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Expression of Chicken Hepatic Type I and Type III Iodothyronine Deiodinases during Embryonic Development*

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ABSTRACT

In embryonic chicken liver (ECL) two types of iodothyronine deiodinases are expressed: D1 and D3. D1 catalyzes the activation as well as the inactivation of thyroid hormone by outer and inner ring deiodination, respectively. D3 only catalyzes inner ring deiodination. D1 and D3 have been cloned from mammals and amphibians and shown to contain a selenocysteine (Sec) residue. We characterized chicken D1 and D3 complementary DNAs (cDNAs) and studied the expression of hepatic D1 and D3 messenger RNAs (mRNAs) during embryonic development. Oligonucleotides based on two amino acid sequences strongly conserved in the different deiodinases (NFGSCTSecP and YIEEAH) were used for reverse transcription-PCR of poly(A^+) RNA isolated from embryonic day 17 (E17) chicken liver, resulting in the amplification of two 117-bp DNA fragments. Screening of an E17 chicken liver cDNA library with these probes led to the isolation of two cDNA clones, ECL1711 and ECL1715. The ECL1711 clone was 1360 bp long and lacked a translation start site. Sequence alignment showed that it shared highest sequence identity with D1s from other vertebrates and that the coding sequence probably lacked the first five nucleotides. An ATG start codon was engineered by site-directed mutagenesis, generating a mutant (ECL1711M) with four additional codons (coding for MGTR). The open reading frame of ECL1711M coded for a 249-amino acid protein showing 58–62% identity with mammalian D1s. An in-frame TGA codon was located at position 127, which is translated as Sec in the presence of a Sec insertion sequence (SECIS) identified in the 3′-untranslated region. Enzyme activity expressed in COS-1 cells by transfection with ECL1711M showed the same catalytic, substrate, and inhibitor specificities as native chicken D1. The ECL1715 clone was 1366 bp long and also lacked a translation start site. Sequence alignment showed that it was most homologous with D3 from other species and that the coding sequence lacked approximately the first 46 nucleotides. The deduced amino acid sequence showed 62–72% identity with the D3 sequences from other species, including a putative Sec residue at a corresponding position. The 3′-untranslated region of ECL1715 also contained a SECIS element. These results indicate that ECL1711 and ECL1715 are near-full-length cDNA clones for chicken D1 and D3 selenoproteins, respectively. The ontogeny of D1 and D3 expression in chicken liver was studied between E14 and 1 day after hatching (C1). D1 activity showed a gradual increase from E14 until C1, whereas D1 mRNA level remained relatively constant. D3 activity and mRNA level were highly significantly correlated, showing an increase from E14 to E17 and a strong decrease thereafter. These results suggest that the regulation of chicken hepatic D3 expression during embryonic development occurs predominantly at the pretranslational level. (*Endocrinology* **138:** 5144–5152, 1997)

IN MOST vertebrates the thyroid secretes predominantly T_4, a prohormone with little or no biological activity. The activation of T_4 occurs in peripheral tissues by outer ring deiodination (ORD) to T_3. Two iodothyronine deiodinases, D1 and D2, are responsible for this conversion. Inactivation of T_4 and T_3 by inner ring deiodination (IRD) to rT_3 and 3,3′-diiodothyronine (3, 3′-T_2), respectively, is catalyzed by D1 and D3 (1). In recent years, the different deiodinases have been cloned from rat (2–4), human (5–7), mouse (8), dog (9), *Rana catesbeiana* (10, 11), and *Xenopus laevis* (12). They exhibit significant sequence homology and contain catalytically essential selenocysteine (Sec) residues. In addition, we have recently characterized D1 and D3 from a teleost fish (tilapia) (13),[1] whereas the cloning of D2 from *Fundulus heteroclitus* has recently also been reported (14). Information concerning other vertebrates is lacking, as bird and reptile deiodinases have not yet been cloned.

It is well known that thyroid hormone plays an important role in many crucial developmental events in all vertebrate classes. In birds, this hormone is essential for yolk sac retraction, functional maturation of the lungs, pipping (penetration of the air sac), and hatching (15). Decuypere *et al.* (16, 17) showed that plasma T_3 increases dramatically at the moment of pipping, when the embryo switches from allantoic to lung respiration. This is correlated with a decrease in hepatic D3 activity rather than with an increased D1 activity,

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[1] Sanders, J. P., S. Van der Geyten, E. Kaptein, V. M. Darras, E. R. Kühn, J. L. Leonard, and T. J. Visser, manuscript in preparation.

suggesting that the peak in plasma T_3 at the end of incubation is caused by a decrease in its hepatic breakdown (18). Later studies showed that the increase in plasma GH, also observed at the end of incubation, plays an important role in the suppression of hepatic D3 activity (19, 20).

In this study we generated complementary DNA (cDNA) fragments by reverse transcription-PCR (RT-PCR) of polyadenylated RNA from embryonic day 17 (E17) chicken liver using primers based on the conserved amino acid sequences NFGSCTSecP and YIEEAH. Using these RT-PCR products as probes, we isolated two cDNA clones from an E17 chicken liver cDNA library, each of which appears to code for a Sec-containing protein. One clone shows highest sequence homology with mammalian D1 cDNAs and expresses characteristic D1 activity after transfection into COS-1 cells. The other clone, which lacks approximately the first 16 codons, shows high homology with mammalian and amphibian D3 cDNAs. These two cDNA clones were used in the study of D1 and D3 expression in chicken liver during embryonic development.

Materials and Methods

Materials

TRIzol reagent was obtained from Life Technologies (Breda, The Netherlands); oligo(deoxythymidine)-cellulose [oligo(dT)cellulose] was purchased from New England Biolabs (Beverly, MA); SuperTaq DNA polymerase was obtained from HT Biotechnology (Cambridge, UK); AMV reverse transcriptase and pCI-Neo were purchased from Promega (Madison, WI); Klenow DNA polymerase was obtained from Boehringer Mannheim (Mannheim, Germany); pCR-II was obtained from Invitrogen (San Diego, CA); synthetic oligonucleotides were obtained from Pharmacia Biotech (Roosendaal, The Netherlands) or Life Technologies; Hybond membranes and [α-^{32}P]deoxy-ATP were purchased from Amersham (Aylesbury, UK); polyethylene glycol (PEG6000) was obtained from Merck (Hohenbrunn, Germany); diethylaminoethyl-dextran and Sephadex LH-20 were purchased from Pharmacia. Nonradioactive iodothyronines were obtained from Henning Berlin R&D (Berlin, Germany), [3′,5′-^{125}I]T_4 (~1200 Ci/mmol) was obtained from Amersham, and [3,5-^{125}I]T_3 (~35 Ci/mmol) was obtained from Mr. R. Thoma (Formula, Berlin, Germany) courtesy of Dr. G. Decker (Henning Berlin). [3′,5′-^{125}I]rT_3 (~2000 Ci/mmol) and [3,5-^{125}I]T_3 sulfate ([3,5-^{125}I]T_3S) were prepared in our laboratory as described previously (21, 22). 6-*n*-Propyl-2-thiouracil (PTU), iodoacetate (IAc), gold thioglucose (GTG), dithiothreitol (DTT), and chloroquine were obtained from Sigma Chemical Co. (St. Louis, MO). All other reagents were of the highest purity commercially available.

