INTRODUCTION
Retinoic acid (RA) and its derivatives play vital roles in the regulation of growth, differentiation, and morphogenesis. During development, several roles have been proposed for RA, including anterior-posterior patterning of the limb, promotion of neural crest outgrowth and survival, and promotion of neurite outgrowth. Traditionally, research in the retinoid field has focused primarily on the effector system, the expression patterns of RA receptors and binding proteins, with less emphasis on synthesis. However, recent findings have found RA synthesis to be under tight spatiotemporal control and thus implicate the importance of RA metabolism in the developing embryo.  \(^5\) The steps of RA metabolism include: (i) the cellular uptake of retinol, followed by a reversible oxidation of retinol to retinaldehyde, and (ii) an irreversible oxidation of retinaldehyde to RA. This second step is mediated by a novel dehydrogenase, retinaldehyde dehydrogenase-2 (RALDH-2), which is specific for retinaldehyde. However, current research concentrating on in vivo studies have not found a direct source of RALDH-2 in the limb proper. Rather, they postulate that RA is produced in the flank and diffuses to the limb. \(^3\) \(^4\) We have used an in vitro model of mesenchymal chondrogenesis in micromass high density limb cell cultures to study the regulation of RA synthesis. We hypothesize that RA is active in the limb bud, not only for patterning, but also differentiation and maturation, specifically in cartilage formation, and that RA synthesis via RALDH-2 is an important regulatory component.

MATERIALS AND METHODS
Chick Limb Bud Micromass Cultures: Fertilized White Leghorn chicken eggs (CBT Farms, Chesterville MD) were incubated at 37°C in a humidified egg incubator until Hamburger and Hamilton stage 23-24 of development. The limb buds were surgically removed from each embryo, pooled and digested. The reaction was stopped and individual cells were isolated from undigested tissue using a cell strainer. The isolated limb bud cells were pelleted and resuspended to a volume of 20x10^6 cells/ml for micromass cultures. Three 10 µl drops were placed into each well of a 6 well tissue culture plate and the cells were allowed to adhere. Alcian Blue Stain: The extent of chondrogenesis in the micromass cultures was assessed by Alcian blue staining. Peanut Agglutinin (PNA) Stain: The presence of pre-cartilage condensations was detected by PNA staining. Whole Mount In Situ Hybridization (WISH): To localize the expression pattern of cRALDH-2 mRNA, an antisense digoxigenin-labeled probe was constructed against cRALDH-2, and micromass cultures were incubated with this probe to allow for hybridization of complementary sequences. The expression pattern was visualized using a nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphosphate (NBT/BCIP) reaction. Enzyme Histochemistry: To determine the spatiotemporal enzymatic activity of cRALDH-2, unfixed micromass cultures were incubated in a staining medium containing retinol, NAD^+*, phenylethosulfate (PMS), and NBT to identify areas of reduced tetrazolium salts by NADH.

RESULTS AND DISCUSSION
The micromass cell culture system is an effective mimic of mesenchymal condensation and chondrogenesis (Fig. 1). This is evident by Alcian blue staining of negatively charged sulfated groups of glycosaminoglycans (GAGS), secreted in the extracellular matrix of cartilage nodules. Additionally, PNA, which binds to cell surface galactosyl epitopes stains cells with chondrogenic potential and are undergoing condensation. Our data reveal that RALDH-2 gene expression is present in the condensing cell cultures (Fig. 2). Additionally, the site of expression is in the nodules of cellular condensation. RALDH-2 enzymatic activity was also localized to the center of the micromass, specifically in the condensing cells (confirmed by counterstaining with Alcian blue, not shown; Fig. 3). A number of previous studies have reported the absence of RA synthesizing enzymes in the limb bud, and suggests that diffusion from the flank is the source of RA for the limb. \(^5\) On the other hand, the endogenous levels of retinol, a substrate for RA synthesis is high in the limb bud. \(^2\) \(^3\) \(^4\) The results provided here strongly suggest that RALDH-2, a key enzyme of RA biosynthesis, is active in the condensing and chondrifying limb mesenchyme, and is likely to be important in specifying the biological activity of RA in limb formation. Additionally, detectable levels of RA have been reported in chick embryonic wing and leg buds, with higher levels in the posterior limbs. \(^3\) Taken together, these results provide strong evidence of RA synthesis in the developing limb.

REFERENCES

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