Retinoid nutritional status differently affects the expression of Japanese quail retinoic acid receptor-β isoform transcripts in a tissue-specific manner

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Abstract

We investigated the effects of vitamin A (VA) nutritional status on the levels of expression of retinoic acid (RA) receptor-β (RARβ) gene in the various tissues of Japanese quail. VA deficiency caused a significant decrease in the mRNA levels of brain, liver, heart, lung and kidney RARβ2/β4, whereas no change was observed in the level of testis RARβ2 transcript. In contrast, reduction in the RARβ1 transcript caused by VA depletion was observed only in the lung, remaining unchanged in the other tissues. The administration of RA to the VA-deficient quail rapidly induced the expression of RARβ2/β4 mRNAs in all the tissues examined, but RA increased the expression of RARβ1 transcript in the liver, heart, lung and kidney at a lower magnitude. RA could not change the expression of the brain RARβ1 transcript, while it induced the expression of the testis RARβ1 mRNA in a temporal way. These results clearly indicate that VA nutritional status differently regulates the expression of RARβ1 and RARβ2/β4 transcripts in a tissue-specific manner.

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Introduction

Retinol (vitamin A (VA)) is essential for normal growth, vision, reproduction and embryonic and fetal development, for the maintenance of numerous tissues, and for overall survival in many species. The indispensability of VA becomes clear by feeding VA-deficient (− VA) diets to animals. In embryonic development, maternal VA deficiency during gestation leads to embryonic malformations including cleft palate and abnormalities of the eyes, heart, circulatory system and urogenital tract (Wilson *et al*. 1953). In postnatal life, VA deprivation causes many abnormalities including retarded growth, widespread substitution of keratinizing squamous epithelium, atrophy of several glandular organs, eye lesions and testis degeneration (Wolbach & Howe 1925, Underwood 1984). These animals eventually die because of infection. The lesions can be reversed by VA administration, firmly confirming the importance of this vitamin as an essential factor in every aspect of life.

Retinoic acid (RA), a VA metabolite, is able to perform most of the actions of VA in vertebrates, because most VA-deficiency symptoms can be prevented or reversed by RA administration. Moreover, the action of RA is more vigorous or quicker than that of VA in many *in vivo* and *in vitro* instances (Heyman *et al*. 1992, Levin *et al*. 1992,

Lohnes *et al*. 1994, Mendelsohn *et al*. 1994b). Thus, it has been generally accepted that RA is the active form of VA. Recently, we also found that RA is more active than VA in the process of sexual maturation of Japanese quail (Fu *et al*. 1997b, 2000).

The biological effects of the RA signal are mediated through its specific nuclear receptors, which act as inducible transcriptional regulatory proteins and belong to the steroid/thyroid hormone superfamily of nuclear receptors (Giguerre *et al*. 1987, Petkovich *et al*. 1987, Evans 1988). There are two distinct classes of retinoid receptors: RA receptors (RARs), which are activated by both all-*trans* and 9-*cis* RA, and retinoid X receptors (RXRs), which bind to and are activated by 9-*cis* RA only. Each class consists of three subtypes of receptor (RARα, β, γ; RXRα, β, γ). From each gene, several isoforms are derived by alternative splicing and the usage of different promoters (Kastner *et al*. 1990, Leroy *et al*. 1991, Zelent *et al*. 1991). The functions of these receptors have been studied using transgenic mice with targeted disruption of a particular receptor or double receptors. Disruption of the single RARα (Lufkin *et al*. 1993), RARγ (Lohnes *et al*. 1993) or RXRβ (Kastner *et al*. 1996) resulted in male sterility in homozygous knockout mice; these effects were similar to those observed in the male mice kept on a − VA diet. Compound null mutations of RARs led to lethality

in utero or shortly after birth and to numerous developmental abnormalities (Lohnes *et al.* 1994, Mendelsohn *et al.* 1994*b*); most of them resembled those observed in the offspring of VA-deficient dams (Wilson *et al.* 1953). These studies strongly support the function of RARs serving as transcriptional transducers of the retinoid signal. Therefore, elucidating how VA nutritional status affects the expression of RARs and/or RXRs will help in understanding the physiological actions of VA.

The influences of VA depletion or repletion on the expression of RARs and RXRs have been examined in various tissues of adult mammals (Ul-Haq *et al.* 1991, Kato *et al.* 1992, Van Pelt *et al.* 1992, Verma *et al.* 1992, Gaemers *et al.* 1997, Akmal *et al.* 1998). Results from these studies demonstrated that, of RARs and RXRs, RARβ was most sensitive to changes of VA nutritional status, suggesting that RARβ functions, at least partly, as a participant in the retinoid-signaling pathway. In birds, the effects of VA deficiency on the expression of RARs have been analyzed only in the very early stages of embryonic development, suggesting the importance of RARβ2 in the mechanism of the action of retinoids in early avian development (Kostetskii *et al.* 1996). What has remained unknown, however, are the molecular mechanisms of retinoid signal transduction pathways that are responsible for the essential physiological functions of VA in birds after hatching. We recently found that RARβ is widely expressed in various tissues of adult Japanese quail (Fu *et al.* 2001). This result suggests that RARβ may play an important role in the maintenance of numerous tissues in Japanese quail.

