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RESEARCH ARTICLE

Developmental regulation of hemoglobin synthesis in the green anole lizard *Anolis carolinensis*

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SUMMARY

Tetrapod vertebrates possess multiple α- and β-like globin genes that are ontogenetically regulated, such that functionally distinct hemoglobin (Hb) isoforms are synthesized during different stages of development. The α- and β-like globin genes of amphibians, birds and mammals are differentially expressed during embryonic development and postnatal life, but little is known about the developmental regulation of globin gene expression in non-avian reptiles. Here we report an investigation into the developmental regulation of Hb synthesis in the green anole lizard *Anolis carolinensis*. We tested two hypotheses derived from comparative genomic studies of the globin gene clusters in tetrapod vertebrates. First, we tested whether the product of the *Anolis* α^D-globin gene is incorporated into embryonic Hb, thereby performing the role that would normally be performed by the embyronic α^E-globin gene (which has been deleted from the green anole genome). Second, we tested whether two 'lizard-specific' β-globin paralogs have independently evolved a division of labor between an early-expressed embryonic gene and a later-expressed adult gene. Results of a proteomic analysis revealed that α- and β-like globin genes of the anole are differentially expressed during embryonic development. However, the same repertoire of α- and β-chain Hb isoforms was expressed during all stages of development and postnatal life, and the ontogenetic shifts in isoform composition were relatively subtle. In contrast to the pattern that has been documented in other tetrapod vertebrates, it appears that the developmental regulation of Hb synthesis in the green anole lizard does not involve discrete, stage-specific switches in gene activation and gene silencing.

Key words: *Anolis*, gene duplication, globin gene family, hemoglobin, reptile genomics.

INTRODUCTION

In vertebrates, the multimeric hemoglobin (Hb) protein binds and transports oxygen and other gaseous ligands in support of cellular aerobic metabolism. The protein carries out these functions under the diverse range of physiological conditions that are encountered during the various stages of prenatal development and postnatal life (Brittain, 2002). The Hb of jawed vertebrates is a heterotetramer composed of two α-chain subunits and two β-chain subunits that are encoded by members of two paralogous gene families. During the course of vertebrate evolution, multiple rounds of gene duplication and divergence have given rise to families of α- and β-like globin genes that are ontogenetically regulated, such that functionally distinct Hb isoforms (isoHbs) are synthesized in embryonic and adult erythroid cells (Collins and Weissman, 1984; Hardison, 1998; Hardison, 2001). For example, the α- and β-like globin genes of *Xenopus* are differentially expressed in the larval and adult stages (Banville and Williams, 1985a; Banville and Williams, 1985b; Fuchs et al., 2006), and the α- and β-like globin genes of birds and mammals are also differentially expressed during embryonic development and postnatal life (Hardison, 2001; Alev et al., 2008; Alev et al., 2009). However, very little is known about the developmental regulation of Hb synthesis in non-avian reptiles.

In the α-globin gene family, the physiological division of labor between early- and late-expressed genes was established in the common ancestor of tetrapod vertebrates and it appears to have been retained in nearly all descendant lineages. The ancestral arrangement of the tetrapod α-globin gene cluster consists of three linked genes, 5′-α^E, α^D, α^A-3′ (Hoffmann and Storz, 2007; Hoffmann et al., 2010). The α^E- and α^A-globin genes originated *via* tandem duplication of an ancestral proto α-globin gene after the stem lineage of teleost fishes split from the stem lineage of tetrapods, and the α^D-globin gene originated subsequently *via* tandem duplication of the proto α^E-globin gene in the common ancestor of tetrapods (Fig. 1A) (Hoffmann and Storz, 2007). The embryonic α^E-globin gene (known as α^L-globin in amphibians, π-globin in birds and ζ-globin in mammals) is exclusively expressed in primitive erythroid cells derived from the yolk sac, and the adult α^A-globin gene is expressed in definitive erythroid cells during later stages of prenatal development and postnatal life (Proudfoot et al., 1982; Higgs et al., 1989; Hardison, 2001). Among tetrapod vertebrates, the green anole lizard (*Anolis carolinensis*) represents the one documented exception to this highly conserved pattern of developmental regulation due to the apparent absence of the α^E-globin gene in this species (Hoffmann et al., 2010). It remains to be seen whether this gene is absent from the genomes of other squamate reptiles as well. As the α^D-globin gene originated *via* duplication of an α-like globin gene that had an ancestral larval/embryonic function (Hoffmann and Storz, 2007), it may be that α^D-chain isoHbs perform the necessary oxygen-binding and oxygen-transport functions during early stages of embryonic development in lizards and other reptiles that do not

