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Molecular Cloning and Developmental Expression of Corticotropin-Releasing Factor in the Chicken

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We have characterized the structure of the chicken corticotropin-releasing factor (CRF) gene through cDNA cloning and genomic sequence analysis, and we analyzed the expression of CRF mRNA and peptide in the diencephalon of the chick throughout embryonic development. The structure of the chicken CRF gene is similar to other vertebrate CRF genes and contains two exons and a single intron. The primary structure of the mature chicken CRF peptide is identical to human and rat CRF. This is the first archosaurian CRF gene to be characterized. We used RIAs to analyze CRF peptide content in the diencephalon and the median eminence and plasma corticosterone during the last week of embryonic development. We also developed a semiquantitative RT-PCR method to analyze the expression of CRF mRNA during the same period. CRF peptide content in the diencephalon increased, whereas peptide content in the ME decreased just before hatching, suggesting that release and biosynthesis are coupled. Plasma corticosterone concentration significantly increased between embryonic d 20 and the first day post hatch. By contrast, CRF mRNA levels in the diencephalon decreased just before hatching. Changes in CRF production just before hatching may be causally related to the regulation of the thyroid and interrenal axes at this stage of chicken development. (*Endocrinology* 146: 301–308, 2005)

DURING THE LAST week of embryonic development in the chicken, the hypothalamic-pituitary-thyroid (HPT) axis and the hypothalamic-pituitary-adrenal (HPA) axis both exhibit increased activity (1, 2). This activity correlates with the important roles that glucocorticoids and thyroid hormones play in the hatching process. Corticotropin-releasing factor (CRF) is a potent secretagogue for both ACTH (3) and TSH (4) in the embryonic chick. As a central regulator of both the HPA axis and the HPT axis, CRF could play a key role in the endocrine control of hatching. In mammals and other vertebrates, CRF has been shown to function as both a neurohormone and a neurotransmitter/neuromodulator (5). In mammals, hypophysiotropic CRF is synthesized in neurosecretory neurons in the paraventricular nucleus (PVN) of the hypothalamus and is transported via axons to the external zone of the median eminence (ME), where it is released into the pituitary portal circulation (6).

Like most neuropeptides, CRF is derived from a larger precursor molecule (proCRF), which is proteolytically processed to yield the mature 41-amino acid peptide by the action of the endopeptidase prohormone convertase 2 (PC2) (7–9). The primary amino acid sequence of proCRF has been elucidated in several species (10–16). In addition, the complete gene has been sequenced in human, rat, sheep, mouse, and the frog *Xenopus laevis* (13, 17–21).

Although CRFs have been characterized in mammals, amphibians, and fishes, to date, there are no reports of CRF gene structures or prohormone sequences in birds or reptiles (including all archosaurs). Knowledge of such sequences is necessary to gain a full understanding of CRF gene evolution in tetrapods and to allow for analysis of the roles of CRF in physiology and development. In this article, we report the gene structure and the deduced amino acid sequence of the chicken (c) CRF prohormone. Given the potential role of CRF in the hatching process (through its regulation of the HPT and HPA axes), we analyzed CRF mRNA expression in the chick diencephalon and CRF peptide content in the diencephalon and ME during the final week of embryogenesis. In addition, we analyzed hypothalamic PC2 mRNA (as a potential indirect measure of CRF prohormone processing), pituitary proopiomelanocortin (POMC) mRNA, and plasma corticosterone concentration (as indirect measures of CRF activity) to determine whether these correlate with measures of CRF expression.

Materials and Methods

Animals

All studies were conducted on broiler chickens (Avibel, Halle-Zoersel, Belgium) purchased as fertilized eggs or 1-d-old chicks of both sexes. Eggs were incubated in a forced-draft incubator at a temperature of 37.8 C with increasing humidity and ventilation from d 14 onward and with continuous lighting and a 45° rotation every hour. The start of incubation

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Abbreviations: c, Chicken; C1, 1-d-old chick; CRF, corticotropin-releasing factor; E1, embryonic d 1; h, human; hn, heteronuclear; HPA, hypothalamic-pituitary-adrenal; HPT, hypothalamic-pituitary-thyroid; ir, immunoreactivity; ME, median eminence; o, ovine; PC2, prohormone convertase 2; POMC, proopiomelanocortin; PVN, paraventricular nucleus; r, rat; RACE, rapid amplification of cDNA ends; RT, reverse transcription; SHRP, stress hyporesponsive period; xCRF, *Xenopus laevis* corticotropin-releasing factor.

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was designated as embryonic d 1 (E1). Hatched chicks were kept under a 14-h light, 10-h dark photoperiod and fed a commercial mixed diet (Hendrix, Merksem, Belgium). Water and food were available *ad libitum*. The Katholieke Universiteit Leuven Ethical Committee for Animal Experiments approved all experimental protocols.

For the ontogeny experiment, samples were taken from embryos on d 16, 18, and 20 of the incubation (E16, E18, and E20) and from 1-d-old chicks (C1). Blood was collected after heart puncture/decapitation and centrifuged at 1800 × *g* at 4 C for 15 min. The plasma was collected and stored at −20 C until assayed for corticosterone. Brain tissue was dissected, frozen in N_2, and stored at −80 C until assayed.

