

## The Expression of Beta ( $\beta$ ) Keratins in the Epidermal Appendages of Reptiles and Birds<sup>1</sup>

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**SYNOPSIS.** The integuments of extant vertebrates display a variety of epidermal appendages whose patterns, morphology and terminal differentiation (epidermal keratins) depend upon interactions between ectodermal (epidermis) and mesodermal (dermis) tissues. In reptiles and birds, appendage morphogenesis precedes terminal differentiation. Studies have demonstrated that appendage morphogenesis influences the expression of the appendage specific keratin genes. However, little is known about the nature of the structural genes expressed by the epidermal appendages of reptiles. How pattern formation and/or appendage morphogenesis influence terminal differentiation of reptilian appendages is not known.

The epidermal appendages of reptiles and birds are characterized by the presence of both alpha ( $\alpha$ ) and beta ( $\beta$ ) type keratin proteins. Studies have focused on the genes of avian  $\beta$  keratins because they are the major structural proteins of feathers. The occurrence of  $\beta$  keratin proteins in the scales and claws of both birds and reptiles and their immunological cross-reactivity suggest that the genes for reptilian  $\beta$  keratins may be homologous with those of birds. In bird appendages, the  $\beta$  keratins are the products of a large family of homologous genes. Specific members of this gene family are expressed during the development of each appendage. Recent sequence analyses of feather  $\beta$  keratins, from different orders of birds, demonstrate that there is more diversity at the DNA level than was implied by earlier protein sequencing studies.

Immunological techniques show that the same antibodies that react with the epidermal  $\beta$  keratins of the chicken (*Gallus domesticus*) react with the epidermal  $\beta$  keratins of American alligators (*Alligator mississippiensis*). Furthermore, a peptide sequence (20 amino acids) from an alligator claw  $\beta$  keratin is similar to a highly conserved region of avian claw, scale, feather, and feather-like  $\beta$  keratins. These observations suggest that the  $\beta$  keratin genes of avian epidermal appendages have homologues in the American alligator. Understanding the origin and evolution of the  $\beta$  keratin gene families in reptiles and birds will undoubtedly add to our understanding of the evolution of skin appendages such as scales and feathers.

### INTRODUCTION

#### *The development of the chicken scutate scale*

It has been proposed that the avian feather evolved from the epidermal scales of reptiles (Rawles, 1963; Kemp and Rogers, 1972; Lucas and Stettenheim, 1972; see also, Feduccia, 1996; Brush, 1996; Brush, 2000; Maderson and Alibardi, 2000). This line of thinking has led to the assumption

that avian scales are remnants of birds' reptilian ancestry. Unfortunately, little comparative developmental genetics exists for the epidermal appendages of birds and reptiles. Figure 1 shows a timeline of scutate scale epidermal development in the White Leghorn chicken. This brief summary demonstrates the level of understanding that has been gained in studying the scutate scales of the chicken. Such studies are not yet available for reptiles.

The scutate scales develop on the anterior metatarsal region of the chicken foot (Figure 1). The simple ectodermal epithelium of the anterior metatarsal region produces the suprabasal peridermal layer

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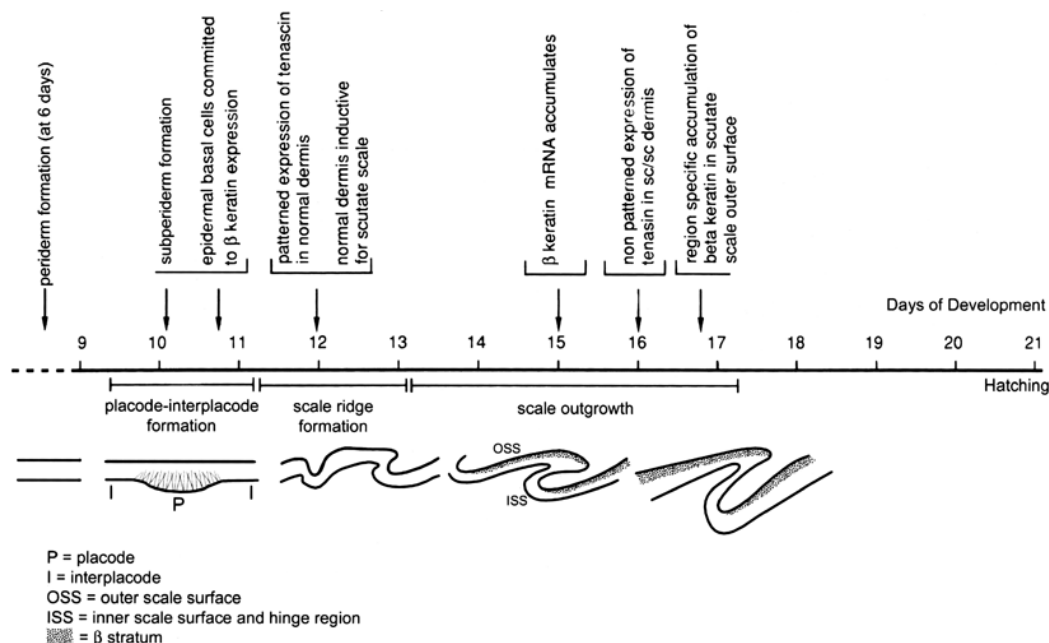