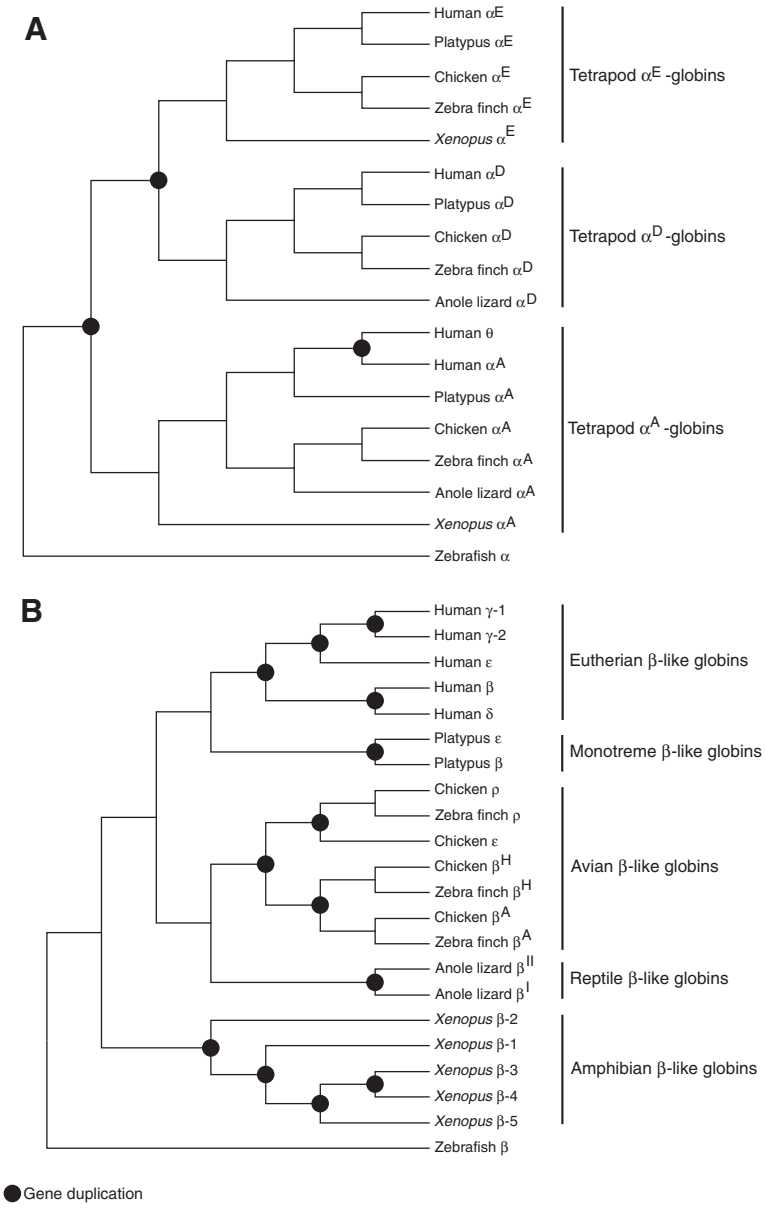

Fig. 1. Schematic cladograms depicting the inferred phylogenetic relationships among members of the α- and β-globin gene families in tetrapod vertebrates [based on data from Hoffmann and Storz (Hoffmann and Storz, 2007), Opazo et al. (Opazo et al., 2008a) and Hoffmann et al. (Hoffmann et al., 2010)]. In each tree, filled symbols denote nodes that represent gene duplication events. (A) Phylogeny of α-like globin genes in tetrapods. Note that the three paralogs (α^E-, α^D- and α^A-globin) are reciprocally monophyletic relative to one another, and that the α^E-globin gene (which is exclusively expressed in primitive erythroid cells during embryonic development) and the α^D-globin gene are products of a duplication event that occurred in the stem lineage of tetrapods. (B) Phylogeny of β-like globin genes in tetrapods. Note that birds, non-avian reptiles, eutherian mammals, monotremes and amphibians each inherited an ortholog of the same proto β-globin gene, which then underwent one or more rounds of duplication and divergence to produce distinct repertoires of β-like globin genes in each descendant lineage.

possess an ortholog of α^E-globin. In some species of birds, for example, the α^D-globin gene is only expressed during embryonic development (Ikehara et al., 1997) whereas other species express α^D-globin in both primitive and definitive erythroid cells (Alev et al., 2008; Alev et al., 2009).