Molecular cloning of cDNAs for chicken proCRF

Total RNA was extracted from the diencephalon of C7 chicks using the Total RNA Isolation System (Promega, Leiden, The Netherlands). The mRNA was isolated from the total RNA using the NucleoTrap mRNA mini kit (BD Biosciences, Palo Alto, CA). This mRNA was subsequently used to isolate CRF cDNA sequences by RT-PCR. First, 150 ng of mRNA was denatured at 75 C for 5 min in the presence of an oligo(dT)-primer (Roche, Mannheim, Germany). Reverse transcription (RT) was performed at 42 C for 60 min using 2.5 U avian myeloblastosis virus reverse transcriptase (Roche). The subsequent PCR amplification was conducted using 5 μl of the RT reaction. The total PCR mix (50 μl) contained PCR buffer (10 mM Tris-HCl, pH 9.0; 1.5 mM $MgCl_2$; 50 mM KCl; 0.1% Triton X-100; and 0.01% gelatin), 1 mM of each deoxynucleotide triphosphate (Roche), 1 μM of each primer, and 1 U SuperTaq DNA polymerase (HT Biotechnology, Cambridge, UK). After denaturation at 94 C for 5 min, a thermocycle of 45 sec at 94 C, 1 min at 55 C, and 2 min at 72 C was conducted for 30 cycles and was followed by a final elongation step at 72 C for 5 min. The primers designed for this PCR were based on the known mammalian CRF sequences of ovine (GenBank accession no. J00803), human (GenBank accession no. V00571), rat (GenBank accession no. X03036), and bovine (GenBank accession no. AF340152) proCRF and were as follows: sense primer, 5′-GTCCTGCTGGTGGCTTCCTGCC-3′ (corresponding to bp 31–53 in Fig. 1A) located in the signal sequence; and antisense primer, 5′-CCTGTTGCTGTGGGCTTGCTG-3′ (corresponding to bp 457–477 in Fig. 1A) located in the conserved mature peptide sequence. This resulted in the amplification of a 447-bp fragment, which was purified from an agarose gel using the QIAEX II Gel Extraction kit (Qiagen, Hilden, Germany) and then subcloned into the pCRII-TOPO vector using the TOPO-TA cloning kit (Invitrogen, Merelbeke, Belgium). The nucleotide sequence was determined by automatic sequencing using the ABI PRISM 310 Genetic Analyzer (Applied Biosystems, Lennik, Belgium).

In an attempt to obtain the full-length cDNA sequence, 5′- and 3′-rapid amplification of cDNA ends (RACE) was performed using both the Marathon cDNA Amplification system (BD Biosciences) and SMART-RACE technology (Clontech, Palo Alto, CA) following the manufacturer's guidelines. Primers were designed based on the sequence of the partial cDNA obtained from the initial RT-PCR described earlier. For 5′-RACE, the antisense primer sequence was 5′-CCCATGCGGAGCAGGACGGGCAGAG-3′ (corresponding to bp 158–182 in Fig. 1A). For 3′-RACE, the sense primer sequence was 5′-GGTGCTGGAGATGGCCCGCGCCGAGC-3′ (corresponding to bp 423–448 in Fig. 1A). The amplified fragments were subcloned and sequenced as described earlier. The sequence was verified by analyzing multiple clones.

cCRF genomic sequence analysis

In addition to the molecular cloning of the cCRF cDNA, we used the first draft of the chicken genome project (http://www.ensembl.org/Gallus_gallus), released March 1, 2004, to assemble a genomic contig that spanned the entire cCRF gene. Potential TATAA and CAAT boxes and the polyadenylation site were identified by searching for their respective consensus sequences.

Putative intron/exon boundaries were deduced by comparing the cCRF genomic fragment with the human (h) CRF genomic sequence (GenBank accession no. V00571). To verify these exon/intron boundaries, a new set of RT-PCR primers was chosen. The sense primer