Animals

Fertilized chicken eggs from a rapidly growing broiler strain (Hybro) were purchased from Euribrid (Aarschot, Belgium) and incubated in a laboratory incubator as described previously (18). The start of incubation was defined as E1. Animals were killed between day 14 (E14) and day 20 (E20) of embryonic development, on day 21 just after hatching (C0), and 1 day after hatching (C1). Of the animals killed on E20, approximately half were in the nonpipping stage, and the other half were in the internal pipping stage. Livers were isolated, frozen in liquid nitrogen, pooled, and stored at −80 C until further processing. Livers were pooled from seven (E14), five (E15), four (E16), three (E17), and two (E18–C2) animals. Blood was collected by cardiac puncture (embryos) or decapitation (chicks). Plasma pools from two animals (E14 and E15) or individual plasma samples (E16–C2) were stored at −20 C until hormone analysis. The experimental protocol was approved by the ethical experimental animal committee of the K. U. Leuven.

Cloning

Total RNA was isolated from embryonic chicken liver (ECL) by homogenization of tissue in TRIzol reagent, and polyadenylated RNA was isolated on oligo(dT)-cellulose. Oligo(dT)-primed cDNA was obtained using AMV reverse transcriptase. PCR was performed using the upstream primer 5′-AATTTTGGCAGTTGTACCTGACC-3′, the downstream primer 5′-RTGIGCTTCCTCIATGTA-3′, and SuperTaq DNA polymerase. The products were isolated and ligated into pCR-II. Two different clones were isolated and sequenced, showing that the inserts were homologous to known deiodinases. An E17 chicken liver cDNA library was constructed in Lambda ZAP-Express (Stratagene, La Jolla, CA). The library was blotted on Hybond-N^+ and screened with the RT-PCR products labeled by extension of the PCR primers using Klenow DNA polymerase in the presence of [α-^{32}P]deoxy-ATP. The phagemids carried in selected positive bacteriophages were excised, generating cDNA clones in pBK-CMV. cDNA inserts were then sequenced manually and by automatic sequencing in both directions using the dideoxy method of Sanger *et al.* (23).

Transient transfection

The cDNA encoding chicken D1 (ECL1711) was subcloned from pBK-CMV into pCI-Neo using *Eco*RI and *Not*I. Both clones isolated (ECL1711 and ECL1715) were 5′-truncated based on alignment with known deiodinases from other vertebrates (2, 3, 5, 6, 8–10, 12), with coding sequences that presumably lacked the first 5 and 46 nucleotides, respectively. A eukaryotic translation start site (24) was engineered in ECL1711 at the *Eco*RI restriction site by site-directed mutagenesis using the oligonucleotide 5′-GCTAGCCTCGAGAAATGGGCACGAGGTTGA-3′ and the MORPH kit (5Prime→3Prime, Boulder, CO), yielding the mutant ECL1711M. This mutation, which was confirmed by sequencing, created an ATG start codon in a Kozak consensus sequence (24), and resulted in a four-amino acid (MGTR) addition to the N-terminus of the amino acid sequence encoded by the cDNA (see Fig. 1). RNA secondary structure prediction was performed using the MFOLD program provided by Dr. M. Zuker (Institute for Biomedical Computing, Washington University, St. Louis, MO) on the Internet (http:/www.ibc.wustl.edu/~zuker) (25).

Expression

ECL1711M was expressed in COS-1 cells grown in DMEM-Ham's F-12 (DMEM/F12) containing 10% FCS (Life Technologies) and 40 nM Na_2SeO_3 (26). One day before transfection, COS-1 cells were seeded at 50% confluency in 55-cm^2 cell culture dishes. ECL1711M in pCI-Neo (7 μg), isolated by alkaline lysis and polyethylene glycol precipitation (27), was added to serum-free DMEM/F12 medium containing 100 μg/ml diethylaminoethyl-dextran. After 2 h, the medium was replaced by serum-free DMEM/F12 medium containing 100 μM chloroquine, and the cells were incubated for an additional 2 h. The medium was then replaced with fresh DMEM/F12 medium containing 10% FCS and 40 nM Na_2SO_3. After 3 days, the cells were rinsed with PBS, collected in 0.3 ml 0.1 M sodium phosphate buffer (pH 6.9) containing 1 mM EDTA and 10 mM DTT, sonicated, snap-frozen on dry-ice/ethanol, and stored at −80 C.

Northern analysis

Total tissue RNA (20 μg/lane) was separated on 1% (wt/vol) formaldehyde-agarose gels and blotted onto Hybond-N membranes. Blots were hybridized at 60 C with random hexamer-primed ^{32}P-labeled cDNA probes in 6 × SSC (standard saline citrate), 0.5% SDS, 5 × Denhardt's solution (1X Denhardt's solution = 0.2% Ficoll, 0.2% BSA, and 0.2% polyvinylpyrrolidone), and denatured salmon sperm DNA (100 μg/ml). Blots were washed twice for 15 min each time at 55 C with 3 × SSC-0.5% SDS and twice for 15 min each time at 60 C with 1 × SSC-0.5% SDS. Autoradiographs were scanned using a Hewlett-Packard Scanjet IIcx (Palo Alto, CA), and signals were quantified using software developed by Dr. R. Docter (Department of Internal Medicine III, Erasmus University Medical School, Rotterdam, The Netherlands). Photographs of the ethidium bromide-stained gels were analyzed similarly, indicating less than 20% variation in the amount of applied RNA.