FIG. 1. Time-line of developmental events during the formation of the scutate scales located on the anterior metatarsus. The transient embryonic layers (periderm and subperiderm) are formed prior to scale ridge morphogenesis and epidermal differentiation. Placode and interplacode regions form around 10 days of development. The epidermal basal cells of scutate scales become committed to  $\beta$  keratin expression prior to scale ridge morphogenesis and epidermal differentiation. Following epidermal placode and interplacode morphogenesis the scale ridge forms, coincident with the patterned expression of tenascin and the inductive ability of the scutate scale dermis. Differentiation of the outer and inner epidermal surfaces of the overlapping scutate scales results in the appearance of  $\beta$  keratin mRNA and protein. For specific references see the INTRODUCTION.

around 6 days of development. This embryonic layer of flattened surface cells is independent of the developing epidermis proper. Between 9.5 and 10 days of development, the epidermis forms the placode and interplacode cell populations of the avian scutate scale (Sawyer, 1972a, b; Sawyer, 1983; Sawyer *et al.*, 1986; Tanaka and Kato, 1983a, b). Morphogenesis of these distinct epidermal cell populations results in the formation of the Definitive Scale Ridge at 12 days of development. At this stage, the dermal ridge is able to induce the formation of scutate scale epidermis when recombined with presumptive feather epidermis (Rawles, 1963; Kato, 1969; Fisher and Sawyer, 1979; Sawyer *et al.*, 1984; Zeltinger and Sawyer, 1991a, b; 1992). The patterned expression of the extra-cellular matrix molecule, tenascin, occurs at this time (Shames *et al.*, 1994). Between 9.5 and 11.5 days of development, a second supra-

basal layer of cells is generated below the periderm which is known as the subperiderm (Sawyer, 1972a, b; Sawyer *et al.*, 1974a, b; Tanaka and Kato, 1983a, b; Sawyer and Goetinck, 1988; Sawyer, 1990). These embryonic layers (periderm and subperiderm) undergo cornification at 17–19 days of development (Shames and Sawyer, 1986; 1987; Knapp *et al.*, 1993), and are sloughed off at the time of hatching. The full complement of scutate scale keratins and embryonic feather keratin are expressed in these embryonic layers (Barnes, 1993; Shames *et al.*, 1988; Knapp *et al.*, 1993). As the Definitive Scale Ridge elongates, from 13 to 15 days of development, the epidermis stratifies and  $\beta$  keratin mRNA appears (Shames and Sawyer, 1986; 1987). By 17 days of development, scutate scale  $\beta$  keratins begin to accumulate along the outer scale surface to form the overlapping plates characteristic of scutate scales

(Sawyer, 1972a, b; Sawyer *et al.*, 1974a, b; Sawyer, 1979; 1983; O'Guin *et al.*, 1982; Sawyer *et al.*, 1986).

