) are labeled with the 5 boxes. The translational initiation codon ATG is underlined.
al. 1990) was sequenced completely and aligned with the previously reported murine CRABP genomic DNA sequence which overlapped with this 2.6 kb fragment for 200 bp. By aligning the two sequences, it is possible to obtain a continuous 3.2 kb DNA sequence (Fig. 1) of the 5'-flanking region immediately upstream from the translational initiation of CRABP gene. Within this region, in addition to the previously identified 5 copies of G/C box sequences (indicated by 5 boxes) near transcriptional initiation, there exist 9 pairs of inverted repeat sequences that are longer than 7 bp, as indicated by bold underlines with arrowheads and numbered from 1 to IX. The restriction sites, EcoRI, PstI and NarI, used to generate DNA fragments for making fusion genes, are labeled under the sequence.

Construction of CRABP-LacZ fusion genes and expression of fusion genes in transgenic embryos

Two DNA fragments from the 5'-flanking region of the murine CRABP gene were fused, separately, to the E. coli LacZ reporter sequence Hox1.3-LacZ as described in Materials and methods. This reporter sequence contained the E. coli LacZ structural gene sequence to which the 5'-untranslated region of the mouse homeobox gene Hox1.3 deleted in its upstream regulatory region from the XhoI site was fused in frame at the translational initiation codon ATG. Thus this Hox1.3-LacZ sequence contained only enough information for the transcriptional initiation and was deleted in its further upstream regulatory region for transcriptional control. Therefore, LacZ expression was absent from transgenic mouse embryos carrying only this Hox1.3-LacZ sequence (Zakany et al. 1988). However, if a cis-acting regulatory DNA fragment was added to its 5'-end, this reporter sequence was able to express LacZ activities in transgenic mouse embryos (Tuggle et al. 1990; Zakany et al. 1988) accordingly.

In CRABP-LacZ fusion gene constructs, the long construct (L-CRABP-LacZ) contained a 3 kb EcoRI-NarI fragment derived from the 5'-flanking region of the mouse CRABP gene and the short construct (S-CRABP-LacZ) contained a 583 bp fragment (PstI to NarI) from this region in front of the reporter sequence (left of Fig. 2). To examine LacZ expression from the fusion gene in transgenic mouse embryos, F₀ embryos at gestation day 12 were fixed and stained for LacZ activities. The expression frequencies in F₀ embryos are shown in the right column of Fig. 2. For the long construct (L-CRABP-LacZ), approximately 66% (6 out of 9) of F₀ transgenic mouse embryos at day 12 show a specific pattern of LacZ expression (see below). In contrast, none from the 12 F₀ transgenic mouse embryos injected with DNA of the short construct (S-CRABP-LacZ) has shown any specific LacZ expression at day 12, indicating that DNA of the short fragment does not contain enough information for the reporter gene LacZ expression. To confirm the lack of LacZ expression from this short construct, more F₀ transgenic embryos of the short fusion gene were generated at different days of gestation, including days 11, 13 and 15, and none of them showed LacZ activities. This also provides a negative control for LacZ expression from transgenic mouse embryos injected with DNA of the long construct. Fig. 3 shows three representative F₀ transgenic embryos (A, B, and C) of the L-CRABP-LacZ construct with a specific LacZ expression pattern at gestation day 12.

The non-transgenic embryos from all the litters have shown no LacZ activities and one (embryo D) is included for comparison. Major areas of LacZ expression at this stage include the eyes, the CNS, the internal organs (heart, liver and intestine), the limbs and, to a less extent, the tip of the nose. Among the nine F₀ transgenic embryos, six are expressors and have shown very similar patterns of LacZ expression at the same stage, although minor differences in the intensity of blue stain exist.

Limited information for the expression patterns of endogenous CRABP is available from studies using in situ hybridization (Dolle et al. 1989; Perez-Castro et al. 1990).

![Fig. 2](image)