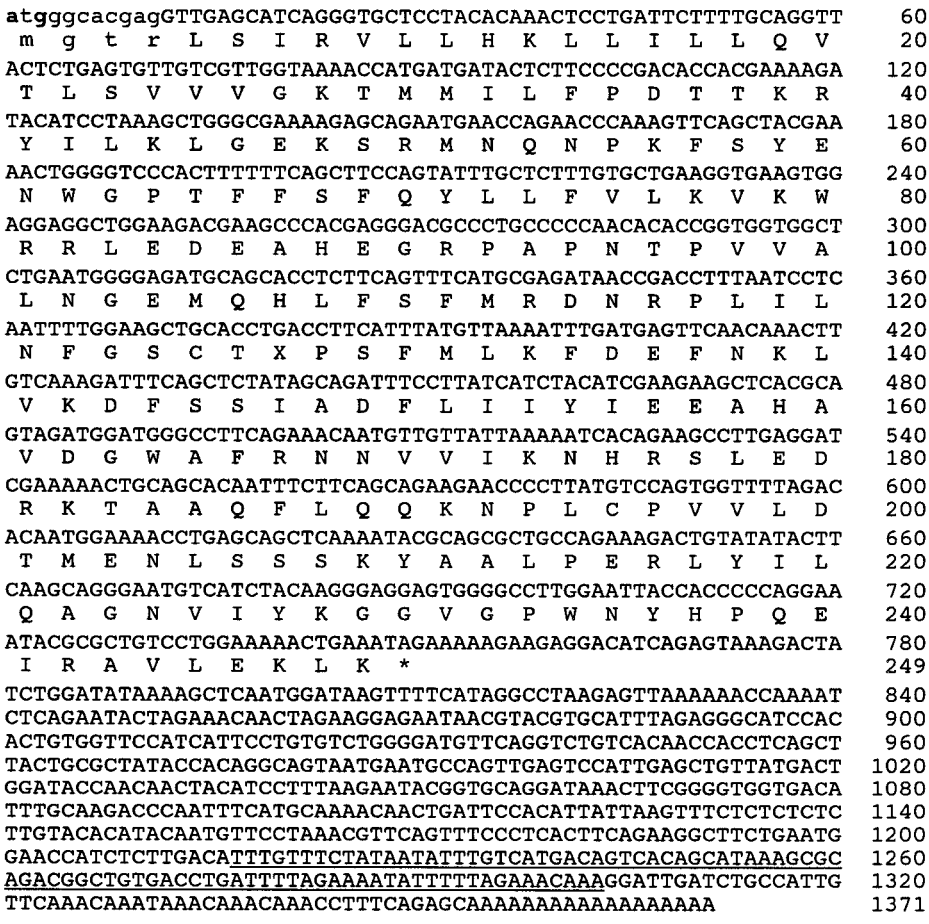

FIG. 1. Nucleotide and deduced amino acid sequence of ECL1711M cDNA. Lower case nucleotides are derived from the vector. The bold nucleotides **a** and **g** were substituted for **t** and **c**, respectively, by site-directed mutagenesis of ECL1711 cDNA as described in *Materials and Methods*. The Sec residue is denoted by X. The putative SECIS element in the 3′UTR is *underlined*.

Enzyme assays

Liver microsomal fractions were prepared as described previously (18). Deiodinase activities of native and recombinant enzyme preparations were determined by measuring the radioiodide released from either [3′,5′-^{125}I]T_4 or [3′,5′-^{125}I]rT_3 by ORD, or from [3,5-^{125}I]T_3 or [3,5-^{125}I]T_3S by IRD (21, 22). In short, 0.5–200 μg tissue or lysate protein were incubated in triplicate for 20–60 min at 37 C with 10 or 100 nM [^{125}I]substrate in 0.2 ml 0.1 M sodium phosphate buffer (pH 7.2), 2 mM EDTA, and 10 mM DTT. Reactions were stopped, and [^{125}I]iodothyronines were precipitated by successive addition of 0.1 ml 5% BSA and 0.5 ml 10% trichloroacetic acid. Radioiodide was further isolated from the supernatant on Sephadex LH-20 minicolumns (21, 22). The characteristics of the deiodinase activity expressed in ECL1711M-transfected COS-1 cells were compared with those determined in E19 chicken liver microsomes. For the developmental study, D1 and D3 activities were assayed as described by Darras *et al.* (18). For D1 activity, incubation mixtures contained 1 μM [3′,5′-^{125}I]rT_3, 50 μg/ml microsomal protein, and 5 mM DTT. For D3 activity, incubations contained 10 nM [3′-^{125}I]T_3, 10 or 500 μg/ml microsomal protein, 1 μM rT_3, 0.1 mM PTU, and 50 mM DTT.

Miscellaneous

Plasma T_3 and T_4 were measured as described by Darras *et al.* (28, 29). Unless indicated otherwise, the results presented are taken from representative experiments and were reproduced in at least two other experiments.

Results

Based on homology between conserved amino acid sequences in known deiodinases (2, 5, 9, 12), oligonucleotide primers were designed corresponding to the conserved amino acid sequences NFGSCTSecP and YIEEAH. These primers were used to amplify two cDNA fragments, ECL17a and ECL17b, by RT-PCR. Both fragments were 117 bp long and showed high homology with corresponding regions of other reported iodothyronine deiodinase nucleotide sequences (2, 5, 9, 12). ECL17a and ECL17b were then used as probes to screen an E17 chicken liver cDNA library (120,000 independent clones). This resulted in the identification of 20 (ECL17a) and 8 (ECL17b) positive clones from a total of 500,000 plaque-forming units of the amplified cDNA library. The longest clones were identified after PCR with vector-specific and RT-PCR product-specific primers. The cryptic pBK-CMV eukaryotic expression plasmids containing the cDNA inserts were excised from the bacteriophage and sequenced.

Chicken D1

The longest cDNA clone identified after screening with ECL17a (clone ECL1711) was 1360 bp long, but it lacked a translation start site (Fig. 1). Alignment with the reported sequences of other deiodinases showed that it shared highest sequence identity with D1s from other species (2, 5, 7, 9), and based on optimal nucleotide alignment, this clone presumably lacked the first five nucleotides of the coding sequence. After subcloning of ECL1711 into pCI-Neo, an ATG start codon in a Kozak consensus sequence (24) was engineered by

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Chi     MGTRLSIRVL-LHKLLILLQVTLSVVVGKTMMILFPDTTKRYILKLGEKSRMNQNPKFSY     59
Rat     ..--..qlw.w.kr.v.f...a.e.at..vl.t...erv.qn..am.q.tg.tr..r.ap     58
Hum     ..--.pqpg.w.kr.wv..e.avh.....vll.....rv..n..am...tg.tr..h..h     58
Dog     ..--.prp..w.rr.wv....avq.a...vflk...arv.qh.vamng.-----..h...     53

Chi     ENWGPTFFSFQYLLFVLKVKWRRLEDEAHEGRPAPNTPVVALNGEMQHLFSFMRDNRPLI    119
Rat     d..v.....i..fw.....r.q....r.ey.gl...ct..r.s.qkcnvwd.iqgs...v    118
Hum     d..i.....t..fw.....r.q....ttel.gl...c...r.s.qrcniwe..qg....v    118
Dog     d..a..ly.m..fw.....q.q....rtep.gl...c...r.s.qrcniwd..qg....v    113

