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The Thyroid Hormone Receptor Gene (c-*erb*Aα) Is Expressed in Advance of Thyroid Gland Maturation during the Early Embryonic Development of *Xenopus laevis*

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The c-*erb*A proto-oncogene encodes the thyroid hormone receptor, a ligand-dependent transcription factor which plays an important role in vertebrate growth and development. To define the role of the thyroid hormone receptor in developmental processes, we have begun studying c-*erb*A gene expression during the ontogeny of *Xenopus laevis*, an organism in which thyroid hormone has well-documented effects on morphogenesis. Using polymerase chain reactions (PCR) as a sensitive assay of specific gene expression, we found that polyadenylated *erb*Aα RNA is present in *Xenopus* cells at early developmental stages, including the fertilized egg, blastula, gastrula, and neurula. By performing *erb*Aα-specific PCR on reverse-transcribed RNAs from high-density sucrose gradient fractions prepared from early-stage embryos, we have demonstrated that these *erb*A transcripts are recruited to polysomes. Therefore, *erb*A is expressed in *Xenopus* development prior to the appearance of the thyroid gland anlage in tailbud-stage embryos. This implies that *erb*Aα/thyroid hormone receptors may play ligand-independent roles during the early development of *X. laevis*. Quantitative PCR revealed a greater than 25-fold range in the steady-state levels of polyadenylated *erb*Aα RNA across early stages of development, as expressed relative to equimolar amounts of total embryonic RNA. Substantial increases in the levels of *erb*Aα RNA were noted at stages well after the onset of zygotic transcription at the mid-blastula transition, with accumulation of *erb*Aα transcripts reaching a relative maximum in advance of metamorphosis. We also show that *erb*Aα RNAs are expressed unequally across *Xenopus* neural tube embryos. This differential expression continues through later stages of development, including metamorphosis. This finding suggests that *erb*Aα/thyroid hormone receptors may play roles in tissue-specific processes across all of *Xenopus* development.

The c-*erb*A gene was first identified as the cellular counterpart of the v-*erb*A viral oncogene (27, 62), one of the transforming genes of avian erythroblastosis virus (for a review, see reference 24). This proto-oncogene encodes the thyroid hormone receptor, a ligand-dependent transcriptional activator (50, 65). Two c-*erb*A genes have been identified in several species, including chicken, human, and rat (4, 19, 29, 41, 50, 65). These closely related genes have been designated c-*erb*Aα and c-*erb*Aβ. Additionally, alternative splicing of the c-*erb*Aα genes produces carboxy-terminal variants in rats and humans (4, 26, 32, 37, 42); these variants encode proteins that do not bind thyroid hormones (26, 29, 32, 37, 52). Expression patterns of the different *erb*A transcripts have been studied in developing chickens (19) and rats (58). While expression of *erb*A RNA seems to be subject to complex regulation, it is not clear to what extent, if any, this regulation influences receptor function.

The functions of c-*erb*A/thyroid hormone receptors in the various tissue and developmental settings is not known. Thyroid hormones affect many cellular processes, both positively and negatively. Some of the responses to thyroid hormones are almost certainly indirect, in that they require relatively long periods of time after hormone application (for example, see reference 54) and/or require other hormones (for example, see reference 40). However, c-*erb*A binding sequences in thyroid hormone-responsive genes have been identified, and these elements bind c-*erb*A protein and function as thyroid hormone response elements in hybrid reporter constructs (3, 13, 18, 21, 22, 29, 31, 45, 46, 49, 67, 69, 71). Interestingly, certain of these elements are positively regulated in vitro by c-*erb*A in the presence of thyroid hormone and negatively regulated by c-*erb*A in the absence of thyroid hormone or by v-*erb*A (which does not bind thyroid hormone). Elements from the growth hormone gene promoter (7), the Moloney murine sarcoma virus long terminal repeat (51), the chicken lysozyme promoter (3), and a synthetic thyroid hormone response palindrome derived from the vitellogenin estrogen response element (14, 24) are examples of bifunctional *erb*A response elements. In erythroid cells, c-*erb*A proteins induce the expression of erythrocyte-specific genes, including carbonic anhydrase and the anion transporter band 3 genes, in the presence of thyroid hormone but suppress these genes in the absence of thyroid hormone, while v-*erb*A proteins are hormone-independent negative regulators of these genes (70). On the other hand, c-*erb*A proteins can negatively regulate certain other genes in the presence of thyroid hormone (10, 11, 13, 17, 67).

We have begun to study the regulation of c-*erb*A expression in *Xenopus laevis* to determine the role of thyroid hormone receptor modulation in developmental processes. Anurans, including *X. laevis*, undergo metamorphosis, a period of morphological, biochemical, and behavioral changes which convert the animal from a strictly aquatic larva into an air-breathing adult. These changes include regression of some larval structures and functions; for example, the larval tail regresses via increased production of proteases and hydrolases and by a decreased rate of protein synthesis and increased metabolism of certain RNAs. In other cases, larval forms are replaced by adult forms, as in the examples of the keratins of the larval versus adult epidermis, the hemoglobins of the larval versus adult blood,

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and the retinal pigments of the eye of the larva versus the adult. Additionally, novel structures are developed; for example, increased protein and nucleic acid synthesis lead to the production of the fore- and hindlimbs (for a review, see reference 20a). This complex process is absolutely dependent on the presence of thyroid hormone as the early addition of exogenous thyroid hormone produces precocious metamorphosis, and early thyroidectomy of embryos prevents metamorphosis (for a review, see reference 20a).

Thyroid hormone binding activity exists in tissues of metamorphosing *X. laevis* (33) and in tissues of another anuran, *Rana catesbeana*, during metamorphosis (20, 20a, 39). Evidence that *Xenopus* tadpoles are competent to respond to thyroid hormone by increasing total RNA synthesis (60) prior to metamorphosis at stage 42 or by inducing adult keratin gene expression at stage 48 (35) has been reported. However, there is little information regarding the nature or the expression of the thyroid hormone receptor gene in early *Xenopus* ontogeny. Therefore, we determined the expression pattern of c-*erb*A transcripts during early *Xenopus* development.

MATERIALS AND METHODS

Genomic cloning and sequencing. An *X. laevis* blood genomic DNA library in EMBL3 (Stratagene) containing 10^5 independent recombinant lambda phage was kindly provided by Michael King. The library contains *Bam*HI-digested, size-selected (10- to 20-kb) DNA inserts. This library was screened with a full-length chicken *erb*Aα cDNA (F1, kindly provided by B. Vennstrom). Hybridization was performed under low stringency using standard techniques (34). Screening of a *Xenopus* genomic DNA library with the chicken *erb*Aα probe yielded three lambda clones, each containing an insert of approximately 16 kb. Restriction maps for the three clones were identical. 5′ (*Bam*HI-*Eco*RV) and 3′ (*Eco*RV-*Eco*RI) chicken *erb*Aα probes were used to further characterize the *erb*A coding regions of the lambda clones. 3′, but not 5′, probes hybridized to a 2.3-kb *Bam*HI-*Sst*I fragment, and this fragment contained all of the *erb*A cDNA-homologous sequences of the genomic clones. This *Xenopus* DNA fragment (Xerb2.3) was subcloned into the Bluescribe(+) vector (Stratagene) and sequenced by dideoxy-chain termination methodologies adapted to automated sequencing with fluorescent primers (Applied Biosystems) using Sequenase polymerase (U.S. Biochemical) (59) and the 370A Applied Biosystems instrument. The partial sequence of this subclone is available upon request. Comparison of the sequence of this subclone with the published *Xenopus erb*Aα cDNA sequence (8) showed that the genomic clone, designated Xerb2.3, contained a small intron and the two exons encoding the 169 C-terminal amino acids of *Xenopus erb*Aα (Fig. 1).