Fig. 2. The structures of fusion genes and their frequencies of expression in F₀ transgenic mouse embryos. S-CRABP-LacZ fusion contains the PstI to NarI fragment and L-CRABP-LacZ fusion contains the EcoRI to NarI fragment (Fig. 1). The LacZ reporting sequence contains the E. coli LacZ structural gene fused in frame to the ATG codon of the mouse Hox1.3 gene where the regulatory sequence (upstream of the XhoI site) has been deleted, and has shown no LacZ expression in transgenic mouse embryos (Zakany et al. 1988). N/X is the junction of blunt-ended NarI and XhoI, arrows indicated by H and P are the sites (HindIII and PstI, respectively) used to generate the probe for the RNAase protection assay. This probe will protect 156 nt long RNA fragments (the bar labeled 156 nt) only from RNA transcribed from the fusion gene. The column on the right shows expression frequencies of these fusion genes in F₀ transgenic mouse embryos at day 12 of gestation.
Fig. 3. LacZ expression pattern of L-CRABP-LacZ transgenic mouse embryos at day 12. Three representative F₀ transgenic mouse embryos of L-CRABP-LacZ, at gestation day 12 (embryos A, B, and C), as well as a non-transgenic embryo (D) from the same litter as embryo A are shown here. A, B, C and D are the whole-mount lateral views; A', B', C' and D' are the internal views of these sagittally sliced embryos.
Fig. 4. Temporal and spatial expression of L-CRABP-LacZ fusion gene in transgenic mouse embryos. Embryos are dissected at different days of gestation and stained for LacZ activities as described in the text. Transgenic embryos, as well as non-transgenic embryos for control, from established lines of L-CRABP-LacZ have been systematically assayed for LacZ activities. Both the whole mount and sagittally sliced embryos are observed after staining. A (day 10), B (day 11), C (day 12), D (day 13), E (day 14) and F (day 15) are pictures of the whole mounts. A', B', C', D', E' and F' are pictures of the internal views from the sagittally sliced embryos. A", B", C", D", E" and F" are pictures of the whole mounts of non-transgenic embryos for each time point after staining. Magnifications for embryos of days 10, 11 and 12 are two-fold higher than those for embryos of days 13, 14 and 15.

Fig. 5. Histological sections of transgenic embryos showing staining in the developing eyes. Stained embryos were embedded in OCT compound and 10 micrometer sections were cut with a cryotome and observed under a microscope. A, B, C and D are from frontal sections of embryos at days 10, 11, 12, and 12.5, respectively and show blue stain in the inner layer of the retina. E is from a cross section of an embryo at day 13, showing expression in the retina (R), lens (L) and the optic stalk (OP). F is the two-fold magnification of E. Magnification 100× for A to E, 200× for F.
Fig. 6. Histological sections of transgenic embryos showing staining in internal organs and the CNS. A and C are from sagittal sections of day 12 embryos, with 15× and 12.5× magnification, respectively. The staining at the tissue level in the boxed areas is seen under higher magnification as shown in B (100×), as well as D (40×) and E (100×). H, heart; L, liver.
Table 1. A summary of L-CRABP–LacZ expression in transgenic mouse embryos at various stages of gestation

<table>
<thead>
<tr>
<th>Gestation day</th>
<th>CNS</th>
<th>Eyes</th>
<th>Internal organs (heart, liver, intestine, vessels, and urogenital structure)</th>
<th>Limbs</th>
<th>Nose</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>11</td>
<td>a</td>
<td>b</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>12*</td>
<td>a</td>
<td>b</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>13</td>
<td>-</td>
<td>+</td>
<td>-c</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>14</td>
<td>-</td>
<td>-</td>
<td>-c</td>
<td>+d</td>
<td>+e</td>
</tr>
<tr>
<td>15</td>
<td>-</td>
<td>-</td>
<td>-c</td>
<td>+d</td>
<td>-</td>
</tr>
<tr>
<td>16–18</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

a Overall expression is decreased as compared to day 10 and day 11.  
b Expression is seen in mesencephalon, myelencephalon and spinal cord.  
c Expression can only be seen in the inner wall of intestine on thin sections, but background staining exists in these areas.  
d Expression is restricted to the digits, especially skin and the distal end which is mainly mesenchymal tissue.  
e Expression is restricted to the hair follicles of the nose.

1989; Vaessen et al. 1990) and immunostaining (Dencker et al. 1990; Maden et al. 1988). When compared to these results, this L-CRABP–LacZ fusion gene expression pattern in transgenic mouse embryos shows discrepancies, most notably in the lack of graded expression in the limb buds and the relatively low levels of expression in the CNS and the nose. Therefore, the 3 kb DNA fragment from the 5'-flanking region of the mouse CRABP gene contains only parts of the regulatory information for its expression in the embryos.

