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CELLULAR RETINOIC ACID BINDING PROTEIN TYPE II WAS PREFERENTIALLY LOCALIZED IN MEDIUM AND POSTERIOR PARTS OF THE PROGRESS ZONE OF THE CHICK LIMB BUD

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The expression and distribution of cellular retinoic acid binding protein II (CRABP II) was examined in chick limb buds. CRABP II was detected in the limb buds at Hamburger and Hamilton (1) stage 21 and the amount of CRABP II was gradually increased during stages 21-27 and thereafter decreased. CRABP II was mainly located in the progress zone, and the dorsal and ventral premuscular mass in the proximal region of the limb buds at stage 23. CRABP II was preferentially localized in the medium and posterior parts rather than the anterior part of the progress zone; The content of CRABP II in the medium and posterior parts was 8-9 times more than that in the anterior part.

Retinoic acid, the most biologically active natural metabolite of vitamin A, was postulated as a natural morphogen determining the digit pattern in chick limb buds (2-5). Since then, great attention has been focused on the distribution of cellular retinoic acid binding protein (CRABP) and retinoic acid receptors (RARs) in the limb buds in the early developmental stages (6-9). With respect to RARs, the expression of these RARs could be spatio-temporally regulated during development of mouse limb buds. Until now, three different mammalian RAR genes, termed α, β and γ, that are members of the erb A superfamily (10-11), have been identified (12).

The presence of isoforms of CRABP was recently demonstrated in chick embryos by us, i.e., chick CRABP I and II (13). CRABP II is specifically expressed in muscle and bone in chick embryos, while CRABP I is expressed

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in the central nervous system (14). Unlike RARs, however, the biological functions of CRABP have not been sufficiently clarified. The limb bud is a model system for investigation of the development of distinct tissue primordia from a mixture of precursor cells of myogenic cells and chondrogenic cells. As an approach to clarifying the biological functions of isoforms of CRABP, we tried to examine the distribution of CRABP II, a major isoform of CRABP (7, 8), in the limb buds in the early developmental stages.

In this study, we demonstrated that CRABP II was located in the dorsal and ventral premuscle masses, and in the medium and posterior parts of the progress zone of the chick limb buds at stage 23.

MATERIALS AND METHODS

Immunoblot analysis

The progress zone of the limb buds was obtained from 400 chick embryos at stage 23 and was dissected into three parts, i.e., anterior, medium and posterior, as shown in Figure 3A. The limb buds at various developmental stages and the progress zone of the limb buds at stage 23 were homogenized with 5 vol. of PBS, followed by centrifugation at 100,000 x g to obtain the supernatants. After the protein value of each sample was estimated by Bio Rad Kit, each sample (100 μg) was subjected to 15 % SDS-polyacrylamide gel electrophoresis according to Laemmli (15). Proteins in the gels were electrophoretically transferred to the nitrocellulose sheets as described by Towbin et al.(16). Anti-CRABP II antisera which specifically react with CRABP II, but not CRABP I (13), were used for the detection of CRABP II. Anti-CRABP antisera, which can not distinguish between two isoforms, were used for the detection of CRABP. Immunoreactivities on the sheets were detected with ABC kit (Vecstastain, Vector, USA) and 4-chloro-1-naphthol as reagents, and then were assigned arbitrary numerical valves based on densitometric scanning. The densitometry was performed on an image analyzer (TIAS-100, ACI, Japan).

Immunohistochemistry

Limb buds of chick embryos at stage 23 were fixed with 4 % paraformaldehyde in PBS, and then embedded into paraffin. The serial sections (5 μm) were used for the immunohistochemical detection of CRABP II. The immunoreactivities of anti-CRABP II on the sections were examined by ABC-kit (Vecstastain, Vector, USA) and 3,3'diaminobenzidine as reagents according to a procedure described previously (17). The specificity of the immunostaining was examined by replacement of the primary antibody with pre-immune serum.

RESULTS

CRABP II was detected in the limb buds during stages 21-23. The amount of CRABP II in the limb buds was increased during stages 21-27 and thereafter gradually decreased during development (Figure 1).

