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Gene Expression During Estivation in Spadefoot Toads, *Scaphiopus couchii*: Upregulation of Riboflavin Binding Protein in Liver

KENNETH B. STOREY,* M. EILEEN DENT, AND JANET M. STOREY
Institute of Biochemistry and Department of Biology, Carleton University, Ottawa, Ontario, Canada K1S 5B6

ABSTRACT A cDNA library constructed from liver of 2-month estivating female spadefoot toads, *Scaphiopus couchii*, was differentially screened to reveal genes that were induced or upregulated during estivation. After two rounds of screening a clone was isolated that showed 60% higher expression in liver of estivating, versus control, toads. The clone possessed a 1.0 kb insert which annealed to a single 0.7 kb band on Northern blots. Sequencing revealed a 1053 nucleotide full-length cDNA; the largest potential open reading frame was 708 nucleotides which encoded a protein of 235 amino acids. A homology search in Genbank indicated that the protein was a riboflavin binding protein (RfBP), a monomeric phosphoglycoprotein produced by the liver of female birds, reptiles, and mammals that functions to bind plasma riboflavin and load the vitamin into eggs or fetus. To our knowledge, this is the first demonstration that RfBP is also present in amphibians. Toad RfBP showed 50% of residues identical with the chicken or turtle liver proteins and many essential structural features were conserved in the toad protein including 18 cysteine residues, two asparagine glycosylation sites, and 6 tryptophan residues. However, a region with eight phosphoserines in the chicken or turtle proteins that functions in RfBP binding to the oocyte membrane contained only three serine residues in toad RfBP, suggesting that recognition and binding to oocyte receptors must be different in toads. Northern hybridization showed that toad RfBP was largely liver-specific; no mRNA transcripts were detected in brain, gut, heart, or kidney but low message levels occurred in hind leg skeletal muscle of estivating, but not control, toads. Upregulation of RfBP in liver of estivating toads may be linked with maturation of eggs in preparation for the explosive breeding that occurs immediately upon emergence from estivation but might also have a role for the adult in "caching" riboflavin to maintain an endogenous vitamin pool over the 9–10 months of each year that toads are dormant. *J. Exp. Zool. 284:325–333, 1999.*

The Couch spadefoot toad, *Scaphiopus couchii*, inhabits arid regions of the American southwest and spends as much as 9–10 months of each year burrowed underground in a state of dormancy, termed estivation (McClanahan, '67). Long-term survival is ensured by strong metabolic rate depression, oxygen consumption decreasing to only 20–30% of the resting rate when aroused (Seymour, '73), and by adaptations that minimize the loss of body water as the soil dries out (McClanahan, '67). Metabolism while dormant is fueled primarily by the consumption of lipid reserves but as water stress becomes severe toads also increase the catabolism of protein (Seymour, '73; Jones, '80). From this point, they accumulate the nitrogenous end product, urea, in amounts up to 300 mM in plasma and other body fluids and this enhances water retention via colligative action (McClanahan, '67; Jones, '80).

Whereas considerable attention has been given to energy metabolism and water balance during amphibian estivation (Seymour, '73; Jones, '80; Flanigan et al., '90; Pinder et al., '92; Shoemaker, '92; Cowan and Storey, '98; Cowan et al., '98) little is yet known about the role of gene expression in promoting survival. Presumably, specific up- or downregulation of selected genes is needed in order to readjust and optimize numerous metabolic processes to sustain long-term dormancy. Indeed, changes in the levels of various proteins (chiefly enzymes) are known to occur during estivation.

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*Correspondence to: Kenneth B. Storey, Institute of Biochemistry and Department of Biology, Carleton University, 1125 Colonel By Drive, Ottawa, Ontario, Canada K1S 5B6. E-mail: kenneth_storey@carleton.ca
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For example, the activity of liver carbamyl phosphate synthetase, an enzyme of the urea cycle, in *S. couchii* emerging from estivation was double that found in active, summer animals (Jones, '80) whereas the maximal activities of a variety of enzymes involved in intermediary energy metabolism or antioxidant defense changed (either rose or fell) in estivating toads, compared with controls (Cowan et al., '98; Grundy and Storey, '98). However, the full range of protein adjustments, and the underlying changes in gene expression, that are needed to support estivation remains elusive.

The techniques of cDNA library construction and differential screening allow broad scans to be made to detect differences in the mRNA populations of tissues between two or more physiological states. Isolation, sequencing, and identification of clones that are positive for the stress state can then highlight the participation of selected genes, and the proteins and cellular functions that they support, as important in the adaptive process. For example, our first application of this technology for an analysis of freeze-induced gene expression in liver of the freeze tolerant wood frog, *Rana sylvatica,* showed strong upregulation of four proteins (α- and γ-subunits of fibrinogen, the mitochondrial membrane ADP/ATP translocase, and a novel protein of 10 kD), all previously unsuspected as having any part in natural freeze tolerance (Cai and Storey, '97a,b; Cai et al., '97).

