Function of the retinoic acid receptors (RARs) during development

(II) Multiple abnormalities at various stages of organogenesis in RAR double mutants

Cathy Mendelsohn*,†, David Lohnes*‡, Didier Décimo, Thomas Lufkin§, Marianne LeMeur, Pierre Chambon¶ and Manuel Mark§

Laboratoire de Génétique Moléculaire des Eucaryotes du CNRS, Unité 184 de Biologie Moléculaire et de Génie Génétique de l’INSERM, Institut de Chimie Biologique, Faculté de Médecine, 11 rue Humann, 67085 Strasbourg Cedex, France

*Should be considered as equal first authors
†Present address: Columbia University, Department of Physiology and Biophysics, 630 W 168th Street, New York, NY 10032, USA
‡Present address: Institut de Recherches Cliniques de Montréal, Laboratoire de Biologie Cellulaire et Moléculaire, 110 Avenue des Pins-Ouest, Montréal, H2W 1R7, Canada
§Present address: Brookdale Center for Molecular Biology, The Mount Sinai Medical Center, 1 Gustave L. Levy Place, New York, NY 10029-6574, USA
¶To whom reprint requests should be sent
§To whom correspondence should be addressed

SUMMARY

Compound null mutations of retinoic acid receptor (RAR) genes lead to lethality in utero or shortly after birth and to numerous developmental abnormalities. In the accompanying paper (Lohnes, D., Mark, M., Mendelsohn, C., Dollé, P., Dierich, A., Gorry, Ph., Gansmuller, A. and Chambon, P. (1994). Development 120, 2723-2748), we describe malformations of the head, vertebrae and limbs which, with the notable exception of the eye defects, were not observed in the offspring of vitamin A-deficient (VAD) dams. We report here abnormalities in the neck, trunk and abdominal regions of RAR double mutant mice, which include : (i) the entire respiratory tract, (ii) the heart, its outflow tract and the great vessels located near the heart, (iii) the thymus, thyroid and parathyroid glands, (iv) the diaphragm, (v) the genito-urinary system, and (vi) the lower digestive tract. A majority of these abnormalities recapitulate those observed in the fetal VAD syndrome described by Joseph Warkany’s group more than forty years ago [Wilson, J. G., Roth, C. B. and Warkany, J. (1953) Am. J. Anat., 92, 189-217; and refs therein]. Our results clearly demonstrate that RARs are essential for vertebrate ontogenesis and therefore that retinoic acid is the active retinoid, which is required at several stages of the development of numerous tissues and organs. We discuss several possibilities that may account for the apparent functional redundancy observed amongst retinoic acid receptors during embryogenesis.

Key words: mouse, ontogenesis, retinoic receptor mutants, neural crest, heart, trachea, aortic arches, lung, kidney, genital tract, functional redundancy

INTRODUCTION

Fetuses from rat mothers reared on vitamin A-deficient (VAD) diets exhibit severe congenital malformations known as the fetal VAD syndrome (Wilson and Warkany, 1948, 1949; Warkany et al., 1948; Wilson et al., 1953). These malformations include abnormalities of the eyes, respiratory tract, heart and great vessels, urogenital system and diaphragm, while cleft palate has been reported in the case of VAD pigs (Hale, 1933). Addition of retinol to the diet of VAD dams during pregnancy reversed nearly all of these malformations, a clear demonstration that vitamin A is critical for normal development. Interestingly, the time of retinol administration during pregnancy was critical in order to prevent the appearance of a given malformation, suggesting that vitamin A could be required at several distinct stages of development (Wilson et al., 1953). It is generally believed that retinoic acid (RA) is the active derivative of vitamin A during development, as it is after birth (except for vision, see Thompson et al., 1964; Wald, 1968); however, it has not been shown that RA can rescue VAD embryos with the exception of cardiovascular development in quail embryos (Dersch and Zile, 1993). Furthermore, it is widely accepted that the two families of retinoic acid nuclear receptors (RARs and RXRs) act as transcriptional transducers of the retinoid signal during development. It is also thought that the main isoforms (α1 and α2, β1 to β4, γ1 and γ2) of the three RAR types (RARα, β and γ), which have distinct patterns of expression during embryogenesis, may possess specific transactivation characteristics enabling them to control the expression of specific subset of genes during development (reviewed in Hofmann and Eichele, 1994; Chambon, 1994; Linney and LaMantia, 1994; Kastner et al., 1994; see Introduction of Lohnes et al., 1994 for additional references).

Thus, the highly diverse effects of the RA signal would be accounted for by the multiplicity of functionally distinct receptors (Leid et al., 1992; Chambon, 1994). This possibility
has been recently tested in vivo by generating null mouse mutants for the various receptors (Li et al., 1993; Lohnes et al., 1993; Lufkin et al., 1993; Mendelsohn et al., 1994). Surprisingly, none of the abnormalities characteristic of the fetal VAD syndrome was observed in these mutants, thus suggesting a high degree of functional redundancy amongst the various RARs. Compound RAR mutant mice were then generated to investigate this possibility, and as reported in the accompanying study (Lohnes et al., 1994), the double mutants died during gestation or immediately after birth. In addition, these mutants exhibited a larger number of malformations, including abnormalities characteristic of the VAD syndrome, thus demonstrating that RA is the retinoid signalling molecule used during development and that RARs are indeed transducers of this signal in vivo. We have reported in the accompanying study (Lohnes et al., 1994), the craniofacial and skeletal abnormalities presented by RAR double mutant fetuses. We describe here the visceral abnormalities, and show that RA is required at many stages of development of a given tissue or organ. The implications of the present results and of those reported in the preceding study are discussed with respect to the multiple roles of RA in development and the apparent functional redundancy amongst the RARs.

MATERIALS AND METHODS

RAR double mutants were generated and analysed as described in the accompanying study (Lohnes et al., 1994).

RESULTS

(A) Oesophagotracheal abnormalities

In 18.5 dpc wild-type (WT) fetuses, the right lung (rL, Fig. 1a,h) is composed of four essentially separate lobes, a cranial (CL), a middle (ML) and a caudal lobe (DL) that are located on the right side of the thorax, and an accessory lobe that originates on the right side, but is predominantly located on the left side of the midline (Fig. 1a, and data not shown). In contrast, the smaller left lung (fL, Fig. 1a,h) is not subdivided. In a majority (6 out of 7) of 18.5 dpc αβ^-2 mutants analyzed on serial histological sections (Table 1), and in one fetus analyzed by whole mount, the left lung was absent (Fig. 1c), whereas the right lung (rL, Fig. 1c;i; Table 1, and data not shown) was markedly hypoplastic and consisted of 3 or 4 lobes. No portion of the right lung ever crossed the midline, due to hypoplasia of the accessory lobe. In one αβ^-2 and in some αβ^-2/^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^
of the tracheobronchial tree and of the alveoli are derived from the endoderm of the foregut, the cartilaginous and smooth muscular components of the trachea and bronchi are derived from the surrounding lateral mesoderm.

All 18.5 dpc double mutants analysed by whole mounts showed tracheal cartilage malformations. However, these defects varied greatly depending on the mutant genotype (Table 1; Fig. 2). As reported (Lohnes et al., 1993), the tracheal rings of RARγ null fetuses were variably fused in the ventral plane (TR, Fig. 2f). Strikingly, the tracheal rings disappeared in α1γ, α1α2γ−γ and αγ double mutants to be replaced by a ventral cartilage and cartilaginous nodules (TR, Fig. 2g-i). Moreover, there was a marked reduction in the intensity of alcian blue staining in αγ mutant tracheas, suggesting a deficiency in cartilage formation (Fig. 2i). In the β2γ mutants, the tracheal rings were severely malformed (TR, Fig. 2b). The tracheal rings were only mildly malformed in α1β2 mutants, but completely disorganized in αβ2 mutants (TR, compare Fig. 2a to c, d and e). RARβ2 transcriptions are expressed both in the foregut endoderm and mesoderm, whereas RARγ expression is restricted to the mesoderm (Dollé et al., 1990; Ruberte et al., 1990; our unpublished results). Consistent with these differences in expression patterns, inactivation of either RARγ or RARβ2 in RARα1 or RARα (all isoforms) mutant backgrounds resulted in different anomalies of the tracheal cartilages; for example, the ventral cartilaginous fusion was present in α1γ, but not in α1β2 mutants (see Fig. 2c,g; see also Fig. 2i,e,d).

The length of the trachea was markedly and specifically reduced in the αγ and αβ2 mutants, with the bifurcation which gives rise to the stem bronchi occurring prematurely (compare e.g. Fig. 2a with d, e and i; arrowheads). Interestingly, this was not seen in the α1γ and α1β2 mutants (compare Fig. 2c with e and g and h with i), thus the removal of the α2 isoform in α1γ and α1β2 mutant backgrounds was responsible for these defects. The left stem bronchus of the αβ2 mutant shown in Fig. 2e was almost absent, reflecting the lack of the left lung (see above); however, the cartilaginous rings of the visible portion of the right bronchus appeared regularly spaced. Further histological analysis revealed that the cartilages of the more caudal portions of the stem bronchi (not visible in whole mounts) were regularly spaced in αβ2, αβ2γ−γ and αβ2 mutants, but fused in the α1γ, α1α2γ−γ, β2γ and αγ mutants (data not shown). Since RARγ transcripts are expressed in the bronchial mesenchyme (Ruberte et al., 1990; Dollé et al., 1990 and our unpublished results), it is likely that this receptor is involved in formation of the bronchial cartilages.

RAR double mutants which exhibited malformed thyroid cartilages, also had malformed and smaller arytenoid cartilages (Table 1). In RARγ single mutants, as in α1γ and β2γ mutants, these cartilages were normal in size and shape (TC and AC, in Fig. 2f,g and b), but were smaller in the α1γα2γ−γ and unrecognizable in αγ mutants (TC and AC in Fig. 2h and star in Fig. 2i). The thyroid and arytenoid cartilages were nearly normal in α1β2 mutants (Fig. 2c), while in αβ2 mutants their structure was severely altered (TC and AC, Fig. 2d,e).