The purposes of the studies described here were to determine how VA deficiency and retinoid administration affect the expression of RARβ isoform transcripts in various tissues of Japanese quail, and to explore the relationship between the growth stages of birds and the effects of retinoid administration on RARβ gene expression.

Materials and Methods

Animals and experimental design

One-day-old male Japanese quail were housed in a brooder and maintained on continual illumination for 3 days, and then were subjected to a 14 h light:10 h darkness cycle. Food and water were available at all times throughout the experiment. Three different diets, a −VA diet, a −VA diet supplemented with 5000 IU/kg diet of all-*trans* retinyl acetate (+VA diet), and a −VA diet supplemented with 4 mg /kg diet of all-*trans* RA (+RA diet), were prepared as described previously (Fu *et al.* 2000). In the −VA diet, other nutrients except VA were sufficient to support normal growth. All three diets were prepared fresh weekly and stored at −20 °C until use. All-*trans* retinyl acetate and all-*trans* RA were purchased

from Wako Purechemical (Osaka, Japan) and Sigma Chemical Co. (St Louis, MO, USA) respectively.

In Experiment 1, 1-day-old quail were randomly divided into two groups (control and VA-deficient groups). The VA-deficient group was fed the −VA diet, while the control one was maintained on the +VA diet. To assess the degree of VA depletion in the VA-deficient group, serum VA and hepatic retinyl palmitate levels were determined at 7, 14, 17 and 21 days of age by HPLC as described previously (Fu *et al.* 1998, 1999). At 14 and 21 days of age, five birds from each group were killed by decapitation, immediately after which their tissues, including brain, liver, heart, lung, kidney and testis, were dissected and frozen in liquid nitrogen and stored at −80 °C until analysis.

In Experiment 2, 1-day-old quail were fed the +VA or −VA diet for 21 days. At this time, five quail from each group were injected i.m. with 0·1 mg RA, 0·1 mg VA or an equal amount of vehicle. RA and VA were dissolved in ethanol (10 mg/ml), and were diluted 5-fold in rapeseed oil prior to injection. The quail were killed 3 or 8 h after treatment and their tissues were dissected and immediately frozen in liquid nitrogen, then stored at −80 °C until analysis.

In Experiment 3, in order to deplete VA and RA as completely as possible, 1-day-old quail were fed the −VA diet for 2 weeks, then the +RA diet for 2 or 4 weeks, and finally the −VA diet for 1 week. Then the quail were treated with VA, RA, or vehicle as described in Experiment 2. The quail were killed 3 h after treatment, immediately after which their tissues were dissected and frozen as described in Experiment 1.

All experiments were performed under the guidelines of the Animal Usage Committee of the Faculty of Agriculture, University of Tokyo.

Lysate RNase protection assay

A 425 bp segment of qRARβ2 cDNA, which consisted of 9 bp of 5′-untranslated region, all of the A and B domains and part (177 bp) of the C domain (corresponding to positions 18–443 bp, GenBank accession No. AF110730), was amplified by RT-PCR. To generate an antisense RARβ cRNA probe, this 425 bp cDNA was subcloned into the pCR II vector (Invitrogen Carlsbad, CA, USA), linearized with HindIII restriction endonuclease, and transcribed by T7 RNA polymerase in the presence of [α-^{32}P]UTP as described previously (Matsumura *et al.* 1996). We have confirmed that an assay using the antisense RARβ2 cRNA probe can distinguish mRNA expression among different isoforms of qRARβ (Fu *et al.* 2001).

Levels of qRARβ mRNA in tissues obtained from Experiments 1, 2 and 3 were analyzed by lysate RNase protection assay as described previously (Fu *et al.* 1997*a*, 2001, Kato *et al.* 1999). Briefly, tissues were homogenized

in five volumes (w/v) of lysis buffer (5 M guanidinium thiocyanate, 0·1 M EDTA, 0·1 M Tris, pH 7·0, 1% β-mercaptoethanol). The homogenate was centrifuged at 10 000 *g* for 5 min at room temperature, and aliquots of 50 μl supernatant were mixed with 5 μl lysis buffer containing 250 000 c.p.m. of probe. After overnight hybridization at 37 °C, 500 μl digestion buffer containing RNase A and RNase T1 were added, and excess probes were digested at 37 °C for 30 min. Twenty microliters 10% sodium N-lauroyl-sarcosinate and 5 μl proteinase K solution (RNA grade; Gibco BRL, Rockville, MD, USA) were added, and tubes incubated at 37 °C for 30 min. The tubes were centrifuged for 2 min at room temperature. The supernatant and 500 μl isopropanol were mixed, kept at −20 °C for 30 min, and then centrifuged for 15 min. The precipitates were dried and resuspended in sample buffer. Hybridized RNA was size-separated on an 8% acrylamide gel. To compare equivalent quantities of RNA, quail β-actin (120 bp) was used as an internal control.