In the present study, we applied the same technology to an analysis of changes in gene expression occurring during long-term estivation in spadefoot toads. Differential screening of a cDNA library made from the liver of 2-month estivated female toads revealed the strong upregulation during dormancy of the gene for riboflavin binding protein. This protein, which is produced by the liver, secreted, and taken up by oocytes, is one of several vitamin-binding proteins that function to load water-soluble vitamins into eggs to provide the vitamins needed for embryonic development. Its upregulation during estivation is probably linked with the maturation of oocytes prior to the explosive breeding of this species which occurs within hours after toads emerge from estivation.

METHODS AND MATERIALS

Chemicals and animals

All chemicals were of molecular biology grade and were obtained from Sigma Chemical Co. (St. Louis, MO) or New England Biolabs (Beverly, MA). Spadefoot toads, *S. couchii*, were captured several days after emergence from estivation in July near Tucson, Arizona and were air-freighted to Carleton University. Animals were washed in a tetracycline bath and then placed in plastic basins at 15°C. Half of the toads were sacrificed within 24 hr as controls; toads were killed by pithing and tissues were rapidly dissected out, frozen in liquid nitrogen, and then transferred to –70°C for storage. Examination of the guts of control toads revealed that all animals had eaten very recently and so the remaining toads were not fed prior to beginning experimental estivation. Remaining toads were placed in basins containing damp soil and were held in an incubator at 15°C (±2°C). Toads burrowed into the soil and were allowed to estivate for 2 months. At the end of this time, soil humidity was 10–12%, and toads had lost 29.4% of their total body mass (=35.7% of total body water if all mass lost was water) (Grundy and Storey, '97). Individual toads were gently dug out of the soil so as not to disturb others and were immediately processed as above. Samples of frozen liver from female estivated toads was shipped on dry ice to Clontech (Palo Alto, CA) for the synthesis of a λ-Zap II cDNA library.

cDNA probe synthesis

Total RNA was extracted from liver samples of control and estivating female toads using Trizol™ reagent (Gibco-BRL, Gaithersburg, MD). Poly(A^+) RNA was purified from total RNA using an oligo-dT cellulose column as per manufacturer instructions (Gibco-BRL). Equal amounts of control and estivating mRNA (measured spectrophotometrically) were used for the synthesis of single-stranded cDNA probes which were ^{32}P-labeled with random primers (Sambrook et al., '89). After scintillation counting, equal numbers of counts of labeled control versus estivating probe were added to hybridizations.

Library screening

A 1:125,000 dilution of the λ library phage was incubated with 200 μL of an overnight culture of XL1-Blue MRF' host cells (OD_{600} = 1.0) (Stratagene, La Jolla, CA) and was then plated onto several 23 cm^2 petri plates containing LB agar supplemented with 0.2% maltose and 10 mM $MgSO_4$. The plates were grown overnight at 37°C. Two replicate lifts were made from of each plate using 0.4 mm pore size HYBond-N membrane (Amersham, Arlington Heights, IL). The membranes were denatured in a solution of 1.5 M

NaCl and 0.5 M NaOH for 7 min and were then neutralized twice for 3 min in a solution of 1.5 M NaCl, 0.5 M Tris-Cl (pH 7.2) and 10 mM EDTA (pH 8.0). The membranes were rinsed in 0.25 M $NaPO_3$ (pH 7.2) and then fixed at a wavelength of 254 nm using a UVP ultraviolet cross linker (VWR Canlab, Montreal, PQ, Canada). Lifts to be probed with the control cDNA probe (primary lifts) were separated from those to be probed with estivated cDNA probe (replicate lifts). Each set of lifts was rolled into a 30 × 5 cm hybridization tube with layers of nylon mesh to separate the membranes.

Lifts were prehybridized in a Labline hybridization oven (VWR Canlab) at 42°C with a solution containing 50% v/v formamide, 0.25 M $NaPO_3$ (pH 7.2), 0.25 M NaCl, 1 mM EDTA (pH 8.0), 7% sodium dodecyl sulfate (SDS) and 8% polyethylene glycol (mw 7000–9000) for at least 30 min. The solution was drained and fresh hybridization solution containing radiolabeled probe (4.0×10^6 cpm/ml) was added. Each set of lifts was hybridized with a cDNA probe derived from either control or estivated toad liver mRNA at 42°C for 16–20 hr. Blots were washed twice for 15 min at 21°C in 2× SSC buffer (0.3 M NaCl, 0.03 M Na_3 citrate, pH 7) plus 2% SDS followed by a 15 min wash in 1× SSC plus 1% SDS at 21°C and finally a wash in 0.5× SSC plus 1% SDS at 55°C for 5–20 min until counts were reduced 400–1500 cpm.