The cricoid cartilage was abnormally fused to the tracheal rings in all double mutants and in RARγ single mutants (RC, αβ2, αβ2γ−γ, αγ and αβ2 mutants).
Fig. 2. Whole mounts of tracheas and hyoid bones derived from 18.5 dpc mutant and wild-type fetuses stained with alcian blue and alizarin red. Note that in f-i, the hyoid bones have been removed. (a) A wild-type trachea with hyoid bone attached. (b) The trachea and hyoid bone from a β2/γ mutant fetus. (c) The trachea and hyoid bone from an αβ2 fetus. The fusion between the thyroid cartilage and the lesser horn of the hyoid bone is denoted by a black wedge. (d) The trachea and hyoid bone from an αβ2 fetus. The premature bifurcation of the tracheal cartilage and the fusion between the lesser horn of the hyoid bone and the thyroid cartilage are indicated by black wedges. (e) The trachea and hyoid bone from an αβ2 fetus. The black wedge indicates the premature bifurcation of the right stem bronchus and the premature termination of the left stem bronchus. (f) A trachea derived from an RARγ null fetus. (g) A trachea from an αγ fetus. (h) A trachea derived from an αα2 γ fetus. (i) A trachea derived from an αγ fetus. The black wedge denotes the premature bifurcation of the left and right stem bronchi. The star designates the cartilaginous mass which replaces the thyroid, arytenoid and cricoid cartilages (j) and (k). Hyoid bones of WT and αγ mutants, respectively. Abbreviations: AC, arytenoid cartilage; RC, cricoid cartilage; TC, thyroid cartilage; TR, tracheal rings; HY, hyoid bone; BO, body of the hyoid bone; LH, lesser horn of the hyoid bone; GH, greater horn of the hyoid bone.
Fig. 2b-i). As for the thyroid and arytenoid cartilages, the shape of the cricoid cartilage in the γ, αβγ2, αγγ and βγ2 mutants was nearly normal, whereas it was clearly abnormal in the αγγ2+/- fetuses, and unrecognizable in αβ2 and αγ2 mutants (compare Fig. 2c). The body of the hyoid bone originates from neural crest cells (NCC) that populate the 2nd and 3rd branchial arches, the greater horn being derived from the 3rd arch and the lesser horn from the 2nd arch. The size and shape of the hyoid bones of 18.5 dpc γ, αγγ and αγγ2 mutants were normal (data not shown). In contrast, the hyoid bone in αγ2 mutants was highly abnormal; the greater horns, body and lesser horns were unrecognizable (BO, LH and GH, compare Fig. 2j with k). In the βγ2, αβ2 and αβ2 mutants, the shape of the hyoid bone was normal, but there was a fusion between the hyoid greater horn and the rostral horn of the thyroid cartilage in all αβ2 and some αγ2 fetuses (arrowheads in Fig. 2c and d).

(C) Diaphragmatic hernia

Posterolateral diaphragmatic defects, which result from a failure of closure of the pleuroperitoneal canals (which is normally complete by 14.0 dpc; Kaufman, 1992), were observed in one of seven normally complete by 14.0 dpc; Kaufman, 1992), were observed in one of seven αβ2 and one out of four of αβ2+/- 18.5 dpc mutants but not in any other double mutants. In the αβ2 mutant, there was an abnormal foramen in the right dorsal portion of the diaphragm (DR) permitting protrusion of a mass of liver (LE) into the right pleural cavity (compare Fig. 1h and i). This herniated mass compressed the right lung (RL, Fig. 1i).

In the affected αβ2+/- mutant, the diaphragmatic defect was observed on the left side. RARβ2 transcripts, but not RARγ transcripts are expressed in the anlage of the diaphragm at 12.5 dpc (our unpublished results), suggesting that RARβ2 could play a role in its formation.

(D) Abnormalities of the heart and outflow tract

The ventricular myocardium, which develops from cardiogenic plate mesoderm, was abnormally thin in two (out of five) 18.5 dpc αγ2 mutant fetuses, (compare Fig. 3a with e and Fig. 3f with g), but not in the other mutants. The deficiency was particularly marked in the subepicardial or compact (outer) layer of the ventricular wall (CMY, Fig. 3f,g), while the trabecular (inner) layer (TMY, Fig. 3f,g) appeared less affected, thus imparting a spongy appearance to the myocardium reminiscent of that seen in 10.5-12.5 dpc normal embryos. In addition, one of these two fetuses lacked the ventricular septum (VS, compare Fig. 3a with e). Myocardial deficiency, occurring as a consequence of a growth arrest, might account for the embryonic lethality in αγ2 mutants (see Lohnes et al., 1994), since neither renal agenesis (see below) or exencephaly (see Lohnes et al., 1994) are usually embryonic lethal in mouse or human (Kreiberg et al., 1993; Franz, 1989 and refs therein).

Between 11.0 and 13.0 dpc, the aortic sac is divided longitudinally by the spiral-shaped aorticopulmonary septum (AP, Figs. 4c), (Fananapazir and Kaufman, 1988; Vuillemin and Pexieder, 1989), a structure derived from rhombencephalic NCC (Kirby et al., 1983), thus giving rise to the ascending aorta (AS, Fig. 4c) and to the pulmonary trunk (PT, Fig. 4c). Failure of this division to take place or to become complete results in a persistent truncus arteriosus (PTA) receiving blood from both ventricles (TA, Figs 3b,d, 4d). This defect was observed in all 18.5 dpc αβ2 (seven) and αγ2 (five) mutants (compare Fig. 3a and b). PTA, which appeared as a single outflow vessel on serial histological sections (TA, Fig. 3b), was connected with the heart through a valve with 4 semilunar cusps (TA:1-4, Fig. 3d), instead of the normal 6 cusps, namely the 3 aortic cusps (AS: 1-3, Fig. 3c) and 3 pulmonary cusps (PT: 1-3, Fig. 3c) found in WT fetuses. The origin of this vessel from the right ventricle alone was evident in five αβ2 and in three αγ2 mutants; in the remaining αβ2 and αγ2 mutants, PTA overrode the ventricular septum (not shown). PTA was also present in two (out of four) αβ2+/- fetuses, and in one (out of three) αβ2 18.5 dpc fetuses (Table 1). In one αγ2 fetus the pulmonary trunk and ascending aorta were septated distally, but undivided proximally; with this exception, however, there was no evidence of partitioning in any part of PTA of αβ2, αβ2+/-, αβ2 and αγ2 mutants.

In all 18.5 dpc mutants with PTA (with the exception of the one αγ2 mutant with PTA in which the ventricular septum was totally absent, see above), the membranous portion of the ventricular septum (or pars membranaceae) was incomplete, resulting in a high ventricular septal defects located (i) just beneath the semilunar cusps of PTA and/or (ii) between the aortic vestibulum and the right ventricle (not shown). It is noteworthy that interventricular septal defects of the first type might in fact correspond to the normally persisting interventricular foramen [i.e. FIV II in the terminology employed by Vuillemin and Pexieder (1989)], which in the heart of WT mice forms the ostium of the aorta. In contrast, the communication between the aortic vestibulum and right ventricle (i.e. FIV III; Vuillemin and Pexieder, 1989) is normally closed by 14.5-15.0 dpc, thus its persistence in the 18.5 dpc double mutants is pathological. Other outflow abnormalities found in RAR double mutants included four cases of dextroposition of the ascending aorta combined with high ventricular septal defect and a case of double outlet right ventricle (Table 1). In dex-
troposed aorta (DA), this vessel arose partially from the right ventricle and thus straddled the ventricular septum (not shown). In double outlet right ventricle (DORV), the ascending aorta (AS, Fig. 3m) and the pulmonary trunk (PT, Fig. 3m,n) arose separately from the right ventricle (rV, Fig. 3m,n). DA and DORV were observed in ααγγαγ−/− and ααγγαα+/− fetuses. In addition, one αβ2 and one αγ2 mutant showed a persistent atrioventricular canal, possibly resulting from the failure of the embryonic canal to divide into a right and a left orifice (not shown). With these two exceptions, inflow abnor-

Fig. 4. Comparison of the aortic arches, and outflow tract of the heart between 11.5 dpc wild-type (a,c,e) and an α−/−β2−/− embryos (b,d,f) and thymus (TH) and thyroid (TY) ectopias in 18.5 dpc mutant fetuses (g-i). (a,b; c,d; and e,f) Transverse sections at comparable levels of the dorsal aortic roots (fAR and rAR) and conotruncus (CT). (g-i) Frontal section through the pharynx (PH). Abbreviations: AP, aorticopulmonary septum; fAR, left aortic root; rAR, right aortic root; AS, ascending aorta; AT, atrium; BC, base of the skull; CR, conotruncal ridges; CT, conotruncus; HY, hyoid bone; O, oesophagus; OT, oesophagotrachea; OTS, oesophagotracheal septum; rP, right pulmonary artery; PH, pharynx; PT, pulmonary trunk; SC, spinal cord; T, trachea; TA, truncus arteriosus; TH, thymus; TH*, aberrant lymphoid tissue; TY, thyroid gland; fV, left ventricle; VE, vertebral column; f6, left 6th aortic arch; r6, right 6th aortic arch. Magnifications: ×74 (a-f,i); ×30 (g,h).
Fig. 5. Diagrammatic representations of the normal embryonic (i.e. 9.5-11.5 dpc) pattern of aortic arches (a), of the normal 18.5 dpc pattern of aortic arch (b) and of some abnormal patterns observed in 18.5 dpc RAR double mutant fetuses (c-i). Note that during development the 5 pairs of aortic arches are never present simultaneously, but they are represented in (a) in cumulative arrangement in order to emphasize their positional relationships. The parts of the embryonic system which have regressed are represented by broken lines in b-i. Abbreviations: L. ao. root, left aortic root; R. ao. root, right aortic root; ao. sac, aortic sac; arch ao., arch of the aorta; L. arch ao, left arch of the aorta; R. arch ao, right arch of the aorta; asc. ao., ascending aorta; L. cerv. arch ao, left cervical arch of the aorta; L. com. car, left common carotid artery; L. car. duct, left carotid duct; L. ceph. ao., left cephalic aorta; R. cerv. arch ao, right cervical arch of the aorta; L. cerv. arch ao, left cervical arch of the aorta; L. subcl, left subclavian artery; L. 7th art., left 7th segmental artery; R. 7th art., right 7th segmental artery.

Malformations were not detected. PTA and heart abnormalities were not observed in αγβ and αγγ fetuses (Table 1).