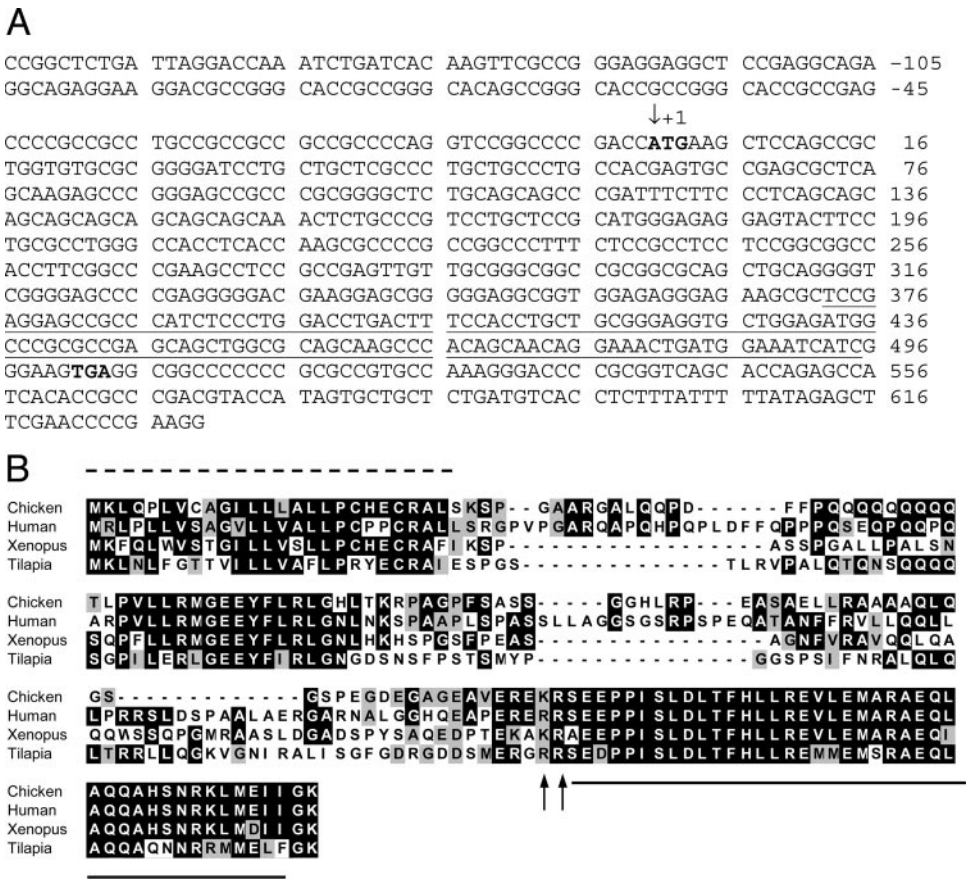

FIG. 1. A, Representation of the chicken CRF cDNA sequence as it was isolated, with the start and stop codon printed in *bold* and the sequence coding for the mature CRF peptide *underlined*. Numbering started at the translation start site. B, The deduced amino acid sequence of proCRF in the chicken, compared with sequences of other vertebrate species. Identical amino acids have a *black background*, and similar amino acids have a *gray background*. The *solid black line* indicates the mature peptide, and the *dashed black line* indicates the signal peptide encoding region. *Arrows* indicate the conserved dibasic cleavage site.

(5′-CCGGCTCTGATTAGGACCA-3′, corresponding to bp −164 to −146 in Fig. 1A) was located in the predicted exon 1, and the antisense primer (5′-TCAGTTTCCTGTTGCTGTGG-3′, corresponding to bp 465–484 in Fig. 1A) was located in the predicted exon 2. Because the PCR product that results from this amplification has a high guanosine-cytosine percentage (70%), we used a PCR kit developed for the amplification of guanosine-cytosine-rich nucleic acids (GC-Rich PCR System, Roche). The amplified fragment was subcloned and sequenced as described earlier. The sequence was verified by analyzing multiple clones.

CRF extraction from tissue

The CRF content in diencephalon and ME was measured using an adaptation of the method of Mastorakos *et al.* (22), which is described by Boorse and Denver (23). Tissue samples from embryos (E16, E18, and E20) and C1 chicks were dissected and weighed. The ME was separated from the rest of the diencephalon. Individual MEs were pooled into groups of five. Ten volumes of boiling acetic acid (2 M) were added (1 ml/0.1 g tissue weight), and samples were boiled for 10 min. This was followed by addition of β-mercaptoethanol to a final concentration of 5% (vol/vol) and incubation of the samples for 10 min at room temperature. Samples were then placed on ice and homogenized. The homogenate was centrifuged for 30 min at 15,000 × *g* (4 C), and the supernatant was transferred to a new microcentrifuge tube. Three volumes of acetone were added (0.3 ml/0.1 g tissue), and the sample was mixed thoroughly. The mixture was then centrifuged (15 min at 15,000 × *g*, 4 C), and the supernatant was dried under vacuum. The residue was dissolved in 500 μl CRF assay buffer [0.1 M $Na_2HPO_4 \cdot 2H_2O$; 0.05 M NaCl; 0.01% (m/vol) NaN_3; 0.1% (m/vol) BSA; and 0.1% (vol/vol) Triton X-100; pH 7.4].

The recovery of CRF during the extraction process was estimated by adding a fixed amount (1000 cpm) of radiolabeled CRF to brain homogenates, and this averaged 47.16 ± 2.18%.