**L-CRABP–LacZ fusion gene expression is temporally and spatially specific in transgenic mouse embryos**

To examine L-CRABP–LacZ expression in transgenic mouse embryos systematically, several transgenic lines were established and the F1 or F2 transgenic embryos at different gestation days were derived from these founders and stained for LacZ activities. Both the external and internal staining of these embryos were examined with a stereoscope. Staining at the level of tissue specificity was observed with a microscope on thin sections. From examination of these transgenic lines, it was found that the temporally and spatially specific patterns of L-CRABP expression in transgenic mouse embryos were very consistent among these lines. The results are shown in Figs 4, 5 and 6, and are summarized in Table 1. Fig. 4 shows the typical L-CRABP–LacZ expression patterns in whole mounts of transgenic mouse embryos from gestation day 10 to day 15. At days 10, 11 and 12, expression is found in many areas, including the eyes, the CNS, the limbs, the nose, internal organs such as heart, liver, intestine and the urogenital structure. At day 12, the level of expression decreases, particularly in the limbs and the nose. As embryos develop, expression is restricted to the digits of the limbs and the hair follicles of the nose, with little expression in the eyes, the CNS or internal organs. By day 15, expression is further restricted to the tip of the nose and the digits of the developing limbs. For embryos from gestation day 15 up to day 18, blue stain is only seen in the intestine (data not shown) which also shows background staining. Very similar and reproducible patterns of expression, both temporally and spatially, have been observed in three out of the four transgenic lines that express the long fusion gene and the results are summarized in Table 1. In one line, LacZ expression is not stable. For more details of tissue-specific expression of this fusion gene, thin sections have been obtained from stained embryos and observed under a microscope. Fig. 5 shows expression in the eyes of embryos from day 10 to day 13. Specific expression in the developing eyes is restricted to the inner layer of the retina opposite to the pigment epithelium during this period of development. By day 14, no significant expression in the eyes is found in the sections (data not shown), which agrees with the results observed from the whole mounts. Interestingly, expression is also seen along the optic stalk (labeled as OS in E and F) and the lens (labeled as L in E). As observed from the sliced embryos with a stereoscope, expression in the internal organs on the sections is found in heart, liver, aorta, the inner wall of intestine and the developing urogenital structure, and the CNS. Fig. 6 shows staining in the heart and the liver (labeled as H and L, respectively, in B), and faint but visible staining in the CNS (indicated by arrows in D and E) of embryos at day 12 of gestation. In contrast, sections from similarly stained non-transgenic mouse embryos do not show Lac activities in these areas (data not shown).

**Detection of transcripts from fusion gene with RNAase protection assays**

To confirm the presence of the LacZ protein expressed from the fusion gene L-CRABP–LacZ, RNAase protection assays (Fig. 7) were conducted to detect RNA transcripts derived from the fusion gene in transgenic embryos. An L-CRABP–LacZ-specific probe was used in a RNAase protection assay on RNA samples prepared from tissues of transgenic embryos,
Fig. 7. RNAase protection assays for expression of the fusion gene and the endogenous CRABP gene. RNA samples include day 12 transgenic embryos (1), the limbs of day 13–15 transgenic embryos (2), the placentae of day 12 transgenic embryos (3), day 12 non-transgenic embryos (4), yeast tRNA (5) and P19 cells induced with RA (6). Lane M is the HpaII-digested pBR322 DNA used as size standard. The amount of RNA used is 100 µg for samples 1–4, and 20 µg for samples 5 and 6. The right portion labeled under fusion gene is the result using the L-CRABP–LacZ-specific probe (see Materials and methods). The protected 156 nt fragment specific for the fusion gene is indicated by an arrow on the right. The left portion labeled under mouse CRABP is the result using the mouse CRABP-specific probe (see Materials and methods). The 147 nt long fragment specific for the mouse endogenous CRABP is indicated by an arrow on the left. The exposure time of X-ray films is for two days.

including whole embryos at day 12 (lane 1), limb buds of day 13–15 embryos (lane 2) and placentae of day 12 embryos (lane 3), from established L-CRABP–LacZ transgenic lines. For control, RNA samples from non-transgenic embryos at day 12 (lane 4) and RA-induced P19 embryonal carcinoma cells (lane 6) which have been shown to contain high levels of CRABP transcripts (Wei et al. 1989, 1990), as well as yeast tRNA (lane 5) were included in the same assay. As shown in the right portion of Fig. 7, RNA fragments of 156 nt length, representing the protected fusion transcripts, are detected in the RNA samples of both day 12 embryos (lane 1) and the limbs of day 13–15 embryos (lane 2) from the transgenic group, but not in RNA prepared from either the non-transgenic embryos (lane 4) or the placentae of transgenic embryos (lane 3). The control groups, P19 RNA (lane 6) and yeast tRNA (lane 5), do not show this specific RNA fragment either. As an internal control, in the left portion of Fig. 7, the mouse CRABP-specific probe protects the correct 147 nt long RNA fragments in RNA samples from both transgenic and non-transgenic groups (lane 1, 2 and 4), and also in RNA from RA-induced P19 cells (lane 6). As expected, the negative control, yeast tRNA, is not protected by this mouse CRABP-specific probe. Therefore, L-CRABP–LacZ fusion gene expression in transgenic mouse embryos is further confirmed at the RNA level and it is concluded that the 3 kb long DNA fragment located 150 nt upstream from the transcription initiation site of the mouse CRABP gene contains certain regulatory information for its spatial and temporal expression in mouse embryos.