After a 3-day exposure to Kodak-AR film (Picker, Highland Heights, OH), the autoradiograms from control and estivated lifts were compared. Plaques generating greater signals with the estivation probe were cored and incubated overnight at 4°C in 500 μL of SM buffer (0.1 M NaCl, 10 mM $MgSO_4 \cdot 7H_2O$, 35 mM Tris-HCl pH 7.5, 2% w/v gelatin) with 5% v/v chloroform added. Phage stocks were re-screened using the procedure described above to confirm the differential expression of the clone and to obtain a pure phage stock.

In vivo excision and plasmid preparation of positive clones

The Ex-Assist helper phage system (Stratagene, La Jolla, CA) was used to isolate all potential differentially expressed inserts within pBluescript phagemids. The cDNA inserts were recovered from the plasmid by EcoR1/Xho1 digestion and electrophoresis in a 0.9% TAE/agarose gel.

Northern blotting

Potential positive inserts were purified from the agarose gel using Geneclean III (Bio 101, La Jolla, CA). Random primer labeling was used to generate [α-^{32}]P-dCTP-labeled probes for Northern blotting (Sambrook et al., '89). Total RNA was extracted from samples of brain, gut, heart, lung, liver, kidney, and muscle from both control and estivating female toads using Trizol™ reagent. Aliquots of each RNA extract (10 μg) and 5 μg of RNA molecular weight ladder (Gibco-BRL, Gaithersburg, MD) were electrophoresed on a 1% agarose formamide-formaldehyde RNA gel and blotted overnight onto 0.2 μm Nytran Plus membranes (Schleicher & Schuell, Keene, NH). After transfer, RNA was fixed onto membranes by heating at 80°C for 2 hr under vacuum. Membranes were pre-hybridized, and then hybridized using probes generated from radiolabeled inserts of the potentially differentially expressed clones, and washed according to the protocol described above for library screening. After X-ray autoradiography, the films were scanned with a Scan Jet3C scanner using DeskScan II v2.2 software and then the surface area of bands was quantified using Imagequant v3.22 software (Innovative Optical Systems Research). RNA transcript sizes were estimated from a graph of RNA molecular weight standards versus migration distance in the gel.

Sequence analysis

Clones that showed a differential signal on the Northern blots were amplified using the alkaline lysis maxiprep procedure followed by PEG purification (Sambrook et al., '89). Insert DNA was dideoxy sequenced using Sequenase 2.0 (US Biochemicals, Cleveland, OH). Plasmid preparations were also sent for automated sequencing at the Core Facility for Protein/DNA Chemistry in the Biochemistry Department at Queen's University (Kingston, ON, Canada). Sequence homologies were obtained from Genbank using the Blast program and additional analyses were performed using Lasergene sequence analysis software (DNASTAR, Madison, WI).

RESULTS

Primary screening of the cDNA library made from liver of estivating female toads yielded ~110 putative positive clones which were reduced to ~20 after secondary screening. Several of these were confirmed as differentially expressed by Northern blotting but cross-hybridization revealed that they all shared the same sequence. The unique clone selected for further characterization possessed a 1.0 kb insert which annealed to a single 0.7 kb band on a Northern blot of total RNA

isolated from liver of control and estivated female toads (Fig. 1A). The level of expression of the mRNA transcript was 60 ± 4% higher (n = 3 different runs) in liver from estivated, compared with control, toads.

Sequencing of the cDNA clone showed that the full-length cDNA transcript was 1053 nucleotides. The nucleotide sequence is shown in Fig. 2 along with the deduced amino acid sequence of the longest (708 nucleotides) open reading frame which begins at nucleotide residue 58. This potentially encoded a protein of 235 amino acids, which would have a predicted molecular weight of 26.6 kD. A homology search in Genbank indicated that the protein sequence was highly similar to that of riboflavin binding protein (RfBP). Fig. 3 shows the sequence alignment comparison of the putative amino acid sequence of the toad protein with RfBP from chicken *Gallus gallus* oviduct (Zheng et al., '88) and turtle *Pelodiscus sinensis* liver (Hamajima and Ono, '95). Toad liver RfBP shared 50.4% identity with the chicken protein and 49.6% homology with the turtle protein, whereas chicken and turtle proteins shared 70.2% sequence homology. The areas of greatest variation between the three proteins were within the 17–18 residue signal sequence (which is cleaved from the mature protein) and in the final 25 residues near the C-terminus. In addition, a highly conserved region containing 8 phosphoserine residues, that is present in the turtle and chicken proteins and responsible for protein recognition and binding to receptors on the oocyte membrane (Soor-yanarayana et al., '98), was much reduced in the toad protein with only three potential serine phosphorylation sites present.

