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Original Article

Characterization of cDNAs encoding two chick retinoic acid receptor α isoforms and distribution of retinoic acid receptor α, ß and γ transcripts during chick skin development

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ABSTRACT The amino acid sequence of the retinoic acid receptors α, ß and γ (RARα, ß and γ) can be divided into six functional domains (A-F), different isoforms arising from the presence of different A domains by differential splicing. In order to address the respective roles of the different RARs during skin morphogenesis in birds, cDNAs encoding two chick RARα isoforms (α1 and α2) have been isolated. While the A1 and B-F domains of the RARα are highly conserved across species, the chick A2 domain contains 50% specific amino acids. The three *RARα, ß* and *γ* genes display specific patterns of expression during chick skin morphogenesis. As in mouse, *RARα* and *γ* transcripts are present in both the dermis and epidermis during the first stages of skin appendage formation. Furthermore, Northern blot analysis suggests that different RARα and γ isoforms could be successively required during feather formation. The *RARγ* gene, continuously expressed in the epidermal cells in both chick and mouse, is thus likely to play a similar role in skin development in these two species. However, *RARα* transcripts, only transiently detected during mouse skin development, still accumulate in epidermis during the later stages of chick skin differentiation. Furthermore, *RARß* transcripts, never detected during normal development in mouse skin, are actually present at the early stages of chick skin morphogenesis. Thus, our results suggest that the role of the three RAR in skin development has not been strictly conserved in the different classes of vertebrates.

KEY WORDS: *Gallus, gene expression, nuclear receptor, RAR isoforms, transcription factor*

Introduction

Retinoic acid (RA) receptors α, ß and γ (RARα, ß and γ) are ligand-inducible *trans*-regulators that control transcription of target genes by interacting with *cis*-acting RA responsive elements (Chambon *et al.*, 1991; Leid *et al.*, 1992, 1993). Their amino acid (aa) sequence can be divided into six domains (A-F) based on homology among themselves and with other members of the hormone nuclear receptor superfamily (Green and Chambon, 1988). The amino-terminal A and B domains contain a ligand-independent transactivation function, different A domains giving rise to different RAR isoforms by differential use of two promoters and alternative splicing. The C domain contains two zinc fingers and is implicated in DNA-binding and dimerization. The E domain contains a ligand-dependent transactivation function and a dimerization interface. The functions of the D and F regions remain poorly understood.

During mouse embryogenesis and in the adult, the three *RAR* genes display specific spatio-temporal patterns of expression (Zelent *et al.*, 1989; Kastner *et al.*, 1990). *RARγ* transcripts are preponderant in cartilage as well as in keratinizing squamous epithelia (Ruberte *et al.*, 1990, 1991; Viallet and Dhouailly, 1994). *RARß* expression appears mutually exclusive with that of *RARγ*, whereas *RARα* expression, particularly the *RARα1* isoform, is almost ubiquitous (Dollé *et al.*, 1990).

RA treatment has marked effects on embryonic skin differentiation, leading to the exchange of one developmental pathway for another, i.e., abnormal feather location on the normally scaled feet of chick embryos (Dhouailly *et al.*, 1980) and

Abbreviations used in this paper: aa, amino acid(s); ff, feather formation; HH, Hamburger and Hamilton; nt, nucleotide(s); PCR, polymerase chain reaction; RA, retinoic acid; RAR, retinoic acid receptor; *RAR*, cDNA or mRNA coding for RAR.

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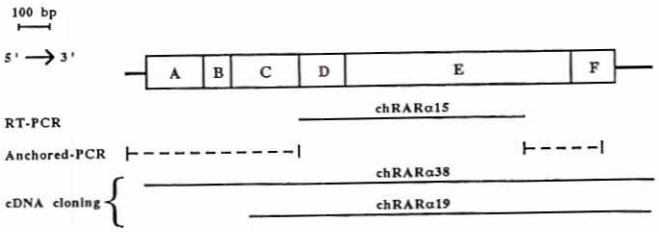


Fig. 1. Chick *RARα* cloning strategy. *Degenerate oligonucleotides (whose position is indicated by arrows in Fig. 2) were derived from evolutionarily well conserved parts of C and E domains of human and mouse RARα genes. They were used as primers to amplify poly(A)⁺-RNA extracted from a mixture of 4-, 7-, 10- and 14-day whole chick embryos (stages 24, 31, 36 and 40 HH, respectively) using RT-PCR. The derived clones (as for example chRARα15) are 666 bp long. Assays were then made to isolate the 5' and 3' part of the RARα coding region by anchored-PCR. It was thus possible to characterize 57 bp of the 5' untranslated region and the sequences encoding the A1, B and C domains, and the end of the E domain plus the 28 first codons of the F region (dashed lines). The chRARα15 clone was then used as a probe to screen 1.5x10⁶ lysate plaques of a cDNA library prepared from 8.5-day (stage 35 HH) embryonic chick skin poly(A)⁺-RNA. The two isolated cDNAs are aligned under the schematic RARα coding region.*

glomerular glands in the place of hair vibrissae in mouse (Hardy, 1968, 1983). In this species, the expression of the *RARα* and *γ* genes occurs in both the dermal and epidermal components during the first stages of hair morphogenesis; later, *RARα* and *RARγ* transcripts are no longer detectable in the dermal cells, whereas *RARγ* transcripts become abundant in the epidermal cells (Kastner *et al.*, 1990; Viallet and Dhouailly, 1994). Thus, it is reasonable to assume that changes in the expression pattern of the three *RAR* genes may be in some way related to the dermal-epidermal interactions which take place during cutaneous appendage morphogenesis (Dhouailly, 1984), each *RAR* gene performing a specific function possibly conserved across the different classes of vertebrates. In order to investigate this possibility, we analyzed the expression pattern of the three *RAR* genes at the main stages of chick skin morphogenesis.

To date, only cDNAs coding for RARß (Noji *et al.*, 1991; Rowe *et al.*, 1991; Smith and Eichele, 1991), and RARγ2 (Michaille *et al.*, 1994) have been isolated in the chick. The aim of the present work was to complete the panel of the available *RARs* in chick and to study *RARα, ß* and *γ* expression during normal chick skin development, that will then allow us to further analyze the respective role of each RAR during avian embryogenesis. Here we report the isolation and characterization of chick cDNAs encoding RARα1 and RARα2. Northern blot analysis suggests that different RARα and γ isoforms could be successively required during feather formation. *In situ* hybridization shows some similarities but also some differences in the distribution pattern of the transcripts of the three *RAR* genes, especially *RARα* and *ß*, during chick and mouse skin differentiation.

