Leptin (*ob* gene) of the South African clawed frog *Xenopus laevis*

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Leptin, the protein product of the *obese* (*ob*) gene, is a type-I cytokine hormone secreted by fat that is integral to food intake regulation and influences almost every physiological system in juvenile and adult mammals. Since the identification of leptin in the mouse in 1994, biologists have searched for orthologous genes in other species with limited success. In this article, we report the identification and functional characterization of leptin and leptin receptor (LR) in *Xenopus*. Despite low amino acid sequence similarity to mammalian leptins (≈35%) the frog protein has a nearly identical predicted tertiary structure and can activate the frog and mouse LRs *in vitro*. We showed that recombinant frog leptin (rxLeptin) is a potent anorexigen in frogs, as it is in mammals, but this response does not develop until midprometamorphosis. However, during early prometamorphosis, exogenous rxLeptin induced growth and development of the hind limb, where LR mRNA is expressed. The rxLeptin also stimulated cell proliferation in cultured hind limbs from early prometamorphic tadpoles, as measured by [^{3}H]thymidine uptake. These findings are evidence that leptin can influence limb growth and differentiation during early development. Furthermore, the isolation and characterization of leptin and its receptor in a nonamniote provides an essential foundation for elucidating the structural and functional evolution of this important hormone.

evolution | metamorphosis | obesity

The protein hormone leptin is a type-I cytokine secreted by adipocytes that acts on the CNS to regulate food intake and metabolism (1, 2). In addition to the regulation of body weight, leptin influences reproduction, growth, stress responses, and thyroid function (3, 4). Actions of leptin are both acute and chronic. For example, postprandial increases in plasma leptin inhibit food intake, whereas daily mean plasma leptin concentrations communicate long-term energy status to the brain (5). These actions are mediated by membrane leptin receptors (LRs), of which six isoforms have been identified in mammals (6). Leptin binding to the long form of the LR (LRb) activates the Janus kinase 2/signal transducer and activator of transcription 3 (STAT-3) signaling pathway (7), which mediates leptin effects on food intake, glucose metabolism, and weight gain but does not affect fertility (8).

The rising prevalence of human obesity and metabolic disorders (9) has focused research on the physiological role of leptin in energy balance and food intake in adult mammals. Recently, links between birth weight and adult-onset metabolic disorders (10) has turned attention to possible relationships among leptin, growth, and development during fetal stages. Circulating leptin is elevated in the human fetus during late gestation and correlates with fat mass and birth weight (11, 12). In the fetal mouse, leptin and LR are expressed in liver, heart, hair follicles, and primordial bone before the formation of adipose tissue (13, 14), and leptin is found in the circulation of fetal sheep (15). Although these expression patterns suggest a role for leptin in the fetus, little is known about leptin functions in early development.

In this article, we report on the molecular cloning of frog homologs of the mammalian *ob* and *ob* receptor (*obr*) genes and the functional characterization of the protein products of these genes in a nonamniote vertebrate. Frog leptin and LR (corresponding to mammalian LRb) are expressed throughout embryogenesis and tadpole development and are widely expressed in the juvenile frog. Intracerebroventricular injection of recombinant frog leptin (rxLeptin) exerted potent anorexigenic effects in the midprometamorphic tadpole and juvenile frog but not in the early prometamorphic tadpole. The LR is expressed in the hind limb of early prometamorphic tadpoles, and rxLeptin treatment induced hindlimb growth and digit formation *in vivo* and stimulated [^{3}H]thymidine uptake *in vitro*. Thus, in addition to its integral role as a regulator of appetite and energy balance, leptin may also serve novel growth factor functions during early development.

Results

Molecular Cloning of Frog *ob* and *obr* Genes. We isolated and sequenced the coding region of a *Xenopus laevis ob* gene, along with the full 5′ UTR and ≈600 bp of the 3′ UTR. The predicted 16.9-kDa protein product of the frog *ob* gene has a 21-aa signal peptide and a 148-aa mature peptide. The frog leptin gene has three exons and two introns, with the coding sequence in exons 2 and 3, similar to the genomic structure of mammalian *ob* genes (Fig. 1*A*). Frog leptin is 35% similar to human, 34% similar to chicken, but only 13% similar to a putative pufferfish leptin (ref. 16; Fig. 1*B*). The predicted tertiary structures of frog, rat, and pufferfish leptins derived from the SWISS–MODEL algorithm (17) are highly conserved despite considerable divergence in the primary structures among species (Fig. 1*C*).