Chi     LNFGSCTXPSFMLKFDEFNKLVKDFSSIADFLIIYIEEAHAVDGWAFRNNVVIKNHRSLE    179
Rat     ..........l....q.kr..d..a.t.............t.....k...d.rq....q    178
Hum     ..........f...q.kr.ie.........v........s.....k..md.r..qn.q    178
Dog     ..........lf...q.kr.ie..c.t.............s.....k...n.rt.qt.q    173

Chi     DRKTAAQFLQQKNPLCPVVLDTMENLSSSKYAALPERLYILQAGNVIYKGGVGPWNYHPQ    239
Rat     ..lr..hl.lars.q....v...q.q..ql.........vi.e.ric...kp.....n.e    238
Hum     ..lq..hl.lars.q....v...q.q..ql..........i.e.ril...ks.....n.e    238
Dog     ..lq..rl.ldra.p....v...r.q..qf........fv..e.ril...kp.......e    233

Chi     EIRAVLEKLK                                                      249
Rat     .v.......cippghmpqf                                             257
Hum     .v.......hs                                                     249
Dog     .v.......hs                                                     244
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FIG. 2. Alignment of deduced amino acid sequences of chicken, rat, human, and dog D1. The Sec residue is denoted by X. Identical amino acids are indicated by *dots*, and gaps are indicated by *hyphens*. Bold amino acids indicate the additional residues engineered to the N-terminus of ECL1711.

site-directed mutagenesis at the *Eco*RI restriction site, expanding the coding sequence by four codons (coding for MGTR), which then is probably two codons longer than the authentic coding sequence. Figure 2 shows the alignment of the deduced amino acid sequence of the mutant, ECL1711M, with those of other D1s, indicating that it has 62% amino acid identity with human D1 (5), 60% identity with rat D1 (2), and 58% identity with dog D1 (9). ECL1711M also contains an in-frame TGA codon at position 127, *i.e.* at the corresponding position as in the other D1s (Fig. 1). UGA usually functions as a translation stop codon, but is translated as Sec when a Sec insertion sequence (SECIS) element is present in the 3′-untranslated region (3′UTR) of selenoprotein-coding messenger RNAs (mRNAs) (30–32). A putative SECIS element is located between nucleotides 1217–1303 in ECL1711M (Fig. 1).

Transient expression of the original ECL1711 carried in pCI-Neo in COS-1 cells did not result in the synthesis of a functional deiodinase. However, COS-1 cells transfected with ECL1711M in the same vector expressed high deiodinase activity. Incubation of lysates of ECL1711M-transfected cells with 10 nM ^{125}I-labeled substrates in the presence of 10 mM DTT resulted in significant ORD of T_4 and rT_3 and IRD of T_3 and T_3S (Fig. 3). Rates of iodothyronine deiodination decreased in the order $rT_3 > T_3S > T_4 > T_3$, which is identical with the substrate preference demonstrated by native chicken D1 in E19 chicken liver microsomes (Fig. 3).

Further characterization of the protein encoded by ECL1711M was performed by determining the effects of substrate analogs and typical D1 inhibitors on the deiodination of the preferred substrate rT_3, and the results were compared with those obtained using E19 liver microsomes as a source of native D1. Increasing concentrations (0.1–10 μM) of rT_3, T_4, and T_3 resulted in the progressive and identical inhibition of the ORD of [3′,5′-^{125}I]rT_3 by ECL17711M-expressed and native D1 activity (Fig. 4). The potencies by which the different unlabeled iodothyronines inhibited the ORD of [^{125}I]rT_3 de-

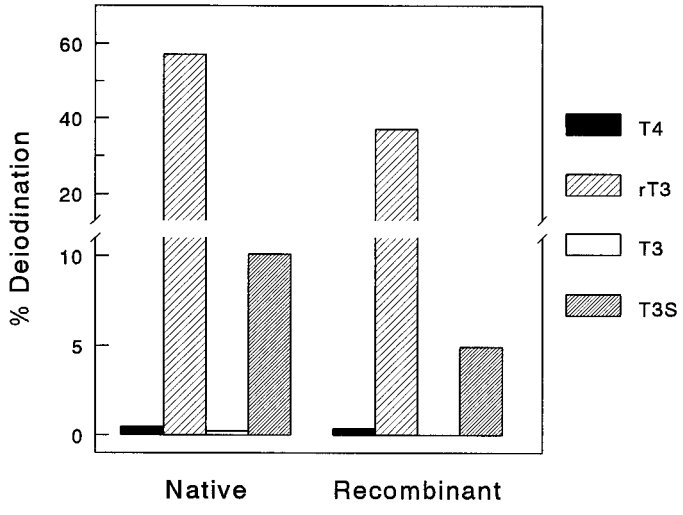

FIG. 3. ORD of T_4 and rT_3 and IRD of T_3 and T_3S by recombinant enzyme expressed in ECL1711M-transfected COS-1 cells and native enzyme in E19 chicken liver microsomes. Assay mixtures contained 10 nM substrate, 10 mM DTT, and 250 (lysate) or 10 (microsomes) μg protein/ml and were incubated for 60 min at 37 C.

creased in the order $rT_3 > T_4 > T_3$, with IC_{50} values of approximately 0.3, 2, and more than 10 μM, respectively. The K_m value for rT_3 calculated from Lineweaver-Burk plots was 0.26 μM for both ECL1711M-expressed enzyme and native chicken D1 (not shown).

Figure 5 shows that addition of 0.1–100 μM PTU or IAc or 0.01–10 μM GTG resulted in the dose-dependent and identical inhibition of the ORD of 0.1 μM rT_3 by the ECL1711M-encoded enzyme and native chicken D1 in the presence of 10 mM DTT. In both cases, the IC_{50} values were about 30 nM for GTG and about 2 μM for both PTU and IAc. Taken together, these data demonstrate that ECL1711 cDNA codes for chicken D1.

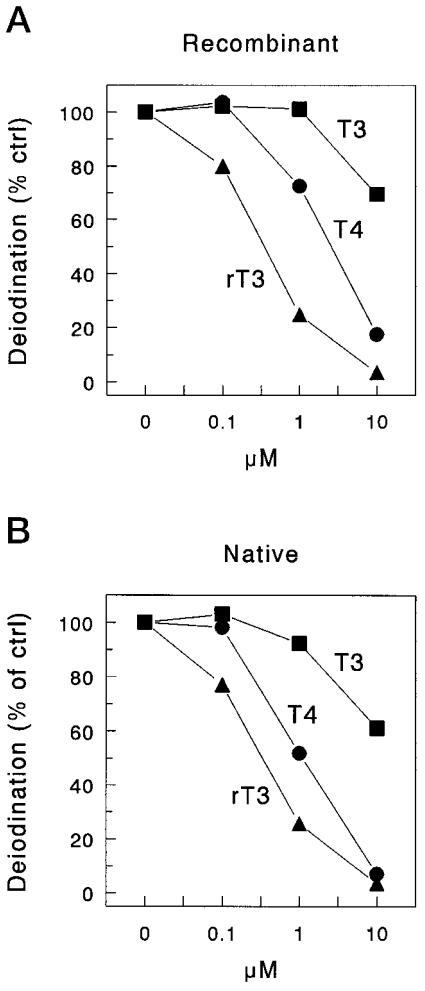

FIG. 4. Inhibition of the ORD of [3′,5′-^{125}I]rT_3 by recombinant enzyme expressed in ECL1711M-transfected COS-1 cells (A) or native enzyme in E19 chicken liver microsomes (B) by 0.1–10 μM unlabeled rT_3, T_4, or T_3. Assay mixtures contained 10 nM [3,5′-^{125}I]rT_3, 10 mM DTT, and 100 (lysate) or 10 (microsomes) μg protein/ml and were incubated for 60 (lysate) or 30 (microsomes) min at 37 C.