As expected from the analysis of 18.5 dpc mutants (see above), no sign of aorticopulmonary septation was observed in a majority (five out of six) of 11.5 dpc αβ2 and αγγ embryos (compare AP, Fig. 4c with d). However, the 2 conotruncal ridges always appeared to be normally developed in all of these mutants (CR, compare Fig. 4e and f). These ridges are located in the ventriculo-arterial (conotruncal) region of the heart where they form upon fusion (by 14.5-15 dpc), the membranous portion of the ventricular septum, dividing the conotruncal lumen into a pulmonary and an aortic vestibulum. Although the conotruncal ridges are continuous with the aorticopulmonary septum, they are not derived from NCC (Noden, 1991). These observations support the conclusions that in mutants: (i) amongst the structures involved in septation of the outflow tract of the heart, only those that are derived from NCC are affected, and (ii) that the high ventricular septal defects seen at 18.5 dpc are most probably occurring secondarily to abnormal aorticopulmonary septation in order to compensate for changes in blood flow. This view is supported by the constant association of PTA, DA or DORV with ventricular septal defects in human patients (Gray and Skandalakis, 1972; Taussig, 1960).
Aortic arch abnormalities were found in all 18.5 dpc double mutants with the exception of\( \alpha \gamma \) and \( \beta \gamma \) fetuses. They showed great individual variations (Fig. 5; Table 2 in which the data are derived from observations made on serial histological sections, and occasionally from whole mounts). For the sake of simplicity in the description, they were classified hereafter into 5 categories: (i) abnormalities characterized by the persistence of the carotid duct, (ii) abnormalities characterized by persistence of the right aortic root, (iii) coarctation of the aorta, (iv) aberrant origin of pulmonary arteries, (v) malformations of the aortic arches secondary to abnormal aorto-pulmonary septation.

(i) Abnormalities characterized by a persistence of the carotid duct

A cervical arch of the aorta (CAA, Figs 1c and 3o; Gray and Skandalakis, 1972) was identified firstly by the proximity of the arch of the aorta to the larynx (see CAA, Fig. 1c) and, secondly, by the fact that the common carotid artery was missing on one side, thus resulting in the internal and external carotid arteries arising separately from the crest of the arch (f 1 and f E, Fig. 3o; Wilson and Warkany, 1949). This is likely caused by a persistence of the carotid duct, i.e. the segment of the cephalic dorsal aorta located between the 3rd and 4th aortic arches (Fig. 5j,k) which should normally regress. Two cases of cervical arch of the aorta (CAA) were observed, one on the left side (Figs 5k, 1c, 3o), and the other on the right side (Fig. 5j). In both cases, the 4th aortic arches had failed to persist, bilaterally. Both mutants with cervical arch of the aorta had additional abnormalities, namely retroesophageal subclavian arteries (e.g. r OS in Figs 1c, 3h), PTA and agenesis of the left pulmonary artery (Fig. 5j,k).

Table 2. Aortic arch and heart outflow tract abnormalities in 18.5 dpc RAR double mutants

<table>
<thead>
<tr>
<th>RAR double mutant genotype</th>
<th>18.5 dpc fetuses</th>
<th>Abnormal arch (see Fig. 5)</th>
<th>Abnormal arch abnormalities</th>
<th>Associated abnormalities of the outflow tract of the heart</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \alpha \beta )</td>
<td>( \alpha \beta )</td>
<td>R. ao. root, dist R6</td>
<td>L4, dist L6</td>
<td>R. 4th arch ao.; L. retroes. subcl.</td>
</tr>
<tr>
<td>A (5i)</td>
<td>B (5e)</td>
<td>R. ao. root</td>
<td>L4</td>
<td>R. 4th arch ao.; Double asymmetrical arch ao.</td>
</tr>
<tr>
<td>C (NI)</td>
<td></td>
<td>dist L6</td>
<td>Isolated absence of ductus art.</td>
<td>PTA</td>
</tr>
<tr>
<td>( \alpha \beta )</td>
<td>( \alpha \beta )</td>
<td>R. ao. root</td>
<td>L. ao. root, dist L6</td>
<td>R. 4th arch ao.</td>
</tr>
<tr>
<td>A (5c)</td>
<td>B (5b)</td>
<td>prox R6, prox L6</td>
<td>R. 4th arch ao.; Double asymmetrical arch ao.;</td>
<td>Aberrant origin of pulm. a rt.</td>
</tr>
<tr>
<td>C (5f)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>D (NI)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>( \alpha \beta )</td>
<td>( \alpha \beta )</td>
<td>R. ao. root</td>
<td>L. ao. root, L6</td>
<td>R. 4th arch ao.; Absence of L. pulm. art.</td>
</tr>
<tr>
<td>A (5d)</td>
<td>B (5h)</td>
<td>R. ao. root, L6, R4</td>
<td>R. retros. subcl.; Absence of L. pulm. art.</td>
<td>PTA</td>
</tr>
<tr>
<td>C (5h)</td>
<td>D (5j)</td>
<td>R. ao. root, dist R6</td>
<td>L4, dist L6, R4</td>
<td>R. cerv. arch ao.; L. retros. subcl.; Absence of L. pulm. art</td>
</tr>
<tr>
<td>E (5h)</td>
<td>F (NI)</td>
<td>R. ao. root</td>
<td>L6</td>
<td>R. retros. subcl.; Absence of L. pulm. art.</td>
</tr>
<tr>
<td>G (5k)</td>
<td></td>
<td>L. car. duct, R. ao. root</td>
<td>L4, R4, L6, L6</td>
<td>Isolated absence of L. pulm. art. and ductus art.</td>
</tr>
<tr>
<td>( \alpha \gamma )</td>
<td>( \alpha \gamma )</td>
<td>R. ao. root</td>
<td>L. ao. root, prox L6</td>
<td>R. 4th arch ao.; Aberrant origin of L. pulm. art.</td>
</tr>
<tr>
<td>A (5i)</td>
<td>B (5c)</td>
<td>R. ao. root</td>
<td>L. ao. root, dist L6</td>
<td>R. 4th arch ao.</td>
</tr>
<tr>
<td>E (NI)</td>
<td></td>
<td>R. ao. root</td>
<td>L4, dist L6</td>
<td>R. 4th arch ao.; L. retros. subcl.</td>
</tr>
</tbody>
</table>

For a given genotype, A-G designate different fetuses; the relevant schematic representation in Fig. 5 is given in parentheses (5a-l panels); similar to Fig. 5g, but with the right pulmonary artery arising normally from the pulmonary trunk. NI, not illustrated; ND, not analysed (these mutants had no PTA, however a detailed analysis of the outflow tract was not possible due to an inappropriate plane of sectioning); R4 and L4, right and left 4th aortic arches, respectively; R. ao. root and L. ao. root, right and left aortic roots, respectively; R. car.duct. and L. car. duct, right and left carotid duct; dist R6, distal portion of the right 6th aortic arch; L6, left 6th aortic arch which gives rise to the proximal part of left pulmonary artery and to the ductus arteriosus; proc L6, proximal portion of the left 6th arch (proximal part of the left pulmonary artery); dist L6, distal portion of the left 6th arch (ductus arteriosus); R. 4th arch ao., right 4th arch of the aorta; R. and L. retros. subcl., right and left retroesophageal subclavian artery, respectively; R. and L. cerv. arch ao., right and left cervical arch of the aorta, respectively; DA, dextroposed aorta; PTA, persistent truncus arteriosus; DORV, double outlet right ventricle.
(ii) Abnormalities characterized by a persistence of the right aortic root

These abnormalities, by far the most frequent (Fig. 5; Table 2), can be subdivided into right 4th arch of the aorta (Fig. 5c-f,i,l) and right retroesophageal subclavian arteries (Fig. 5g,h). In the simplest case of right 4th arch of the aorta, the left arch of the aorta was absent and replaced by the corresponding vessel on the right side (rAAA, Figs 1b, 3i; 5c,d). The arrangement of major arteries derived from the right 4th arch of the aorta (i.e. subclavians, carotids) exhibited a mirror image of the normal pattern. In addition to the right sided arch of the aorta, the two mutants shown in Fig. 5c,d exhibited a PTA and one had an agenesis of the left pulmonary artery (Fig. 5d). A third variant is illustrated in Fig. 5e: as in the two preceding examples, the right dorsal aortic root failed to regress, and the abnormal right-sided arch of the aorta gave rise to the left and right common carotid arteries. Here, however, the left dorsal aortic root (i.e. the normal vessel) persisted, giving rise (as normally) to the left subclavian artery, and connecting the heart with the descending dorsal aorta via the pulmonary trunk and the left ductus arteriosus (i.e. the normal distal portion of the 6th arch). This is one of the many possible forms of double arch of the aorta (Wilson and Warkany, 1949; Gray and Skandalakis, 1972; Taussig, 1960). In the last variant of this group (Fig. 5f), both the left and the right 4th aortic arches and aortic roots had persisted and formed a symmetrical double arch of the aorta, each component of the double arch gave rise to a common carotid and to a subclavian artery (Fig. 5f); additional defects included a PTA, and the left and right pulmonary arteries arose abnormally from this double arch of the aorta (see below).

In normal embryos, the left subclavian artery arises entirely from the 7th left segmental artery which is connected to the left dorsal aortic root. In contrast, the proximal portion of the right subclavian artery is formed in part from the right 4th aortic arch, whereas its distal portion is formed from right 7th segmental artery (Barry, 1951; Fig. 5a,b). The right subclavian artery normally arises from the innominate (brachiocephalic) artery (Fig. 5b). In the simplest case of right retroesophageal subclavian artery, this vessel arose as the most distal rather than as the most proximal branch from an otherwise normal left arch of the aorta (Fig. 5g,h). The subclavian artery (r OS, Fig. 3h) passed to the right behind the oesophagus (O, Fig. 3h) and in front of the vertebral column (VE, Fig. 3h) to reach its normal destination, namely the right forelimb. This abnormality is probably caused by the abnormal regression of the right 4th aortic arch and the persistence of the right aortic root which normally disappears (Table 2). In the variant shown in Fig. 5g, the aortic arch pattern is otherwise normal, whereas that shown in Fig. 5h has additional malformations, namely PTA and agenesis of the left pulmonary artery. Note that a mirror-image abnormality (i.e. retroesophageal left subclavian) existed in some cases of persistent right 4th arch of the aorta (f OS, Fig. 3i; see also Fig. 5i). Right and left retroesophageal subclavian arteries were also observed in mutants with cervical arch of the aorta (see above and Fig. 5j,k).