Embryo culture. Eggs were obtained from adult *Xenopus* females after injection with 500 to 800 U of human chorionic gonadotropin (Quad) and fertilized with minced testes. Embryos were grown in 0.1× modified Barth's saline (MBS; 1× MBS is 88 mM NaCl, 1 mM KCl, 1 mM $CaCl_2$, and 5 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid [HEPES], pH 7.8 [Sigma]) at 15°C or at room temperature. Early embryos were dejellied in 2% cysteine (pH 7.5) for approximately 5 min and then extensively washed in 0.1× MBS.

PCRs of DNAs and RNAs. The polymerase chain reaction (PCR) was performed on a variety of templates. Chicken *erb*Aα, human *erb*Aβ, and rat *erb*Aβ plasmid cDNAs were used as input DNAs, as was the *Xenopus erb*Aα genomic

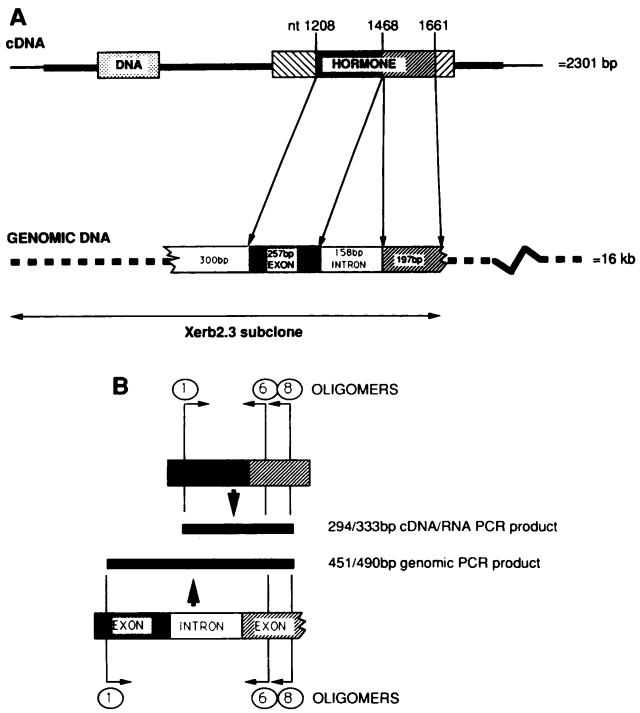

FIG. 1. (A) Comparison of *X. laevis* c-*erb*Aα cDNA clone TRα1 (8) and genomic clones (not to scale). The cDNA was isolated from an oocyte library and encodes a protein of 418 amino acids with a domain structure including a putative DNA binding domain (stippled) and thyroid hormone binding domain (diagonal lines). The cDNA clone includes 5′ and 3′ untranslated regions (thin horizontal lines). Nucleotide positions are noted relative to the translation start site. The 16-kb genomic clone corresponds to the 3′ end of the cDNA clone and includes two exons from the hormone binding domain (diagonally lined boxes) and two introns (open boxes) as well as unsequenced regions (dashed horizontal lines). The 5′ end of the 300-bp intron and the 3′ end of the 197-bp exon have not been mapped (broken-end boxes). The region contained in the Xerb2.3 genomic subclone is also shown. nt, Nucleotides. (B) Schematic representation (as in panel A) of the cDNA/RNA *erb*Aα versus genomic *erb*Aα regions amplified by PCR in the analyses reported in the text. Positions of the 5′ sense (oligonucleotide 1) and 3′ antisense (oligonucleotides 6 and 8) PCR primers are indicated by circled numbers. The orientations of the PCR primers are shown by arrows. Oligomer sequences are noted in Material and Methods. PCR product sizes of 295 and 453 bp are the expected result of PCR with primers 1 and 6 from cDNA and genomic DNA, respectively; similarly, PCR products of 333 and 490 bp are the expected result of PCR with primers 1 and 8.

plasmid described in this report. Xerb2.3 RNA was transcribed in vitro from the *Xenopus* genomic plasmid (containing an intron), using T7 RNA polymerase (Promega) as described by the enzyme supplier. The in vitro RNA was treated with DNase two to three times until it produced no PCR product in the absence of reverse transcription. Dilutions of the in vitro RNA were then made on the basis of A_{260} measurements of stock solutions. Total RNAs were prepared from frog embryos by a modification of the acid phenol extraction method (11). LiCl (3 M) was used to further selectively precipitate RNA from DNA when necessary. Avian myeloblastosis virus reverse transcriptase (5 U/μg; Life Sciences) was used to produce first-strand cDNAs from 1 to 2 μg of total RNA in 20-μl reaction volumes containing 50 mM KCl, 10 mM Tris (pH 8.3), 16 mM $MgCl_2$, 1 U of

RNasin (Promega) per μl, 500 μM deoxynucleoside triphosphates, and 50 to 100 pmol of random primers (Pharmacia) or oligo(dT) or *erb*Aα-specific oligonucleotide 8. Reactions were continued at 42°C for 1 to 2 h, stopped at 95°C for 5 min, and held on ice. Then 1 to 5 μl of this reaction mix was added to a PCR reaction mix containing the buffer described above, 2 U of *Taq* polymerase (Perkin-Elmer Cetus), 50 pmol of each specific primer, and a final dNTP concentration of 100 to 200 μM.

For *erb*Aα-specific PCR, the 5′ (sense) primer used was designated oligonucleotide 1. Its sequence is 5′-GACCCT AACGCTGAGCGG-3′ as at nucleotide position 839 of the protein-coding sequence of the *Xenopus erb*Aα cDNA (8). The 3′ (antisense) primers are designated oligonucleotide 6, which corresponds to nucleotide position 1116 (5′-CCCAAACTCCTAATGAAG-3′), and oligonucleotide 8, which corresponds to nucleotide 1155 (5′-GCTGGCATGG CATGCCCC-3′). On the basis of the positions of the oligonucleotide sequences in *Xenopus* genomic DNA and cDNA clone sequences, the sizes of PCR fragments obtained with these primers were predicted: the primer pair including oligonucleotides 1 and 6 (hereafter called 1/6) was predicted to produce fragments of 451 bp from genomic DNA (with intron) and 294 bp from cDNA (without intron), while the primer pair including oligonucleotides 1 and 8 (hereafter called 1/8) was predicted to produce 490- and 333-bp PCR fragments from genomic DNA and cDNA respectively (Fig. 1B).