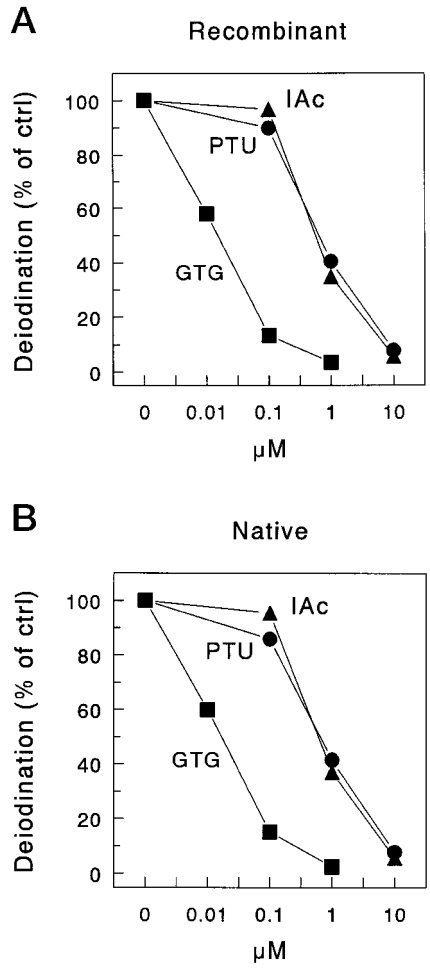

FIG. 5. Inhibition of the ORD of [3′,5′-^{125}I]rT_3 by recombinant enzyme expressed in ECL1711M-transfected COS-1 cells (A) or native enzyme in E19 chicken liver microsomes (B) by 0.1–100 μM PTU or IAc or 0.01–10 μM GTG. Assay mixtures contained 0.1 μM [3′,5′-^{125}I]rT_3, 10 mM DTT, and 100 (lysate) or 2.5 (microsomes) μg protein/ml and were incubated for 60 min at 37 C.

Chicken D3

Eight positive clones were identified by screening of the E17 chicken liver cDNA library with the ECL17b PCR fragment. The longest cDNA clone (ECL1715) was sequenced and found to be 1366 bp long, but unfortunately, it also lacked a translation start site (Fig. 6). Alignment of this sequence with the reported sequences of other deiodinases showed that ECL1715 shared sequence identity with D3s from other species (3, 6, 10, 12), and that the coding sequence presumably lacked the first 46 nucleotides. Alignment of the deduced amino acid sequence from ECL1715 with the sequences of other D3s indicated 62% amino acid identity with rat D3 (3), 63% identity with human D3 (6), 72% identity with *X. laevis* D3 (12), and 71% identity with *R. catesbeiana* D3 (10) (Fig. 7). The homology between ECL1715 and the other D3 sequences includes the presence of a Sec-encoding TGA codon at a corresponding position as well as a SECIS element in the 3′UTR (Fig. 7).

The 5′-truncation of ECL1715 was presumed to be too large to expect the expression of a functional deiodinase, even if a translation start site was engineered immediately upstream of this truncated sequence as was performed for the ECL1711 clone described above. To obtain a full-length clone, an additional 500,000 plagues were screened, but no clones longer than ECL1715 were identified. Attempts to complement the nucleotide sequence of ECL1715 by rapid amplification of 5′-cDNA ends also failed. However, the finding that the putative protein coded for by ECL1715 shows much greater homology with D3 than with D1 or D2 variants from other species (2–14) strongly suggests that it represents a near-full-length cDNA coding for chicken D3.

Ontogeny of D1 and D3 in the chicken

During the last week of embryonic development, important changes in circulating thyroid hormone were observed. Plasma T_3 levels increased dramatically after E18 until the moment of internal pipping (E20IP) and decreased somewhat until hatching (C0; Fig. 8). Plasma T_4 levels gradually increased from E14 until E18, and remained relatively con-