In contrast to the ancient functional diversification of the α-globin gene cluster, the physiological division of labor between early- and late-expressed genes in the β-globin gene cluster appears to have evolved independently in several different tetrapod lineages (Hoffmann et al., 2010). Each of the main lineages of tetrapods inherited an ortholog of the same proto β-globin gene, which then underwent one or more rounds of duplication and divergence to produce distinct repertoires of β-like globin genes in each descendant lineage (Fig. 1B) (Opazo et al., 2008a; Opazo et al., 2008b; Patel et al., 2008; Hoffmann et al., 2010). For example, an inventory of globin genes in the green anole genome revealed that this species possesses a pair of highly distinct β-like globin genes, β^I and β^{II}, that are distinguished from one another by 25% sequence divergence at the amino acid level (Hoffmann et al., 2010). Phylogenetic analysis of reptile β-like globin genes revealed that orthologs of the *Anolis* β^I- and β^{II}-globin genes are shared by other lizards, but it is not yet clear whether they are shared more widely among other lepidosaurs, and nothing is known about the developmental expression profiles of these genes. Given that the developmental regulation of β-like globin genes has evolved independently in amphibians, birds and mammals (Hoffmann et al., 2010), it is possible that the β^I- and β^{II}-globin paralogs of lizards have evolved a functionally similar physiological division of labor between an early-expressed embryonic gene and a later-expressed adult gene.

Here we report the results of a developmental study of Hb synthesis in the green anole lizard *A. carolinensis*. We tested two main predictions that were derived from our current understanding of globin gene family evolution in tetrapod vertebrates. The first prediction is that the product of the *Anolis* α^D-globin gene is incorporated into embryonic Hb, thereby performing the role that would normally be performed by the embryonic α^E-globin gene (which is missing from the green anole genome). The second prediction is that the two β-globin paralogs of *Anolis* (which are products of a lineage-specific duplication event) are developmentally regulated such that one of the two paralogs is expressed exclusively

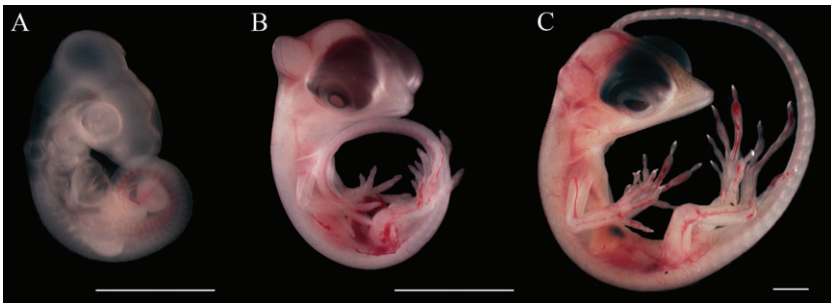

Fig. 2. Representative developmental stages of *Anolis carolinensis*. Pictures in panels A, B and C correspond to stages 5, 11 and 17, respectively. Scale bars=2 mm.

in primitive erythroid cells of early-stage embryos, while the other paralog is expressed in definitive erythrocytes during later stages of prenatal development and postnatal life.

MATERIALS AND METHODS

Specimen collection

To test our predictions regarding the developmental regulation of Hb synthesis in *Anolis* we collected tissues from embryonic specimens and blood samples from adult specimens of *Anolis carolinensis* Voigt 1832. We sampled early, mid- and late-stage embryos corresponding to stages 5, 5/6, 11 and 17 of *Anolis* development (Fig. 2) (Sanger et al., 2008a). Relative to most avian eggs, *A. carolinensis* eggs are laid at a relatively advanced embryonic stage, represented by stage 5. At this stage, limb buds, hind-, mid- and forebrain segments, heart and many other structures are already present in the embryo (Fig. 2A). Blood is visible in the embryo several stages prior to hatching, but peripheral blood vessels are not yet visible in the extremities. By stage 11 the embryonic liver is yellow–brown in color, and blood is easily visible in the heart and in the extremities. After approximately 30 days the embryo is fully developed and capable of living outside of the egg. We sampled just before this time at stage 17.