(iii) Coarctation of the aorta

In one mutant with coarctation of the aorta (Table 2), the arch of the aorta between the ductus arteriosus and the left common carotid artery was markedly narrowed, possibly as a result of an abnormal incomplete regression of the left 4th aortic arch (compare AA, Fig. 3j and k; Barry, 1951).

(iv) Agenesis and aberrant origin of pulmonary arteries

A case of aberrant origin of left and right pulmonary arteries from their respective ipsilateral arches of the aorta between the origins of the subclavian and carotid arteries is illustrated in Fig. 5f. This is likely due to the abnormal regression of the proximal segments of the left and right 6th aortic arches and abnormal persistence of the distal segment of the right 6th aortic arch. An aberrant origin of the left pulmonary artery from the subclavian artery (Fig. 5f) represents a variant of the preceeding condition.

Agenesis of the left pulmonary artery was always observed in αβ2 and αβ2+/− fetuses with agenic left lungs (Fig. 5d,h,j,k). In this case, the absence of the left pulmonary artery is likely to be secondary to lung agenesis.

(v) Some malformations of the aortic arch pattern likely to be secondary to abnormal or aberrant aorticopulmonary septation

An aberrant origin of the right pulmonary artery from the
ascending aorta was observed in one mutant (rP, Fig. 3l; Fig. 5g), whereas the left pulmonary artery arose from the pulmonary trunk, as usual. The developmental fault underlying this condition must have been improper alignment of the aorticopulmonary septum with respect to the 6th arch primordia (Wilson and Warkany, 1949; Gray and Skandalakis, 1972).

The ductus arteriosus was absent in all of the mutants with a PTA (see examples in Fig. 5c,d,j,k). Absence of the ductus arteriosus is almost invariably associated with PTA in mice and humans (Franz, 1989; Gray and Skandalakis, 1972). It is currently assumed that absence of significant blood flow through the ductus arteriosus due to the presence of a much larger aorticopulmonary communication permits the distal portion of the left 6th aortic arch (which normally regresses only in the immediate postpartum) to undergo involution earlier in fetal life (Gray and Skandalakis, 1972; Taussig, 1960). However, the possibility that alteration of the hemodynamic pattern caused by an outflow tract abnormality such as PTA might explain all of the other aortic arch abnormalities appears unlikely, since it is clear from the present data that these two types of malformations can occur independently. Both types of malformations are also known to exist independently in rats (Wilson and Warkany, 1949) and humans (Gray and Skandalakis, 1972).

(2) Early abnormalities of the aortic arch pattern

The most frequent aortic arch abnormalities found in 18.5 dpc mutants were associated with abnormal persistence of the right dorsal aortic root and abnormal regression of the left 6th arch (Table 2). In WT embryos at 11.5 dpc, i.e. when the aortic arch system is still bilaterally symmetrical and prior to complete aorticopulmonary septation, the left and right aortic roots receive blood from the heart via the ipsilateral 3rd, 4th and 6th arches (Kaufman, 1992). In mutant embryos, the 3rd and 4th aortic arches were bilaterally present in all 11.5 dpc αβ2 (four), 11.5 dpc γγ (two) and 10.5 dpc αβ2 (two) mutants examined. However, in three of these embryos, the calibres of both the left 4th arch and of the ipsilateral aortic root (which together represent the normal anlage of the arch of the aorta) were markedly reduced compared to the same structures on the right side (compare rAR and lAR, Fig. 4a with b). The left and right 6th aortic arches were barely visible in 11.5 dpc mutants examined (r6 and f6, compare Fig. 4c with d), and their distal portions (on the left side, the future ductus arteriosus) were never identified in γγ and αβ2 mutants at later developmental stages (i.e. from 12.5 to 18.5 dpc, data not shown). Lastly, in one 10.5 dpc αβ2 mutant, the calibres of both the right 4th arch and ipsilateral aortic root were reduced compared to their left homologues (not shown).

Taken together, these data clearly indicate that at least some of the aortic arch abnormalities observed in 18.5 dpc fetuses are determined before the stage at which specific arches normally start to regress. Since the 6th aortic arch becomes first visible at a stage when the septation of the aortic sac is already underway, it is not possible to assess whether their small size in the 11.5 dpc mutants corresponds to a primary effect of the mutations or is secondary to PTA. In any event, our findings indicate that, in mutant embryos with PTA, the ductus arteriosus might disappear early in development, before 12.5 dpc.

(F) Abnormalities of the thyroid, parathyroid and thymus glands

Rhombencephalic NCC together with the endoderm of the primitive pharynx give rise to the thyroid, parathyroid and thymus glands (Le Douarin, 1981). These gland anlagen subsequently ‘migrate’ caudally to their definitive locations. In 18.5 dpc WT fetuses, the thyroid gland is seen in close contact with the lower part of the larynx and the first three cartilaginous tracheal ‘rings’. The parathyroid glands are embedded in the posterolateral part of each lobe of the thyroid gland. The two lobes of the thyroid are located beneath the sternum, within the superior part of the anterior mediastinum. Most mutants exhibited at least one form of glandular ectopia (Table 3), including cervical thymus (TH, Fig. 4g), thyroid beneath the hyoid bone (TY, Fig. 4g and h), and rostrally displaced ectopic parathyroids not associated with the thyroid gland (not shown). In addition, aberrantly located lymphoid tissue was found in the larynx of some double mutant fetuses (Table 3; TH*, Fig. 4i), resembling in this respect the lymphoid nodules that have been proposed to correspond to ectopic accessory thymus glands in humans (Gray and Skandalakis, 1972).

(G) Abnormalities of the urinary system

Abnormalities of the kidneys and ureters were found in αβ2, αβ2, αγ2 and αγ mutants at 18.5 dpc (Table 4). Renal abnormalities fell into 3 broad categories: renal agenesis, aplasia and hypoplasia (Gray and Skandalakis, 1972). Renal agenesis and renal aplasia were observed specifically and consistently in γγ mutants, and these two abnormalities eventually coexisted in the same 18.5 dpc fetus: from a total of five fetuses, two fetuses had bilateral agenesis, two exhibited bilateral aplasia, and the last one showed agenesis on one side and contralateral aplasia (Table 4, and data not shown). In agenesis, no trace of renal tissue existed. This abnormality was always associated with agenesis of the ipsilateral ureter. In renal aplasia, small retroperitoneal masses of tissue containing randomly dispersed kidney tubules and glomeruli were found caudal to the normal location of the kidney. The renal pelvis was always absent. The ureters were absent or atretic, i.e. had a markedly reduced caliber and lacked the caudal portion. In addition, the urinary bladder was absent in one 18.5 dpc γγ mutant fetus.

Embryos were examined to investigate the possible origin of renal agenesis in 18.5 dpc γγ mutants. In 11.5 dpc WT embryos, the epithelial ureteric bud (U, Fig. 6c), which arises from the caudal part of mesonephric duct (Wolffian duct), had come into contact with the condensed metanephric mesenchyme (N, Fig. 6c). In the two 11.5 dpc γγ mutants, the ureteric bud was absent on one or both sides, and when present failed to reach the metanephric blastema (U, compare Fig. 6c and d). At this time, the nephric mesenchyme was bilaterally present (N, Fig. 6c,d) and showed no evidence of increased apoptosis (see Kreidberg et al., 1993). The parenchyma of 12.5 dpc WT kidneys contain numerous tubules (arrowheads in Fig. 6e). Analysis of a 12.5 dpc γγ mutants revealed that the nephric mesenchyme was bilaterally present (N, Fig. 6f), but showed no signs of histological differentiation. In addition, a large number of pycnotic nuclei were present (not visible at the magnification of Fig. 6f). Interestingly, this mutant had no ureters (compare U, Fig. 6e and f). In one 14.5 dpc γγ fetus (not
shown), a rudimentary kidney was present on one side, while on the other side there was no trace of nephric tissue and no ureter.

In renal hypoplasia, the renal parenchyma at 18.5 dpc, although conspicuously smaller than normal (compare K, Fig. 6a and b, and Fig. 6g and h), was often subdivided into cortical and medullary regions (CO and ME, compare Fig. 7e and f). This abnormality was found bilaterally in all αβ2 mutants and in a majority of cases (seven out of ten), it was associated with dilation of the renal pelvis (hydronephrosis; compare PL in Fig. 6g and h) and of the ureter (hydroureter; e.g., compare fU in Fig. 6i and j), indicating that some renal excretory function must have occurred. The hypoplastic kidneys were often ectopic, i.e. located in the lower lumbar or sacral regions instead of the normal upper lumbar site (compare K, in Fig. 6a and b). At the histological level, the cortical region of the hypoplastic kidneys contained abnormally large glomeruli (GL) and convoluted tubules (TU) (compare Fig. 7e and f); moreover, this cortical region always lacked the peripheral nephrogenic zone (Z, compare Fig. 7e and f), where nephrons continue to be produced after birth. Bilateral renal hypoplasia was also conspicuous in three (out of four) α1γ mutants, at 18.5 dpc but was always less severe than in the αβ2 mutants (Table 4).

Agenesis of caudal ureters and ectopic ureteral openings constitute anatomical and functional obstructions respectively, resulting in the accumulation of urine in the urinary tract, leading to hydronephrosis and hydroureter. All αβ2 mutants (seven out of ten, see Table 4) exhibiting hydronephrosis and hydroureter displayed abnormalities of caudal ureter. Ectopically terminating ureters (e.g. fU, Fig. 7b) opened into the urethra instead of into the base of the urinary bladder, their normal termination site. Agenesis of caudal ureter was characterized by a failure of one or both ureters to join any part of the lower genito-urinary tract (compare rU in Fig. 6i and j). Hydronephrosis and hydroureter with an otherwise normal genito-urinary tract was observed in one β2γ mutant.