We also isolated a full-length cDNA corresponding to a putative *obr* gene from *Xenopus tropicalis* lung. The predicted frog *obr* gene (Fig. 2*A*) spans 87.3 kb of genomic DNA and consists of 26 exons, 8 exons of 5′ UTR (826 bp) and 18 exons of coding sequence (3,436 bp; 1,145 aa) and partial 3′ UTR (129 bp). The predicted frog LR protein is 37.5% identical to the human LR; sequence similarity is greatest in and around the ligand binding domain, transmembrane region, and the intracellular C-terminal region (see supporting information, which is published on the PNAS web site). This sequence includes the terminal 922-bp exon that corresponds to the extended mammalian terminal exon, which codes for the C-terminal intracellular domain of the LR long form. In mammals, two tyrosine residues (Tyr-985 and Tyr-1138 in mouse) within this exon are essential for intracellular signaling (7), and these residues are conserved in the frog *obr* (see supporting information). The ProDom algorithm (18) confirmed that this cDNA sequence is a frog homolog of mammalian *obr* genes (at $P < 0.0001$), and

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Abbreviations: LRs, leptin receptors; STAT-3, signal transducer and activator of transcription 3; LRb, the long form of the LR; rxLeptin, recombinant frog leptin; RTqPCR, quantitative RT-PCR; NF, Nieuwkoop–Faber.

Data deposition: The sequences reported in this paper have been deposited in the GenBank database [accession nos. AY884210 (*X. laevis* obese mRNA), DQ149644 (*X. laevis* leptin receptor partial cDNA), and DQ401069 (*X. tropicalis* leptin receptor mRNA)].

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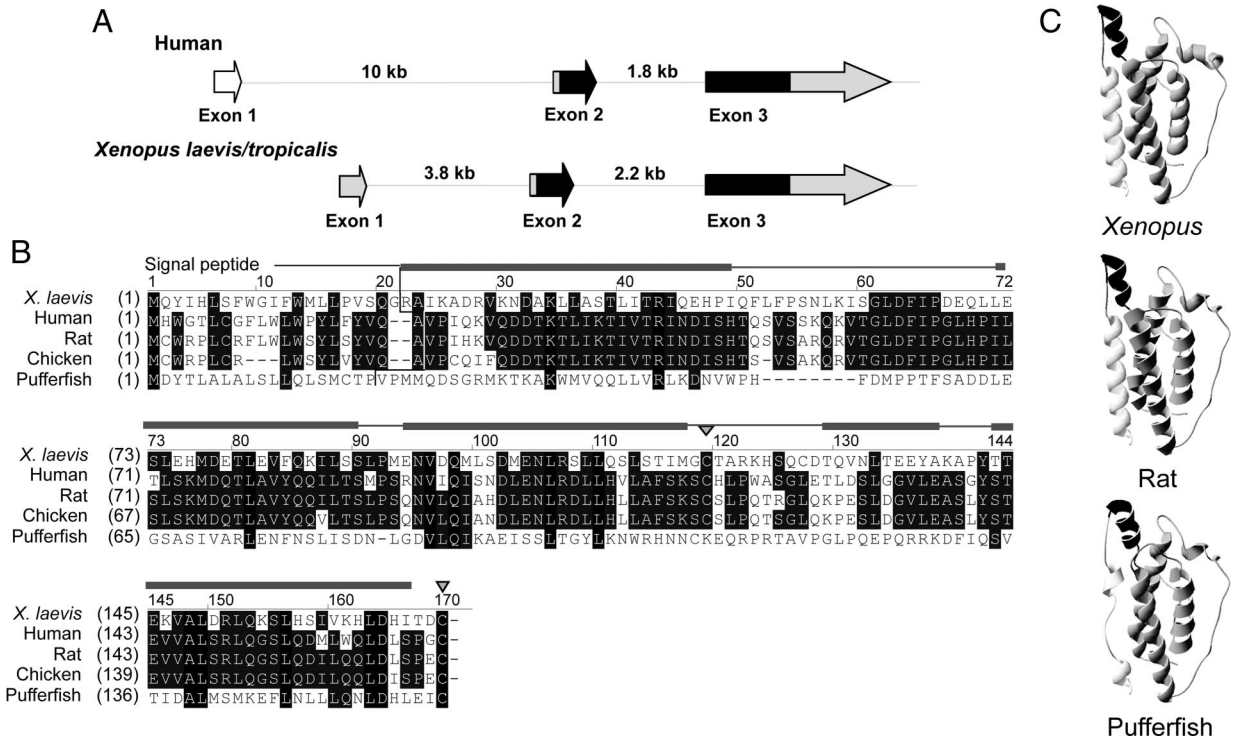

Fig. 1. Molecular characterization of frog leptin. (*A*) The predicted genomic structure of the frog *ob* gene is conserved with human. Dark shading shows coding regions. (*B*) Amino acid alignment of vertebrate leptins. Arrowheads show conserved cysteines. GenBank accession nos. are as follows: *X. laevis*, AY884210; human, NP000221; rat, BAA08296; chicken, AF012727; pufferfish (*Tetraodon*), AB193549. (*C*) Ribbon diagrams of predicted tertiary structures of *X. laevis*, rat, and pufferfish leptins (SWISS–MODEL automated protein homology-modeling server; based on human leptin Protein Data Bank structure file 1AX8.pdb).

neighbor-joining analysis (19) comparing amino acid sequences of LR and similar cytokine receptor proteins (prolactin, growth hormone, IL-6, IL-12, and chemokine) grouped the frog LR within the vertebrate LR clade (Fig. 2*B*). SIGNALP 3.0 (20) predicted a signal peptide in the first 21 bp of the coding sequence, which is equal in size to the signal peptide of the human LR.