Lizard husbandry and embryo collection were performed in accordance with guidelines approved by the Institutional Animal Care and Use Committee of Harvard University (IACUC# 28-14 and 26-11), and details are provided by Sanger et al. (Sanger et al., 2008a; Sanger et al., 2008b). Briefly, sexually mature *A. carolinensis* lizards were collected from the wild and purchased from Candy's Quality Reptiles (La Place, LA, USA). To collect embryonic tissues, we housed 3–5 females in a standard rat cage with one male and several sticks to act as perches. A potted plant within each cage was checked every one to three days for new eggs. We incubated eggs in coarse-grained vermiculite at 27°C and approximately 75% humidity. We collected embryos of the appropriate stage by submerging the egg in phosphate-buffered saline (PBS) in a Petri dish. To remove the embryo from the egg, a shallow incision was made over the embryo using #5 watchmaker's forceps allowing the eggshell to be folded away. The embryo was then cleared from the yolk and rinsed in fresh PBS. Early embryos (stages 5 and 5/6) were immediately flash frozen. The liver was dissected from later stage embryos (stages 11 and 17) by opening the abdomen and chest cavity using 3 mm spring scissors while the embryo was submerged in fresh PBS. After being flash frozen, all tissues were stored at –80°C until the time of processing. Blood samples from four adult lizards were collected by filling a capillary tube from a small incision along the ventrum of the tail of a partially anesthetized lizard (Sellers et al., 1980).

Characterization of isoHb diversity

In the case of the adult *A. carolinensis* specimens, the isoHb composition of mature erythrocytes was characterized by means of isoelectric focusing (IEF; PhastSystem, GE Healthcare Bio-Sciences, Piscataway, NJ, USA). After separating native Hbs by means of IEF, gel bands were excised and digested with trypsin. The resultant peptides were then identified by means of tandem mass spectrometry (MS/MS) (cf. Nakachi et al., 2008; Campbell et al., 2010; Storz et al., 2010). The peak lists of the MS/MS data were generated by Distiller (Matrix Science, London, UK) using the charge state recognition and de-isotoping with default parameters for quadrupole time-of-flight data. Database searches of the resultant MS/MS spectra were performed using Mascot (Matrix Science, v1.9.0, London, UK). Specifically, peptide mass fingerprints derived from the MS/MS analysis were used to query a custom database of *Anolis* α- and β-globin sequences. These amino acid sequences were derived from conceptual translations of all annotated α- and β-globin genes from the current assembly of the green anole genome (release 54 of the Ensembl database).

A previous examination of the green anole genome (Hoffmann et al., 2010) revealed two putatively functional α-like globin genes (located in scaffolds 2790:1-15038 and 1188:1-163036). Phylogenetic reconstructions based on coding sequence indicated that one of the genes is orthologous to the α^{A}-globin gene of other tetrapods, and the other gene is orthologous to the α^{D}-globin gene of other amniotes (see Fig. 1A). However, there was no trace of the embryonic α^{E}-globin gene in the current green anole genome assembly or in independent EST databases. Hoffmann et al. also annotated two putatively functional β-like globin genes (β^{I}- and β^{II}-globin, located in scaffolds 7008:1-4809 and 3777:1-10310) (Hoffmann et al., 2010). Phylogenetic reconstructions indicated that the β^{I}-globin genes of the green anole and common iguana are 1:1 orthologs, as are the β^{II}-globin genes from the same species pair (Hoffmann et al., 2010). The β^{I}- and β^{II}-globin genes of the green anole and iguana appear to be products of a lizard-specific or squamate-specific duplication event. In the reference database for the MS/MS analysis, we also included the α- and β-globin sequences from one amphibian (*Xenopus tropicalis* Daudin 1802), two additional squamate reptiles (common iguana, *Iguana iguana* Linnaeus 1758, and Indian python, *Python molurus* Linnaeus 1758), one sphenodont reptile (tuatara, *Sphenodon punctatus* Gray 1842), two crocodilians (Nile crocodile, *Crocodylus niloticus* Laurenti 1768, and American alligator, *Alligator mississippiensis* Daudin 1802), two testudines (loggerhead turtle, *Caretta caretta* Linnaeus 1758, and Galapagos tortoise, *Geochelone nigra* Quoy and Gaimard 1824), two birds (chicken, *Gallus gallus* Linnaeus 1758, and zebra finch, *Taeniopygia guttata* Vieillot 1817) and two mammals (human, *Homo sapiens* Linnaeus 1758, and platypus, *Ornithorhynchus anatinus* Shaw 1799). The following search parameters were used for the MS/MS analysis: no restriction on protein molecular weight or isoelectric point, and methionine oxidation allowed as a variable peptide modification. Mass accuracy settings were 0.15 Da for peptide mass and 0.12 Da for fragment ion masses. We identified