Northern blots were run using total RNA isolated from other tissues of estivating and control toads (brain, gut, heart, kidney, leg skeletal muscle) and probed with the liver clone (termed *tRfBP*) to ascertain whether this gene was expressed in other organs besides liver (Fig. 1B). Transcripts of *tRfBP* were detected only in hind leg skeletal muscle from estivating toads; other tissues, as well as control muscle, showed no *tRfBP* transcripts. Transcript levels in muscle of estivated animals were estimated to be only about 10% of the corresponding amount in liver of the same animals, for equal amounts of RNA loaded on the gels.

DISCUSSION

Differential screening of the cDNA library prepared from liver of estivating spadefoot toads with probes from control versus 2-month estivated toads resulted, after two rounds of screening, in the isolation of one clone that was expressed much more strongly in liver of estivating females than in controls. The isolation of only a single clone was somewhat surprising as we had expected that a number changes in gene expression would be found in estivating animals in support of the biochemical adjustments needed for estivation. However, a lack of other upregulated genes during estivation might be explained in one of two ways. First, the overall lower metabolic rate in the esti-

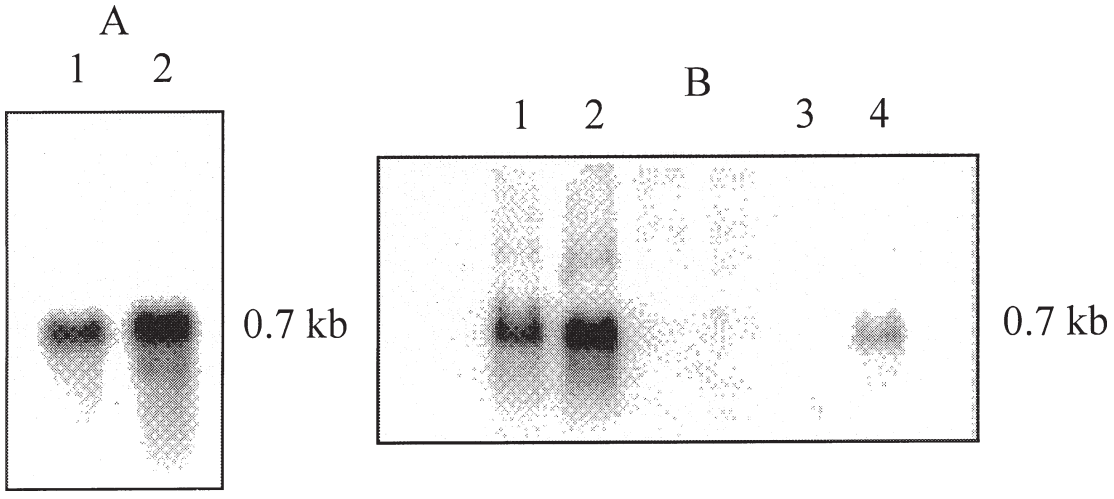

Fig. 1. Northern blots of total RNA (10 μg per lane) from tissues of control and estivated toads probed with *tRfBP*. (**A**) Total RNA from liver of control (lane 1) and estivated (lane 2) toads. (**B**) Total RNA from liver of control (lane 1) and estivated (lane 2) toads and skeletal muscle of control (lane 3) and estivated (lane 4) toads. Representative blots from n = 3 trials are shown.