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CGGCCGCCTGCATCCTCCTCTTTCCCCGCTTCCTGCTCACCGCTGTGATGCTCTGGCTC      59
   A  A  C  I  L  L  F  P  R  F  L  L  T  A  V  M  L  W  L      19
CTGGATTTTCTGTGCATTCGCAAGAAGATGCTGACGATGCCCACGGCGGAGGAGGCGGCC    119
 L  D  F  L  C  I  R  K  K  M  L  T  M  P  T  A  E  E  A  A     39
GGAGCCGGCGAGGGGCCGCCCCCCGACGACCCTCCGGTCTGCGTGTCCGACTCCAACCGC    179
 G  A  G  E  G  P  P  P  D  D  P  P  V  C  V  S  D  S  N  R     59
ATGTTCACGCTGGAGTCGCTCAAGGCCGTGTGGCACGGGCAGAAGCTGGACTTCTTCAAG    239
 M  F  T  L  E  S  L  K  A  V  W  H  G  Q  K  L  D  F  F  K     79
TCGGCGCACGTGGGCTCGCCTGCCCCCAACCCCGAGGTGATCCAGCTGGACGGGCAGAAG    299
 S  A  H  V  G  S  P  A  P  N  P  E  V  I  Q  L  D  G  Q  K     99
AGGCTCCGCATCCTCGACTTCGCCCGCGGCAAGAGGCCCCTCATCCTCAACTTCGGCAGC    359
 R  L  R  I  L  D  F  A  R  G  K  R  P  L  I  L  N  F  G  S    119
TGCACCTGACCCCCGTTCATGGCCCGCCTGAGGTCCTTCCGGCGCCTGGCCGCGCACTTC    419
 C  T  X  P  P  F  M  A  R  L  R  S  F  R  R  L  A  A  H  F    139
GTGGACATTGCCGACTTCCTGCTGGTGTACATCGAAGAAGCGCACCCCTCCGACGGCTGG    479
 V  D  I  A  D  F  L  L  V  Y  I  E  E  A  H  P  S  D  G  W    159
GTCAGCTCGGACGCTGCCTACAGCATCCCCAAGCACCAGTGCCTCCAGGACAGGCTGCGG    539
 V  S  S  D  A  A  Y  S  I  P  K  H  Q  C  L  Q  D  R  L  R    179
GCGGCGCAGCTGATGCGGGAAGGGGCGCCCGATTGCCCCCTGGCCGTGGACACCATGGAC    599
 A  A  Q  L  M  R  E  G  A  P  D  C  P  L  A  V  D  T  M  D    199
AACGCTTCCAGCGCTGCCTACGCGGCCTACTTCGAGCGGCTCTACGTCATCCAGGAGGAG    659
 N  A  S  S  A  A  Y  A  A  Y  F  E  R  L  Y  V  I  Q  E  E    219
AAGGTGATGTACCAGGGCGGCCGAGGACCGGAGGGCTACAAGATCTCGGAGCTGCGGACG    719
 K  V  M  Y  Q  G  G  R  G  P  E  G  Y  K  I  S  E  L  R  T    239
TGGCTAGACCAGTACAAAACCCGGCTCCAGAGCCCCGGCGCGGTGGTCATCCAAGTGTAA    779
 W  L  D  Q  Y  K  T  R  L  Q  S  P  G  A  V  V  I  Q  V  *    259
AAAGGACCCGGCAGAAGGCGCGGGGGGAGAGGGGAGGGCGGGCGGAGGAACGCGGGGGGG    839
CTCCGCGCCGGGGCTGCGTGCGGGCGAGATGTGCCATAGTCCTTTCTCTATTCAAATCAT    899
GTTGATTTGCCAGCCACGCTCTGTCAATACTGTATTTCCATGTGCGTTTTGTAAATAACT    959
CCTTTTTTTTCTTTTTTCTTTTTTTTTTTTTTCTTTCCTTTTGAGAAGCGTTTACTATTTT   1019
TTAAGGAGGCTCTCTTCCTTCGGGATCTGTTCCCAGCTGCGTGTGTGCGCGGGTGTGTGT   1079
CTGAAAAGTTGTGTACAAGTGCTCCGTGCTGCCTAGCAAGTGCTAACTGGGATTTCTAGT   1139
ATTTCTTTGTGATGACCGATTTTGAAATGGGTTTCTCTAATGCCAGGAAATCGTGTCTGA   1199
TGTTGTCAAGTACTAGAACTGCCAATAGCCAGAGCTGAACGGAATGTCCTATTTATGGGG   1259
GGGGTTTTGTAAGACGTTCGTTCACCTTTTTTTTTTTTTTTTTAATGAATTTTTTAAAAT   1319
AATAAAGGTTGAGTAAATACATAAAAAAAAAAAAAAAAAAAAAAAA                 1366
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FIG. 6. Nucleotide and deduced amino acid sequence of ECL1715 cDNA. The Sec residue is denoted by X. The putative SECIS element in the 3′UTR is *underlined*.

stant thereafter (Fig. 8). In comparison, Fig. 9 shows the ontogeny of hepatic D1 activity, catalyzing the ORD of T_4 to T_3 and of rT_3 to 3,3′-T_2, and Fig. 10 shows the ontogeny of hepatic D3 activity, catalyzing the IRD of T_4 to rT_3 and of T_3 to 3,3′-T_2. D1 activity gradually increased from E14 until a maximum was reached 1 day after hatching (C1). D3 activity increased between E14 and E17 and rapidly decreased between E18 and E20IP. When the profiles of the mean plasma thyroid hormone levels (n = 7–24) and mean hepatic iodothyronine deiodinase activities (n = 4–5) were compared, plasma T_3 was positively correlated with D1 activity ($r = 0.700$; $P < 0.05$) and negatively correlated with D3 activity ($r = -0.767$; $P < 0.01$). Despite the increase in the plasma levels of the precursor T_4, plasma T_3 remained low between E14 and E17, and this was associated with a doubling of hepatic D3 activity. Between E18 and E20IP, plasma T_4 remained almost constant, but plasma T_3 increased sharply, which was associated with a dramatic decrease in hepatic D3 activity. No correlation was found between plasma T_4 levels and hepatic D3 activity ($r = -0.359$; P = NS). However, a strong positive correlation was found between plasma T_4 levels and hepatic D1 activity ($r = 0.833$; $P < 0.01$); both showed a gradual increase during ontogeny (Figs. 8 and 9).

The expression of D1 and D3 mRNA in chicken liver during embryonic development was determined by Northern analysis of total RNA isolated from liver samples between E14 and C1 using ^{32}P-labeled probes from either ECL1711 or ECL1715 cDNA (Figs. 9 and 10). Densitometric analysis of these blots revealed that D1 mRNA levels remained relatively constant during embryonic development. In contrast, D3 mRNA levels showed a modest increase from E14 to E17 and a strong decrease thereafter to almost undetectable levels on E20IP. The mean D3 mRNA levels (n = 2) and mean D3 activities (n = 4–5) were highly significantly correlated ($r = 0.977$; $P < 0.001$).

Discussion

Our findings clearly show that ECL1711 cDNA and ECL1715 cDNA are near-full-length clones coding for chicken D1 and D3, respectively. The amino acid sequence deduced from the ATG-equipped ECL1711M mutant shows high homology with D1 of rat (60%), human (62%), and dog (58%), including an in-frame TGA codon at position 127. RNA secondary structure prediction (25) also reveals a putative SECIS element in the 3′UTR of ECL1711M (30–32). This SECIS element is essential for Sec incorporation at UGA, which otherwise functions as a stop codon (30–32). Although we have not directly demonstrated that the enzyme encoded by ECL1711 cDNA is a selenoprotein, the high homology among the different deiodinases around the UGA codon and the presence of a 3′UTR SECIS element strongly suggest that this UGA codon is not a stop codon, but, instead, encodes Sec. Furthermore, expression experiments in COS-1 cells showed

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Chi                          AACILLFPRFLLTAVMLWLLDFLCIRKKMLTMPTAEEAAGA---     41
Xen     mlhcagphtgklvkqv...c..l......gl.......q...rrv.ltare.st.eh---     57
Ran     mlpaphtccrllqqll-..c..l......vll......p.v.rrvirgake.dpgap---     56
Rat     mlrslllhslrlcaqt.s.lv......g..f...........hf.rrrhpdhpepevel     60
Hum     mlhslllhslrlcaqt.s.lv......g..f...........hf.grrrrgkpepevel     60

Chi     ---GEGPPPDDPPVCVSDSNRMFTLESLKAVWHGQKLDFFKSAHVGSPAPNPEVIQLDGQ     98
Xen     ----.-----...l........c.v...r.........y.....l.cs...t..vm.e.r    108
Ran     --er.-----...l....t...c.........y...........l.gg...t..vt.e..    109
Rat     nse..em......i....d..lc..a..............q..e.g....s..vrp..f    120
Hum     nse..ev......i....d..lc..a..............q..e.g....s..vlp..f    120

