

Distribution of endogenous retinoids, retinoid binding proteins (RBP, CRABPI) and nuclear retinoid X receptor β (RXR β) in the porcine embryo

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(Received 7 December 2001; accepted 15 May 2002)

Abstract — Retinoids are important signalling molecules in the development of limbs and in the determination of the anterior-posterior orientation of the embryo. The present study examined the content and distribution of retinoic acid, retinol and retinyl esters in porcine embryos during early gestation (gestation days 22–30) macroscopically and microscopically by its autofluorescence and by HPLC. Macroscopically, the yellowish-greenish autofluorescence characteristic of vitamin A was observed in tissues affected by morphogenesis, such as the limbs, in a spatial and temporal manner. Changes in the intensity of autofluorescence in the limbs paralleled changes in the concentration of retinoids in these structures. In the limbs and the body, retinol, retinyl palmitate, and all-*trans*-retinoic acid but neither the isomers of all-*trans* retinoic acid nor other retinoid metabolites were detected. In addition, the distribution of specific retinoid-binding proteins was investigated; these are involved in vitamin A transport, metabolism and signal transduction. Immunoreactive retinol-binding protein as well as cellular retinoic acid binding protein type I were only localised in the mesonephros, while the retinoid X receptor β was widely distributed in most of the tissues and organs of the embryo throughout the time period investigated. The combination of autofluorescence and HPLC analysis allowed for the first time to attribute the yellowish-greenish autofluorescence in specific regions of the embryo to vitamin A, and offers a method to study the local cellular distribution of retinol and/or retinyl esters as well as their concentrations in embryonic tissues.

retinoid / vitamin A / binding protein / nuclear receptor / autofluorescence / embryo / pig

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1. INTRODUCTION

In addition to its essential role in vision, reproduction, differentiation, and maintenance of differentiated tissues, vitamin A has long been recognised to be an essential factor in embryonic development. One of the first observations of the possible function of vitamin A in embryonic development concerned specific congenital abnormalities as a consequence of prenatal vitamin A deprivation in rats [17]. Today, it is well established that retinoic acid is involved in signal transduction pathways via retinoid receptors, which regulates embryonic development [19, 21, 39]. Its role as a signalling molecule in the morphogenic process has been shown in much detail in the development of limbs and the establishment of the primary body axis that extends between the head and the tail [20, 26, 31]. This was first shown by the implantation of exogenous all-*trans* retinoic acid into the anterior limb mesoderm of chicks, which resulted in digit pattern duplications, thus mimicking the activity of the posteriorly positioned zone of polarising activity (ZPA) [33]. Subsequent studies demonstrated that all-*trans* retinoic acid (all-*trans*-RA) is endogenously present in the chick limb bud with higher concentration in the posterior part [30]. It was assumed that digit formation is induced by a concentration gradient (high posteriorly) of all-*trans*-RA along the anterior-posterior axis; all-*trans*-RA is synthesised through the sequential oxidation of retinol and retinaldehyde [31]. The gradient of all-*trans*-RA is strengthened by a reverse gradient (high anteriorly) of cytosolic retinoic acid binding protein (CRABP), which is supposed to lower the concentration of free all-*trans*-RA available for binding to the nuclear receptor [14, 28, 29]. In addition, 3,4-didehydroretinoic acid (ddRA) and its precursor 3,4-didehydroretinol (ddROH) were identified and quantified in chick limb buds [28, 32]. However, numerous differences between chicks and mice exist with respect to the endogenous retinoid profile

in embryonic limb buds. Although all-*trans*-RA and retinol (ROH) were also found in the limb buds of mouse embryos, neither a posterior nor anterior gradient of the concentrations of these retinoids was found, nor were ddRA, ddROH or retinyl esters detected in the mouse limb buds [25, 28], indicating possible differences between mammals and birds.