Discussion

We have identified a 3 kb DNA fragment from the 5'-flanking region of the mouse CRABP gene, which contains regulatory information for most of the spatially and temporally specific expression of this gene in developing mouse embryos.

We have first determined this 3 kb DNA sequence which extends the 5’-end of the promoter region of this gene from previous studies. Analyses on the DNA sequence in this region reveal nine pairs of inverted repeat sequences in addition to the five copies of GC boxes that have been identified previously (Wei et al. 1990). By taking the advantage of a well-characterized LacZ reporter sequence, which has been successfully used to study the regulatory elements for mouse homeobox gene expression during embryonic development (Tuggle et al. 1990; Zakany et al. 1988), we have constructed fusion genes containing DNA fragments from the 5'-flanking region of the mouse CRABP gene
fused upstream to the LacZ reporter sequence. Transgenic mouse embryos of the large construct (L-CRABP—LacZ) containing the 3 kb DNA fragment of the mouse CRABP genomic sequence have shown spatially and temporally specific LacZ expression, whereas those of the short construct (S-CRABP—LacZ) containing only a 583 bp fragment (PstI—Narl) from the same region of the CRABP gene have not shown any specific LacZ expression. Hence, it is tempting to speculate that this 3 kb DNA fragment contains temporal and spatial information for the expression of this gene in mouse embryos and that the regulatory region must extend further upstream of the PstI site in this region.

The expression frequency of the long construct is reasonably high, with greater than 60% of independent transgenic lines showing the consistent LacZ expression pattern for either the F0 embryos or embryos from the established lines. The patterns of expression are relatively stable as three out of the four transgenic lines have shown very reproducible expression patterns in more than 95% of their F1 or F2 embryos. Thus, this transgene can be stably inherited and its expression is consistent in the mouse embryos from different lines throughout the development.

The overall pattern of expression of the CRABP gene as detected with this LacZ reporter is similar to those detected with either in situ hybridization (Dollé et al. 1989; Perez-Castro et al. 1989; Vaessen et al. 1990) or immunological staining (Dencker et al. 1990; Maden et al. 1988). However, there are certainly discrepancies in the results obtained with different methods. The most apparent discrepancy is the relatively low levels of expression in the limbs, nose and CNS, as well as the lack of graded expression in the limb buds, of these transgenic embryos particularly at day 12. Observed from the sections of transgenic mouse embryos, expression in the limbs and nose is mainly in the skin and the frontal ends (the tips) and no gradient of expression in the limb buds as detected by immunostaining is found. It is possible that information for graded expression in the limbs and for the high levels of expression in the nose and the CNS resides outside this 3 kb DNA fragment, or the information is encoded at other levels of gene regulation such as translation. Another possibility is that strong expression as detected with either antibodies or RNA probes but not with this LacZ reporter system in these areas is in fact encoded by other homologous CRABP genes. A second CRABP has been recently identified in neonatal rats (Bailey and Siu, 1988) and chick embryos (Kitamoto et al. 1989), and one CRABPII cDNA has been cloned from a mouse embryonic cDNA library (Giguere et al. 1990), which is highly homologous to this mouse CRABP studied here. Finally, during the construction process of these fusion genes, four out of the five GC boxes near the transcriptional initiation site have been deleted due to the lack of restriction sites in the region between the last GC box and the translational initiation site, which may also account for the relatively low level of expression in some tissues as detected with this fusion gene approach. These possibilities are currently under investigation by using fusion genes containing larger pieces of DNA fragments from the genomic sequence.

Expression of LacZ from the fusion gene in transgenic embryos is confirmed further by a RNAase protection assay that detects the specific RNA fragments of 156 nt length from the fusion transcripts. An interesting observation is that, from both the transgenic and non-transgenic groups, the endogenous CRABP transcripts are present at similar levels in day 12 embryos (lane 1 and 4 of the left portion in Fig. 7). This would suggest that the presence of the transgene, and hence extra copies of the 3 kb long 5'-flanking region of CRABP gene, is not competing out all the trans-acting factors for regulating endogenous CRABP transcription. However, it is still possible that this 3 kb DNA fragment is not sufficient to trap all the regulatory factors involved in transcription for this gene.

This is the first study to report a DNA fragment conferring spatial and temporal information for the expression of the mouse CRABP gene during embryonic development. Using this system, it is now possible to dissect specific regulatory elements involved in the expression of this gene during embryonic development.

This work was supported by NMRP046 from Chang Gung Medical College, and NSC79-0412-B182-01 and NSC80-0412-B182-09 from NSC of R.O.C. to L.-N.W.

References


(Accepted 25 March 1991)