Cell Transfection. We found that rxLeptin activated the mouse LRb with a potency similar to recombinant human leptin (ANOVA, $F = 25.71$, $P < 0.0001$; $EC_{50} = \approx 5$ nM; Fig. 2*C Left*). Similarly, we found that both rxLeptin and recombinant human leptin activated the frog LR expressed in COS-7 cells with similar potencies in the low nanomolar range (ANOVA, $F = 6.742$, $P = 0.014$; $EC_{50} = \approx 5$ nM; Fig. 2*C Right*). There were no statistically significant differences in the responses between replicate experiments.

Tissue Distribution of Frog Leptin and LR mRNAs. Using quantitative RT-PCR (RTqPCR), we found leptin mRNA in diverse frog tissues, including fat and liver, where leptin is primarily expressed in mammals and chicken (1, 21), but also in brain, pituitary gland, heart, gastrointestinal tract, lungs, kidney, and gonads (Fig. 3*A*); we obtained similar results with semiquantitative RT-PCR (data not shown). Expression levels varied among tissues, with the highest in the brain and heart. We detected leptin mRNA in unfertilized oocytes and whole embryos starting at Nieuwkoop–Faber (NF) stage 23 (Fig. 3B).

We also analyzed the tissue distribution of LR mRNA by RTqPCR. In mammals, alternative splicing results in at least six LR isoforms that differ in their C termini (6). Only one of these isoforms, the LRb, possesses the C-terminal region known to be necessary for intracellular signaling. We therefore used two primer sets, one to target the extracellular ligand binding region (expected to be present in all forms of the frog LR, exons 14/15) and the other to target the intracellular C-terminal region known to be necessary for signaling in mammals (exons 25/26). LR mRNA is widely distributed in the frog and is expressed in tissues where leptin is not (e.g., skin, pancreas, spleen, and skeletal muscle). Transcripts containing exons 25/26 were expressed in all tissues analyzed (except blood) and variation in their relative levels among tissues paralleled that of transcripts containing exons 14/15 (only data for RTqPCR analysis of exons 25/26 is shown in Fig. 3*A*; similar results were obtained with semiquantitative RT-PCR; data not shown). We found the highest LR mRNA expression in the pituitary followed by the brain. High levels of expression were also observed in skin, muscle, and testis; lower but detectable levels of expression were found in different visceral organs and the ovary (note the different scales of the graphs in Fig. 3*A Left* and *Right*).

Food Intake Assays. Intracerebroventricular injection of rxLeptin was potently anorexigenic in juvenile frogs, with a dose of 0.02 ng/g of body weight causing maximal inhibition of food intake ($n = 4$; ANOVA, $F = 11.44$, $P = 0.0002$; Fig. 4 *Left*). rxLeptin (i.c.v.) had no effect on foraging time in early prometamorphic tadpoles ($n =$ 5–6 tadpoles per treatment) but produced a dose-dependent inhibition of feeding in midprometamorphic (Gosner stage 36–37) tadpoles ($n = 5$–6 tadpoles per treatment; ANOVA, $F = 31.58$, $P < 0.0001$; Fig. 4). Stage-dependent effects of i.c.v. rxLeptin injections on appetite are further supported by our finding that daily i.p. injections of rxLeptin for 1 week did not affect body weight (relative to saline-injected controls) in fed or fasted early prometamorphic tadpoles (data not shown). However, rxLeptin injections caused significant body weight reductions in midprometamorphic tadpoles [fed: saline 3.73 ± 0.48 g, rxLeptin 3.24 ± 0.35 g, $P = 0.054$ (*t* test); fasted: saline 2.90 ± 0.35 g, rxLeptin 2.41 ± 0.48 g, $P = 0.043$; $n =$ 7–9 per group].

Effects of rxLeptin on Hind-Limb Development *in Vivo* and [^{3}H]Thymidine Uptake *in Vitro*. Although injections of rxLeptin did not affect feeding or body growth in early prometamorphic tadpoles, we