(H) Abnormalities of the genital tract and lower digestive tract

Male genital ducts develop mainly from mesodermal epithelium of the Wolffian (or mesonephric) duct (epididymis, vas deferens and seminal vesicles) and from endoderm of the urogenital sinus (e.g. prostate). Abnormalities of the genital tract were observed only in the two α1γ, and in one (out of two) α1γαβ+/- 18.5 dpc mutant males examined (see Table 4). Bilateral agenesis of epididymis, vas deferens and seminal vesicles was found in one α1γ mutant male and was associated with bilateral renal agenesis. Bilateral dysplasia of epididymis and vas deferens was observed in one α1γ mutant and in one α1γαβ+/- male: these two abnormal structures displayed a succession of dilated and stenotic portions. Moreover, in both mutants, the vas deferens stopped at a short distance from its normal opening in the urethra, whereas the seminal vesicles appeared normal (α1γαβ+/- mutant) or were missing (α1γ mutant) (Table 4, and data not shown). However, prostate buds were present in these males. Interestingly, the caudal-most Wolffian duct was always lacking in α1γ mutants examined at 11.5 dpc (two), 12.5 dpc (one) and 14.5 dpc (one) (W, compare Fig. 6e and f).

In αβ2+/-, αβ2, α1γ and β2γ mutant males, the genital tract was apparently normal. The testes appeared histologically normal in all mutants (data not shown).

The female genital ducts develop from mesodermal epithe-

---

**Table 4. Uro-genital tract abnormalities in 18.5 dpc RAR double mutants**

<table>
<thead>
<tr>
<th>RAR mutant genotype and number of 18.5 dpc fetuses examined (males : females)</th>
<th>α1β2 1 : 2</th>
<th>αβ2+/- 4 : 0</th>
<th>αβ2 4 : 6</th>
<th>α1γ 2 : 3</th>
<th>α1γαβ2+/- 2 : 3</th>
<th>α1γ 2 : 3</th>
<th>α2γ 2 : 3</th>
<th>β2γ 1 : 2 VAD</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Kidney abnormalities</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Renal agenesis (uni- or bilateral)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>3/5</td>
<td>0</td>
</tr>
<tr>
<td>Renal aplasia (uni- or bilateral)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>3/5</td>
<td>0</td>
</tr>
<tr>
<td>Renal hypoplasia (bilateral)</td>
<td>2/5</td>
<td>3/4</td>
<td>10/10</td>
<td>0</td>
<td>2/5</td>
<td>0</td>
<td>0</td>
<td>1/3</td>
</tr>
<tr>
<td>Hydronephrosis</td>
<td>2/5</td>
<td>0</td>
<td>7/10</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1/3</td>
</tr>
<tr>
<td><strong>Ureter abnormalities</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Agenesis (partial or total, uni- or bilateral)</td>
<td>2/3</td>
<td>0</td>
<td>0</td>
<td>6/10</td>
<td>0</td>
<td>0</td>
<td>5/5</td>
<td>0</td>
</tr>
<tr>
<td>Ectopia (uni- or bilateral)</td>
<td>2/3</td>
<td>0</td>
<td>0</td>
<td>5/10</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Absence of anal canal</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><strong>Abnormalities of the genital tract</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Agenesis of the uterus total</td>
<td>0</td>
<td>NA</td>
<td>6/6</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Agenesis of the cranial vagina</td>
<td>2/2</td>
<td>NA</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>3/3</td>
<td>0</td>
</tr>
<tr>
<td>Male</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bilateral agenesis or dysplasia of the vas deferens</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1/2</td>
<td>2/2</td>
</tr>
<tr>
<td>Bilateral agenesis of seminal vesicles</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>2/2</td>
<td>0</td>
</tr>
<tr>
<td>Pseudohermaphroditism</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Agenesis of urinary bladder</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1/5</td>
<td>0</td>
</tr>
</tbody>
</table>

*The body of the uterus was lacking, but the uterine horns were uni- or bilaterally present. NA, not applicable; NR, not reported.
Wolffian ducts were complete in all αβ2 mutants examined at 12.5 dpc (two) and 13.5 dpc (two).

Agenesia of the anal canal was observed only in (five out of seven) 18.5 dpc αβ2 mutants; the terminal end of the rectum was separated from the skin by a thick layer of mesenchyme (not shown).

**DISCUSSION**

**(A) RA is required for several steps in morphogenesis of foregut derivatives**

The endoderm of the primitive thoracic foregut and the associated lateral mesoderm represent the putative anlagen of the larynx, pharynx, oesophagus, tracheobronchial tree and lung parenchyma. Some abnormalities of thoracic foregut derivatives found in RAR double mutants can be ascribed to developmental arrests (i.e. agenesis of the left lung and/or of the oesophagotracheal septum), growth retardation (hypothesis of the left and right lung) or impaired cellular differentiation (ciliated metaplasia of the stratified squamous oesophageal epithelium). However, the malformations of the laryngeal cartilages and tracheal rings have no counterparts during normal embryogenesis.

Left lung agenesis, hypoplasia of the left and/or right lungs and agenesis of the oesophagotracheal septum, which were constantly associated in αβ double mutant fetuses, have been reported in VAD rat fetuses (Warkany et al., 1948; Wilson et

**Table 5. Aortic arch abnormalities in RAR double null mutants and vitamin A-deficient fetuses**

<table>
<thead>
<tr>
<th>Aortic arch abnormalities</th>
<th>RAR mutants (Fig. 5)</th>
<th>VAD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Right cervical arch of the aorta</td>
<td>5j</td>
<td>+</td>
</tr>
<tr>
<td>Left cervical arch of the aorta</td>
<td>5k</td>
<td>+</td>
</tr>
<tr>
<td>Right 4th arch of the aorta</td>
<td>5c,d,e, f, i, l</td>
<td>+</td>
</tr>
<tr>
<td>Right retroesophageal subclavian artery</td>
<td>5g,h,k</td>
<td>+</td>
</tr>
<tr>
<td>Left retroesophageal subclavian artery</td>
<td>5i,j</td>
<td>NR</td>
</tr>
<tr>
<td>Double aortic arch symmetrical and asymmetrical</td>
<td>5e,f</td>
<td>+</td>
</tr>
<tr>
<td>Absence of left pulmonary artery</td>
<td>5d,h,j,k</td>
<td>+</td>
</tr>
<tr>
<td>Aberrant origin of pulmonary arteries from ipsilateral arch of the aorta</td>
<td>5f</td>
<td>NR</td>
</tr>
<tr>
<td>Aberrant origin of left pulmonary artery from the left subclavian artery</td>
<td>5l</td>
<td>NR</td>
</tr>
<tr>
<td>Aberrant origin of right pulmonary artery from the ascending aorta</td>
<td>5g</td>
<td>+</td>
</tr>
<tr>
<td>Coarctation of the aorta</td>
<td>NI</td>
<td>NR</td>
</tr>
</tbody>
</table>

NI, not illustrated; NR, not reported.
RARs in ontogenesi
al., 1953). Left and right lung defects are determined at two stages, through alterations of distinct RA-dependent processes. Analysis of 11.5 and 12.5 dpc αβ2 mutants indicates that left lung agenesis and hypoplasia of both lungs result from the absence of left lung budding and altered bronchial branching, respectively. Both processes are dependent on interactions between the foregut endoderm and its surrounding mesoderm (Spooner and Wessels, 1970). However, they are initiated at two stages (i.e. 10.0 dpc for lung budding and 11.0 dpc for bronchial branching) and exhibit different mesodermal inductive requirements: in vitro, lung budding can be triggered by a variety of embryonic mesenchymes, whereas bronchial branching is strictly dependent on bronchial mesenchyme (Spooner and Wessels, 1970; Schuger et al., 1993). Furthermore, vitamin A must be administrated to VAD rat embryos before the equivalent of 9.5 dpc in the mouse to prevent lung

![Fig. 7. Comparison of the kidney and of the genito-urinary tract between (a,c,e) wild-type (WT) and (b,d,f) αβ2 fetuses at 18.5 dpc (a,b,e,f) and at 13.5 dpc (c,d). (a,b) Sections at similar levels of the urethra (UT) and rectum (R). Note that the mutant left ureter (fU) was found to terminate in the urethra at a short distance caudal to this plane of section. (c,d) Sections at similar levels of the gonads (GO). Abbreviations: CO, cortical region of the kidney; GC, Gartner's cyst; GL, glomeruli; GO, gonad; M, Müllerian duct; ME, medullary region of the kidney; PL, renal pelvis; R, rectum; TU, convoluted tubules; UT, urethra; fU, left ureter; V, cranial vagina; W, Wolffian duct; Z, nephrogenic zone. The large arrow in e points towards the renal pelvis. Magnifications: ×79 (a,b); ×158 (c-f).]
agensis, whereas lung hypoplasia can be prevented by vitamin A administration at 10.5 dpc, i.e. after the onset of lung budding (Wilson et al., 1953).

In WT embryos, lung budding and formation of the oesophagotracheal septum occur almost concomitantly. Both RARα and RARβ2, but apparently not RARγ, play a role at early stages of respiratory tract development. Agensis of the oesophagotracheal septum was associated with normal lungs in one RARαβ2 mutant, supporting the conclusion that lung budding and the formation of the oesophagotracheal septum also represent distinct RA-dependent events. However, disruption of the second RARβ2 isoform allele in a RARαβ2+/− background is sufficient to generate agensis of the left lung and lack of formation of the oesophagotracheal septum (compare RARαβ2−/− and αβ2 double mutants in Table 1). Consistent with a distinct role of RARβ2, in situ hybridization results show that RARβ2, but not RARγ, is expressed in the foregut endoderm (Ruberte et al., 1991; Dollé et al., 1990 our unpublished results). Interestingly, RARβ2 has also been shown to be expressed at early stages in the foregut endoderm of chick embryos (Smith, 1994). In the mouse, RARβ2 expression, which begins at 9.5 dpc, i.e. prior to lung bud formation, continues in tracheal and lung bud epithelia. However, by 11-12 dpc, RARβ2 is not detected in the epithelium of the most distal branching bronchi, nor in the closely associated mesenchyme, but its expression remains in the epithelium of more proximal bronchi and in peripheral lung mesenchyme (our unpublished results). Thus, during lung branching RARβ2 may act to generate a mesenchymal signal acting at a distance on bronchial branching. In any event, it is clear that RARγ, which is weakly expressed in lung mesenchyme (Dollé et al., 1990; our unpublished data), cannot replace the function of RARβ2 during branching morphogenesis.