PCR primers used in control experiments for assessment of levels of *XA1* RNA corresponded to sequences within a long open reading frame in a head-specific cDNA cloned from a library produced after differential hybridization of head and nonhead polyadenylated RNA-derived sequences (57). The 5′ (sense) oligonucleotide was designated *XA1*-1 and has the sequence 5′-AACCGAGGCATGGCAGGC-3′; the 3′ (antisense) oligonucleotide was designated *XA1*-3 and has the sequence 5′-TTCATCATGGGTAGCAAGGG-3′. The *Mix.1* PCR primers corresponded to 3′ sequences in the full-length 2A7 cDNA insert (48): *Mix*-1 is the 5′ primer (nucleotide position 881 of the coding sequence) with the sequence 5′-TACAAATATGATGGAGACGG-3′; *Mix*-4 is the 3′ primer (nucleotide position 1064) with the sequence 5′-TTGTTGACATGTGACACGG-3′. *EF1*α PCR primers corresponded to 3′-sequences at nucleotide positions 1088 to 1113 and 1283 to 1308 of the protein-coding sequence of the cDNA clone pXef1 (30) and were designated 9 (5′-CTGAAT CACCCAGGCCAGATTGGT-3′) and 14 (5′-GAGGGTAT GAGAAGCTCTCCAC-3′).

Thirty (*erb*, *XA1*, and *Mix.1*) or 19 (*EF1*) cycles of PCR were performed using a Perkin-Elmer Cetus Thermocycler with cycles typically consisting of 30 s of denaturation at 94°C, 1 min of annealing at 50 to 55°C, and 2 min of extension at 72°C. PCR products were analyzed by separation on agarose or polyacrylamide gels followed by ethidium bromide staining or by Southern blotting and probing with radioactive chicken *erb*Aα probes. Alternatively, PCR reactions were performed in the presence of approximately 0.5 μCi of added [^{32}P]dCTP, and products were separated on polyacrylamide gels and detected by autoradiography and densitometry using the Bioimage analysis system. Densitometric data for embryonic *erb*Aα expression were normalized relative to the known Xerb2.3 genomic DNA-derived RNA input, as determined in spectrophotometry of concentrated Xerb2.3 RNA solutions. The different lengths and CTP content of the genomic plasmid-derived (451-bp) and the cDNA-derived (294-bp) PCR fragments were factored into calculations of mean expression of *erb*Aα RNA, which are presented as picograms of *erb*Aα RNA per microgram of total embryonic RNA.

Direct sequencing of double-stranded PCR products. A modification of the Winshop (66) dideoxy-chain termination technique was used. Briefly, PCR products were excised from a gel, electroeluted, and reamplified by PCR. Then 30 to 50 ng of PCR product was added to an annealing mixture held on ice containing 20 pmol of 5′ or 3′ PCR primer (oligonucleotide 1 or oligonucleotide 6), 1× Sequenase buffer (U.S. Biochemical) (59), and 10% dimethyl sulfoxide (DMSO). This mixture was boiled for 2 min and then snap-cooled on dry ice. Labeling mix containing Sequenase (U.S. Biochemical), [^{35}S]dATP, and dithiothreitol at a final concentration of 10 mM was added to the annealing mix, which was immediately divided into termination tubes. Termination mixes contained 10% DMSO, 50 mM NaCl, and 80 μM three dNTPs and either 8 μM ddCTP, ddGTP, or ddTTP or 0.08 μM ddATP. Extension/termination reactions were continued at 37°C for 2 min, snap-cooled on dry ice, and then chased in the presence of 10% DMSO and approximately 100 μM each dNTP for 2 min at 37°C. Loading buffer was added, and samples were boiled for 2 min and loaded onto a 5% sequencing gel containing urea.

Polysome isolation and RNA preparation. As described by Dworkin et al. (17), 50 to 100 staged embryos were homogenized on ice in a Dounce-type homogenizer with a tight-fitting pestle in 1 to 2 ml of homogenization buffer (300 mM KCl, 2 mM $MgCl_2$, 20 mM Tris-HCl [pH 7.4], 0.35% Nonidet P-40, 4 μg of polyvinyl sulfate per ml, and RNase inhibitor [300 U/ml; Bethesda Research Laboratories]). Cycloheximide at 0.1 mM or puromycin at 450 μg/ml (for negative controls) was added to half of each sample, along with deoxycholate to 0.35%. The homogenates were centrifuged at 15,000 × *g* for 10 min, and 0.5 to 1 ml of supernatants was loaded onto the top of 15 to 50% linear sucrose gradients in polysome buffer without polyvinyl sulfate or RNase inhibitor and centrifuged in a Beckman SW41 rotor for 2 h at 37,000 × *g*. Gradients were then analyzed, and 1-ml fractions were collected by using an Isco model 185 density gradient fraction collector and analyzer. Total RNA was prepared from 400-μl samples from each fraction in the presence of 2% sodium dodecyl sulfate, using a modified acid phenol extraction procedure. Approximately 1/100 (*EF1* and stage 25 *erb*) or 1/10 (stage 11 *erb*) of each RNA sample (<1 μg) was reverse transcribed with oligo(dT) (*EF1*) or *erb*A-specific primers (*erb*A); 1/20 of each of the cDNA samples was analyzed by *EF1* PCR, and 1/5 was analyzed by *erb*Aα-specific 1/6-primed PCR.

RESULTS

Two PCR fragments are obtained with genomic DNA input. c-*erb*Aα-specific sequences were chosen from within the 5′ and 3′ exonic regions of the Xerb2.3 *Xenopus erb*Aα genomic clone (see Materials and Methods and Fig. 1). Corresponding synthetic oligonucleotides were prepared appropriate for priming PCRs. When these experiments were begun, the expression of only the α form of c-*erb*A/thyroid hormone receptor had been demonstrated in *Xenopus* cells by the cloning of a *erb*Aα cDNA (7). The 1/6 and 1/8 oligonucleotide pairs were predicted to preferentially recognize *erb*Aα, but not *erb*Aβ, *Xenopus* coding sequences since the two *erb*A genes were known to diverge in the regions corresponding to these sequences in the human, rat, and chicken *erb*A genes. To test this hypothesis, PCR reactions were