GCG	GCC	GCG	TCG	ACC	ATC	ACA	TTC	CTT	TCC	AGC	AAA	GTG	ATA	42
					Met	**Lys**	**Leu**	**Ala**	**Leu**	**Val**	**Ile**	**Leu**	**Val**	**9**
GGA	GTT	GAA	GCC	AAT	ATG	AAG	CTT	GCA	TTG	GTG	ATC	CTA	GTA	84
Ala	**Gly**	**Tyr**	**Phe**	**Cys**	**Ala**	**Val**	**Ser**	**Cys**	**His**	**His**	**Ser**	**Cys**	**Leu**	**23**
GCT	GGC	TAC	TTT	TGT	GCT	GTG	TCC	TGC	CAC	CAT	AGC	TGT	CTG	126
Gln	**Gly**	**Pro**	**Asn**	**His**	**Lys**	**Ala**	**Ser**	**Pro**	**Gly**	**Pro**	**Glu**	**Ile**	**Gly**	**37**
CAA	GGG	CCA	AAT	CAC	AAA	GCG	TTC	CCA	GGC	CCG	GAG	ATC	GGC	168
Phe	**Gln**	**Glu**	**Cys**	**Phe**	**Leu**	**Tyr**	**Ala**	**Glu**	**Asp**	**Ser**	**Cys**	**Cys**	**Tyr**	**51**
TTT	CAG	GAA	TGC	TTC	TTG	TAT	GCT	GAA	GAT	TCT	TGT	TGT	TAC	210
Ala	**Asn**	**Phe**	**Thr**	**Glu**	**Lys**	**Leu**	**Ala**	**Gln**	**Ser**	**Pro**	**Val**	**Ile**	**Glu**	**65**
GCT	AAC	TTT	ACC	GAG	AAG	CTG	GCT	CAG	TCC	CCT	GTT	ATA	GAG	252
Val	**Asp**	**Asn**	**Tyr**	**Tyr**	**Trp**	**Asn**	**Arg**	**Cys**	**Gly**	**Asn**	**Leu**	**Ser**	**Lys**	**79**
GTG	GAT	AAC	TAT	TAC	TGG	AAC	AGA	TGT	GGG	AAC	CTC	AGC	AAA	294
Ser	**Cys**	**Glu**	**Asp**	**Tyr**	***Met***	**Lys**	**Lys**	**Leu**	**Glu**	**Cys**	**Phe**	**Tyr**	**Gln**	**93**
AGT	TGT	GAA	GAT	TAT	ATG	AAG	AAG	TTA	GAA	TGC	TTT	TAC	CAG	336
Cys	**Ser**	**Pro**	***Met***	**Thr**	**Ala**	**His**	**Trp**	**Val**	**His**	**Pro**	**Asn**	**Val**	**Ser**	**107**
TGT	TCC	CCA	ATG	ACA	GCT	CAC	TGG	GTT	CAC	CCC	AAT	GTA	TCA	378
Asp	**Ala**	**Val**	**Gln**	**His**	**Ile**	**Pro**	**Leu**	**Cys**	**His**	**Ser**	**Phe**	**Cys**	**Asp**	**121**
GAT	GCA	GTA	CAG	CAT	ATT	CCC	CTC	TGC	CAT	TCC	TTC	TGT	GAC	420
Ser	**Trp**	**Phe**	**Glu**	**Ala**	**Cys**	**Lys**	**Ser**	**Asp**	**Leu**	**Val**	**Cys**	**Ala**	**Arg**	**135**
AGC	TGG	TTT	GAA	GCC	TGT	AAG	TCA	GAT	TTG	GTC	TGT	GCT	CGG	462
Asn	**Trp**	**Ile**	**Ser**	**Asp**	**Trp**	**Ile**	**Ile**	**Asp**	**Glu**	**Asn**	**Gly**	**Asn**	**His**	**149**
AAC	TGG	ATA	TCT	GAC	TGG	ATT	ATT	GAT	GAA	AAT	GGA	AAC	CAC	504
Cys	**Lys**	**Asn**	**Asp**	**Cys**	**Ile**	**Pro**	**Phe**	**His**	**Glu**	***Met***	**Tyr**	**Ala**	**Asn**	**163**
TGT	AAA	AAT	GAC	TGC	ATC	CCA	TTC	CAT	GAG	ATG	TAT	GCG	AAT	546
Gly	**Thr**	**Asp**	**Leu**	**Cys**	**Gln**	**Ser**	**Ala**	**Trp**	**Gly**	**Glu**	**Ser**	**Phe**	**Val**	**177**
GGC	ACT	GAC	CTA	TGC	CAG	AGT	GCA	TGG	GGT	GAA	TCC	TTC	GTA	588
Val	**Ser**	**Ser**	**Ser**	**Pro**	**Cys**	**Arg**	**Cys**	**Leu**	**Asp**	***Met***	**Thr**	**Glu**	**Thr**	**191**
GTC	TCC	TCT	TCA	CCA	TGC	CGC	TGC	CTG	GAC	ATG	ACT	GAG	ACA	630
Asp	**Lys**	**Lys**	**Val**	**Ile**	**Lys**	**Tyr**	**Ile**	**Leu**	**Asp**	**Asp**	**Asp**	**His**	**Ser**	**205**
GAC	AAA	AAA	GTG	ATC	AAG	TAC	ATT	TTG	GAT	GAC	GAC	CAC	TCA	672
Glu	**Glu**	**Ser**	**Ser**	**Glu**	**Lys**	**Lys**	**Asp**	**Cys**	**Lys**	**Pro**	**Gly**	**Leu**	**Gln**	**219**
GAG	GAG	AGC	AGT	GAA	AAG	AAG	GAC	TGC	AAA	CCC	GGA	CTG	CAG	714
Lys	**Pro**	**Lys**	**Asp**	**Lys**	**Glu**	**Gly**	**Glu**	**Gly**	**Glu**	**Glu**	**Gly**	**Glu**	**Glu**	**233**
AAA	CCT	AAA	GAT	AAA	GAA	GGA	GAA	GGA	GAA	GAA	GGA	GAA	GAA	756
Gly	**Arg**	**.**												**235**
GGA	AGA	TGA	AGG	GGA	TGA	CTG	AGT	GTG	AGT	GTG	GGC	ATC	CAA	798
TGG	GAA	GGA	GCG	TTG	GTG	GAG	ATA	AAC	TGA	CAG	TGA	TAA	AGT	840
CTT	GTT	GGG	GAA	TAC	TCC	GAA	GAG	TTT	ACA	TAA	TAT	TGG	TGA	882
AAC	ATC	CAG	AAG	GTT	CTC	CTA	CAT	CCC	ATT	CAA	GTT	TTA	TCA	924
GTA	CAT	CAA	ACC	ATG	GAA	CAG	TCA	GAA	GAT	CCA	GGA	ATT	CTG	966
GGC	CTC	AGG	GTA	CCG	TCT	TTT	CTG	ATT	TAC	TAA	TAA	CAC	AAT	1008
AAA	AAC	AGT	TGA	AAT	GAA	AAA	AAA	AAA	AAA	AAA	AAA	AAA	AAA	1050
AAA														1053