Data analysis

Hybridized blots were imaged and analyzed using MacBAS-2000 (FujiPhoto Film, Tokyo, Japan). All values for mRNA levels are given as a relative value within tissue in an experiment. In cases where the s.e.m. is given, the means reflect five samples, and the statistical significance was determined by one-way or repeated measures ANOVA. *Post hoc* analysis was performed with Tukey's multiple comparisons test.

Results

VA deficiency affects the expression of qRARβ isoform mRNAs in tissue/isoform-specific manners (Experiment 1)

Being fed the −VA diet for 14 days did not retard the growth of the quail, but significantly reduced their growth during the next 7 days ($P<0·05$) (Fig. 1). The process of VA depletion in plasma and liver of the −VA birds during this period is shown in Fig. 2. The VA in plasma (df=4,24; $F=330·6$; $P<0·001$) and total retinyl palmitate in liver (df=4,24; $F=57·4$; $P<0·001$) declined rapidly with age when the quail were fed the −VA diet, and both reached a not-detected level on day 21. In contrast, serum VA levels in the control group stayed almost constant (about 110 μg/dl), and abundant storage of retinyl palmitate in the liver was also confirmed (data not shown).

Then, we examined how VA deficiency influences the expression of qRARβ isoform transcripts in various tissues. Levels of qRARβ isoform mRNAs were measured by the RNase protection assay in many tissues on day 14 and day 21, when the quail were under different degrees of VA deficiency. As illustrated in Fig. 3, two major bands were present in all tissues examined. They represented qRARβ1 and β2 transcripts respectively, according to our
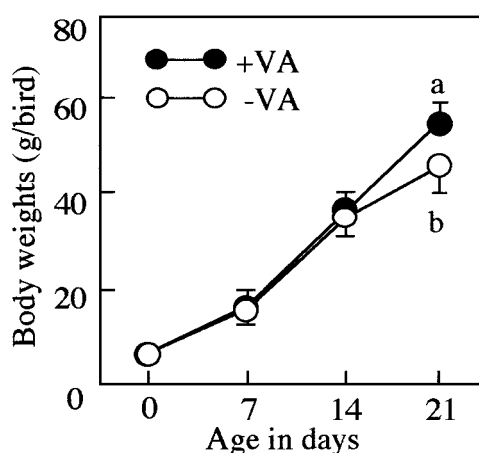

Figure 1 Feeding Japanese quail a −VA diet for 3 weeks retarded their growth. One-day-old quail were fed a −VA diet or this −VA diet supplemented with 5000 IU/kg diet of VA (+VA). Data represent means ± s.e.m. (*n*=5). Means not having the same letters within the same week differ significantly (*P*<0·05).

previous results (Fu *et al.* 2001). An additional, weak band existing between the β1 and β2 is considered to be qRARβ4, based on our recent results from analysis of the structure of the qRARβ gene (Z W Fu, T Noguchi & H Kato, unpublished observations).
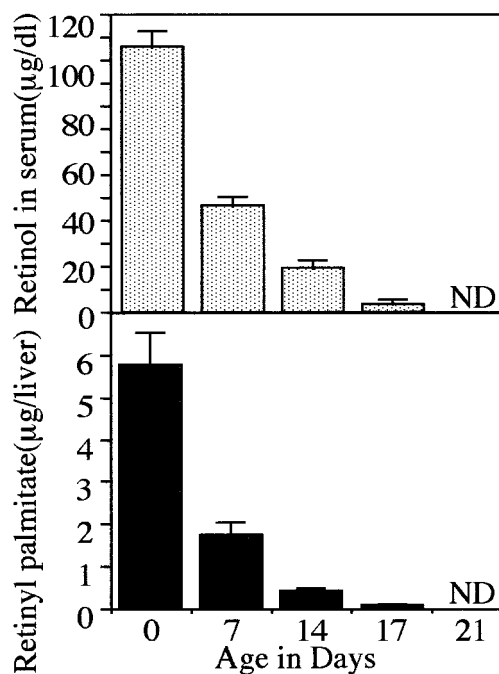