At stages 23, CRABP II-positive regions were gradually, but not completely, being separated into proximal and distal regions during

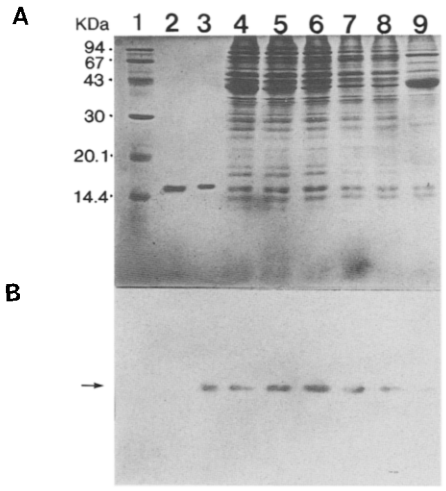

Figure1.
The developmental changes of the expression of CRABP II in the chick limb buds. (A) SDS polyacrylamide gel (15 %) electrophoresis of the cytosolic proteins (100 μg) of chick limb buds during development. Proteins were stained with Coommassie Brilliant Blue. (B) Immunoblot analysis of CRABP II in the chick limb buds during development. An arrow shows the position of CRABP II. 1 μg and 0.1 μg of CRABP I and II were used for the detection by Coommassie Brilliant Blue staining (A) and by immunoblot analysis (B), respectively. Lane 1, the molecular weight markers of proteins; lane 2, CRABP I; lane 3, CRABP II; lane 4, stage 21-23; lane 5, stage 24-25; lane 6, stage 26-27; lane 7, stage 28-29; lane 8, stage 30-31; lane 9, stage 32-34.

development of condensation of the cartilage in the limb bud. In the distal regions, a proximodistal gradient of CRABP II was clearly observed (Figure 2). CRABP II was mainly located in the progress zone (Figure 2A, 2C, 2E) and was most strongly expressed in the region just behind the apical ectodermal ridge (AER), but it was weakly positive in the AER. In the proximal regions, CRABP II was positive in the dorsal and ventral areas (Figure 2C, 2G), into which somitic cells will be migrated from the myotome. The central core of the proximal region of the limb bud, in which the cartilage began to condense, was CRABP II-negative (Figure 2C, 2F, 2G). The distribution of CRABP II in the three-dimensions of the limb bud at stage 23 was reconstructed on the basis of the immunostainings of the sagittal, longitudinal and transverse serial sections of limb buds as shown in Figure 2H.

In addition to the proximodistal gradient, a gradient of CRABP II across the anteroposterior axis was observed (Figure 2A). Unlike the gradient of CRABP previously reported (6), CRABP II was preferentially localized in the medium and posterior parts of the progress zone. The content of CRABP II in the medium and posterior parts was 8-9 times higher than that in the anterior part (Figure 3B), while that of CRABP was almost equal in the anterior,