In the present study, the distribution of endogenous retinoids in early porcine embryos at the Witisch stages 27–31 (days 22 to 30 in gestation) was investigated. For the first time, the identification as well as the localisation of retinoids was possible due to the combination of autofluorescence, a characteristic of retinol and retinyl esters which allowed the localisation of retinoids, and high-performance liquid chromatography (HPLC) which allowed the quantification of individual retinoids. In addition, specific retinoid-binding proteins, such as retinol-binding protein (RBP), cellular retinoic acid binding protein I (CRABP I) and the retinoid X receptor β (RXR β), were determined by immunohistochemistry in the embryos of pigs during early gestation.

2. MATERIALS AND METHODS

2.1. Animals and experimental design

A total of 30 crossbred primiparous gilts (German Landrace x Piétrain) of similar age, weight and genetic background were housed in individual pens. The gilts were inseminated and then slaughtered between days 22 and 30 of gestation, which correspond to the Witisch stages 27–31 at two day intervals (3–4 animals per group) (1st insemination = day 0). Immediately after slaughter at a local abattoir, the uteri were recovered from each sow, cut open, and the embryos were collected. A total of 5 to 16 embryos were collected per gilt. All embryos intended for HPLC analysis were immediately frozen in liquid nitrogen and stored at -80°C until analysis.

2.2. Autofluorescence in whole embryos and whole-mounted cryosections of embryos

Autofluorescence of the embryos was observed macroscopically and microscopically under UV exposure (excitation wavelength: 312 nm) as described previously [27]. Briefly, for fluorescence microscopy, sections (6 μm) were prepared from native frozen tissues with a freezing microtome at $-20\text{ }^{\circ}\text{C}$. The unfixed and unstained specimen was observed with a reflected light fluorescence microscope (Olympus, Model BX40). A 330–385 nm exciter filter and a 420 nm barrier filter were used (Cube U-MWU, Olympus). Frame and focus were controlled under visible light. Autofluorescence was documented by photography (Fujichrome Provia, ASA 1600). The association of the autofluorescence with lipids was tested by treatment of cryosections with *n*-hexane [3].

2.3. Immunohistochemistry

The immunolocalisation of RBP, CRABPI and RXR β was performed in embryonic samples, which were placed immediately after slaughter in a 4% formaldehyde solution. The samples were dehydrated through a graded series (50–96%) of ethanol, embedded in paraffin, sectioned at 2–4 μm , and mounted on glass slides (Super-Frost, Fa. Menzel, Braunschweig, Germany). RBP, CRABP I and RXR β were localised using the peroxidase anti-peroxidase method (PAP) as described previously [27]. Normal rat serum, monoclonal (mouse) anti-retinoid X receptor beta antibody (IgG₁, MOK 13–17, 2 mg·mL⁻¹ in PBS), monoclonal (mouse) anti-cellular retinoic acid binding protein I antibody (IgG2b, C-1), affinity pure rat anti-mouse IgG (H+L; 1.2 mg·mL⁻¹) and mouse PAP (20 mg·mL⁻¹) were purchased from Dianova GmbH (Hamburg, Germany); normal swine serum, rabbit anti-human retinol-binding protein (8.2 g·L⁻¹), swine anti-rabbit serum (160 g·L⁻¹) and rabbit PAP were purchased

from Dako Diagnostica (Hamburg, Germany). All antisera were used as recommended by the company.

2.4. HPLC analysis of retinoids

For HPLC analysis, embryos were dissected free of extraembryonic membranes and separated into liver, forelimbs, hindlimbs and the remaining body. Samples of a varying number of embryos were pooled before assay in order to provide samples of adequate size for analysis.

The samples were analysed by a reversed-phase HPLC method with a gradient elution following sample enrichment with liquid-liquid and solid-phase extraction (thoroughly described by [4]; modified by [35]). All analytical procedures were performed in dark rooms under dim yellow light to prevent photoisomerisation. Shortly, three volumes of isopropanol were added to an accurately weighed amount of sample; however, very small embryonic samples (< 100 mg) were filled up to 100 mg with water before addition of 0.3 mL isopropanol. The tissues were disrupted by pulsed sonication on ice for about 20 seconds, the mixtures were shaken at room temperature for 2 minutes, and precipitated protein was pelleted by short centrifugation at 6500 g. The supernatants were then extracted on solid-phase extraction cartridges prior to introduction into the HPLC system. Separation of the retinoids was achieved within 25 minutes by use of a linear gradient formed from 60 mM aqueous ammonium acetate and methanol. For detection, the UV absorbance of the eluate was measured at 340 and 356 nm by a two-channel SPD-10AV detector (Shimadzu, Duisburg, Germany). HPLC peaks were identified on the basis of the coincidence of their retention times and absorbance ratios (i.e., ratio of peak areas) at the two wavelengths of detection with those of the authentic retinoids. Standard retinoids were kindly provided by Hoffmann-La Roche (Basel, Switzerland). Water for HPLC was purified using a Milli-Q water purification system (Millipore,