The scaleless (*sc/sc*) line of chickens carries an autosomal, recessive gene (*sc*) that in the homozygous condition results in the bird being mostly featherless and completely scaleless on its feet and legs (Abbott and Asmundson, 1957). The epidermal placode and interplacode cell populations of the scutate scales do not develop (Sawyer and Abbott, 1972; Sawyer *et al.*, 1974b; Sawyer, 1979, 1983, 1990). Dhouailly and Sawyer (1984) demonstrated that although the scaleless limb bud ectoderm is defective early in morphogenesis (3 days of development) (Sengel and Abbott, 1963), the scutate scale dermis becomes defective at 9.5 days of development. Thus, the scaleless dermis becomes unable to participate in normal scutate scale morphogenesis at the same time that the epidermal placodes fail to form. As indicated in Figure 1, the extra-cellular matrix molecule, tenascin, occurs at the Definitive Scale Ridge stage of normal scale development, but is not seen until much later in the development of the scaleless skin (Shames *et al.*, 1994).

In 1979, Maderson and Sawyer examined the developing scales of alligator embryos, histologically, and did not find the epidermal placode and interplacode cells populations that characterize the scutate scales of chick embryos. The relationships between scale morphogenesis and appendage-specific  $\beta$  keratin gene expression are unknown for the alligator. Now, developmental studies of the American alligator, using immunological and biochemical approaches, indicate that even though the American alligator does not form epidermal placode and interplacode cell populations during scale formation, it does express  $\beta$  keratin polypeptides that are homologous with the avian  $\beta$  keratins.

#### *Biochemical and immunological characterization of scales and feathers*

In 1972, Kemp and Rogers demonstrated that the reduced and S-carboxymethylated (SCM) proteins of scales differ from those of feathers when analyzed by polyacrylamide gel electrophoresis. However, pro-

teins of scales are expressed in feathers, and antiserum made against the soluble (SCM) keratins of adult feathers reacted with both feather and scale keratins. To localize the  $\alpha$  and  $\beta$  keratins in developing scales and feathers of birds, O'Guin *et al.* (1982) generated non-cross-reacting antisera to the  $\alpha$  and  $\beta$  keratins (Tables 1 and 2). The antisera were made by injecting rabbits with either soluble SCM skin  $\alpha$  keratins or soluble SCM feather  $\beta$  keratins (see O'Guin and Sawyer, 1982), which had been separated by one-dimensional polyacrylamide gel electrophoresis. These antisera have been used in numerous studies of avian and reptilian skin development (see references in Sawyer *et al.*, 1986). Even though the anti- $\beta$  keratin antibody (designated 2 anti- $\beta$  1, O'Guin *et al.*, 1982) was generated against feather  $\beta$  keratins, it reacts with both scale (O'Guin *et al.*, 1982; O'Guin, 1984; Sawyer *et al.*, 1986), and feather  $\beta$  keratins (Haake *et al.*, 1984; Haake, 1985). It also reacts with the  $\beta$  keratins of the lingual epithelium of the chicken tongue (Carver and Sawyer, 1989; Carver *et al.*, 1990), the epidermal  $\beta$  keratins of lizard scales (Carver and Sawyer, 1987), the shell and claws of turtles (Carver, 1988), and the epidermal  $\beta$  keratins of the American alligator (Mays, 1998). Using immunoelectron microscopic methods, Shames *et al.* (1988; 1989) have demonstrated that the anti- $\beta$  keratin antisera recognize the bundles of 3 nm  $\beta$  keratin filaments in scutate scales.

#### *Identification of scale $\beta$ keratin polypeptides*

To further analyze the differentiation of the scutate scale epidermis during development, Shames and Sawyer (1986, 1987) followed the pattern of mRNA expression using a cDNA probe (pCSK-12) specific for scale  $\beta$  keratins (Wilton, 1984; Wilton *et al.*, 1985). Shames and Sawyer (1987) determined through hybrid selection of total scale mRNA, followed by *in vitro* translation of the selected mRNA, that the scale gene represented by pCSK-12 directed the synthesis of a single polypeptide. This polypeptide ( $\beta$  3 of the  $\beta$  2–3 doublet) reacted strongly with the anti-beta keratin antiserum of O'Guin *et al.* (1982). A 260 base