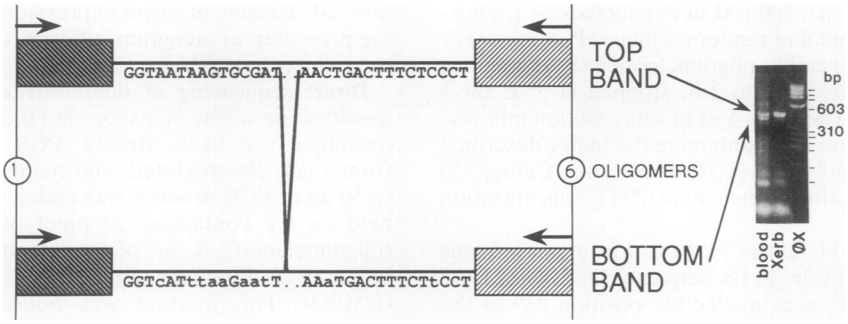

FIG. 2. Comparison of the PCR products from Xerb2.3 genomic plasmid and blood DNA inputs and partial schematic comparison of the intronic sequences of the two genomic DNA-derived PCR products. PCR was performed on DNA inputs with the 1/6 primer pair, and products were separated by agarose gel electrophoresis, identified by ethidium bromide staining, and sized relative to the gel mobility of *Hae*III-digested ϕX174 DNA size standards, as shown. Double-strand products were sequenced directly after gel purification. Sequence data were obtained for all but approximately 50 bases at each of the ends of these fragments. The sequence derived from the PCR fragments in the top band was identical to the sequence from the same region in the Xerb2.3 subclone in both exonic (diagonal lines in block, as in Fig. 1) and intronic (line between blocks) segments. The sequence derived from the PCR fragments in the bottom band was identical to the sequences derived from the top band in the exonic segments but divergent in the intronic segments. The bottom band fragments were 36 bp shorter (represented by converging lines between top-band and bottom-band sequences) and contained nucleotide nonidentities (lowercase letters in the bottom-band sequence) as compared with the top-band fragments.

performed on Xerb2.3 plasmid DNA input, using the 1/6 oligonucleotide pair as primers, and the predicted genomic DNA-sized 451-bp fragment was obtained (Fig. 2). However, two fragments were amplified from *Xenopus* blood genomic DNA, one of 451 bp and another smaller fragment (Fig. 2). Direct double-strand sequencing of the genomic DNA-derived fragments showed that the larger fragment had the same sequence as the Xerb2.3 genomic clone. The smaller fragment was identical to Xerb2.3 genomic plasmid sequence in the exonic segments but contained a shorter, divergent intron (results summarized in Fig. 2).

Comparison of DNA content and chromosome number in various *Xenopus* species has led to the conclusion that *Xenopus* is an essentially tetraploid genus (5). The results of control experiments lead us to conclude that the second of the two *X. laevis* genomic DNA PCR fragments was derived from a second *erb*Aα gene rather than from an *erb*Aβ gene. First, two PCR fragments were amplified with both the 1/6 (Fig. 2) and 1/8 oligonucleotide pairs as primers (data not shown). As mentioned above, all three oligonucleotide sequences are from exonic regions that diverge between *erb*Aα and -β genes of other species and would not be expected to hybridize to *erb*Aβ sequences. Second, as expected, the 1/6 oligonucleotide pair yields a PCR product of the predicted size from chicken *erb*Aα cDNA but not from rat or human *erb*Aβ cDNA (data not shown).

Relative levels of *erb*Aα transcripts from *Xenopus* embryos of different stages can be quantitated. Total RNAs from early-stage *Xenopus* embryos, including fertilized egg, and stage 9, 14, 17, 21, 35, and 48 embryos (Table 1) were analyzed for *erb*Aα expression. RNA concentrations were normalized by ethidium bromide staining, and then RNAs were reverse transcribed and subjected to *erb*Aα PCR amplification using the 1/6 oligonucleotide pair as primers. PCR fragments of 294 bp were predicted to be amplified from *erb*Aα cDNA inputs (which lack introns). Fragments of this size were evident in all embryonic PCR samples after ethidium bromide staining compared with the mobility of DNA molecular weight standards (data not shown). Direct double-strand sequencing of these fragments confirmed that the PCR fragments from all embryonic stages contained *erb*Aα sequences (data not shown). To further characterize the *erb*A RNA expression in early *Xenopus* development, we performed quantitative, radiolabeled PCR assays. Again, even RNA samples from early embryonic stages were shown to contain *erb*Aα RNAs (Fig. 3A). Comparisons of the

TABLE 1. Selected embryonic and larval stages of *X. laevis* development examined in these experiments[a]

Stage	Descriptor	Relevant characteristics	Size (mm)	Time at 22°C
Egg	Fertilized egg		1.4–1.5	0 h
9	Blastula		1.4–1.5	7 h
12	Late gastrula		1.4–1.5	13 h
14	Neural plate		1.5–1.6	16 h
17	Late neural fold		1.5–1.6	19 h
21	Closed neural tube		1.9–2.0	22 h
35	Hatching, late tailbud	Thyroid anlage discernible	5.3–6.0	2 days, 2 h
48	Feeding tadpole	Forelimb bud visible, hindlimb growing, thyroid of 4–6 lobes (follicles at stage 50)	14–17	7.5 days
58	Prometamorphic	Forelimb emergent, hindlimb with paddles and claws	50–60	24 days
62	Late prometamorphic	Head narrowing, extending forelimbs	50–60	49 days
64	Metamorphic climax	Head narrower than trunk, regressing tail, adult-type skin	50–60	53 days

[a] From reference 43.