CRF RIA

To measure tissue CRF content, we adapted a RIA protocol developed for analysis of *X. laevis* CRF (xCRF) (23). The xCRF differs from cCRF, rat (r) CRF, and hCRF in only three of 41 residues (Fig. 1B), and the r/hCRF exhibits identical potency and parallelism with the xCRF standard curve in the xCRF RIA (23). The tracer, [^{125}I]Tyr^0-r/hCRF (cCRF and r/hCRF are identical; Fig. 1B), was purchased from PerkinElmer (Wellesley, MA). Synthetic r/hCRF (NeoMPS, Strasbourg, France) was used to produce a standard curve (0.05–6.25 ng/ml, diluted in CRF assay buffer). Standard and sample solutions (100 μl) were incubated with 200 μl CRF assay buffer and 100 μl anti-xCRF (diluted 1:1500 in CRF assay buffer) in polystyrene tubes for 24 h at 4 C. Subsequently, radioiodinated r/hCRF (100 μl; 15,000 cpm/tube) was added, and the tubes, with a final volume of 500 μl, were incubated for another 24 h at 4 C. Separation of free and bound label was achieved by immunoprecipitation using 100 μl Sac-cell antirabbit γ-globulins (IDS, Boldon, UK). After 1 h of incubation at room temperature and centrifugation (1500 × *g* for 15 min, 4 C), the radioactivity of the precipitate was measured in a γ-counter.

The intraassay coefficient of variation (1.63%) was determined by measuring three different samples (extracts from diencephalon; one high, one medium, and one low concentration of CRF) 10 times in the same assay (same standard curve and antibody dilution). For the interassay coefficient of variation (4.05%), the same three samples were measured in five separate assays. For further validation of the CRF RIA, we set up displacement curves from serial dilutions (1:10, 1:20, 1:40, 1:80, and 1:160) of extracts of diencephalon, and these showed good parallelism with the standard curve. The cross-reactivity was tested for rat urocortin, human urocortins I and III, and sauvagine. Only the heterologous peptide sauvagine exhibited measurable competition in the RIA (<10%).

Semiquantitative RT-PCR analysis

Semiquantitative RT-PCR was used to measure the relative proCRF, PC2, and POMC mRNA levels during the last week of embryonic development. Total RNA was extracted from embryonic tissues (E16, E18, and E20) and from C1 chicks using the Total RNA Isolation System (Promega). ProCRF and PC2 mRNA levels were measured in individual diencephalons, whereas pituitaries were pooled five per sample to determine POMC mRNA levels. For each age, three replicate samples were studied. The integrity of the RNAs was verified by 1.5% agarose gel electrophoresis followed by ethidium bromide staining. To rule out the possibility that PCR products would result from the amplification of genomic DNA contaminating the RNA sample, RNA samples were treated with DNase I using the DNA-Free kit (Ambion, Austin, TX). As an extra control, a PCR was performed on the total RNA samples that did not undergo a RT, which did not produce any DNA bands on a 1.5% agarose gel. For RT-PCR, 1 μg of total RNA was denatured at 75 C for 5 min in the presence of an oligo(dT)-primer followed by RT with avian myeloblastosis virus reverse transcriptase (2.5 U) at 42 C for 60 min. The subsequent PCR amplification was done on 5 μl of the RT reaction mix. The PCR reaction (50 μl) contained PCR buffer (components as described above), 1 mM of each deoxynucleotide triphosphate, 1 μM of each primer, and 1 U SuperTaq DNA polymerase (HT Biotechnology). After denaturation at 94 C for 5 min, a number of cycles (35 for proCRF, 30 for PC2, and 25 for POMC), including denaturation for 30 sec at 94 C, annealing of the primers for 1 min at 55 C (proCRF and PC2) or 70 C (POMC), and extension at 72 C, were performed, followed by a final elongation step at 72 C for 5 min. The primers (Table 1) designed for the semiquantitative RT-PCR analyses are based on the known sequences of chicken proCRF (present study; GenBank accession no. AJ621492), chicken PC2 (GenBank accession no. BM491499), and chicken POMC (GenBank accession no. AB019555) (24). The number of cycles used for each gene was empirically determined using a linear amplification range beginning at 20 cycles and increasing to 50 cycles in five-cycle increments. The midpoint of the linear part of the amplification curve was chosen as the optimal number of cycles for each gene. PCR products were visualized on a 1% agarose gel containing ethidium bromide (0.5 μg/ml). Stained bands were captured digitally, and densitometric analyses were conducted using image analysis software developed by Dr. R. Docter (Department of Internal Medicine III, Erasmus University Medical School, Rotterdam, The Netherlands). Levels of chicken β-actin mRNA (GenBank accession no. K02173) were analyzed in parallel to correct for variations in the amount of total RNA applied (25). In short, this RT-PCR protocol for β-actin is the same as described for POMC with an annealing temperature of 56 C. The primers used in this RT-PCR are also listed in Table 1.

Corticosterone RIA

Plasma concentrations of corticosterone, the primary glucocorticoid in the chicken (26), were determined using a commercial RIA kit (ICN Biomedicals, Irvine, CA), which has been adapted and validated for use in chicken plasma (27).

Statistics, data analysis, and software

Statistical analysis was conducted using the SAS program (SAS Institute, Cary, NC). Statistical significance was assessed by one-way ANOVA using the general linear models procedure followed by Scheffé's test ($P < 0.05$).