TABLE 1. *Summary of antisera against  $\beta$  keratins.*

Name of antiserum	"2 anti- $\beta$ 1"	"anti- $\beta$ 1"	"FKc"
Source of antigen	SCM $\beta$ keratins isolated from 1-d gel (chicken)	$\beta$ 1 polypeptide isolated from 2-d gel (chicken)	FKc polypeptide isolated from 2-d gel (chicken)
Responsive tissues	Epidermal stratum corneum of beak, claw, tongue, scale, shell, spur, feather, and embryonic layers	Epidermal stratum corneum of beak, egg tooth, claw, scale, feather, and embryonic layers	Epidermal stratum corneum beak, claw, feather, and embryonic layers
Responsive taxa	Turtles, snakes and lizards, alligators, and birds	Alligators and birds	Alligators and birds
References	O'Guin <i>et al.</i> , 1982; O'Guin, 1984; Sawyer <i>et al.</i> , 1986; Haake <i>et al.</i> , 1984; Haake, 1985; Carver, 1988; Carver and Sawyer, 1989; Carver <i>et al.</i> , 1990; Mays, 1998; Shames and Sawyer, 1987; Shames <i>et al.</i> , 1988; 1989	Shames <i>et al.</i> , 1991; Knapp <i>et al.</i> , 1993; Mays, 1998; Knapp, Shames, and Sawyer, unpublished data	Barnes, 1993; Mays, 1998; Shames and Sawyer, unpublished data

pair RNA recombinant probe generated from pCSK-12 was shown to localize, specifically, to the cells of the *Beta Stratum* of the scutate scale epidermis (Shames and Sawyer, 1987; Shames *et al.*, 1988).

Furthermore, sequence analysis has shown that the 3' coding ends of the  $\beta$  keratin genes are highly conserved (Gregg and Rogers, 1986; Gregg *et al.*, 1983, 1984). Again, Shames *et al.* (1988) used hybrid selection (with a synthetic oligonucleotide probe to the conserved 3' region) and *in vitro* translation to identify seven scale  $\beta$  keratin polypeptides. All of these polypeptides reacted with the anti- $\beta$  keratin antise-

rum. The avian claw  $\beta$  keratin gene does not contain this 3' conserved region (Whitbread *et al.*, 1991). Figure 2 is a diagrammatic representation of the scutate scale polypeptides separated by two-dimensional gel electrophoresis. The diagram shows the alpha ( $\alpha$ ) keratins (O'Guin *et al.*, 1987), the beta ( $\beta$ ) keratins (Shames *et al.*, 1988; 1991; Knapp *et al.*, 1993), the embryonic feather  $\beta$  keratin (FKc) (Barnes, 1993), the cytokeratin-associated proteins (CAPs) (Knapp *et al.*, 1991), and the histidine-rich proteins (HRPs) (Barnes, 1993; Barnes and Sawyer, 1995) also known as the Fast Proteins (Powell and Rogers, 1979).

#### *Individual scutate scale $\beta$ keratins as immunogens*

To take advantage of the immunological approach, the seven scale  $\beta$  keratin polypeptides, and other polypeptides (Barnes, 1993; Barnes and Sawyer, 1995; Knapp *et al.*, 1991; 1993), were isolated from two-dimensional gels and prepared as immunogens for use in rabbits and mice (unpublished data, LWK, RBS and RHS; Shames *et al.*, 1988, 1991). Unlike the S-carboxymethylated polypeptides used as immuno-

TABLE 2. *Antiserum against  $\alpha$  keratins.*

Name of antiserum	"3 anti- $\alpha$ 1"
Source of antigen	SCM $\alpha$ keratins isolated from 1-d gel (chicken)
Responsive tissues	All epidermises
Responsive taxa	Amphibians, fishes, snakes and lizards, turtles, alligators, birds, and mammals
References	O'Guin <i>et al.</i> , 1982; 1987; Sawyer <i>et al.</i> , 1986; Mays, 1998; Knapp, O'Guin and Sawyer unpublished data