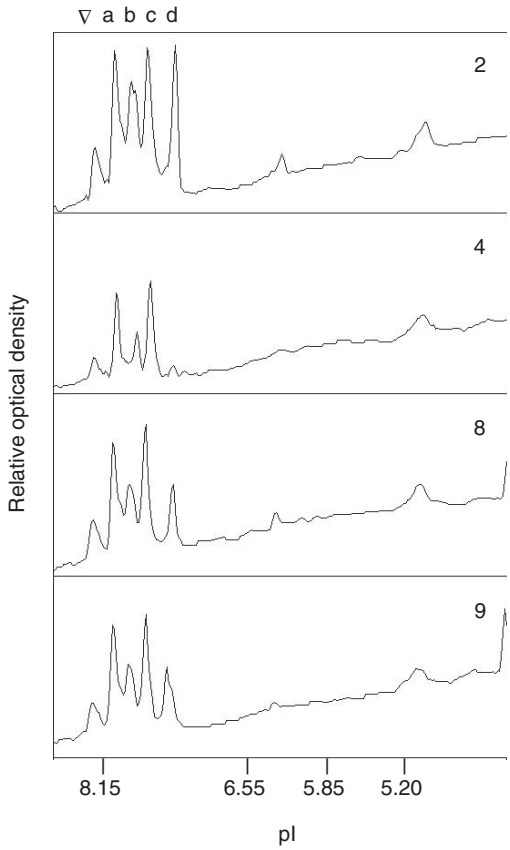

Fig. 3. Densitometric scans of isoelectric focusing (IEF) gels showing hemoglobin isoform (isoHb) diversity in the red blood cells of adult *Anolis carolinensis* (*N*=4; specimens 2, 4, 8 and 9). The four major peaks in each trace (labeled a, b, c and d) represent CO-derivatives of structurally distinct isoHb tetramers. The ∇ symbol denotes the position of the loading well in the IEF gel.

all significant protein hits that matched more than one peptide with $P<0.05$.

Quantitative proteomic analysis of Hb expression

In the case of Hb components separated by IEF, the relative concentrations of the different isoHbs were quantified densitometrically using Image J (Abramoff et al., 2004). In the case of the *A. carolinensis* embryos, we pooled whole embryos (stages 5 and 5/6) or dissected liver tissues (stages 11 and 17) for trypsin digests and subsequent MS/MS analyses. Tissues from 5–10 embryos were pooled for each of the four developmental stages. To estimate the relative abundance of different isoHbs in each sample, we measured the exponentially modified protein abundance index, emPAI, using the number of identified peptides per protein (Ishihama et al., 2005).

RESULTS

The IEF analysis revealed that adult lizards express four distinct isoHb components (Fig. 3), and the MS/MS analysis revealed that each of the subunit components represent products of the previously annotated α- and β-like globin genes in the *Anolis* genome assembly (Fig. 4). There were no peptide matches corresponding to the products of genes other than the α^A-, α^D-, β^I- and β^{II}-globin genes of *Anolis*. The MS/MS analysis revealed that adult lizards express each of the four possible tetrameric $\alpha_2\beta_2$ subunit isoHb combinations, which were present in the following rank order of protein abundance: $\alpha^A{}_2\beta^I{}_2>\alpha^D{}_2\beta^{II}{}_2\geq\alpha^D{}_2\beta^I{}_2>\alpha^A{}_2\beta^{II}{}_2$. In the mature erythrocytes of adult lizards, the mean ratio of α^D/α^A-chain isoHbs was 1.13 (range=1.09–1.17), and the mean ratio of β^I/β^{II}-chain isoHbs was 1.38 (range=1.30–1.52; *N*=4 individuals)

In the case of the developmental study, results of the MS/MS analysis revealed that the α- and β-globin genes of the green anole are differentially expressed during the course of embryonic development (Fig. 5). However, the same subunit isoHbs that were identified in the mature erythrocytes of adult lizards were also expressed throughout the entire course of prenatal development. Thus, although expression levels undergo subtle changes during the course of development, the MS/MS data demonstrate that the α^A-, α^D-, β^I- and β^{II}-globin genes were expressed in both primitive and definitive erythroid cells.