We used the Vector NTI Suite version 5.5 (Informax, Inc., Frederick,

TABLE 1. Primers used for the semiquantitative RT-PCR analysis

	Sense	Antisense
proCRF	5′-TCTCCCTGGACCTGACTTTC-3′ (bp 389–408, Fig. 1A)	5′-GAGGTGACATCAGAGCAGCA-3′ (bp 580–599, Fig. 1A)
PC2	5′-AAATGATGGACGGACAGCTC-3′	5′-TGGTTCCTTTTGGAGGTGAG-3′
POMC	5′-CAGGCGTGTGCCAAGGCATGCCG-3′	5′-CTCCATGGGGTAACTCTCAGCCGAC-3′
β-Actin	5′-CAAAGCCAACAGAGAGAAGA-3′	5′-TCACGCACAATTTCTCTCTC-3′

MD) and DNAMAN version 2.51 (Lynnon Biosoft, Vandreuil, Quebec, Canada) software packages to conduct sequence alignments.

Results

Analysis of the structure of the cCRF gene and prohormone

RT-PCR using primers based on proCRF sequences in different species, together with 5′- and 3′-RACE, allowed us to isolate a cDNA spanning the entire coding region of the cCRF gene (Fig. 1A). The deduced amino acid sequence of the cCRF prohormone showed that it is structurally similar to the known vertebrate CRF prohormones (Fig. 1B). The N terminus contains a signal peptide sequence that is followed by the cryptic peptide and the mature peptide (Fig. 1B). There is one conserved dibasic cleavage site in the chicken proCRF that is expected to result in the production of a mature peptide of 41 amino acids similar to other vertebrate CRFs. The primary sequence of the mature cCRF peptide is identical to r/hCRF.

Using the chicken cDNA sequence, we analyzed the first draft of the chicken genome sequence released on March 1, 2004 (http://www.ensembl.org/Gallus_gallus), and were able to assemble a sequence that spanned the cCRF gene (contig 299.7.1.2353; coding sequence: bp 114528083–114528559 on chromosome 2). The cCRF gene is located on chromosome 2 and contains two exons and a single intron of 200 bp in length (Fig. 2, A and B). The presence of this intron was verified by a RT-PCR using a sense primer in exon 1 and an antisense primer in exon 2. This PCR reaction resulted in a 648-bp DNA fragment. Comparison of this DNA fragment with the genome sequence showed the presence of the intron and allowed us to determine the exact intron/exon boundaries, which are indicated in Fig. 2B. We found a putative TATAA and CAAT box upstream from the prohormone coding region and two potential polyadenylation sites downstream (Fig. 2B). Figure 2B also shows the found putative conserved *cis*-acting DNA elements, which included two cAMP-responsive elements in the promoter area and a repressor element-1 silencing transcription factor or neuron-restrictive silencer factor in the intron sequence.

The cDNA sequence obtained by the total of all RT-PCR and RACE reactions has been submitted to the European Molecular Biology Laboratory database under accession number AJ621492.

Developmental changes in CRF peptide content in the diencephalon and ME

The CRF peptide content in the diencephalon without the ME exhibited significant changes during the last week of