Results

Isolating chick RARα1 and RARα2 encoding cDNAs

Poly(A)⁺-RNAs were isolated from 4-, 7-, 10- and 14-day whole chick embryos (i.e., Hamburger and Hamilton [HH] stages 24, 31, 36 and 40, respectively, 1951). Several degenerated primers were synthesized from the evolutionarily most conserved regions of the C (DNA-binding) and E (ligand-binding) domains. RT-PCR (reverse transcriptase polymerase chain reaction) cloning from a mixture of these poly(A)⁺-RNAs gave us specific 666-base pairs (bp) amplification products (as for example the *chRARα15* cDNA, Fig. 1) extending from the first codon of the C domain to the 176th codon of the E domain of the chick *RARα*. Several rounds of anchored-PCR experiments were then needed for isolating the sequences encoding the A1, B and E domains, and the first 28 codons of the F domain. However, we were unable to obtain the end of the F domain using this technique. Therefore, we screened 1.5x10⁶ clones of a cDNA library prepared from 8.5-day (stage 35 HH) embryonic chick skin, using as a probe the *chRARα15* cDNA. Two different clones were thus isolated (Fig. 1). The 1494 bp-*chRARα38* cDNA contains 9 nucleotides (nt) of the 5' untranslated region, a region encoding an A2-like and the B-F domains, and 123 nt of the 3' untranslated region (Fig. 2). The 1186 bp-*chRARα19* cDNA contains a region encoding the C (except the first 16 aa) to F domains, and 112 nt of the 3' untranslated region.

Interspecies comparison of the aa sequences of the RARα

The aa sequences deduced from our clones were aligned with the RARα sequences described in other species (Fig. 3). The sizes of the A1 and B-F domains are well conserved, especially among chick, mouse and human. The corresponding aa sequences are also highly conserved (Fig. 3a,c), except the middle part (known as the variable region) of the D domain and the beginning of the F domain. As a consequence, the B-F domains, shared by all RARα isoforms, contain only 6 specific aa in chick (2 in the D and 4 in the F domain, respectively).

In contrast, the aa sequence of the putative chick A2 domain, encoded by the *chRARα38*, appears more divergent (Fig. 3b). The presence of a tryptophan residue (position 47 in Fig. 3b) found at a corresponding position in the A2 domain of all known RARs as well as that of a serine (position 53) as a first residue in the B domain give a strong indication that this RAR is more similar to RARα2 than to other isoforms (Leroy *et al.*, 1991). This deduction is supported by the presence of 14 other aa highly conserved among the different species: glutamic acid 3, valine 7, prolines 13 and 15, methionine 19, aspartic acid 20, arginine 26, cysteine 28, leucine 29, prolines 37 and 41, arginine 43, histidine 46 and serine 50. Several other aa are also found at a corresponding position in RARα2 of one or several other species. This aa sequence conservation strongly suggests that the *chRARα38* cDNA actually encodes the chick RARα2. But it is noteworthy that as much as 26 positions (i.e., 50%, including one insertion and two deletions) of the A2 domain are chick-specific, i.e., most probably avian-specific (dots in Fig. 3b).

Comparative expression of the RARα, ß and γ genes during chick skin differentiation

The distribution of the *RARα, ß* and *γ* transcripts at four main steps of feather morphogenesis (7, 7.5, 8.5 and 14.5 day, i.e. stages 31, 32, 35 and 40 HH, respectively) was studied by *in situ* hybridization. For a better comparison with the corresponding stages of hair formation in mouse (Hardy, 1968), these four stages (Fig. 4) were respectively referred to as stage ff1, ff2, ff3 and ff7 (i.e., stage 1, 2, 3 and 7 of feather formation, respective-