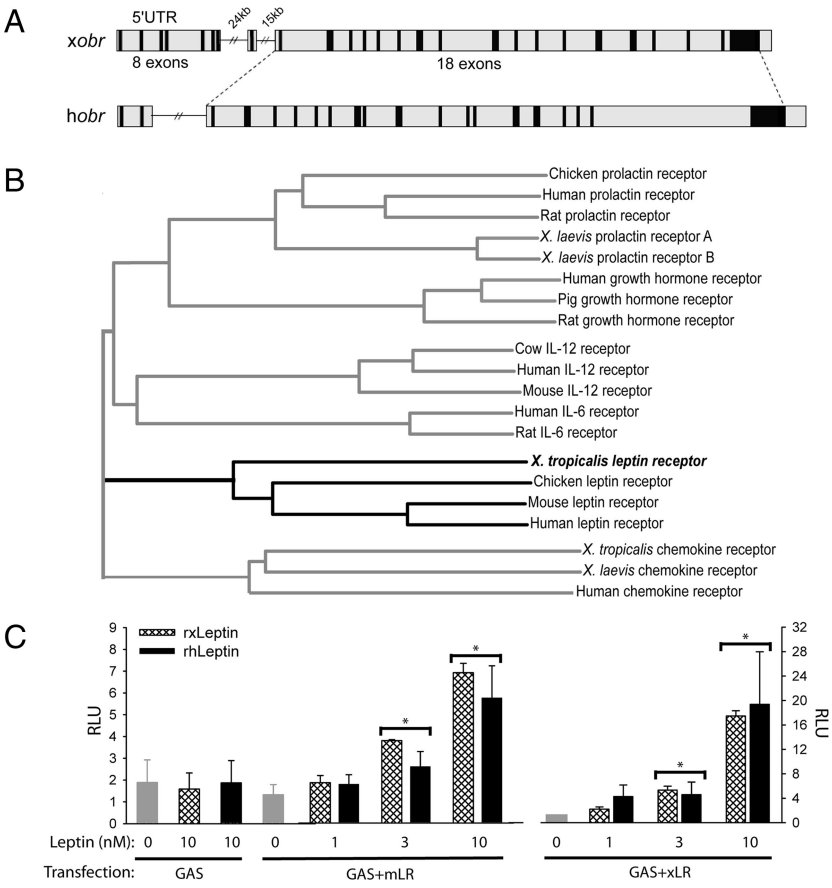

Fig. 2. Molecular and functional characterization of frog leptin receptor. (*A*) Predicted gene structure of the frog *obr* gene (x*obr*) and comparison with the human *obr* gene (h*obr*). The frog *obr* gene spans 87.3 kb. (*B*) Neighbor-joining phylogenetic tree of amino acid sequences of leptin receptors and related cytokine receptor genes. We used the Align X module within VECTOR NTI SUITE (v. 5.5; Informax, Bethesda) to conduct the analysis. GenBank accession numbers are provided in supporting information. (*C*) rxLeptin activates the mouse and the frog LR in transient transfection assays. COS-7 cells were transfected with a STAT-3-responsive luciferase reporter plasmid (GAS) or with both GAS plus pcDNA3.1-mLR (mouse leptin receptor) (*Left*) or pcDNA3.1-xLR (frog leptin receptor; separate experiments) (*Right*), then exposed to different doses of rxLeptin or human recombinant leptin (hrLeptin). Bars represent means ± SEM (n = 4–6 per treatment; a representative experiment is shown, and each experiment was repeated three times). Asterisks indicate significant differences between leptin treated and untreated controls (Fisher's least significant difference test; $P < 0.05$). RLU, relative light units.

found a significant increase in hind-limb growth and differentiation of the digits in both fed [ANOVA hind limb: $F = 3.95$, $P = 0.035$; analysis of covariance (ANCOVA) stage: $F = 3.64$, $P = 0.030$] and food-deprived (ANOVA hind limb: $F_{3,36} = 8.9$, $P = 0.0002$; ANCOVA stage: $F = 8.95$, $P = 0.001$) animals (Fig. 5 *A–C*); tail and body lengths were not affected by rxLeptin treatment (data not shown). At this developmental stage, the hind limb is the only tissue in which cartilage and bone are formed *de novo*. We found LR but not leptin mRNA in the hind limb of early prometamorphic *X. laevis* tadpoles (NF stage 54–56; Fig. 5*D*). rxLeptin stimulated [^{3}H]thymidine uptake by cultured hind limbs from early prometamorphic *X. laevis* tadpoles (NF stage 54–56) in a dose-dependent manner (control, 0.74 ± 0.24; 1 ng/ml rxLeptin, 2.36 ± 0.75; 10 ng/ml rxLeptin, 3.56 ± 0.91; ANOVA, $F = 6.557$, $P = 0.013$; ratios $\log_{10}$ transformed). Taken together, our results show that leptin, probably acting via the LR, can promote limb growth and differentiation during early postembryonic development.

Discussion

Here, we report the definitive molecular and functional characterization of a leptin and a leptin receptor in an ectothermic vertebrate, and our findings provide an essential foundation for elucidating the structural and functional evolution of this important protein hormone. Despite low amino acid sequence similarity to mammalian leptins, the frog leptin has a conserved tertiary structure that probably confers specific LR binding affinity, which was confirmed by rxLeptin activation of both the mouse and the frog LRs *in vitro*. Our findings show that the role of leptin as a regulator of food intake arose early in vertebrate evolution, before the divergence of the amniotes. This anorectic action develops during metamorphosis in the amphibian, a time when the hypothalamus, a major feeding control center in vertebrates, undergoes maturation in response to thyroid hormone (22). We also report a previously uncharacterized action of leptin as a growth factor for limb morphogenesis in the tadpole.