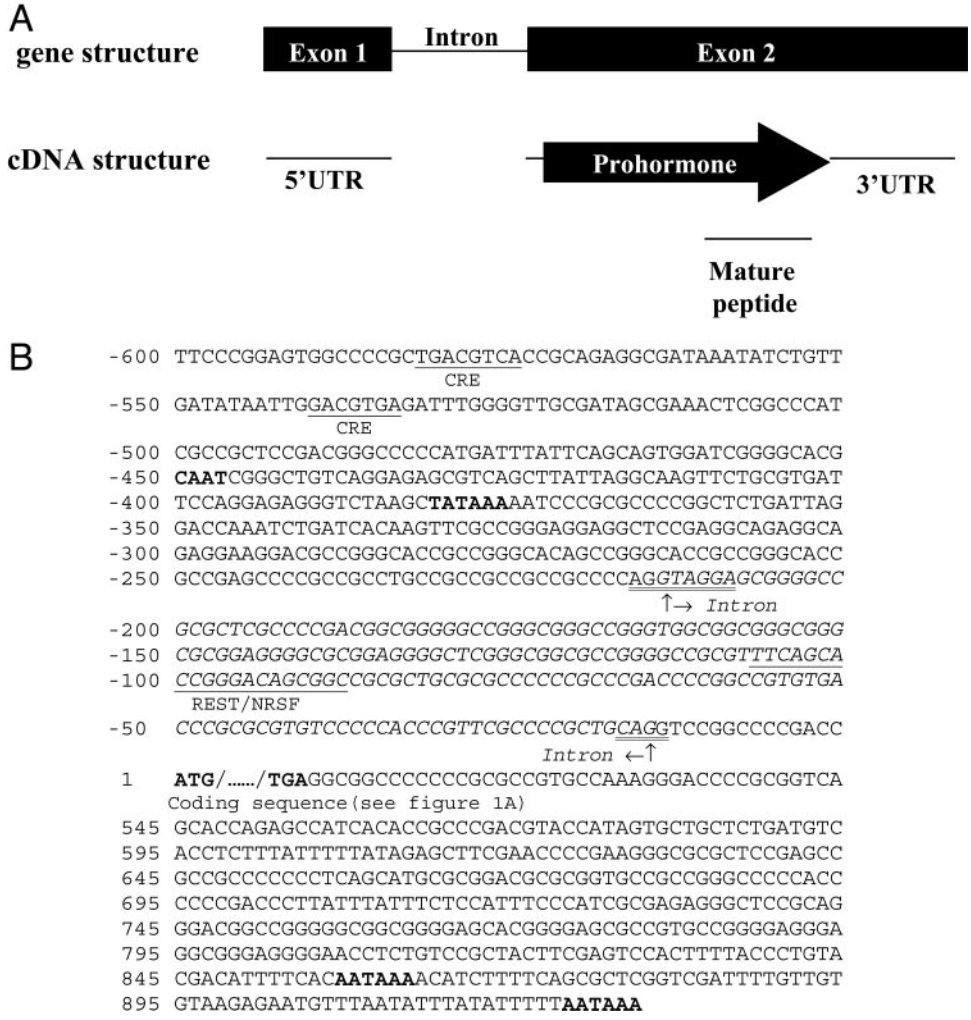

FIG. 2. Schematic representation of the gene and cDNA structure of chicken proCRF (A) and the CRF gene nucleotide sequence (B) as it was determined by *in silico* analysis of the chicken genome database. Start and stop codons and the potential consensus sequences (TATAA and CAAT box in the 5′UTR and polyadenylation sites in the 3′UTR) are printed in *bold*. Putative conserved *cis*-acting DNA elements are *underlined* [cAMP-responsive elements (CREs) in the promoter area and repressor element-1 silencing transcription factor (REST)/neuron-restrictive silencer factor (NRSF) in the intron sequence]. The intron sequence is printed in *italic*. The conserved splice sites at the exon/intron boundaries are *double underlined*. For coding sequence, see Fig. 1A. UTR, Untranslated region.

embryonic development (F = 15.70, $P = 0.0001$; ANOVA). CRF peptide content increased significantly just before hatching on E20 and remained elevated after hatching (Fig. 3A). By contrast, there was a striking decrease in CRF content in the ME (F = 19.24, $P = 0.0001$; ANOVA) from E16 through E20. This was followed by a robust increase in ME CRF content after hatching to a level approximately 2 times that observed on E16 (Fig. 3B).

ProCRF, PC2, and POMC mRNA expression in the chick diencephalon during the last week of embryonic development

The proCRF mRNA level in the chick diencephalon exhibited significant changes during the last week of embryonic development (F = 7.55, $P = 0.0135$; ANOVA). As shown in Fig. 4, CRF mRNA levels in the diencephalon remained constant from E16–E18 but then decreased significantly on E20. CRF mRNA levels on the first day after hatching (C1) were comparable to those on E16 and E18. By contrast, PC2 and POMC mRNA levels exhibited no statistically significant changes during the last week of embryonic development (data not shown).

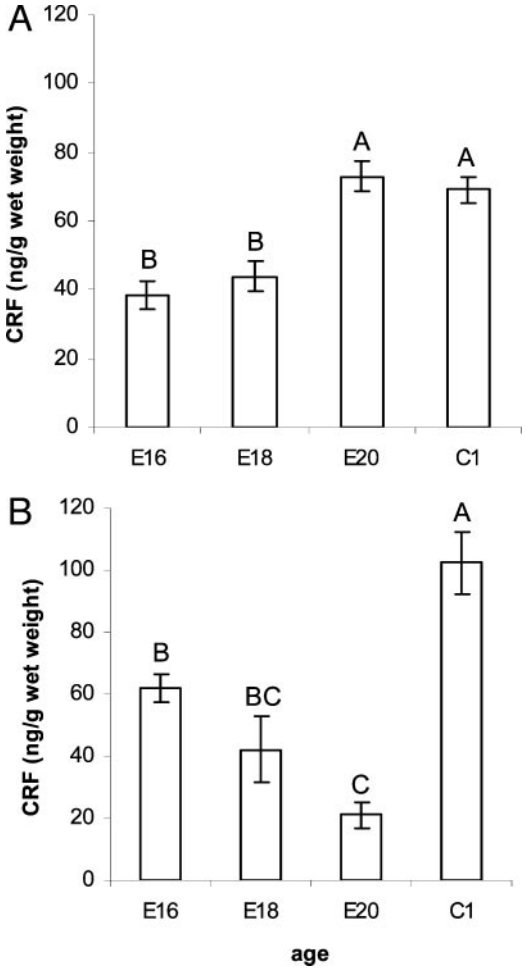

FIG. 3. Amount of CRF (ng/g wet weight) in the diencephalon without ME (n = 8–10) (A) and in the ME (n = 3–5 pools of 5) (B) during the last week of chicken embryonic development. Data shown represent the mean ± SEM. Data with a *common letter* are not significantly different (Scheffé's test, $P < 0.05$).