During late fetal life and early postpartum (15.0 dpc to day 4), the oesophageal lumen is lined with non-ciliated and ciliated cells, the former being much more abundant. At 16.0 dpc and later, most of the non-ciliated cells are of the squamous (i.e. adult) type (Calvert et al., 1991). In all 18.5 dpc αβ2 and in one αβ2 fetuses, the differentiation into squamous cells was impaired, since the entire oesophageal lumen and the cardiac and proventricular portions of the stomach were lined exclusively with ciliated cells, which was never observed in RARγ double mutants. Since both RARβ2 and RARγ transcripts are expressed in the oesophageal epithelium (Dollé et al., 1990 and our unpublished results), RARβ2 may be specifically required for oesophageal epithelium differentiation. However, since this abnormality was always associated with a complete lack of oesophagotracheal septation, it cannot be excluded that it is related to an abnormal contact with the amniotic fluid known to contain soluble factors involved in epithelial cell maturation in the digestive tract (Calvert et al., 1991 and refs therein). Note that, in contrast, vitamin A deficiency in adult animals induces a squamous metaplasia of the normally ciliated tracheal epithelium (Wolbach and Howe, 1925; Boren et al., 1974). Interestingly, the RARαβ2 mutants undivided oesophagotracheal tube lined with ciliated epithelial cells resembled the gut tube of more primitive vertebrates (e.g. fishes), suggesting that RA has been instrumental for the appearance of new foregut derivatives during evolution.

(B) Abnormalities of the heart and aortic arch-derived great vessels

Abnormalities of heart and great vessels found in RAR double mutants consisted of myocardial deficiency, interventricular septal defects, outflow tract abnormalities, persistent atroventricular canal and abnormal aortic arch pattern. All of these abnormalities have been described in the offspring from VAD rats (Wilson and Warkany, 1949; Wilson et al., 1953; Tables 1 and 5). Thus, the present data demonstrate that RARs are transducing the retinoid signal necessary for proper myocardial growth, aorticopulmonary and ventricular septation, and patterning of aortic arches.

The heart outflow tract abnormalities found in 18.5 dpc RAR double mutants included PTA, dextroposed aorta (DA) and double outlet right ventricle (DORV). PTA, which was the most frequent defect found in all αβ2 and γ2 and in some αβ2 and αβ2+/− fetuses, but not in αγ2 and βγ2 fetuses, was due to the lack of formation of the aorticopulmonary septum at 11-13 dpc. In agreement with this, PTA could be prevented in VAD rat fetuses by administration of vitamin A at the equivalent of mouse 10.5 dpc (Wilson et al., 1953).

(1) An initially asymmetrical aortic arch system may be responsible for the abnormal and highly variable definitive aortic arch pattern of RAR double mutants

In 18.5 dpc WT fetuses, the arrangement of the great arteries located near the heart is remarkably constant (see Fig. 5). In 18.5 dpc, αβ2−/−, αβ2+−, αγ2−/−, αγ2+− and γ2−/− fetuses, but not in αγ2 and βγ2 fetuses, this arrangement was remarkably variable and with a few exceptions abnormal, including (i) hypoplasia (i.e. coarctation of the aorta) (ii) agenesis (i.e. absence of the ductus arteriosus, of the innominate artery, of the left or right common carotid arteries or of the normal systemic arch) (iii) aberrant origins (e.g. retroesophageal subclavian artery, aberrant origin of the pulmonary artery), and (iv) malposition of the systemic arch, (i.e. cervical arch of the aorta, right arch of the aorta). In a majority of cases, multiple arterial abnormalities were present in the same fetus (see Fig. 5 and Table 5).

In WT embryos, the aortic arch system is bilaterally symmetrical up to 11.5 dpc. Between 12.0 and 13.5 dpc, specific segments of this system (i.e. the left and right carotid ducts, the right dorsal aortic root and the distal segment of the right 6th aortic arch) regress and disappear yielding an asymmetrical pattern (Kaufman, 1992). From the first time of their appearance, the aortic arches are functional since they connect the heart with the dorsal aorta and issue major arterial stems.

As blood is pumped through the asymmetrical aortic arch system a complex hemodynamic pattern of blood pressures and velocities evolves by putting different stresses on the various segments of the arterial vessels in such a manner as to determine their subsequent persistence or involution (Moffat, 1959; Rychter, 1962; Pexieder, 1969). In this respect, it is noteworthy that abnormalities of the aortic arch pattern can be detected in αβ2 and αβ2 mutant embryos as early as 10.5 and 11.5 dpc, i.e. at a time when the WT pattern is still bilaterally symmetrical. These early abnormalities consist of hypoplasia of aortic arches or segments of the dorsal aorta: (i) reduction of the calibre of the left 4th aortic arch and aortic root (as compared with their right homologues) (ii) reduction of the
calibres of both the right 4th aortic arch and aortic root (as compared with their left homologues) and (iii) concomitant reduction of the calibre of left and right 6th aortic arches (as compared with WT counterparts). Smaller calibres of both the left 4th aortic arch and left dorsal aortic root may deviate the blood flow towards the right side, thus preventing the normal involution of the right arch of the aorta. The left systemic arch may subsequently involute (resulting in a mirror-image configuration of the normal definitive pattern) or persists (resulting in a definitive double arch of the aorta), while the formation of aberrant left or right cervical arch of the aorta may be due to an initial reduction of the calibre of both right and left 4th aortic arches. A decrease of the calibre of a 4th aortic arch but not of the ipsilateral aortic root may lead to aberrant retrooesophageal subclavian arteries. The initial bilateral narrowing of the 6th aortic arches observed in 11.5 dpc mutants was systematically associated with, and is likely to be secondary to, the absence of septation of the aortic sac (PTA). Since the distal portion of the right 6th arch normally disappears before 13.5 dpc and both the proximal portions of the left and the right 6th aortic arches normally make only minor contributions to the pulmonary arteries (Moffat, 1959), this early abnormality, per se, is likely to result solely in the absence of the ductus arteriosus.

(2) Abnormalities of aorticopulmonary septation and aortic arches are consistent with a cardiac neural crest deficiency

It has been shown in quail-chick chimeras that rhombencephalic neural crest cells (NCC), which originate from the neural folds at an axial level corresponding to the mid-otic placode (the future rhombomere 6) to the caudal, unsegmented, portion of the rhombencephalon (3rd somite level), form the tunica media of the 3rd, 4th and 6th aortic arches (LeLièvre and Le Douarin, 1975; Noden, 1991; Kirby and Waldo, 1990 and refs therein). After reaching the pharyngeal arches 3, 4 and 6, some NCC continue their migration towards the aortic sac where they participate in its septation. These cranial NCC, which contribute to the walls of the aortic arches arteries 3 to 6 and to the aorticopulmonary septum, are referred to as cardiac NCC. Excision of premigratory cardiac NCC in the chick results in a high incidence of (i) heart outflow tract defects, including PTA, DA and DORV, (ii) aortic arch abnormalities, including left arch of the aorta (in birds, the definitive systemic arch is the right one), double aortic arch, coarctation of the aorta and abnormal origin of the subclavian and pulmonary arteries and (iii) high interventricular defects (Kirby et al., 1983; Nishibatake et al., 1987; Kirby et al., 1985; Bockman et al., 1987; Kirby and Waldo, 1990 and refs therein). Inflow abnormalities, such as persistent atroventricular canal were also detected. From these cardiac NCC ablation studies, it was concluded that a mesodermal cell deficiency was directly responsible for PTA and most of the aortic arch abnormalities. The other outflow tract defects (i.e. DA and DORV) and all the inflow abnormalities as well as high interventricular septal defects were ascribed to alterations in the blood flow caused by a PTA or an abnormal aortic arch pattern (Kirby and Waldo, 1990 and refs therein). That the heart outflow tract and aortic arch abnormalities observed in chick fetuses following ablation of cardiac NCC are strikingly similar to those found in our RAR double mutant fetuses strongly suggests that the cardiac NCC may be quantitatively and/or qualitatively deficient in RAR double mutants.

It is unlikely that these cardiac NCC deficiencies are already determined at the premigratory stage. Vitamin A can indeed prevent the appearance of aortic arch abnormalities and PTA in VAD rat offspring provided that it is administered before the equivalent of mouse 9.5 dpc and 10.5 dpc, respectively (Wilson et al., 1953), whereas all cardiac NCC have already migrated away from the mouse neural tube at 9.0 dpc (Serbedzija et al., 1992). Thus the RA signal may be important for cardiac NCC late in their migration or at the time of formation of the aortic arch tunica media and/or of the aorticopulmonary septum. In RAR double mutants, RA-dependent processes will be impaired, and more or less pronounced cardiac NCC deficiencies may occur in the various arches on a stochastic basis, leading subsequently to aberrant patterns. Preventing the RA signal to be transduced might therefore be equivalent to ablation of the cardiac NCC. The spatial expression patterns of RARs are also consistent with a role in controlling the fate of migratory or postmigratory rather than premigratory NCC (see Lohnes et al., 1994).

It is noteworthy that ablation of cardiac NCC also results in thyroid, parathyroid and thymic ectopia and hypoplasia (Bockman and Kirby, 1984; reviewed in Kirby and Waldo, 1990), similar to those seen in RAR mutants. In contrast, the ectopic cartilage nodules that were observed in the heart valves of RAR mutants may correspond to a defect in the specification of some cardiac NCC, since similar ectopic cartilage nodules have been found in the heart valves following grafting of mesenchephalic NCC at the level of cardiac NCC (Kirby, 1989).

(C) Abnormalities of the kidney and ureter are generated at distinct developmental stages

The definitive kidney (metanephros) and the ureter develop from both the mesenchymal metanephric blastema (i.e. the caudal portion of the intermediate mesoderm) and the epithelial ureteric bud, which arises from caudal mesonephric duct (Wolffian duct). The metanephric mesenchyme gives rise to excretory metanephric units (nephrons) comprising the kidney tubules (i.e. the convoluted tubules and the loop of Henle) and the glomeruli. In contrast, all the components of the drainage system including ureters, renal pelvis and collecting tubules originate from the ureteric buds (reviewed in Torrey, 1985). The essential features of metanephric development are (i) ingrowth and dichotomous branching of epithelial ureteric buds into metanephric mesenchyme and (ii) conversion of the mesenchyme into epithelial kidney tubules (reviewed by Saxên, 1987). It is well established that kidney histomorphogenesis requires reciprocal epithelial-mesenchymal interactions; branching of the ureteric bud is dependent upon some specific inductive signal generated by the metanephric blastema, whereas kidney tubule differentiation within the metanephric blastema is triggered by an inductive stimulus from the ureteric bud (Saxên, 1987).