Fig. 2. Nucleotide sequence of the *tRfBP* cDNA clone and the deduced amino acid sequence (shown above the corresponding nucleotides) of the longest open reading frame which starts at nucleotide residue 58. Nucleotide and amino acid (in bold) residues are numbered on the right; methionine residues are italicized and the polyadenylation signal is underlined.

vating state could mean that most genes are downregulated compared with their status in control animals and, indeed, our visual analysis of the results of primary screening showed hundreds of plaques with equal or lesser intensity on the estivating, versus control, lifts. If many genes are downregulated during estivation, a functional "upregulation" of selected others could be achieved by simply not changing their level of expression in the estivating, compared with control, states. Secondly, upregulation of the expression of those genes that facilitate the establishment of dormancy or the initiation of estivation-specific functions (e.g., urea synthesis) would likely have been completed long before two months had passed such that toads were in a steady-state dormancy by this time. For example, blood urea levels in these animals had already risen to 207 ± 4.8 μmol/ml (from 14.5 ± 5.2 μmol/ml in controls) (Grundy and Storey, '98), suggesting that estivation-induced elevation of the activities of urea-synthesizing enzymes must have already occurred in the liver. In addition, it must be remembered that cDNA library screening detects only changes in mRNA levels which we assume are generally accurate reflections of corresponding changes in both preceding (gene transcription) and subsequent (protein levels) events but other regulatory factors could also be in play that could produce estivation-induced changes in selected proteins without detectable changes in mRNA levels.

Hence, the elevated levels of RfBP message (60% higher than in controls) found in liver of 2-month estivated toads suggests that very few metabolic processes are actively upregulated during long-term dormancy and that those that are, such as RfBP, may have functions that prepare the organism for arousal. We presume that el-