Figure 2 Depletion of VA in serum and liver of Japanese quail. One-day-old quail were fed a −VA diet, and concentrations of serum VA and hepatic retinyl palmitate were measured by HPLC as described in Materials and Methods. ND means undetected level. Data represent means ± s.e.m. of five samples.
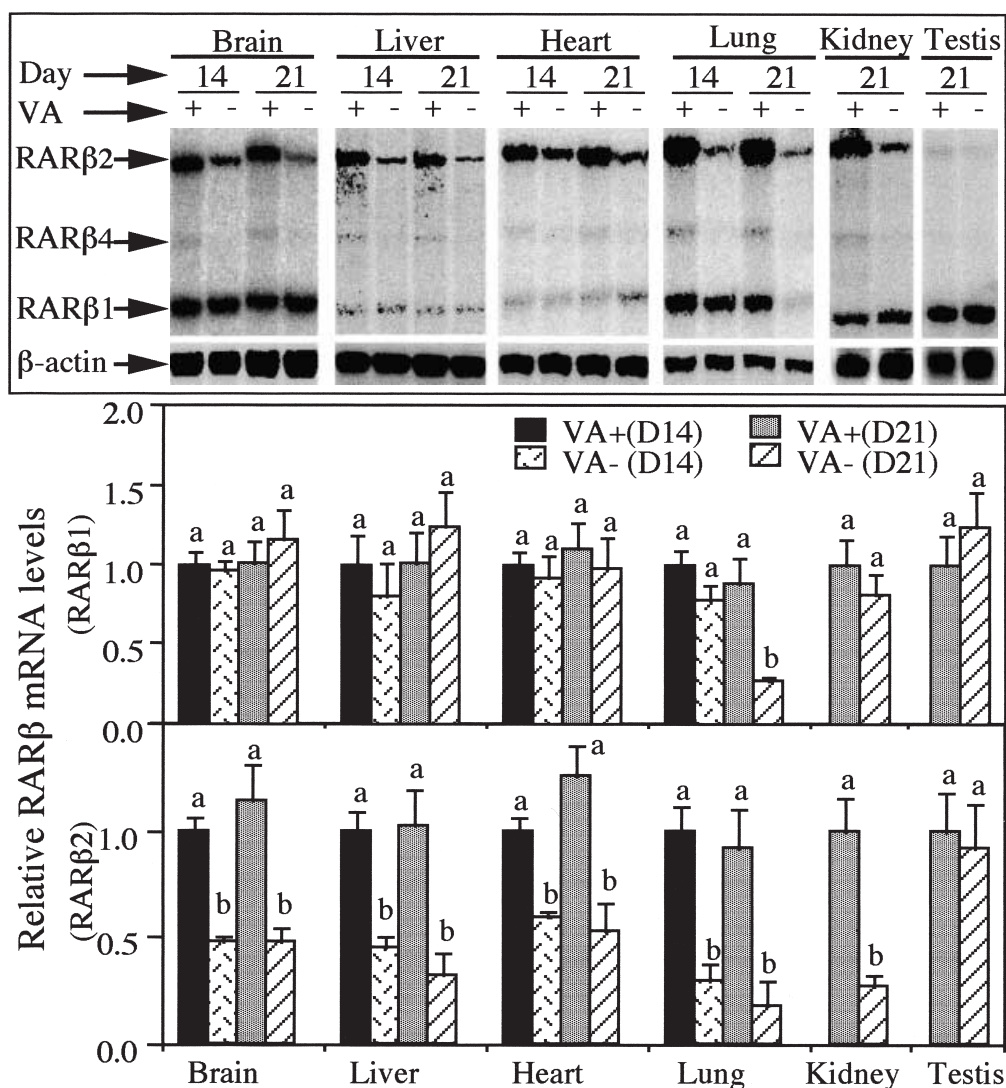
Figure 3 VA deficiency mainly reduces the expression of RARβ2 and β4 transcripts. One-day-old Japanese quail were fed a −VA diet or a −VA diet supplemented with VA as control. At 14 and 21 days of age, five birds from each treatment group were killed by decapitation. Representative results obtained by the lysate RNase protection assay from the selected tissues are shown in the upper panel. The quantitative representations of multiple results expressed as values relative to the abundance in the control (day 14 or 21) are presented in the lower panel. Means not having the same letters within tissues differ significantly (P<0·05).

The qRARβ1 mRNA levels in brain, liver, heart and kidney were not affected by VA deficiency regardless of the degree of vitamin depletion. The expression of qRARβ1 mRNA in lung, however, decreased significantly (P<0·05) in the VA-deficient group from day 14 to day 21. In contrast, expression of qRARβ2 mRNA was much more sensitive to the change of VA status. Feeding quail the −VA diet for 14 or 21 days significantly reduced the levels of qRARβ2 mRNA in all tissues examined except testis. In the testis, however, neither the qRARβ1 mRNA nor the qRARβ2 mRNA level was influenced by VA deficiency. In addition, the expression of qRARβ4

mRNA in many tissues showed a similar response to VA deficiency as seen in that of qRARβ2 mRNA (data not shown). These results clearly indicate that VA deficiency affected the expression of qRARβ isoform mRNAs in a tissue- and isoform-specific manner.

RA rapidly induces the expression of qRARβ isoform transcripts (Experiment 2)