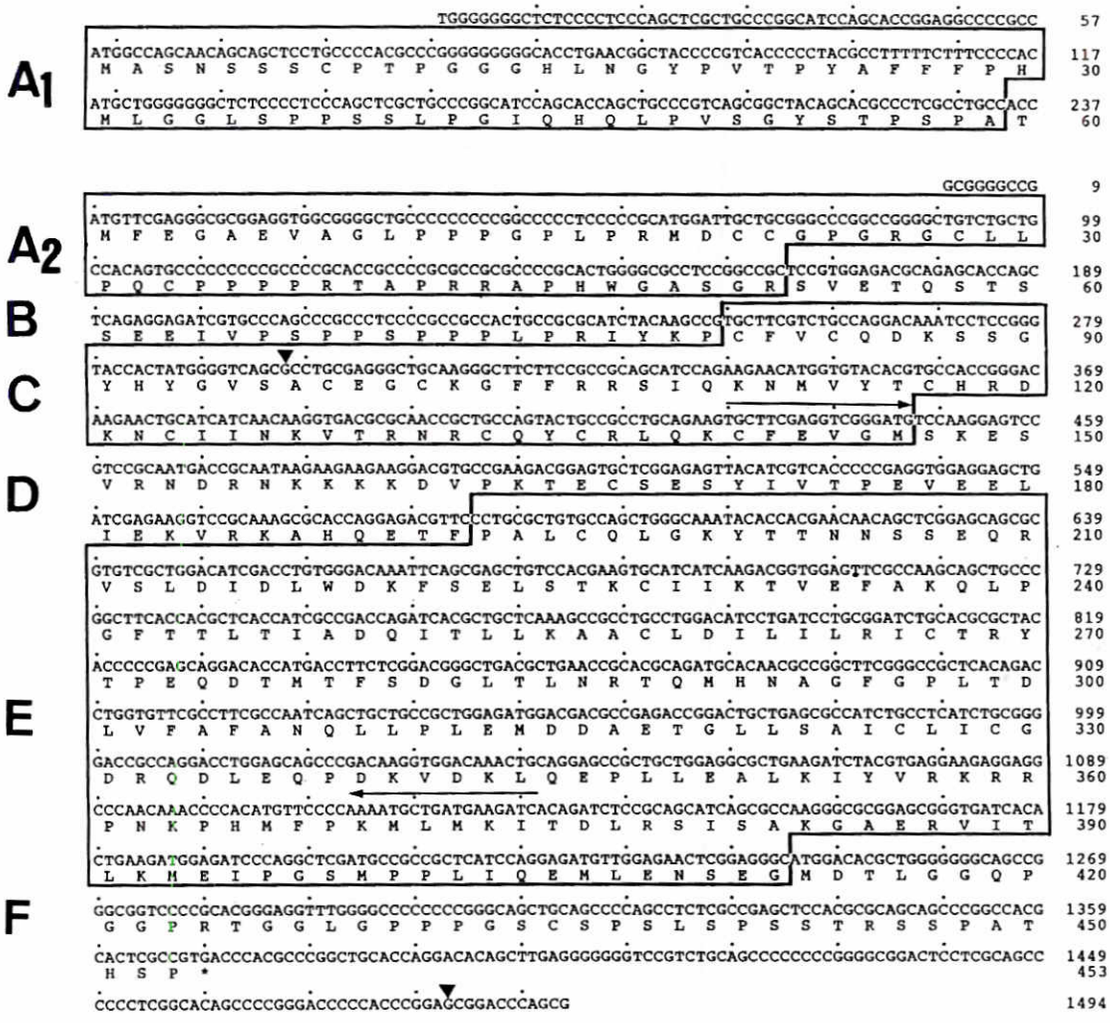
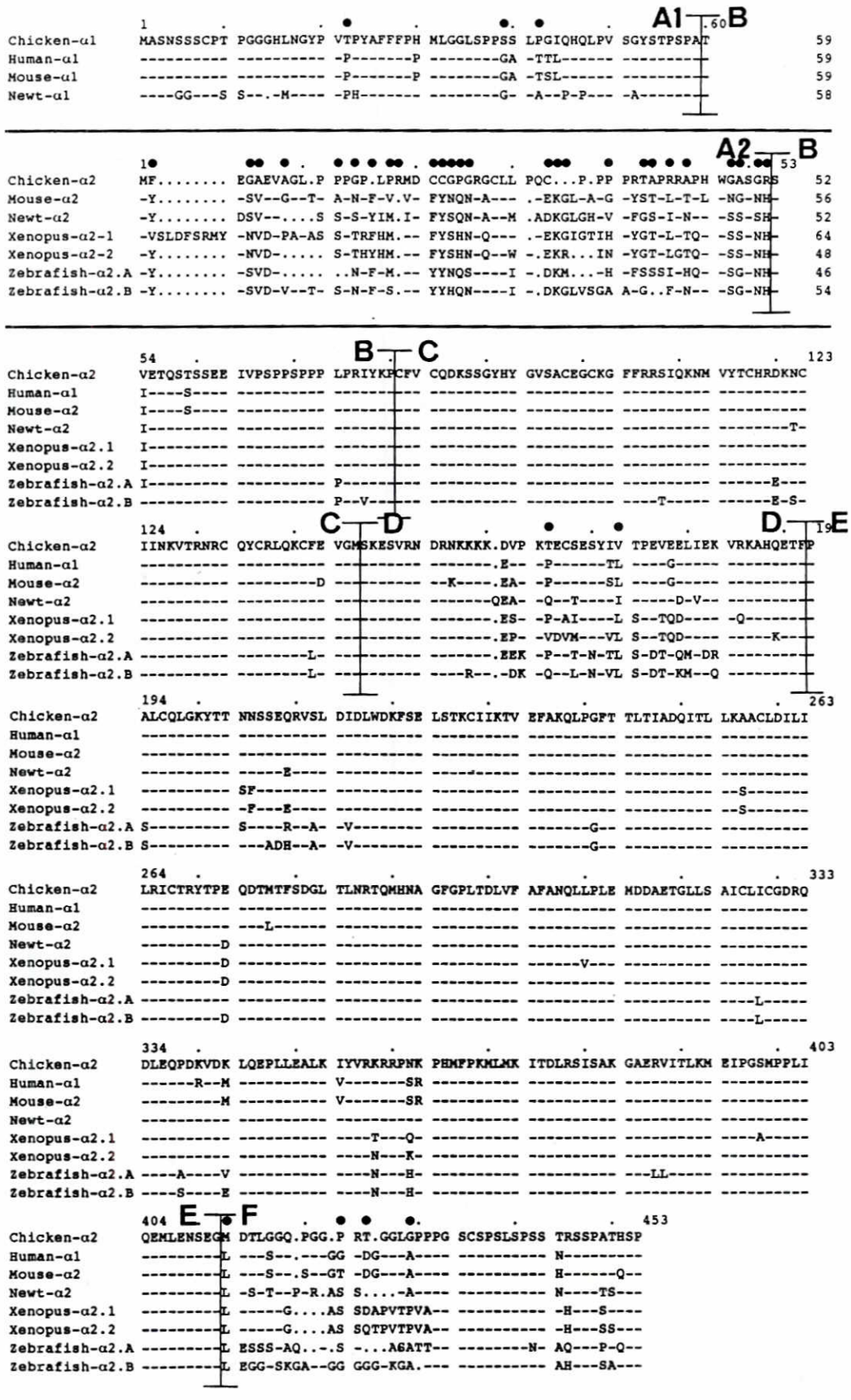


Fig. 2. Nt sequence of the chick *RARα1* and *α2* isoforms. *The nt sequence encoding the A1 domain of the chick RARα, plus the sequence of the first codon of the B domain, obtained by anchored-PCR, is given on the top of the figure* **(A1)**. *The complete nt sequence of the chRARα38 cDNA is given on the bottom of the figure* **(A2-F)**. *The predicted aa sequences are given under the nt sequences. The limits between the A-F domains were deduced from the comparison with the previously published RARα. The arrowheads (positions 298 and 1483) indicate respectively the 5' and 3' ends of the chRARα19 cDNA. The arrows give the position of the two internal degenerated oligonucleotides used in RT-PCR cloning.*

ly). Serial dorsal skin sagittal sections were hybridized using as a probe either (i) the 1494 bp-*chRARα38*, (ii) a 675 bp-cDNA containing the sequence encoding the D domain and the first 225 aa of the E domain of the chick RARß, or (iii) the 3' part of the *chRARγ10* cDNA (about 850 bp in length), which contains the last 69 codons of the E domain, the F domain and about 600 bp of the 3' untranslated region of the chick *RARγ* gene (Michaille *et al.*, 1994). Each of these probes permits specific detection of all the transcripts of the corresponding *RAR* gene, but does not discriminate between different *RAR* isoforms.

At stage ff1, when the dermal cells condense under the feather placode, the dermal and epidermal cells display abundant transcripts of the three *RAR* genes, as shown for the *RARß* (Fig. 5a,b). At stage ff2, when the epidermal cap begins to arise in connection with the dermal condensation, *RARß* transcripts can only be detected in the epidermis (Fig. 5c,d), whereas the epidermal cap and dermal condensation still display abundant *RARα* and *γ* transcripts (Fig. 5e,f and g,h, respectively). At stage ff3, when the feather bud individualizes, *RARα* and *γ* transcripts are still present in both the dermal and epidermal layers (Fig. 6a,c), the epidermal transcripts appearing preponderant at its caudal tip. In contrast, the *RARß* transcripts are evenly distributed in the ectoderm of both the feather bud and interbud regions (Fig. 6b). Furthermore, some *RARß* transcripts are also detected in the anterior sagittal part of the feather bud dermis (compare the two buds of Fig. 6b). At stage ff7, when the feather follicle forms, *RARα* and *γ* transcripts are still abundant in the epidermal cells, especially in the feather barb ridges (Fig. 7d,f), whereas *RARß* transcripts can only be detected in the inner epithelial sheath of the feather filament (Fig. 7e).