Structural Characterization of Frog *ob* and *obr* Genes. The predicted leptin protein of *X. laevis* shares ≤35% sequence similarity with mammalian leptins, which is in striking contrast to the 95% sequence similarity reported between the mouse and chicken *ob* genes (greater similarity than that among several mammalian leptins) (21, 23). Despite the low sequence similarities between the ectotherm and mammalian leptins, molecular modeling shows a conserved four-helix structure that is not only characteristic of mammalian leptins but also of other long-chain helical cytokines (24). Thus, despite divergence in amino acid sequence among species, a conserved tertiary structure has been maintained by natural selection and is presumably constrained by the structure of the receptor binding pocket. Leptins of frog and human origin exhibited similar potencies on mouse and frog LRs when tested in transfection assays, and human and frog leptins had similar potencies in inhibiting food intake in juvenile *X. laevis* (E.J.C., unpublished data). Based on these structural and functional similarities with mammals, we have designated the gene that we isolated from frog leptin.

Vertebrate leptins, including frog and pufferfish, differ from other long-chain helical cytokines in that they possess two conserved cysteine residues predicted to form disulfide bonds known to be required for bioactivity of human leptin (24, 25). The frog and pufferfish sequences also have conserved Leu (163) and Glu (164) residues at the C terminus of helix D that contribute to the unique conformation of the leptin protein (24). Unlike the pufferfish, frog leptin has regions of higher conservation (50–70%) with mammalian leptins that correspond to receptor binding domains (e.g., midregions of helix A and C, AB loop, and the N terminus of helix

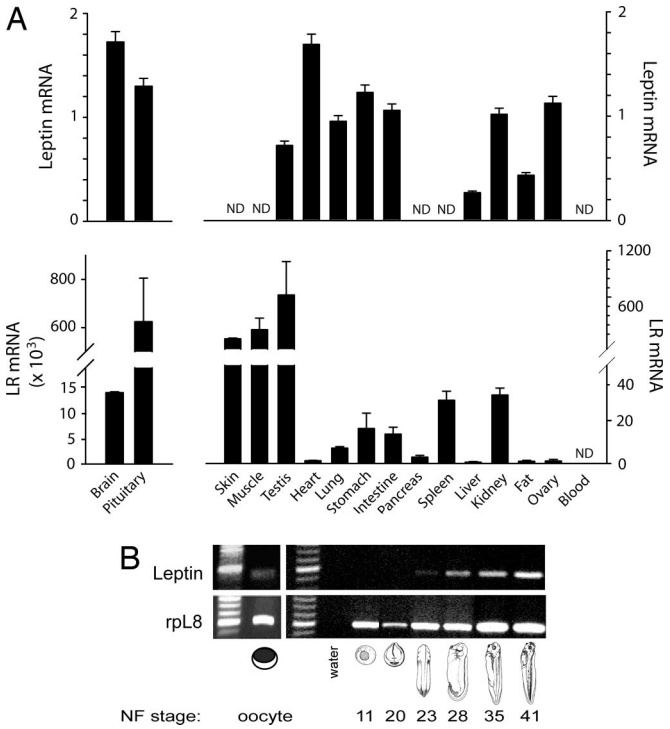

Fig. 3. Expression of leptin and LR mRNAs in the frog. (*A*) The tissue distribution in juvenile frogs of leptin and LR mRNAs was analyzed by quantitative RT-PCR (see *Methods*). Leptin and LR mRNA levels were normalized to the expression of the ribosomal protein L8 gene (rpL8). (*B*) The developmental expression of leptin mRNA in *X. laevis* was analyzed by semiquantitative RT-PCR (see *Methods*).

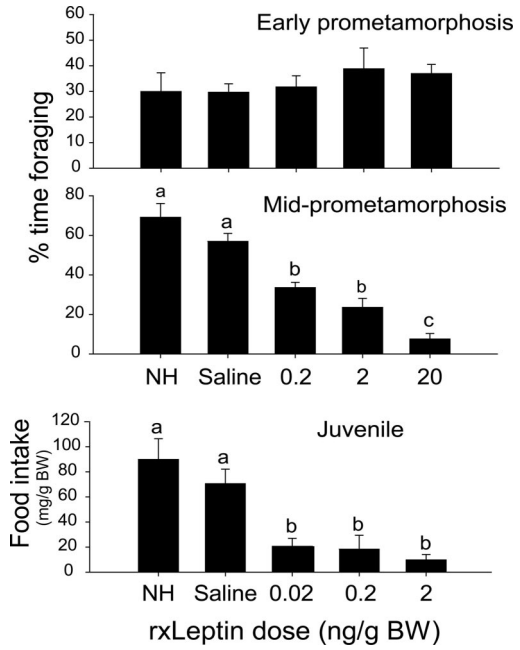

Fig. 4. Effects of i.c.v. injection of rxLeptin on time spent foraging in early prometamorphic or midprometamorphic *S. hammondii* tadpoles and on meal size in *X. laevis* juveniles. Letters indicate Duncan's pairwise differences among treatments ($P < 0.05$).