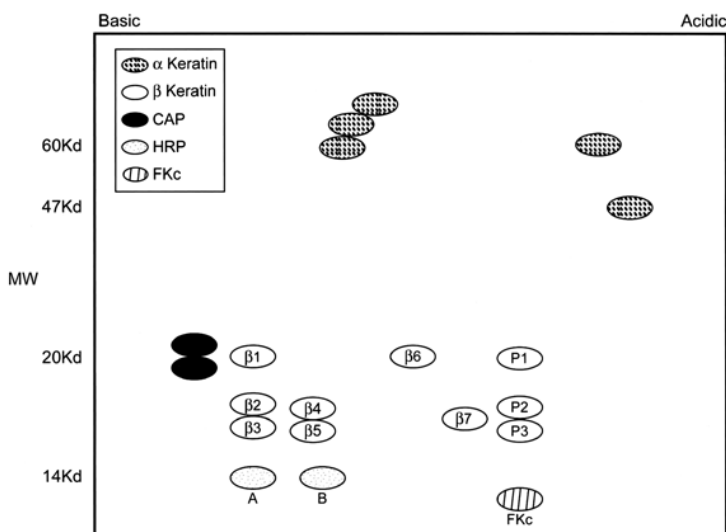


FIG. 2. Separation of epidermal polypeptides extracted with Triton-X 100/ 1.5 M KCL (Franke *et al.*, 1979) from chicken epidermis (see Shames *et al.*, 1988; 1991; Knapp *et al.*, 1993). The alpha ( $\alpha$ ) keratins of the chicken have been characterized by O'Guin *et al.* (1987). The cytokeratin-associated proteins (CAPs) are non-keratin polypeptides and have been analyzed by Knapp *et al.* (1991). Barnes and Sawyer (1995) have studied the histidine-rich proteins (HRPs), also known as Fast Protein (Powell and Rogers, 1979). The seven scutate scale beta ( $\beta$ ) keratins ( $\beta$  1–7) and their phosphorylated forms (P 1–3) have been characterized by Shames *et al.* (1989;1991). In addition to characterizing the HRPs, Barnes (1993) also identified the FKc polypeptide as an embryonic feather  $\beta$  keratin (see also Mays, 1998).

gens by O'Guin *et al.* (1982), the epidermal polypeptides, used as immunogens by Shames *et al.* (1988; 1989), were extracted by the Triton-X 100/1.5 M KCL method of Franke *et al.* (1979) and separated by two-dimensional SDS-polyacrylamide gel electrophoresis (O'Farrell *et al.*, 1977). Attempts to generate monoclonal antibodies to the various scale beta keratins ( $\beta$  1–7, and P 1–3) have been unsuccessful to date. However, a small amount of monospecific antiserum was obtained from a mouse injected with the polypeptides,  $\beta$  2 and 3, which occur as a doublet on two-dimensional gels. This antiserum was highly specific for the scale  $\beta$  2–3 polypeptides on Western blots, localized exclusively to the suprabasal epidermal cells of the *Beta Stratum* of the outer scutate scale surface, and specifically decorated the 3-nm  $\beta$  keratin filament bundles of the *Beta Stratum* (Shames *et al.*, 1988). These results suggested that it might be possible to generate monospecific polyclonal antisera for the individual  $\beta$  keratin polypeptides.

#### The anti- $\beta$ 1 antiserum

The individual scale beta keratins ( $\beta$  1–7) were used as immunogens to generate polyclonal antibodies in rabbits (unpublished data, LWK, RBS, and RHS). Thus far, characterization of these antibodies indicates that they all react with most of the scale  $\beta$  keratin polypeptides even though they were raised against individual polypeptides. For example, the anti- $\beta$  1 antiserum raised against the  $\beta$  1 polypeptide of scutate scales has been shown to cross-react on Western blots with all known scale  $\beta$  keratins (Shames *et al.*, 1991; Knapp *et al.*, 1993). Shames *et al.* (1991) used this antiserum to show that different scutate scale  $\beta$  keratins are present in the chick egg tooth, beak, and claw (Table 1).

#### The HRP and FKc antisera

Using the above approach (see Shames *et al.*, 1988, 1991), Barnes (1993; Barnes and Sawyer, 1995) generated antiserum (anti-HRP) against the histidine-rich protein (HRP), also known as Fast-protein (Powell



and Rogers, 1979). The anti-HRP reacts specifically with the HRP polypeptide on two-dimensional Western blots, and decorates the feather barb ridge cells of feathers (Barnes and Sawyer, 1995). The anti-HRP antiserum reacts with only the embryonic layers (Periderm and Subperiderm) of the scutate scale.

The embryonic feather  $\beta$  keratin (FKc) was likewise isolated by two-dimensional gel electrophoresis of Triton-X 100/1.5 M KCL extracts of embryonic feathers (see Haake, 1985). The amino acid composition of FKc indicates that it is a member of the feather  $\beta$  keratin family, as does its molecular weight of 10.5–11.0 kilodaltons. Antiserum was generated against the FKc polypeptide. This antiserum (anti-FKc) reacts with the FKc polypeptide and a second polypeptide that appears to be a more acidic form of FKc (Barnes, 1993). On Western blots, it does not react with the histidine-rich proteins, the cytokeratin associated proteins or any of the scutate scale beta keratins (Barnes, 1993). In feather tissues, anti-FKc specifically localizes to the barb ridge cells (Barnes, 1993). This antiserum also localizes to epidermal cells in the stratum corneum of the embryonic beak and claw (Barnes, 1993).