Injection (i.m.) of RA (0·1 mg/bird) for 3 h into the VA-deficient quail dramatically increased the levels of
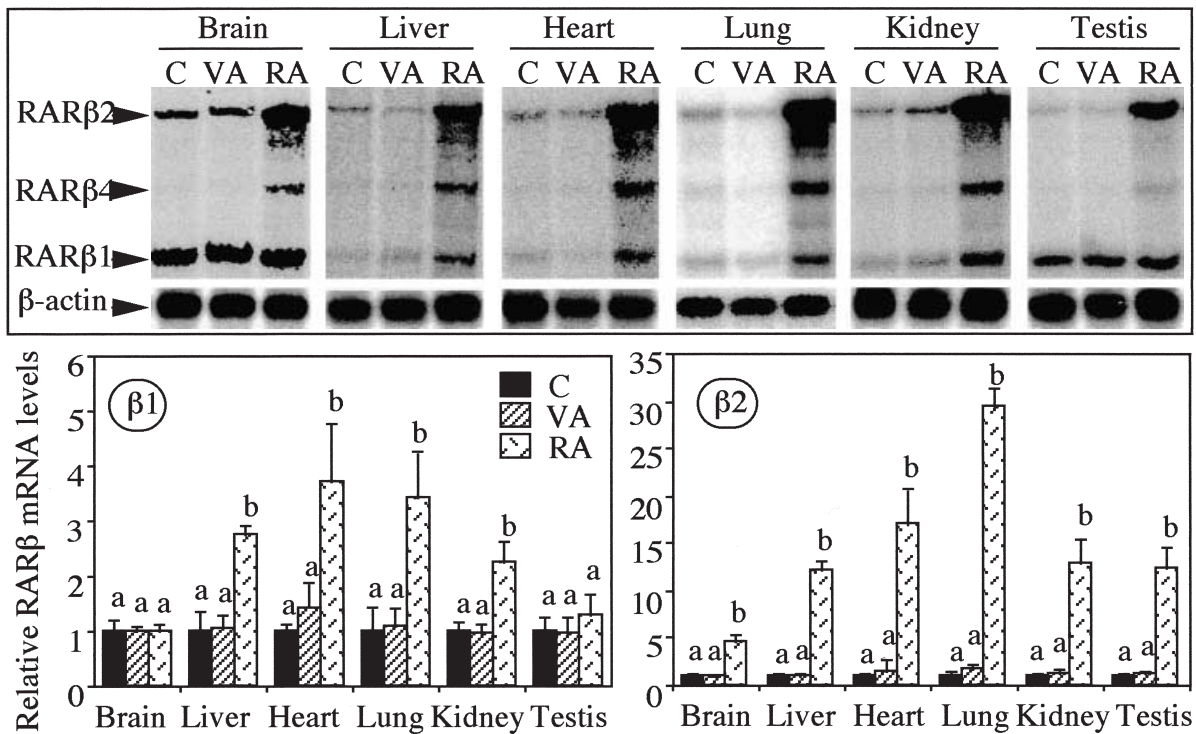
Figure 4 RA induces the expression of RARβ isoform transcripts in a tissue-specific manner. Japanese quail were depleted of VA by feeding them a −VA diet for 3 weeks. They were killed 3 h after treatment with 0·1 mg/bird of VA or RA, and their tissues were processed for measuring the expression of RARβ isoform mRNAs by the lysate RNase protection assay. A typical result of the assay is shown in the upper panel, and the quantitative representation of multiple results expressed as values relative to the abundance in the vehicle treatment (C) are presented in the lower panel. Data represent means ± S.E.M. of five samples. Means not having a common letter are significantly different ($P<0·05$).

qRARβ2 mRNA in all tissues examined, with a large variation (Fig. 4). As compared with the expression in the control quail (with vehicle injection), the expression of qRARβ2 was increased more than 25-fold in the lung and heart, while only a 5-fold increase was observed in the brain. With respect to the change in the qRARβ1 expression, a tissue-specific response to the RA treatment was clearly observed (Fig. 4). Administration of RA caused a significant increase in the levels of qRARβ1 mRNA in the lung, heart, kidney and liver, whereas no change could be detected in the brain and testis. Additionally, the expression of qRARβ4 could also be induced by RA administration in a manner similar to that of qRARβ2 (the upper panel of Fig. 4).

In contrast to RA treatment, administration of an equimolar amount of VA (0·1 mg/bird) to the VA-deficient quail did not alter the levels of qRARβ1 and β2/4 mRNAs in any tissue examined after 3 h (Fig. 4). This might be partly due to the time lag for conversion of VA to RA, the active form of VA. To prove this hypothesis, we measured the expression of qRARβ isoform mRNAs in several tissues 8 h after administration of

VA or RA to the VA-deficient quail. As shown in Fig. 5, an injection of VA also induced the expression of the qRARβ2 mRNA in the selected tissues, although the magnitude of induction was less than that by RA. Interestingly, the qRARβ1 mRNA levels in this case were not influenced by treatment with VA after 8 h, whereas the changes in the qRARβ1 mRNA levels responding to the RA treatment exhibited similar tissue-specific patterns to those shown in Fig. 4.