D; ref. 26). However, there are two stretches of 16–20 aa in frog leptin that have almost no sequence similarity to mammalian leptins, including the C terminus of the helix A/AB loop region and the CD loop/helix E region (which does not interact with the LR but may play a role in appetite regulation; ref. 26). The conspicuous similarity of residues 61–66 (GLDFIP; Fig. 1*B*) in the AB loop among mammalian and frog leptins suggests that this sequence may be important for receptor binding.

The predicted frog LR protein shares only 37% amino acid sequence similarity with human LRb, but the frog gene structure is similar to mammalian *obr* genes, and phylogenetic analysis clearly placed the frog gene within a clade of mammalian and chicken *obr* genes. Stretches of highest sequence similarity correspond to regions that are known to be important for mammalian LR signaling. The sequences of the corresponding ligand binding and transmembrane domains show greater conservation, as do specific residues in the C-terminal domain of the mammal LRb shown to be necessary for intracellular signaling (6). Our *in vitro* transfection assays confirm that leptin binding to the frog LR activates STAT-3 signaling. The number of exons encompassing the 5′ UTR is greater in frog compared with human (8 vs. 2 exons). In the human, the first two exons also encode a protein (leptin receptor gene-related protein; ref. 27), which has been identified in *Xenopus* (GenBank accession no. AW766133; the first four exons of the frog LR gene overlap with the N-terminal sequence of the leptin receptor gene-related protein). Based on conserved structure and function, we conclude that the frog gene that we isolated is homologous to mammalian LR.

Tissue Distribution of Leptin and LR mRNAs. The tissue distribution of leptin mRNA is broader in the juvenile frog compared with mammals. It is noteworthy that the expression levels in liver and fat, the two primary sites of leptin expression in mammals were lower than those in other frog organs that express leptin.

We found that LR transcripts containing exons that code for the C-terminal region of the molecule necessary for intracellular signaling are widely distributed, which suggests that leptin may have widespread and diverse physiological roles in amphibians. High expression levels were detected in the frog brain, which is a main site of LRb expression in mammals (6). We detected highest levels of expression of LR mRNA in the pituitary gland, which may be a target for leptin action in mammals (28). The relative levels of LR transcripts detected with both RTqPCR assays (targeting the ligand binding domain and the C terminus) were comparable among tissues. The LR was expressed in all of the same organs as leptin, suggesting that leptin may have a paracrine function in these tissues. By contrast, an exclusively hemocrine action of leptin is predicted in tissues where only the receptor is expressed.

Effect of rxLeptin on Food Intake Throughout Amphibian Development. Leptin had potent inhibitory effects on appetite in midprometamorphic tadpoles and juvenile frogs. Murine leptin inhibited food intake in a fish (29), but our results in the frog show that a homologous leptin in an ectotherm has anorectic activity. Repeated rxLeptin injections also caused food-deprived midprometamorphic tadpoles to lose more weight than vehicle-injected controls, suggesting that leptin also increases energy expenditure as it does in mammals (2). Thus, the function of leptin as a regulator of food intake and energy balance evolved before the emergence of amniote vertebrates.

The anorectic action of leptin in the amphibian does not develop until midprometamorphosis, which corresponds to the formation of discrete fat bodies (E.J.C., unpublished data) and the maturation of the neuroendocrine system under the influence of thyroid hormone (22). Earlier, we showed that inhibitory inputs to hypothalamic feeding control centers involving corticotropin-releasing factor (CRF) neurons are formed at this stage of development (30). The inhibitory effect of leptin on food intake is at least partially mediated by CRF signaling in mammals (31); therefore, it is likely