Eschborn, Germany); other solvents and chemicals were of HPLC or high purity commercial grade (Merck, Darmstadt, Germany). The preparation and storage conditions of retinoid stock solutions was recently described [36].

3. RESULTS

3.1. Autofluorescence of whole embryos and in whole-mount cryosections

When whole pig embryos were viewed under UV-light (312 nm excitation), a bright yellowish-greenish autofluorescence was observed macroscopically in all embryos from day 22 of gestation onward. Differences in the distribution and in the intensity of autofluorescence were found. At all stages of development examined, fluorescence was localised in the liver, the eye cup and the

neural tube. In the forelimbs and hindlimbs fluorescence appeared faint and to be more uniformly distributed at day 22. From day 26 onward, two to three distinct bands were observed first in the forelimbs (Fig. 1A) and two days later more pronounced in the hindlimbs as well (Fig. 1C). In general, the highest intensity of fluorescence was encountered in embryos from day 22, with greatest intensity at days 26 and 28. At days 30 and 32, the intensity of the autofluorescence was less intensive than at earlier stages. The autofluorescence faded under continuous UV-irradiation of whole embryos within 20 minutes (Figs. 1B and 1D) and in cryosections in less than 1 minute.

In cryosections of embryos at different stages of development, fluorescence microscopy showed a typical yellowish-greenish fluorescence in the liver, the mesonephros, the eye cup, the neural tube, spinal ganglions and in the non differentiated mesenchyme of the forelimb and

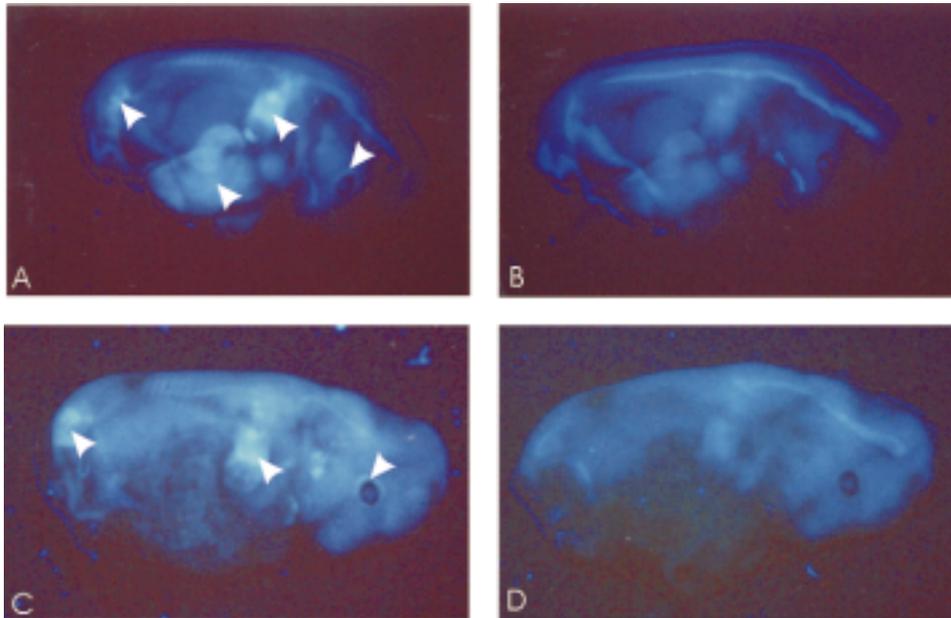


Figure 1. Visualisation of the autofluorescence of vitamin A in whole porcine embryos at gestational days (GD) 26 (A) and 28 (C) before UV-exposure (A, C) and after 20 minutes of continuous exposure (B, D). Arrows indicate the localisation of autofluorescence in the forelimb, the hind limb, liver and eye cup.