To determine whether the inductive effect of RA on the expression of the qRARβ isoform transcripts depends upon retinoid status, we carried out an experiment with the VA-sufficient quail that was similar to the experiment done with the VA-deficient quail. Regardless of retinoid status, an injection of RA (0·1 mg/bird) into the VA-sufficient quail also dramatically increased the levels of both qRARβ1 and β2 mRNAs in the lung, and the level of qRARβ2 mRNA in the brain (Fig. 6). The magnitude of induction was less than that seen in the VA-deficient quail, reflecting the differences in the basal mRNA levels of the two control groups (+VA vs −VA).
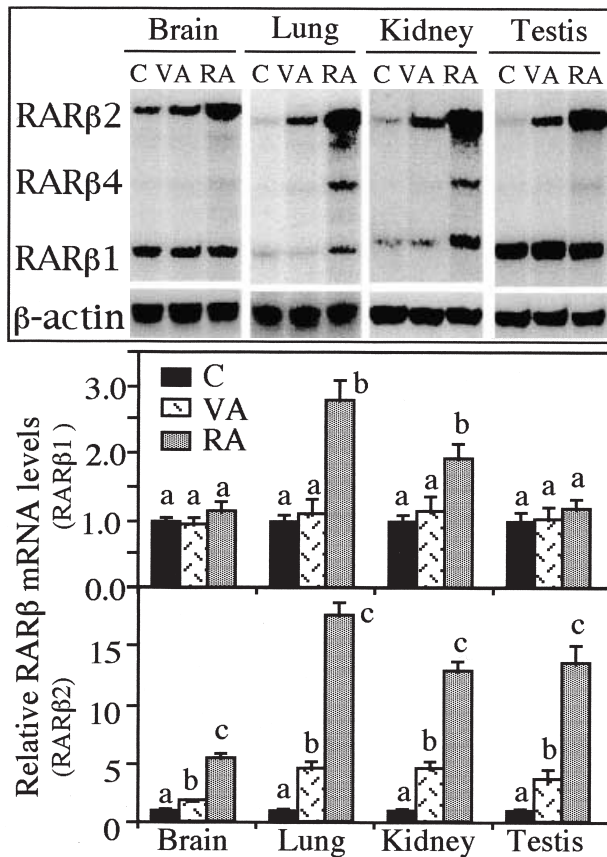
Figure 5 RA affects expression of RARβ2/4 mRNA more efficiently and more quickly than VA does. Japanese quail were depleted of VA by feeding them a −VA diet for 3 weeks. They were killed 8 h after treatment with 0·1 mg/bird of VA or RA, their tissues were processed for measuring the expression of RARβ mRNA by the lysate RNase protection assay. Data are shown as the relative abundance to those of the vehicle treatment (C). Data represent means ± S.E.M. of five samples. Means not having a common letter are significantly different ($P<0·05$).

Figure 6 An excess of RA induces the overexpression of the RARβ isoform transcripts in Japanese quail. RA (0·1 mg/bird) was i.m. injected into the VA-sufficient quail (3 weeks of age). After 3 h these quail were killed and tissues were dissected for measuring the RARβ isoform mRNAs by using the lysate RNase protection assay. Typical results of the assay in the brain and lung are shown in the upper panel, and the relative abundance of RARβ1 or β2 mRNA to that of the vehicle treatment (C) (means ± S.E.M., $n=5$) respectively is shown in the lower panel. Means not having a common letter are significantly different ($P<0·05$).

RA induces the expression of the testis qRARβ1 transcript in a temporal manner (Experiment 3)

In our previous study, we found that the expression of qRARβ1 mRNAs clearly exhibits the developmental changes in the reproductive system of Japanese quail (Fu *et al.* 2001). Thus, we hypothesized that the inductive effect of RA on the expression of qRARβ1 isoform transcript, as shown above, may differ to some extent depending on the growth stage. To elucidate this matter, we examined the effects of retinoid administration on the expression of qRARβ isoform mRNAs in various tissues of 5- and 7-week-old quail, whose degree of sexual maturation obviously differed. At 5 weeks of age, when sexual maturation was in process and the expression of qRARβ1 mRNA was at the maximum level, the administration of RA did not enhance the expression of
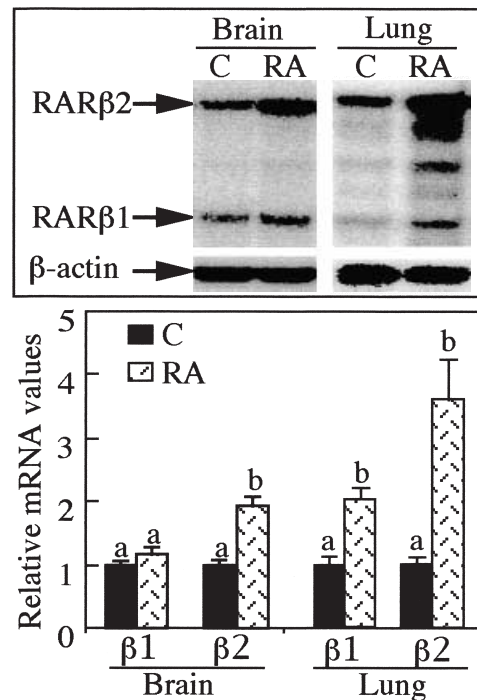
qRARβ1 mRNA in the testis and brain as seen in Experiment 2 (data not shown). However, administration of RA to quail at 7 weeks of age, when sexual maturation was completed and the level of qRARβ1 mRNA in the testis lowered to about 50% of the 5-week-old value (data not shown), significantly increased the level of the qRARβ1 mRNA 2-fold (Fig. 7). The expression of the qRARβ1 in the brain was still not changed at this stage (Fig. 7). These results indicate that the regulation of the qRARβ1 transcript by RA may depend upon the developmental stage of the testis.