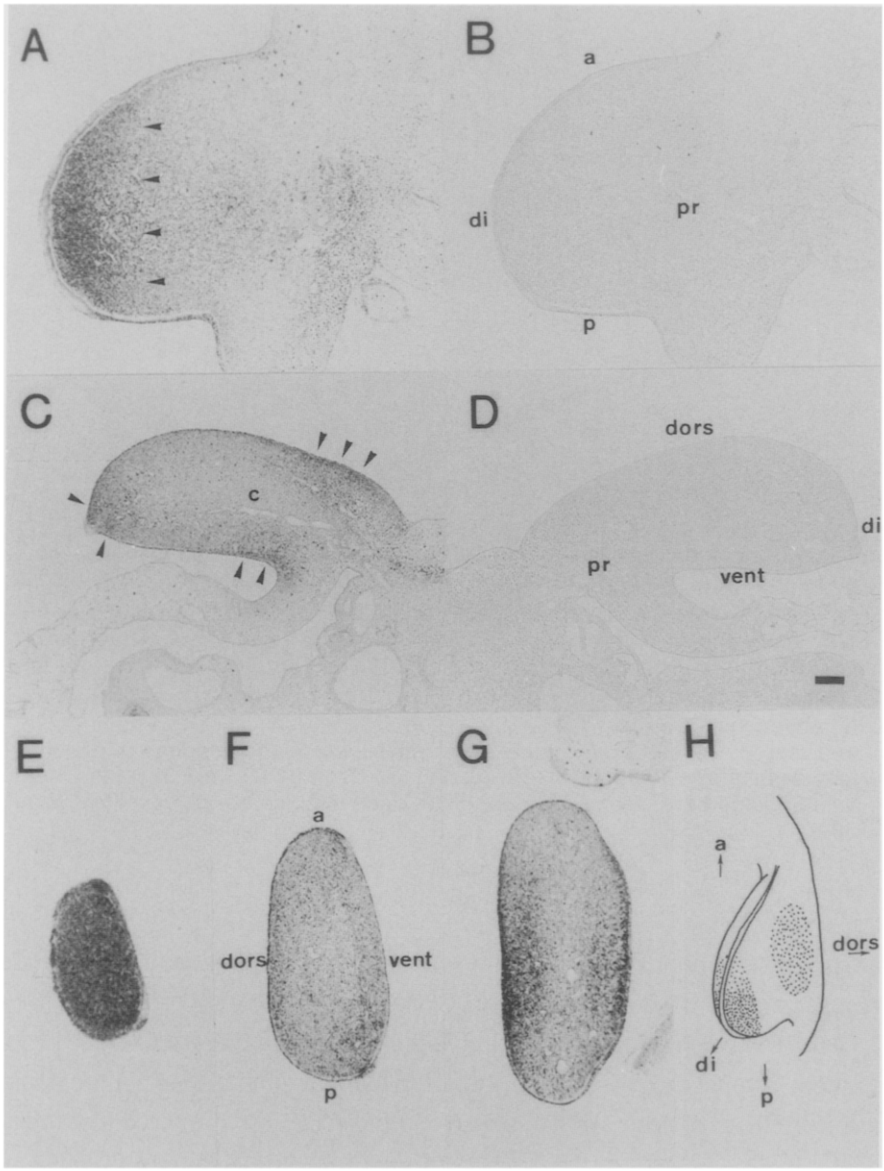

Figure 2.
Distribution of CRABP II in a chick limb bud at stage 23. A sagittal (A, B), longitudinal (C, D) and transverse (E, F, G) sections of a chick limb bud at stage 23 were immunostained with anti-CRABP II (A, C and E-G), and with pre-immune serum (B, D). Transverse sections (E, F and G) show distal, that is the progress zone, middle and proximal region of the limb bud, respectively. Three-dimensional distribution of CRABP II in the limb bud at stage 23 (H). Bar indicates 100 μm. Arrowheads indicate the CRABP II-positive regions in limb buds. a, anterior; di, distal; dors, dorsal; p, posterior; pr, proximal; vent, ventral directions; C, region of cartilage condensation.

medium and posterior parts (Figure 3C, 3D); i.e., the content of CRABP II in the anterior, medium and posterior parts was 0.8, 7.4 and 6.9, respectively, while that of CRABP in these three parts was 6.6, 7.8 and 7.2, respectively.