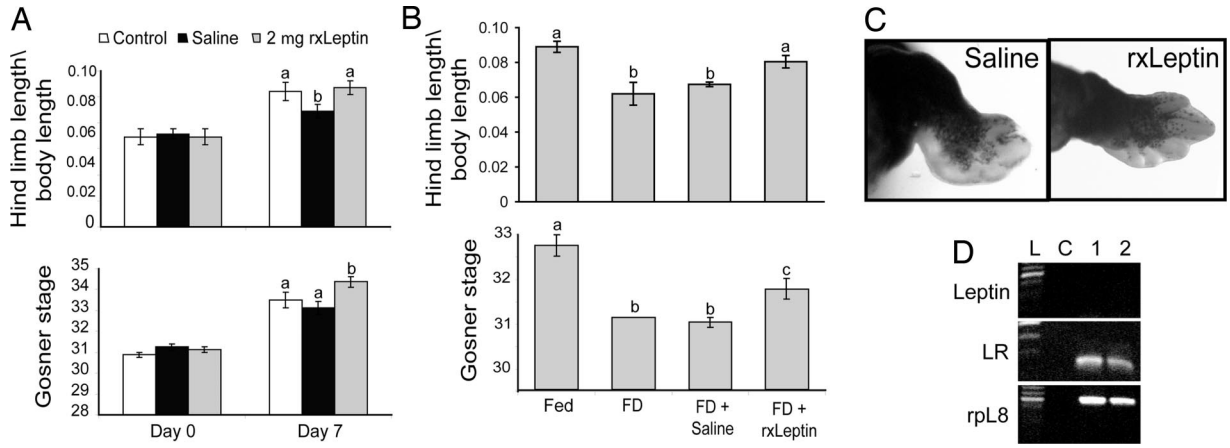

Fig. 5. Effects of rxLeptin injections on hind-limb growth and development in early prometamorphic *S. hammondii* tadpoles. (*A*) Mean ± SEM hind-limb length divided by body length and Gosner stage before and after rxLeptin injections (i.p. every other day for 7 days; $n = 8$ per treatment). Letters indicate Duncan's pairwise differences among treatments ($P < 0.05$). (*B*) Mean ± SEM hind-limb length divided by body length and Gosner stage of tadpoles fed or food-deprived (FD) and given 2 μg of rxLeptin i.p. every other day for 6 days ($n = 10$). Letters indicate significant differences between experimental groups (Tukey's multiple comparisons test; $P < 0.05$). (*C*) Representative hind limbs of tadpoles given saline or rxLeptin injections as described above. (*D*) RT-PCR analysis of leptin and LR expression in two independent hind-limb samples from *X. laevis* NF stage 56 (four hind limbs pooled per sample). rpL8, ribosomal protein L8; C, water control; L, DNA ladder.

that the maturation of CRF neurons in brain regions controlling appetite is linked to the development of leptin's effects on food intake in amphibians.

Leptin Function in Early Development of Amphibians. The expression of leptin mRNA in frog oocytes and embryos before feeding stages and before adipose tissue formation suggests that leptin may have adipocyte-independent roles in early development. Leptin and LR are expressed in cartilage/bone, heart, and liver in fetal mouse and sheep (13, 15); but, specific actions of leptin have not been described at these early stages of mammalian development. Our findings that rxLeptin injections advanced tadpole hind-limb growth and development and that rxLeptin increased [^{3}H]thymidine uptake by cultured tadpole hind limbs supports the view that leptin plays a role as a growth factor during vertebrate development. Furthermore, we found LR but not leptin mRNA in the tadpole hind limb, suggesting that leptin of hemocrine origin mediates tadpole limb development.

There is evidence that leptin plays a role in mammalian limb development. Leptin and LR mRNAs are expressed in bone and cartilage of the femur and hind-limb digits of 13.5-day postcoitus fetal mouse (13, 32). Also, leptin injections enhanced limb bone mass and density in juvenile leptin-deficient ob/ob mice (33), although others found that leptin had negative effects on bone growth that are mediated by the hypothalamus (34). Gat-Yablonski and colleagues (35) showed that leptin treatment restored starvation-induced decreases in limb bone mass in juvenile mice, which is consistent with our finding that leptin injections restored hind-limb growth and development in food-deprived tadpoles. Taken together, these findings highlight the potential for adipocyte-independent functions of leptin in early development.

Methods

Molecular Cloning of Frog Homologs of Mammalian *ob* and *obr* Genes. We first identified a presumptive frog leptin sequence in the *X. tropicalis* genome database (JGI v. 3.0; Joint Genome Institute) by searching for amino acid sequences with similarity to human leptin, designing oligonucleotides based on these regions of similarity, and amplifying cDNA fragments from *X. laevis* brain, liver, and fat using PCR. We used random amplification of cDNA ends (SMART RACE Kit; CLONTECH) to isolate the 5′ and 3′ ends of the molecule, and subsequently designed primers to amplify a partial cDNA. We determined the genomic structure of the frog *ob* gene by aligning the amplified *X. laevis* cDNA with *X. tropicalis* genomic sequences.

We identified a putative frog *obr* gene in the *X. tropicalis* genome database (JGI v. 4.1) by BLAST searching with specific mammalian exon amino acid sequences. These searches yielded multiple sequences within a 100,000-bp span of genomic DNA on Scaffold 4; we used GENESCAN (version 1.0; ref. 36) to predict exon/intron boundaries. To locate the 5′ UTR, we conducted BLAST searches of the *X. tropicalis* genome using the human *obr* exon 1 and 2 sequences. We then designed primers to amplify the entire LR coding sequence from *X. tropicalis* lung RNA.

We subcloned the entire coding region of frog leptin into pET 151/D-TOPO and the entire coding region of the frog LR into pcDNA3.1/D-TOPO following the manufacturer's instructions (Invitrogen). These vectors were subsequently used for protein expression in *Escherichia coli* and transient transfection in mammalian cells, respectively.