Further information was obtained by Northern blot analysis (Fig. 7). Poly(A)$^+$-RNA extracted from skin or heart at 8.5 or 14.5 day (stages 35 and 40 HH, respectively) were tested with the same three probes. At 8.5 day, the *RARα* probe only detected a transcript of about 2.1 kilobases (kb) in skin and two approximately 2.0 and 6.0 kb-transcripts in heart (Fig. 7a). At 14.5 day, the 2.1 kb-transcript disappeared from skin. It was replaced by the 2.0 and 6.0 kb-transcripts and a new 2.4 kb-one. In contrast, only the 2.0 kb-transcript was still detected in heart. At 8.5 day, two *RARß* transcripts, whose sizes were estimated to about 3.2 and 3.6 kb, were faintly detected in skin (Fig. 7b). They were also present in heart. At day 14.5, the 3.6 kb-transcript was the major


a
b
c
A1
A2
B
C
D
E
F
Chicken-α1
Human-α1
Mouse-α1
Newt-α1
Chicken-α2
Mouse-α2
Newt-α2
Xenopus-α2-1
Xenopus-α2-2
Zebrafish-α2.A
Zebrafish-α2.B
Xenopus-α2.1
Xenopus-α2.2

RARβ transcript in both these tissues. In contrast, while a 2.0 kb-transcript was detected by the *RARγ* probe in 8.5 day-skin (Fig. 7c), two other transcripts (1.9 and 3.0 kb in length, respectively) were present at day 14.5. By comparison, the two same *RARγ* transcripts were detected in heart, the relative proportion of the 3.0 kb-transcript greatly increasing between days 8.5 and 14.5.

Discussion

The sequences encoding the A1, the B-E and the first 28 codons of the F domain of the chick RARα were determined using RT-PCR and anchored-PCR experiments. The inability to characterize the end of the F domain and other isoforms using this technique may in part be related to the high richness in C+G nt of the corresponding regions, i.e., respectively 84 and 78% for the A2 and F-encoding sequences, and 79% for the 112 nt-3' untranslated region. Thus, the incomplete amplification products derived from anchored-PCR experiments probably arose from internal priming of the poly(dC)-oligonucleotide used as a primer. By possibly interfering with the reverse transcription process, the high content in C+G nt may also explain the lack of isolated sequences containing more than 123 nt of the 3' untranslated region, either from our skin cDNA library or from another cDNA library prepared from RNA extracted from chick hematopoietic progenitor cells actually expressing the *RARα* (Gandrillon *et al.*, 1994).

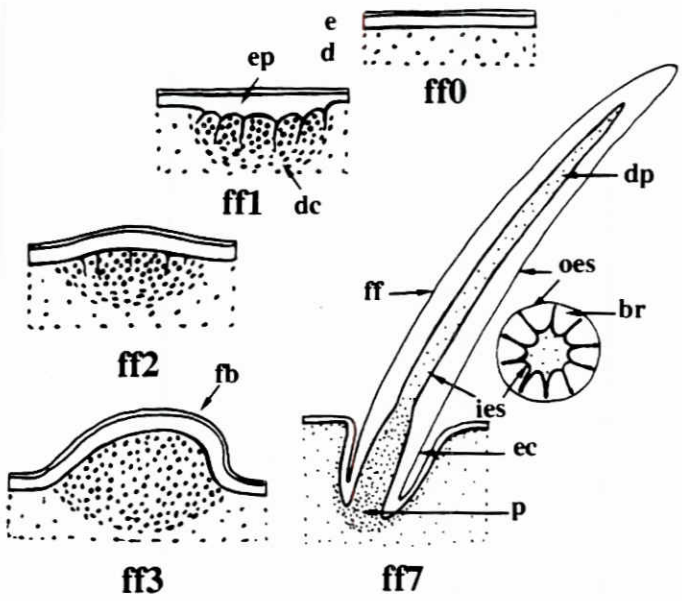


Fig. 4. The main stages of feather formation (ff0, 1, 2, 3 and 7) in chick. *br, barb ridge; d, dermis; dc, dermal condensation; dp, dermal pulp; e, epidermis; ec, epidermal collar; ep, epidermal placode; fb, feather bud; ff, feather filament; ies, inner epithelial sheath; oes, outer epithelial sheath; p, papilla.*

Special features of the chick RARα2

Comparison of the aa sequences deduced from our cDNAs with RARα from other species shows that both the sizes and the sequences of the A1 and B-F domains are highly similar. It is thus probable that most of the RARα1 functions have been evolutionarily conserved. This is in good agreement with the fact that the accumulation of the *RARα1* transcripts is almost ubiquitous, as already shown in mouse (Dollé *et al.*, 1990). However, the size of the A2 domains varies from 46 to 64 aa. The chick A2 domain displays 26 (50%) specific positions (including one insertion and two deletions), most of them are not conservative. It has lost all but two (threonine 39 and serine 50) hydroxylated aa, and almost all the aminated and aromatic residues. The otherwise well conserved 5 aa-central motif (positions 21-25) is also missing. In contrast, the chick A2 domain contains 6 basic aa, four of which are clustered between positions 38 and 52. Thus, this domain appears to contain three subdomains: (i) an amino-terminal subdomain (positions 1-20) which mostly contains hydrophobic residues, including 5 prolines; (ii) a central subdomain (positions 21-36) which contains only one charged residue (the conserved arginine 26), four cysteines (versus one in all other A2 domains) and 5 prolines; and (iii) a basic carboxy-terminal subdomain. The most remarkable feature of the chick A2 domain is the presence of a total of 13 prolines (25%) versus 7 in mouse (Leroy *et al.*, 1991), 5 in newt (Ragsdale *et al.*, 1992), and 4 in both the A2 domains described in *Xenopus* (Sharpe, 1992) and zebrafish (Stachel and Kushner, 1993). Furthermore, these prolines mostly appear as two PPPGPLPR and PQCPPPPR motif. Such a proline-rich motif is also found at the end of the B domain of all described RARα [PSPPSPPP(L/P)PR], β [PSPPSPLPPPR], and γ [P(N/S)SPSPPPPPR]. As the A and B domains are both involved in the ligand-independent transactivation function of the RARs (Leid *et al.*, 1992, 1993), it suggests that the two motives contained in the chick A2 domain may in some way synergize with the corresponding motif of the B domain. Mutational analysis and subdomain swapping experiments should help to address the functional significance of such features.