Discussion

Feeding 1-day-old quail the −VA diet caused rapid decreases in the levels of both serum VA and hepatic retinyl palmitate, and consequently reduced the growth rate. To elucidate the physiological changes underlying the alteration of VA status in birds, we examined the expression of RARβ gene in the various tissues of both
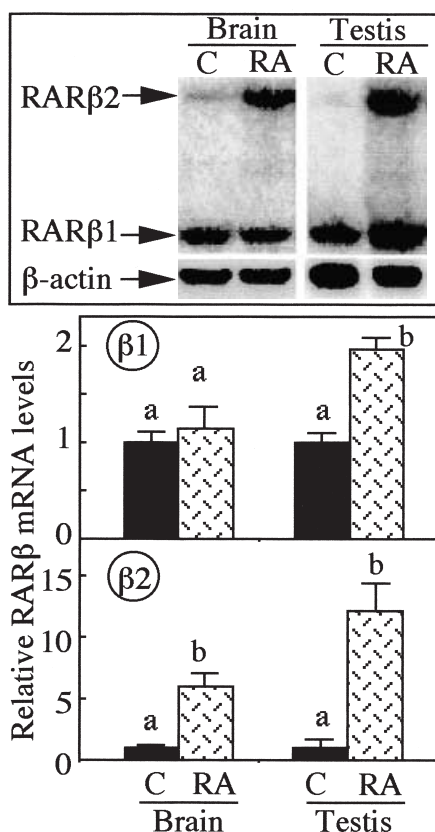
Figure 7 RA affects the expression of RARβ1 mRNA in a stage-specific manner in the testis. Japanese quail were depleted of VA by feeding them a −VA diet for 2 weeks, −VA supplemented with RA for 4 weeks, and then −VA for 1 week. These quail were killed 3 h after they were administered 0·1 mg/bird of RA or the vehicle (as control, C), and their tissues were processed for assaying RARβ mRNA. Typical results in the brain and testis are shown in the upper panel. The relative abundance of RARβ1 or β2 mRNA to that of the control group (means ± S.E.M., $n=5$) is shown in the lower panel. Means not having a common letter are significantly different ($P<0.05$).

normal and VA-deficient quail, before and after retinoid administration, since RARβ has been thought to be involved in the retinoid signal transduction pathways in mammals (Lohnes *et al.* 1993, 1994, Lufkin *et al.* 1993, Mendelsohn *et al.* 1994*b*).

The results presented here clearly indicate that retinoid nutritional status specifically modulates the expression of each qRARβ isoform transcript. VA deficiency significantly reduced the expression of qRARβ2 and β4 mRNAs in the tissues examined except testis, whereas the levels of the qRARβ1 transcript in these tissues were almost unchanged. The absence of the effect of VA deficiency in the testis qRARβ2 mRNA might be partly due to the lower level of this isoform expressed in this

tissue. Thus, these results strongly indicate that RARβ2 and β4 rather than β1 is VA-dependent during quail growth. Previously, several research groups (Ul-Haq *et al.* 1991, Kato *et al.* 1992, Verma *et al.* 1992, Gaemers *et al.* 1997) have shown that the expression of RARβ is greatly reduced in many VA-deficient rat or mouse tissues. However, those groups described only the effect of VA deficiency on the expression of the pan-RARβ transcripts, without addressing the question of which mRNA isoforms were more susceptible to VA deficiency. Our present results clearly demonstrate for the first time that the expression of RARβ1 in intact animals is regulated differently by VA than is the expression of RARβ2 and β4. In addition, both qRARβ1 and β2 mRNAs decreased in the VA-deficient lung, in agreement with the results for the lung of VA-deficient rats (Takeyama *et al.* 1996) and those for VA-deficient quail embryos (Kostetskii *et al.* 1996). Taken together, these results clearly indicate that VA regulates the expression of RARβ isoform transcripts in an isoform-specific manner, and also in a tissue-specific manner.

The effect of VA deficiency on the levels of RARβ expression was transiently reversed by the administration of RA. A single dose of RA to the VA-deficient quail rapidly increased the levels of the qRARβ2 mRNA in all tissues examined. In the case of the qRARβ1 mRNA, however, the inductive effect of RA was exhibited in a tissue-specific manner. RA could induce the expression of the qRARβ1 in the liver, heart, lung and kidney, while no change was observed in the levels of qRARβ1 mRNA in the brain and testis. These results further confirmed that RARβ2 is more sensitive to changes in retinoid status. Similar induction of RARβ transcripts has been characterized in mammalian tissues (Kato *et al.* 1992, Gaemers *et al.* 1997) and in several cell lines (Clifford *et al.* 1990, Hu & Gudas 1990, Zelent *et al.* 1991). Moreover, we have shown that administration of RA to the control (VA-sufficient) quail resulted in the overexpression of the RARβ gene. This rapid increase in the RARβ mRNA levels in the tissues of whole animals by RA treatment, together with the down-regulation of the qRARβ transcripts in the tissues of VA-deficient quail, suggests an important role for RARβ in mediating the physiological actions of VA in Japanese quail.