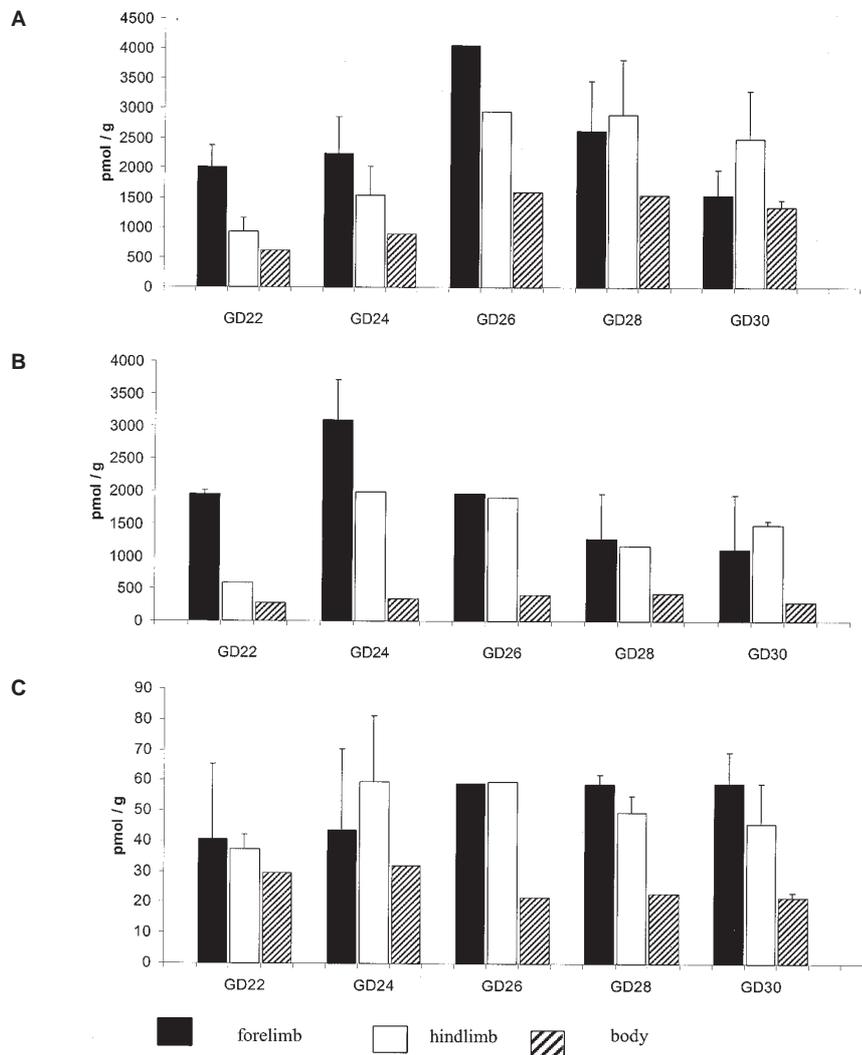


Figure 2. Endogenous concentrations of retinol (A), retinyl palmitate (B), and all-*trans*-retinoic acid (C) in the forelimbs, hindlimbs, and body of porcine embryos from gestational days (GD) 22 to 30. Bars indicate means \pm S.D. ($n = 3$ for forelimb and hindlimb samples, except $n = 2$ for GD 26; $n = 2$ for body samples, except $n = 3$ for GD 30 and $n = 1$ for GD 26).

hindlimb. To investigate the association of the fluorescence with tissue lipids, cryosections of whole embryos were treated prior to microscopy with the organic solvent *n*-hexane in order to remove these lipids. This treatment resulted in the complete disappearance of

the autofluorescence characteristic for vitamin A (data not presented).