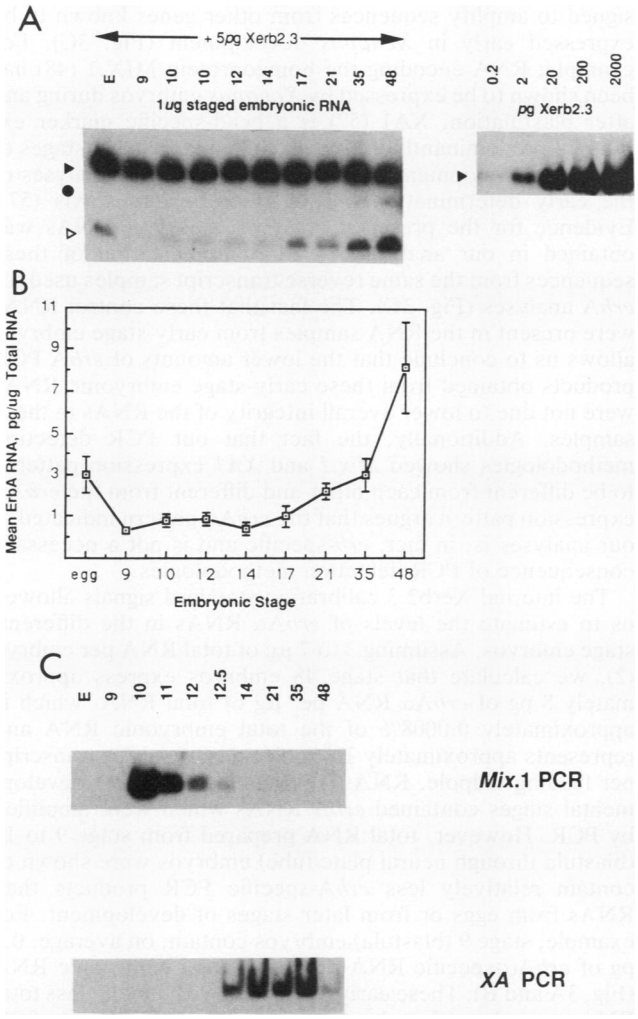

FIG. 3. Quantitation of c-*erb*Aα RNA in early stages of *Xenopus* development. (A) RNA samples were prepared from whole embryos at the indicated stages and normalized by ethidium bromide staining of rRNAs separated by agarose gel electrophoresis. Samples (5 pg) of Xerb2.3 transcripts prepared in vitro were added to normalized RNAs (1 μg) and then reverse transcribed with random priming. Aliquots were subjected to PCR amplification with the *erb*Aα-specific 1/6 primer pair. Trace amounts of [^{32}P]dCTP were added to PCRs and incorporated into PCR products to allow densitometric quantitation after polyacrylamide gel electrophoresis and autoradiography. A representative experiment is shown. Also shown are data from a linearity control experiment in which increasing amounts of Xerb2.3 transcript input were added to reverse transcriptions prior to addition of aliquots to *erb*A-specific PCRs. Polyacrylamide gels were dried, and autoradiography was performed. Sizes of PCR products were determined relative to the migration of DNA size markers (data not shown). As expected, a 451-bp genomic plasmid-derived band (filled triangles) and a 294-bp embryonic RNA-derived band (empty triangle) were noted. In some cases, *erb*-specific PCR yielded an additional band (circle) which was the result of genomic DNA contaminating embryonic RNA samples. The contribution of genomic DNA to the genomic plasmid-derived 451 bp signal was subtracted in quantitative assessments of *erb*Aα RNA expression (B). Means and standard deviations were calculated for each embryonic stage from data obtained in three independent PCR experiments, using two different RNA preparations (for example, stage 10 shown here). (C) Aliquots of reverse transcription reactions equal to those used in *erb*Aα-specific PCR were added to *Mix.1* and *XA-1* PCRs, which were performed as described for panel A; 183-bp *Mix*-specific and 180-bpA *XA*-specific PCR products were recovered. A representative experiment is depicted.

autoradiographic densities of *erb*Aα RNA-derived PCR products from different-stage embryos revealed a greater than 25-fold range in *erb*Aα RNA expression across early development, as expressed relative to 1 μg of total RNA (Fig. 3A).

We were able to internally calibrate the efficiency of the reactions and to quantitate the embryonic *erb*Aα message levels by using Xerb2.3 genomic plasmid-derived *erb*A RNA as an internal standard (63). Xerb2.3 RNA was produced by in vitro transcription and treated repeatedly with DNase. Its concentration was determined by spectrophotometry, and known amounts of Xerb2.3 RNA were added to RNA samples from each embryonic stage. That reverse transcription and PCRs were subsaturating was established in a series of control reactions. Increasing amounts of embryo and Xerb2.3 RNAs were added to reverse transcription reactions, and increasing amounts of reverse transcripts produced in the first reactions were added to PCRs containing trace amounts of radioactive deoxyribonucleotide. The PCR products were separated by gel electrophoresis and quantitated by densitometry of autoradiographs. When 1/20 to 1/10 fractions of the reverse transcription reactions containing 0.5 to 2 μg of embryonic RNA and 1 to 5 pg of Xerb2.3 RNA were then added to PCR mixtures, final products proportional to the amount of nucleic acid input were obtained. Control experiments showed that as much as 40 pg of *erb*A-specific RNA (from embryonic RNA and/or Xerb2.3 RNA sources) could be reverse transcribed and PCR amplified within the linear range of these reactions. Figure 3A includes data from one such control experiment in which 0.2 to 200 pg of Xerb2.3 *erb*Aα RNA was shown to be subsaturating in sequential reverse transcription and PCR reactions. Additionally, Fig. 3A demonstrates that the quantity of *erb*-specific PCR products derived from 5 pg of Xerb2.3 RNA was not significantly affected by the presence of increasing amounts of embryonic RNA-derived *erb*Aα-specific sequences. Experiments in which 0.5 and 1 μg of stage 9 total embryonic RNA and 1 and 2 μg of stage 40 embryonic RNA were added to the Xerb2.3-standardized reactions yielded quantitative assessments expected for results within the linear range of these reactions (data not shown). Other quantitation experiments were performed with 1 or 2 pg of Xerb2.3 RNA as an internal standard, with quantitation results for the embryonic *erb* RNAs similar to those with 5 pg of Xerb2.3 RNA, shown in Fig. 3A. These results are included in Fig. 3B and, together with the results of the control experiments just described, make us confident of quantitations of 0.2 to 40 pg of *erb*Aα-specific PCR products. The reproducibility of the PCR-based quantitation of *erb*Aα-specific RNA in mixed RNA populations is exhibited in the similarly intense stage 10 embryo RNA-derived PCR signals in Fig. 3A and in the statistical summary in Fig. 3B.

Because exon-specific PCR primers were used in these quantitative assays, the PCR products derived from embryo RNA versus genomic plasmid-derived sources could be readily distinguished by size due to the presence or absence of intron sequences as outlined in Fig. 1. As expected, a 451-bp genomic plasmid-derived band and a 294-bp embryonic RNA-derived band were noted in samples containing both Xerb2.3- and embryo-derived cDNAs (Fig. 3A and Fig. 4). In some cDNA samples, *erb*-specific PCR yielded an additional band which corresponded to the shorter PCR fragments recovered from *erb*-specific PCR of genomic DNA (Fig. 2). This band represented genomic DNA contaminating embryonic RNA samples. As such contamination could be

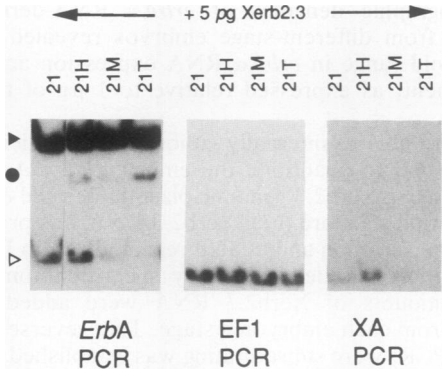

FIG. 4. Evidence that *Xenopus erb*Aα is expressed with region-specific differences. RNAs were prepared from total embryo, head (H), trunk (M), and tail (T) segments of stage 21, neural tube embryos. PCR experiments were performed after reverse transcription, and data from a representative experiment are depicted with symbols as in Fig. 3A. *erb*A PCR inputs were normalized by *EF1* PCR. Segmental identities of stage 21 embryo sections were confirmed by *XA* PCR of samples from the same reverse transcriptions used for *erb*Aα PCR.

readily distinguished and as genomic DNA produced two signals of equal intensity, the contribution of genomic DNA to the 451-bp standardizing signal was subtracted in quantitative assessments. Xerb2.3 plasmid-derived and embryo-derived PCR products were quantitated by densitometry, and the levels of embryonic *erb* RNA were expressed relative to the known amounts of Xerb2.3 RNA input.

Negative controls (PCR reactions without added RNA and/or PCR reactions containing yeast tRNA) were included in each experiment (data not shown). An *erb*A-specific signal was never obtained from these negative control samples, demonstrating that the low amounts of *erb*A PCR products obtained from early-stage *Xenopus* RNA samples were not due to background *erb*-specific amplification and were not simply due to cross-contamination from other samples containing larger amounts of *erb*A transcripts. We assume that the variability in the *erb*Aα-specific PCR amplification from the early-stage embryonic RNAs is due to the fact that the levels of *erb*Aα RNAs in the early embryos are at the lower detection limit of these analyses, as shown in control titrations (for example, Fig. 3A). We believe it is unlikely that *erb*Aα sequences were degraded differentially during the preparation of RNAs from the different-stage embryos, but we have not rigorously excluded the possibility that *erb*A RNAs were differentially recovered from the early-stage embryos. Developing oocytes are surrounded by follicle cells in *Xenopus* ovaries, and when mature oocytes are obtained surgically, they can be contaminated by the adult follicle cells to variable degrees. Brooks et al. (8) suspected that the *erb*Aα cDNA that they obtained from a *Xenopus* oocyte library was follicle cell derived. However, eggs are naturally defolliculated during ovulation, and we further dejellied early-stage embryos in cysteine before isolating RNA. Therefore, we believe that the *erb*A sequences amplified from early-stage RNA samples were not adult cell derived.