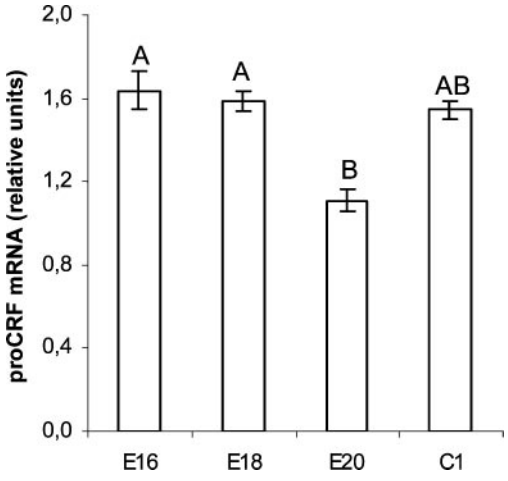

FIG. 4. Levels of proCRF mRNA in the diencephalon (n = 3) during the last week of chicken embryonic development. Data shown represent the mean expression of proCRH mRNA ± SEM. These data are expressed in relative units because they represent the proCRF expression after normalization with β-actin mRNA expression. Data with a *common letter* are not significantly different (Scheffé's test, $P < 0.05$).

Developmental changes in plasma corticosterone

Figure 5 shows the concentration of corticosterone in the plasma of chicken embryos during the last week of embryonic development. There is a trend for a decrease from E16–E20, but this was not statistically significant. However, we found a statistically significant ($P < 0.05$) increase in plasma corticosterone from E20 to C1.

Discussion

Earlier studies showed that the chicken HPT and HPA axes increase their activity during the last week of embryonic development (1, 2). Because CRF has been shown to be a central regulator of both the HPT and HPA axes in the chicken (3, 4), we hypothesized that this neurohormone might play a role in the endocrine control of the hatching

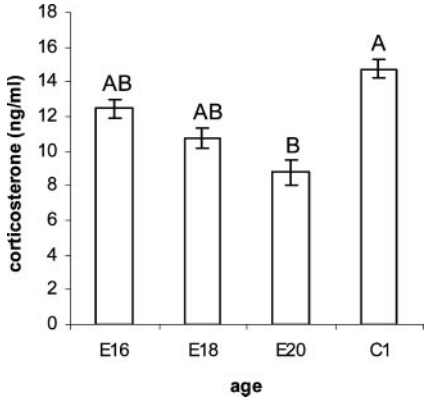

FIG. 5. Concentration of corticosterone in the plasma of chickens during the last week of chicken embryonic development. Data shown represent the mean ± SEM (n = 8–10). Data with a *common letter* are not significantly different (Scheffé's test, $P < 0.05$).

process. Therefore, we have characterized the cCRF gene and have analyzed its mRNA and peptide expression during the last week of embryonic development. Molecular cloning and genomic analysis showed that the structure of the cCRF gene is similar to other vertebrate CRF genes, containing two exons separated by a single intron. The chicken intron (200 bp) is considerably smaller than the proCRF introns in other vertebrates (*e.g.* human, 798 bp; and *Xenopus*, 577 bp). Upstream from the coding region, we found two putative cAMP-responsive element signals and a potential CAAT and TATAA box. In the intron, a possible repressor element-1 silencing transcription factor/neuron-restrictive silencer factor sequence was detected, and downstream from the stop codon, we found two putative polyadenylation sites. Based on the deduced amino acid sequence derived from the cDNA, we found that the primary structure of the cCRF prohormone is similar to other known vertebrate proCRFs. Translation of the cDNA showed that the N terminus contains a signal peptide sequence (25 amino acids) that is highly conserved throughout tetrapod evolution. A 99-amino acid long cryptic peptide follows, containing a highly conserved region of 15 amino acids with an unknown function. The mature peptide (41 amino acids) is located at the C terminus and is separated from the cryptic peptide by a conserved dibasic cleavage site (Lys-Arg). The primary structure of the mature cCRF peptide was found to be identical to r/hCRF. We thus designate the mature peptide r/h/cCRF. Compared with the amino acid sequence of ovine (o) CRF, the peptide that was commonly used in earlier experiments in chickens, we find an 83% sequence similarity (seven of 41 amino acids of oCRF are different from r/h/cCRF). It is unlikely that these amino acid differences in oCRF would result in differences in the tertiary structure of the peptide (compared with r/h/cCRF) because the individual amino acid differences are predominantly conservative (Gln for Glu, Thr for Ala, Lys for Arg, Asp for Glu, Leu for Met, and Ala for Ile). Nevertheless, future experiments with CRF in chickens should ideally be conducted using the homologous and commercially available r/h/cCRF.

Recently, the distribution of CRF throughout the chicken brain was studied using an antibody raised in rabbit against the entire h/rCRF sequence (28, 29). CRF-immunoreactivity (ir) was found in hypothalamic and extrahypothalamic regions of the brain. The presence of CRF-ir perikarya in the PVN and of a dense CRF-ir field in the ME confirms that CRF is present in the avian hypothalamic-pituitary system. The extrahypothalamic avian distribution of CRF-ir perikarya and fibers parallels that described for mammals and suggests that, as in mammals, CRF may be directly involved in the control of some aspects of stress and anxiety responses in birds, in addition to its role in the control of the HPA. We can conclude here that CRF has been extremely conserved throughout the tetrapod evolution, not only concerning its sequence, but also concerning its distribution throughout the brain and, therefore, most probably also its biological functions.