3.2. Retinoids in porcine embryos

Analysis of porcine embryonic samples by HPLC showed the physiological

occurrence of all-*trans*-RA, retinol and retinyl palmitate in all embryonic structures examined. Retinol and its esters were the most abundant retinoids present, whereas concentrations of all-*trans*-RA were generally one to two orders of magnitude lower (Fig. 2). Neither any other isomer of RA (13-*cis*, 9-*cis*-, or 9,13-di-*cis*-RA) nor other retinoid metabolites such as the didehydroretinoids ddROH and ddRA were detected. Due to the limited number of samples available, statistical evaluation of the data was not possible, and the results should therefore be interpreted cautiously. In the forelimbs, retinol levels were apparently higher on day 26 than on the other days, whereas in both the hindlimbs and body (except limbs and liver) elevated concentrations of retinol were observed on days 26 to 30 compared to the levels on days 22 and 24. Concentrations of retinyl palmitate increased to highest levels from day 22 to day 24. Thereafter, the retinyl palmitate decreased more rapidly in the forelimb compared to the hindlimb. The levels in the body remained rather constant. Finally, endogenous concentrations of all-*trans*-RA in all three tissues (range of average: 7–20 ng·g⁻¹) remained essentially unchanged during the time period examined, except from a slight increase occurring between days 22 and 26 in the forelimb and the hindlimb. Interestingly, concentrations of endogenous retinoids in the forelimbs and hindlimbs were considerably higher than in body samples. This was more pronounced for retinyl palmitate (e.g., its levels in the limbs were in most cases 3- to 5-fold of those in the body – Fig. 2B), rather than for retinol and all-*trans*-RA.

3.3. Immunohistological distribution of RBP, CRABPI and RXR β in whole-mount sections

Immunoreactive RBP and CRABPI were observed at all investigated days only in the epithelial cells of the peripheral situated

tubuli of the mesonephros. The cytoplasmic localization of RBP and the nuclear localization of RXR β in the mesonephros is shown in Figures 3A and 3B. Immunoreactive RXR β was observed in all investigated porcine embryos at all developmental days in most tissues of ectodermal (neural epithelium, placode of the eye, epidermis), mesodermal (heart, kidney, somites, gonads) and endodermal (gut epithelium, lung, liver) origin (Figs. 3C and 3G)

4. DISCUSSION

The importance of vitamin A as an essential micronutrient in embryonic development has long been recognised, first by the observation of a spectrum of congenital malformations caused by vitamin A deficiency and then through the teratogenic effects caused by acute or chronic intoxication [38]. In pigs, the effects of vitamin A deprivation on specific periods of embryonic development were shown by adding vitamin A to the diet of vitamin A deficient pregnant pigs at specific days of gestation [22]. No malformations were observed when pigs were kept deficient until the day of implantation of the embryo (day 12 of gestation). If the deficiency persisted until day 16, however, malformations were induced in the heart and the thyroid gland. Deficiency until day 18 resulted in additional malformations in the eye, until day 25 in additional malformations in the limb, liver, kidneys and reproductive organs and until day 36 in additional malformations in the pharynx, lungs, diaphragm and gut. These findings demonstrated the importance of retinoids for normal embryonic development in a stage-dependent and tissue-specific manner. The great advance of knowledge through the discovery of retinoid binding proteins and nuclear receptors has further helped to explain the molecular control of these events [13, 19, 39].