#### *The keratins of embryonic alligators*

Recently, we initiated studies to examine the expression of  $\alpha$  and  $\beta$  keratins during the development of epidermal appendages in the American alligator (*Alligator mississippiensis*). We found that the antisera generated against chicken  $\alpha$  and  $\beta$  keratins cross-reacted with the keratin polypeptides in the scale and claw epidermal tissues from the alligator embryos (Mays, 1998; Shames and Sawyer, unpublished observations). The anti- $\beta$  keratin antisera also reacted with keratin polypeptides separated by polyacrylamide gel electrophoresis of Triton-X 100/1.5 M KCL extracts of alligator scale and claw epidermis. In addition, Mays (1998) discovered that the anti-FKc antiserum, which was generated against an embryonic feather  $\beta$  keratin (Barnes, 1993), reacts with the suprabasal cells in the claw epidermis of the embryonic alligator. Anti-FKc antiserum reacts with a single band on

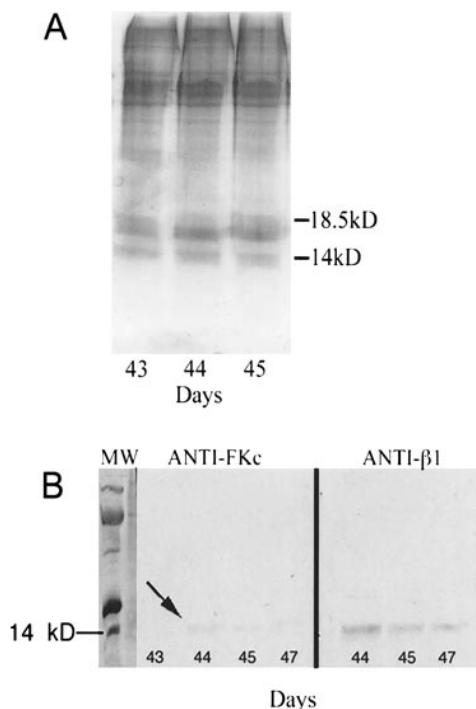


FIG. 3. A. Triton-X 100/1.5M KCL extracted alligator claw keratins separated by polyacrylamide gel electrophoresis and stained with 0.1% Coomassie Brilliant blue. At 43, 44, and 45 days of development, polypeptides are present in the molecular weight range of 14 to 18.5 kilodaltons (kD). B. Western blots of alligator claw keratins separated as in (A) and transferred to nitrocellulose paper. The polypeptides were reacted with either the anti-FKc antiserum or the anti- $\beta$  1 antiserum. Both antisera react with polypeptide bands in the 14 kD molecular weight range.

Western blots of alligator claw extracts (Fig. 3).

#### *DNA and amino acid sequences among birds*

A current focus of our laboratory is the investigation of the diversity of DNA sequences leading to the keratin proteins, especially the feather  $\beta$  keratins. Direct amino acid sequencing of feather  $\beta$  keratins has been accomplished for the chicken (Walker and Rogers 1976, Molloy *et al.* 1982), emu (O'Donnell, 1973), gull (O'Donnell and Inglis, 1974), pigeon (Arai *et al.*, 1986) and duck (Arai *et al.*, 1986). The DNA sequences and expression of several chicken keratin genes have been investigated by George Rogers' group (*e.g.*, Molloy *et al.*, 1982;