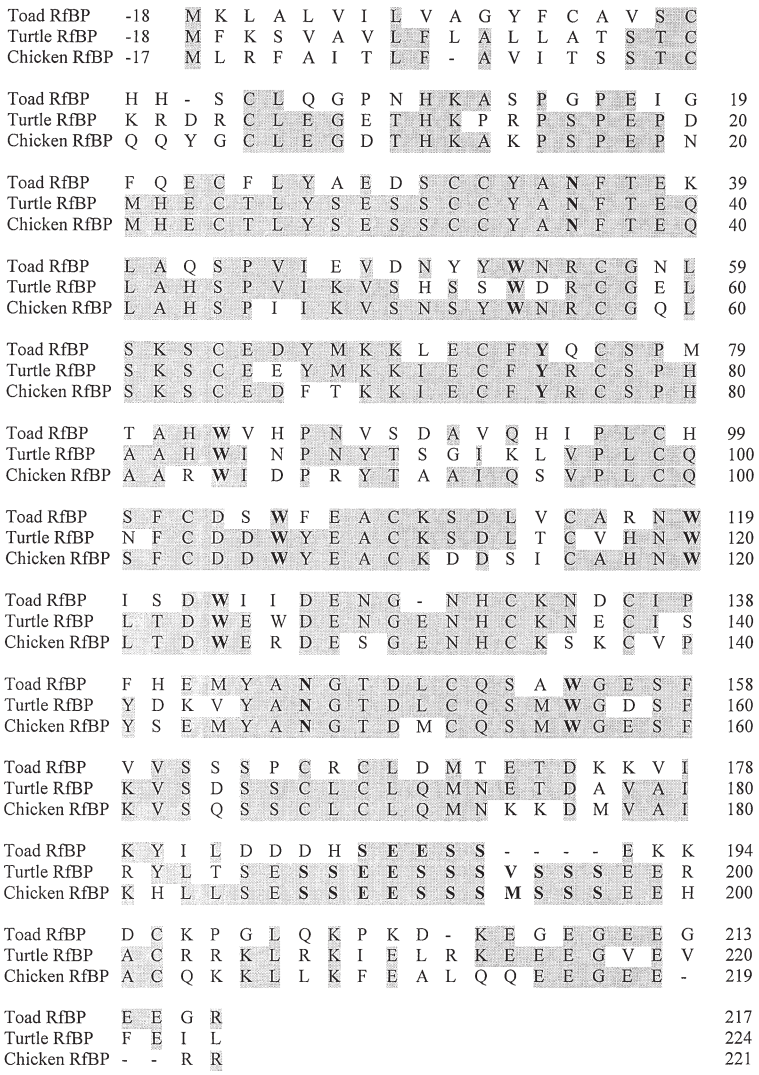

Fig. 3. Comparison of the deduced amino acid sequences of RfBP from spadefoot toad liver (*Scaphiopus couchii*; Genbank accession no. AF102545), Chinese softshell turtle liver (*Pelodiscus sinensis*; Genbank accession no. D49954; Hamajima and Ono, '95), and domestic chicken oviduct (*Gallus gallus*; Genbank accession no. J03922; Zheng et al., '88). Identical residues are boxed and conserved asparagine residues (glycosylation sites), tryptophan residues (involved in ligand binding), and phosphoserine cluster (recognition site for uptake by oocyte) are shown in bold. Amino acids in the mature protein are numbered on the right hand side and are preceded by the 17–18 residue signal sequence that is cleaved from the mature protein. A four-residue insertion after the phosphoserine cluster was needed to align the C-terminal section of the toad protein with that of the other two species.

evated levels of RfBP message in liver are consistent with increased RfBP synthesis and secretion into plasma where it can bind riboflavin and load it into the maturing eggs of the female toads. *Scaphiopus* species are explosive breeders; the first heavy rains of the summer bring thousands of toads to the surface and most breeding activity is over within the first 24 hr after emergence. Hence, eggs must be fully matured by the end of the estivating period. Another possible function of increased RfBP levels (and maybe also other vitamin-binding proteins) might be to allow the toads to cache vitamins that might be released as part of a wasting of skeletal muscle mass during extended dormancy and so prevent their excretion. Jones ('80) found that *S. couchii* consumes 17% of its body protein reserves during estivation and that this accounts for 24% of the total energy budget. Much of this apparently comes from skeletal muscles since only about 50% of the total fuel reserves used up in estivation came from stores in liver and fat body. Hence, considerable

catabolism of the skeletal muscle mass appears to occur during dormancy. In this regard, the results of Northern blotting that showed the presence of RfBP mRNA in leg muscle of estivated, but not control, toads are instructive.

The identification of riboflavin binding protein (RfBP) as being upregulated in liver of estivating spadefoot toads is highly interesting. To our knowledge, the protein has never before been reported as present in an amphibian species. The only previous attempt to find RfBP in an amphibian was negative when Northern blots (probing with chicken liver RfBP cDNA) were used to search for RfBP mRNA in liver and oviduct of *Xenopus laevis* (Hamajima and Ono, '95). The presence of mRNA transcripts for RfBP in toad liver but not in brain, gut, heart, or kidney is consistent with the distribution of the protein elsewhere, being liver-specific in reptiles and mammals but produced by both liver and oviduct in birds. As noted above, however, the low levels of RfBP transcripts in leg skeletal muscle of estivating animals is intriguing and has not been reported in other species.