The expression of the three RAR genes is developmentally regulated during chick skin differentiation

The pattern of transcripts accumulation of the three *RAR* genes varies according to the stage of feather formation. At stage ff1, transcripts of the three RAR genes are present in both

Fig. 3. Interspecies RARα comparison. (a) *The aa sequence of the A1 domain and the first aa of the B domain of the chick RARα1 were aligned with those of human (Krust et al., 1989), mouse (Giguere et al., 1987) and newt (Ragsdale et al., 1989). The aa number in each A1 domain is indicated to the right of the sequence.* **(b)** *The aa sequence of the A2 domain and the first aa of the B domain of the chick RARα2 were aligned with the corresponding parts of the mouse (Leroy et al., 1991) and newt (Ragsdale et al., 1992) RARα2, and with those of both the RARα2 isoforms described in Xenopus (Sharpe, 1992) and zebrafish (Stachel and Kushner, 1993). The aa number in each A2 domain is indicated to the right of the sequence.* **(c)** *The B (except the first aa) to F domains of the chick RARα were aligned with the corresponding domains of the human RARα1 (Krust et al., 1989), mouse RARα2 (Leroy et al., 1991), newt RARα2 (Ragsdale et al., 1992), Xenopus RARα2.1 and α2.2 (Sharpe, 1992), and zebrafish RARα2.A and α2.B (Stachel and Kushner, 1993). Amino acids are numbered according to the chick RARα1* **(a)** *or RARα2* **(b and c)** *respectively used as references. Gaps (indicated by dots) were introduced to maximize homologies. Dashes indicate aa homologies with chick RARα1* **(a)** *and α2* **(b,c)**. *The closed circles* **(a-c)** *point out chick-specific aa.*

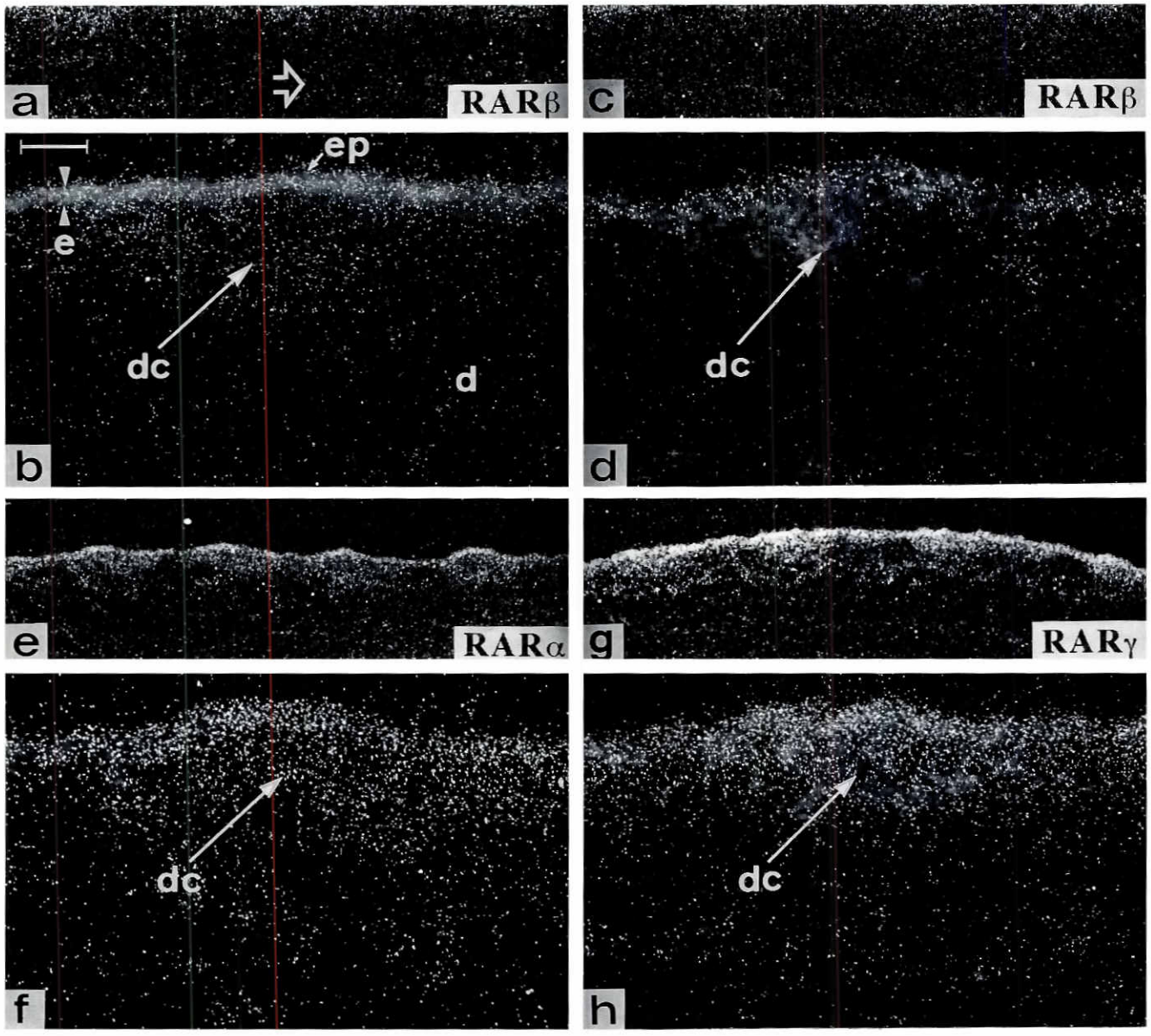


Fig. 5. Distribution of *RARα, ß* and *γ* transcripts at early stages of chick dorsal skin development. *Serial sagittal sections were prepared from 7-* **(a,b)** *and 7.5-day* **(c-h)** *embryos (stages ff1 and ff2, respectively). The antisense RNA probes were respectively synthesized from* **(a-d)** *a 675-bp cDNA containing the sequence encoding the D domain and the first 225 aa of the E domain of the chick RARß (our unpublished results),* **(e,f)** *the 1494-bp chRARα38 cDNA, or* **(g,h)** *the 3' part of the chRARγ10 cDNA (about 850 bp long), which contains the 69 last codons of the E domain, the F domain and about 600 bp of the 3' untranslated region of the chick RARγ gene (Michaille et al., 1994). These three probes allow to specifically detect all the transcripts of the corresponding RAR gene, but do not discriminate between different RAR isoforms.* **(a,c,e,g)** *Darkfield illumination. The arrowheads in* **(a)** *point to the dermal condensations, while the open arrow indicates the anterior-posterior (i.e., cephalo-caudal) orientation. Bar, 250 μm.* **(b,d,f,h)** *Simultaneous darkfield and fluorescence illumination. Bar, 45 μm. At stage ff1* **(a,b)**, *when the feather placodes form, RARß transcripts are present both in the dermal condensation and the epidermis. At stage ff2* **(c-h)**, *the RARß transcripts* **(c,d)** *are no more detected in the dermal condensation, whereas the RARα* **(e,f)** *and γ* **(g,h)** *genes are still expressed in both skin components. d, dermis; dc, dermal condensation; e, epidermis; ep, epidermal placode.*

the dermis and epidermis. At the following stage (ff2), *RARß* transcripts can no more be detected in the dermal cells but still remain present in the epidermal cells. In contrast, *RARα* and *γ* transcripts are present in both the dermis and epidermis at stages ff1 and ff2, their distribution pattern in the epidermis being asymmetrical by stage ff3. Namely, at this stage, the corresponding transcripts are much more abundant in the cells of the posterior (i.e., caudal) tip of the buds. The same type of asymmetrical distribution in the epidermal cells has previously been observed at the corresponding stage for the *c-myb* and *c-myc* transcripts (Desbiens *et al.*, 1991), and also for the N-CAM protein, with the difference that no N-CAM was detected in the ante-