Chi     KRLRILDFARGKRPLILNFGSCTXPPFMARLRSFRRLAAHFVDIADFLLVYIEEAHPSDG    158
Xen     rlck....sq.....vv..............qay.....qh.g.................    168
Ran     rlc.....sk.h...v..............qayq....qrl.f.............c..    169
Rat     qsq....y.q.t...v..............msa.q..vtkyqrdv...ii..........    180
Hum     qsqh...y.q.n...v..............msa.q..vtkyqrdv...ii..........    180

Chi     WVSSDAAYSIPKHQCLQDRLRAAQLMREGAPDCPLAVDTMDNASSAAYAAYFERLYVIQE    218
Xen     .l.t..s.q..q........a.....lq...g.rvv......s.n...g.......ivl.    228
Ran     .l.t....q..t..............lq...g.rvva...t...n...g.........ld    229
Rat     ..tt.sp.v..q.rs.e..vs..rvlqq...g.a.vl...a.s..s..g..........s    240
Hum     ..tt.sp.i..q.rs.e..vs..rvlqq...g.a.vl...a.s..s..g..........s    240

Chi     EKVMYQGGRGPEGYKISELRTWLDQYKTRLQSPGA---VVIQV                    258
Xen     g..v................m..e..qqg.mgtkgsgq.....                    271
Ran     g..v..............g...n.....q..atgn..--l-....                    269
Rat     gti........d..qv.......er.deq.hgtrprrl                          278
Hum     gti........d..qv.......er.deq.hgarprr-.                          278
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FIG. 7. Alignment of deduced amino acid sequences of chicken, *X. laevis*, *R. catesbeiana*, rat, and human D3. The Sec residue is denoted by X. Identical amino acids are indicated by *dots*, and gaps are indicated by *hyphens*.

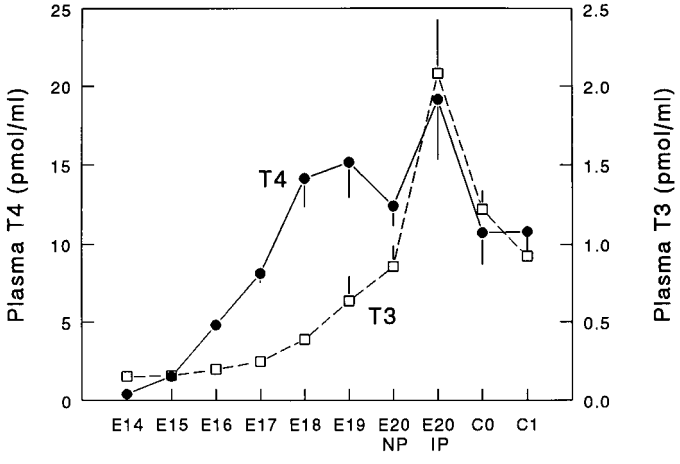

FIG. 8. Plasma T_4 (●) and T_3 (□) levels during embryonic development. Results are presented as the mean ± SEM of 7–24 plasma samples. NP, Nonpipping; IP, internal pipping.

that ECL1711M cDNA codes for an enzyme with identical substrate specificity ($rT_3 > T_3S > T_4 > T_3$) and sensitivity to inhibitors (GTG > PTU ~ IAc) as native chicken D1. The conclusion that ECL1711M is derived from chicken D1 mRNA is also supported by the finding that recombinant and native chicken D1 show identical K_m values for rT_3 (0.26 μM), which is similar to values found for rat (33) and human (34) D1. Since the completion of our study, an expressed sequence tag (EST) cloned from chicken T cells was entered in the GenBank/EMBL Data Bank (accession no. AA495711) with an almost identical nucleotide sequence as ECL1711. The deduced protein is six amino acids shorter at the N-terminus than ECL1711M and has Ala instead of Thr at position 37 and Leu instead of Met at position 131.

The conclusion that ECL1715 is a partial cDNA clone coding for chicken D3 is based on indirect evidence, as expression studies were not possible. The deduced amino acid sequence shows strong homology with rat (62%), human (63%), *X. laevis* (72%), and *R. catesbeiana* (71%) D3. The weak homology of ECL1715 with mammalian, amphibian, and fish D2 sequences (30–45%), in contrast to the strong homology among these D2 sequences (68–90%) (4, 7, 11, 14), strongly suggests that ECL1715 does not encode chicken D2. This is supported by the findings of Galton and Hiebert (35) and ourselves (unpublished observations) that D2 activity is not expressed in embryonic chicken liver. Finally, the strong correlation between hepatic D3 activity and ECL1715-hybridizing mRNA expression during embryonic development represents convincing evidence that ECL1715 is a partial cDNA clone coding for chicken D3.

The availability of chicken D1 and D3 cDNA clones enables the detailed investigation of the regulation of these enzymes under different physiological conditions and in different developmental stages. Birds, in general, and chickens, in particular, are excellent models for studying the role of thyroid hormones in development. In contrast to mammals, which experience intrauterine development, the chicken embryo develops in a closed compartment deprived of the influx of maternal endocrine factors. Circulating thyroid hormone in the embryonic chicken represents the remainder of what was deposited in the yolk before laying (36) and, to increasing extents, hormone synthesized by the embryo itself. During chicken embryonic development, important changes occur in circulating thyroid hormone levels. Plasma T_3 levels increase dramatically at the moment of pipping, when the embryo perforates the air chamber and switches from allantoic to lung respiration, and remain high until