Abnormalities of the kidney found in RAR double mutant fetuses consisted of developmental arrests (i.e. renal agenesis, aplasia and hypoplasia) and ectopia. Renal agenesis and aplasia were found exclusively in αγ mutants, and renal agenesis was always associated with agenesis of the ipsilateral ureter. Histological analysis of 11.5, 12.5 and 14.5 dpc αγ embryos indicated that renal agenesis is most probably caused by a
failure of the ureter anlage to bud from the caudal Wolffian duct or, if budding, to reach the metanephric blastema, thus resulting in the disappearance of the latter, as suggested by the presence of a large number of pycnotic nuclei. These early defects of the ureteric bud were always associated with, and in some case might be secondary to, the agenesis of the caudal-most Wolffian duct (see results section and below). Thus, renal agenesis might be determined at 11.0 dpc, which corresponds to the onset of ureteric bud formation, or even earlier at 10.5 dpc when the caudal Wolffian duct normally reaches the cloaca. From the current knowledge on kidney organogenesis (see above), we also conclude that complete ureteric regression most probably occurred in the 18.5 dpc γα mutant fetus, which exhibited kidney aplasia and no ureter.

Hyposplastic kidneys, often ectopically located caudal to their normal position, were observed in all αβ2 and in some αβ2+/−, α1β2 and α1γ2+/− mutants. The size of the αβ2 metanephric blastema and its relationship with the ureteric bud appeared normal at 11.5 dpc (data not shown). That the mutant kidneys stopped growing at some stage of development is indicated by the absence of the nephrogenic zone at 18.5 dpc. Thus, in αβ2 mutants, the ureteric bud reaches the metanephric mesenchyme to generate nephrons, but branching morphogenesis is arrested at some later stage. These data indicate that RA and specific pairs of RARs are required for two steps of kidney morphogenesis (formation of the ureteric bud and growth of the metanephric blastema), as well as for the ascent of the kidneys from their original sacral location to a lumbar site. RARγ appears to play a distinct role at early times, whereas RARβ2 appears to be more important at later stages. In this respect, we note that RARβ2 transcripts are selectively located in metanephric mesenchyme at the time of branching morphogenesis (D. Décimo and P. Dollé, unpublished results). Interestingly, agenetic or hypoplastic kidneys have not been reported in the fetal VAD syndrome, whereas ectopies were frequently observed.

Ectopic ureteric openings in the urethra or agenesis of the caudal ureter, which were observed in all αβ2 and some α1β2 fetuses, often coexisted in the same fetus. Ectopic ureteric openings most probably result from a failure of the caudal end of the ureters to migrate cranially toward the vesical portion of the urogenital sinus (Monie, 1975), whereas partial agenesis must be attributed to regression rather than incomplete formation of caudal ureters, since both kidneys and cranial ureters were present. These two abnormalities also appear to be determined at different stages, since administration of vitamin A to VAD embryos reduced the incidence of ureteric interruptions up to the equivalent of mouse day 12.0 dpc, whereas ureteric ectopia was less frequent following vitamin A administration up to 14.0 dpc (Wilson et al., 1953). Interestingly, RARβ2 but not RARγ transcripts were detected in both the mesenchyme and the epithelium of the urogenital sinus adjacent to the ureter, but not in the ureter itself (our unpublished observations). Thus, the defect in ureter migration/attachment reflects the lack of a RA-induced signal emanating from the urogenital sinus or associated mesenchyme, whose generation would involve RARβ2 (or RARα), but not RARγ.

(D) Several steps in morphogenesis of the male and female genital tracts are affected in RAR mutants

The Wolffian duct first appears at 9.0 dpc in the upper thoracic region, then grows caudalwards until it reaches the cloaca at 10.5 dpc (Kaufman, 1992). The agenesis of the seminal vesicles and caudal vas deferens seen in day 18.5 dpc γα mutants is likely to reflect the absence of the caudal Wolffian ducts seen in 11.5 dpc γα embryos. Interestingly, although the rostral Wolffian ducts were always present in the analysed 11.5 dpc γα embryos, their derivatives (the epidiymidis and rostral vas deferens) were absent (γα fetuses) or severely malformed (γα and α1γ2+/− fetuses) at 18.5 dpc. In VAD rats, Wolffian ducts were always complete, but seminal vesicles were often agenic and this defect could be prevented by vitamin A administration up to the equivalent of mouse 12.0 dpc (Wilson et al., 1953). Thus, RA is most probably required at two stages of male genital duct development, i.e. before 11.5 dpc for the formation of at least the caudal Wolffian duct, and after 11.5 dpc for the morphogenesis of the epidiymidis and rostral vas deferens.

The female genital ducts develop from the Müllerian ducts and from the vaginal plate. The cranial portion of the Müllerian ducts becomes identifiable at 12.5 dpc, and its caudal growth is completed at 14.0 dpc. The midline union of the Müllerian ducts which is complete at 17.0 dpc (Thiedemann, 1987) forms the body of the uterus and the cranial vagina. All abnormalities of the genital ducts observed in 18.5 dpc αβ2 female fetuses might have resulted from either a defect in the formation or a failure in the persistence of the Müllerian ducts. Since Müllerian ducts were not identifiable in 12.5 dpc αβ2 mutant embryos, abnormalities of the genital ducts observed in 18.5 dpc αβ2 female fetuses result from a defect in the formation of the Müllerian duct, most probably occurring before the possible expression of the Müllerian inhibiting substance by the embryonic gonad (Münsterberg and Lovell-Badge, 1991). Furthermore, our results indicate that this defect is not due to a primary defect in the formation of Wolffian ducts which are known to be required for the induction and growth of Müllerian ducts (Gruenwald, 1941; Didier, 1971 and refs therein). The less severe phenotype observed in α1β2 and γα female fetuses, namely agenesis of the body of the uterus and of the cranial vagina, reflects the absence of caudal Müllerian ducts only. This absence may possibly occur through different mechanisms in these two instances, since caudal Wolffian ducts were lacking in γα mutants, but not in α1β2 mutants.

Absence of the uterus and cranial vagina were also observed in VAD fetuses, in which they were related to the agenesis or incomplete development of the Müllerian ducts or to the failure of these ducts to unite caudally (Wilson and Warkany, 1948). That vitamin A administration to VAD embryos prevented the agenesis or incomplete formation of Müllerian ducts only up to the equivalent of mouse 9.5 dpc (Wilson and Warkany, 1953) strengthens the view that there is an early requirement for RA for their formation. However, vitamin A administration at the equivalent of mouse 12.0 dpc could still prevent the failure of Müllerian ducts to unite. These observations indicate that RA is required for multiple steps in the morphogenesis of the female reproductive tract, i.e. formation of the rostral and caudal portions of the Müllerian duct at early stages, and midline union of the caudal Müllerian duct at a later step.

Abnormal persistence of Wolffian ducts in females and Müllerian ducts in males resulting in pseudohermaphroditic tendency was observed in offspring of VAD rats (Wilson and Warkany, 1948), but not in RAR mutants. Together with a
shorter ventral retina (see Lohnes et al., 1994), this is the only aspect of the fetal VAD syndrome which was not recapitulated in the RAR double mutants analyzed in this and in the accompanying study (Lohnes et al., 1994).

CONCLUSIONS

RAR mutant abnormalities, RA target genes and pattern of expression of RARs

One fundamental question regarding the retinoid signalling pathway is how a simple compound such as RA can induce so many diverse and complex responses. Our study (see also Lohnes et al., 1994) clearly shows that these responses are elaborated through the RARs. Our mouse RAR mutants should prove invaluable models to elucidate further this signalling cascade, both regarding the nature of the involved developmental events and the RA-target genes controlling these events.

The congenital abnormalities described here and in the accompanying study (Lohnes et al., 1994) could result from alterations in several cell autonomous or non-autonomous developmental processes, including: axial patterning, as illustrated by homeotic transformations of cervical vertebrae; specification of cell fate and/or migration, as exemplified by the appearance of ectopic cartilaginous nodules likely derived from NCC, and by malformations of most cranial and cardiac mesectodermal derivatives; cell proliferation and/or differentiation, which is likely to be the case for the myocardial cell deficiency and limb defects; epithelial-epithelial inductive interactions involved in the formation of ectodermal placode-derived structures, as evidenced by defects in derivatives of the otic and lens placodes; and morphogenetic processes which rely on epithelial-mesenchymal inductive interactions, such as lung budding and bronchial branching, and kidney and salivary gland branching morphogenesis. There are a number of RA-responsive genes that are likely involved in the above processes (see Gudas et al., 1994, for review). These include transcription factors (e.g. Hox genes, N-myc), cell adhesion molecules (e.g. laminin B1), growth factors (e.g. PDGF, TGFBs, BMPs) and growth factor receptors (e.g. EGF receptor, PDGF receptor).

The vertebral homeotic transformations, which are strikingly restricted to the cervical region, may provide one example of a cell autonomous effect of retinoids. As discussed (see Lohnes et al., 1994), the similarity of these malformations to those observed in some Hox-deficient animals suggests that RA may control axial patterning through direct control of expression of certain Hox genes. The chondrification of the meninges and other structures such as the persistent retrolentalicular mesenchyme in RAR mutants offers an example of RA involvement in cell fate specification, and suggests that factors involved in the initiation of chondrogenesis may be aberrantly expressed in RAR mutants. One possible candidate could be the chondrogenic factor BMP4, which is known to be down-regulated by RA treatment in embryocarcinoma cells (Rogers et al., 1992).

Cellular migration depends on the expression of cell adhesion and extracellular matrix proteins (Erickson and Perris, 1993). Retinoids have been shown to regulate the expression of a number of these proteins, including N-CAM (Husmann et al., 1989) and laminin B1 (Vasios et al., 1989, 1991). Perturbation of the expression of such proteins could lead to abnormal NCC migration (Bronner-Fraser et al., 1991) and contribute to the malformations of NCC-derived elements seen in RAR double mutants. Alternatively, these NCC defects could be attributed to postmigratory events, possibly involving growth factor or growth factor receptors. In this respect, there are striking similarities between the phenotypes of Patch (which harbor a deletion of the PDGF receptor α locus) and RAR mutant mice. In both types of mutants, the defects are observed primarily in cardiac and cranial NCC derivatives (e.g. skull bones, thymus, cornea, aortico-pulmonary septum), but not in gangliogenic NCC (Morrison-Graham et al., 1992).