RTqPCR Analysis of Frog Leptin and LR mRNAs. We used RTqPCR to determine the tissue distribution of leptin and LR mRNAs and to compare relative expression levels in juvenile frogs. We isolated RNAs from frog tissues using the TRIzol reagent (Invitrogen) and synthesized cDNA using Superscript II (Invitrogen) following the manufacturer's instructions. We developed TaqMan assays and analyzed samples on an ABI 7500 fast real-time PCR machine using TaqMan Universal PCR Master Mix (Applied Biosystems). The primer/probe sets were designed to span exon/intron boundaries and are described in supporting information. Standard curves were generated by using cDNAs from tissues that exhibited the highest expression level for each gene. Leptin and LR mRNAs were normalized to the level of L8 mRNA.

For semiquantitative RT-PCR, we used HotStar *Taq* polymerase (Qiagen, Valencia, CA) following the manufacturer's protocol. Oligonucleotide primers and the PCR conditions used are described in supporting information.

Production of Recombinant Frog Leptin. We produced recombinant *X. laevis* leptin in *E. coli* using the pET 151/D-TOPO expression vector (Invitrogen) transformed into BL21 Star (DE3) cells (Invitrogen). We purified rxLeptin from inclusion bodies by fractionating bacterial lysate on a 12% SDS/PAGE gel, electroeluting

into 20 mM Tris base/150 mM glycine/0.01% SDS (pH 7.0), and dialyzing overnight against 10 mM ammonium bicarbonate.

Cell Transfection. We first determined whether rxLeptin could activate the mouse LRb in transfection assays. We then used transfection assay to test whether the frog LR that we isolated was functional. We cotransfected COS-7 cells (40,000 cells per well; 24-well culture plates) with either the pcDNA3.1 mouse or frog LR-receptor expression vector (200 ng) and a STAT-3 responsive luciferase reporter construct (GAS; 100 ng; ref. 37) using FuGENE 6 transfection reagent (Roche Biosciences). We also transfected cells with *Renilla* luciferase reporter construct (2 ng; Promega) for normalization of transfection efficiency. After transfection, cells were serum-deprived for 14–15 h before the addition of different concentrations of either recombinant human or rxLeptin. After 6 h, we harvested cells and measured both firefly and *Renilla* luciferase activities using a dual-luciferase reporter assay (Promega).

Food Intake Assays. We administered i.c.v. injections of rxLeptin (0.2, 2, 20 ng/g body weight in PBS) into the third ventricle of juvenile *X. laevis* and monitored food intake as described in ref. 38. We also tested the effects of rxLeptin on food intake in amphibian larvae using tadpoles of the Western spadefoot toad *Spea hammondii* in which food intake (foraging) can be easily quantified. Using the protocol of Crespi and Denver (30), we tested animals at two stages of metamorphosis, early prometamorphosis when hindlimb buds are first visible (Gosner stage 30) and midprometamorphosis (Gosner stage 36–37). We measured the percent time foraging within a 2-min observation period and used ANOVA to detect treatment effects. We also injected 2 μg of rxLeptin/50 μl of PBS into the peritoneum (i.p.) every other day for 6–7 days in daily fed and food-deprived tadpoles to assess the effects of prolonged rxLeptin treatment on growth and development rate ($n = 8$ per treatment) during early and midprometamorphic stages. We analyzed the ratio of hind limb to body length with ANOVA and ln-transformed Gosner stage with ANCOVA (body weight as a covariate). We compared posttreatment body weight of saline- and rxLeptin-injected tadpoles with *t* tests. All animal experiments were conducted following institutional guidelines for animal care and use.

[^{3}H]Thymidine Uptake Assay. Hind limbs were harvested from *X. laevis* tadpoles (NF stage 54–56) and cultured individually in a 24-well plate. One limb served as the control (culture medium alone, $n = 5$), whereas the other served as the experimental treatment (culture medium plus 1 or 10 ng/ml rxLeptin; $n = 5$ and $n = 4$, respectively). We cultured limbs in L-15 medium (containing penicillin, streptomycin, and thyroid hormone-stripped FBS; diluted 1:1.5) under a humidified atmosphere of 5% CO_2 and 95% O_2 at 25°C with gentle shaking for 48 h. We then added [^{3}H]thymidine (0.75 μCi per well; PerkinElmer) to each well and incubated for an additional 16 h before washing tissues with ice-cold PBS, fixing in 5% trichloroacetic acid for 20 min at 4°C, and lysing in 1 M NaOH. After 30 min of gentle shaking at 65°C, we measured radioactivity in cell extracts by liquid scintillation counting.

Note Added in Proof. Boswell *et al.* (39) recently reported the isolation of a leptin-like gene in the tiger salamander that shares 60% sequence similarity with *X. laevis* leptin.

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