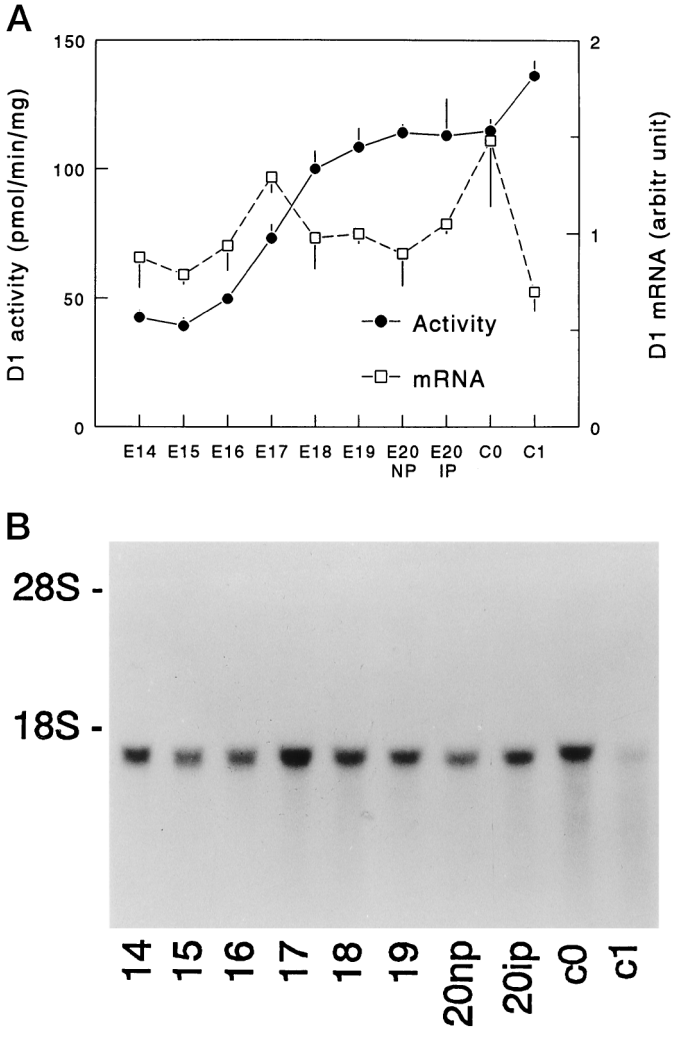

FIG. 9. A, Hepatic D1 activity (●) and D1 mRNA level (□) during embryonic development. Results are presented as the mean ± SEM of four or five (activity) or two (mRNA) liver pools. B, Northern blot of chicken liver RNA hybridized with radiolabeled ECL1711 cDNA.

hatching. These data are in accordance with the work of Decuypere *et al.* (16, 17) and Darras *et al.* (18). Plasma T_4 levels increase more gradually, reaching a maximum at hatching. Whereas plasma T_4 was not correlated with hepatic D3 activity, a negative correlation was found between plasma T_3 and D3 activity. Similar results by Borges *et al.* (37) and Darras *et al.* (18) suggest that the rise in plasma T_3 in embryonic chickens at the end of incubation is at least in part caused by a decreased breakdown due to the decrease in hepatic D3 activity. In recent years it has become clear that this decrease in hepatic D3 activity is at least in part caused by the simultaneous increase in plasma GH (19, 20). It is also interesting to point out that hepatic D1 expression is positively correlated with both plasma T_3 and T_4 during embryonic development. Although it is unlikely that the prohormone T_4 directly induces D1 expression, the positive correlation between plasma T_3 and D1 activity is a true "chicken and egg" problem, as T_3 is not only a product but also a stimulator of D1 expression, at least in rats (38).

These results demonstrate that iodothyronine deiodinases, especially hepatic D3, are acutely regulated during embryonic development. To find out whether this regulation occurs at the transcriptional or the translational level, we decided to investigate D1 and D3 mRNA expression in chicken liver. We did not standardize D1 and D3 mRNA levels relative to actin or GAPDH mRNA levels, because expression of the latter may change significantly during embryonic development. However, it should be stressed that D1 and D3 mRNA levels were determined in the same RNA preparations, indicating completely different ontogenic patterns. Whereas hepatic D1 activity gradually increases during embryonic development from E14 onward, the D1 mRNA level remains relatively constant. As D1 activity and mRNA level are standardized relative to microsomal protein and total RNA levels, respectively, it cannot be firmly concluded that the ratio of D1 activity to mRNA changes and, thus, that regulation takes place at the translational or posttranslational level. In contrast, hepatic D3 activity and D3 mRNA level were very strongly correlated; both showed an increase between E14

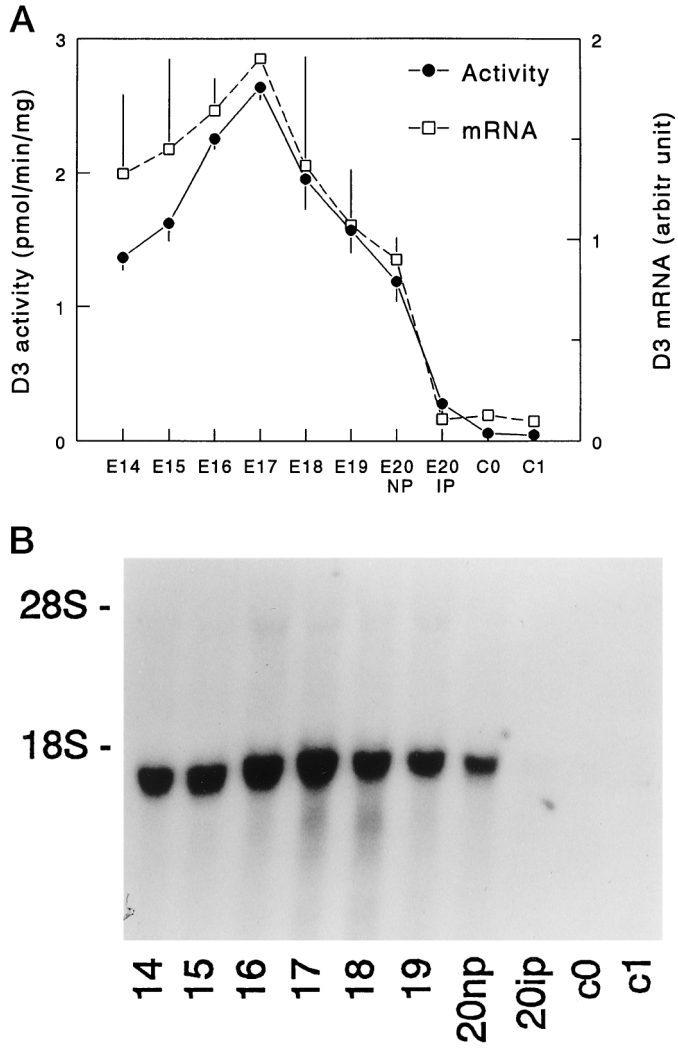

FIG. 10. A, Hepatic D3 activity (●) and D3 mRNA level (□) during embryonic development. Results are presented as the mean ± SEM of four or five (activity) or two (mRNA) liver pools. B, Northern blot of chicken liver RNA hybridized with radiolabeled ECL1715 cDNA.

and E17, followed by a dramatic decrease. These results strongly suggest that the regulation of hepatic D3 expression during embryonic development occurs predominantly at the pretranslational level. Whether these changes are due to alterations in transcription rate or mRNA stability requires further investigation. Also, the role of GH in this mechanism remains to be elucidated.

In conclusion, 1) ECL1711 and ECL1715 are near-full-length clones for chicken D1 and D3, respectively; 2) both cDNAs contain an in-frame TGA codon and a consensus SECIS element in the 3′UTR, making them members of the Sec-containing iodothyronine deiodinase family; and 3) the regulation of hepatic D3 expression in the chick during embryonic development largely represents a pretranslational mechanism.

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