The magnitude of induction of RARβ isoforms by the RA treatment seemed to be higher in the more highly vascularized tissues such as liver, heart and lung (Fig. 4). Thus, this could raise the possibility that the tissue-specific changes in RARβ isoforms are due to variations in blood cell components or vascularization among different tissues. In our sampling protocol, quail were killed by decapitation, and bled as completely as possible before the tissues were dissected. It has been reported that the expected yield of total RNA from the animal blood cell is far lower than that from any tissue (for example, about 15–20 μg/ml of whole blood vs 6–10 mg/g of liver). Moreover, we have

found that each qRARβ isoform was almost not expressed in the quail blood cells, and that the RA treatment could not induce their expression (data not shown). In addition, we have also found that the level of each qRARβ isoform mRNA in blood vessels including aorta, pulmonary trunk and superior and inferior vena cava was not higher than those in the heart or lung, and that the inductive magnitude in the blood vessel by the treatment of RA was not greater than those observed in either heart or lung (data not shown). From these results, the possibility mentioned above seems to be low.

In this study, we also examined the influence of VA on RARβ gene expression. Compared with RA, an equimolar amount of VA was not able to induce the expression of each qRARβ isoform transcript 3 h after administration to the VA-deficient quail. However, a significant increase was observed 8 h after administration of VA, although the inductive magnitude was lower than that of RA treatment. These results indicated that the induction of RARβ gene expression by VA is delayed and less strong than that by RA. Several comparable results were also reported in mammals (Kato *et al.* 1992, Van Pelt *et al.* 1992, Gaemers *et al.* 1997). These results suggest that the delay and weak effect may be partly due to the time lag for the conversion of VA to RA, or partly due to differences in peak RA exposure caused by differences in metabolite transport or delivery kinetics to target organs. Recently we have found that when Japanese quail were fed diets depleted of VA but supplemented with RA the growth of their testes was faster than in VA-fed controls (Fu *et al.* 1997*b*). Furthermore, we also observed that RA exerts the same effect on the maturation of the female reproductive system in Japanese quail as it does in the male (Fu *et al.* 2000). In addition, we have found that RA is easily taken up by the testis of Japanese quail (Fu *et al.* 1997*b*), in contrast to the case with mammalian reproductive organs (Kurlandsky *et al.* 1995). Taken together, these results suggested that the accelerative effects of RA on the maturation of the reproductive organs in quail may be an action of RA through its nuclear receptors to alter the expression of genes that relate to sexual maturation.

It has been reported that RARβ1 and RARβ2/4 are generated by the use of two different promoters in mammals (Zelent *et al.* 1991, Mendelsohn *et al.* 1994*a*). RA response element (RARE) has been identified in the promoter region of mouse RARβ2/4 (Zelent *et al.* 1991), whereas no RARE exists in the RARβ1 promoter region (Mendelsohn *et al.* 1994*a*). *In vitro* study has shown that RA modulation of RARβ2/β4 transcripts is achieved at the level of transcriptional initiation via RARE present in the RARβ2/β4 promoter (Mendelsohn *et al.* 1991). In contrast, RARβ1 promoter activity is not modified by RA treatment, and the RA-induced accumulation of RARβ1 transcript

may occur via an RA-dependent release of a block in RNA chain elongation (Mendelsohn *et al.* 1994*a*). We recently analyzed the structure of the qRARβ gene and found that the DNA sequence of the qRARβ2/4 5′-untranslated region was highly conserved compared with that in human and mouse RARβ2/4. Furthermore, RARE and a TATA box also existed in the qRARβ2/4 promoter region (Z W Fu, T Noguchi & H Kato, unpublished observations), as is seen in mammals. Therefore, the differences of RARβ1 and β2/4 transcripts in their responses to changes in retinoid status might be due to the use of different promoters in the qRARβ gene.

An additional interesting observation reported here is that the inductive effect of RA on the testis qRARβ1 transcript depended upon the growth stage. RA could not induce the expression of testis qRARβ1 transcript at 5 weeks of age, while it caused a 2-fold increase at 7 weeks of age. Meanwhile, the level of the qRARβ1 transcript decreased by about 2-fold during this period of testicular development, which is in agreement with our previous result (Fu *et al.* 2001). These results mean that the RA-induced accumulation of the testis qRARβ1 transcript was almost equal to the decreased amount, which occurred during testicular maturation. Moreover, the absence of the inductive effect of RA on the brain qRARβ1 transcript at any developmental stage examined might be due to the constant expression of this isoform throughout the whole development (Fu *et al.* 2001). Therefore, from these results we hypothesize that retinoid treatment has an inductive effect only when the RARβ1 mRNA level is below the maximum.

Analysis of RARβ gene structure in mammals further suggested that the 5′-untranslated region is involved in the tissue-specific expression of this gene (Zelent *et al.* 1991, Mendelsohn *et al.* 1994*a*, Zimmer *et al.* 1994). Therefore, further analysis of the qRARβ gene structure will surely help the understanding of the mechanism underlying the effect of retinoid status on the tissue-specific expression of each isoform.

In conclusion, the present study clearly demonstrates the diverse regulation of RARβ isoforms by VA nutritional status in the quail and suggests distinct roles for the RARβ isoforms in the physiological actions of VA.

Acknowledgements

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