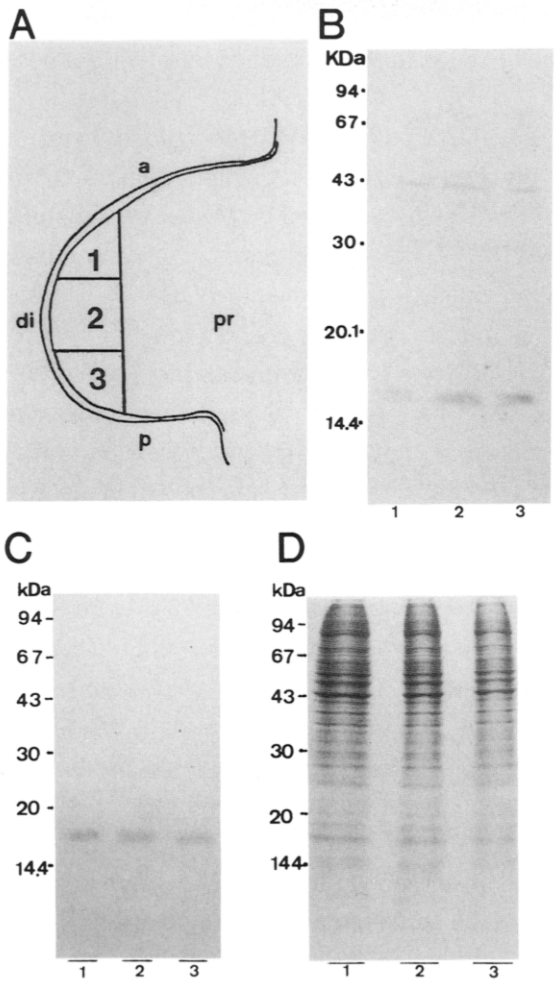

Figure3.
Expression of CRABP II and CRABP in the anterior, medium and posterior part of the progress zone of chick limb bud at stage 23. The progress zone of the limb buds at stage 23 were dissected into three parts, anterior (1)-, medium (2)- and posterior (3)-part as shown in (A). Cytosolic protein (100 μg) samples from the three parts of the progress zone of limb buds were subjected to SDS-polyacrylamide gel (15 %) electrophoresis and immunoblot analysis using anti-CRABP II (B) and anti-CRABP (C). The proteins in the SDS-polyacrylamide gels were stained with Coommassie Brilliant Blue (D). Lane 1, anterior part; lane 2, medium part; lane 3, posterior part.

DISCUSSION

Two different functions of CRABP have been proposed; CRABP may act as a shuttle, transferring retinoic acid from cytosol to nuclei (18), or it may compete with the nuclear receptors for free retinoic acid and regulate the intracellular concentration of free retinoic acid (6). The latter was proposed on the base of the observation that CRABP is spatially distributed

with the concentration gradient along the anteroposterior axis of limb buds in the opposite direction to the concentration gradient of retinoic acid (5).

However, several contradictory observations have been reported on the concentration gradient of CRABP in the limb bud. A CRABP gradient from anterior to posterior was firstly reported by Maden et al. before discovery of the isoforms of CRABP (6). Dolle et al. did not find an anteroposterior gradient of CRABP I transcript but found a gradient of CRABP I transcript along the proximodistal axis of mouse limb buds (9). On the other hand, we and Giguère et al. found that CRABP II is highly expressed in chick and mouse limb bud, respectively (7, 8, 19). In the present study, we found that CRABP II was preferentially localized in the medium and posterior parts rather than the anterior part of the progress zone of the limb bud at stage 23 in addition to the proximodistal gradient (Figure 2, 3B), but could not find any significant difference between the content of CRABP in the anterior and posterior parts of the progress zone of the limb buds at stage 23 (Figure 3C, 3D).

These results suggested that the isoforms of CRABP were distributed with different anteroposterior gradient in the chick limb bud. The gradient of CRABP from anterior to posterior demonstrated by Maden et al. may be due to the distribution of CRABP I alone or the distribution overlapped with CRABP I, II and the unidentified isoforms. If so, we should reconsider the respective functions of CRABP I and II in the limb buds during development. With regard to this, it is noteworthy that the gradient of CRABP II was consistent with that of the concentration of retinoic acid (5), although CRABP II was not localized in the zone of polarizing activity, from which the morphogen is considered to be produced; The concentration of retinoic acid in the posterior part is 2.5-fold higher than that in the anterior part (5). The expression of chick hox 4 cluster genes, which are regulated by retinoic acid, are also consistent with that of CRABP II in the limb bud; Chick hox 4 cluster genes are expressed as concentric circles with the posterior-distal region of the progress zone as the core (20). In contrast with CRABP, RARs are evenly distributed throughout the limb buds of chick embryos. There was no difference between the contents of RARs in the anterior, medium and posterior parts of the limb buds (8). Thus, CRABP I and II may be involved in the production of the anteroposterior gradient of retinoic acid in the progress zone and may give respective positional information on the digit pattern of the limb bud.

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