A number of structures in RAR mutants appear to have been growth-arrested, among which is the myocardium. Several retinoid target genes have been implicated in normal cardiovascular development, including TGFβs and N-myc (reviewed in Gudas et al., 1994). Evidence supporting a role for these target genes include the finding that fetal VAD leads to down regulation of TGFβ1 in the developing heart (Mahmood et al., 1992). Furthermore, mice deficient for N-myc exhibit a myocardial deficiency similar to that observed in RARγ double mutants (Moens et al., 1993; Charron et al., 1992). That RA plays a role in branching morphogenesis is indicated by the defects in the lungs and kidneys of RAR mutants. Retinoid-responsive genes that are likely involved in lung branching morphogenesis include N-myc (Moens et al., 1993; Stanton et al., 1992), laminin B1 (Schuger et al., 1991) and the EGF receptor (Schuger et al., 1993). Interestingly, the mitogenic effect of RA appears to originate from the mesenchymal population of developing lungs (Schuger et al., 1993; see below).

From the above examples, it is clear that RA can play a role in many developmental processes through the regulation of a broad number of genes with diverse functions. Although the target genes and target cells/tissues directly affected in the RAR mutants remain to be determined, many of the candidate gene products (e.g. growth factors, extracellular matrix molecules) can act at a distance and in a number of distinct morphogenetic events. It is therefore not surprising that the site of expression of RARs and the localization of the affected structures do not always coincide in the mutants. The defects in branching morphogenesis of the lung may correspond to such a situation (see above). In all of these instances tissue recombination experiments will be required to determine which tissue is primarily responding to RA.

Many structures whose development is affected by administration of RA excess and is believed to require retinoids during ontogenesis (e.g. some regions of the CNS, neurogenic NCC; for reviews see Morris-Kay, 1991, 1993; Maden, 1994; Linney and LaMantia 1994) appear unaffected in the mutants examined to date. Since RARα1 and β3 isoforms (in addition to RARβ2) are expressed in some of these structures (our unpublished results), RARβ (all isoforms) and RARγ compound mutants are required to assess the possible role of RA in their development. Alternatively, retinoids may not be required for some of these RAR-positive tissues to develop properly (Linney and LaMantia, 1994). In this respect, it is worth remembering that even though RARγ is expressed in all precartilage condensations, irrespective of their embryological origin (Ruberte et al., 1990), only a subset of cartilage-derived
structures are affected in RARγα mutants, clearly indicating that, in some cases, the expression of RARγ is gratuitous with regard to cartilage histomorphogenesis.

Redundancy and variations in penetrance and expressivity

Taken together, the high degree of interspecies conservation of each individual RAR isoform throughout vertebrate evolution, the differential distribution of their transcripts during embryogenesis and in the adult (particularly for RARβ and γ transcripts which are often mutually exclusive), and the apparent specificity of their transactivation functions in transfected cells, have suggested that each RAR isoform may control the expression of a specific subset of RA target genes, thus contributing to the highly diverse effects of RA (see Chamblin, 1994 for a review). Unexpectedly, homozygous null fetuses for the RARα1, RARβ2 or RARγ2 isoforms or all RARα isoforms (Mendelsohn, 1994; Lohnes et al., 1993; Li et al., 1993; Lufkin et al., 1993) did not display any obvious abnormalities, and the congenital abnormalities exhibited by RARγ null fetuses were much more discrete than anticipated from its pattern of expression (Ruberte et al., 1990). The results reported here and in the accompanying study (Lohnes et al., 1994) support our earlier suggestion that there could be considerable functional redundancies between members of the RAR family (Lohnes et al., 1993; Lufkin et al., 1993).

It is particularly striking that in a number of cases the same malformations were apparently generated in different double mutants (e.g. RARγα and RARαβ2, see Tables in this and in the accompanying study of Lohnes et al., 1994). These abnormalities cannot be attributed simply to a lack of normal expression of the RARβ isoforms (see Lohnes et al., 1994). Thus, at one extreme, our present observations may be interpreted as indicative of complete redundancy between all RARs for transcriptional control of all RA target gene expression. In this scenario, the conservation of RAR isoform A/B regions would not correspond to a requirement for isoform specific AF-1 transactivation functions (for refs see Leid et al., 1992; Kastner et al., 1994; Chamblin, 1994). The only requirement would be to reach a certain threshold level of RAR in a given cell at a given time, which could be achieved through any combination of RAR isoforms. Multiple RAR genes with multiple promoters and specific patterns of expression would have evolved only to fulfill these purely ‘quantitative’ spatiotemporal requirements. Thus, in order to elicit a developmental defect in a cell expressing both RARα, RARβ2 and RARγ, it might be sufficient to inactivate either α and β2, α and γ, or β2 and γ to be below a critical threshold, whereas in a cell expressing RARα at a low level and RARγ at a high level, it might be sufficient to inactivate RARγ to be below this threshold. Interestingly, in several instances, an abnormality that is not presented by RARαγ γ mutants appears in RARαγ αβ2−/− mutants and, furthermore, an abnormality not present in RARαγ αβ2−/− mutants may be generated in RARβ (all isoforms) RARαγ double mutants (see Tables). Generating RARαγ double mutants is necessary to determine whether these RARαγ dosage effects correspond to a quantitative requirement for RARα1 and RARα2 isoforms or rather reflect a specific function of RARα2.

In view of the importance of inductive interactions during vertebrate ontogenesis, it seems more likely that the generation of the same malformation in two different double mutants reflects an ‘interaction’ between two RA-dependent events involved in a given developmental pathway. For instance, the generation of the same malformation in RARγα and RARαβ2 double mutants may correspond to the control of a growth factor receptor by RARγα in a given cell type, and of the corresponding growth factor in another cell type, with RARα and γ, and RARα and β2 being functionally redundant. Alternatively, the appearance of the same malformation in two different double mutants may correspond to two sequential defects in a given cell type, the results of which cannot be phenotypically distinguished.

At the present time, functional redundancies appear to provide the simplest explanation to account for the appearance of the VAD fetal abnormalities only in RAR double mutants. However, our results do not exclude more complex developmental scenarios in which multiple molecular defects generated by non-redundant receptors, each acting on specific subsets of RA target genes, would have to be combined to generate visible (phenotypic) abnormalities (see Thomas, 1993). In this respect, we note that the RARα1 and RARβ2 mutations, which on their own have no detrimental effects during development and in the adult, results in a number of fetal abnormalities and death shortly after delivery, when combined. It should also be kept in mind that the RARα and RARγ mutations are ultimately lethal at various stages after birth (Lohnes et al., 1993; Lufkin et al., 1993), and that the strong conservation of each RAR isoform across vertebrates suggests that each likely possesses at least one specific function. Examining the expression of a battery of putative RA-responsive genes in the various RAR mutants is necessary to determine whether the apparent phenotypic redundancy truly reflects a functional redundancy at the molecular level. Ultimately, the substitution in the mouse genome of one RAR isoform by another is required to determine whether, and to which extent, these receptors are functionally redundant.

For a given mutation, variability in the penetrance of a given abnormality between different animals, and variability of its expressivity within an animal, were very frequently encountered in the present and accompanying study of Lohnes et al. (1994) (see Tables). These variabilities may be related, at least in part, to variations in the levels of expression of redundant RARs. Variability in penetrance may be due to variations in the genetic background of the present null mutants, which is not homogenous. Increase in the penetrance of some abnormalities observed in RAR mutants upon sequential removal of RARγ, RARα1 and RARβ2 (i.e. RARγ, RARα1γ, RARα1γα2−/− and RARγα mutants; see Tables) might reflect variations in the expression of functionally redundant RARγ, RARα1 and RARβ2 in different animals depending on their genetic background. Alternatively, or concomitantly, variations in the levels of factors (e.g. transcription factors) which may act synergistically with RARs, may also account for the observed variations in penetrance. Variations in expressivity are particularly striking. Some of them probably result from stochastic variations in the amount of redundant receptors in contralateral cells of symmetrical structures. For instance, agenesis of the Harderian glands is either unilateral or bilateral in RARγα mutants (Lohnes et al., 1993), whereas it is always bilateral in RARα1γ and β2 mutants (see Table 4 of Lohnes et al., 1994); RARγ null cells with RARα1 or RARβ2 below
certain threshold levels may not respond to the RA signal, thus leading to the agenic phenotype, whereas cells with sufficient levels of RARα or β2 would respond. In other cases, stochastic variations of the levels of other synergistic factors may account for variations in expressivity. This may be the case for the dramatic variations in expressivity that are observed in the forelimbs of RARγ mutants (see Lohnes et al., 1994). It is likely that these frequent variations in penetrance and expressivity of the phenotypes exhibited by the various RAR mutants is a reflection of the complexity of the molecular mechanisms that underlie the developmental events controlled by retinoic acid.

We would like to thank Dr T. Pexieder for a critical reading of the manuscript, Dr A Dierich and P. Dollé for their collaboration and all members of the retinoic group for useful discussions; B. Weber, C. Fischer and V. Giroult and the technical staff of the animal facility for excellent help; B. Boulay, J. M. Lafontaine and C. Werlé for the illustrations and the secretarial staff for assembling the manuscript. C. M. was supported by a fellowship from the NIH (5F32 GM13597-03) and from the ARC. D. L. was a recipient of a fellowship from the MRC Canada and T. L. was a recipient of a fellowship from the American Cancer Society (PF-2919) and from the Fondation pour la Recherche Médicale. This work was supported by funds from the Institut National de la Santé et de la Recherche Médicale, the Centre National de la Recherche Scientifique, the Centre Hospitalier Universitaire Régional, the Association pour la Recherche sur le Cancer, the Human Frontier Science Program and the Fondation pour la Recherche Médicale.

REFERENCES


Hale, F. (1933) Pigs born without eyeballs. J. Hered. 24, 105-127


(Received 15 July 1994)