Due to difficulties of methodology, however, little information is available

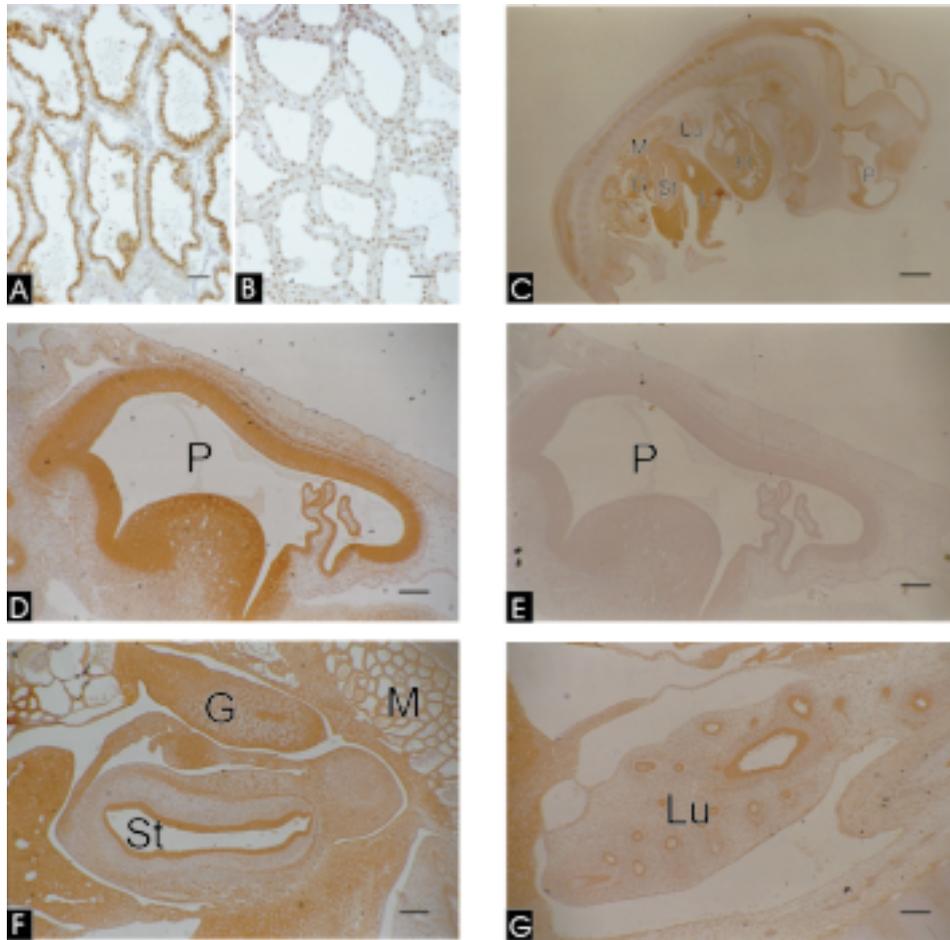


Figure 3. Avidin-biotin staining of porcine embryos incubated with rabbit anti-human RBP (A), RXR β (B-G). **A, B:** RBP and RXR β in the mesonephros at gestational days (GD) 28, showing cytoplasmatic and nuclear localization, respectively (40 \times); **C:** the sagittal section of a GD 28 embryo showing the distribution of RXR β (P – prosencephalon; H – heart; Lu – lung; Li – liver; St – stomach; G – germ epithelial; M – mesonephros, 6.3 \times); **D:** Distribution of RXR β in the prosencephalon (P) at GD 30 and negative control (**E**, 40 \times); **F:** RXR β in the mesonephros (M), germ epithelial (G) and stomach (St) at GD 24 (40 \times), **G:** RXR β in the lung (Lu) at GD 30 porcine embryo (40 \times). Bars represent 159 and 25 μ m for 6.3 \times and 40 \times , respectively.

concerning the tissue distribution and especially the exact localisation of retinoids in embryonic tissue. An easily applicable method to determine the localisation of vitamin A (retinol and retinyl esters) would be helpful for the determination of the distribution of these components within the

developing embryo. Autofluorescence of vitamin A was first noted by von Querner [37] and was extensively studied in the tissues of humans and rats [23]. The results presented in this study are the first application of this technique to whole embryos and to cryosections of embryos. The evidence

supporting the hypothesis that the yellowish-green fluorescence observed in specific regions of the embryo is caused by vitamin A (retinol and retinyl esters) has been recently discussed in detail [27].

The results of the present study suggest that there is vitamin A-dependent autofluorescence in the tissues involved at certain periods tissue or organ development. This is the most obvious in the limbs, which develop during days 22–28 of gestation, starting with the forelimb and followed by the hindlimb. In accordance with autofluorescence, HPLC-derived concentrations of retinol and retinyl esters were higher in the forelimbs and hindlimbs than in the remaining embryonic body (without the liver) and showed the same temporal pattern as the intensity of the autofluorescence, which can be attributed to the amount of retinol and retinyl esters present, but not to retinoic acid. In the limbs, the localisation of the autofluorescence is very close to the developing skeletal elements. For chicks it has been shown that substantial amounts of endogenous retinoids are present in the perichondrial tissue, as has been shown in this study by the localisation of autofluorescence. These retinoids have been shown to influence chondrocyte maturation and enchondral bone formation [11].