The overall integrity of early-stage RNAs was demonstrated, not only by staining total RNA samples with ethidium bromide and normalizing to rRNA fluorescence (data not shown) but also by performing PCR reactions with these same normalized samples using oligonucleotide primers designed to amplify sequences from other genes known to be expressed early in *Xenopus* development (Fig. 3C). For example, RNA encoding the homeoprotein MIX.1 (48) has been shown to be expressed by *Xenopus* embryos during and after blastulation. XA1 (57) is a head-specific marker expressed predominantly during blastula and gastrula stages of *Xenopus* development which has been useful in analyses of the early determination of the anteroposterior axis (57). Evidence for the presence of *Mix.1* and *XA1* RNAs was obtained in our analyses by PCR amplification of these sequences from the same reverse transcript samples used for *erb*A analyses (Fig. 3C). The fact that these control RNAs were present in the RNA samples from early-stage embryos allows us to conclude that the lower amounts of *erb*A PCR products obtained from these early-stage embryonic RNAs were not due to lower overall integrity of the RNAs in these samples. Additionally, the fact that our PCR detection methodologies showed *Mix.1* and *XA1* expression patterns to be different from each other and different from the *erb*Aα expression pattern argues that the *erb*Aα pattern indicated in our analyses is, in fact, *erb*-specific and is not a necessary consequence of PCR detection methodologies.

The internal Xerb2.3 calibration standard signals allowed us to estimate the levels of *erb*Aα RNAs in the different-stage embryos. Assuming 3 to 7 μg of total RNA per embryo (2), we calculate that stage 48 embryos express approximately 8 pg of *erb*Aα RNA per μg of total RNA, which is approximately 0.0008% of the total embryonic RNA and represents approximately 10^8 molecules of *erb*Aα transcript per feeding tadpole. RNAs from even the earliest developmental stages contained *erb*A RNAs which were amplified by PCR. However, total RNA prepared from stage 9 to 17 (blastula through neural plate/tube) embryos were shown to contain relatively less *erb*A-specific PCR products than RNAs from eggs or from later stages of development. For example, stage 9 (blastula) embryos contain, on average, 0.3 pg of *erb*Aα-specific RNA per μg of total embryonic RNA (Fig. 3A and B). These early-stage embryos contain less total RNA per embryo than do stage 48 embryos. Estimates of the increase in total RNA content of embryos across early *Xenopus* development range from approximately 2-fold (9) to as much as 14-fold (2). Our own results concerning total RNA per embryo vary between preparations, with as much as a 10-fold difference between total RNA per stage 9 versus stage 48 embryos (data not shown). Such variation is undoubtedly influenced by the efficiency of recovery of RNAs from embryo lysates. However, the point can be made that *erb*Aα transcript number per embryo increases across premetamorphic *Xenopus* development. This increase is at least 24-fold (8 pg/μg in stage 48 versus 0.3 pg/μg in stage 9), assuming constant total RNA content per embryo across this period of development, and could be as much as 300-fold, assuming the larger estimate of increase in total RNA content per embryo during this period.

The number of cells per embryo clearly does increase more substantially across early *Xenopus* development than does the amount of total RNA per embryo (15), from approximately 20,000 cells in stage 12 (late gastrula) embryos to approximately 10^5 cells in stage 21 (closed neural tube) embryos to approximately 10^6 cells in stage 48 (feeding tadpole) embryos. Given these estimates of cell number per embryo, our data suggest that the average cellular level of *erb*Aα transcripts does not increase significantly after neurulation and until late tadpole stages (Fig. 3A and B). We estimate that there are approximately 50 to 200 *erb*Aα transcripts per cell in stage 21 to 48 embryos, assuming that

the transcripts are evenly distributed across the embryo. However, RNA isolation and PCR amplification of normalized RNA templates from dissected stage 21 (Fig. 4) and stage 35 (data not shown) embryos showed that head regions of these animals expressed more *erb*A RNA than did trunk or tail sections. The input of RNAs from these samples to 1/6-primed *erb*A PCRs was normalized by ethidium bromide staining and by PCR amplification of sequences encoding *Xenopus* elongation factor EF1α, which is found in all cells after mid-blastula transition (30). The assignment of these samples with regard to body position was confirmed by *XA1* (head-specific) PCR. These data lead us to conclude that the number of *erb*A molecules per cell varies across the embryo and suggest that *erb*Aα function may vary, at least in degree, across embryos during their development.

As expected, *erb*Aα expression was demonstrated in all tissues obtained from metamorphosing animals (Table 1), although subtle tissue-specific differences in levels of *erb*A RNA relative to total RNA were noted, and larger differences may exist on a per-cell basis. The metamorphic tissues demonstrated minimal increases in the percentage of *erb*Aα-specific RNAs compared with the relative *erb*A expression in the stage 48 (feeding tadpole) embryos (data not shown).

***erb*Aα transcripts are polysome associated.** The assessments of the relative levels of *erb*Aα RNAs in embryos at different stages of development were similar when *erb*Aα-specific PCR was performed after reverse transcription of RNAs with random priming (Fig. 3), oligo(dT) priming (data not shown), or *erb*Aα-specific priming (data not shown). That cDNAs produced in each of these ways contained approximately equal numbers of *erb*A-specific PCR templates supports the validity of our quantitative evaluation of *erb*Aα RNA expression across *Xenopus* development. As there are no strings of adenylate nucleotides within the 3′ *erb*A coding sequence assayed in our PCR analyses, the oligo(dT)-primed templates amplified by *erb*A-specific PCR must have been primed in the poly(A) tracts of the 3′ untranslated regions of these *erb*A transcripts. That oligo(dT)-primed cDNAs contained *erb*Aα sequences therefore strongly suggests that even early-stage embryonic embryos contain *erb*Aα transcripts which are polyadenylated.

Given the extremely low levels of *erb*Aα RNA in early stages of *Xenopus* development (Fig. 3A and B) (1, 68), it is not surprising that detection of *erb*A protein by immunoprecipitation or Western immunoblotting has been unsuccessful (data not shown). In *X. laevis*, polyadenylation of RNA transcripts is required for recruitment to polysomes and subsequent translation (36). The demonstration that messages are localized to polysomes is generally accepted as strong evidence that an RNA is translationally active. We therefore made use of the great amplification of signal unique to PCR to assess the polysomal recruitment of *erb*Aα RNAs and to provide evidence of the presence of *Erb*Aα proteins in early frog embryos. Cytoplasmic lysates from staged *Xenopus* embryos were prepared and fractionated on sucrose gradients (Fig. 5A), using standard polysome isolation techniques (17). Total RNAs were then prepared from each fraction and analyzed for the presence of *erb*Aα and *EF1*α RNA by reverse transcription and PCR (Fig. 5B) as described above.