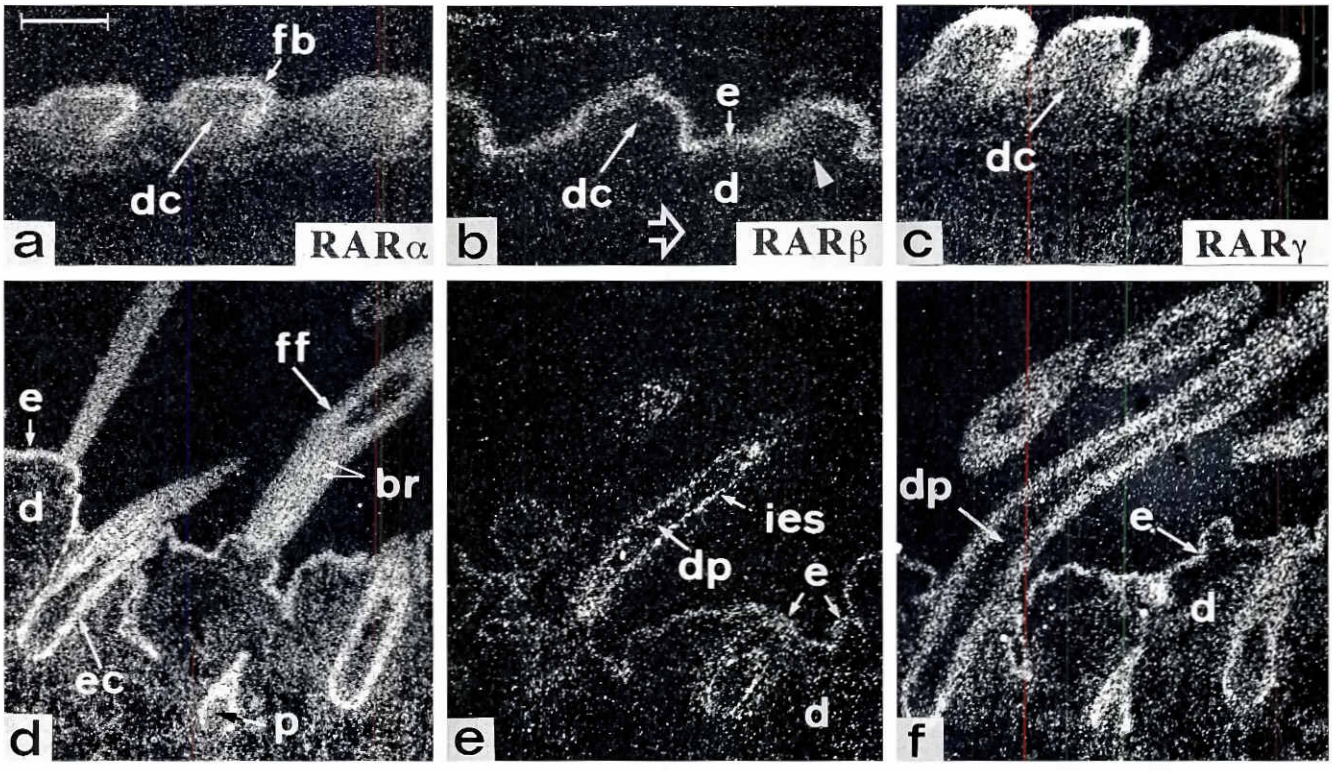


Fig. 6. Distribution of *RARα, ß* and *γ* transcripts at later stages of feather morphogenesis. *Darkfield illumination. Serial sagittal sections of chick dorsal skin were prepared from 8.5-* **(a-c)** *or 14.5-day* **(d-f)** *embryos (stages ff3 and ff7, respectively). The antisense RNA probes are respectively specific of the RARα (a, d), RARß (b, e), or RARg (c, f) transcripts. The open arrow in* **(b)** *gives the cephalo-caudal orientation. Bar, 250 μm. At stage ff3, when the feather buds form, RARα and γ transcripts are localized both in the dermal and epidermal cells. The RARß transcripts are mainly restricted to the epidermis, although a few can be detected at the anterior part of the dermal condensation (arrowhead in* ***b****) as long as the section is nearly sagittal according to the bud structure (compare the two feather buds in* ***b****). Note that the signal obtained with the RARα and γ probes in the epidermis is more intense at the caudal tip of the feather buds. At stage ff7, when the proximal part of the feather filaments invaginates, RARα and γ transcripts are still present in all the epidermal cells, while the RARß ones appear restricted to the inner epithelial sheath. br, barb ridges; d, dermis; dc, dermal condensation; dp, dermal pulp; e, epidermis; ec, epidermal collar; fb, feather bud; ff, feather filament; ies, inner epithelial sheath; p, papilla.*

rior (i.e. cephalic) part of the epidermis (Jiang and Chuong, 1992). In contrast, the distribution of the *RARß* transcripts in the epidermal cells remains homogenous at stage ff3, as does that of the L-CAM and Hox C6 proteins (Chuong *et al.*, 1990). However, a few *RARß* transcripts are also present in the anterior part of the feather bud mesoderm, according to the position of the buds along the anterior-posterior axis of the body, as previously described for the tenascin and N-CAM proteins, and also for the Hox C6 and D4 homeoproteins (Chuong *et al.*, 1990; Chuong, 1993). Likewise, transcripts of the *Hox C8, D9, D11* and *D13* genes differentially accumulate during hair follicle development in the different body regions of mouse embryo (Kanzler *et al.*, 1994). Taking into account the fact that RA can modify *Hox* gene expression in a concentration-dependent way (Simeone *et al.*, 1990), this could possibly explain why RA can modulate the phenotype of skin appendages both *in vivo* (Dhouailly *et al.*, 1980) and *in vitro* (Hardy, 1968, 1983; Chuong *et al.*, 1992, 1994). At stage ff7, *RARß* transcripts are only present in the inner root sheath of the feather filament. In contrast, *RARα* and *γ* transcripts, still detected in all the epidermal cells, are more abundant in the barb ridges, at the origin of the feather barbs and barbules.