	1	0	2	3	4	5	6
Chicken FBK A	MSCYDLCRPS----	APTPLANSNEPCV-	RQCQDSRVVIQPS	PVVVTLPGPILSS	FPQNTAVG-		
Chicken FBK B	MSCYDLCRPC----	GPTPLANSNEPCV-	RQCQDSRVVIQPS	PVVVTLPGPILSS	FPQNTAVG-		
Chicken FBK D	MSCFDLCRPC----	GPTPLANSNEACV-	RQCQDSRVVIQPS	PVVVTLPGPILSS	FPQNTLVG-		
Quail	MSRYDLCRPC----	GPTPLANSNEPCV-	RQCQDSRVVIQPS	PVVVTLPGPILSS	FPQNTAVG-		
WoodStork	MSCYDLCRPC----	GPTPLANSNEPCV-	RQCQDSRVVIQPS	PVVVTLPGPILSS	FPQNTAVG-		
Pigeon	?SCYDLQPC----	GPTPLANSNEPCV-	RQCQDSRVVIQPS	PVVVTLPGPILSS	FPQNTAVG-		
Duck	?SCYDLCRPC----	GPTPLANSNEPCV-	RQCQDSRVVIQPS	PVVVTLPGPILSS	FPQNTAVG-		
Emu	?SCYNPLPRSSC-	GPTPLANSNEPCV-	RQCQDSRVVIQPS	PVVVTLPGPILSS	FPQNTVVG-		
C. Feather-Like	MSFHVLP-----	PTPLANSNEPCV-	RQCQDSRVVIQPS	PVVVTLPGPILSS	FPQNTVVG-		
Chicken Scale	MSCYDLCPPTSCIS	RQPIADSGNEPCV-	RQCQDSRVVIQPS	PVVVTLPGPILSS	FPQNTVVG-		
Chicken Claw	MSCSSLCAP_ACVAT	PTPLADSGNEPCV-	RQCQDSRVVIQPS	PVVVTLPGPILSS	FPQNTVVG-		
Alligator	-----	-----	STVVIQPPSVVTIP	GAILS-----			

FIG. 4. This figure shows the alignment of feather  $\beta$  keratins for a variety of birds including: direct protein sequences from emu (O'Donnell 1973), pigeon (Arai et al. 1986), and duck (Arai et al. 1986), and protein sequences inferred from DNA sequences for chickens (Presland et al. 1986a), quail (unpublished data, J.O.F.) and woodstork (unpublished data, J.O.F.). These sequences are very similar to other keratins from chickens including claw, scale, feather, and feather-like  $\beta$  keratins (Presland et al., 1989a, b; Whitbread et al., 1991; Wilton et al., 1985; Rogers, 1985). The similarity even extends to 20 amino acids directly determined from alligator claw  $\beta$  keratin (unpublished data, Y. I. and R.B.S.).

Gregg *et al.*, 1984, Rogers, 1985; Presland *et al.*, 1989a, b; Whitbread *et al.*, 1991). We used the known feather  $\beta$  keratin sequences described in Presland *et al.* (1989a) to design primers for the polymerase chain reaction (PCR) to amplify the DNA sequences of feather  $\beta$  keratins from different birds. These primers amplify feather  $\beta$  keratins from a variety of avian taxa. The protein sequences inferred from our DNA sequences and the sequences of Presland *et al.* (1989a) are compared with protein sequences derived from direct amino acid sequencing in Figure 4. These sequences clearly demonstrate that the PCR primers reliably amplify feather  $\beta$  keratin DNA sequences and that all of the keratins are closely related.

#### Amino acid sequence of alligator claw keratin

We have had limited success obtaining direct amino acid sequence data on alligator scale and claw keratins extracted with Triton-X 100/1.5 M KCL and separated by two-dimensional gel electrophoresis. However, a partial amino acid sequence (20 amino acids in length) was determined for a peptide obtained from the most abundant alligator claw keratin (unpublished data, YI and RBS). The peptide sequence can be easily aligned with avian  $\beta$  keratins (Fig. 4). The alligator claw peptide sequence is 85% similar to the chicken claw sequence, 85% similar to the chicken scale sequence, 80%

similar to the chicken feather-like sequence, and 75% similar to the feather  $\beta$  keratin consensus sequence. This high level of amino acid sequence similarity suggests that the alligator claw protein is closely related to the avian  $\beta$  keratin family.

#### SUMMARY AND CONCLUSIONS

Numerous studies have been published dealing with the development of the scales and feathers of the chicken, and much is known about their structural proteins, the  $\beta$  keratins. On the other hand, little is known about the developmental mechanisms or the structural proteins of reptilian epidermal appendages. If we wish to understand the evolution of feathers, then we must gain a better understanding of the relationships between the reptilian and avian epidermal appendages, including the developmental expression of genes responsible for pattern formation and structural proteins.

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