*EF1*α is translated at high levels after the mid-blastula transition, and *EF1*α RNA has been readily detected in Northern (RNA) analyses of RNAs from embryos as early as stage 10 (30). As expected, in three independent assays, *EF1*α PCR products were recovered from high-density fractions obtained from all embryonic stages analyzed in our

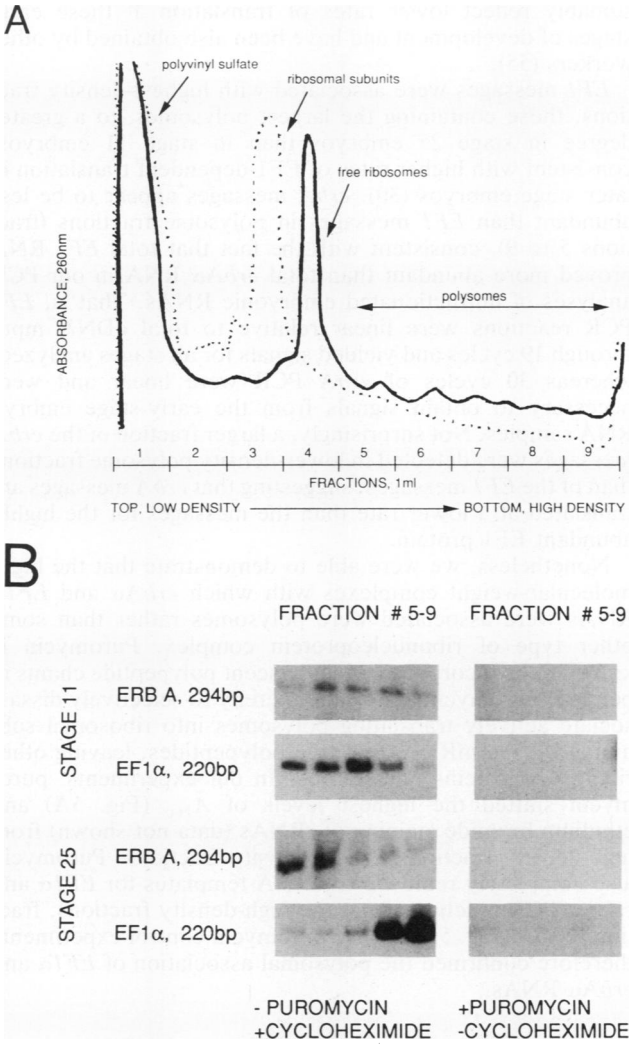

FIG. 5. Evidence that *erb*Aα transcripts are polysome-associated. Cellular fractions were prepared from 100 stage 11 and 50 stage 25 embryos as described in Materials and Methods. These were separated in, and fractions were collected from, a 15 to 50% sucrose gradient while an A_{260} profile was recorded. (A) The position of the absorbance of polyvinyl sulfate was determined empirically to be at the top of the gradient, while the positions of absorbance of ribosomal subunits, free ribosomes, and polysomes are noted as in reference 55. A_{260} profiles of stage 25 lysates treated with cycloheximide (solid line) or with puromycin (dotted line) are shown. (B) Total RNAs were prepared from equal aliquots of each cycloheximide and puromycin fraction. An appropriate aliquot (see Materials and Methods) of each RNA sample (<1 μg) was reverse transcribed with an oligo(dT) (*EF1*α) or *erb*A-specific primers. (*erb*A). The cDNA samples were then analyzed by *EF1* PCR and by *erb*Aα-specific 1/6-primed PCR. Radiolabeled PCR products are shown.

experiments, including those from stages 11, 13, 21, 25, and 41 (Fig. 5B and data not shown). *Erb*Aα PCR products were produced from RNA in these same high-density fractions. Representative A_{260} profiles of sucrose density gradients prepared from stage 25 lysates (Fig. 5A), and the results of representative *EF1* and *erb*A PCR analyses of RNAs in high-molecular-weight fractions (fractions 5 to 9) from stage 11 and stage 25 lysates are shown (Fig. 5B). The rather shallow polysome peaks in early-stage embryo profiles pre-

sumably reflect lower rates of translation at these early stages of development and have been also obtained by other workers (55).

EF1 messages were associated with highest-density fractions, those containing the largest polysomes, to a greater degree in stage 25 embryos than in stage 11 embryos, consistent with higher rates of EF1-dependent translation in later stage embryos (30). *erb*A messages appear to be less abundant than *EF1* messages in polysome fractions (fractions 5 to 9), consistent with the fact that total *EF1* RNA proved more abundant than total *erb*Aα RNA in our PCR analyses of unfractionated embryonic RNAs. That is, *EF1* PCR reactions were linear relative to total cDNA input through 19 cycles and yielded signals for all stages analyzed, whereas 30 cycles of *erb*A PCR were linear and were necessary to obtain signals from the early-stage embryo RNA samples. Not surprisingly, a larger fraction of the *erb*A messages were detected in lower-density polysome fractions than of the *EF1* messages, suggesting that *erb*A messages are translated at a lower rate than the messages for the highly abundant EF1 protein.

Nonetheless, we were able to demonstrate that the high-molecular-weight complexes with which *erb*Aα and *EF1*α RNAs were associated were polysomes rather than some other type of ribonucleoprotein complex. Puromycin is known to be incorporated into nascent polypeptide chains in peptidyl-puromycin bonds and thereby to selectively dissassociate actively translating polysomes into ribosomal subunits (6), free mRNA, and free polypeptides, leaving other ribonucleoproteins undisturbed. In our experiments, puromycin shifted the highest levels of A_{260} (Fig. 5A) and ethidium bromide staining of rRNAs (data not shown) from high-density fractions to low-density fractions. Puromycin also completely removed the RNA templates for *EF1*α and *erb*Aα PCR reactions from the high-density fractions, fractions 5 to 9 (Fig. 5B). These puromycin control experiments therefore confirmed the polysomal association of *EF1*α and *erb*Aα RNAs.

DISCUSSION

The results described in this report provide evidence for the expression and polysomal recruitment of *erb*A transcripts during very early *Xenopus* development. We have used extremely sensitive PCR techniques to analyze *erb*A expression quantatively. Our data show that polyadenylated *erb*A RNAs are present at low but reproducibly measurable levels as maternally derived transcripts in the fertilized egg and during blastulation. No stable increase in the steady-state levels of *erb*A mRNA is apparent directly after the onset of new transcription at the mid-blastula transition or during gastrulation. *erb*Aα transcripts accumulate during tadpole development prior to metamorphosis, such that the proportion of *erb*A transcripts increases relative to total RNA until stage 48, after which little if any further relative increase per embryo is noted. Regarding *erb*Aα-specific RNA expression in early *Xenopus* development, we have presented several lines of evidence supporting our conclusions: (i) assessment of the linear range of the quantitative PCR analyses; (ii) use of an internal *erb*Aα reverse transcription and PCR quantitation control; (iii) use of negative RNA controls; (iv) determination of embryo and RNA integrity by PCR amplification of other, previously characterized, RNA sequences expressed at early stages of development; and (v) identification of PCR products as *erb*Aα sequences by nucleotide sequencing.