With regard to the α-like globin genes, the α^D-chain isoHbs were most highly expressed during the earliest stages of embryogenesis, and the relative abundance of α^D-chain isoHbs consistently exceeded that of α^A-chain isoHbs over the course of development (Fig. 5). The ratio of α^D/α^A-chain isoHbs decreased from 1.44 at day 1 post-oviposition (stage 5) to 1.03 at day 21 (stage 17). Compared with the embryos at stage 17, the ratio of the two α-chain isoHbs remained remarkably similar to the ratio measured in the mature erythrocytes of adult lizards.

With regard to the β-like globin genes, the β^I-chain isoHbs were most highly expressed during the earliest stages of embryonic development, exhibiting a twofold increase in relative abundance at day 4 post-oviposition (stage 5/6), followed by a gradual decline up to the pre-hatching stage (stage 17). Aside from the early spike in the relative abundance of the β^I-chain isoHb, the ratios of β^I/β^{II}-chain isoHbs during the remaining stages of embryonic development were quite similar to the ratio measured in the mature erythrocytes of adult lizards.

DISCUSSION

By characterizing the developmental regulation of Hb synthesis in the green anole lizard, we were able to test two hypotheses derived from comparative genomic studies of the globin gene clusters in tetrapod vertebrates. First, we tested whether the product of the *Anolis* α^D-globin gene is incorporated into embryonic Hb, thereby performing the role that would normally be performed by α^E-globin. Second, we tested whether the two 'lizard-specific' β-globin paralogs are developmentally regulated such that one of the two paralogs is expressed exclusively in primitive erythroid cells of early-stage embryos, while the other paralog is expressed in definitive erythrocytes during later stages of embryonic development and postnatal life. Below we describe tests of both predictions in turn.

Expression of α-chain isoHbs

Results of the MS/MS analysis revealed that the highest relative expression of α^D-chain isoHbs occurred during the earliest post-oviposition stage of embryogenesis (Fig. 5). This suggests that the product of α^D-globin may play a key role in oxygen uptake and/or oxygen scavenging in early-stage lizard embryos. However, in contrast to the α^E-globin gene of other tetrapods, the α^D-globin gene in the green anole is also expressed at a high level throughout the course of embryonic development, and α^D-chain isoHbs are present at a slightly higher concentration than α^A-chain isoHbs in the mature erythrocytes of adult lizards. The high-level expression of α^D-globin during postnatal life is quite different from the typical pattern