In chick (Michaille *et al.*, 1994) as in mouse (Zelent *et al.*, 1989; Viallet and Dhouailly, 1994), *RARγ* appears predominantly expressed in skin. In contrast, the presence of *RARß* transcripts in chick feather buds (stage 3), as well as the detection of *RARα* transcripts at stage 7, constitute the major differences with the results previously reported in mouse. Namely, *RARß* transcripts, not detected during hair vibrissae normal development (Viallet and Dhouailly, 1994), only appeared in dermal cells in the case of RA-induced epidermal metaplasia (Viallet *et al.*, 1991), and *RARα* transcripts were not detected in hair vibrissae follicle at stage 7 (Viallet and Dhouailly, 1994). The pattern of expression of the three *RAR* genes during cutaneous appendage development has thus not been strictly conserved during evolution, particularly after stage 3, i.e., when the main morphological difference between chick and mouse skin appendage differentiation takes place. At this stage, hair and feather anlagen indeed undertake distinct developmental pathways when the placode develops into a bud. The feather placode protrudes above the skin forming an outgrowth which subsequently elongates into an epidermal tube, i.e., the feather filament. In contrast, at the same stage, the epidermal basal cells of the hair placode proliferate and form a downgrowth protruding into the dermis. The compact epidermal peg elongates

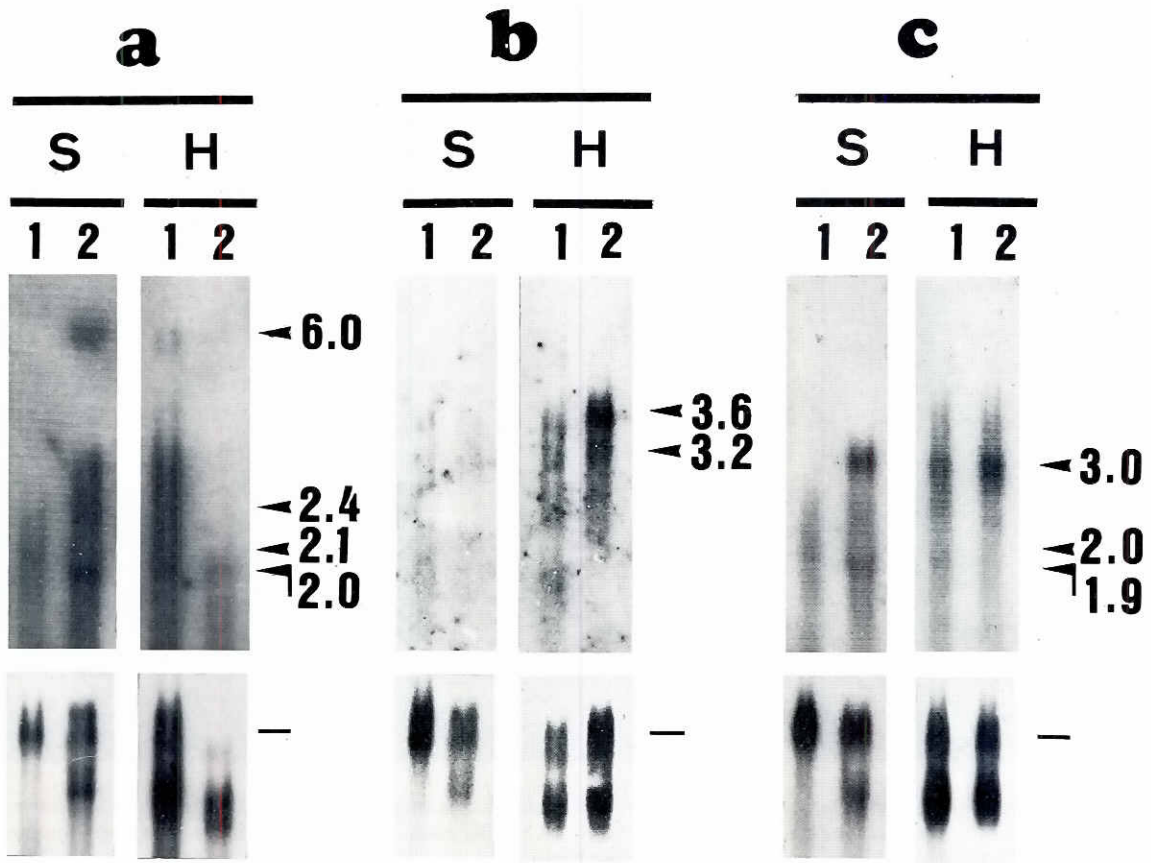


Fig. 7. Northern blot analysis of developmental expression of the chick *RARα, ß* and *γ* genes. *Three Northern blots were prepared as described in materials and methods with 8 μg of poly(A)⁺-RNA extracted from chick dorsal skin (S) or heart (H) at (1) day 8.5 (stage 35 HH) or (2) day 14.5 (stage 40 HH) of incubation. The DNA probes were labeled by random priming. Top: The blots were probed with* **(a)** *the 1494-bp chRARα38 cDNA,* **(b)** *a 675-bp RARß cDNA, or* **(c)** *the 3' part of the chRARγ10 cDNA (about 850 bp in length). These three probes do not discriminate between the different transcripts of the corresponding RAR genes. Bottom: The same blots were rehybridized with a 1030-bp mouse ß-actin cDNA as a control. The position of the chick ß-actin mRNA is indicated by the bar. Chick 28S and 18S rRNAs, and E. coli 23S and 16S rRNAs (4.6, 1.8, 2.9 and 1.5 kb, respectively) and α- and ß-actin mRNAs (1.6 and 2.0 kb, respectively) were used as size markers. The exposure time was two weeks for the RARα, ß and γ probes and two days for the ß-actin probe.*

and then differentiates into seven concentric layers, forming the hair follicle. Further analysis is thus needed to define more precisely the respective role of each RAR in the building of class-specific skin appendages.