In contrast to earlier investigations of the limbs of chickens and mice, the concentrations of all-*trans* retinoic acid (12–19 ng·g⁻¹ corresponding to 43–63 nM) and retinol (0.27–1.18 µg·g⁻¹ corresponding to 939–4115 nM) were higher in the limbs of pigs [28, 30, 32]. The same was true for retinyl palmitate, which has been found in chick limb buds but not in those of mice [24]. Comparable to the situation in mice [28] and rats [5], neither ddRA nor ddROH were detected in pig embryonic tissues. ddRA has previously been detected in the embryos of chickens [7, 32] and rabbits [34]. These variations might be due to differences in the developmental stages examined and the tissue source of samples and/or might

reflect species specific differences [10]. It can be assumed that retinyl esters as well as retinol might serve as a local source for the formation of retinoic acid. The enzymes for the formation of retinoic acid, retinol dehydrogenase, retinaldehyde dehydrogenase, and different isoforms of the alcohol dehydrogenase have been shown to be expressed in the tissues of rodent embryos, which require retinoic acid for normal development e.g. in the heart, the gut and the limbs [1, 2, 8, 9]. The observed tendency of a decrease of retinol and retinyl esters in the limbs with the progress of embryonic development, as indicated by the reduced intensity of autofluorescence and the apparently lower concentrations determined by HPLC, may either be due to a reduced accumulation of these retinoids in these structures and/or a higher rate of oxidation into the active metabolite retinoic acid during embryonic development.

At the early time of embryonic development in pigs, we were able to detect immunoreactive RBP and CRABPI only in the cells of the mesonephros. In the postnatal RBP metabolism, the kidney plays an important role by synthesising RBP in the S₂-Segments and reabsorbing glomerular filtrated RBP in the S₁-Segments of the proximal tubulus [15]. The presence of RBP in the mesonephros at the stage of embryonic development examined may indicate an important role of RBP for embryonal and postnatal renal development [18]. In contrast to the restricted expression of CRABP I in pig embryos, both CRABP I and II are widely distributed in the mesenchymal tissues of mouse embryos of gestational days 8.5–11.5 [12]. On the contrary, RXRβ was widely distributed in pig embryonic tissue. On the contrary to the RAR receptor family, only a few studies have addressed the distribution of the RXRβ receptor in embryonic tissue [6]. In accordance to our results, studies using in-situ hybridisation analyses have shown broad but unique expression patterns of RXRs, which only partially overlapped with the expression

patterns of RARs, suggesting that the RXR family plays critical roles in the diverse aspects of development, from embryo implantation to organogenesis and differentiation [16].

In conclusion, this study presents for the first time results on both the amount and the localisation of vitamin A in the embryos of pigs and shows that the yellowish-greenish autofluorescence in pig embryos may be attributed to vitamin A. Despite some limitations arising mainly from the instability of vitamin A when exposed to UV-light, thereby causing problems to localise the vitamin A precisely in tissue if present at lower concentrations and in more complex structures, the macroscopic and microscopic investigation of the autofluorescence of vitamin A in combination with immunological techniques may provide additional power to study the distribution of vitamin A and its metabolism at a morphological level. It could prove helpful for understanding of the co-ordinate function of retinoid ligands and receptors during embryonic development in species other than the pig.

ACKNOWLEDGMENTS

The authors wish to thank the staff of the experimental farm of the faculty in Oberholz for their support in handling of the animals and Ms. Claudia Plum for her assistance with HPLC analysis. The study was supported by Hoffmann-La Roche Basel, Switzerland and Grenzach-Whylen, Germany. Part of the work reported here was performed at the Department of Physiology, Veterinary Faculty, University of Leipzig and the Institute of Clinical Pharmacology and Toxicology, Freie Universität Berlin, Berlin, both Germany.

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