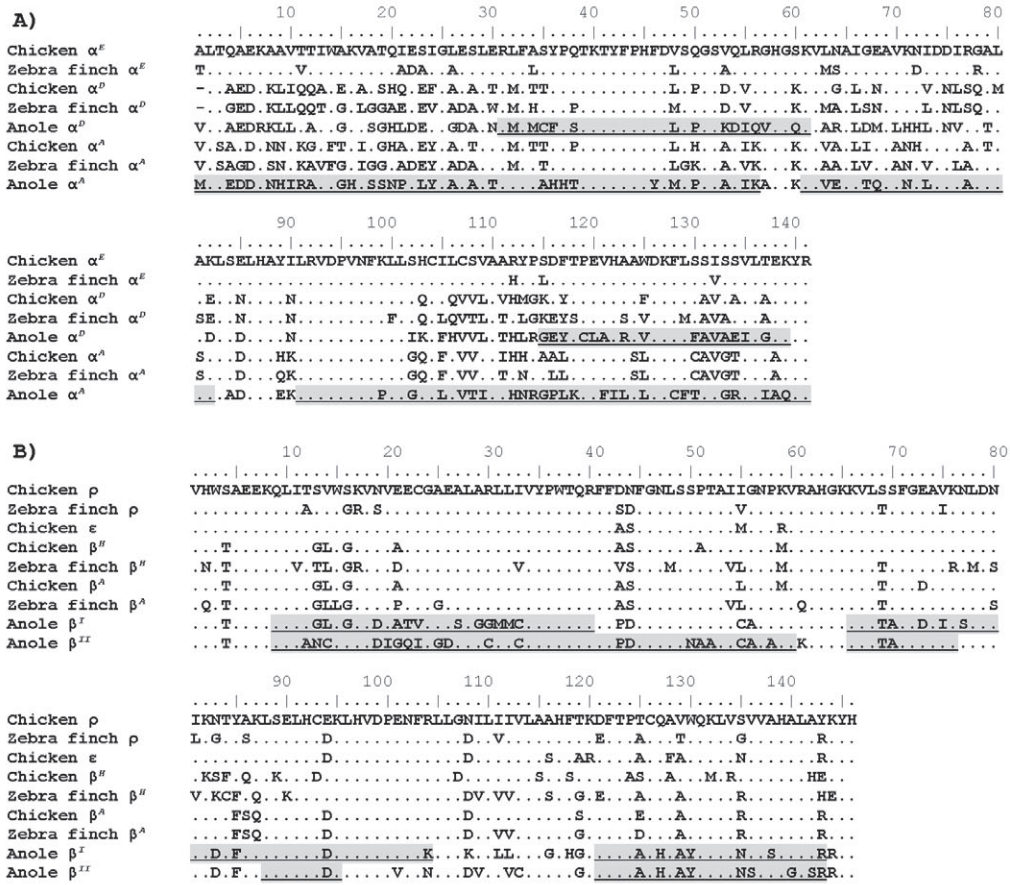

Fig. 4. Alignment of amino acid sequences representing the complete repertoire of α- and β-like globin genes from *Anolis carolinensis* and two bird species, chicken and zebra finch. Sequences of α- and β-like globin genes are shown in panels A and B, respectively. The highlighted portions of each *Anolis* sequence denote the coverage of matched peptides in the mass spectrometry analysis (see text for details).

observed in birds. In the definitive erythroid cells of adult birds, the α^D-chain isoform typically constitutes the minor fraction of adult Hb and the α^A-chain isoform typically constitutes the major fraction, although the relative abundance of the two isoforms is quite variable among species (Borgese and Bertles, 1965; Brown and Ingram, 1974; Hiebl et al., 1987; Ikehara et al., 1997).

The function of α^D-Hb has always been something of an enigma, and its evolutionary origin and phylogenetic affinities have only recently been illuminated (Hoffmann and Storz, 2007). In mammals, the α^E- and α^A-globin genes are often present in multiple copies, but the α^D-globin gene has been deleted independently in multiple lineages (Hughes et al., 2005; Cooper et al., 2006; Hoffmann et al., 2008). The α^D-globin gene has also been secondarily lost from *Xenopus* (Fuchs et al., 2006; Hoffmann and Storz, 2007), and it remains to be seen whether this gene is absent from the genomes of all amphibians. Products of the α^D-globin gene have not been recovered from the erythrocytes of adult crocodilians (reviewed by Gorr et al., 1998), but *in lieu* of a complete genome sequence it remains possible that crocodilians possess a transcriptionally active α^D-globin gene that has thus far evaded detection because it is exclusively expressed during embryonic development. In summary, our experimental results suggest that α^D-chain isoHbs may play an especially important role during the earliest stages of embryonic development in the green anole, as this is the stage where it is most highly expressed. However, unlike the embryonic α^E-globin gene of other tetrapods, the α^D-globin gene of the green anole does not appear to be performing a specialized function that is specific to early embryogenesis, as the same gene is highly expressed during all stages of development and postnatal life.