A shift of RARα and γ transcripts, but probably not of the ß ones, seems correlated with the differentiation of the feather anlage into a feather filament

The presence of different *RARα, ß* and *γ* transcripts during skin appendage formation was studied by Northern blotting. *RARß* transcripts were hardly detected using this technique, even after long exposure (two weeks), whereas the *ß-actin* probe did not suggest a particular RNA degradation. Two about 3.2 and 3.6 kb-transcripts, quite abundant in heart, were thus very faintly detected in skin at days 8.5 and 14.5 of incubation. This suggests that these transcripts are rather rare in skin, especially since at both these stages, *RARß* transcripts are almost only found in the epidermis, which contains less than one fourth of the skin cell population. These two *RARß* transcripts should correspond to the 3.2 and 3.4 kb-transcripts described by Smith and Eichele (1991), which represent two non-*RARß2* isoforms. However, this should not mean that there is no *RARß2* transcripts in 8.5- or 14.5-day skin. Namely, these authors showed that a 4.6 kb-transcript (i.e., the chick *RARß2* isoform) was very poorly detected onto Northern blots, although it was actually present in limb bud at about the same concentration than the other *RARß* transcripts, as demonstrated by RNase protection experiments. Accordingly, although the 4.6 kb-transcript did not appear onto our blots, we isolated cDNAs encoding the entire RARß2 from our 8.5-day skin cDNA library (our unpublished results).

The 8.5-day skin mostly contained a 2.0 kb-*RARα* transcript. At day 14.5, the most abundant *RARα* transcripts in skin were 6.0, 2.4 and 2.1 kb long. More than two *RARα* isoforms are thus likely to be produced in chick, as previously shown in mouse (Leroy *et al.*, 1991). On the other hand, the 2.0 kb-*RARγ* transcript present in 8.5-day skin was not detected at day 14.5, where two 1.9 and 3.0 kb-transcripts were present. The results obtained with the control *ß-actin* probe, as well as the rehybridization of the same blots with non-*RAR* probes (results not

shown), suggest that the lack of some *RARα* and *γ* transcripts in 8.5- or 14.5-day skin does not result from RNA degradation, but more likely arises from a shift of isoforms. This shift could be correlated with the transformation of the feather buds into feather filaments. In contrast, our results give no evidence that a shift of *RARß* transcripts could also occur during feather differentiation.

In conclusion, the present isolation of *RARα*-encoding cDNAs, which completes the panel of the available *RARs* in chick, will facilitate a further analysis of the respective role of the different RARs during avian embryogenesis. For the moment, the targeted expression of a RARα dominant-negative in mouse skin was shown to inhibit the epidermal maturation (Saitou *et al.*, 1995). The alteration of the pattern of *RARα, ß* and *γ* expression after RA treatment as well as the comparative expression of mutated forms of the three RARs in the dermis or the epidermis should allow us to understand better the functional implication of each RAR during chick skin morphogenesis.

Materials and Methods

RNA isolation

Dorsal skin or heart were ground up under liquid nitrogen. RNA was extracted in RNA extraction buffer (4 M guanidine isothiocyanate, 0.05 M Tris-HCl (pH 7.6), 0.01 M EDTA, 1% ß-mercaptoethanol, and 0.5% sodium lauryl sarcosine) and purified on a 5.7 M cesium chloride cushion according to Sambrook *et al.* (1989). Poly(A)$^+$-RNA was prepared by affinity chromatography using oligo(dT)-cellulose.

PCR cloning

Single strand cDNAs were synthesized by reverse transcription using a poly(dT)-oligonucleotide as a primer. Two successive PCR amplifications were then performed using oligonucleotides derived from evolutionarily well-conserved parts of the C and E domains of human and mouse *RARα* genes. The first amplification was performed with two external degenerate oligonucleotides: a sense [5'-GCCGCAGCATCCA(AG)AA(AG)AA(TC)ATG-3'] and an antisense one [5'-GGCATCGGGCC TGG(AGT)AT(TC)TCCAT-3']. The second round of PCR was performed from the obtained amplification product using as primers a pair of internal degenerated oligonucleotides bearing synthetic restriction sites, whose position is indicated by arrows on Fig. 2: a sense [5'-TGCTTCGAGGT (TCGA)GG(TCGA)ATG-3'] and an antisense one [5'-GATCTTCAT(TCGA)AGCAT(TC)TT-3']. Chick specific oligonucleotides were then designed to be used in anchored-PCR experiments as described by Loh *et al.* (1989). A 675-bp cDNA containing the sequence encoding the D domain and the first 225 aa of the chick RARß was also isolated using the same technique.

cDNA cloning

An 8.5-day (i.e., stage 35 HH) embryonic chick skin cDNA library was constructed in the bacteriophage λZAP II (Stratagene). Approximately 10^6 independent recombinant clones were amplified. Clones were isolated by the screening of $1.5x10^6$ lysate plates using as a probe one of the cDNAs previously obtained by PCR (*chRARα15*, Fig. 1).

Sequence analysis

Sequence analysis was performed using the ^{T7}-Sequencing™ Kit from Pharmacia, according to the manufacturer's instructions. At least four independent PCR clones were sequenced to avoid PCR artefacts. The sequence of the chick *RARα1* (derived from anchored-PCR experiments) and that of the *chRARα38* cDNA (encoding the whole RARα2) have been submitted to the EMBL/GenBank Data Libraries and received the accession numbers X73972 and X78335, respectively.

Northern blot analysis

RNAs were fractionated in formaldehyde 1% agarose gels which were run for 14 h at 45 volts and transferred to Nylon membranes (Amersham). ^{32}P-labeled probes were prepared by random priming using the "Ready To Go" kit of Pharmacia. Hybridization conditions were as described by Sambrook *et al.* (1989): prehybridization at 42°C in 50% formamide, 5xSSC (SSC: 150 mM NaCl, 15 mM sodium citrate), 2xDenhardt (Denhardt: 0.01% Ficoll [Type 400], 0.01% polyvinylpyrrolidone), 1 mM EDTA, 1% SDS and 100 µg/ml denatured herring DNA; hybridization at 42°C in the same buffer with the addition of $1x10^6$ cpm/ml labeled probe; and washes including 2xSSC, 0.1% SDS at room temperature twice for 15 min, 2xSSC, 0.1% SDS at 65°C twice for 15 min followed by 0.1xSSC, 0.1% SDS once at 65°C for 15 min.

In situ hybridization

In situ hybridization was modified from Décimo *et al.* (1995). Embryos were embedded in Tissue Tech® and serial 10 µm sections were air dried and treated serially with acetone, 4% formaldehyde at 4°C, 0.1 M triethanolamine/0.25% acetic anhydride, 50% formamide/1xSSC at 60°C and a series of ethanol washes. The hybridization buffer included 50% formamide and $5x10^6$ cpm of the ^{35}S-labeled antisense RNA probe or a control ^{35}S-labeled sense RNA probe. All slides were treated with RNase for 30 min at 37°C and then washed with 2xSSC in 50% formamide for 1 h at 55°C, 0.1xSSC for 15 min at room temperature followed by ethanol dehydration. Slides were dipped in Kodak NTB-2 nuclear track emulsion and exposed for about three weeks before developing. Sections were stained with propidium iodide and photographed with an Olympus BH-2 microscope using both darkfield illumination and fluorescence.

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