This is the first study to analyze changes in CRF peptide content in the diencephalon and ME of the chicken. We found an increase in CRF peptide content in the diencephalon and a decrease in the ME just before hatching. The increase in the diencephalon could reflect increased CRF biosynthesis, whereas the decrease in the ME could reflect increased secretion. This would suggest that the rate of CRF biosynthesis increases as CRF release increases. The increase in plasma corticosterone in C1 chicks would be consistent with this interpretation. In mammals, CRF neurons have been shown to be activated in response to a variety of stressors, and this activation results in an increase in peptide content in CRF perikarya, a decrease in ME peptide content, and an increase in CRF gene transcription (30–33). During the final days before hatching, a rise in plasma T_4, T_3, and corticosterone has been reported in literature (1, 2). Therefore, the increase in CRF in the diencephalon and the decrease in the ME reported in our experiments can be causally related to the regulation of the HPT and HPA axes at this stage of chicken development.

We used the cCRF cDNA sequence to develop a semiquantitative RT-PCR assay to measure CRF mRNA levels in chicken tissues. Contrary to the CRF peptide measurements discussed earlier, we observed a decrease in CRF mRNA in the diencephalon just before hatching. Steady-state mRNA levels reflect a balance between transcription and mRNA turnover. It is likely that the mRNA level is an imperfect measure of CRF gene transcription in the chicken, as has been shown in mammals. For example, *in situ* hybridization histochemistry using intronic probes to detect CRF heteronuclear (hn) RNA has demonstrated robust stress-induced transcriptional activation of CRF genes in rodents (31, 33, 34). However, the strong increases in hnRNA are not paralleled by similarly large increases in mRNA levels. It is also possible that the decrease in CRF mRNA that we observed reflects an increased rate of turnover associated with a higher rate of translation (as evidenced by the increased CRF peptide content at this time). Future studies of CRF hnRNA synthesis and mRNA turnover in the chicken will be required to address these issues.

The developmental pattern observed for CRF mRNA in the diencephalon can also be correlated with the so-called stress hyporesponsive period (SHRP) described in rodents just before and after birth. In rats, hypothalamic CRF mRNA levels showed a marked decrease during the perinatal period and increased again after the first postnatal week (35, 36). It has been suggested that the SHRP functions as an adaptive mechanism to tightly control glucocorticoid levels in the neonate. In chickens, a lack of response to stress in the newly hatched chick has also been reported, with the response being slowly re-established after 1–2 d (37). This short period of low CRF mRNA levels is, however, not consistent with the increasing levels of CRF protein we observed in the diencephalon. We could postulate that CRF is differentially regulated in distinct brain nuclei during development, which has been observed in rodents. In the mouse PVN, a perinatal decrease in CRF mRNA was found, correlating with the SHRP (38). In Barrington's nucleus, however, CRF expression remained high during the SHRP. CRF located in nuclei other than the PVN, like Barrington's nucleus, is thought to act as a neuromodulator or neurotransmitter. The presence of CRF in extrahypothalamic regions of the brain is also described in birds (29), suggesting, as stated earlier, that avian CRF might also play a role in physiological and be-

havioral responses other than the control of ACTH release by the pituitary. Therefore, it is possible that, in our experiment, the levels of CRF protein and mRNA show a different developmental pattern in very precise regions of the brain that cannot be detected when measuring CRF in the total diencephalon. Therefore, in future experiments, it would be interesting to investigate levels of CRF mRNA and protein in different nuclei of the brain.

No significant changes were observed in PC2 and POMC mRNA levels during the last week of embryonic development in the chicken. PC2 mRNA levels were measured as a potential measure of CRF prohormone processing, which is probably not affected during the final week before hatching. POMC mRNA levels were measured as a proxy for changes in ACTH, but we recognize that this may be an imperfect measure given that mRNA levels may neither accurately reflect differences in transcription nor include translation or peptide processing. The increase in plasma corticosterone in C1 chicks suggests that circulating ACTH increased, although this was not reflected in a change in POMC mRNA levels in the diencephalon. However, it is possible that small changes in its expression cannot be detected by semiquantitative RT-PCR because the steady-state levels of POMC mRNA in the anterior pituitary are very high.

In summary, we have deduced the amino acid sequence of proCRF in the chicken, which is the first archosaur from which the CRF gene has been characterized. The cCRF is 100% identical to r/hCRF. During the final week before hatching, the CRF peptide content increased in the diencephalon but decreased in the ME, suggesting that release and biosynthesis are coupled. This was followed by an increase in plasma corticosterone in C1 chicks. However, CRF mRNA levels in the diencephalon decreased just before hatching. We hypothesize that this reflects increased mRNA turnover associated with increased proCRF translation, although this could also reflect a state of stress hyporesponsiveness. Changes in CRF production just before hatching may be causally related to the regulation of the thyroid and interrenal axes during the final days before hatching.

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