The deposition of many water-soluble vitamins into the eggs of birds and reptiles or into the developing mammalian fetus is facilitated by vitamin-binding proteins. RfBP was first identified in avian eggs (in both yolk and white) (Rhodes et al., '59; Ostrowski et al., '62) and also occurs in liver and plasma of laying birds (White and Merrill, '88). The yolk protein is synthesized by liver, whereas RfBP in the white is produced by the oviduct. The protein is also produced by the liver of reptiles and occurs in the yolk of reptilian eggs (but not the white) (Abrams et al., '88, '89; White and Merrill, '88; Hamajima and Ono, '95) and is pregnancy-induced in mammals (Merrill et al., '79). Properties of chicken RfBP have been deduced and summarized by a number of authors (Hamazume et al., '84; Zheng et al., '88; Rohrer and White, '92; Monaco, '97). RfBP from both avian and reptile sources is a monomeric phosphoglycoprotein of about 30 kD (Hamazume et al., '84; Abrams et al., '88). The yolk protein is synthesized by liver (under estrogen control), secreted into plasma where it binds riboflavin (1:1 vitamin:protein) with high affinity (dissociation constant ~1.3 nM) and is then deposited into the yolk. Once inside, 11–13 amino acids are cleaved from the C-terminus, presumably ensuring that the protein, and its bound riboflavin, remain in the yolk where riboflavin concentrations may rise as much as ninefold higher than plasma levels (Norioka et al., '85). Avian egg white RfBP is synthesized by the oviduct (coded by the same gene as in liver) but has different carbohydrate moieties attached to it and does not undergo C-terminal cleavage as in yolk. Some authors have proposed that its role in egg white is antibacterial, scavenging and binding riboflavin so that this nutrient is unavailable for bacterial growth; the observation that egg white RfBP is only about 35% saturated with riboflavin, in contrast with the fully saturated yolk RfBP, supports this (Tranter and Board, '82). A similar function might be important in amphibian eggs that are laid without a shell in water and a further analysis of the distribution of RfBP mRNA levels in oviduct versus liver and RfBP in yolk versus white of amphibian eggs would be instructive.

The structure of toad liver RfBP is highly similar to that of the chicken (oviduct RfBP amino acid sequence is shown in Fig. 3 but liver RfBP is the same; MacLachlan et al., '93) and turtle proteins (Fig. 3), the predicted amino acid sequence of toad RfBP showing 50% of residues identical with those of the chicken or turtle proteins (toad and chicken RfBPs share 70% identity). In all three cases, the open reading frame of the cDNA coded for a protein of 235–242 amino acids and included a 17–18 amino acid signal sequence, starting with methionine and ending with cysteine, that is cleaved from the mature protein. The chicken cDNA also coded for two arginine residues at the C terminus that are not present in the mature protein of 219 amino acids. Whether such C terminal processing also occurs with toad or turtle RfBP is not known and, in addition, both toad and turtle RfBPs contained an additional three amino acid residues just before the C terminus so that the mature protein in these species would be 215–217 residues in toad or 222–224 residues in turtle. Amino acid residues that are known to be essential to protein function are all conserved in toad RfBP. Eighteen cysteine residues that form nine disulfide bridges (Monaco, '97) are conserved in all three proteins as well as two asparagine glycosylation sites (Asn35 and 145 in toad, Asn36 and 147 in chicken and turtle) and 6 tryptophan residues that are thought to be involved in ligand binding (Blankenhorn, '78) (Fig. 3). The ligand binding domains runs from the N-terminus to about Cys169 in chicken RfBP (Monaco, '97) (Cys167 in toad) and is followed by a highly phosphorylated region that extends from amino acids 186 to 197 in the chicken and turtle

protein and contains eight phosphoserines interspersed with glutamate residues. It is in this region that the only major structural difference between the toad protein and the avian and reptile proteins was seen. The toad protein exhibited only three serine residues in this region, spaced by two glutamate residues. RfBP transport across the yolk membrane in avian eggs is facilitated by a specific carrier which recognizes the region of eight phosphoserines; studies have shown that dephosphorylation of these serine residues or competition from synthetic phosphopeptides of the same sequence strongly inhibits riboflavin uptake into the egg (Sooryanarayana et al., '98). Hence, with the much smaller sequence of serine residues present in the toad protein, it is probable that the recognition and binding of RfBP to toad oocytes is quite different in amphibian systems.

Overall, then, the current study presents the first use of cDNA library screening technology to investigate the changes in gene and protein expression that facilitate amphibian estivation. Our findings show the enhanced expression of the gene for riboflavin binding protein in female spadefoot toads after two months of dormancy. The data provide the first confirmation of the presence of RfBP in an amphibian species and suggest a probable role for the protein in vitamin loading into the eggs so that these are fully mature in anticipation of the period of breeding and egg-laying that must take place immediately after the toads are aroused from dormancy.

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