Expression of β-chain isoHbs

As the two β-globin paralogs of *Anolis* are products of a lizard- or squamate-specific duplication event and are therefore not orthologous to embryonic β-like globin genes of other tetrapods (Hoffmann et al., 2010), we tested whether the two genes independently evolved different stage-specific expression patterns. The MS/MS analysis revealed ontogenetic shifts in the relative abundance of β^I- and β^{II}-chain isoHbs. However, both isoHbs were

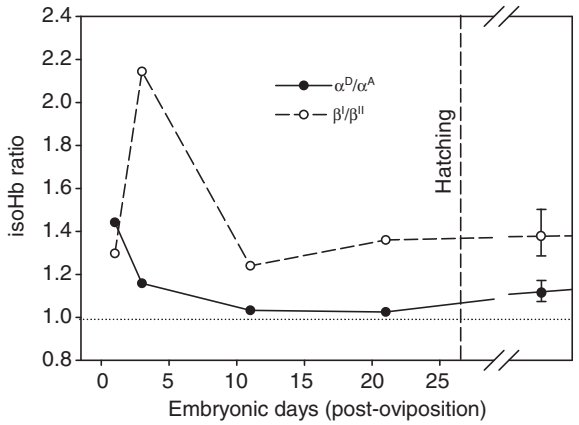

Fig. 5. Changes in the relative abundance of α- and β-chain hemoglobin isoforms (isoHbs) during embryonic development and postnatal life in *Anolis carolinensis*. Mean isoHb ratios from red blood cells of four adult lizards are shown to the right of the axis break, and the bars denote the minimum and maximum values.

also co-expressed in the mature erythrocytes of adult lizards. Thus, although the high level of amino acid sequence divergence between the two lizard-specific β-globin paralogs suggests that they may have evolved some form of physiological division of labor (cf. Weber et al., 2000; Storz et al., 2008; Runck et al., 2009), the isoHb differentiation is not associated with any discrete differences in the developmental timing of expression. The two lizard-specific β-globin paralogs exhibit subtle changes in expression during the course of prenatal development, but the pattern appears to be qualitatively different from the more discrete gene switching that characterizes the ontogenetic regulation of Hb synthesis in other tetrapod vertebrates (Banville and Williams, 1985a; Banville and Williams, 1985b; Hardison, 2001; Nagel and Steinberg, 2001; Fuchs et al., 2006; Nakazawa et al., 2006; Alev et al., 2008; McIntyre et al., 2008; Nagai and Sheng, 2008; Alev et al., 2009).

Conclusions and future directions

Results of the MS/MS analysis revealed that α- and β-like globin genes of the green anole lizard are differentially expressed during the course of embryonic development. However, the same repertoire of α- and β-chain isoHbs was expressed during all stages of embryonic development and postnatal life, and the ontogenetic shifts in isoHb composition were relatively subtle. In birds and mammals, by contrast, the embryonic α- and β-like globin genes are exclusively expressed in primitive erythroid cells derived from the yolk sac, and they are not re-activated in definitive erythroid cells during postnatal life. Contrary to the pattern that has been documented in other tetrapod vertebrates, it appears that the developmental regulation of Hb synthesis in the green anole does not involve discrete, stage-specific switches in gene activation and gene silencing.

Developmental changes in blood–gas transport have been documented in almost all tetrapod species that have been examined in sufficient detail. In some taxa, developmental changes in blood–oxygen affinity are at least partly attributable to the stage-specific expression of functionally distinct isoHbs. This Hb switching has been documented in a number of birds (Borgese and Nagel, 1977; Baumann et al., 1982), crocodilians (Grigg et al., 1993), snakes (Pough, 1977; Birchard et al., 1984; Ragsdale and Ingermann, 1991) and turtles (Wells and Baldwin, 1994). In some taxa, such as the viviparous lizard, *Sphenomorphus quoyii* (Grigg and Harlow, 1981), developmental changes in blood–oxygen affinity are primarily attributable to changes in the red cell concentrations of organic phosphates or other allosteric cofactors that modulate Hb–oxygen affinity. It remains to be seen whether oviparous lizards like *Anolis* conform to this same pattern. In the future, it will be important to characterize functional properties of the various α- and β-chain isoHbs that are differentially expressed during prenatal development and adulthood in the anole lizard. In *A. carolinensis*, products of the α^{A}- and α^{D}-globins are distinguished by 75 amino acid substitutions (53% amino acid sequence divergence), and products of the β^{I}- and β^{II}-globins are distinguished by 36 amino acid substitutions (25% divergence; Fig. 4). Given the high levels of amino acid divergence between paralogous genes that encode the same subunit types, there would seem to be ample scope for functional isoHb differentiation in these lizards. It will also be important to assess whether developmental changes in blood–gas transport are attributable to changes in red cell pH and/or changes in the intracellular concentration of organic phosphates such as ATP and GTP that are known to modulate Hb–oxygen affinity in other non-avian reptiles.

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