While our studies were in progress, Baker and Tata (1) presented slot blot analyses of *Xenopus* embryo total RNAs probed with antisense RNA from the 3′ coding region (ligand binding domain) of chicken *erb*Aα. Using conditions that the authors suggest would produce signals linearly related to *erb*A RNA input, they obtained data consistent with expression of *erb*Aα RNA as early as stage 30. While this report was being reviewed, Yaiota and Brown (68) reported the detection of the expression of *erb*Aα RNA at and after stage 38 by the implementation of a modified primer extension method. They were unable to detect *erb*Aα RNA in earlier stages. In our study, the sensitivity of PCR amplification techniques allowed us to measure *erb*Aα expression even during blastulation and gastrulation, well before stage 30. We believe that the greater overall difference in the levels of *erb*A RNA expression across development noted in our studies (greater than 25-fold) is due to the greater sensitivity of our analyses. The results of Yaiota and Brown (68) are consistent with our own with regard to the increase in the relative level of *erb*Aα RNA prior to metamorphosis, confirming that the PCR method that we have used in our experiments can yield data analogous to those obtained by using more traditional assays of RNA expression. Yaoita and Brown (68) also presented evidence of multiple *erb*Aα genes in *X. laevis*, in agreement with our findings in PCR assays.

Thyroid hormones have effects on various developmental and metabolic functions in different tissues. Some evidence supports the idea that the expression of RNA for the β more than for the α form is correlated with the ability of rat tissues to bind triiodothyronine (T3) in in vitro assays (41, 58). Both forms, when expressed in vitro, are capable of binding T3 (21, 26, 31) and of binding and transactivating thyroid hormone response elements (46, 71). Whether *erb*Aα, *erb*Aβ, or both types of *erb*A proteins are functional with regard to developmentally significant processes is not clear. Our studies do not address *erb*Aβ expression. Yaoita and Brown (68) emphasized the close correlation of *erb*Aβ RNA levels detected in their assays with the presence of measurable levels of circulating thyroid hormones and the onset of metamorphosis. It will be of interest to apply the more sensitive PCR approach to determine whether *erb*Aβ is also expressed at low levels early in *Xenopus* development.

The most interesting conclusion from our studies is that polyadenylated *erb*Aα RNA is expressed in *Xenopus* cells at substantial, measurable levels and recruited to polysomes for translation well in advance of the completion of thyroid gland maturation (43) and in advance of stage 47, when detectable levels of the circulating thyroid hormones are present in metamorphosing *X. laevis* (33). Whether or not low levels of hormone are contributed to the *Xenopus* embryo by the egg or might be supplied by the surrounding environment is unknown at this time. Using radioimmunoassays, two groups (38, 47) were unable to detect circulating thyroid hormones before premetamorphosis in other anuran amphibians, including *R. catesbeana*. At the beginning of *Rana* metamorphosis, 5 ng of T3 per 100 ml (approximately 70 pM) was detected (47). This level is below the average K_D (10^{-10}) reported for *erb*A/thyroid hormone receptors binding this thyroid hormone (50, 65). It will be important to determine whether functional *erb*Aα (and perhaps *erb*Aβ) thyroid hormone receptors are present in *X. laevis* at the early developmental stages analyzed in our experiments. Previous studies were unable to detect significant thyroid hormone binding activity in developing frogs prior to the initiation of metamorphosis (20b, 39), at which time circulating thyroid

hormones are first detected, as mentioned above. If *erb*Aα transcripts are recruited to polysomes but do not produce functional *erb*A/thyroid hormone receptors early in development, then posttranslational controls of *erb*A function will be added to an already long list of regulatory mechanisms associated with this family of proteins.

The timing of the earliest known responses of specific genes to thyroid hormones is later than the expression and recruitment to polysomes of *erb*Aα transcripts as measured in our studies. Albumin and its RNA are synthesized in *R. catesbeana* tadpole liver in response to thyroid hormone at the onset of metamorphosis (53). The response of the *Xenopus* adult keratin gene to exogenous thyroid hormone can be moved forward to stage 48, but no earlier, in advance of its normal induction at stage 51 by circulating hormone (35). Other factors besides the constituents of the thyroid hormone/thyroid hormone receptor system may be involved in the timing of the responsiveness of the keratin gene to T3. In fact, the thyroid hormones seem to be part of complex response pathways in that full responses to hormone often require the presence of other inducing agents. For example, the maximal induction of vitellogenin at metamorphosis in *Xenopus* liver requires both thyroid hormone and estrogen (28, 64). Therefore, even the presence of completely functional *erb*A/thyroid hormone receptor molecules may be insufficient for the regulation of some thyroid hormone-specific responses.

In vivo, during *R. catesbeana* metamorphosis when circulating thyroid hormone levels are high, thyroid hormone both negatively regulates DNA synthesis (in epithelial cells of the regressing tail) and positively regulates DNA synthesis (in cells of the body epithelium), via as yet unknown gene expression networks (44). In transient expression assays, ErbA proteins can act as thyroid hormone-dependent transcriptional transactivators or repressors as summarized in the introduction. However, in the absence of thyroid hormones, *erb*A/thyroid hormone receptors repress the same genes that they transactivate in the presence of their specific ligand and they can interfere with the responses of these genes to other intracellular transactivators. For example, the synthetic thyroid hormone response element derived from the vitellogenin estrogen response element can be transactivated by both c-*erb*A/thyroid hormone and retinoic acid receptors in the presence of their respective hormones (23, 25). The negative regulation of this element by c-*erb*A in the absence of thyroid hormone can be dominant to the positive transactivation of the element by retinoic acid-bound retinoic acid receptor (25). Both retinoic acid and thyroid hormone affect the in vitro differentiation of v-*sea*-transformed chicken erythrocytes, and high amounts of exogenous c-*erb*Aα, in the absence of added thyroid hormone, can negatively affect the activities of retinoic acid in this system (4a).

Our data demonstrate a low level of c-*erb*Aα RNA during early *Xenopus* development. ErbA protein present during this developmental period would be expected to have negative regulatory activities since its presence would precede measurable circulation of thyroid hormones. It is of considerable interest that a window of retinoic acid teratogenesis in early *Xenopus* development (16, 57) correlates well with the developmental period during which embryos express relatively less *erb*Aα transcripts, as noted in our studies. Additionally, the anterior structures of early embryos are especially sensitive to the teratogenic effects of retinoic acid such that retinoic acid-treated embryos do not develop head structures (16, 56). We have shown that early embryos express more *erb*Aα RNA in their heads than in the rest of their bodies. Perhaps retinoic acid plays an essential role in normal embryo patterning processes and this activity requires that negative modulation functions of *erb*A proteins be down-regulated. We are currently addressing the possible interaction between these two gene regulation systems in early *Xenopus* development.

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